Stoichiometric and metabolomic shifts of organisms under environmental changes

PhD Thesis by

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to be eligible for a Doctor of Ecology degree

Supervised by

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Autonomous University of Barcelona, September 2013.

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Can you see, can you feel
all the beauty that we have in this world?
There's so much to see, forever
Ignorance, arrogance
keep us from being ourselves
So we just follow our leaders, why?

[Mother Gaia – Stratovarius (2000)]

You can look the other way
or you can face the light
Although it seems so far away
Freedom's worth the fight

[Outcry – Dream Theater (2011)]

Once I saw the eagle fly
it was the sound of peace
beauty dies in agony
crushed under debris

She was so beautiful
she was a dream
and when I cry for her
I hear them scream

[To Mother Earth – Gamma Ray (2007)]

To my mother

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Thesis abstract

Carbon (C), nitrogen (N) and phosphorus (P) are the principal elements that constitute the living systems and their uptake from the environment is essential for all organisms. Ecological stoichiometry aims to explain the organism life style and the ecosystem structure and function in relation to the environment and organism C:N:P ratios. C:N:P biomass ratios have proven to be important in several ecological processes, such as decomposition of litter, the capacity of N_2 fixation, plant-herbivore-predator relationships, ecosystem-specific composition and diversity and the capacity to adapt to environmental stress. One of the central paradigms of ecological stoichiometry is the growth rate hypothesis (GRH). The GRH proposes that growing organisms must increase their allocation of P to RNA to meet the elevated demands for the synthesis of proteins required for growth. Low ratios of environmental N:P and C:P under not limiting conditions favor species with very high rates of growth, which may induce shifts in species communities. Recently, the evidences of those relationships between the abiotic media and organismic C:N:P ratios conferred to ecological stoichiometry a central role in ecological research.

Most elements, especially C, N and P, the most studied elements in ecological stoichiometry, do not actuate as themselves but as molecular compounds. The coupling of techniques to identify and quantify metabolites in organism such as metabolomics to stoichiometry studies help to understand the response of organisms under biotic and non-biotic stresses, and the ontogenetic and physiological processes, and to disentangle the allocation of different nutrients (C, N, P) to the different physiological functions such as growth, storage or defense. Metabolomics aims to analyze the metabolome, the total set of metabolites of an organism in a specific moment and has demonstrated a great sensitivity in detecting the phenotypic mechanisms and key molecules underlying organism responses to environmental changes. This “holistic” method enables unbiased exploration and examination of sample molecular biochemistry.

In this thesis we reviewed the state of the art of ecological stoichiometry and metabolomics applied to the field of ecology and physiology. After that, we optimized a NMR-based method for metabolomic analyses and conducted metabolomics and stoichiometric analyses in different wild plant species living in field plots under different climatic conditions and cyanobacteria growing under different nutrient deprivation.

Our experiments showed that the different environmental conditions of the different seasons of the year in Mediterranean climate make plants to shift their metabolomes. These metabolomic shifts were also accompanied by stoichiometric changes demonstrating the

relationship between the shifts of foliar C:N:P:K and the shifts of metabolomes. For example, *Erica multiflora* shrub presented higher foliar concentrations of metabolites related to growth such as sugars and amino acids in spring, the growing season. These increments in the concentrations of these compounds were accompanied by lower foliar N:P ratios as expected in the frame of the GRH. However the GRH does not seem to be totally fulfilled in big trees that present large wood structures that act as a reservoir of nutrients as in the case of *Quercus ilex*. In terrestrial ecosystems there are several factors to take into account that make the relationship between low N:P body ratios and growth rate less immediate such as the skeleton, the storage necessity, the defense compounds or other mechanisms of stress avoidance.

Mediterranean plants under drought conditions presented higher levels of phenolic compounds with antioxidant function and other osmoregulants such as sugars or choline. These shifts were also accompanied by higher levels of foliar K to prevent water losses. This trend is most common in summer drought than in experimentally induced droughts. Drought seems also to stimulate the folivory activity by shifting the foliar metabolomes of plants by increasing foliar sugars and antioxidant concentrations. It could have thus a further indirect impact on trophic webs.

Cyanobacetria changed their metabolism under P and iron (Fe) deprivation. Cells shifted from aerobic to anaerobic metabolism by increasing the cellular lactic acid concentrations among other metabolomic changes. The anaerobic metabolism produces less energy and reduces cell growth. This growth reduction may produce a further shift in species composition and biodiversity of communities in aquatic ecosystems and of controls of ecosystem production and CO_2 sequestration.

This thesis has thus helped to make the first steps in application of metabolomics and stocichiometry to better understand the responses of organisms and ecosystems to environmental changes. The results of the conducted studies warrant and open challenging prospects to further research in ecometabolomics.

GENERAL INTRODUCTION

General Introduction

The introduction summarizes the state of the art of ecological stoichiometry and ecological metabolomics, a new emerging field of ecological studies which couple matabolomic analytical techniques with ecological studies. Three literature reviews on those topics were conducted at the beginning of the PhD thesis. They are attached in the chapter 1 of this PhD thesis.

1. Ecological stoichiometry.

a. General concepts.

Ecological stoichiometry aims to explain the organism life style and the ecosystem structure and function in relation to the environment and organism C:N:P ratios. Carbon, Nitrogen and Phosphorus are the principal elements that constitute the living systems and their uptake from the environment is essential for all organisms. The C:N:P biomass ratios of an organism could be considered its elemental phenotype. C:N:P biomass ratios have proven to be important in several ecological processes, such as decomposition of litter (d'Annunzio et al. 2008; Güsewell and Gessner 2009), the capacity of N_2 fixation (Sanudo-Wilhelmy et al. 2001), plant-herbivore-predator relationships (Kagata and Ohgushi 2006; Ngai and Jefferies 2004; Tibbets and Molles 2005), ecosystem-specific composition and diversity (Güsewell et al. 2005; Olde Venterink et al. 2003; Roem and Berendse 2000) and the capacity to adapt to environmental stress (Sardans and Peñuelas 2007; Woods et al. 2003). Recently, the evidences of those relationships between the abiotic media (water and soils) and organismic C:N:P ratios conferred to ecological stoichiometry a central role in ecological research (Elser et al. 1996; Güsewell 2004; Sterner and Hessen 1994). Aquatic and terrestrial ecosystems have been studied separately since the ecological mechanisms of the C:N:P of ecosystems act in different ways in each medium (Sardans et al. 2012a).

b. Aquatic ecosystems.

Ecological stoichiometry began to be studied in aquatic ecosystems. In 1963, Redfield measured the elemental composition of plankton from few stations in the Atlantic Ocean. He proposed a C:N:P ratio of 106:16:1 as an average of species-specific plankton community. Different organisms can strongly differ in their C:N:P biomass ratios. There are several mechanisms that are able to explain these differences between organisms even of the same

species. In 2004, (Klausmeier et al. 2004) proposed that such shifts in C:N:P biomass in plankton community might be due to the trade-off between competitive equilibrium, which favours greater allocation to P-poor resource-acquisition machinery and therefore to a higher N:P ratios, and exponential growth that favors greater allocation to P-rich assembly machinery and therefore lower N:P ratios. Furthermore, it has been demonstrated that nitrogen fixers play an important role in marine ecosystems as they exert a buffer effect on N:P ratio. When marine N:P ratios fall, nitrogen fixation increases by augmenting the biomass of N_2 fixers increasing then the water N:P ratios. Conversely, when the ratio rises, N_2 fixation decreases by P limitation and water N:P ratios decrease, implying that P exerts a primary control of oceanic production (Tyrrell 1999). On the other hand, the enzymatic N_2 fixation is depenent of other elements such as Fe or Mo (Howarth et al. 1988; Lenton and Watson 2000; Wu et al. 2000), demonstrating that not only N or P are the key elements to regulate the C:N:P biomass of oceans. Adding to it, Fe is also considered a key limiting nutrient for phytoplankton growth and photosynthesis and continues in the forefront of oceanographic studies (Behrenfeld et al. 1996; Martin et al. 1994; Timmermans and van der Wagt 2010) even though it is the fourth most abundant element in the Earth's (Bibby et al. 2001).

Redfield ratio is not fixed across all oceans' surface. Near the coasts, the N:P ratio is more variable due the N and P loadings from human activity throughout continental waters (Slomp and Van Cappellen 2004) where waters can induce phytoplankton blooms (Mei et al. 2005) directly related to the global change drivers of humans. Nevertheless, it has been reported a progressive adjustment of C:N:P ratios close to Redfield's values of 106:16:1 from coastal waters to open oceans (Doering et al. 1995; Fox et al. 1985; Hopkinson and Vallino 2005). This adjustment could be due some processes such as the trade-off between competitive equilibrium and growth rate, the buffering effect of N_2 fixers and the oceanic geochemical cycles of N and P. These changes of C:N:P ratios in marine communities demonstrate the high stoichiometric flexibility of organisms and communities to shift their biomass stoichiometries, which can produce cascade effects throughout the trophic webs.

The C:N:P ratios of water, plankton and seston in rivers and lakes are more variable than oceans and vary greatly around the world (Dobberfuhl and Elser 2000; Hecky et al. 1993). It is mainly due to the much smaller size of continental waters systems than oceans and the environmental C:N:P ratios of these systems are mainly determined by the particular traits of the surroundings, such as rock type or human impacts. The homeostatic capacity to adjust the C:N:P biomass ratios to narrow range is limited and is often determined by P availability (Cross et al. 2007; Elser et al. 2005; Fitter and Hillebrand 2009).

c. Terrestrial ecosystems.

In terrestrial ecosystems, the strong heterogenic interaction of the systems with bedrocks and the atmospheric dynamics at different regions of the planet explains the inexistence of any fixed optimum Redfield C:N:P biomass ratio as in marine ecosystems (Sardans et al. 2012a). For example, it has been reported that N:P ratios of soil, litter and plants generally increase from cool and temperate to tropical ecosystems despite large variations within each climatic area (Kerkhoff et al. 2005). The recent historic glaciations are one of the main factors that explain this great variability across different biomes. Nitrogen is often unlimited in many regions of the world, the N_2 inputs from the atmosphere to ecosystems are continuously increasing but it does not take place with P since it is derived primarily from the weathering of rock and the inputs to the ecosystems are decreasing (Peñuelas et al. 2012). The formation of new current soils has occurred more in higher latitudes than in lower latitudes that have not been affected by recent glaciations. Glaciations are the main natural drivers to weather the superficial layers of soils exposing the rock to the atmosphere. For this reason, the lack of recent glaciations in tropical regions make their soils often limited in P (Chadwick et al. 1999; Walker and Syers 1976) and also it is important to take into account the possible terminal state of P depletion in these regions (Vitousek et al. 2010).

Terrestrial organisms tend to present also a C:N:P biomass stoichiometry more constrained than the stoichiometry of its environment. Terrestrial organisms present homeostatic mechanisms such as resorption of nutrients from leaves in the case of plants buffering their biomass stoichiometries ratios. Even though, the homeostatic capacity is not a general trend in all living terrestrial organisms (Chrzanowski et al. 2010). For example, the N:P body ratio of heterotrophs is more homeostatic than is on autotrophs that present high stoichimetric flexibility (Sistla and Schimel 2012). This greater variation is mainly due to the variability of nutrient richness in habitats (Bridgham et al. 1995), the autotrophic turnover rate (Sterner 1995) and the balance between light and nutrients (Sterner et al. 1997).

d. Stoichiometry, species traits and ecosystem structure and function.

i. *Growth rate hypothesis*

The growth rate hypothesis (GRH) proposes that elevated growth rates depends on high P availability to supply the P-rich ribosomal RNA (r-RNA) (Elser et al. 2000a, 2000b, 2000c; Sterner and Elser 2002). The high demand of protein synthesis under high growth rates requires the increase of P allocation to rRNA for the translation process in cells (Hessen et al. 2007). Then, as expected, organisms with high growth rates are only possible under low N:P environmental ratios

(Main et al. 1997). The relationships between the growth rate and N:P ratios have been also observed in unicellular organisms (Karpinets et al. 2006), zooplankton (Carrillo et al. 2001; DeMott et al. 2004; Hessen et al. 2007; Main et al. 1997) and fish (Pilati and Vanni 2007; Tanner et al. 2000).

In freshwater ecosystems N:P availability appears to determine web trophic structures (Andersen & Hessen 1991). Lakes with four trophic levels (phytoplankton, zooplankton, herbivorous fish and piscivorous fish) are dominated by large-bodied zooplankton and the crustacean *Daphnia* (P-rich, low N:P biomass ratio). On the other hand, lakes with three trophic levels are dominated by zooplankton with low-P levels (high N:P ratios) (Sterner et al. 1998).

The GRH is not only proposed in aquatic ecosystems. Also, several studies suggest that the observations made in aquatic ecosystems can be extended to terrestrial ecosystems. For example, terrestrial insect herbivores present the same C:N:P biomass stoichiometry than their freshwater counterparts (zooplankton) and relationships between the growth rate and low C:P and N:P biomass contents have been reported (Hambäck et al. 2009; Schneider et al. 2010; Watts et al. 2006; Woods et al. 2004). Also in plants and animals, there are some studies that showed a relationship between low N:P ratios with high growth rates (Apple et al. 2009; Cernusak et al. 2010; Elser 2006; Elser et al. 2003; Kay et al. 2006; Niinemets and Kull 2003; Niklas and Cobb 2005; Rivas-Ubach et al. 2012; Visanuvimol and Bertram 2010; Zhang and Han 2010). Even so, the terrestrial food webs are built on an extremely nutrient-poor autotrophic basis (high C:P and C:N), although the N:P ratios are nearly identical to aquatic ecosystems (Elser et al. 2000b).

There are some concerns about generalizing the GRH. Not all studies are consistent with the GRH as due by many factors described below, thus preventing its generalitzation to all situations and types of organism. First, Vrede et al. (1999, 2004) reported that high growth rates are generally constrained to low N:P body ratios but at low growth rates the N:P ratios can differ substantially as a consequence of various patterns of allocation to RNA, protein and other potential biochemical constituents. Also, it is important to take into account the N and P availability (Sterner and Elser 2002). Low N:P environmental ratios may be found under N limitation and the GRH is not consistent in this situation as organisms do not present higher growth rates (Acharya et al. 2004). In environments where some nutrients may be limiting, a greater homeostatic capacity to adjust body N:P ratio should be particularly needed (Sterner and Elser 2002).

Second, the quality of food is not only explained by the C:N:P biomass ratios. It has been demonstrated that the allocation of nutrients to different molecules and structures is also an important factor (Anderson et al. 2004). For example, in the case of algae, both C:N:P ratios and polyunsaturated fatty acids (PUFA) are important variables of quality of food for zooplanktonic

growth (Ferrão-Filho et al. 2003; Gulati and DeMott 1997; Park et al. 2002; Saikia and Nandi 2010) but C:N:P ratios and PUFA are not fully correlated (Gladyshev et al. 2007; Viso and Marty 1993) and could shift under environmental changes (Leu et al. 2007). Also, the allocation of nutrients to other metabolites or structures such as defensive tissues or toxins can affect the efficiency of growth of the predators (Mitra and Flynn 2005). Then, C:N:P ratios of organisms could thus be linked to different compounds and nutritional qualities.

The third issue stems from the fact that rRNA is not always the most significant sink of P, especially in large organisms such as vertebrates and plants. It has seen a lower allocation of P to rRNA as larger is the animal (Gillooly et al. 2005). The skeleton of organisms, the storage necessity, respiration rate, digestion capacity, immune function, defense tissues or compounds, or the mechanisms of stress avoidance are factors to take into account making the relationship between low N:P body ratios and growth rate of organisms less immediate (Sardans et al. 2012a). For example, recently Sardans and Peñuelas 2013 have observed that forests tend to accumulate P in higher proportions than N in wood with tree aging. In this case, the N:P of the total aboveground biomass decreases across time without any relation with tree growth rate capacity.

ii. *Ecosystem structure and function*

Changes in N:P stoichiometry of primary producers induce shifts in the community of species (Conde Porcuna et al. 2002; de Eyto and Irvine 2007; DeMott and Tessier 2002; Hassett et al. 1997; Makino et al. 2002; Smith 1983). The species composition with different growth rate is related by the environmental N:P and C:P ratios in aquatic environments (Elser and George 1993; Elser and Urabe 1999; Hassett et al. 1997; Sardans et al. 2012a; Smith 1983; Vanni et al. 1997) and the dynamics of producers-herbivore dynamics can be predicted when considering the quality (C:P ratio) and quantity (absolute C algal content) of food (Andersen 1997; Nelson et al. 2001), but these investigations are still poor in terrestrial ecosystems. Mulder and Elser (2009) found that the higher the P availability in soils (lower C:P ratios) the larger biomass of large-bodied invertebrates relative to small invertebrates, which may have important implications on soils since invertebrates of different sizes present different effects on soils. As Mulder and Elser stated, other factors may be involved, the association between soil-element stoichiometry and the body-size spectrum of the food web, though, might not simply result from a faster growth of the different organisms of the food web in response to low C:P and protein:RNA ratios, as predicted by the GRH.

In aquatic ecosystems, nutrient availability and grazing interaction might even determine the richness of species of primary producers (Liess et al. 2009) but there are also some incoherencies to establish general relationships. Some studies have observed an increase of

species diversity under low C:P and C:N ratios of community organisms (Liess et al. 2009) but others have observed no clear results (Evans-White et al. 2009; Singer and Battin 2007; Striebel et al. 2009). In terrestrial ecosystems it have been seen an increase of species diversity under high P availability (Güsewell et al. 2005; Seastedt and Vaccaro 2001) but increases in N availability increases body N:P ratios and reduces species diversity (Güsewell et al. 2005; Roem and Berendse 2000; Seastedt and Vaccaro 2001).These results are in accordance with the previously noted in aquatic ecosystems, high P availability allow the existence of large trophic webs (Sterner et al. 1998), although at certain low levels of N:P ratios, N can also limit the production capacity of ecosystems affecting the species richness (Sardans et al. 2012a). Although the N:P ratio could determine the length of trophic webs, other factors could be more important in determining the niche dimension such as competence (Olde Venterink and Güsewell 2010) or resources limitation (Harpole and Tilman 2007; Interlandi and Kilham 2001).

The C:N:P ratios thus seem to have an important role in controlling trophic relationships, structure and species composition of communities in both terrestrial and aquatic ecosystems. It has been suggested that herbivores prefer biomasses with high nutrient contents and low C:N (Ngai and Jefferies 2004) but some studies observed that folivory attack is more closely related with the foliar N concentration than with the C:N biomass ratios (Kagata and Ohgushi 2006, 2007). Matsumura et al. 2004 observed that predators present lower C:N ratios than their herbivorous prey and are able to compensate N concentreations by increasing the consumption of more nitrogen-rich intraguild prey. But others did not observe any change in N content of predators after the intragild predation (Fagan and Denno 2004; Kagata and Katayama 2006) showing that C:N ratio is less crucial in the transfer of matter through trophic levels than N:P ratio.

e. C:N:P of organisms and ecosystems in a changing world.

Several factors promoting global change predicted by the IPCC 2007 such as the increase of CO_2, global warming or eutrophication have been demonstrated to interact with the C:N:P stoichiometry of ecosystems (Elser et al. 2009; Elser et al. 2010; Ferreira et al. 2010; Funk and Vitousek 2007; Reich et al. 2006; Sardans and Peñuelas 2008; Sardans et al. 2012b). Global change drivers are able to affect the biomass C:N:P stoichiometries of organisms and ecosystems mainly through two ways. First, by the direct effect of global change driver on C:N and C:P ratios of primary producers altering the food quality and producing a cascade effect to the whole trophic web. Second, by the changes in N:P of ecosystems that could lead to a shift in ecosystem composition by altering the competitiveness of a species depending on its growth rate and lifestyle (Elser et al. 1996; Elser and Hamilton 2007).

In terrestrial ecosystems the elevated concentrations of CO_2 ($[CO_2]$) increase the plant C:N ratios of tissues in many terrestrial plants making a N dilution in tissues (Cotrufo et al. 1998; Gifford et al. 2000; Hungate et al. 1996; Lindroth 2010; Peñuelas and Matamala 1990; Sardans et al. 2012b; Stiling and Cornelissen 2007) (overall 22% of C:N increase as observed in a meta-analysis). The phenomenon is particularly evident in C3 grasses, but is absent in C4 grasses. Elevated atmospheric $[CO_2]$ also lead to an average 38% higher foliar C:P ratios in C3 plants.

In aquatic ecosystems, the effects of elevated $[CO_2]$ on C:N:P ratios are considerable. It increases C:N and C:P biomass ratios of phytoplankton (Finkel et al. 2010) but the effects on plankton vary considerably among species (Fu et al. 2007, 2008; Iglesias-Rodriguez et al. 2008) and even can increase the N and P uptake of phytoplankton (Hutchins et al. 2007). The studies on N:P biomass ratios under high CO_2 exposure are very scarce and inconclusive since different responses have been observed in phytoplankton; decreases (Tortell et al. 2002), increases (Fu et al. 2007; Hutchins et al. 2007) and no effects on N:P biomass ratios (Bellerby et al. 2008; Fu et al. 2008).

The effects of high $[CO_2]$ levels may have consequences at the ecosystem levels by altering the trophic webs. The reduction of food quality (high C:N) caused by the CO_2 have an indirect effect of increasing herbivore feeding rates to compensate the N supplies (Johnson and McNicol 2010). Also, the low quality food favors some herbivorous insect species, which can alter the composition of the insect community (Stiling et al. 2009). The level of pathogen infestation in plants and the size and aggressiveness of those pathogens may be increased under high $[CO_2]$ (Chakraborty and Datta 2003; Mitchell et al. 2003). Additional effects on trophic webs include changes in the composition of plant's defensive chemical compounds at high levels of atmospheric $[CO_2]$ (Matros et al. 2006; Peñuelas and Estiarte 1998).

The N fertilization studies showed that N addition alters the C:N:P ratios of aboveground biomass of plants, leaves, litter, soils and aquatic ecosystems. The addition of N causes a reduction of foliar C:N (25% average decrease of foliar C:N ratio). Even though the effects of N fertilization on N:P ratios are not as clear as the effects on C:N ratios, it has been observed an increase on N:P ratios of total aboveground biomass but not on leaves, litter or organic soils (Sardans et al. 2012b). On the other hand, the reductions in the abundance of N_2-fixers in response to increased N-deposition can have compensatory effects on N:P ratios but, typically, these effects are insufficient to prevent an increase in the N:P ratios of organisms and ecosystems. Moreover, according to the GRH, higher N:P ratios produced by nitrogen deposition have proven to favor species that have low growth rates (Elser et al. 2000a, 2000b).

The excessive application of N and P as fertilizers, mainly from animal slurries, to maintain the croplands throughout the world have reduced soil N:P ratios because, typically, the

N:P ratios of animal slurries are low, and because P is less soluble than N and, therefore, the former has a stronger tendency to accumulate in soil (Cech et al. 2008; Peñuelas et al. 2009). Intensive fertilization results in N leachates from fields, which has consequences for continental aquatic ecosystems increasing the N:P ratios. The N deposition has thus been demonstrated to play a central role in the ecosystem structure since as mentioned above, in general it has been observed an increase of N:P ratios of wild ecosystems and a reduction in crops (Peñuelas et al. 2012). The shifts of N:P ratios have the potential to alter the species composition of communities by reducing the community of N_2-fixers and species diversity in both aquatic and terrestrial ecosystems (Jefferies and Maron 1997).

Climate change drivers such as drought and warming are also potential sources of changing the C:N:P of ecosystems. The effects of drought differ among ecosystems and species. In Mediterranean semid-arid areas, drought reduced C:N ratios of *Quercus ilex* roots due the higher allocation of N to roots to improve the water absorption capacity (Gonzalez et al. 2010; Sardans et al. 2008). On the other hand, the foliar C:N and C:P ratios increase by drought due the increases in sclerophylly with high contents of lignin (Bussotti et al. 2000; Sardans et al. 2006). In temperate heatlands, drought reduces the C:N ratio of plants (Larsen et al. 201). It is due mainly by the reduction in water uptake, the reduction of mobility of N in soils and the reduction in the N released from soil organic matter. In wet-temperate ecosystems, the C:N ratio can decrease under moderate drought because plants increase N uptake and reduce growth (Lu et al. 2009a, 2009b). These evidences suggest that drought tends to increase the C:N ratios of photosynthetic tissues in semi-arid ecosystems but the effects are not as clear in wet ecosystems where drought can affect multiple aspects of plant and soil microbes, which can differentially affect soil N and its availability (Rennenberg et al. 2009). In dry regions, the increases in the C:N ratios in litter caused by drought can be due to a combination with the effects of increases in CO_2 concentrations slowing the N and P mineralization and cycling, reducing food quality and N and P availability for plants. It is translated thus to decreases in N and P biomass concentrations. The number of studies regarding N:P and drought are quite scare and the results have seen inconclusive, thus, there is a need to examine the effects of drought, in combination with other global change drivers such as CO_2, on N:P ratios and trophic webs, concurrently, in a range of climates from semi-arid to wet areas.

In aquatic ecosystems, warming can increase water stratification (Feng et al. 2008; Finkel et al. 2009), which impedes the rise of nutrients from the sediment to the surface, increasing thus the C:N and C:P ratios of ocean food webs (Sarmiento et al. 2004; van de Waal et al. 2010). However, warming with increases in atmospheric CO_2 is able to increase the N_2-fixation and reduce dissolved CO_2 levels in oceans, having thus a compensatory effect on the C:N ratios. Those

compensatory mechanisms might explain why the C:N ratios of aquatic organisms did not change (Iglesias-Rodriguez et al. 2008) or were reduced (Fu et al. 2007) under elevated [CO_2].

In terrestrial ecosystems, warming can increase, decrease, or have no effects on the C:N ratios of plants depending on the type of plant and the climate. Some studies observed no changes in C:N plant ratios submitted to warming and high levels of CO_2. The increases in temperature cause higher rates of C-mineralization by higher respiration rates, which have a countereffect on the C-storage capacity increase due higher CO_2 atmospheric levels (Ferreira et al. 2010; Finkel et al. 2010). In addition, respiration responses to warming can depend also on the light and water availability, so the responses can difference among species and plant organs (Atkin et al. 2005). Even so, the evidences suggest that the increases in temperature will increase plant C:N and C:P ratios in warm-dry and temperate climate though water stress resistance mechanisms or increases in Nitrogen Use Efficiency (Sardans et al. 2008). On the other hand, in cold ecosystems the effects of warming on plant C:N ratios are not well understood. Studies showed very different results regarding the C:N ratios, some demonstrated a reduction of C:N ratios by the increase of nutrient uptake (Aerts et al. 2009; Welker et al. 2005) while others showed an increase of C:N associated with a dilution effect by an increase in biomass production (Day et al. 2008). Some other studies did not find any effect of warming in cold ecosystems (Larsen et al. 2011).

The current results suggest that warming and drought can increase C:N and C:P ratios in warm-dry and temperate-dry terrestrial ecosystems because of an increase in nutrient use efficiency or an increase in resistance to drought (a protective mechanism associated with an increase in water use efficiency and the prevention of water loss). The effect can be exacerbated when warming and drought coincide in semi-arid or mesic environments, and furthermore by the effects of increases in atmospheric [CO_2]; however, the effect can be counterbalanced by an increase in respiration in response to increased warming.

2. Metabolomics in ecology.

a. Ecometabolomics.

Metabolomics, which aims to analyze the metabolomes, the total set of metabolites of an organism in a specific moment (Fiehn 2002), is gaining importance in ecological studies (ecometabolomics) because of the increasing use of new technical advances, such as modern nuclear magnetic resonance (NMR) spectrometers or gas/liquid chromatography coupled to mass spectrometry (LC-MS/GC-MS) (Peñuelas and Sardans 2009; Sardans et al. 2011). Metabolomics demonstrated a great sensitivity in detecting the phenotypic mechanisms and key molecules underlying organism responses to environmental changes. When it is not required or possible to identify every metabolite of organisms, especially in ecology it is often sufficient to rapidly classify samples according to their origin or their ecological or ecophysiological relevance. This process is called *metabolic fingerprinting*. This "holistic" method enables unbiased exploration and examination of sample molecular biochemistry, and through suitable interpretation can be used to study plant responses to environmental changes (Gidman et al. 2005, 2006). When the aim is to obtain information about the whole metabolome (the total number of metabolites in one biological system) by identifying and quantifying as many metabolites as possible it is necessary to conduct different complement analysis approaches with NMR, LC-MS or GC-MS that enables to determine and quantify the maximum number of metabolites. Since such an approach reveals the maximum information of metabolome as possible of the biological system under study, this approach is called metabolic profile.

The possibility of using progressively metabolomic techniques in ecophysiological and ecological studies has opened up a new way to advance knowledge of the structure and function of organisms and ecosystems in all scales. It is able to provide the phenotypical response at the metabolic level in a particular environmental circumstance and it demonstrated to be a powerful tool to monitor the phenotypic variability of one genotype in response to environmental shifts in drought (Fumagalli et al. 2009; Rivas-Ubach et al. 2012), nutrient availability (Hirai et al. 2005, 2005), pollutants (Bundy et al. 2008; Jones et al. 2007), salinity (Fumagalli et al. 2009), temperature (Michaud and Denlinger 2007; Rivas-Ubach et al. 2012) and biotic interactions (Choi et al. 2006), among other factors.

Ecometabolomic applications are not only limited to the ecophysiology of organisms and it is possible to apply this technique from individual to population and ecosystem levels (Figure 1). For example, it has demonstrated that metabolite fingerprinting and profiling is sufficiently sensitive to be able to identify the metabolic differences between populations of *Arabidopsis petraea* (Davey et al. 2008). A principal component analysis of metabolite fingerprints revealed

different metabolic phenotypes for each population. At the landscape level, it has shown that different metabolic fingerprints measured in tissue samples of *Galium saxatile* are correlated with a gradient of N deposition across the entire UK landscape (Gidman et al. 2006). Metabolomics thus allows the investigation of complex ecological systems and provides a rapid and sensitive indicator of ecosystem health.

Metabolomics has the advantage that all extracted compounds are measured at once in only one step, rather than put through iterative purification steps and unstable compounds are more likely to be detected and measured. It has been proposed that the measurement of multiple metabolites at the same time instead of individual metabolites can provide a more robust assessment of the metabolic health of an organism, characterising the health of multiple species will provide a more complete assessment of the ecosystem responses to environmental stressors, and even of the nature of the stressor (Viant 2007). The modern improvements in analytical methods and the computer hardware and software to interpret and visualize large data sets (Gehlenborg et al. 2010) have multiplied the possibilities of rapidly identifying and quantifying a big number of compounds (e.g., carbohydrates, amino acids, lipids, phenolics and terpenoids). These advances will enable to take "static pictures" and to "film" the dynamic nature of the organisms. Ecometabolomics can thus serve as a powerful indication for defining organism lifestyle.

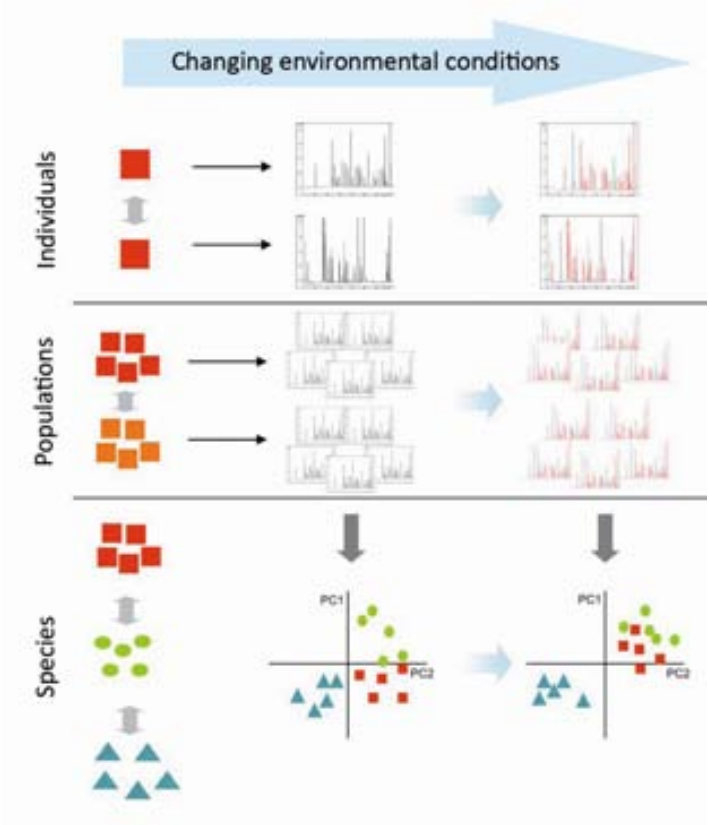

Figure 1. Metabolome analyses can be used to identify phenotypic differences between individuals, between populations and between species, and to follow such differences through time in response to environmental changes. Multivariate analyses of large datasets are needed to deal with metabolomic studies. The lower panels of the figure depict schematically the results of principal component analyses of metabolites (depicted in upper panels) from different individuals of different species in an ecosystem at different times and under different environmental conditions (modified from Peñuelas and Sardans 2009).

b. Analytical techniques.

Currently no single analytical method or combination of methods can detect all metabolites (estimated to be between 100.000 and 200.000 in the plant Kingdom) within a given biological sample. The most used analytical techniques are GC-MS, LC-MS and NMR (Figure 2) presenting the best capacity to determine the widest ranging sets of metabolites. GC-MS has proven to be a robust tool for the study of volatile organic compounds (Degen et al. 2004; Llusià et al. 2010; Ozawa et al. 2008) but GC-MS analyses of extracts containing other analytes such as organic acids, sugars, aminoacids, and steroids is complicated. Many metabolites are non-volatile and must be derivatized prior to GC-MS analysis (Gullberg et al. 2004) and thermolabile compounds may be lost. LC-MS is of particular importance to study a great number of metabolic pathways at once since plant metabolism embodies a huge range of semi-polar compounds, including many key groups of secondary metabolites, which are better separated by HPLC systems (High Pressure Liquid Chromatography) (Allwood and Goodacre 2009). Thus, while GC-MS is best suited for compound classes appearing mainly in primary metabolism (frequently after derivation) i.e. aminoacids, fatty acids and sugars or volatile compounds, LC-MS is more adequate to determine the overall biochemical richness of plants including several semi-polar groups of secondary metabolites. On the other hand, it is difficult to elucidate the unknown structures of metabolites by using GC-MS or LC-MS alone.

^{1}H-NMR has proven to be an appropriate tool for structure elucidation and for untargeted analyses. It has the advantage that it can be applied to determine polar, semi-polar and non polar metabolites, and that it produces signals linearly correlated with compound abundance (Lewis et al. 2007). However, NMR spectroscopy has intrinsic low sensitivity for low concentrations of metabolites and signal overlapping for complex mixtures. This can at times be problematic for structure elucidation of a metabolite at low concentrations. The use of low temperatures to stabilize the detector by modern cryogenically cooled devices can improve the sensitivity up to a factor of five by reducing the thermal noise from the electronics of the NMR spectrometer. Two dimensional (2D) NMR spectroscopy methods and high-resolution magic angle spinning (Sekiyama et al. 2010) further improve the sensitivity.

Figure 2. Different analytical instruments for metabolomic analyses.

3. Objectives of this PhD thesis: Coupling stoichiometry and metabolomics in ecology.

As seen above, there are several questions to answer in the ecological stoichiometry context. Since most elements, especially C, N and P, the most studied elements in ecological stoichiometry, do not actuate as themselves but as molecular compounds, the coupling of metabolomic techniques to stoichiometry studies help to understand the response of organism under biotic and non-biotic stresses, and even the ontogenetic physiological or other natural processes, in the allocation of different nutrients (C:N:P) to the different physiological functions such as growth, storage or defense. It may also provide the elemental and metabolic budgets for different species along gradients from low to fast growth, which would allow a better test of the links between C:N:P ratio, growth rate and body-size spectrum.

In this thesis we aimed to test the effects of different environmental stresses on both stoichiometry and metabolomics to different organisms growing in field and laboratory conditions. We were aiming to make a step forward understanding how the stoichiometric shifts of organisms are linked to metabolomic changes and how this helps to understand the ecosystem structure and function. In particular, this PhD thesis has thus the following main objectives:

Objective 1. Summarize the most-recent ecological stoichiometric and ecometabolomic bibliography.

Objective 2. Understand the relationships of the stoichiometric shifts with the metabolome shifts of organisms under environmental changes, specially testing the GRH at both stoichiometric and metabolomic level.

Objective 3. Apply and test the sensitivity and reproducibility of the new emerging metabolomic techniques such as NMR and LC-MS in ecological studies.

Objective 4. Study the effects of seasonal-ontogeny and climate change drivers such as drought and warming on the foliar C:N:P:K stoichiometry. Understand the crucial role of K in plants under natural or experimental drought.

Objective 5. Study the effects of seasonal-ontogeny and climate change drivers such as drought and warming on the foliar metabolomes.

Objective 6. To observe the potential casacade effects of drought, one of the most important global change drivers in the Mediterranean basin, trough trophic webs by using ecometabolomics and stoichiometric techniques.

Objective 7. To understand and identify the metabolic strategies that phytoplankton use to cope with phosphorus and iron limitations.

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Thesis structure

Chapter 1. Review of the State of the Art of Ecological Stoichiometry and ecological Metabolomics.

The first task in this PhD thesis consisted in reviewing stoichiometric and metabolomic studies applied in the field of ecology to uptade the knowledge on these areas and to discern the research needed to make a step forward in both stoichiometric and ecometabolomic fields. Theis resuled in three synthesis articles published in three different journals attached in the first chapter.

Chapter 2. Ecometabolomics: Optimized NMR-based method.

The lack of a standard protocol for ecometabolomic studies, especially plants collected from the field, encouraged us to develop one. We developed a NMR-based protocol for such ecometabolomic analyses, where wild organisms present large differences in elemental composition, metabolism, phenology, and life style, much more so than do entries in a laboratory model. This implies that the number of individuals to be analyzed should be sufficiently large for a consistent statistical analysis. This protocol was thus developed to provide an overall analysis of the main metabolites in field samples, including secondary and nonpolar compounds, and which allows the detection and identification of those metabolites that play a key role in an organism's response to environmental change.

Chapter 3. Strong relationship between elemental stoichiometry and metabolome in plants.

In this chapter we demonstrated the existence of the relationship between the shifts of stoichiometry and the shifts of metabolomes. We considered the different year seasons to obtain the metabolomic and stoichiometric "film" of the ontogeny of the plants across the different marked seasons of Mediterranean climate and drought and warming as climate change drivers.

Chapter 4. Drought stress enhances folivory by shifting foliar metabolomes in *Quercus ilex* trees.

In this chapter we studied the potential role of drought stress in Mediterranean mature forests of *Quercus ilex* to produce cascade effects throughout the trophic webs. Drought shifted foliar stoichiometry and metabolism and resulted in increased folivory activity.

Chapter 5. *Synechocystis.*

In this chapter we aim to identity and understand the metabolic strategies that phytoplankton use to cope with different types of nutrient limitations. In this case, the shifts of stoichiometric and metabolomes of the cyanobacteria *Synechocystis* sp. *PCC6803* growing under P and Fe limitation were studied in laboratory conditions. The metabolomes of cyanobacteria tended to increase the concentrations of lactic acid since increased the fermentation metabolic pathways.

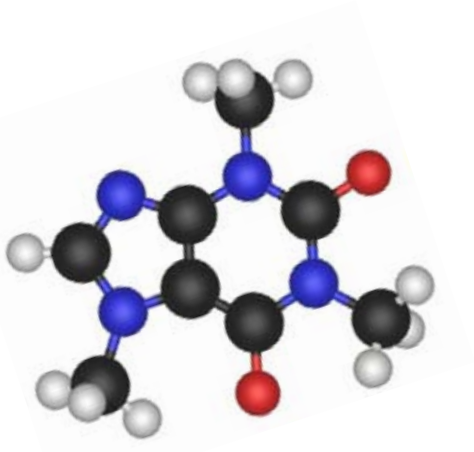
Chapter 1.

Review of the State of the Art of Ecological Stoichiometry and ecological Metabolomics

· **The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives.**

· **The C:N:P stoichiometry of organisms and ecosystems in a changing world: A review and perspectives.**

· **Ecological metabolomics: overview of current developments and future challenges.**



The elemental stoichiometry of aquatic and terrestrial ecosystems and its relationships with organismic lifestyle and ecosystem structure and function: a review and perspectives.

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Biogeochemistry (2012) 111 : 1-39.

Abstract

C, N and P are three of the most important elements used to build living beings, and their uptake from the environment is consequently essential for all organisms. We have reviewed the available studies on water, soils and organism elemental content ratios (stoichiometry) with the aim of identifying the general links between stoichiometry and the structure and function of organisms and ecosystems, in both aquatic and terrestrial contexts.

Oceans have variable C:N:P ratios in coastal areas and a narrow range approximating the Redfield ratio in deep water and inner oceanic areas. Terrestrial ecosystems have a general trend towards an increase in soil and plant N:P ratios from cool and temperate to tropical ecosystems, but with great variation within each climatic area. The C:N:P content ratio (from now on C:N:P ratio) is more constrained in organisms than in the water and soil environments they inhabit. The capacity to adjust this ratio involves several mechanisms, from leaf re-absorption in plants to the control of excretion in animals. Several differences in C:N:P ratios are observed when comparing different taxa and ecosystems. For freshwater ecosystems, the growth rate hypothesis (GRH), which has consistent experimental support, states that low N:P supply determines trophic web structures by favoring organisms with a high growth rate. For terrestrial organisms, however, evidence not yet conclusive on the relevance of the GRH. Recent studies suggest that the N:P ratio could play a role, even in the evolution of the genomes of organisms.

Further research is warranted to study the stoichiometry of different trophic levels under different C:N:P environment ratios in long-term ecosystem-scale studies. Other nutrients such as K or Fe should also be taken into account. Further assessment of the GRH requires more studies on the effects of C:N:P ratios on anabolic (growth), catabolic (respiration), storage and/or defensive allocation. Combining elemental stoichiometry with metabolomics and/or genomics should improve our understanding of the coupling of different levels of biological organization, from elemental composition to the structure and evolution of ecosystems, via cellular metabolism and nutrient cycling.

Keywords Body size · climate gradients · competition · diversity · elemental stoichiometry · growth rate · herbivore-predator · plant-herbivore · Redfield's ratio · soil composition · temperature gradients · trophic webs.

Introduction

C:N:P biomass ratios are significant in several important ecological processes, such as decomposition of litter (d'Annunzio et al. 2008; Güsewell and Gessner 2009), capacity of N_2 fixation (Sanudo-Wilhelmy et al. 2001), plant-herbivore-predator relationships (Kagata and Ohgushi 2006; Ngai and Jefferies 2004; Tibbets and Molles 2005), ecosystem-specific composition and diversity (Güsewell et al. 2005; Olde Venterink et al. 2003; Roem and Berendse 2000) and the capacity to adapt to environmental stress (Sardans and Peñuelas 2007; Sardans et al. 2008; Woods et al. 2003). Ecological stoichiometry aims to explain organismic lifestyle and ecosystem structure and function in relation to the C:N:P stoichiometry of abiotic media (water, soil) and organisms. Over the last twenty years, experimental evidence of the relationships between soils, water and organismic C:N:P ratios with essential ecological traits has increased, and these relationships now play a central role in ecological research (Elser et al. 1996; Elser et al. 2000a; Raubenheimer and Simpson 2004; Sterner and Elser 2002; Sterner and Hessen 1994).

Here we have conducted a wide review of studies that investigate the relationships of the stoichiometric composition of organisms with aquatic and terrestrial environmental gradients, traits of taxa such as growth rate and body size, community diversity and trophic chain structure. In addition, we propose new experimental approaches for investigating ecological stoichiometry, highlighting the possibility of coupling it with recent advanced methodologies such as metabolomics and genomics.

Stoichiometry in environmental gradients

Aquatic ecosystems

In oceans, well-constrained C:N:P ratios have been found in planktonic biomass. In 1934, Alfred Redfield wrote a classic paper in which he proposed that the N:P ratio of plankton and deep waters of open oceanic areas was 16:1. He later extended this to a C:N:P ratio of 106:16:1 on a molar basis (the *Redfield ratio*) in the statistical sense (Redfield et al. 1963). This ratio was originally derived from measurements of the elemental composition of plankton and the NO_3^- and PO_4^{3-} content of seawater from a few stations in the Atlantic. Hundreds of other independent measurements in deep oceanic waters throughout the world (Falkowski and Davis 2004; Goldman 1986; Hecky et al. 1993; Ho et al. 2003; Tyrrell 1999), usually in phytoplankton (Ho et al. 2003; Klausmeier et al. 2008), subsequently supported these results. Despite generations of biochemists and physiologists reporting the plasticity of the elemental composition of marine phytoplankton in the field and in laboratory cultures (Geider and Roche 2002; Hecky et al. 1993), values close to N:P Redfield ratios in marine phytoplankton (Ho et al. 2003; Klausmeier et al. 2008) and in inorganic dissolved matter in deep oceans have been observed throughout the

world (Hecky et al. 1993). Geochemists use a C:N:P stoichiometry of 106:15:1 based on the co-variation of nitrate, phosphate and a non-calcite contribution to total inorganic C in deep seawater (Broecker and Peng 1982), whereas biologists use a ratio of 106:16:1 based on Fleming's analysis of the average elemental composition of marine organisms (Goldman et al. 1979). Different organisms can strongly differ in their C:N:P ratio, thus the Redfield N:P ratio of 16:1 is not a universal biochemical optimum but instead represents an average of species-specific community N:P ratios (Klausmeier et al. 2004). These differences might be due to the trade-off between competitive equilibrium, which favors greater allocation to P-poor resource-acquisition machinery and therefore to a higher N:P ratio, and exponential growth that favors greater allocation to P-rich assembly machinery and therefore lower N:P ratios (Klausmeier et al. 2004). The N:P ratio in marine ecosystems could thus change through time with the variation in the proportion of organisms growing exponentially and those in equilibrium within the community. The N:P ratio could also change in coastal areas due to spatial changes in the N:P availability ratio by loadings from continental waters (Klausmeier et al. 2004).

The strongest support for the Redfield ratio comes from the fact that the NO_3^-:PO_4^{3-} ratio in the deep open areas of all major oceans is remarkably similar to the N:P ratio of plankton (16:1). The maintenance of the Redfield ratio is favored by the long duration of residence of these two elements in the ocean (10^4 years) relative to the ocean's circulation time (10^3 years) (Falkowski and Davis 2004). Biological processes also underlie Redfield ratios, in addition to the geochemical causes. N_2 fixation can play an important role in the homeostasis of the marine N:P ratio (Ganeshram et al. 2002; Holl and Montoya 2008; Kenesi et al. 2009; Lenton and Watson 2000; Tyrrell 1999; Weber and Deutsch 2010) (Figure1 and Table 1). When marine N:P ratios fall, nitrogen fixation increases by augmenting the biomass of N_2 fixers. When these N_2 fixers decompose or are eaten, their N-rich organic matter releases soluble ammonium and nitrates, thereby increasing water N:P ratios. Conversely, when the ratio rises, N_2 fixation decreases by P limitation, thereby restoring a lower N:P ratio of the water. This process implies that P exerts primary control of oceanic production (Tyrrell 1999). This control has been experimentally demonstrated in lakes (Howarth et al. 1988; Smith 1983) but not in oceans, where results of some studies are inconclusive (Howarth et al. 1988; Lenton and Klausmeier 2007), irrespective of Redfield's ratio being generally attained in oceans (Figure 1 and Table 1). On the other hand, denitrification counteracts N_2 fixation and represents a mechanism of venting excess N. When N_2 fixation provides new N, the increases in production enhance the demands for O_2 needed for respiration, leading to O_2 depletion and denitrification (Hong et al. 2011; Koeve and Kähler 2010; Ward et al. 2009) and anammox (Mills and Arrigo 2010). These processes occur at very different intensities in different oceanic areas (Gruber and Sarmiento 1997), with some areas acting as a

source of N, e.g. the Mediterranean and northern tropical Atlantic, and other areas acting as N sinks, e.g. the Arabian Sea and eastern tropical Pacific (Gruber and Sarmiento 1997). However, large oceanic currents mix waters of different areas, fostering similar N:P ratios throughout the world (Weber and Deutsch 2010).

The buffer effect of N_2 fixers around Redfield's ratio is dependent on the availability of elements other than N and P such as Fe and Mo, which are necessary for the enzymatic fixation of N_2 (Howarth et al. 1988; Lenton and Watson 2000; Wu et al. 2000). The effect of these micronutrients on N_2 fixation can be different in freshwater and marine ecosystems. In eutrophicated lakes, significant N_2 fixation by planktonic organisms generally occurs when the N:P ratios of the nutrient loading are near or below Redfield's ratio. In contrast, N_2 fixation in estuaries is low even when the N:P ratio is low. In the more oxic oceanic water of estuaries, Fe and Mo are mainly in a state of high oxidation, and their availabilities are low, negatively affecting N_2 fixation by limiting nitrogenase capacity, which finally hinders the establishment of Redfield's ratio (Howarth et al. 1988).

The Redfield ratio has not been found in certain oceanic areas where the water circulation induces a phytoplanktonic bloom (Mei et al. 2005). These phenomena, however, are generally short-term, cyclic and local (Gismervik 1997; Körtzinger et al. 2001; Mei et al. 2005; Redfield et al. 1963). Most deviations from the ratio of 16:1, such as local variations within the range 5-34:1, have been observed mainly in coastal waters (Duarte et al. 2004; Geider and Roche 2002) where large variations in N:P ratios occur due to loadings from continental waters (Harrison et al. 2008; Li et al. 2010; Ruiz-Fernández et al. 2007). Several studies suggest a progressive adjustment of aquatic particulate matter to Redfield's ratio from coastal waters to open oceans (Doering et al. 1995; Downing 1997; Fox et al. 1985; Hopkinson and Vallino 2005; Lavrentyev et al. 1998). Most studies have thus observed a trend of approaching Redfield's ratio from the coast to the open ocean, despite the large variations in N:P concentration ratios in coastal waters, which are mainly linked to freshwater inputs of N and P from human activity (Slomp and Van Cappellen 2004; Yin and Harrison 2007) or linked to local sediments (Fox et al. 1985). The main causes of this trend, without disvaluing geophysical and geochemical contributions such as oceanic currents (Weber and Deutsch 2010), are biological processes, such as N_2 fixation, denitrification and the trade-off between competitive equilibrium and exponential growth (Gruber and Sarmiento 1997; Klausmeier et al. 2004; Lenton and Klausmeier 2007; Tyrrell 1999) (Figure 1 and Table 1).

Some studies suggest that the adsorption of P to particles (Sanudo-Wilhelmy et al. 2004) or organisms (Fu et al. 2005) can be very significant and should be considered in studies that test Redfield's ratio since they could explain certain deviations in results (Sanudo-Wilhelmy et al. 2004). These studies, however, have not been independently confirmed or refuted. Moreover,

the effect of P limitation in coastal areas could be currently underestimated on a global scale because such studies have frequently been conducted in relatively polluted coastal waters. The possible role of P in limiting primary production could be larger than currently thought (Downing et al. 1999).

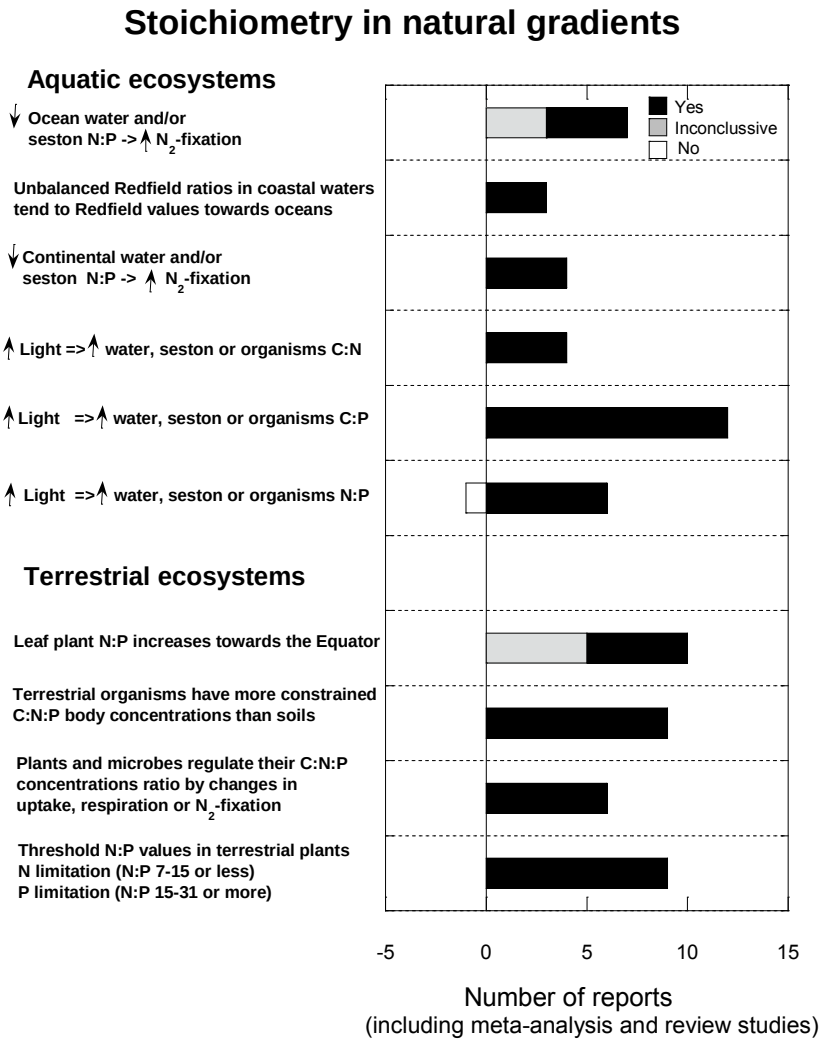

Figure 1. Number of reports in the literature supporting and not supporting the established statements on stoichiometric regularities in natural gradients in aquatic and terrestrial ecosystems (ISI Web of Knowledge review to November 2010). Detailed information for each study is reported in Table 1.

Most studies generally support Redfield's ratio in oceans as being the result of biogeochemical constraints: the trade-off between competitive equilibrium and growth and the buffer effect of N_2 fixers and denitrification. Several questions, though, remain. Fish and marine mammals represent a significant portion of the oceans' biomass, and yet general information on their C:N:P ratios and their role in Redfield ratios is lacking. Another question that remains to be answered conclusively in marine ecosystems is the relative contribution of an optimal biological function underlying the existence of the Redfield ratio. Moreover, the relationships between the tendency for general ecosystems to maintain constant C:N:P ratios and the plasticity of community species in time and space are not well known. Interest in these questions is growing

because human activity is threatening the C:N:P homeostasis of wide oceanic areas by loading continental water with C:N:P ratios that differ from Redfield's ratio (Harrison et al. 2008; Ruiz-Fernández et al. 2007). In any case, Redfield's ratio in marine ecosystems seems to be the result of the equilibrium between certain biogeochemical processes. Nevertheless, whether the ratio is purely a consequence of this equilibrium or whether the process is driven to reach the optimal C:N:P ratio still requires clarification. A recent mathematical model has suggested that the origin of Redfield's N:P = 16 could be the consequence of the most advantageous ratio of protein:RNA under optimal growth conditions for microbes and algae (Loladze and Elser 2011). This model opens a promising future line of research to investigate the feasibility of this link.

The available studies show that the causes that determine C:N:P ratios in freshwater are different, at least to some extent, from the causes in oceans. The lower volume of water in lakes compared to oceans means these ratios are more likely to be influenced by the particular traits of the surrounding environment such as rock type or human impact. These factors can strongly determine the traits of water chemistry, mainly through the differences in oxidizing power that affects C:N:P stoichiometry. In comparisons of C:N:P stoichiometry of particulate matter in lakes and oceans throughout the world, C:P and N:P ratios are more variable and generally higher in lake particles than in marine particles (Dobberfuhl and Elser 2000; Hecky et al. 1993). Quan and Falkowski (2009) have recently observed that $[O_2]$ in water is positively correlated with water-soluble inorganic N:P concentration ratios only below 100 □ in μM L^{-1}. At higher $[O_2]$, water-soluble inorganic N:P concentration ratios are highly variable. Moreover, a closer negative relationship between total N:total P ratio and total P content has been observed throughout the world's lakes (Sterner 2011), suggesting that despite the greater variability of N and P contents in lakes compared to oceans, N and P contents in lakes are somehow related. As an example, Lake Superior has been described as an "end member" of this relationship (Sterner 2011). The lake's large NO_3^- content, which has been continuously increasing during recent decades, cannot be solely explained by the increase in NO_3^- deposition from human activities (Finlay et al. 2007) but derives also from other processes. Most of the NO_3^- comes from nitrification within the lake (Finlay et al. 2007; Sterner et al. 2007). Also, the low productivity of this lake due to low P availability leads to a lack of organic C in the anoxic deep waters, which prevents denitrification (Sterner 2011).

A recent analysis of more than 2000 observations in aquatic ecosystems, including lakes, oceanic coastal and off-shore marine surface areas, has reported a C:N:P ratio of 166:20:1 across the entire data set (Sterner et al. 2008). This ratio implies higher C sequestration in surface waters than that observed for the Redfield ratio. These authors also observed that increasing sestonic abundance negatively correlated with sestonic C:N and C:P ratios at a regional scale in

both freshwater and marine habitats. This contrast between the global data set and subsets of global data indicates that values of C:N:P ratio are scale dependent, namely that Redfield-like constancy is a function of very large-scale systems, e.g. connecting oceanic basins (Sterner et al. 2008).

Despite the large variability of C:N:P ratios in continental waters and the resident organisms, an organism's capacity to adjust the C:N:P ratio to narrower ranges than those of their food sources has frequently been reported (Hall et al. 2005; Sterner and George 2000). This homeostatic capacity, though, is limited, and freshwater organisms change their C:P and N:P ratios in response to large changes in P availability (Cross et al. 2007; Elser et al. 2005; Fitter and Hillebrand 2009).

Light availability can also exert a significant effect on the C:N:P ratios of organisms and ecosystems by means of its effect on the capacity of C fixation. The light nutrient hypothesis (LNH) links light availability with the nutrient content of tissue producers (Diehl 2007; Urabe and Sterner 1996). High light intensity increases C:N and/or C:P ratios in planktonic communities (Andersen et al. 2007; Danger et al. 2008; Hessen et al. 2008a), lowering the nutritional quality of algae, limiting planktonic herbivore growth (Andersen et al. 2004; 2007; Jäger et al. 2008; Liess et al. 2009; Sterner et al. 1998; Urabe et al. 2002a; 2002b) and also increasing C:N and C:P ratios in sestonic and soluble materials (Dickman et al. 2006; Spears et al. 2008; Sterner et al. 1997) (Figure 1 and Table 1). Over longer timescales, grazers may ameliorate this effect through their impact on nutrient cycling, showing grazer-mediated shifts in producer composition (Hall et al. 2007; Urabe et al. 2002a).

Table 1. Stoichiometry regularities in natural gradients.

Variables studied	Medium, species and communities studied	Experimental type	Results	References
AQUATIC ECOSYSTEMS				
N_2 fixation when N:P concentration ratios of water or seston decrease in oceans				
N:P	N_2 fixing cyanobacteria	Estuaries	Not conclusive	Howarth et al. (1988)
N:P	Water and phytoplankton organisms	Ocean waters and culture studies	↑	Holl and Montoya (2008)
N:P	N_2 fixing organisms	Ocean water manipulation	↑	Karl and Letelier (2008)
N:P	*Cylindrospermopsis racioborskii*	Culture experiments	↑	Kenesi et al. (2009)
N:P	N_2 fixing and no-fixing phytoplankton organisms	Ocean waters	Not conclusive	Lenton and Klausmeier (2007)
N:P	N_2 fixing organisms	Oceans waters	Not conclusive, the	Lenton and Watson

			capacity depends on Fe availability	(2000)
N:P	Phytoplankton community	Mesocosm experiment	↑	Vrede et al. (2009)
N_2 fixation when N:P concentration ratios of water or seston decrease in freshwater ecosystems.				
N:P	N_2 fixing cianobacteria	Lakes	↑	Howarth et al. (1988)
N:P	N_2 fixing cianobacteria	Lakes	↑	Smith (1983)
N:P	Water and phytoplankton	Mesocosm simulating lake	↑	Vrede et al. (2009)
Unbalanced Redfield ratios of water, seston or organism in coastal areas progressively tend to reach Redfield ratios towards ocean.				
N:P	Water and seston	Simulated estuarine gradient	↑	Doering et al. (1995)
N:P	Water and seston	Mississippi estuary	↑	Fox et al. (1985)
C:N:P	Dissolvent organic matter	Transects from continental shelf to continental slope and central ocean areas	↑	Hopkinson and Vallino (2005)
N:P	Seston	Florida Bay	↑	Lavrentyev et al. (1998)
Light availability effects on N:P and C:P body concentration ratios				
C:P	Plankton	Review study	↑ C:P	Andersen et al. (2004)
C:P	Algal Selenastrum capricornutum	Laboratory	↑ C:P	Andersen et al. (2007)
C:P and C:N	Phytoplankton	Forest ponds	↑ C:P ↑ C:N	Cáceres et al. (2008)
C:P and C:N	Periphyton	Field (Lake)	↑ C:P ↑ C:N	Danger et al. (2008)
C:P, C:N and N:P	Phytoplankton	Field manipulation experiment (lake and reservoirs)	↑ C:P ↑ C:N ↑ N:P	Dickman et al. (2006)
C:P and C:N	Seston	Laboratory	↑ C:P ↑ C:N	Dickman et al. (2008)
C:P	Seston	Mesocosms	↑ C:P	Hall et al. (2004)
C:P	Phytoplankton	Lake enclosures	↑ N:P	Jäger et al. (2008)
N:P	Water dissolved fraction	Laboratory	↑ N:P	Leonardos and Geider (2004a)
N:P	*Chaetoceros mulleri*	Culture studies	↓ N:P	Leonardos and Geider (2004b)
C:P and N:P	Phytoplankton organisms	Stream channels	↑ N:P ↑ C:P	Liess et al. (2009)
C:P	Periphyton	Lake manipulation	↑ C:P	Qin et al. (2007)
N:P	Water dissolved fraction	Field (lake) and laboratory	↑ N:P	Spears et al. (2008)
C:P	Seston	Review metadata analysis	↑ C:P	Sterner et al. (1997)
C:P	Seston and plankton community	Indoor plankton towers	↑ C:P	Sterner et al. (1998)
C:P	Algal	Laboratory (aquatron)	↑ C:P	Urabe et al. (2002a)
C:P	Algal	Lake enclosures	↑ N:P	Urabe et al. (2002b)
TERRESTRIAL ECOSYSTEMS				

N:P leaf concentration ratios increase towards the Equator, and in general with increasing Mean Annual Temperature (MAT) and Mean Annual Precipitation (MAP).				
N:P	Leaf concentrations	Metaanalyses on China flora	No conclusive results	Han et al. (2005)
N:P	Leaf concentrations	Metaanalyses on China flora	No conclusive results	He et al. (2008)
N:P	Leaf concentrations	World scale concentrations metadata	↑	Kerkhoff et al. (2005)
N:P	Leaf concentrations	Study in Mangrove ecosystems of different latitudes	No conclusive results	Lovelock et al. (2007)
N:P	Leaf and litter concentrations	World scale concentrations metadata	↑	McGroddy et al. (2004)
N:P	Leaf concentrations	World scale concentrations metadata	↑	Ordoñez et al. (2009)
N:P	Leaf concentrations	World scale concentrations metadata	↑	Reich and Oleksyn (2004)
N:P	Leaf concentrations	Within tropical zone concentrations metadata	No conclusive results	Townsend et al. (2007)
N:P	Leaf concentrations	World scale concentrations metadata	↑	Yuan and Chen (2009)
N:P	Leaf concentration	Analysis of 126 in loess Plateau of China	No conclusive results	Zheng and Shangguan (2007)
Terrestrial organisms have more constrained C:N:P body concentration ratios than soil.				
N:P	Leaf	Community plant species across a soil gradient	↑	Castle and Neff (2009)
N:P	Leaf	Plant species mixtures growing at different nutrient supply	↑	Güsewell and Bollens (2003)
N:P	Leaf	Natural wetlands and greenhouse experiments	↑	Güsewell and Koerselman (2002)
N:P	Shots and roots	Grasses growing different nutrient supply	↑	Güsewell et al. (2005)
N:P	Insect	*Drosophila* spp.	↑	Jaenike and Markow (2003)
N:P	Microbes	Escherichia coli at growing at different C:N:P ratios	↑	Makino et al. (2003)
N:P	Insect	*Drosophila* spp.	↑	Markow et al. (1999)
N:P	Leaf	Community plant species across a soil gradient	↑	Neff et al. (2006)
N:P	Shot	Grasses growing different nutrient supply	↑	Shaver and Melillo (1984)
The leaf or plant N:P threshold values for N limitation are N:P < 14 or similar and for P limitation are N:P > 16 or similar. ↑ = agree.				
N:P	Plant biomass analyses	European lowland fens	↑ N limitation N:P < 8-15, P limitation are N:P > 23-31	Boeye et al. (1997)
N:P	Leaf analyses	Sarracenia purpurea plants in wetland environments.	↑ N limitation N:P < 14	Bott et al. (2008)

N:P	Plant analyses	*Trifolium repens* growth in soils at different N and P availability	↑ N limitation N:P < 10	Chen et al. (2010)
N:P	Plant analyses	Review and modelling study of crop plants	↑ N limitation N:P < 7, P limitation are N:P > 14	Greenwood et al. (2008)
N:P	Plant analyses	Review	↑ N limitation N:P < 10, P limitation are N:P > 20 in the most cases	Güsewell (2004)
N:P	Shoot biomass analyses	Different Dutch wetlands	↑ P limitation are N:P > 20	Güsewell et al. (2003a)
N:P	Plant analyses	Review study of wetland communities	↑ N limitation N:P < 14, P limitation are N:P > 16	Koerselman and Meuleman (1996)
N:P	Leaf analyses	Understory and forest vegetation. Experimental and review data	↑ N limitation N:P < 14, P limitation are N:P > 16	Tessier and Raynal (2003)
N:P	Leaf analyses	Herbaceous wet vegetation	↑ N limitation N:P <14, P limitation are N:P > 16	Verhoeven et al. (1996)
Buffer effect of N:P concentration ratios in terrestrial plants and ecosystems by N_2 fixation or by changes in N or P reabsorption and uptake.				
N:P	Prairie soils	Field study	↑ Soil N:P was negatively correlated with N_2 fixing	Eisele et al. (1989)
N and P	Shoot analyses	Increases of N deposition and N availability increases in different plant functional species	↑ N deposition increased P uptake	Phoenix et al. (2004)
N:P	Temperate rainforest	Different levels of P supply	↑ P availability decrease increased proficiency P resorption capacity	Richardson et al. (2008)
N and P	*Brachypodium pinnatum* and *Dactylis glomerata*	Different levels of N and P supply	↑ N and P availability increased P and N uptake capacity	Ryser and Lambers (1995)
N and P	Soil N content and N_2 fixing microbes	Review study	↑ Low N and high P soil contents increased N_2 soil fixation	Smith (1992)
N:P	*Alnus incata*, *Frankia* spp.	Different N and P supply	↑ Soil N:P was negatively correlated with N_2 fixing	Wall et al. (2000)
Buffer effect of terrestrial animals and microbes. The range of body N:P ratios is narrower in animals' body than in their food source.				
N:P	*Drosophila* species	Laboratory study	↑	Jaenike and Markow (2003)
C:P and N:P	*Escherichia coli*	Laboratory study	↑	Makino et al. (2003)

Terrestrial ecosystems

In contrast to marine ecosystems, terrestrial ecosystems are subject to wide variation in climate and soil bedrock, which strongly influence the elemental stoichiometry of both ecosystems and organisms. Several studies have investigated C:N, C:P and/or N:P biomass ratios

across climatic and environmental gradients (Figure 1 and Table 1). The temperature-related physiological process hypothesis predicts that most biogeochemical processes cause leaf N and P contents to increase monotonically with temperature around the globe (Kerkhoff et al. 2005). On the other hand, the soil substrate age hypothesis claims that older tropical soils might be more P-limited than younger soils of cooler regions due to the observed general trend of soils evolving from N-limiting to P-limiting over time (Chadwick et al. 1999; Walker and Syers 1976). Since P is derived primarily from the weathering of rock, ecosystems begin their existence with a fixed complement of P from which even very small losses cannot readily be replenished. Regions of high latitudes have experienced recent glaciations leaving a young landscape without soils. The formation of current soils has thus begun more recently in higher than in lower latitudes that have not been affected by recent glaciations. Regions at higher latitudes with young soils therefore have a greater capacity to release P from parental minerals than older soils with long-term meteorization of surface parental materials. Consequently, ecosystems with old soils can become P depleted. These assumptions were corroborated by Walker and Syers (1976) in studies of soil chronosequence in New Zealand. Over the last several million years, ecosystems at high latitudes have been systematically and cyclically rejuvenated by cyclic glaciations and related processes. In contrast, no glaciers have existed in the lowland tropics for hundreds of millions of years, and so a larger fraction of tropical sites could approach the Walker and Syers (1976) terminal state of P depletion (Vitousek et al. 2010). This hypothesis was thereafter applied to explain the results of several global-scale meta-analyses that have observed a general trend towards decreases in leaf and litterfall N and P contents and increases in N:P ratios as latitude decreases and mean annual temperatures (MAT), mean annual precipitation (MAP) and length of growing season increase (Kerkhoff et al. 2005; McGroddy et al. 2004; Ordoñez et al. 2009; Reich and Oleksyn 2004; Yuan and Chen 2009; among others, see Figure 1 and Table 1). In a recent metadata analysis of foliar N and P content of woody plants of the American continent, Elser et al. (2010) have observed a strong latitudinal gradient in P and a weaker gradient in N, with lower P content towards the equator.

Several studies of nutrient cycling and soil chemistry further confirm the increases in P limitation towards areas of tropical climate. A global dataset of 2800 observations shows that the rate of litter decomposition is primarily controlled by its nitrogen content, which affects rates of microbial activity (Manzoni et al. 2008). In this context, the high N:P ratio of litter in tropical ecosystems favors faster nutrient recycling and rapid uptake by plants, preventing nutrient losses by leaching out of the ecosystem (Lovelock et al. 2007; Ratnam et al. 2008). C:P ratios of leaf litter in tropical forests are the main factors controlling the rate of its decomposition (Kaspari and Yanoviak 2008; McGlynn et al. 2007). Observations of the nitrogen cycle over the past half

century indicate that intact tropical forests tend to accumulate and recycle large quantities of N compared to temperate forests. This difference is due to frequent P limitations, abundance of N_2-fixing plants and sustained export of bioavailable N at the ecosystem level (Hedin et al. 2009). Within the tropical zone, use efficiency of photosynthetic P and resorption efficiency of N and P decrease with increasing latitude, further confirming that low availability of P in the tropics has been an important selective pressure shaping the evolution of plant traits (Lovelock et al. 2007). In the secondary succession of Amazonian forests following agricultural abandonment, the soil N:P ratio is initially low but rapidly increases to values typical of mature tropical forests, and a conservative P cycle typical of mature lowland forest re-emerges. This behavior indicates an increase in mechanisms of P conservation and a decrease in mechanisms of N conservation with soil age (Davidson et al. 2007). In tropical ecosystems, a greater proficiency in the resorption of P is the main mechanism for plants to retain P in poor soils (Hättenschwiler et al. 2008; Richardson et al. 2008), and soils have still proved to develop high N:P loss ratios over time (Hedin et al. 2003). Also, carnivorous capacities in tropical plants have been suggested to be an adaptation to enhance P uptake in P-poor soils (Wakefield et al. 2005). Similarly to plants, *Escherichia coli* increases cellular organic C, C:P and N:P biomass ratios in response to increasing temperature by allocating an increased proportion of its P cell quota towards machinery of resource acquisition (membranes) (Cotner et al. 2006).

Not all studies, however, have detected general patterns of N:P ratios between and within the climatic areas (Vitousek et al. 2010). No relationships were observed between N:P ratios and either latitude or mean annual precipitation within tropical areas (Lovelock et al. 2007; Townsend et al. 2007), which is likely due to different regional N and P contents in the bedrock. For example, Tripathy et al. (2008) suggested that dry tropical ecosystems in India are more N-limited than P-limited in contrast to other tropical regions. Australia has soils very poor in P compared to those of other continents (Orians and Milewski 2007). He et al. (2008) investigated the changes in N:P ratios of 213 plant species across 199 research sites located on a climatic gradient. They observed that climatic variables had very little relationship to leaf P content and N:P ratios, with growing-season precipitation together with temperature explaining less than 2% of the variation. In contrast, inter-site differences and intra-site phylogenetic variation explained 55 and 26% of the total variation of leaf P and N:P ratios, respectively. A study based on a national data set including 753 plant species across China reported that leaf N and P contents increased with increasing latitude (decreasing mean annual temperature), but the N:P ratio did not show significant changes (Han et al. 2005). Contrary to the predictions of the soil age hypothesis, however, increases in leaf N:P ratios with latitude have been observed in a screening of 126 higher plants of the Loess Plateau in China (Zheng and Shangguan 2007). These observations might not be

comparable with the global data sets of Reich and Oleksyn (2004) because of the cold climate of the Tibetan Plateau and the extreme altitudinal and continental gradients of China. Although foliar N:P ratios tend to be higher towards the tropical areas, foliar N:P ratios are thus more variable within climate latitude areas due to soil and topographic variability.

Plant stoichiometry has lower levels of variation than soil and/or bedrock stoichiometry (Castle and Neff 2009; Neff et al. 2006). Several biogeochemical mechanisms are involved in this buffering capacity on N:P stoichiometry. Some of these processes are changes in plant N:P ratio uptake (Güsewell et al. 2003a; Phoenix et al. 2004; Ryser and Lambers 1995), in reabsorption (Ratnam et al. 2008; Richardson et al. 1999; 2008; Shaver and Melillo 1984) and in N_2 fixation (Eisele et al. 1989; Smith 1992; Wall et al. 2000), with all varying according to the N:P supply ratio in a form that tends to adjust the plant N:P ratio to an optimal value (Figure 1 and Table 1). Moreover, increases in N availability enhance P uptake capacity by stimulating root-surface phosphatase activities in all functional types of plants studied (Phoenix et al. 2004) or by increasing plant growth (Perring et al. 2008). Conversely, vegetation growing on soils with a low P content is less likely to sequester N in additional production of biomass (Britton and Fisher 2007). In terrestrial ecosystems, C:N:P ratios of plants thus change significantly when the availability of one or more of these elements changes due to differences in soil C:N:P ratios. This is especially so where some elements are limiting, such as observed in studies of fertilization (Esmeijer-Liu et al. 2009; Feller et al. 2007), but overall C:N:P stoichiometry tends to be adjusted to a narrow optimal value related to optimal production capacity.

All these previous issues lead to the question of whether terrestrial plants have optimal N:P ratios, similar to Redfield's ratio from phytoplankton in oceans. Studies based on theoretical considerations and on experiments in plant nutrition generally suggest that terrestrial plants require nutrients in the same ratios as planktonic organisms (Knecht and Göransson 2004). Several studies with different plant types, communities and taxon have shown that N is limited when the N:P ratio is < 14, and P is limited when the ratio is > 16 (Boeye et al. 1997; Bott et al. 2008; Güsewell and Bollens 2003; Koerselman and Meuleman 1996; Verhoeven et al. 1996) (Figure 1 and Table 1). More recently, Güsewell (2004) proposed a broader range of ratios for co-limitation of plant communities stating that N is limiting when the N:P ratio is < 10, and P is limiting when N:P is > 20. Several studies conducted in agronomics and natural ecosystems further confirm that biomass growth is constrained by N:P leaf ratios (Das et al. 2006; Van Duren and Pegtel 2000; Vitousek and Howarth 1991). Most reviews of studies of fertilization have supported the use of threshold ratios to predict N or P limitation (Greenwood et al. 2008; Tessier and Raynal 2003). When studying several different groups of plants together, leaf N:P ratios range from 5-65 with a mean of 28-30 (Elser et al. 2000b; 2010). Despite these findings, a few

studies have reported that N:P values of terrestrial plants present greater variability than plankton and other ecological groups such as microbial communities in soil. Margaris et al. (1984) have observed that on average the leaf N:P ratio differs markedly, being on average 45 in Australian Mediterranean plants and 8.2 in Chilean Mediterranean plants, coinciding with the gradient of soil and rock P content in Mediterranean areas, with intermediate values in Californian and Mediterranean basin plants (Margaris et al. 1984).

Minimum and maximum threshold values of N:P ratios are apparent. Lower N:P ratio values can have a minimum threshold due to P "toxic" effects. Greater P availability increases the possibility of Zn deficiency (Loneragan et al. 1979; 1981), and higher N:P ratios reach a maximum as observed by Bragazza et al. (2004) in a study of N deposition gradient in Northern Europe, probably due to an effect of P limitation. Thus, although some studies have observed wider ranges of N:P ratios where N and P are co-limiting (Ågren 2008; Boeye et al. 1997) or have found weaker relationships between site N:P ratios and response to fertilization (Craine et al. 2008; Güsewell et al. 2003b), the trends towards an average optimal range of the leaf N:P ratio have strong experimental support. When comparing the N:P ratio in area basis at the community level (g m^{-2}) of several vegetal types, the observed change in the N:P ratio was not related to the amount of standing biomass. This observation suggests that different vegetal types, such as forest, shrubland and grassland, do not differ in N:P ratio at the community level (Kerkhoff et al. 2005), similar to the observations of Klausmeier et al. (2004) in marine ecosystems.

Although the data in the literature suggest a possible trend in terrestrial vegetation towards a certain narrow C:N:P ratio, much data is in disaccord. In contrast to aquatic ecosystems where water is not limiting, terrestrial ecosystems strongly differ in their levels of availability of water. This difference implies very different physiological responses and adaptations between plants of different climatic ecosystems, which very probably affect their C:N:P allocation. In drier environments, the allocation of C:N:P within and between plant tissues should be strongly focused on enhancing the efficient use of water, whereas in wet environments, the efficient use of water should be less important, and C:N:P allocation could be more directly related to growth rate and competitive efficiency.

Comparing stoichiometry in different taxon and trophic levels.

When comparing the C:N:P stoichiometry of terrestrial and aquatic plants, C:P and C:N ratios are higher in terrestrial plants than in aquatic plants, reflecting the greater allocation to low-nutrient structural organs. Foliar elements, including nutrients, micronutrients and trace elements, have been found to be different among the dominant species of community plants in Mediterranean forests and shrublands and characterize a different biogeochemical niche for each

species (Peñuelas et al. 2008). This differentiation into biogeochemical niches could be a mechanism to avoid interspecific competition. Despite species-specific differences within communities, N:P ratios are, on average, similar in aquatic and terrestrial plants (Elser et al. 2000b). The N:P ratio is less variable than C:N and C:P ratios but is also variable among several ecological and functional plant groups. Herbaceous plants have lower N:P ratios than woody plants (An and Shangguan 2010; Kerkhoff and Enquist 2006; Ratnam et al. 2008), and graminoids have higher ratios than do forbs (Güsewell 2004). Differences in N:P ratios have also been observed between sprouting and non-sprouting Mediterranean species (Saura-Mas and Lloret 2009); between polyploid and non-polyploid ferns (Amatangelo and Vitousek 2008); among stress tolerators, competitors and ruderal plant types (Güsewell 2004; Willby et al. 2001); among different herbaceous crops (Sadras 2006); between leguminous and non-leguminous trees (Powers and Tiffin 2010) and among different functional groups of trees (Ratnam et al. 2008). For example, the N:P ratios in green leaves on the African savanna were lowest for grasses (8.6), intermediate for broad-leaved trees (13.7) and highest for fine-leaved trees (18.0) (Ratnam et al. 2008). The N:P ratio was highest in legumes, lowest in oilseed crops and intermediate in cereals, as observed by Sadras (2006) in a meta-analytical study. When comparing the N:P ratio of whole communities of several vegetal types, the observed changes in N:P ratios were not related to the amount of standing biomass, thus suggesting that forests, shrublands and grasslands do not differ in N:P ratio at the community level (Kerkhoff et al. 2005). Similar observations were made by Klausmeier et al. (2004) in marine ecosystems.

As in plants, animals and microbes show lower variation in C:N:P stoichiometry than their food sources. Studies of *Drosophila*-host stoichiometry are a good example (Jaenike and Markow 2003; Markow et al. 1999). Aquatic invertebrates have a more homeostatic C:P body ratio than terrestrial invertebrates (Persson et al. 2010). Makino et al. (2003) observed that *Escherichia coli* has only two-fold variations in biomass C:P and N:P ratios when the supply C:P and N:P ratios varied by two orders of magnitude, suggesting the existence of homeostatic regulation of the cellular C:N:P ratio. Some contrary results, though, have also been reported. Chrzanowski et al. (2010) have recently observed that a two-fold variation in the range in the N:P stoichiometry of the bacterial prey *Pseudomonas fluorescens* translates to a three-fold variation in the range in the bacterivorous *Ochromonas danica* feeding on it. Some studies of terrestrial ecosystems suggest that microbial community N:P ratios are related to plant community and soil N:P ratios (Cleveland and Liptzin 2007; Kerkhoff and Enquist 2006). Close relationships between microbial and soil C:N ratios across a soil C:N gradient have been also observed, for example in Scandinavian boreal forests (Högberg et al. 2006). Some studies indicate that microbial enzymes adjust their activities to mineralize C, N and P in a proportion to adequately maintain optimal

body C:N:P ratios and growth (Högberg et al. 2006). In microbes, each heterotrophic bacterial strain and phylogenetic group regulates its elemental composition homeostatically within a relatively narrow range, but when different bacterial strains and phylogenetic groups are compared, different C:N:P ratios are observed (Cleveland and Liptzin 2007; Makino et al. 2003). On a global scale, the average C:N:P ratio is 186:13:1 in soil and 60:7:1 in microbial communities (Cleveland and Liptzin 2007). The available data, though, suggest that a trend towards an optimal C:N:P stoichiometry in soil microbial decomposer communities is not yet clear at the global scale. Further studies are thus necessary in this field to obtain a more definitive understanding.

The fate of C in organisms, food webs and ecosystems is to a major extent regulated by principles of mass-balance and the availability of key nutrients. Comparing different trophic levels, autotrophs and decomposers have flexible but generally high C:nutrient ratios, and consumers have lower C:nutrient ratios and tighter stoichiometric regulation. Nutrient stoichiometries may also help us to understand the evolution of diet through trophic levels. Animals frequently have a lower C:N ratio than their food sources because much of the assimilated C is used as an energy source. Comparing C:N in plants and in arthropod herbivores and predators in terrestrial ecosystems, $C:N_{plants} >> C:N_{herbivores} > C:N_{predators}$. Higher organisms have more allocation of nutrients to structural organs (skeleton, wood), which can be poorer in N. A similar tendency has been observed for C:P ratios (Fagan et al. 2002; Sterner et al. 1998; Zhang and Han 2010). Nonetheless, the efficiency of C use (the proportion of C used in biological functions such as growth or respiration relative to the total C, including C in structural organs and storage) in food webs may increase when C:element ratios in food approaches those of consumers. This tendency influences the capacity of sequestration of C in the ecosystem, since more C will be diverted to detritus in soils and sediments when the efficiency of C use is low due to a stoichiometric imbalance (Hessen et al. 2004). Moreover, the N:P body ratio of heterotrophs is more homeostatic than that of autotrophs, despite the variation between different heterotrophic taxonomic groups (Persson et al. 2010). This greater variation in autotrophs is due to the variability of nutrient richness in habitats (Bridgham et al. 1995), the autotrophic turnover rate (Sterner 1995) and the balance between light and nutrients (Sterner et al. 1997). Moreover, some important groups of heterotrophs such as bacteria and fungi can absorb N and P from organic and also from mineral sources, an ability that endows these groups with more possibilities to control their body C:N:P stoichiometry (Manzoni et al. 2008). Furthermore, predators, which are usually nitrogen-limited, can increase their nitrogen consumption by expanding their diet to include other N-rich predators, suggesting that the divergence in nutrient stoichiometries between herbivores and predators is an important factor in the evolution of omnivores (Denno and Fagan 2003). Endotherms need plant material with a higher C:N ratio than

do ectotherms because they have much higher requirements for carbon in order to maintain their higher metabolic energy (Klaassen and Nolet 2008).

In aquatic ecosystems, bacteria are frequently the richest organisms in nutrients, followed by fish, invertebrate predators, invertebrate herbivores, primary producers and fungi (Cross et al. 2005; Christian et al. 2008), with a similar tendency to have scaling trophic webs with changing C:nutrients ratios like those observed in terrestrial ecosystems (Sterner et al. 1998). Within trophic levels, P limitation is equally strong in freshwater, marine and terrestrial ecosystems, as observed in a review of experimental studies of N and P enrichment (Elser and Hamilton 2007). In native forests, both foliage and litter N:P ratios are more variable than in marine particulate matter when comparing different forest biomes (tropical, temperate broadleaf and temperate coniferous). Within each forest biome, though, the ratios were as constrained as marine ratios (McGroddy et al. 2004). The available data thus suggests that C:nutrient ratios tend to be lower and narrower in higher trophic levels of the web. Contrary to some prevailing paradigms, aquatic and terrestrial ecosystems can have quite similar levels of N and P limitation.

The phylogenetic relationships of N:P ratios, however, remain inconclusive. Whereas some studies have not found phylogenetic relationships (Broadley et al. 2004; Peñuelas et al. 2010), others have detected significant relationships (He et al. 2006). Perhaps no clear phylogenetic relationships with N:P ratios have been observed in previous studies because such relationships only matter at some spatial scales but not at others, and studies have differed in their scales of inquiry. More studies on this topic are necessary to understand the degree to which N:P body composition is related to genetic evolution.

Elemental stoichiometric relationships with species traits and community structure.

Growth rate hypothesis

Several studies have linked elemental stoichiometry to species traits and community structure. The hypothesis that has occupied the central role in studies of ecological stoichiometry has been the growth rate hypothesis (GRH). The GRH proposes that elevated growth rates are linked to elevated demands for P for the synthesis of P-rich ribosomal RNA (rRNA) (Elser et al. 2000a; 2000b; 2000c; Sterner and Elser 2002). The principle is that organisms must increase their allocation to P-rich rRNA in order to meet the elevated demand for protein synthesis required for rapid growth. N:P ratio and growth rate are thus linked via the intimate connections between P allocation to ribosomes and N allocation to protein synthesis (Hessen et al. 2007). Greater allocation to P-rich rRNA is possible under low N:P environmental ratios. These conditions allow high growth rates and thus favor species with higher growth rates (Main et al. 1997).

Andersen and Hessen (1991) observed that lakes with four dominant trophic levels (phytoplankton, zooplankton, planktivorous fish and piscivorous fish) are generally dominated by large-bodied zooplankton and the P-rich (low N:P ratio) crustacean *Daphnia*, whereas lakes with three dominant trophic levels (lacking piscivores) are dominated by low-P (high N:P ratios) zooplankton (Sterner et al. 1992). High algal C:P ratios inhibited rather than stimulated the growth of zooplankton (Sterner et al. 1998). These characteristics have also been observed in freshwater planktonic communities (DeMott et al. 2004; Makino et al. 2002). The GRH was then proposed to explain these differences in the limitation of nutrients in zooplankton and its relation to the differences in ecosystem structure (Elser et al. 1996; Elser and Hamilton 2007; Sterner and Elser 2002).

Several studies have also observed this relationship between growth rate and N:P ratios in diverse unicellular organisms (Karpinets et al. 2006), zooplankton (Carrillo et al. 2001; Hessen et al. 2007; Main et al. 1997) and fish (Pilati and Vanni 2007; Tanner et al. 2000). Decreases in sestonic N:P ratios lowered the N:P ratio in phytoplankton and enhanced growth in zooplankton due to the high nutritional quality of phytoplankton (Elser et al. 2000a; 2001; Hessen et al. 2007; Jeyasingh and Weider 2005), thus supporting the GRH.

Further ramifications of the GRH propose that higher growth rate is related to intergenic spacer length in ribosomal DNA (rDNA) (Gorokhova et al. 2002; Weider et al. 2004; 2005). Some studies have also observed an increased demand for phosphorus in organisms with high rRNA content, from microbes to metazoans (Makino et al. 2003; Weider et al. 2005). Other studies have observed a link between genome size and the RNA:DNA content ratio. A small genome may be an evolutionary consequence of allocation from DNA to RNA under P deficiency to maximize growth rate. This hypothesis has received experimental support when tested in different zooplanktonic groups such as *Caenis* spp. and *Daphnia* spp. (Frost and Elser 2002; Hessen et al. 2008b; Weider et al. 2004). The link between high RNA:DNA ratio and high growth rate has recently been observed in higher plants (Reef et al. 2010). The connection between an organisms' growth rate and the allocation to P-rich rRNA supports the proposed stoichiometric bridge between ecosystem ecology and evolutionary biology (Elser 2006). Moreover, changes in food quality in zooplanktonic species affect the reproductive output, neonate body size and body C:N:P stoichiometry (Frost et al. 2010). *Daphnia* mothers consuming P-poor algae produced smaller neonates with higher C:P ratios. Also, neonates from P-stressed mothers fed on P-poor algae grew slower and had delayed reproduction compared to neonates from non-stressed mothers (Frost et al. 2010), showing a further effect of food C:N:P stoichiometry across generations.

Less information about the GRH is available for marine ecosystems. We have already mentioned the work of Sterner et al. (2008). Their analysis included more than 2000 observations of particulate matter from large lakes and near- and off-shore oceanic waters and showed that sestonic abundance negatively correlates with sestonic C:N and C:P ratios, indicating scale-dependent values of the C:N:P ratio. In accordance with these observations, the narrow C:N:P Redfield ratios observed in several oceanic regions could occur because of the narrow range of sestonic content in several oceanic areas (Sterner et al. 2008). The GRH probably thus applies similarly to open oceans as to other ecosystems, being valid mostly for small organisms in optimal conditions for growth.

Several studies also suggest that the observations made in aquatic ecosystems can be extended to terrestrial ecosystems. Elser et al. (2000b) observed that terrestrial food webs are built on an extremely nutrient-poor autotrophic base with C:P and C:N ratios higher than in lake particulate matter, although the N:P ratios are nearly identical. Other studies have also detected links between lower body N:P stoichiometry and higher growth rates in terrestrial plants (Cernusak et al. 2010; Elser et al. 2003; Niinemets and Kull 2003; Niklas and Cobb 2005; Zhang and Han 2010) and also in terrestrial animals (Apple et al. 2009; Elser 2006; Kay et al. 2006; Visanuvimol and Bertram 2010). Terrestrial herbivores (insects) and their freshwater counterparts (zooplankton) are nutrient rich and indistinguishable in C:N:P stoichiometry. Frequent relationships between body C:P and N:P ratios and growth rate have been reported in insects (Hambäck et al. 2009; Schneider et al. 2010; Watts et al. 2006; Woods et al. 2004). An extensive study of European wetlands has observed that stress-tolerant plants (slow growth) have consistently lower nutrient contents and higher N:P ratios than ruderal species (fast growth) (Willby et al. 2001). This result is important and suggests a general link between N:P ratio and species' lifestyle in terrestrial plants and warrants future studies in other groups of organisms.

Several studies have reported a relationship between large body size and low N:P body ratio (Méndez and Karlsson 2005), but several other studies have found no clear relationships between body size and N:P ratio (Bertram et al. 2008; Dantas and Attayde 2007; Martinson et al. 2008). Smaller insects frequently have a higher relative growth rate and lower C:P and N:P ratios than larger insects in accordance with the GRH (Elser et al. 2006). General relationships cannot therefore be established between body size and body N:P ratio. As previously postulated by Elser et al. (2000a; 2000b), lower N:P ratios and environments with greater P availability do not directly favor species with larger body sizes but rather favor species with higher relative growth rates irrespective of their adult body size.

Furthermore, some available data are not consistent with the GRH, thus preventing its generalization to all situations and types of organism. An issue has been raised by Vrede et al.

(1999; 2004). They reported that at high growth rates, body stoichiometry is mainly constrained to low N:P ratio, but at low growth rates, N:P stoichiometry can differ substantially as a consequence of various patterns of allocation to RNA, protein and other potential biochemical constituents. This absence of relationship between growth rate and N:P ratio should be particularly apparent in nutrient-poor aquatic ecosystems. For example, when N is limited, the N:P body ratio is not correlated with growth rate in *Daphnia* spp (Acharya et al. 2004). Elser et al. (2003) have observed that the relationships among organism P content, P-rich rRNA and growth rates are relaxed when P is limiting for growth in microbes, insects and crustaceans. Moreover, fish adapted to P-deficient water can have higher fitness when eating zooplankton with high rather than with low C:P ratios (Schoo et al. 2010). In accordance with these studies and despite the large number of studies that have observed results supporting GRH in freshwater ecosystems, some reports suggest that the GRH can fail or does not fully apply in certain cases, often when N and P are extremely limiting. In environments where one of these nutrients is limiting, a greater homeostatic capacity to adjust body N:P ratio should be particularly desirable, as has been suggested by Sterner and Elser (2002). Under such conditions, body N and P should be allocated preferentially to physiological mechanisms such as excretion, anti-stress mechanisms and/or storage and allocated less to growth-related anabolism. Some studies have observed no relationships between the source of P or body N:P composition and zooplanktonic growth rate under these conditions (DeMott and Pape 2005; Hall et al. 2004; Hessen et al. 2007) (Figure 2, Table 2).

A second general concern about generalizing the GRH is illustrated by the fact that the C:N:P ratio of food is not the unique variable that explains the food's quality. The different allocations of N and P to different molecules and structures are also important (Anderson et al. 2004). *Daphnia* sp. growth has been found to be inversely correlated to body C:P ratio but not to the supply of P, showing that factors such as differences in digestion resistance of phytoplankton can preclude a direct translation of a greater availability of P to a higher capture of P through trophic chains (DeMott and Tessier 2002; Ferrao Filho et al. 2005). Minor changes in the C:N:P composition of herbivores can be associated with significant changes in the nutritional quality of their prey (changes in allocation to defensive tissues or toxins, storage allocation) that can have a disproportionate effect on the efficiency of growth of the predator (Mitra and Flynn 2005). Some studies have observed that both algal C:N:P ratios and polyunsaturated fatty acids (PUFA) are both important variables of food quality for zooplanktonic growth (Ferrão Filho et al. 2003; Gulati and DeMott 1997; Park et al. 2002; Saikia and Nandi 2010). Although some links have been observed between the C:N:P ratios and the PUFA content in different algae, they are not fully correlated (Gladyshev et al. 2007; Viso and Marty 1993) and change in different proportions in

response to environmental changes (Leu et al. 2007). Similar C:N:P ratios of organisms can thus be linked to different molecular structures and nutritional qualities of the organisms.

A third issue stems from the fact that rRNA is not always the most significant sink of P, especially in large organisms. The larger an organism, the less P is allocated to rRNA (Gillooly et al. 2005). As a consequence, the linkage between N:P ratio, protein-rRNA content and growth rate is weaker. Organs such as the skeleton in fish store large amounts of P (Hendrixson et al. 2007), making the GRH less applicable in its current format. Also, the stoichiometry of food in freshwater ecosystems can affect other biological functions with ecological implications apart from just growth rate. Other traits of species such as respiration rate (Hall et al. 2004), herbivore digestion capacity (Anderson et al. 2004), digestion resistance of food (Ferrao-Filho et al. 2005), investment in reproduction (Færøvig and Hessen 2003; Ventura and Catalan 2005), immune function (Frost et al. 2008a; 2008b), skeleton building in fish (Hendrixson et al. 2007) and investment in storage tissues and N-rich chemical defenses such as some N-rich alkaloids like nicotine (Raubenheimer et al. 2009; van der Stap et al. 2007) must also be considered in studies of the causes and consequences of C:N:P ratios in organisms and their sources of food. If some of these traits are different in two different organisms of the same trophic level, they may need resources with a different C:N:P ratio (Hall et al. 2004).

Similarly, some recent studies of terrestrial ecosystems do not fully support the GRH. For example, when studying temperate *Pinus* spp., no clear relationship between protein:RNA ratios and N:P ratios was observed when comparing leaves of the same species growing at low and high rates (Matzek and Vitousek 2009). These authors found that the faster-growing plants had higher RNA contents, higher N and P contents and lower protein:RNA ratios, but not consistently lower N:P ratios. Similarly, other recent experiments have reported higher growth rates at high N and P contents but inconsistent growth rate changes in relation with the N:P ratio in mycorryzal plants (Chen et al. 2010; Lawniczak et al. 2009). Several possibilities can explain why P content is not greater in fast growing plants; for instance, unlike zooplankton, terrestrial plants can store P in vacuoles, allocate N to the production of chemical defenses (Peñuelas and Sardans 2009a) or invest different N:P ratios in vegetative and in reproductive organs (Méndez and Karlsson 2005).

All these results suggest that to reach a more global and comprehensive understanding of the role of N:P stoichiometry in all types of ecosystems and under different scenarios of resource availability, we must consider more than just the allocation of N and P to growth. Further assessments of the GRH will thus require many more studies of the effects of C:N:P ratios on the ratios of different metabolic products. Only a few studies to date have coupled stoichiometry with molecular composition. P and N form part of several compounds related to growth, but also of others related to storage, defense and/or reproduction. The emerging approach of

metabolomics (Peñuelas and Sardans 2009b) constitutes a way to go forward. By coupling ecological stoichiometry studies to metabolomic studies, we can discern whether the change in N:P ratios is due to changes in defensive, storage, anabolic (growth) or catabolic (respiration) allocation. Improved analytical methods and computerized interpretation of large data sets have transformed the task of determining and quantifying more compounds — carbohydrates, amino acids and peptides, lipids, phenolics, terpenoids, alkaloids and so on — and associating them with their corresponding elemental stoichiometries. Such research will improve our understanding of the coupling between different levels of biological organization, from cellular metabolism to the structure of ecosystems and nutrient cycling. We need to integrate the analysis of the pools and flows of chemical elements and energy studied in ecosystem ecology with assessments of genetic fitness and the biochemical products of the genome considered in evolutionary ecology (Peñuelas and Sardans 2009a).

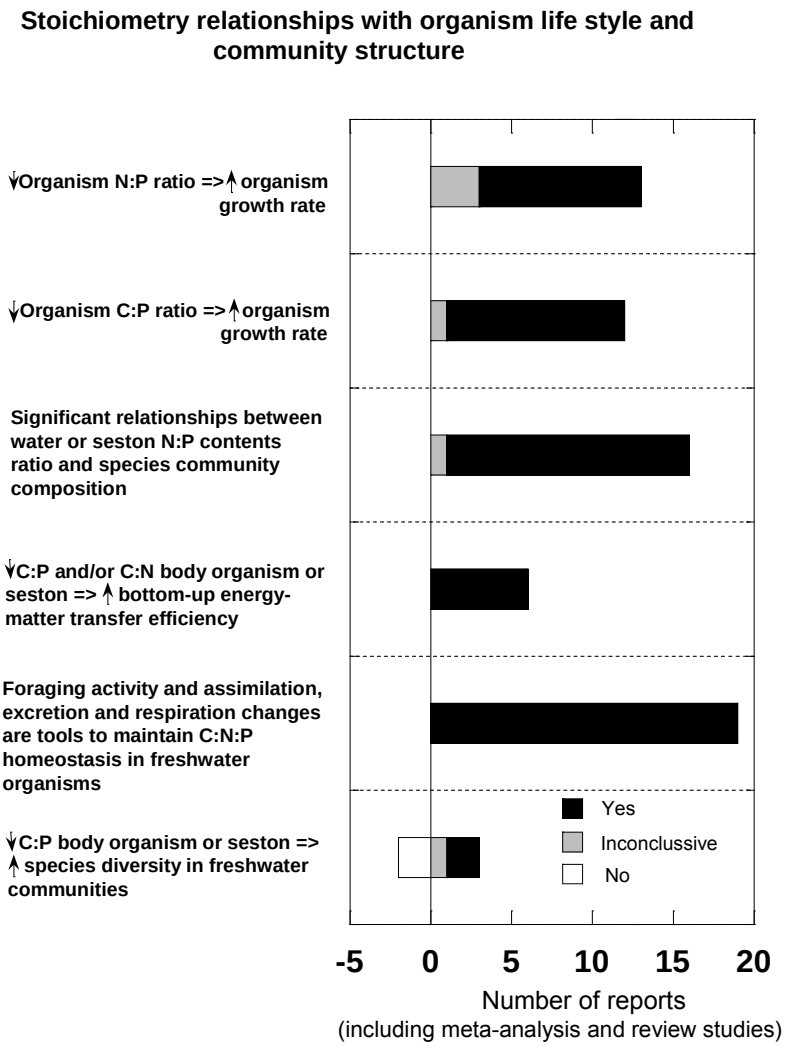

Figure 2. Number of reports in the literature supporting and not supporting the established statements on the relationship of stoichiometry to species growth rate and lifestyle and community structure. (ISI Web of Knowledge review to November 2010). Detailed information for each study is reported in Table 2.

All in all, the overview of the current reports on the GRH shows that N:P ratios exert a determining role in small organisms under non-limiting availability of resources. When organisms are large, though, and allocate P to structural organs and more complex functions rather than to rRNA, and when resources become limiting and the allocation to functions other than growth become more relevant (Peñuelas and Estiarte 1998), the GRH is less applicable to whole organisms. Sufficient results, however, suggest that in some moments in life (Pilati and Vanni 2007) and/or in certain organs directly related to the capacity for growth, such as leaves in plants, N:P ratio and growth rate could be linked to predictions of the GRH (Elser et al. 2003; Niklas et al. 2005).

Table 2. Stoichiometry in relation to species' growth rate and style of life, and community and ecosystem structure

Variables studied	Medium, species and communities studied	Experimental type	Results	References
Lower N:P and/or C:P body or seston concentration ratios are related to higher growth rates				
N:P and C:P	*Daphnia galeata, Daphnia pulicaria*	Laboratory	↓ N:P => ↑ GR ↓ C:P => ↑ GR Except when N was limiting	Acharya et al. (2004)
N:P	*Mixodiaptomus laciniatus*	Field (lake)	↓ N:P => ↑ GR	Carrillo et al. (2001)
C:P	*Daphnia* sp.	Laboratory	Not conclusive	DeMott and Pape (2005)
C:P	*Daphnia* sp	Field (lake)	↓ C:P => ↓ GR	DeMott and Tessier (2002)
N:P and C:P	*Daphnia pulicaria*	Field (lakes)	↓ N:P => ↑ GR ↓ C:P => ↑ GR	Elser et al. (2000a)
C:P	*Daphnia sp*, *Ceriodaphnia reticulata*	Laboratory	Not conclusive	Ferrao-Filho et al. (2005)
C:P	*Daphnia* sp	Laboratory	↓ C:P => ↑ GR	Ferrao-Filho et al. (2007)
C:P and N:P	Micoalgae	Review	Not conclussive	Flynn et al. (2010)
C:P	*Pasteuria ramosa* (parasite), *Daphnia magna* (host)	Laboratory	↓ N:P => ↑ GR	Frost et al. (2008b)
C:P	*Daphnia magna*	Laboratory	↓ C:P => ↑ GR	Frost et al. (2010)
C:P	*Daphnia* sp	Laboratory	Not conclussive	Hall et al. (2004)
C:P	*Selenastrum capricornutim, Daphnia magna*	Laboratory	↓ C:P => ↑ GR	Hessen et al. (2002)
N:P and C:P	*Brachionus calyciflorus* (rotifer), *Selenastrum capricornutum* (algal)	Laboratory	↑ [P] => ↑ GR Not conclusive	Hessen et al. (2007)
C:P	Cladocerans and copepod comparison	Review	↓ C:P => ↑ GR	Hessen et al. (2008b)
N:P and C:P	Diatom spp.	Circular stream channels	↓ N:P => ↑ GR ↓ C:P => ↑ GR	Liess et al. (2009)

N:P	*Daphnia* sp.	Laboratory	↓ N:P => ↑ GR	Main et al. (1997)
C:P and N:P	Bacteria community	Laboratory	↓ N:P => ↑ GR ↓ C:P => ↑ GR Only at low [P]	Makino and Cotner (2004)
C:P	*Daphnia* sp	Laboratory	↓ C:P => ↑ GR	Seidendorf et al. (2010)
C:P	*Brachionus calyciflorus*	Laboratory	↓ C:P => ↑ GR	Strojsova et al. (2008)
N:P	Fishes species	Field (lakes)	↓ N:P => ↑ GR	Tanner et al. (2000)
N:P and C:P	*Spirogyra fluviatilis*	Field (river)	↓ N:P => ↑ GR ↓ C:P => ↑ GR	Townsend et al. (2008)
C:P	*Daphnia magna*	Laboratory	↓ C:P => ↑ GR	Urabe et al. (1997)
C:P	*Daphnia* sp	Laboratory	Not conclusive	Van Geest et al. (2007)
N:P and C:P	*Mixodiaptomus laciniatus*	Field (lake)	↓ N:P => ↑ GR ↓ C:P => ↑ GR In different moments of copepod life	Villar-Argaiz et al. (2002)
Significant relation (↑) between species community composition and water or seston N:P concentration ratio				
N:P in water dissolved fraction	Bacterial and algal comunities	Field manipulation (lake)	↑	Carrillo et al. (2008)
N:P in water dissolved fraction	Zooplankton community	Field (water reservoir)	↑	Conde-Porcuna et al. (2002)
N:P gradient in water (dissolved fraction)	Phytoplankton and bacteria communities	Laboratory	↑	Danger et al. (2007)
N:P of zooplankton community	Zooplankton community	Field (lakes)	↑	Dobberfuhl and Elser (2000)
Body C:P and N:P	Lake community	Field (lakes)	↑	Elser et al. (2000a)
N:P in water dissolved fraction	Diatoms and cianobacteria	Field manipulation	↑	Elser et al. (2005)
C:P and N:P of dissolved and suspended factors	Zooplankton community	Field (lakes)	↑	Findlay et al. (2005)
Supply C:P	*Daphnia* sp	Laboratory	↑	Hall et al. (2004)
Seston C:P and N:P	Zooplankton community	Field (lakes)	↑	Hassett et al. (1997)
N:P gradient in water (dissolved fraction) and periphyton	Lake food web structure	Lake enclosure	↑	Liess et al. (2006)
N:P in Phytoplankton	Phytoplankton community	Field (lake)	↑	Makarewicz et al. (1998)
C:P manipulation by fertilization	Plankton community	Field (lakes)	Not conclusive	Persson et al. (2008)
N:P and C:P changes by pollution	Invertebrate community	Natural gradient of pollution in a river	↑	Singer and Battin (2007)
Epilimnetic N:P	Blue-green algal dominance	Data of 17 lakes throughout the world	↑	Smith (1983)
N:P body composition	Lake food web structure	Review of field experiments	↑	Sterner et al. (1992)
N:P in water dissolved fraction	Cyanobacteria, bacterial and algal comunities	Field manipulation (lake)	↑	Teubner et al. (2003)
N:P in total water column	Lake food web structure	Lake enclosure	↑ By changes in fish and large zooplankton abundance	Vanni et al. (1997)
N:P in water dissolved fraction	Phytoplankton community	Mesocosm	↑	Vrede et al. (2009)
Decreases of C:P and/or C:N increases bottom-up energy-matter transfer efficiency (TE) in trophic web				
C:P and C:N		Review study	↓ C:P => ↑ TE ↓ C:N => ↑ TE	Boersma et al. (2008)

C:P	Lake trophic web (*Scenedesmus obliquus*), primary consumer (*Daphnia magna*) and larval of rainbow trout (*Oncorhynchus mykiss*	Field manipulation (lake)	↓ C:P => ↑ TE	Boersma et al. (2009)
C:N and C:P	Detritus based stream food web	Field (monitorized head streams)	↓ N:P => ↑ TE ↓ C:P => ↑ TE	Cross et al. (2007)
N:P	Plankton community	Field (lake)	↓ N:P => ↑ TE	Elser and Foster (1998)
C:P	Lake web structure	Field manipulation (lake)	↓ C:P => ↑ TE	Elser et al. (1998)
C:P	Periphyton snails (*Elimia livescens*) web structure	Laboratori	↓ C:P => ↑ TE Only at low food quantity	Stelzer and Lamberti (2002)
Assimilation, excretion and/or respiration adaptations are tools to maintain C:N:P homeostasis in organisms. Effects on trophic web bottom-up and top-down relationships of freshwater ecosystems.				
C:N:P	Diverse organisms	Review	↑ (Excretion, respiration)	Anderson et al. (2005)
C:P	Body composition ratios of different freshwater organisms	Review study	↑ (Excretion)	Boersma and Elser (2006)
C:P	Algae and *Daphnia*	Field study and laboratory	↑ (Absorption)	Boersma and Wiltshire (2006)
C:N, C:P and N:P	Mussels	Field (streams)	↑ (Excretion)	Christian et al. (2008)
C:P	*Daphnia magna*	Laboratory	↑ (Excretion, respiration)	Darchambeau et al. (2003)
N:P and C:P	Zooplankton community	Field (reservoirs)	↑ (Excretion)	Darchambeau et al. (2005)
N:P and C:P	Algal-zooplankton system	Review	↑ (Excretion)	Elser and Urabe (1999)
N:P and C:P	Bacterial and phytoplankton	Field (lakess)	↑ (Excretion)	Elser et al. (1995)
C:N and C:P	Trophic web	Laboratory	↑ (Excretion)	Evans White and Lamberti (2006)
C:N and C:P	Fish *Dorosoma cepediamum*	Field (reservoirs)	↑ (Digestion-absorption)	Higgins et al. (2006)
C.P and C:N	Rotifer *Brachionus calciflorus*	Laboratory	↑ (Excretion)	Jensen et al. (2006)
N:P	Omnivorous fish *Dorosoma copedianum* and the zebra fish *Danio rerio*	Field study and laboratory	↑ (Excretion)	Pilati and Vanni (2007)
C:P	*Daphnia magna*	Laboratory	↑ (Grazing activity)	Plath and Boersma (2001)
N:P	Periphyton and the mayfly larvae *Cinygmula sp.*	Field manipulation	↑ (Excretion)	Rothlisberger et al. (2008)
C:P	*Daphnia pulex*	Laboratory	↑ (Grazing activity)	Schatz and McCauley (2007)
C:P	*Daphnia pulicaria*	Laboratory	↑ (Excretion)	Shimizu and Urabe (2008)
C:P and N:P	Body organisms concentration ratios of Cyprinid fish	Field (lake)	↑ (Absorption)	Sterner and George (2000)
N:P	Body composition ratios of different fish	Field	↑ (Excretion)	Torres and Vanni (2007)
N:P	Fish	Field (streams)	↑ (Excretion)	Vanni et al. (2002)
C:P versus species diversity (SD)				
C:P	Macroinvertebrate richness	Field (streams)	↓ C:P ↓ (SD)	Evans-White et al. (2009)

C:N and C:P	Periphyton and gastropod grazers community	Field manipulation	No conclusive results	Liess et al. (2009)
C:P	Invertrebate community	Field (stream)	↓ C:P ↓ (SD)	Singer and Battin (2007)
C:P	Plankton community	Laboratory	↓ C:P ↑ (SD)	Striebel et al. (2009)
C:P	Mixture of *Daphnia* spp	Laboratory	↓ C:P ↑ (SD)	Weider et al. (2008)

More than the GRH

Relationships between N:P body ratio and growth rate other than those predicted by the GRH have also been proposed in terrestrial ecosystems. These models are more complex and take into account factors other than just production capacity of protein to explain growth rate. For example, they also consider body metabolism (respiration) and the investment in structural organs. Ågren (2004; 2008) developed a mathematical model that predicted that the growth rate, estimated as the rate of assimilation of C, is directly dependent on N content, assuming that it is a function of enzyme concentration, whereas increases in N in proteins are a function of P content, assuming that P-rich rRNA is the key to explain rates of protein synthesis. The model predicts that the C:N ratio is a hyperbolic function of growth rate, asymptotically going to zero, and the C:P ratio is a declining function of growth rate that is also asymptotically going to zero. As a result, the N:P ratio increases at a relatively low growth rate, passes a maximum and then decreases at high relative growth rates. Changes in other environmental factors, such as temperature, light and availability of water that affect growth rate of autotrophs, interact with nutrient supply in a way that leads to no simple rules for N:P ratio change regarding relative growth rate (Ågren 2004). These predictions were experimentally verified by the authors in observations of a freshwater alga, *Selenastrum minutum*, a tree seedling, *Betula pendula*, and more recently in species of tropical trees and lianas (Cernusak et al. 2010). This model for terrestrial plants is reminiscent of those we have mentioned in freshwater ecosystems (Vrede et al. 1999; 2004). In both aquatic and terrestrial plants, growth rate can be related to low body N:P ratio. This last relationship is mainly evident in nutrient-rich environments and generally in resource-rich and stable environments. In contrast, the trend in nutrient-poor environments can differ due to the necessity to allocate elements to other, more conservative functions, addressed to improve uptake and efficiency of resource use. This possible slant on the GRH in low-resource ecosystems merits more study.

Another approach linking N:P stoichiometry with plant growth rate in terrestrial ecosystems has been built by using Kleiber's scale of relationships between body size and other organismic characteristics. This relationship states that basal metabolic rates and annual growth

rates of plants scale as the three-quarter power of body mass (Kerkhoff and Enquist 2006; Niklas 1994). Niklas et al. (2005) have proposed that the N leaf content scale is a power function of P leaf content to maintain optimal capacity for growth, showing a relationship between P content and relative rates of plant growth (Niklas 2006). Their findings further support the evidence that the N leaf content scale is, on average, a 3/4 power of P leaf content (Niklas and Cobb 2005; Niklas et al. 2005). Niklas' approach successfully predicted the relative growth of 131 species of vascular plants. In the same way, Reich et al. (2010), in a review of leaf N to P ratio in the major groups of plants, have reported that N scaled as a 2/3 power of P when leaf N and P content were log transformed along rising N, P, leaf mass area (LMA), photosynthetic capacity and growth rate. These findings relate the leaf N:P ratio with the "leaf economic spectrum", a paradigm that claims that plants with higher growth capacity have more productive leaves (lower LMA and higher photosynthetic capacity) (Reich et al. 2010; Wright et al. 2004). Other studies have already observed a close relationship between leaf N or P content and leaf economic traits such as photosynthetic rates or LMA (Peñuelas et al. 2010; van de Weg et al. 2009; Wright et al. 2004; 2005; Wright and Westoby 2003), and in some cases a positive relationship between N:P ratios and LMA has been observed (Paoli 2006). These results taken together suggest that leaf economic spectrum traits (mainly LMA and photosynthetic rates) and other plant traits related to plant lifestyle could be correlated with the N:P ratio differently in nutrient-poor sites compared to nutrient-rich sites. This suggestion coincides with the observations of Vrede et al. (1999; 2004), and is an area warranting further research.

Stoichiometry and ecosystem structure and function

C:N:P stoichiometry exerts a determining role in the functions and structure of ecosystems. A lower sestonic N:P and C:P ratio increases the presence of fast-growing species by favoring higher growth rates (DeMott and Tessier 2002; Dobberfuhl and Elser 2000). The consequent changes in N:P stoichiometry induce shifts in the community of species (Conde-Porcuna et al. 2002; de Eyto and Irvine 2007; DeMott and Tessier 2002; Hassett et al. 1997; Makino et al. 2002; Smith 1983). Increased community domination by fast-growing species in aquatic environments low in C:P and N:P ratios, and vice versa, have been observed in several studies (Carrillo et al. 2008; Danger et al. 2007; Elser and George 1993; Elser et al. 2000a; Elser and Urabe 1999; Hassett et al. 1997; Smith 1983; Teubner et al. 2003; Vanni et al. 1997; among others, see Figure 2 and Table 2). Models that considered the quality (C:P) and quantity (absolute C algal content) of food have been shown to predict the observed dynamics of plant-herbivore system (Andersen 1997; Nelson et al. 2001). Investigations of such relationships in terrestrial ecosystems are only now beginning. Mulder and Elser (2009) recently analysed the mass-abundance and body-size

spectra of all soil invertebrates, fungi and bacteria in 22 grassland soils in the Netherlands. They found that the higher the availability of P in the soil (lower C:P ratio), the steeper was the slope of the faunal biomass size spectrum — namely, the biomass of large-bodied invertebrates became greater relative to that of small invertebrates. This relationship has broad implications because soil invertebrates of different sizes have different effects on soil processes. The relationship connects biogeochemistry with the structure of food webs, and, if it holds true in different settings, opens a way to develop a more general law of the structure and dynamics of terrestrial ecosystems similar to the GRH in freshwater ecosystems. Hence Mulder and Elser's results are promising. The association between soil-element stoichiometry and the body-size spectrum of the food web, though, might not simply result from a faster growth of the different organisms of the food web in response to low C:P and protein:RNA ratios, as predicted by the GRH. As Mulder and Elser state, other factors may be involved. For example, the shift in the body-size spectrum might have resulted from the change from one main source of food (bacterial cells) to another (fungal remains) that Mulder and Elser found in their soils as C:P ratios decreased (Mulder et al. 2009). The shift between these two different energy channels (Rooney et al. 2006), which differ in both productivity and rate of turnover, could have driven the shift in invertebrate dominance from microfauna (nematodes), which cope better with P-limited conditions, to mesofauna (microarthropods). Nonetheless, another recent study has shown relationships between N:P ratios in litter and the food web structure of litter invertebrates (Kaspari and Yanoviak 2009). Species with a high growth rate should thus dominate in nutrient-rich environments in freshwater and terrestrial ecosystems. At intermediate nutrient levels, species can coexist at scale equilibrium, or alternative stable states may emerge, depending on how the grazers affect their resources (top-down effect) (Hall 2004).

The C:N:P ratios thus seem to have an important role in controlling trophic relationships, structure of food webs and species composition in communities in terrestrial ecosystems. Similar to that observed in freshwater ecosystems, an increase in carbon biomass in plants (e.g. in response to more light) may sufficiently dilute the content of the limiting nutrient in plant tissue that the rate of nutrient intake by the herbivore decreases. Consequently, the herbivore's growth rate also decreases. This phenomenon has been termed the paradox of energy enrichment (Loladze et al. 2000).

Herbivores prefer plant biomasses with high nutrient contents and lower C:N ratios (Ngai and Jefferies 2004), but some studies have observed that the degree of herbivore attack on leaves can be dependent on the levels of leaf N but not on the C:N ratio (Kagata and Ohgushi 2006; 2007). Predators on average have a higher N content and lower C:N ratio than their potential herbivorous prey (Matsumura et al. 2004). Many predators thus grow by

supplementing a diet of herbivores with more nitrogen-rich intraguild prey (Matsumura et al. 2004). Some studies, however, did not observe increases in the body N content of insect predators after intraguild predation (Fagan and Denno 2004; Kagata and Katayama 2006). All these results suggest that C:N ratio is less crucial in the transfer of matter through trophic webs than N:P stoichiometry. Moreover, these results highlight the lack of information about the role of predators in ecosystem stoichiometry, and vice versa.

Nutrient availability and grazing interaction in aquatic ecosystems might even determine the richness of species of primary producers (Liess et al. 2009). The experimental data to date, however, does not allow us to establish a general relationship for body and water C:N:P stoichiometry with species richness. Whereas some reports suggest a negative relationship between species diversity and the C:P and C:N ratios of community organisms (Liess et al. 2009), other studies observe no clear and even contrary relationships (Evans-White et al. 2009; Singer and Battin 2007; Striebel et al. 2009). Some studies of terrestrial ecosystems show that N availability increases body N:P ratios and reduces species diversity of communities (Güsewell et al. 2005; Roem and Berendse 2000; Seastedt and Vaccaro 2001), whereas increases in P availability have contrary effects (Güsewell et al. 2005; Seastedt and Vaccaro 2001). These results are in accordance with those previously noted in freshwater ecosystems. Food sources with lower N:P ratios increase energy transfer to higher trophic levels, further favoring the existence of larger trophic webs. This negative relationship between N:P stoichiometry and species richness, though, cannot be so simple. At certain low levels of N:P ratios, N can limit the production capacity of ecosystems and thus limit matter transfer through trophic webs and species richness. The resource balance hypothesis predicts a unimodal relationship between N:P stoichiometry and species richness with an optimal N:P ratio in which species richness reaches a maximum, as observed in some studies (Sasaki et al. 2010). This concept is consistent with studies that show that competing plant species can eliminate their competitors depending on the availability of soil N:P ratio (Olde Venterink and Güsewell 2010). Although the previous results suggest a tendency towards more complex ecosystems under low C:P and N:P ratios and that at intermediate levels the possible coexistence of species leads to a maximum diversity, other studies suggest other links between stoichiometry and diversity. Some studies of grassland and phytoplanktonic communities have shown that species diversity is correlated with the number of limiting resources (Harpole and Tilman 2007; Interlandi and Kilham 2001), thereby suggesting that high C:N and C:P ratios should be related to greater species diversity. All these data together suggest that the N:P ratio could determine the length of the trophic web and the rate of resource transfer along it, but that other factors could be more important in determining niche dimension.

Stoichiometry, nutrient cycling and nutrient stoichiometry homeostasis

Food chain efficiency (FCE) transfer through food webs has also been demonstrated to be constrained by the stoichiometry of food (C:N and C:P ratios) in aquatic ecosystems (Dickman et al. 2008). High C:P ratios in particulate matter at the base of the food web decrease the matter transfer throughout the food web (bottom-up effect) (Cross et al. 2007; Elser et al. 1998). Conversely, decreases in C:P and N:P ratios in water increase the contribution of consumers to C, N and P cycling, altering the proportion of C inputs metabolized by consumers and potentially leading to a reduced ecosystem-level storage of C (Cross et al. 2007) (Figure 2 and Table 2). P content of herbivores in freshwater ecosystems is a key factor for growth of carnivores. If the C:P ratio of herbivores is high, carnivores can compensate the excess carbon by reducing growth, resulting in no significant changes in C:N:P ratios, consistent with the GRH (Stelzer and Lamberti 2002; Sterner and George 2000).

Considerable evidence supports such top-down effects by grazers and predators on N:P ratio in water, seston and producers. These effects are due to the capacity of grazers and predators to accumulate N and P and also due to excretion (Danger et al. 2009; Elser et al. 2005; Hillebrand and Kahlert 2001; Sterner and George 2000; Urabe 1995; among others, see Figure 2 and Table 2). Increased pressure by grazers has been reported to further enhance N and P availability by excretion, indicating that higher regeneration of N by grazers leads to lower C:N and C:P ratios in grazed than in ungrazed algae, thus improving algal nutritional quality (Hillebrand et al. 2008; 2009; Urabe et al. 2002a) and generating a faster cycling of N and P. Despite frequently representing only a smaller part of the ecosystem biomass, predators can thus significantly influence the stoichiometry of lower trophic levels and the environment (soil and water) by their intensive trophic activity and capacity to structure lower trophic levels.

The effects of the decrease in the C:N and mainly in the C:P ratio may be larger in terrestrial ecosystems than in aquatic ecosystems due to the generally higher C:P and C:N ratios in terrestrial plants, as has been recently suggested by Cebrian et al. (2009). Some studies confirm that grazers induce the shift from N-limited plants to P-limited plants by increasing the N content of leaves (Carline et al. 2005; Frank 2008). The herbivore C:P ratio is thus also linked to the C:P ratio of the soil through the influence of soil nutrient availability on foliar nutrient content, which can constrain herbivore investment in P-rich molecules necessary for growth (Apple et al. 2009; Schade et al. 2003). For example, variations in the availability of P in desert soils are linked to the abundance of a herbivorous insect, *Sabinia setosa*, by the influence of soil P availability on the C:P ratios of the host plant, *Prosopis velutina* (Schade et al. 2003) (bottom-up effect).

All these results suggest a general trend towards a homeostatic control of the C:N:P stoichiometry of soils through soil-plant-herbivore relationships that deserve further research. At a low soil N:P ratio, the biomass of grazers increases, shifting the soil N:P availability ratio to a higher value (top-down effects) by decreasing soil P availability through the sequestration of grazers. These changes further limit plant growth and consequently herbivore growth (bottom-up effect) from reaching a state of equilibrium. Also, when plant stoichiometry changes, the non-specialist grazers can adjust their C:N:P stoichiometry by the intake of varying proportions of the different plant species on which they feed (Jonas and Joern 2008). These feed-back mechanisms within ecosystems mediated by different trophic levels suggest a trend for different types of ecosystems to reach an average optimal C:N:P community stoichiometry, despite an array of organisms presenting different body C:N:P stoichiometry. Some recent studies comparing litter and humus composition support this hypothesis by suggesting that the uptake and cycling of bioelements with covalent chemistry such as C, N and P are mainly under biological control (Ladanai et al. 2010), and that therefore their mutual stoichiometry should vary little. More studies of terrestrial ecosystems under different climatic conditions, though, are necessary to clearly establish this trend. The capacity to change the C:P ratios of food tissues can be a key driver in an organism's response against herbivores or predators with further consequences in organism's structure and biotic relationships. The macrophyte *Potamogeton perfoliatus* retranslocates P from leaves (food tissue) to buds (non-food tissues) as a defense mechanism against the leaf herbivore *Acentria ephemerella* (Miler and Straile 2010).

Such top-down and bottom-up effects, though, can also be mediated during their development phases by nutrient availability For example, the introduction of fish could cause a greater impact in N-limited lakes than in other lakes because N-excretion by fish plays a key role in phytoplanktonic growth (Danger et al. 2009). In freshwater trophic web cascades, fish exert a notable top-down control in nutrient stoichiometry by contributing to an increase in the ecosystem's capacity to retain P (Griffiths 2006; McIntyre et al. 2007; Vanni et al. 1997) and N (McIntyre et al. 2007). The nutrient recycling by zooplanktivorous fish will therefore tend to alleviate the limitation of P for phytoplanktonic growth (Schindler and Eby 1997) leading to greater abundance of the latter (Findlay et al. 2005). The contribution of fish to the P cycle diminishes with increasing P content of water (Griffiths 2006). Taken together, these results are consistent with a relationship between the cascade of trophic interactions and elemental stoichiometric shifts that imply changes of species with different rates of growth. The results are also consistent with a relationship between large trophic webs where fish tend to increase the rates of nutrient cycling and the capacity to retain N and P on an ecosystem level.

The homeostatic capacity to regulate N:P and C:P ratios within each trophic level independently from the stoichiometry of food sources (Small and Pringle 2010) involves resorption, the digestive-absorptive process, excretion, increases in grazing activity and changes in respiration (Christian et al. 2008; Darchambeau et al. 2003; 2005; Plath and Boersma 2001; Torres and Vanni 2007; among others, see Figure 2 and Table 2). This homeostatic capacity therefore has an energetic cost that decreases growth and reproductive capacity (Boersma and Elser 2006). Several investigations consistent with the stoichiometric homeostatic capacity of ecosystems show that N:P excretion ratios of fish are more closely related to food N:P ratios than to body N:P ratios (Pilati and Vanni 2007; Torres and Vanni 2007) (Figure 2 and Table 2). Excretion systems could thus have the same stoichiometric significance in animals as leaf resorption and retranslocation have in plants, by adjusting the N:P ratios to the species' optimum. These mechanisms of homeostatic control of body stoichiometry have important consequences for the balance of autotrophic and heterotrophic processes in the species composition of ecosystems and communities (Anderson et al. 2005). If the optimal body C:N:P stoichiometry differs markedly from the C:N:P stoichiometry of the food source, the cost of excretion or respiration can be excessive, leading to a shift towards species with C:N:P stoichiometries more similar to the C:N:P stoichiometries of their food sources. This hypothesis is very important because it is related to the aforementioned prevalence of Redfield ratios in communities of marine plankton (Klausmeier et al. 2004). Further experiments should investigate the capacity of aquatic ecosystems to "resist" changes in water C:N:P stoichiometry without changing the species composition and the mechanism underlying this possible capacity.

Having higher N and P contents than their prey confers predators a high capacity to slow the cycling of nutrients because predators sequester rather than recycle nutrients. This feature can lead to unusual results such as a decrease in the density of prey (Grover 2003). Indeed, the extent to which predators recycle or sequester limiting nutrients for their prey is of critical importance for the stability and response to enrichment of prey-predator systems (Grover 2003) and the dynamics of ecosystems. Further studies could contribute to a better understanding of the role of predators in the sequestration and recycling of nutrients in terrestrial ecosystems and the impacts of predators on the N:P stoichiometry of the trophic web (top-down effect).

Stoichiometry and species evolution

Environmental and organism's stoichiometry can impact the molecular structure and evolution of organisms at different levels. On short time scales, environmental stoichiometry can affect transcriptional expression and metabolomic structure; on longer timescales, it can determine phenotypic selection; and on the longest time scales, it can affect genomics. Some

investigations suggest some of these possibilities, but very few reports have coupled stoichiometric with "omic" studies from an ecological perspective.

Evidence suggests that variation in the environmental supply of P affects the expression of highly conserved genes (e.g. the phosphate transporter system) and that the acquisition, assimilation and allocation of P is genetically variable (Jeyasingh and Weider 2007). Transcriptomic studies in algae (Grossman 2000), microbes (Baek and Lee 2007), terrestrial plants (Morcuende et al. 2007) and more recently in herbivores (Jeyasingh et al. 2011) have also observed that the availability of P influences genetic expression. Such effects of the supply of P should impinge on fitness and drive evolutionary change. Jeyasingh et al. (2009) observed that genetic variation in phosphoglucose isomerase (Pg) indicated that Pg heterozygotes of *Daphnia pulicaria* outcompeted Pg homozygotes under conditions of low C:P ratios, whereas the opposite was observed under conditions of high C:P ratios.

Acquisti et al. (2009) have developed an interesting hypothesis that relates the availability of N directly with the plant genome. Under extremely limited availability of N, selection would favor the use of N-poor nucleotides in plant genomes. Comparing the genome compositions of *Oryza sativa* and *Arabidopsis thaliana* and using data from the TIGR database and available protein sequences of many plants, these authors observed higher N contents in genomes and proteomes of domesticated plants than of undomesticated plants. These results suggest a possible role for the availability of N in the evolution of plant genomes and link genomic evolution with the chemical composition of the environments within which this biota evolved. This process could occur similarly with the availability of P. The N:P ratio can thus become an driver of evolution that affects the structure of ecosystems at medium and longer timescales. All these results suggest that the natural gradients of N:P availability ratios can exert a chemical control over the evolution of species. This control could be indirect through effects such as competitive pressure for the uptake of N and P, a change in an organism's rate of growth, or an adaptation to each situation of N:P availability. The control of evolution could also be direct through affecting the quality of matter (different N:P ratios) supplied to build a genome and achieve the optimal allocation of N and P to DNA/RNA for maximizing fitness. This issue merits further study.

Conclusions

Synthesis of current knowledge

1. Several data suggest a progressive adjustment of aquatic particulate matter to values close to Redfield's ratio from coastal waters to open oceans. This adjustment may be based on several

biogeochemical processes: the trade-off between competitive equilibrium and growth rate, the buffering effect of N_2 fixers and the oceanic geochemical cycles of N and P. In marine ecosystems, Redfield's ratio may thus exert a large deterministic control linked to biogeochemical processes.

2. C:N:P ratios of water, plankton and seston in rivers and lakes, due to the lower volumes of water compared to oceans, are more likely to be determined by the particular traits of the surroundings, such as rock type or human impact. Therefore their C:N:P ratios vary greatly around the world.
3. In terrestrial ecosystems, N:P ratios of soil, litter and plants generally increase from cool and temperate to tropical ecosystems despite large variations within each climatic area.
4. Within the plant kingdom, herbs have lower N:P ratios in photosynthetic tissues than do woody plants.
5. Within the animal kingdom, endotherms have higher C:nutrients ratios than do ectotherms, due to the higher demands for C-rich sources such as sugars to maintain a high energy production.
6. An organism's C:N:P stoichiometry tends to be more constrained than the stoichiometry of its environment as a result of the organism's homeostatic capacity to adjust its C:N:P stoichiometry by different mechanisms, such as leaf reabsorption in plants and excretion and pre-absorption mechanisms in animals.
7. In woody plants, the relationship between leaf N:P ratio and plant size is scaled. This relationship highlights a disproportionate increase in leaf P compared to leaf N when both leaf N and P contents rise.
8. C:N ratios tend to decrease in scaling trophic webs.
9. In freshwater ecosystems, N:P availability appears to determine web trophic structures. Low N:P ratios favor organisms with a high growth rate and a higher slope of the size/mass spectrum, and, in contrast, higher N:P ratios favor species with lower growth rates. These relationships are particularly observed in nutrient-rich waters but are less clear in nutrient-poor aquatic ecosystems and probably in perturbed ecosystems where N:P ratios can also depend on processes other than growth rate, such as storage or defense.
10. Strong experimental support indicates that high C:P and N:P ratios at the base of trophic webs decrease matter transfer throughout the food web and potentially lead to reduced levels of C storage in freshwater and to a lesser extent in terrestrial ecosystems.
11. The N:P ratio is more critical than the C:N ratio in explaining bottom-up cascade effects through multiple trophic levels and shifts in species composition.

12. Studies of terrestrial ecosystems do not lead to a consistent conclusion about the role of the GRH hypothesis in the structure and function of ecosystems. The main difficulty is that, unlike phytoplankton and zooplankton, most terrestrial animals and higher terrestrial plants invest much N and P in strong mechanical structures (skeletons, wood) and in storage and defense, making the relationship between the N:P ratio and protein:RNA allocation less immediate. Moreover, water is limited at different levels in several terrestrial ecosystems, which impacts N and P availability, making comparisons of experiments across different types of ecosystem more difficult. Furthermore, the differences in bedrock N and P content further complicate the comparison of stoichiometric studies throughout terrestrial ecosystems.

Perspectives for future research

1. In contrast to aquatic ecosystems where water is not limiting, terrestrial ecosystems are often water-limited. The C:N:P stoichiometry of organisms in drier terrestrial ecosystems should evolve towards optimizing efficiency of water use. In wetter ecosystems where water is less limiting, organisms should allocate resources and reach a body C:N:P ratio that primarily maximize growth and competitive ability. This role of water availability should be considered when designing and interpreting stoichiometric studies of terrestrial ecosystems.
2. Plants should be studied from a different perspective than with animals when studying the GRH because of the larger role in plants of secondary metabolism and structural tissues.
3. Meta-analysis of current knowledge has shown that N and P demands are usually closely balanced (Elser et al. 2007). Several questions about the role of the N:P ratio in ecosystem performance, though, remain unsolved. We have no mechanistic understanding of how the availability of one resource affects the supply and demand of another (Davidson et al. 2007).
4. Although other elements such as K or Fe have received less attention, some studies have shown the importance of their availability in the function of N and P availability, and their roles may be underestimated. For example, Timmemans and van der Wagt (2010) have recently observed that Fe availability strongly affects the body C:N:P ratios in diatoms through its effects on growth-rate capacity and body size. In terrestrial ecosystems, the availability of other elements can also change plant N:P stoichiometry. Although the mechanisms are not totally understood, increases in S availability decrease plant N:P ratios (Robroek et al. 2009). Some important ecosystems around the world such as meadows or boreal forests are frequently K-limited (Nilsen and Abrahamsen 2003; Olde Venterink et al. 2001), and the plant N:K ratio can account for variations in the composition and diversity of species of ecosystems (Roem and Berendse 2000). Some studies of the optimization of agricultural production show that a maximum plant yield (Mairapetyan et al. 1999) and better adaptation to drought (Sardans and Peñuelas 2007) requires an optimal N:P:K ratio. Future ecological stoichiometric

research should also evaluate nutrient ratios other than those involving C, N, and P. This research should focus mainly on Fe in aquatic ecosystems and K in terrestrial ecosystems.

5. Some previous studies have observed different N:P stoichiometries in species with different lifestyles (ruderals, stress-tolerators and competitors). Further studies should be conducted to investigate whether or not this tendency is universal.
6. Questions concerning stoichiometry need longer-term ecosystem-scale studies; most studies have been of short duration relative to the life cycles of the organisms and lack a community and ecosystem perspective.
7. A key question to investigate is the extension and limit of the ability of plant species to control their C:N:P stoichiometry, and the relative role of genotypic versus phenotypic physiological mechanisms.
8. Some studies highlight the possibility that natural gradients of N:P availability ratios have exerted a chemical control of species evolution by indirect or direct effects, e.g. through the differential supply of N and P to build a genome and to achieve the optimal allocation of N and P to DNA/RNA to maximize fitness. Further studies are warranted to better understand the possible driving role of N:P stoichiometry in the evolution of species. Experiments coupling stoichiometric with genomic or metabolomic studies should advance our understanding of the mechanisms driving genotype-environment interactions.
9. Some studies of terrestrial ecosystems have reported a close relationship between leaf N or P content and other economic traits of leaves such as photosynthetic rates or LMA related to metabolic capacity and the plant's style of life. These traits can be correlated with the N:P ratio differently in nutrient-poor and nutrient-rich sites. This possibility merits further research to reach a general overview of the GRH in freshwater and terrestrial ecosystems.
10. In terrestrial plants, the relationship observed between leaf N and P content (with a potential factor of 3/4, 2/3 or 1/2 depending on the studies) suggests a determining relationship between stoichiometry and metabolic-physiological requirements that warrants further research.
11. Studies are needed involving the stoichiometry of trophic levels in webs comprising the main animal groups, such as insects and mammals in terrestrial ecosystems or fish and gastropods in aquatic ecosystems, and their predators and sources of food. The C:N:P shifts among trophic levels under different equilibrium can then be evaluated, studying both bottom-up and top-down effects together. This work could be done by studying trophic webs in different seasons or through natural spatial gradients.
12. The role of predators in nutrient cycling in ecosystems remains inconclusive. Predators often represent a small part of the total biomass of an ecosystem but have great trophic activity and

can play an important role in the stoichiometry of lower trophic levels. The challenge is to know whether they increase nutrient cycling by reducing C:N and C:P ratios in the base of the trophic web through excretion. More studies are necessary to gain a better knowledge of this topic.

13. New experiments must be designed to investigate N:P relationships with organism's functions other than growth. Further assessments of the GRH require more studies of the effects of C:N:P ratios on the ratios of different metabolic products, such as proteins and RNA, and on growth rates in different taxa and ecosystems. By coupling studies of plant, herbivore or predator stoichiometry to metabolomic studies, we will improve our understanding of the coupling between different levels of biological organization and will reach a better understanding of the role of C:N:P stoichiometry in environments of contrasting nutrient richness or/and water availability. Moreover, the study of the possible role of C:N:P ratios in the evolution of organisms by using "omic" techniques also enables advancement of our knowledge. Such techniques provide information about the mean C:N:P ratios and the evolution of organisms and species in short-term (phenotypic expression, transcriptomics, proteomics and metabolomics), medium-term (genotype selection, genomics and transcriptomics) and long-term (shifts in DNA composition, genomics) periods.

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The C:N:P stoichiometry of organisms and ecosystems in a changing world: a review and perspectives

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Abstract

This study examined the literature in ISI Web of Science to identify the effects that the main drivers of global change have on the nutrient concentrations and C:N:P stoichiometry of organisms and ecosystems, and examined their relationship to changes in ecosystem structure and function. We have conducted a meta- analysis by comparing C:N:P ratios of plants and soils subjected to elevated [CO_2] with those subjected to ambient [CO_2]. A second meta-analysis compared the C:N:P ratios of plants and soils that received supplemental N to simulate N deposition and those that did not receive supplemental N. On average, an experimental increase in atmospheric [CO_2] increased the foliar C:N ratios of C3 grasses, forbs, and woody plants by 22%, but the foliar ratios of C4 grasses were unaffected. This trend may be enhanced in semi-arid areas by the increase in droughts that have been projected for the coming decades which can increase leaf C:N ratios. The available studies show an average 38% increase in foliar C:P ratios in C3 plants in response to elevated atmospheric [CO_2], but no significant effects were observed in C4 grasses. Furthermore, studies that examine the effects of elevated atmospheric [CO_2] on N:P ratio (on a mass basis) are warranted since its response remains elusive. N deposition increases the N:P ratio in the plants of terrestrial and freshwater ecosystems, and decreases plants and organic soil C:N ratio (25% on average for C3 plants), reducing soil and water N_2 fixation capacity and ecosystem species diversity. In contrast, in croplands subjected to intense fertilization, mostly, animal slurries, a reduction in soil N:P ratio can occur because of the greater solubility and loss of N. In the open ocean, there are experimental observations showing an ongoing increase in P-limited areas in response to several of the factors that promote global change, including the increase in atmospheric [CO_2] which increases the demand for P, the warming effect that leads to an increase in water column stratification, and increases in the N:P ratio of atmospheric inputs. Depending on the type of plant and the climate where it grows, warming can increase, reduce, or have no effect on foliar C:N ratios. The results suggest that warming and drought can increase C:N and C:P ratios in warm-dry and temperate-dry terrestrial ecosystems, especially, when high temperatures and drought coincide. Advances in this topic are a challenge because changes in stoichiometric ratios can favor different types of species and change ecosystem composition and structure.

Keywords Climate change · CO_2 · diversity · drought · elemental stoichiometry · eutrophication · freshwater · oceans · Redfield's ratio · soil · species invasion · trophic webs · UV · warming.

Introduction

Ecological stoichiometry examines the relationships between organism and ecosystem structure and function with environment and organisms C:N:P stoichiometry (Elser et al. 1996 and 2000a; Sterner and Hessen 1994). C:N:P biomass ratios can be associated with important ecological processes such as litter decomposition (d'Annunzio et al. 2008; Güsewell and Gessner 2009), N_2 fixation capacity (Sañudo-Wilhelmy et al. 2001), plant-herbivore-predator relationships (Ngai and Jefferies 2004; Tibbets and Molles 2005; Kagata and Ohgushi 2006), ecosystem-specific composition and diversity (Roem and Berendse 2000; Venterink et al. 2003; Güsewell et al. 2005), and with the ability of organisms to adapt to environmental stresses (Woods et al. 2003). An organism's C:N:P stoichiometry can be strongly influenced by environmental stoichiometry (Sterner and George 2000; Güsewell et al. 2003; Hall et al. 2005; Castle and Neff 2009), despite an organism's considerable capacity to maintain their body stoichiometry within ranges than are narrower than the stoichiometries of their environments and their food sources (Elser et al. 2005; Feller et al. 2007; Cross et al. 2007; Fitter and Hillebrand 2009).

Several factors that promote global change such as the increase in atmospheric CO_2, climate change, eutrophication, and species invasiveness, which are predicted to increase in the near future (IPCC 2007), have been shown to interact with the stoichiometry of ecosystems (Reich et al. 2006; Funk and Vitousek 2007; Sardans and Peñuelas 2008; Elser et al. 2009a and 2010; Ferreira et al. 2010). Those drivers of global change might alter ecosystem structure and function, indirectly, through their effects on ecosystem stoichiometry. Global change can affect the stoichiometries of organisms and ecosystems through two pathways. First, the effect of global change on the C:P and C:N ratios of producers can alter food quality, affect nutrient cycling and nutrient transfer through the trophic web, which can alter the C:N and C:P ratios in consumers, and change ecosystem composition from nutrient-rich to nutrient-poor species or vice versa. Second, changes in organism and ecosystem N:P ratios caused by global change might alter the competitiveness of a species depending on its growth rate and lifestyle (Elser et al. 1996; Elser and Hamilton 2007), which can lead to a shift in ecosystem composition, increasing the extinction risk of some species, and produce other unpredictable effects that might result from the interactions among different global change drivers.

In this study, we investigated the relationships between the drivers of global change and the stoichiometry and composition of organisms and ecosystems. The analysis was based on the records in the Web of Science database as of 31 July 2011. The search included all of the observational and experimental studies on the effects of increases in atmospheric [CO_2], climatic change and N eutrophication on the C:N:P stoichiometries of organisms and ecosystems. The large number of experimental studies of the effects of atmospheric [CO_2] and N eutrophication

on leaf C:N and N:P ratios that included controls and manipulated atmospheric [CO_2] (in plants) and added N (in plants and soils) permitted to conduct a meta-analyses, which were performed using Metawin 2.0 (Sinauer Associates, Inc., Sunderland, MA, USA) (Rosenberg et al. 2000). The overarching objective of the study was to identify what is known about the effects of drivers of global change on the C:N:P stoichiometries of organisms and ecosystems and their effects on growth rate, nutrient availability, trophic chain structure, and ecosystem performance. Finally, we propose new experimental ways to approach these ecological stochiometry issues.

Increases in atmospheric CO_2

Terrestrial ecosystems

C:N ratios

Elevated concentrations of atmospheric CO_2 (hereafter, [CO_2]) increase the aboveground plant C:N ratios in many terrestrial plants (Hungate et al. 1996; Cotrufo et al. 1998; Gifford et al. 2000; Stiling and Cornelissen 2007; Lindroth 2010) (Figure 1 and Table S1 of Appendix 1). In our review of available reported studies (ISI Web of Science until 31 July (2011) we found that most of them showed that the C:N ratios in various plant organs and ecosystem compartments were higher at elevated atmospheric [CO_2] (> 500 ppm) than at current atmospheric [CO_2] (Figure 1 and Table S1 of Appendix 1). More than 215 studies provided C:N:P data collected in environments that had realistic increases in [CO_2], but only 34 studies provided enough information to be included in a meta-analysis of the effects of elevated [CO_2] on leaf C:N ratios. In 156 of the 180 experimental results provided by bibliography, leaf C:N ratios increased in response to elevated atmospheric [CO_2]. In 21 studies, there was no significant effect and, in three studies, elevated atmospheric [CO_2] led to a reduction in leaf C:N ratios (Figure 1). A meta-analysis indicated that elevated [CO_2] increased significantly the leaf C:N ratios in woody plants ($P < 0.05$, $n = 40$), forbs ($P < 0.05$, $n = 31$), and C3 grasses ($P < 0.05$, $n = 25$), but not C4 grasses ($P > 0.05$, $n = 8$). On average, C3 plants at elevated atmospheric [CO_2] had leaf C:N ratios that were 22% higher than those of plants at current atmospheric [CO_2] ($P < 0.05$, $n = 96$) (Figure 2), and the increases were similar in the experiments that used chambers or Free Air CO_2 Enrichment (FACE). Despite these general results increasing leaf C:N ratios, the effects of [CO_2] on N dilution in plants vary significantly among species (Körner et al. 2005). The mechanism by which elevated [CO_2] can cause reductions in N uptake is not clear (Taub and Wang, 2008), but there is good evidence that changes in the capacity for nitrate assimilation, growth, transpiration, and plant morphology can cause a reduction in N uptake and assimilation under high [CO_2]. In C3 plants, elevated [CO_2] can inhibit nitrate photo-assimilation (Smart et al. 1998; Bloom et al. 2002 and 2010; Searles and Bloom 2003). Furthermore, when grown in elevated [CO_2], a reduction in the efficiency of

photosynthetic nitrate fixation in some important C3 crops reduces the nutritional quality, e.g., protein concentration (Bloom 2009), and, thereby, reduces food security for an increasing human population. For those reasons, the prudent use of nitrogen fertilizer and a shift from nitrate-based to ammonium-based fertilizers are imperative (Bloom 2009). The Dilution Hypothesis posits that, under high [CO_2] levels, the accumulation of carbon in tissues is greater, particularly when the increase in CO_2 is greater than the increase in N (Peñuelas and Matamala 1990; Taub and Wang 2008). The C-enrichment Effect is involves the increment of C-rich secondary compounds such as phenolics and lignin, and a reduction in N-rich metabolites (Peñuelas and Estiarte 1997 and 1998; Liao et al. 2002; Ainsworth and Long 2005; Matros et al. 2006; Kasurinen et al. 2006). The higher rates of C assimilation at high [CO_2] have a dilution effect on N, thereby, increasing the C:N ratios of plant tissues (Coûteaux et al. 1991; Coleman et al. 1993; Maillard et al. 1999; Steinger et al. 2000; Riikonen et al. 2005). Furthermore, an enhanced Rubisco Efficiency leads to a reduction in the amount of N allocated to enzyme synthesis and, thus, N can be invested in other functions such as reproduction (Ward and Kelly, 2004). That process can increase Nitrogen Use Efficiency (NUE), the amount of C fixed per unit of N uptake (Stitt and Krapp 1999, Gifford et al. 2000), because plants can maintain growth rates under low [N] and high [CO_2] (Taub and Wang 2008), which increases their NUE (Dijkstra et al. 2010).

Many experiments have shown that the enhancement of NUE by the roots and the reduction in N uptake caused by the reduction in plant transpiration lead to higher C:N ratios at high [CO_2] (Polley et al. 1999; Del Pozo et al. 2007). Furthermore, changes in root architecture and the uptake capacity of individual roots can reduce the N-uptake capacity of plants (Berntson 1994; Pritchard and Rogers 2000; BassiriRad et al. 2001). The significance of the stoichiometric responses of roots to high levels of [CO_2] is unclear because there are limited and no conclusive data on the subject (Luo et al. 1999; McDonald et al. 2002; Taub and Wang 2008) (Figure 1 and Table S1 of Appendix 1). That said, in no study did elevated levels of [CO_2] lead to reduced root C:N ratios (Figure 1 and Table S1 of Appendix 1). Even so, there are some studies that found no changes in the aboveground C:N ratios or N concentrations under elevated [CO_2] (Figure 1 and Table S1 of Appendix 1) (Barnes and Pfirrmann 1992; Griffin et al. 1996; Finzi et al. 2001; Kassem et al. 2008). Increases in assimilated C under elevated [CO_2] would have not translated into increases in C concentrations in plants because C would be allocated to functions such as respiration (Griffin et al. 1996) and root exudates (Kassem et al. 2008), that imply C losses. Some studies have suggested that changes in N uptake in response to elevated [CO_2] might depend on differences in transpiration and photosynthesis, which can differ depending on the growth stage of the plant (Shimono and Bunce 2009). Typically, young plants have the highest N uptake capacity, and this might require more carbohydrate supply than do older plants, which results in

a greater responsiveness to the photosynthetic enhancement caused by elevated [CO_2], while, in older plants, the reduction in transpiration under elevated [CO_2] might reduce N uptake capacity (Shimono and Bunce 2009).

Some studies have suggested that there can be buffering effects under high [CO_2] such as increase in N uptake (Finzi et al. 2007; Lagomarsino et al. 2008), and increases in the use of deep soil N sources (McKinley et al. 2009). The proportion of roots colonized by mycorrhizal can rise under high [CO_2] (Treseder 2004) and mycorrhizal plants have typically more N content (BassiriRad et al. 2001); however, most of the research on this subject is inconclusive (Staddon et al. 2002) and there is evidence that mycorrhiza can be a nutrient sink under elevated [CO_2] and, consequently, there can be reduced nutrient translocation to the plants (BassiriRad et al. 2001).

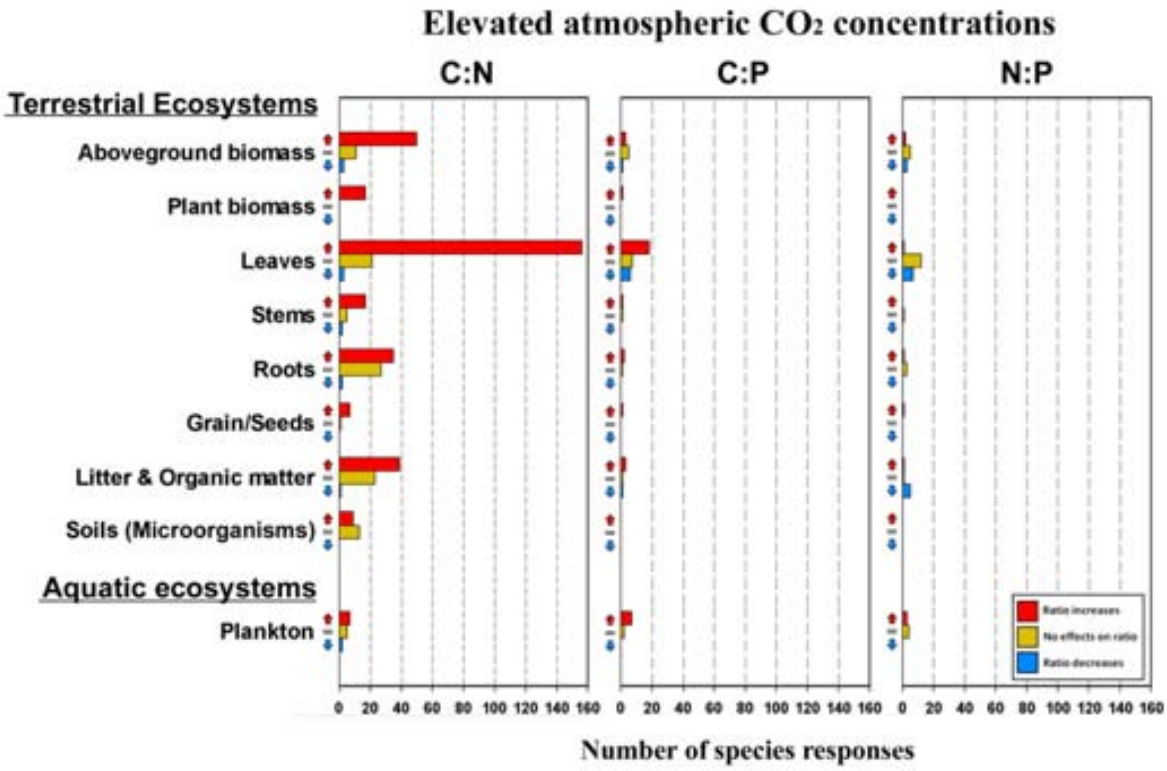

Figure 1. Number of reported increases, decreases, and no changes in the C:N, C:P, and N:P ratios of different ecosystem components in response to elevated atmospheric CO_2 concentrations. Some C:N and C:P ratios are derived from the N and P concentrations. (Detailed data in Table S1 of Appendix 1).

C:P and N:P ratios

Few studies have reported the responses of C:P and N:P ratios to elevated [CO_2] (Figure 1). Nevertheless, studies have shown that elevated [CO_2] can lead to an increase in aboveground plant C:P ratios (Peñuelas and Matamala 1993; Syvertsen et al. 2000; Blank and Derner 2004;

Luomala et al. 2005; Milla et al. 2006; Novotny et al. 2007) (Figure 1 and Appendix. 1). Far fewer studies examined the effects of elevated atmospheric [CO_2] (> 500 ppm) on C:P ratios than examined the effects on C:N ratios (Figure 1 and Table S1 of Appendix 1). Only for terrestrial plant leaves there are several studies that report effects of elevated [CO_2] on C:P ratios. In 19 of 32 species, leaf C:P ratio increased in response to elevated atmospheric [CO_2]; in eight species, the C:P ratio did not change significantly, and, in five species, the ratio decreased (Figure 1). A meta-analysis indicated that plants in elevated atmospheric [CO_2] had leaf C:P ratios that were, on average, 38% higher than those of the plants at current atmospheric [CO_2] ($P < 0.05$, $n = 10$) (Fig 2); however, no significant differences were found in C4 grasses ($P > 0.05$, $n = 6$).

Furthermore, communities can change their overall C:P ratio in response to elevated CO_2 without necessarily changing at the species level. For example, Polley et al. (2011) found that elevated [CO_2] had a limited effect on the N and P concentrations of each species in a C4 grass community but, because the species that had the lowest N and P element increased their proportion of the community biomass and cover, the overall effect of elevated [CO_2] at the community level was a reduction in N and P concentrations.

In general, the effects of elevated [CO_2] on tissue P concentrations are more variable than are the effects on N concentrations (Gifford et al. 2000). Furthermore, the amount of N available can affect the change in the C:P ratio under elevated [CO_2] (Gifford et al. 2000). The effects of elevated [CO_2] on the availability, plant uptake, and use of N and P can vary because of the differential effects of elevated [CO_2] on soil enzymes related to P and N mineralization (Gifford et al. 2000) or by the differential use of N and P by plants, with N more closely associated with C-assimilation capacity, while high P concentrations and low N:P ratios are more closely associated with growth rate capacity (Elser and Sterner 2002). The effects of high environmental [CO_2] levels on plant C:P and, particularly, N:P biomass ratios are still unclear (Johnson et al. 2003; Finzi et al. 2004; Novonty et al. 2007) (Figure 1) and warrant further study because nitrogen and phosphorus are essential in living systems and their ratio is associated with changes in ecosystem structure and function (Sterner and Elser 2002). Indeed, it is important to increase our understanding of the changes in mineralization and N and P stocks in vegetation and soil under elevated [CO_2] levels because of the importance of these elements in the composition of soil litter and decomposition rates (Billings et al. 2010).

Other nutrients

Although at least 31 chemical elements are required by all life on Earth (Melillo et al. 2003), the effects of high [CO_2] levels on many nutrients have received far less attention. The concentrations of nutrients in plants tend to decline because carbohydrates accumulate in plant

tissues (Peñuelas et al. 1997; Loladze, 2002; Duval, et al. 2010). The patterns of nutrient allocation can change in response to elevated [CO_2] levels depending on the type of plant or ecosystem and, consequently, specific nutrients might operate differently depending on their chemical properties and biological function (Peñuelas et al. 2001; Finzi et al. 2007).

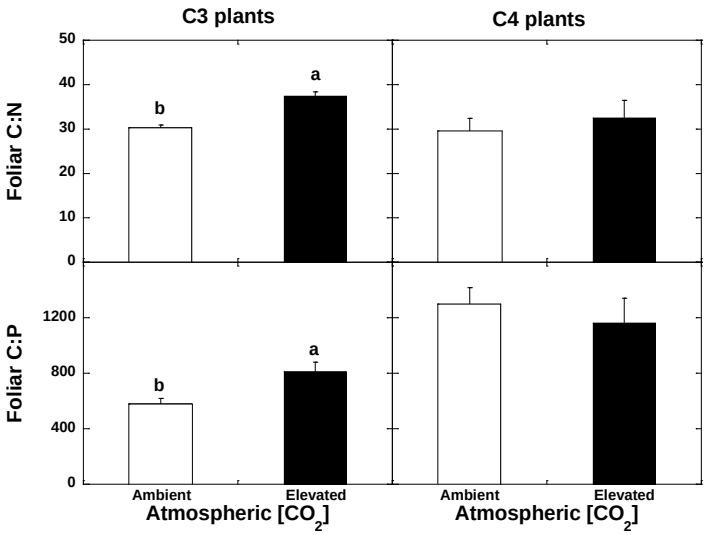

Figure 2. Leaf C:N and C:P ratios of plants that were raised at ambient or elevated levels of atmospheric [CO_2]. Meta-analyses of 96 experiments that evaluated leaf C:N ratios and 16 experiments that evaluated leaf C:P ratios. Only those studies that provided the mean (± S.E.) leaf C:N and C:P ratios of plants that were raised in ambient or elevated levels of atmospheric [CO_2] were included in the meta-analyses. The meta-analyses were performed using the MetaWin Package (Rosenberg et al. 2000). Different letters indicate statistically significant differences ($P < 0.05$).

Cascade effects

Changes in plant stoichiometry in response to elevated [CO_2] levels might have consequences at the ecosystem level, which merit further study. The C-enrichment caused by elevated [CO_2] levels might affect a plant's trophic interactions. Elevated atmospheric [CO_2] levels enhance the growth and N_2 fixing capacity of legumes, and increase mycorrhizae community richness (Johnson et al. 2003; Johnson and McNicol 2010). In addition, it has the indirect effect of increasing herbivore feeding rates, which suggests compensatory behaviour by herbivores in response to low-quality food (Johnson and McNicol 2010). In a shrub oak community, after long-term (from 1996 to 2007) exposure to high atmospheric [CO_2] levels, the food quality of plants decreased, which reduced the density of herbivorous insects. Nevertheless, the low quality food favoured some herbivorous insects, which altered the composition of the insect community (Stilling et al. 2009). In addition, fungal pathogen loadings in plants (Mitchell et al. 2003) and the size and aggressiveness of plant pathogens (Chakraborthy and Datta 2003) increase in response to elevated atmospheric [CO_2] levels. Additional effects on trophic webs include changes in the

composition of a plant's defensive chemical compounds at high levels of atmospheric [CO_2] (Peñuelas and Estiarte 1998; Matros et al. 2006).

Some studies reported that elevated [CO_2] levels increased the C:N ratios in soil organic matter and litterfall (Norby et al. 2001; Luo et al. 2006), but others found no effect on litter C:N ratios (Hirschel et al. 1997; Arnone and Hirschel 1997; Allen et al. 2000; King et al. 2001), and a study of *Betula pendula* found that elevated [CO_2] levels reduced litter C:N ratios (Ferreira et al. 2010). A reduction in litter quality; i.e., an increase in C:N ratio slows nutrient cycling in terrestrial ecosystems, which increases the accumulation of recalcitrant soil organic matter that has a higher C:N ratio (Billings and Ziegler 2005; Gill et al. 2006). Increased plant and litter C:N ratios, do not, however, always lead to changes in N mineralization rates (Coûteaux et al. 1999).

Studies suggest that herbivores and consumers may compensate for a reduction in plant food quality when atmospheric [CO_2] levels are high by increasing feeding rates. The medium- and long-term consequences of a mismatch between the C:N:P ratios of plants and consumers are unclear. Typically, herbivores have C:N and, often, C:P ratios that are lower than those of the plants they consume (Sterner et al. 1998; Fagan et al. 2002; Zhang and Han 2010). The differences in the ratios of herbivores and plants caused by high levels of atmospheric [CO_2], which increase the C:N ratio of plants, might favour the development of consumer communities that have a higher optimum C:N ratio. The C:N homeostatic costs for consumers that have a low C:N ratios might be untenable when the difference between their optimum C:N ratios and that of their food reaches a threshold (Anderson et al. 2005).

Aquatic ecosystems

C:N:P ratios

More than 30% of the CO_2 emitted into the atmosphere over the last 200 yr because of the burning of fossil fuels and changes in land use is stored in the oceans (Raven and Falkowski 1999; Raven et al. 2005). In marine ecosystems, elevated [CO_2] levels can increase the C:N and C:P biomass ratios of phytoplankton (Finkel et al. 2010) (Figure 1 and Table S1 of Appendix 1). The effects of high [CO_2] levels on the C:N ratios of plankton vary considerably among species (Iglesias-Rodríguez et al. 2008; Fu et al. 2007 and 2008). Hutchins et al. (2007) observed that high [CO_2] levels increased the N and P uptake and growth of phytoplankton.

Relatively few studies have examined the effects of elevated [CO_2] levels on the N:P stoichiometry plankton and the results are inconclusive (Figure 1). Tortell et al. (2002) observed changes in the composition of plankton that were directly related to changes in the use of nutrients when exposed to elevated [CO_2] levels; specifically, a reduction the N:P ratios of phytoplankton that grew under high [CO_2] levels, which suggests that elevated [CO_2] levels can

alter organism N:P ratios and affect nutrient cycling in aquatic ecosystems. Some studies reported that the N:P ratios of some cyanobacteria increased in response to elevated environmental [CO_2] levels (Fu et al. 2007; Hutchins et al. 2007), but other studies did not observe this effect (Fu et al. 2008; Bellerby et al. 2008). Thus, the effects of increases in atmospheric [CO_2] on the N:P ratios in aquatic ecosystems remain unclear. Research should be directed toward the study of the effects of elevated [CO_2] levels on N_2-fixation and the N:P ratios in ocean communities. Ideally, studies will examine the effects of elevated [CO_2] levels and elevated global temperatures, concurrently. Widespread change in the N:P ratios of ocean plankton and seston might cause some large ocean areas to become P-limited and others to become P-saturated, which would alter trophic web turnover (Main et al. 1997).

The effects of elevated [CO_2] levels on water pH and the calcification-decalcification equilibrium in oceans are not fully understood. Increases in carbonic acid can exacerbate decalcification and the release of [Ca^{2+}] from sediments, which alters the availability and precipitation of phosphate and, consequently, the C:P and N:P stoichiometries of ocean plankton. There are reports of a reduction in the calcification of corals (Kleypas et al. 1999) and other organisms, e.g., coccolithophores (Riebesell et al. 2000; Zondervan et al. 2001), probably, because the decalcification reaction is favoured at high [CO_2] levels ($CO_2 + H_2O + CaCO_3 \rightarrow Ca^{2+} + 2HCO_3^-$); however, other studies have shown that calcification in oceans increased in response to elevated [CO_2] levels (Iglesias-Rodríguez et al. 2008) and was the direct result of the enhancement of photosynthesis efficiency under high [CO_2]. If the global balance is a decalcification, increases in [Ca^{2+}] might lead to phosphate saturation, which would reduce the availability of P and further increase C:P and N:P ratios. If the global balance is calcification, the opposite result is expected. To understand better the effects of elevated [CO_2] levels on the stoichiometry of oceans, studies should examine the indirect effects of elevated [CO_2] levels through changes in water pH and phosphates solubility.

The environmental impact of elevated [CO_2] levels can act in parallel to other factors that promote global change (e.g., warming, UV-B radiation, eutrophication), which can affect C and N cycling in ecosystems in several ways (Beardall and Raven 2004; Kuijper et al. 2005). The predicted widespread increases in C:N and C:P ratios caused by increases in atmospheric [CO_2] (Finkel et al. 2010) might be exacerbated by the the stratification of water caused by warming. This stratification, impedes the vertical mixing of the water column and reduces the availability of N and P in superficial waters (Beardall and Raven 2004; Tortell et al. 2008; Feng et al. 2008). In that situation, an increase in N availability through N_2 fixation is likely to lead to a reduction in the availability of P and, consequently, an increase in the N:P ratios in oceans and large lakes. Indeed, that phenomenon is already common in most ocean areas (Thingstad et al. 2005).

Research is needed to determine whether those changes have effects on the species composition of communities because they favour species that have low growth rates or invest heavily in conservation mechanisms, and to elucidate further the ecosystem responses of trophic webs through top-down and bottom-up effects.

Cascade effects

For zooplankton, high [C:nutrient] ratios in phytoplankton reduce the food quality, which leads to changes in trophic webs that favour species that are less nutrient demanding (van de Waal et al. 2010). Plankton growth (C-storage) and reductions in food quality (increases in plankton C:N and C:P ratios) can reduce the efficiency of mass transfer to herbivores (Urabe et al. 2003). Furthermore, the high C:N and C:P ratios in food can induce increased respiration in consumers, which increases CO_2 production and the amount of C lost to the atmosphere (Hopkinson and Vallino 2005); however, some herbivores can mitigate that effect by choosing phytoplankton species that have low C:nutrient ratios (Urabe and Waki 2009). Thus, it appears that elevated atmospheric [CO_2] levels have two negative effects on species that have low C:nutrient ratios: a direct effect of elevated [CO_2] levels, which favour high C:nutrient ratios, and an indirect effect through the selection of phytoplankton that have low C:N ratios by herbivores. Atmospheric [CO_2] continues to increase; therefore, species that have relatively high C:N ratios are expected to be favoured, which will alter the species composition of communities.

Eutrophication

Increases in the quantities of nutrients, especially N, in the environment are some of the main drivers of global change (Vitousek et al. 1997); thus, it is important to understand the anthropogenic effects of those increases on N concentrations and N:P of ecosystems (Galloway et al. 1994; Falkowski et al. 2000).

Terrestrial ecosystems

In Jul 2011, the ISI Web of Science database contained at least 48 studies that presented C:N:P data under realistic scenarios of future nitrogen deposition (Table S2 of Appendix 1). Eight and nine studies provided enough information (including variance) to perform meta-analyses of the effects of adding N on the C:N and N:P ratios of herbaceous plants, respectively, by analyzing photosynthetic tissues (leaves and aboveground biomass). In 25 of 31 experiments, C:N ratios in photosynthetic tissues decreased in response to supplemental N; in five experiments, C:N ratios did not change significantly and, in one study, the ratio increased (Figure 3). A meta-analysis demonstrated that added N reduced significantly the C:N ratios of the photosynthetic tissues of

woody ($P < 0.05$, $n = 25$) and herbaceous ($P < 0.05$, $n = 6$) plants (Figure 4). On average, supplemental N reduced the C:N ratios of photosynthetic tissues by 25% ($P < 0.05$, $n = 31$) (Fig 4). In 20 of 36 species, the addition of N increased the N:P ratios of photosynthetic tissue; in 15 species, N:P ratios did not change significantly and, in one species, the ratio decreased (Figure 3). The addition of N significantly increased the N:P ratios of the photosynthetic tissues of woody ($P < 0.05$, $n = 10$) and herbaceous ($P < 0.05$, $n = 12$) plants (Figure 4) and, on average, supplemental N decreased leaf C:N ratios by 25% ($P < 0.05$, $n = 22$) (Fig 4). Supplemental N reduced significantly ($P < 0.05$, $n = 19$) the C:N ratios of the organic soil horizons (Figure 4), but not those of the mineral layers ($P > 0.05$, $n = 12$) (Figure 4).

In northern and central Europe and North America, increases in N deposition have reduced the C:N ratios and increased the N:P ratios of soils, plants (Bobbink et al. 1998; Yesmin et al. 1996; Bragazza et al. 2004; Pardo et al. 2007), and freshwater ecosystems (Elser et al. 2009a) (Figure 3 and Table S2 of Appendix 1). In the last century, the concentrations of P and, especially, N have increased in the plants of terrestrial ecosystems in the Mediterranean Basin (Peñuelas and Filella 2001). Increases in the deposition of N contribute to nutrient imbalances because the increases are not matched by increases in the uptake of other nutrients, e.g., P, Mg, and Ca. Reductions in the C:N ratios in plants can improve their palatability for herbivores (Jefferies and Maron, 1997).

Plant species can respond to increases in the abundance of N by increasing P uptake or by increasing P reabsorption capacity (Phoenix et al. 2004; Fujita et al. 2010); however, long-term N-deposition can increase the N:P ratio in plants (Fujita et al. 2010), which probably contributes to changes in the species composition of terrestrial and aquatic ecosystems (Jefferies and Maron 1997; Smith et al. 1999); e.g., by depleting the soil N_2 fixation community (Compton et al. 2004). Changes in the composition of communities might occur because species that have low growth rates are at an advantage when N availability is high according to the growth rate hypothesis (GRH) (Elser et al. 2000b and 2000c) or because of a reduction in soil N heterogeneity under high rates of N deposition; e.g., in the herbaceous communities of North American forests (Gilliam, 2006).

Increases in N deposition might act in concert with forecasted increases in atmospheric [CO_2] to increase the growth and productive capacity of forests (Maurer et al. 1999); apparently, however, P limitations can hinder significantly the N fertilization effect that increases C storage in biomass by increasing growth. Largely because of anthropogenic inputs, ~ 48-54 Mtones N yr^{-1} and ~ 63.5 Mtones N yr^{-1} are deposited in oceans (Duce et al. 2008: Schlesinger, 2009) and terrestrial ecosystems (Galloway et al. 2004), respectively. Global P deposition is 3-3.5 Mtones $year^{-1}$, mostly as wind-eroded particles (Smil 2000; Mahowald et al. 2008) and aerosols from burnt biomass (Àvila et al. 1998; Baker et al. 2006), of which ~90% is deposited on land and only

~10% in oceans (Graham and Duce, 1979). Thus, the N:P deposition ratio (on a molar basis) is > 20 times the Redfield Ratio (Redfield et al. 1963; Falkowski and Davis 2004) and 2-3 times the soil N:P ratios that are optimal for the growth of most terrestrial plants (Knecht and Göransson 2004).

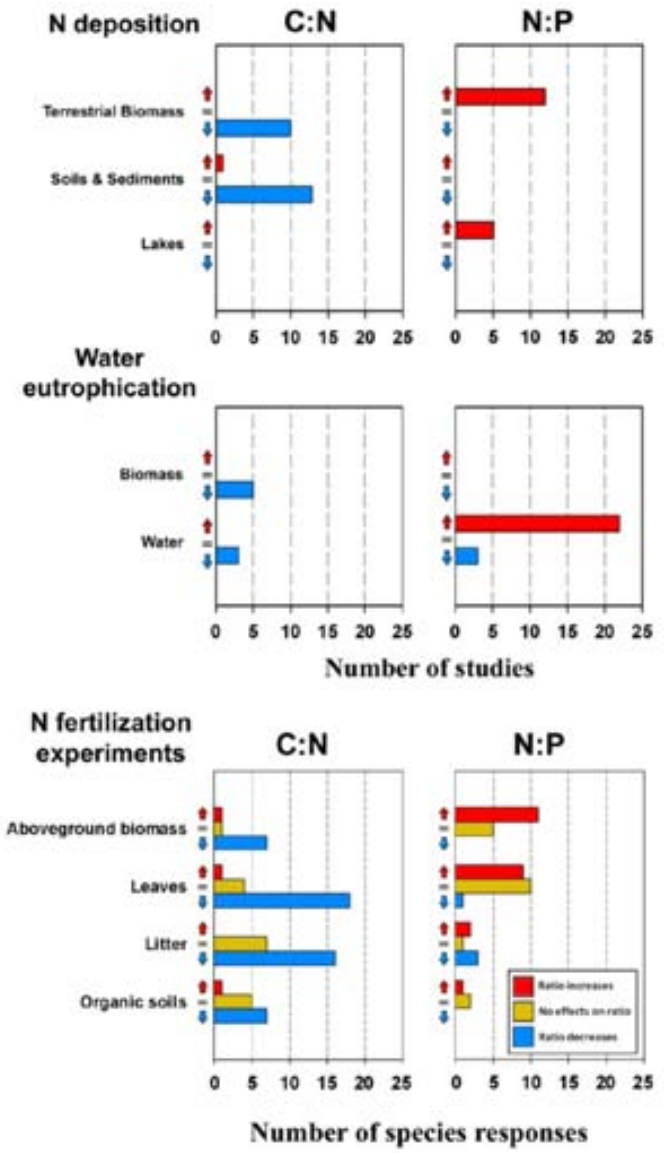

Figure 3. Number of reported increases, decreases, and absence of change in C:N and N:P ratios in response to N deposition, water eutrophication, or N fertilization experiments. (Detailed data in Table S2 of Appendix 1). [*See comments for Figure 1*]

Remote ecosystems such as low alpine heathlands, which often are N and P co-limited are less likely to sequester N inputs in the production of additional biomass and, therefore, such ecosystems might be the most vulnerable to the effects of N saturation and to the movement of N into surface waters (Britton and Fisher 2007). Other consequences of the increases in the N:P ratios of ecosystems in response to N deposition involve interactions with the other factors that promote global change. For instance, increases in the N:P ratio caused by N deposition can increase the success of alien plant species (Burke and Grime 1996).

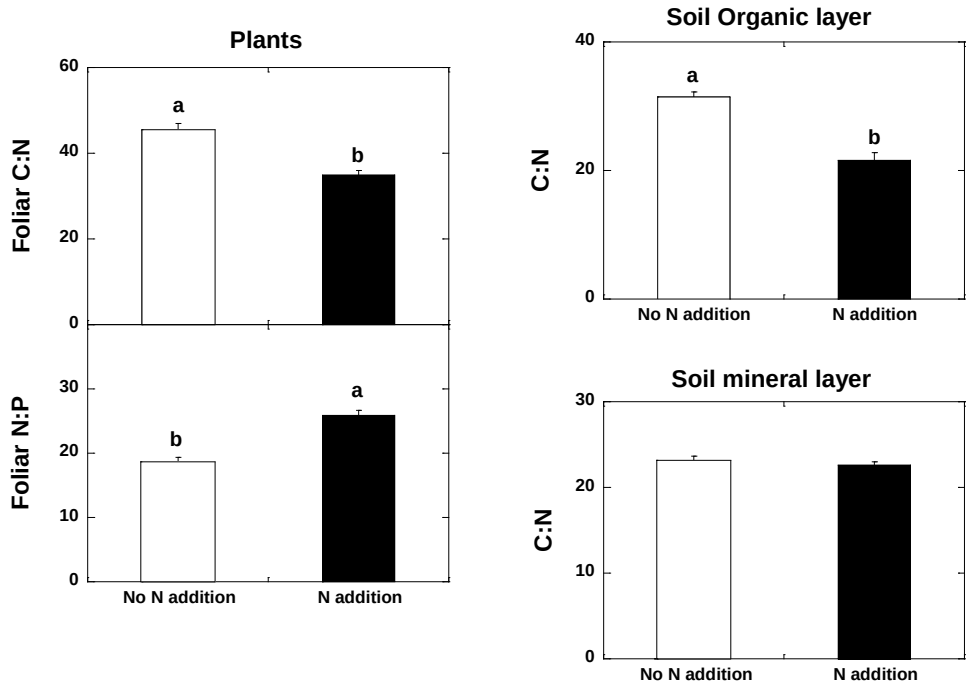

Figure 4. Leaf C:N and N:P ratio of plants growing at ambient and in N addition treatment. Metadata-analysis of 31 different experimental results in the case of leaf C:N ratio and of 22 different experimental results in the case of leaf N:P ratio. Soil organic and inorganic C:N ratios at ambient and in N addition treatment. Only those studies that provided the mean (± S.E.) of leaf C:N and C:P ratios of plants, and soil C:N ratios growing at ambient and in N addition treatment were taken into account. We conducted meta-analyses by using MetaWin Package (Rosenberg et al. 2000), that is based in the knowledge the control and treatment results (mean ± SD) in each study (considering as study each species studied). Different letters indicate statistically different values ($P < 0.05$).

In crop soils subjected to intensive fertilization, e.g., continuous fertilization using pig slurry or livestock excrement, eutrophication can be substantial (Cech et al. 2008; Peñuelas et al. 2009) (Table S2 of Appendix 1). Excessive applications of N and P tend to reduce soil N:P ratios because animal slurries have low N:P ratios, and P is less soluble and tends to accumulate in the soil more so than does N. The N:P ratio of pig slurry is low (2.0-3.8) and its continuous use has reduced the soil N:P ratio in several Spanish crop soils (Peñuelas et al. 2009). Intensive fertilization has increased the P and, especially, N leachates from fields (Gundersen et al. 2006). In contrast, areas that are dominated by crops that are subjected to heavy industrial fertilization without important presence of pasture have streams and lakes that have high N:P ratios (Arbuckle and Downing 2001). Those fertilization processes have affected the continental aquatic ecosystems in

agricultural areas, including changing the C:P and N:P ratios of seston and sediment, which has affected the species composition and growth of phytoplankton, zooplankton, and fish (Arbuckle and Downing 2001; Turner et al. 2003; von Schiller et al. 2007, Pilati et al. 2009; Frost et al. 2009). P leaches from soils, mainly, as particulate organic matter, and most nitrogen leaches in dissolved forms (Inoue and Ebise 1991). In areas that have torrential rainfalls and soil erosion, large amounts of N and P can be leached out.

Aquatic ecosystems

In several coastal areas (including those along large lakes), the N and P inputs delivered by continental streams are the main cause of the shifts in the C:N:P of waters and ecosystems. In freshwater and, especially, ocean ecosystems, however, atmospheric inputs of N and P often are the main causes of changes in C:N:P ratios. Although the extent of the changes in N:P ratios varied considerably, the N:P ratios in aquatic ecosystems tended to increase.

In recent decades, the amount of anthropogenic waste received by coastal areas via rivers has increased (Robertson et al. 2008). Changes in the N:P ratios of the waste products delivered by continental waters have led to changes in eutrophication and the species composition of ecosystems near the mouth of large rivers in marine coastal areas and in large lakes (Kufel and Kufel 1997; Pinto-Coelho 1998; Johnson et al. 2006; King and Richardson 2007; Xu et al. 2008; Santos et al. 2008; Teichberg et al. 2008; Lucea et al. 2005). Anthropogenic waste has reduced the N:P ratios of inorganic particles in several coastal areas (Ryter and Dunstan 1971; Ruiz-Fernández et al. 2007) (Table S2 of Appendix 1) and, in those area approximately half of the P present cannot be used by algae because rivers that have high concentrations of P-detergent have low N:P ratios; however, the replacement of P by N in detergents is unlikely to slow the eutrophication of coastal marine waters that receive waste that has a low N:P ratio and it might even exacerbate the situation because N is the limiting factor (Rhyter and Dunstan 1971).

In other coastal areas, eutrophication is influenced by high N:P ratios in waste (e.g., Harrison et al. 2008; Li et al. 2010, Table S2 of Appendix 1). Recent studies have shown that changes in the N:P stoichiometry of coastal waters subjected to eutrophication are associated with high N:P ratios in water and sediments (Figure 3 and Table S2 of Appendix 1), which is related to increases in the N:P ratios of the streams of continental waters in agricultural areas (see below). By altering the N:P ratio, eutrophication often favors algal species that can compete for the limiting nutrient, which gives them the potential to become dominant and form blooms (Granéli et al. 2008). That problem is particularly serious for harmful algal species that excrete dangerous allelopathic organic compounds (Granéli et al. 2008; Teichberg et al. 2008).

In freshwater ecosystems, N deposition can reduce the C:P and C:N ratios of epilithon, change phytoplankton species composition (Liess et al. 2009), and reduce the population of N_2 fixers (Liess et al. 2009; Vrede et al. 2009), which can have a compensatory effect on N:P ratios (Vrede et al. 2009). In lakes, increases in N:P ratios in response to long-term N deposition have paralleled shifts from a N-limiting to a P-limiting environment (Elser et al. 2009a, 2009b), and, the more N-limited the lake, the greater the impact of eutrophication (Abell et al. 2010). Types of terrestrial vegetation can differ in their response when N deposition changes the threshold for N leaching into continental waters (Rowe et al. 2006). In continental streams, eutrophication can reduce C:P ratios (Frost and Elser 2002; Dang et al. 2009). A low C:P ratio favors fast-growing species that out compete and exclude slow-growing taxa (Frost and Elser 2002), which diminishes the ecosystem's biodiversity (Evans-White et al. 2009).

In the open ocean, experiments have shown that increases in the N:P ratios of the atmospheric inputs can increase the amount of P-limited areas (Thingstad et al. 2005), a phenomenon that can be enhanced by elevated [CO_2] and by greater water column stratification caused by warming altogether increasing P demands and N:P ratios.

Aquatic ecosystems can buffer changes in N:P ratios through processes such as N_2 fixing (Scott et al. 2008; Vrede et al. 2009). In addition, reductions in the C:N ratios of dissolved organic matter and the sediments in lakes, lagoons, and estuaries increase denitrification rates (Herbert 1999). Grazers can buffer moderate eutrophication by selectively consuming algae species that have low C:N ratios (Worm et al. 2000).

Climate change

Warming

In dry terrestrial ecosystems, warming can have no effect or can increase the leaf C:N ratios of the predominant shrub species in Mediterranean shrublands and in temperate, non-Mediterranean grasslands (Figure 5 and Table S3 of Appendix 1); probably, because of increases in water stress in areas that have a Mediterranean climate (Sardans et al. 2008a) and increases in NUE, which is associated with increases in biological activity in temperate grasslands (An et al. 2005). Increases in ambient temperature can increase respiration rates, which increases organic-C mineralization and, thus, counterbalance the direct effect of an increase in the C-storage capacity of an ecosystem caused by an increase in atmospheric [CO_2] (Finkel et al. 2010; Ferreira et al. 2010). That might explain why several studies did not detect an effect of elevated ambient temperatures on the C:N ratios of some plants (Figure 5 and Table S3 of Appendix 1). Typically, carbon cycling models assume that respiration increases exponentially in response to increases in

temperature and that plants do acclimatize to long-term increases in ambient temperature, however, plant respiration can increase after short- and medium-term increases in temperature (Atkin et al. 2000; Hartley et al. 2006), and plant species differ in their capacity to acclimatize their respiration to long-term increases in temperature (Atkin et al. 2000). In addition, respiration responses to warming can depend on the availability of light, water, and CO_2, and responses can differ among species and plant organs (Atkin et at., 2003). Thus, evidence suggests that the increases in temperature predicted by climate models will increase plant C:N and C:P ratios in warm-dry and temperate climates through water stress resistance mechanisms or increases in NUE. This increased C:N and C:P ratios, which might be exacerbated when warming and drought coincide in semi-arid or mesic environments, e.g., Mediterranean Basin, Sahel or might be counterbalanced by the effects of an increase in respiration.

In cold, terrestrial ecosystems that are not water-limited, however, the effects of warming on plant C:N ratios are not well understood. While some studies have demonstrated that warming reduced plant C:N ratios by increasing the production capacity, biological activity, and nutrient uptake of plants (Welker et al. 2005; Aerts et al. 2009), others in cold grasslands did not find effects (Larsen et al. 2010) or reported an increase in the C:N ratio associated with a dilution effect by an increase in biomass production (Day et al. 2008) (Table S3 of Appendix 1).

In oceans and large lakes, warming can increase water stratification (Finkel et al. 2009; Feng et al. 2008), which impedes the rise of nutrients from the sediment to the surface. Consequently, ocean production capacity is diminished and the C:P and C:N ratios of ocean food webs are increased (Sarmiento et al. 2004; van de Waal et al. 2010); however, warming can have a compensatory effect on the C:N ratio by reducing dissolved CO_2 levels and increasing N_2-fixation in oceans. N_2-fixation by heterocystous cyanobacteria occurs mainly in tropical waters at temperatures $\geq$ 25 ºC (Stal 2009). An increase in the area in oceans where temperatures are >25 ºC can increase the N_2-fixation capacity of oceans, worldwide. Increases in atmospheric [CO_2] have increased the N_2-fixing capacity of aquatic organisms such as cyanobacteria (Levitan et al. 2007; Kranz et al. 2010) and diatoms (Wu et al. 2010); however, reductions in N_2-fixation capacity in response to long-term high CO_2 concentrations have been observed in ocean ecosystems (Levitan et al. 2007). Those compensatory mechanisms might explain why the C:N ratios of aquatic organisms did not change (Iglesias-Rodríguez et al. 2008) or were reduced (Fu et al. 2007) under elevated [CO_2] levels.

The extent to which N_2 fixation can be increased can be limited by the availability of P in terrestrial and aquatic ecosystems (Hungate et al. 2004; van Groenigen et al. 2006; Weber and Deutsch 2010). In oceans, increases in atmospheric CO_2 concentrations can increase the N:P ratios in phytoplankton (Fu et al. 2007) because P is not as easily incorporated from other

sources as is N. In aquatic ecosystems, simulated warming of enzymes increased the P demands for the synthesis of protein and RNA, which can make P limited (Persson et al. 2010). The capacity of ocean ecosystems to store C and, therefore, to buffer increases in atmospheric CO_2 concentrations might be hampered significantly by a reduction in N_2-fixation capacity when P is limited. Increases in temperature can also alter the diffusion of nutrients in water because of changes in the boundary layer around organisms, which can cause changes in an organism's stoichiometry (Raven and Geider 1988).

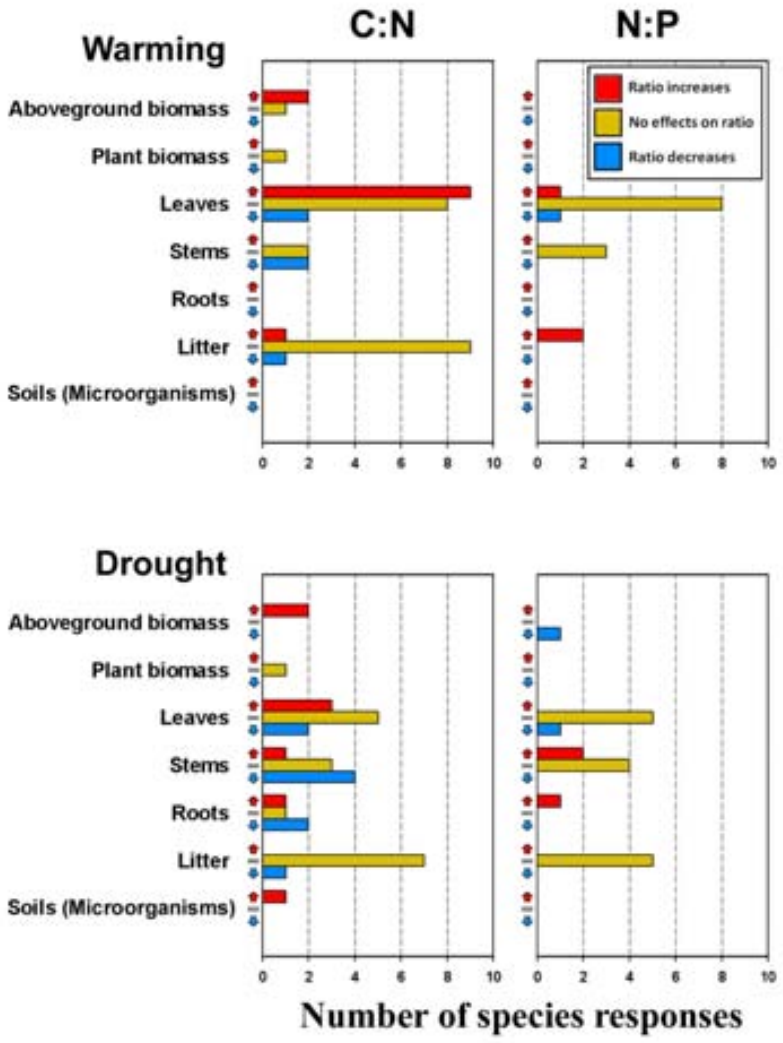

Figure 5. Number of reported increases, decreases, and absence of change in C:N and N:P ratios in response to warming or drought. (Detailed data in Table S3 of Appendix 1).

Drought

Models predict an increase in the extent of drought over large areas of the world which will threaten areas that are already dry, such as the Mediterranean Basin and the Sahel (IPCC 2007). The effects of increased drought differ among ecosystems and species. In semi-arid areas in the Mediterranean Basin, drought reduced the C:N ratios in *Quercus ilex* roots, and

proportionately more N was allocated to the root system which was due to an increase in water absorption capacity (Sardans et al. 2008b, González et al. 2010b). Drought increased the C:N and C:P ratios of the leaves of Mediterranean shrubs and trees, which are protective mechanisms (Inclan et al. 2005; Sardans et al. 2008a and 2008c; Matías et al. 2010) associated with sclerophylly and increases in lignin (Bussotti et al. 2000; Sardans et al. 2006a). In temperate heathlands, drought increased the C:N ratio (Larsen et al. 2010). Reduced water uptake, lower N soil mobility, and a reduction in the N released from soil organic matter are some of the factors that contribute to the reduction in N uptake. The transpiration stream can play a role in increasing the movement of soil N towards the rhizosphere (González-Dugo et al. 2010).

In wet-temperate ecosystems, the C:N ratio can decrease under moderate drought because plants increase N uptake and reduce growth (Lu et al. 2009). Thus, the evidence suggests that drought tends to increase the C:N ratios of photosynthetic tissues in semi-arid environments, but the effects are no as clear in wet ecosystems (Table S3 of Appendix 1), where drought can affect multiple aspects of plant and soil microbes, which can differentially affect soil N and its availability (for a review, see Rennenberg et al. 2009).

The effects of the increase in drought predicted by climate models on N:P ratios in terrestrial ecosystems are not clear. Few studies have actually measured the N:P ratios in plants that have been subjected to experimental drought treatments in the field at the level predicted by climatic models, and those studies were inconclusive.

In dry regions, the increases in the C:N ratios in litter caused by drought can combine with increases produced in response to elevated [CO_2] levels, which suggests a synergy that increases litter C:N and, probably, C:P ratios, slows N and P cycling by reducing food quality, and reduces N and P availability and N and P concentrations in standing biomass. Some studies suggest that medium-term drought can increase N and P concentrations in soils and reduce their concentrations in standing biomass (Sardans and Peñuelas 2004 and 2007; Sardans et al. 2008a, 2008b and 2008e). This contrasting response in plants and soils is aggravated further by the direct impact of drought, which reduces soil enzyme activity and nutrient availability (Sardans and Peñuelas 2005 and 2010; Sardans et al. 2006b and 2008d). All these effects are likely to reduce water use efficiency and, ultimately, plant fitness by reducing the plant's capacity to adapt to drought. Thus, there is a need to examine the effects of elevated [CO_2] and drought on N:P ratios and food webs, concurrently, in a range of climates from semi-arid to wet areas.

Other global change drivers

Other drivers of global change such as species invasiveness, UV radiation, and changes in land use can alter ecological stoichiometry, but the possible effects of those factors have not

been studied extensively. Nevertheless, studies have shown that those factors can have a significant effect on the C:N:P ratios of organisms and the environment (Elser et al. 2003; Mulder and Bowden 2007; Siemann and Rogers 2007).

Although the success of invasive species in nutrient-rich environments is associated with low body C:P and N:P ratios, high nutrient uptake capacity, and rapid growth, in nutrient-poor environments, the success of alien species is associated with high nutrient use efficiency and high, but variable, C:P and N:P ratios (Funk and Vitousek 2007; González et al. 2010a). The immediate effects of plant invasiveness are changes in the stoichiometry of soils and detritus; however, some studies have shown that invasive plant litter (Hughes and Denslow 2005; Whitcraft et al. 2006) and soils (Saggar et al. 1999; Domènech et al. 2006) had reduced C:N ratios in response to alien plant invasion, while others observed contrary results (Evans et al. 2001; Drenovsky and Batten 2007).

UV radiation can reduce algal N:P ratios because it increases P uptake, probably, as a response to increased demands for nucleotide repairs necessitated by UV radiation stress (Hessen et al. 2008). In addition, some studies suggest that UV radiation can alter the C:N and N:P stoichiometries of organisms and ecosystems by reducing N_2-fixation capacity (Solheim et al. 2006).

Often, changes in land use involve change in vegetation type, which can have a significant influence on the C:N:P stoichiometry of ecosystems. The substitution of aquatic riparian vegetation by non-riparian vegetation increased the C:N ratios of herbivorous aquatic invertebrates in rivers because of the higher C:N ratios in the non-riparian than riparian plants as food source (Blanch et al. 2000; Deegan and Ganf 2008). This changed C:N ratios can imply further changes in the composition of plankton species (Scheffer et al. 2001; Deegan and Ganf 2008). The clearing of tropical rainforests for cattle pastures is a widespread change in land use, especially, in South America. In one study, dissolved and inorganic N:P ratios were lower in streams in pastures than they were in the streams in forests and grasslands (Neill et al. 2001).

Stoichiometry in response to global change: knowledge and perspectives

Current knowledge

Effects of Elevated [CO_2]

In terrestrial ecosystems, many (but not all) studies have reported that increases in atmospheric [CO_2] lead to higher plant C:N ratios, especially, in photosynthetic tissues (overall 22% more as observed in the meta-analysis). The phenomenon is particularly evident in C3 grasses,

but is absent in C4 grasses. Elevated atmospheric [CO_2] also lead to on average 38% higher foliar C:P ratios in C3 plants.

In areas of open ocean and in many other aquatic ecosystems, experiments have shown that the extent of P-limited areas has increased in response to several drivers of global change. Increases in atmospheric [CO_2] increase P uptake demands, warming increases water column stratification and N_2-fixation capacity, and atmospheric inputs increase N:P ratios.

Eutrophication effects

Available studies show a 25% average decrease of foliar C:N ratio in response to simulated experimental N deposition. N deposition also increases the N:P ratios of plants in terrestrial and freshwater ecosystems, which has the potential to alter the species composition of communities, reduce the community of N_2-fixers in soil and water, and reduce species diversity. Reductions in the abundance of N_2-fixers in response to increased N-deposition can have compensatory effects on N:P ratios but typically, these effects are insufficient to prevent an increase in the N:P ratios of organisms and ecosystems. High N:P ratios favor species that have low growth rates and high rates of N deposition a reduce the heterogeneity of soil N..

In several croplands throughout the world, the excessive application of N and P , mainly, from animal slurries, has reduced soil N:P ratios because, typically, the N:P ratios of animal slurries are low, and because P is less soluble than N and, therefore, the former has a stronger tendency to accumulate in soil. Intensive fertilization results in N leachates from fields, which has consequences for continental aquatic ecosystems because these leachates increase N:P ratios in the freshwater ecosystems of those croplands.

Effects of climate change

Warming can increase, decrease, or have no effects on the C:N ratios of plants depending on the type of plant and the climate. The current results suggest that warming and drought can increase C:N and C:P ratios in warm-dry and temperate-dry terrestrial ecosystems because of an increase in nutrient use efficiency or an increase in resistance to drought (a protective mechanism associated with an increase in water use efficiency and the prevention of water loss). The effect can be exacerbated when warming and drought coincide in semi-arid or mesic environments, and furthermore by the effects of increases in atmospheric [CO_2]; however, the effect can be counterbalanced by an increase in respiration in response to increased warming. In cold or temperate ecosystems where water is not limiting, warming tends to reduce the C:N ratio.

Prospects for future studies

Effects on the structure and functions of terrestrial and aquatic ecosystems

Under high atmospheric [CO_2] levels, the C:P ratios of plants tend to increase, and plants that have high C:P and N:P ratios often have relatively slow growth rates (Elser et al. 2000b and 2003). Thus, in a high [CO_2] atmosphere, the species composition of forests might shift towards a predominance of slow-growing species that have high C:P ratios, with a reduction in C transfer (and N and P) from the soil and atmosphere into aboveground biomass. Such shifts might be particularly important in tropical rainforests, where N and, especially, P are limiting.

P can be a key limiting resource

P can become progressively limiting because it influences plant production capacity, directly, and limits N_2 fixation. The widespread use of fertilizers results in an increase in the N:P ratios of the loadings in natural terrestrial ecosystems and aquatic ecosystems, and, in some cropland ecosystems, the N:P ratios of soils can be reduced because of the high solubility of N. Thus, in general, the importance of P as a limiting factor in both terrestrial and aquatic ecosystems is increasing (Peñuelas et al. 2011). On the other hand, increases in N deposition can act in concert with increases in atmospheric [CO_2] to increase the capacity of some natural terrestrial and aquatic ecosystems to store C.

C:N:P homeostatic capacity

Several studies suggest that, to varying degrees, organisms have the capacity to control their stoichiometry (Sterner and Elser 2002; Andersen 1997; Yu et al. 2010). To predict changes in the composition of ecosystems in response to changes un C:N:P ratios caused by global change, more needs to be known about the C:N:P homeostatic regulation capacity of species (Elser et al. 2010). Species that have a homeostatic stoichiometry predominate in stable environments, but not in environments where the stoichiometry is highly variable because of the high energetic costs of maintaining a stable body stoichiometry in these environments (Sterner and Elser 2002). Thus, the rapidly increasing global levels of [CO_2] and nutrient disequilibrium are likely to increase the instability of ecosystems, which will favor species that have low homeostatic stoichiometry. It is important to know whether species that have the capacity for rapid growth, high nutrient concentrations, and low N:P ratios have more or less homeostatic capacity and are more or less able to cope with changes in environmental stoichiometry than are species that are slow growing, have low nutrient concentrations, and high N:P ratios.

Asymmetrical responses in terrestrial ecosystems

The effects of the drivers of global change on the structure and function of terrestrial ecosystems through their effects on C:N:P stoichiometry can be asymmetrical at the global scale depending on water availability. In regions such as the Mediterranean Basin, where water is strongly limiting and an increase in drought is predicted, changes in C:N:P might be very important. Moreover, the importance of understanding changes in C:N:P will increase as large areas of the world become drier (IPCC 2007). A reduction in the N and P concentrations in standing biomass has been observed in response to drought in these already dry areas. N and P are associated with water use efficiency, and therefore, their concentration reductions are likely to reduce a plant's capacity to adapt to drought. The role of elevated atmospheric [CO_2] and drought in increasing the C:N and C:P ratios of the photosynthetic tissues of plants in semi-arid environments suggests additional negative effects on the performance and viability of ecosystems, such as the accumulation of N and P in soils and losses caused by torrential rainfall. Thus, the synergistic effect of drought and elevated atmospheric [CO_2] levels might slow N and P cycling by reducing food quality.

New techniques

To assess the causes underlying the stoichiometric changes caused by global change and their effects on trophic webs, ecological stoichiometric studies should be complemented with molecular studies of sugars, amino acids, proteins, RNA… Elements do not act alone; rather, they act as molecular components. Thus, changes in elemental composition can alter an organism's molecular composition. By coupling studies of the stoichiometric changes in plants, herbivores, and predators with metabolomic studies, it will be possible to determine whether changes in C:N:P ratios are caused by or lead to changes in defensive, storage, anabolic (growth), or catabolic (respiration) allocations (Peñuelas and Sardans 2009a). Improved analytical and data treatment methods have eased the task of determining and quantifying compounds and identifying their roles in elemental stoichiometries. Metabolomics will aid in the interpretation of the stoichiometric responses to the drivers of global change, such as the changes in the allocation of resources to growth, storage, and defense, and help in understanding the processes that underlie an organism's adaptation to these factors (Peñuelas and Sardans 2009b). Furthermore, that should help in understanding the impact of stoichiometric changes within trophic webs because changes in C:P or C:N ratio do not provide an accurate measure of the change in the food quality because the latter also depends on the molecular composition.

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Ecological metabolomics: overview of current developments and future challenges

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Abstract

Ecometabolomics, that aims to analyze the metabolome, the total number of metabolites and its shifts in response to environmental changes is gaining importance in ecological studies due to the increasing use of new technical advances, such as modern HNMR spectrometers and GC-MS coupled to bioinformatic advances. We review here the state of the art and the perspectives of ecometabolomics. The studies available demonstrate ecometabolomic techniques have great sensitivity in detecting the phenotypic mechanisms and key molecules underlying organism responses to abiotic environmental changes to biotic interactions. But such studies are still scarce and in most cases they are limited to the direct effects of a single abiotic factor or of biotic interactions between two trophic levels under controlled conditions. Several exciting challenges remain to be achieved through the use of ecometabolomics in field conditions, involving more than two trophic levels, or combining the effects of abiotic gradients with intra and inter-specific relationships. The coupling of ecometabolomic studies with genomics, transcriptomics, ecosystem stoichiometry, community biology and biogeochemistry may provide a further step forward in many areas of ecological sciences, including stress responses, species life style, life history variation, population structure, trophic interaction, nutrient cycling, ecological niche and global change.

Keywords Abiotic relationships · Atmospheric changes · Biotic relationships · Competition · Ecology · Ecophysiology · Eutrophication · GC-MS · Global change · HPLC-MS · Invasiveness · Metabolome · NMR · Nutrients · Plant-animal · Pollution · Stoichiometry · Trophic webs · Water.

Introduction

The possibility of using progressively improved metabolomic techniques in ecophysiological and ecological studies has opened up a new way to advance knowledge of the structure and function of organisms and ecosystem. Metabolomics is the analysis of the complete metabolome (all the metabolites that one organism produces) at one moment (Fiehn 2002). It provides phenotypical response at metabolic level in a particular environmental circumstance. Moreover, it is also a powerful tool to monitor the phenotypic variability of one genotype in response to environmental changes in drought (Fugamalli et al. 2009), nutrient availability (Hirai et al. 2004 and 2005), pollutants (Jones et al. 2007; Bundy et al. 2008), salinity (Fugamalli et al. 2009), temperature (Michaud and Delinger 2007) and biotic interactions (Choi et al. 2006) among other ecological factors. These studies are especially adequate in plants because metabolomic studies enable the simultaneous analysis of primary compounds together with secondary compounds which have a defensive and protective function.

Metabolomics provides a better analysis of the different response capacities conferred by the phenotypic plasticity of each species allowing to ascertain what metabolic pathways are involved in a phenotypic response. Moreover, this facilitates the transcriptomics change research (Hirai et al. 2004; Brosché et al. 2004; Fukushima et al. 2009a). Metabolomics can also be coupled to genomic studies for faster determination of the genes involved in adaptative responses (Bino et al. 2004; Oksman-Caldentey and Saito 2005). This approach has therefore a great potential to elucidate gene function and to establish data networks. In this way, it advances our knowledge of the development of phenotypic plasticity (Via et al. 1995; Pigliucci 2005) and its evolutionary consequences (Agrawal 2001). In this regard, metabolomics has several advantages compared to more conventional methods of analyses in chemical ecology (extract fractionation, purification and bioassays). Since all compounds are measured at once in only one step, rather than put through iterative purification steps, unstable compounds are more likely to be detected and measured. Metabolomics can be also used as a preliminary screening study of the metabolome response. This does not exclude the simultaneous or posterior use of target chemical analyses.

Recent rapid improvements in analytical methods and in the ability of computer hardware and software to interpret and visualize large datasets (Gehlenborg et al. 2010) have multiplied the possibilities of rapidly identifying and quantifying simultaneously an increasing number of compounds (e.g. carbohydrates, aminoacids and peptides, lipids, phenolics, and terpenoids). These advances will enable us not only to take ‘static pictures’ or snapshots of the metabolome, but also to capture and to ‘film’ its dynamic nature. Ecological metabolomics can thus serve as a powerful indication for defining organism lifestyle. All in all, we may now be able to achieve a

dynamic, holistic view of the metabolism and health of an organism, a population, or an ecosystem, and in this fashion open the door to exciting new insights in ecology.

This study reviews the state of the art of ecological applications of metabolomic techniques giving an overview of the current findings reached by its use in ecophysiological and ecological studies. We also discuss the possible future contribution of metabolomics to progress in ecophysiology and ecology. To achieve such progress, we highlight the need to couple metabolomic studies with other omic studies such as genomics, transcriptomics and proteomics. The aim is to reach an overview of organism response to environmental changes at different time scale and from genotype to phenotype. We also discuss the ecological topics where metabolomics could be more successfully used. Among these topics we highlight ecosystem stoichiometry, plant-herbivore-predator systems, parasitism, climate change, and invasive species, among others (Figure 1).

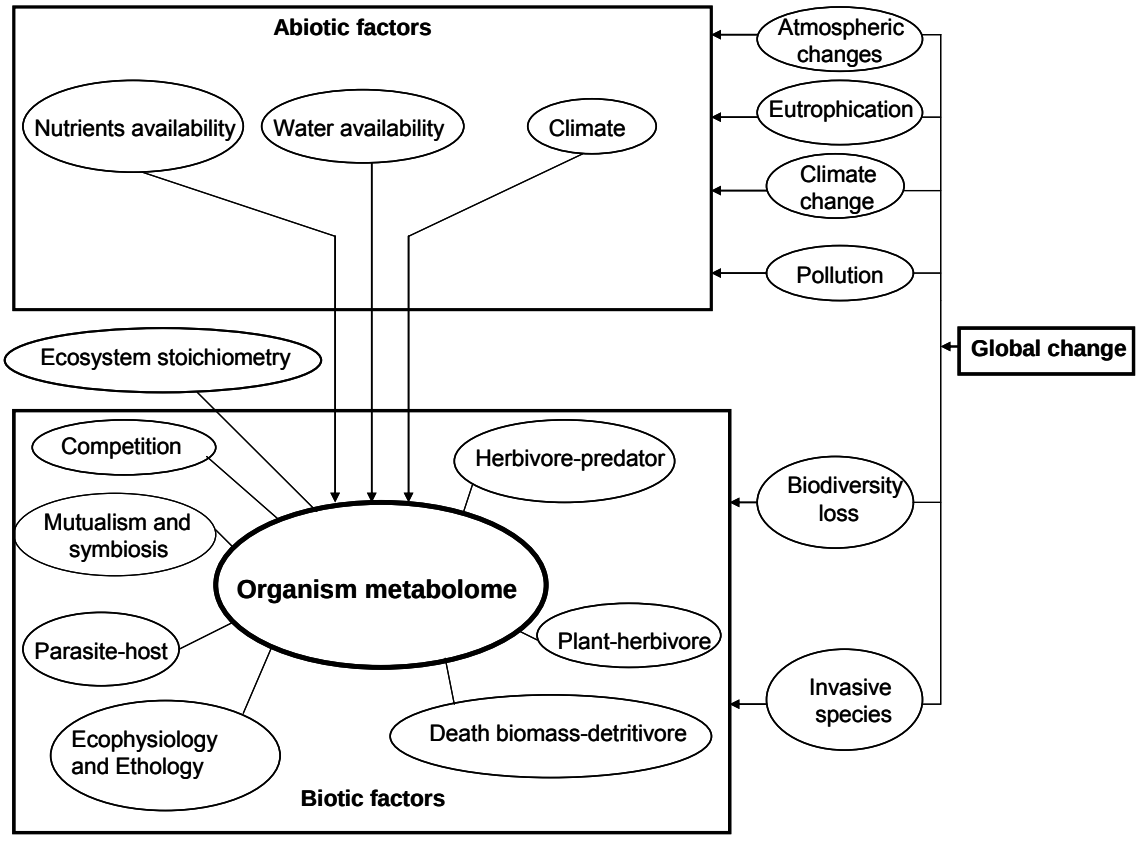

Figure 1. Ecological topics where ecometabolomics should represent a direct tool to advance.

Ecometabolomics

We use here terminology based on that of Fiehn (2002) and Schripsema (2010) but simplified for metabolomic techniques applied to ecological studies. Briefly, the study type is called metabolomic profiling when the aim is the quantitative analysis of a set of metabolites of a selected number of metabolic pathways or of metabolite types (e.g. polar, semipolar or non-polar) with or without its identification. We propose to call partial ecometabolomic studies (PEM) those metobolomic profiling studies applied to ecophysiologycal or ecological studies that aim to elucidate the effects of biotic or abiotic factors on a specific whole pathway or intersecting pathways by identifying the metabolite or set of metabolites involved in organism response to environmental changes by using general qualitative and quantitative analytical techniques such as NMR or GC-MS and data mining statistical analyses. In this way the target studies using more target techniques such as HPLC focused in the study of some limited set of metabolites are not considered ecometabolomic studies.

When it is not required or possible to identify every metabolite, it is often sufficient to rapidly classify samples according to their origin or their ecological or ecophysiological relevance. This process is called metabolic fingerprinting, that now in an ecological context, we propose to name ecometabolic fingerprinting. This "holistic" method enables unbiased exploration and examination of sample molecular biochemistry, and through suitable interpretation can be used to study plant responses to environmental changes (Gidman et al. 2005 and 2006). When the aim is to obtain information about the whole metabolome (the total number of metabolites in one biological system) by identifying and quantifying as many metabolites as possible we must conduct an analysis approach such as NMR or GC-MS that enables to determine and quantify the maximum number of metabolites. Since such an approach reveals the maximum information of metabolome as possible of the biological system under study, this approach is called metabolomics. In an ecological context, when the objective is to discern the global metabolomic response of an organism to environmental changes, we propose to call it ecometabolomics. In fact, this latter approach, ecometabolomics, is the most appropriate to comprehend the complete response of an organism to environmental changes.

Analytical techniques

In metabolomic studies it is very important to take into account the pre-analysis treatment. To prevent post-sampling hydrolysis of some compounds, it is important to immediately freeze the sample (most frequently by introducing it immediately in N_2 liquid) and thereafter lyophilize it to completely dry it until extraction process (see Kim and Verpoorte 2009 for more details on sample preparation). Since all metabolites can provide information about species' responses to

environmental changes, ecometabolomic studies should be designed to detect as many metabolites as possible. As no single solvent allows all metabolites to be extracted, combinations of several different solvents can be used. Kim and Verpoorte have tested different extraction methods for NMR plant metabolomics. The use of a two phase solvent system, composed of a mixture of chloroform, methanol and water (2:1:1, v/v), has proved to be the most advisable method (Choi et al. 2004). For detailed information about the different extractant properties see Kim and Verpoorte (2009) and Kaiser et al. (2009).

Currently no single analytical method or combination of methods (i.e. chromatography combined with mass spectroscopy) can detect all metabolites (estimated to be between 100000 and 200000 in the plant kingdom) within a given biological sample. Gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance spectrometry (NMR) are the procedures with the best capacity to determine the widest-ranging sets of metabolites. GC-MS has proven to be a robust tool for the study of volatile organic compounds (Degen et al. 2004; Ozawa et al. 2008; Llusia et al. 2010) but GC-MS analyses of extracts containing other analytes such as organic acids, sugars, aminoacids, and steroids is complicated. Many metabolites are non-volatile and must be derivatized prior to GC-MS analysis (Gullberg et al. 2004). In such cases, thermolabile compounds may be lost. Moreover, it is difficult to elucidate the unknown structures of metabolites by using GC-MS alone. LC-MS is of particular importance to study a great number of metabolic pathways at once since plant metabolism embodies a huge range of semi-polar compounds, including many key groups of secondary metabolites, which are better separated and detected by LC-MS (Allwood and Goodacre 2009). Thus, while GC-MS is best suited for compound classes appearing mainly in primary metabolism (frequently after derivation) i.e. aminoacids, fatty acids and sugars or volatile compounds, LC-MS is more adequate to determine the overall biochemical richness of plants including several semi-polar groups of secondary metabolites. To gain structural elucidation power, the method of collision-induced dissociation can be used (Jennings 2000). The parent ions providing electrospray ionization (ESI) are isolated and accelerated in mass spectrometry using mass filters, so as to collide with molecules of bath gas, giving rise to the fragment spectrum (MS/MS method). Fourier transformation ion cyclotron resonance mass spectrometry (FT-ICR-MS) and ultra pressure liquid chromatography mass spectrometry (UHPLC-MS) can be used to further increase the number of detectable metabolites. FT-ICR-MS is a very high resolution technique in that masses can be determined with very high accuracy. This is due to the great sensitivity of this method to separate compounds with different mass-to-charge ratios (m/z) by their different cyclotron frequency in a fixed magnetic field. This method together with previous chromatographic methods has been scarcely used in ecometabolomic studies until now (Hirai et

al. 2004; Haesler et al. 2008), but the results are promising. UHPLC-MS constitutes an improvement in the power of separating compounds during the chromatographic phase (high resolution capacity) with respect to conventional HPLC-MS method. This is achieved by the application of great pressure to the carrier solution that reduces the dispersion of each chemical compound in the separation column. Moreover, UHPLC permits shortening the time of the separation phase, fact specialy interesting in ecometabolic studies where great number of samples must be processed.

^{1}H-NMR has proven to be an appropriate tool for untarget analyses. It has the advantage that it can be applied to determine polar, semi-polar and non polar metabolites, and that it produces signals that directly and linearly correlated with compound abundance (Lewis et al. 2007). However, NMR spectroscopy has intrinsic low sensitivity for low concentrations of metabolites and signal overlapping for complex mixtures. This can at times be problematic for structure elucidation of a metabolite at low concentrations. The use of low temperatures to stabilize the detector by modern cryogenically cooled devices can improve the sensitivity up to a factor of five by reducing the thermal noise from the electronics of the NMR spectrometer. Two dimensional (2D) NMR spectroscopy methods and high-resolution magic angle spinning (Sekiyama et al. 2010) further improve the sensitivity. 2D NMR spectroscopy provides an increased signal dispersion thus enabling the detection of connectivity between signals and hence helping to identify metabolites. This method includes the total angular momentum (J) resolved method (^{1}H-^{1}H 2D J resolved), correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY). ^{1}H-^{1}H 2D J resolved yields information on the multiplicity and coupling patterns of resonances which reduces the degree of spectra complexity but retains all the chemical shifts and the relative intensity of spectral peaks. COSY and TCOSY provide ^{1}H-^{1}H spin-spin coupling connectivities, providing information as to which hydrogens in a molecule are closer in terms of chemical bonds. In the high-resolution magic angle spinning approach, ^{13}C is detected indirectly using the more abundant ^{1}H by using spin-spin interaction ^{13}C-^{1}H. This yields both coordinated ^{13}C- ^{1}H-NMR shifts that are useful for identification purposes. Moreover, the stable isotopes ^{13}C, ^{15}N and ^{31}P have been successfully employed in NMR in vivo metabolomic studies in ecophysiological studies (Lundberg and Lundquist 2004; Kikuchi et al. 2004). When for the aim of an study it iis important to elucidate chemical structures that probably are unknown as for example in plant herbivore relationships, NMR based metabolomic studies are the most adequate tool. (see recent review on this topic by Leiss et al. 2011). For a more detailed information of the advantages and disadvantages of each method see Summer et al. (2003), Kopka et al. (2004), Moco et al. (2007) and Verpoorte et al. (2009).

Recently, HPLC separation, Diode Array detector (DAD), MS detection, solid phase extraction (SPE) for enrichment of a metabolite and NMR, namely HPLC-DAD-MS-SPE-NMR (Tang et al. 2009) have been combined, enabling good separation, sensitivity and molecular structure elucidation all at once (Tang et al. 2009). For a further description of data acquisition methods in metabolomics see Hall (2006), Allwood and Goodacre (2009) and Lindon and Nicholson (2008).

Ecometabolic responses to abiotic factors

Several studies have investigated the responses of some metabolic pathways in organisms to changes in abiotic factors such as climate (temperature and water availability), nutrient availability, salinity or pollution (Tables 1 and 2). Changes in the composition of some metabolite groups have been described using analytical target methods in response to changes in several environmental factors, such as drought (Llusia et al. 1999; 2008; Peñuelas et al. 2008), temperature (Peñuelas and Llusia 1999; Filella et al. 2007), pollutants (Peñuelas et al. 1999; Broeckling et al. 2009), irradiance (Peñuelas and Llusia 1999), or CO_2 (Peñuelas and Llusia 1997). Moreover, several studies have reported that the metabolites produced in response to abiotic or biotic environmental changes further interact with other abiotic and/or biotic ecosystem constituents, e.g. terpene emissions that affect the climatic and atmospheric conditions (Andreae and Crutzen 1997; Kavouras et al. 1998; Peñuelas and Llusia 2003; Peñuelas et al. 2009; Peñuelas and Staudt 2010). Now ecometabolomic studies bring the possibilities to reach a step forward in the knowledge at the level of global organism response to environmetal changes. Some reports have already begun to explore the possibilities of the use of metabolomic approaches in ecological studies.

Climatic factors

Most work conducted in plants using partial ecometabolomic techniques to investigate metabolic changes under **cold stress,** has involved analyzing the polar metabolites in Arabidopsis sp. grown in controlled conditions (Kaplan et al. 2004; Cook et al. 2004; Gray and Heath 2005; Kaplan et al. 2007; Davey et al. 2009; Maruyama et al. 2009; Korn et al. 2010) although other plant species have also been studied (Janda et al. 2007) (Table 1). The main responses to low temperatures included increases in metabolites related to aminoacid-protein and soluble carbohydrates recognized as cryoprotectors (Carpenter and Crowe 1988). In addition other studies of partial ecometabolomics mainly focusing on lipids have reported an increase in the degree of lipid unsaturation related to cellular membrane adaptation to low temperatures (Cyril et al. 2002; Lindberg et al. 2005). When partial metabolomic studies have been conducted with transcriptomics, it has been observed that the changes in transcript levels of some metabolic

processes were not correlated with shifts observed at metabolic levels (Kaplan et al. 2007). This indicates that metabolome shifts are more sensitive than transcriptome changes to detect phenotypic responses to cold stress. Different genotypes of Arabidopsis presented significant differences in the metabolite set that increased under cold acclimation (Kaplan et al. 2004; Davey et al. 2009). The metabolomic technique showed greater sensitivity in detecting genotypic and phenotypic differences to cold response than transcriptomic techniques.

Ecometabolomic studies of polar metabolites in animals in response to cold stress have reported similar results than those observed in plants. E.g. increases in glucose concentration coupled to decreases in glycogen have been observed in *Lumbricus rubellus* (Bundy et al. 2003) (Table 1). These results further confirm the increase in sugars and aminoacids as cryoprotectors under low temperatures (Overgaard et al. 2007). Michaud and Denlinger (2007) conducted an ecometabolomic study using both GC-MS and ^{1}H NMR, including both polar and non-polar metabolites. This gave a clearer picture showing the enhancement of some metabolic pathways was related to the simultaneous decrease in other metabolic pathways. Apart from sugars, pyruvate and urea also increase at low temperatures. All these metabolites have been related to cell membrane cryoprotection (Storey and Storey 1983; Lee 1991).

On the contrary to cold stress, **warming** stress increases the saturation level of fatty acids (Larkindale and Huang 2004) and similarly to cold stress, warming stress increases aminoacid and soluble sugar concentrations in plants (Kaplan et al. 2004; Rizhsky et al. 2004, Yamakawa and Hakata 2010), fungi (Pluskal et al. 2010) and in endo- and exometabolome of soil microbes (Coucheney et al. 2008) (Table 1). In animals a decrease in metabolic conditions, i.e. lower ATP and glucose concentrations (Viant et al. 2003) and a rapid increase in concentration of some aminoacids with a subsequent decrease (Malmendal et al. 2006) have been linked to an increase in “heat shock” proteins synthesis (Feige et al. 1996). Other recent study of Folsomia candida showed a decrease of different aminoacids contents in response to a heat stress as a result of up-stream down-regulation of transcription and translation (Waagner et al. 2010). However, the possible immediate increase of other aminoacid contents was not measured. An ecometabolomic fingerprinting study of *Oncorrhynchus mykiss* eggs submitted to warming shock permitted the detection of differences in some groups of metabolites showing the sensitivity and suitability of fingerprinting the metabolome as a screening method prior to conducting ecometabolome profile studies (Turner et al. 2007). Pluskal et al. (2010) also detected many changes in Schizosaccharomyces pombe secondary metabolism, including decreases in urea cycle intermediates and increases in acetylated compounds.

Some studies have monitored the changes in the concentration of water soluble plant metabolites in response to water availability to study **drought** effects using partial

ecometabolomic techniques in vitro or in the lab (Rizhsky et al. 2004; Pinheiro et al. 2004), greenhouse (Cramer et al. 2007; Charlton et al. 2008; Lugan et al. 2009) and also in field experimental conditions (Semel et al. 2007; Mane et al. 2008; Alvarez et al. 2008) (Table 1). An increase in soluble sugars, mainly glucose and sucrose and/or in aminoacids or their derivates (citric acid, threonine, homoserine, valine, proline, malate, □-aminobutyrate) is frequently found under drought, confirming that the reduction in photosyntheses is accompanied by a pronounced mobilization of sugars in soluble form and by a synthesis of soluble aminoacids. Both these mechanisms adjust osmotic potential to prevent water losses. These compounds may also protect cellular components such as membranes and enzymes (Shen et al. 1997). A similar observation has been reported by Michaud et al. (2008) in a partial ecometabolomic study of the polar metabolites of the insect *Belgica antartida* raised in vitro at different moisture levels (Table 1) Unfortunately, significant plant secondary metabolites, such as phenolics, that can minimize the oxidative stress associated with drought (Hura et al. 2007), were not included in such studies. For this reason the ecometabolomic studies that aimed to study primary but also secondary metabolites at once are necessary to reach a general knowledge of plant response to drought.

The effects of **seasonality** on the leaf content of some metabolites have been observed in some target studies (Riipi et al. 2004) in mountain birch trees (*Betula pubescens*). Ecometabolomic studies should allow to improve the knowledge of global metabolism shifts linked to annual phenological changes in both animals and plants in field conditions.

Nutrient deficiency

Ecometabolomic studies that have aimed to investigate metabolome changes under different scenarios of nutrient availability are scarce (Table 1). **N deficiencies** in plants have been proven to decrease certain aminoacid concentrations, whereas the concentration of several sugars, phosphoesters and secondary metabolites increases (Urbanczyk-Wochniak and Fernie 2005; Bölling and Fiehn 2005) (Table 1). There are also shifts from biosynthesising some aminoacids to synthesising others (Howarth et al. 2008). These effects were also observed in the earthworm *Eisenia veneta* (Warne et al. 2001). Partial ecometabolomic studies of polar metabolites found that **P stress** induced the root accumulation of polyols, which are stress related metabolites (Hernández et al. 2007). In addition plants modify carbohydrate metabolism in order to reduce P consumption and remove P from many metabolites (glucose 6-P, fructose 6-P, inositol 1-P and glycerol 3-P) thus reducing the levels of organic acids involved in the tricarboxylic acid cycle (TCA) (Huang et al. 2008) (Table 1). Partial ecometabolomic studies in plants have shown that **S deficiency** tends to increase the concentration of some N rich metabolites such as certain aminoacids and purines (Nikiforova et al. 2005a; Howarth et al. 2008).

Rochfort et al. (2009) have observed that changes in both polar and lipophylic metabolites composition in earthworms provides information on the fertility of the soil in which they have been living.

Salt stress

Plant salt stress is increasing globally due to climate change and inadequate water use by humans and for field management (Ellis and Mellor 1995). Current reports using metabolomic techniques have detected the global metabolic shift under salt stress observing the sets of metabolites that more frequently increased enhancing the plant osmotic potential. Inorganic solutes (Gagneul et al. 2007) and sugars, aminoacids and polyols (Kim et al. 2007; Fumagalli et al. 2009; Widodo et al. 2009; Behrends et al. 2010) are the metabolites that most frequently increase under salt stress (Table 1). Some studies have investigated the metabolomic fingerprinting studies of salt-stressed plants, (Smith et al. 2003; Johnson et al. 2003) and animals (Bussell et al. 2008) and have been able to identifi the molecular groups changing under such stress (Table 1).

Hypoxia

Lack of oxygen is an important ecological trait in some ecosystems such as benthonic communities in intertidal coastal areas. Increases in succinate and valine and decreases in leucine and isoleucine have been observed in ^{1}H NMR partial ecometabolomic studies of polar metabolites in the mussels *Mytilus edulis* and *Mytlus galloprovincialis* grown at different hypoxia levels (Hines et al. 2007; Tuffnail et al. 2009) (Table 1). In the fish *Oryzias latipes* has shown increases of soluble phosphates and decreases in ATP and phosphodiesters in response to hypoxia in a PEM of phosphorilated soluble metabolites by ^{31}P NMR (Pincetich et al. 2005).

Global change drivers

Change in atmospheric composition (CO_2, O_3, NO_x), warming, drought, human driven pollution, trace elements pollution and UV radiation can have an strong impact on organisms metabolomes. The effects of these abiotic factors on organism metabolites have been currently investigated in some ecometabolomic studies. Metabolites linked to carbohydrate biosynthesis and partitioning, aminoacid metabolism, cell wall and hormone biosynthesis pathways were the most affected by **[CO_2] increases** in different genotypes of *Arabidopsis thaliana* submitted to higher [CO_2] (Li et al. 2006). Thus, the responses of the most polar metabolite groups were different depending on the genotypes. However, the use of transcriptome allows to detect that there were a small number of signature transcripts that appears as a common response

mechanism of all Arabidopsis ecotypes to [CO_2] increases irrespective of their underlying genetic diversity and evolutionary adaptation to different habitats (Li et al. 2006). Thus, these results highlight the advantage that suppose the use of metabolomic with other omic techniques at once to reach a general and integrate overview of the responses of organisms in response to environmental changes.

Increases of phenolic compounds contents have been observed in target analysis at high [CO_2] exposure (Huttunen et al. 2008) (Table 1). This linkage between high [CO_2] environment concentrations and high C-rich compounds concentrations warrants to be studied altogether with the whole organism metabolome. Such rises in C-rich secondary metabolites in plant tissues seem related to the increases in the plant C:N concentration ratio observed as general plant response to higher [CO_2] (Peñuelas et al. 1997; Novotny et al. 2007).

Ecometabolomic fingerprinting using Fourier transformed-infrared spectroscopy FT-IR has been used to investigate patterns of plant metabolome changes due to **N deposition**. This was done both in an experimentally manipulated N deposition gradient under common garden conditions using *Calluna vulgaris* and in a natural gradient across the United Kingdom (Gidman et al. 2005; 2006) (Table 2). In both studies FT-IR fingerprinting was able to correlate metabolome changes with N deposition levels. The results showed that the spectra regions corresponding to N-H, C-N, proteins and polysacarid vibrational bands had a positive relationship with higher N-deposition, suggesting an enhancement of N-rich metabolites synthesis and of sugars anabolism. These results highlight the possiblities of the use of this technique as an useful tool for preliminary studies in metabolome research applied to field ecological studies.

Partial ecometabolomic studies allow the detection of the metabolic pathways affected by the pollution produced by different **chemotoxic pollutants** and also the key metabolites that improve the organism resistance both in plants (Trenkamp et al. 2009; Kluender et al. 2009) and in animals (Warne et al. 2000; Bundy et al. 2001; Viant et al. 2006a and 2006b; Samuelsson et al. 2006; Bon et al. 2006; Jones et al. 2008a; Ekman et al. 2008; Mckelvie et al. 2009; Tuffnail et al. 2009; Hansen et al. 2010) (Table 1). For instance, Bundy et al. (2002) have used ecometabolic fingerprinting NMR studies of the earthworm *Eisenia veneta* to ascertain initially the metabolites that change their concentration when the worm was submitted to three different xenobiotics: 4-fluoroaniline, 3,5-difluoroaniline and 2-fluoro-4-methylaniline. In a later step they identified these changing compounds by using HPLC-MS and ^{13}C NMR. This experiment is an example of the utility of metabolic fingerprinting studies to detect the molecular groups which change in the metabolome in response to an abiotic factor as a first step prior to molecular qualitative determination analyses. Using this approach avoids the expense and time consumption that qualitative analyses of the whole metabolome require. Hansen et al. (2010) showed the shifts in

metabolism of the marine copepod *Calanus finmarchicus* in response to diethanolamine (DEA) exposure as a relevant chemical widely used in industrial, agriculture and pharmaceutical applications. The main result of this study was a decrease of cholines, taurine, sarcosine and some aminoacids. Likewise the capacity of ecometabolomic fingerprinting to detect metabolomic responses to different herbicide exposures has been recently reported in *Lemna minor* (Aliferis et al. 2009). In the metabolomic studies of toxicity effects on organism's metabolome (polar and non-polar metabolites), these using GC-MS have been able to detect more compounds than those using in ^{1}HNMR. In contrast, studies using ^{1}HNMR have greater power to elucidate the compound structure through its great number of sources of qualitative determination (COSY, TOCSY and high-resolution magic angle spinning). Thus if the aims are also to determine possible novel structures, the use of ^{1}HNMR is advisable. For example, Kluender et al. (2009) using GC-MS detected 283 metabolites but determined only 39, whereas Jones et al. (2008a) using ^{1}HNMR detected and determined 32 analytes in a similary study on chemotoxic pollutants effects on invertebrate metabolome. Jones et al. (2008a) using both techniques to analyze the same samples detected and determined 32 molecules using ^{1}HNMR and detected 51 but only determined 42 molecules using GC-MS.

A set of recent partial ecometabolomic studies of polar metabolites has also permitted the identification of the metabolites and metabolic pathways affected by certain **trace elements** polluting plants (Bailey et al. 2003; Roesner et al. 2006; Sarry et al. 2006; Le Lay et al. 2006; Sun et al. 2008) and animals (Gibbs et al. 1997; Griffin et al. 2001; 2000; Bundy et al. 2007; 2004; Jones et al. 2008b; Taylor et al. 2009; Guo et al. 2009) (Table 2). These studies have allowed the identification of some metabolites (acetilspermidine, glucose, histidine, l-methylhistidine, mannose) that can be used as biomarkers for Cu, Cd and Zn pollution in various animal species (Bundy et al. 2004; 2007; 2008; Taylor et al. 2009; Guo et al. 2009). In rats, in addition to the changes in aminoacid and sugars contents, a further abnormal metabolome composition has been observed as a result of Cd and Cu intake (Jones et al. 2007, Lei et al. 2008), and changes in lipid metabolism under high levels of As have also been observed (Griffin et al. 2000). A tendency to increase the production of AMP, ADP and N-□-methylhistidine, and to generate an imbalance in metabolites related to energetics by reducing ATP levels has been reported in *Lumbricus rubellus* (Bundy et al. 2007; 2008). In a transcriptomics and ecometabolomic study of Cu toxicity in *Lumbricus rubellus*, Bundy et al. (2008) observed a decrease in small-molecule metabolites (glucose and mannose) related to an over-expression of transcripts of enzymes involved in oxidative phosphorylation, thus showing that Cu interferes with energy metabolism. In addition such studies have advanced the knowledge about the metabolites involved in phytochelant mechanisms in plants (Sarry et al. 2006). The pollution by ^{133}Cs induced higher amino acid

content in cells of *Arabidopsis thaliana* suggesting an induction of the synthesis of abnormal or unwanted proteins under higher levels of ^{133}Cs in the medium. On the other hand, the synthesis increase of enzymes involved in the degradation of short-lived intracellular proteins was detected by proteomic studies (Le Lay et al. 2006). These effects were lower at high levels of K in the growth medium. Altogether these results highlighted the goodness of coupling different omics approaches together with the simultaneous study of different environmental variables such as pollution and nutrient availability levels in the Le Lat et al. (2006) study. Similarly, an increase of some amino acids content has been observed in *Silene cucubalus* and *Arabidopsis thaliana* submitted to Cd pollution (Bailey et al. 2003; Sun et al. 2008) and in *Hordeum vulgare* submitted to B pollution (Roessner et al. 2006) all them supporting the Le Lay et al. (2006) study results.

High levels of **UV radiation** can be expected in a future if stratospheric O_3 levels decreased. This type of radiation is able to damage DNA and other macromolecules (Harm 1980; Jagger 1985). Several target studies have already observed that *Quercus ilex* and *Rhododendrum ferrugineum* change their pigment composition and leaf morphology in response to UV radiation in a latitudinal range (Filella and Peñuelas 1999) demonstrating that a deep metabolic change can also occur but these studies do not allow to reach a global picture of metabolome shift under UV ratiation enhamcement. Partial ecometabolomic studies carried out exposing Arabidopsis thaliana to high UV radiation showed that flavonoids (Lois 1994), □-glicerophosphates (Broecling et al. 200), and phenylpropanoids (Lake et al. 2009) increased their concentrations, thus suggesting that these compounds have a photo protective role (Table 2). In a partial ecometabolomic study of polar metabolites under light starvation using GC-MS and ^{13}C NMR, a decrease in primary metabolism has been observed in roots of *Phaseolus vulgaris* (Bathelier et al. 2009). But in this field there is a lack of global ecometabolomic studies aiming to study polar and non polar metabolites at once to improve the knowledge of the plants and animals capacity and mechanisms to response to increasing levels of UV radiation.

Some interesting ecometabolomic studies including polar and non-polar metabolites have already begun to contribute to provide some information about the effects of **ozone** (**O_3**) on the organism metabolome of different taxa. In an ecometabolomic study of rice (*Oryza sativa*) using electrokinetic chromatography, increases in O_3 concentrations were found to enhance □-aminobutiric acid (GABA), some aminoacids and glutathione (Cho et al. 2008) (Table 1). However, when ecometabolomic studies were conducted using GC-MS in the tree *Betula pendula* as the target species growing in common garden conditions, phenolics and compounds related to the leaf cuticular wax layer were found to be the main metabolites involved in metabolic adaptation to increasing O_3 levels (Kontunen-Soppela et al. 2007; Ossipov et al. 2008) (Table 1). These are the first contributions to understanding what metabolic compounds and metabolic pathways are

involved in phenotypic responses to O_3 pollution and they highlight that plants can respond to O_3 pollution at different metabolic levels which can vary between species.

Remaining questions

This overview of the studies that have used ecometabolomic techniques to study ecophysiological responses to changes in abiotic variables shows there are several key questions still to be tackled. (i) The abiotic variables studied have been mainly manipulated in controlled conditions. Few studies have been conducted observationally in natural gradients or experimentally in field setups. (ii) There is a lack of ecometabolomic studies with polar and non-polar metabolites and also considering the secondary metabolism in plants. (iii) There is a lack of information about the response of several taxa or ecotypes, such as vertebrates, trees or shrubs, and there is an insufficient number of comparable studies enabling more general conclusions. (iv) Although there are preliminary studies, such as detecting changes in the metabolome in different soils and climatic conditions (Wallenstein et al. 2010), ecometabolomic studies of multifactorial effects are just in their beginning.

Table 1. Ecometabolomic studies that have focused on abiotic effects on organism metabolism. The studies which aimed to analyze the entire metabolite spectrum (both polar and non-polar metabolites) are highlighted in bold type.

Species	Analytical techniques, study type	Main results	Reference
COLD			
Dendrobaena spp. *Aporrectodea* spp.	^{1}H NMR, PEM polar metabolites	↑ Glucose	Bundy et al. 2003
Arabidopsis sp.	GC-MS, PEM polar metabolites	↑ Several aminoacids, glucose, fructose, galactinol	Cook et al. 2004
Paspalum vaginatum	GC-MS, PEM lipids	↑ Linoleic acid (triunsatured), fatty acids	Cyril et al. 2002
Arabidopsis lyrata	MS, MF	Sugars was the main discriminate metabolite group	Davey et al. 2009
Arabidopsis spp.	HPLC-MS, MF	Changes in metabolome fingerprinting	Gray and Heath 2005
Triticum aesticum	GC-MS, PEM lipids	↑ Fatty acids insaturation	Janda et al. 2007
Arabidopsis thaliana	GC-MS, PEM polar metabolites	↑ Several aminoacids and sugars, fosforic acid ↓ Xylitol, manitol	Kaplan et al. 2004 and 2007
Arabidopsis thaliana	GC-MS, PEM polar metabolites	↑ Raffinose, glucosa, galactose, sucrose, proline, glycine, maltitol, fumaric acid, succinic acid, galactinol, itaconic acid, ethanolamine	Korn et al. 2010
Beta vulgaris	Thin layer chromatography,PEM lipids	↑ Fatty acids insaturation	Lindberg et al. 2005

Arabidopsis sp.	^{1}H NMR, HPLC-UV, PEM polar metabolites	↑ Tetrahalose, maltose, alanine, sucrose, glutamine	Lugan et al. 2009
Arabidopsis thaliana	GC-MS, HPLC-IT-MS, PEM polar metabolites	↑ Starch degrading pathways, sugar alcohols synthesys, galactinol, Raffinose, kaempfenol, 7-rhammoside.	Maruyama et al. 2009
Sarcophaga crassipolpis	**GC.-MS and ^{1}H NMR, polar and non-polar metabolites**	↑ Urea, sorbitol, glutamine, ↓ □-alamine, ornithine, trehalose	Michaud and Denlinger 2007
Belgica antarctica	GC-MS, PEM polar metabolites	↓ Serine	Michaud et al. 2008
Drosophila melanogaster	^{1}H NMR, PEM polar and semipolar metabolites	↑ Sugars	Overgaard et al. 2007
WARMING			
Different soils, soil metabolome (both of bulk soil and of microbes)	GC-MS, PEM polar and semipolar metabolites	↑ Acetic acid, furanacetic acid, xylulose, phosphoric acid (in bulk soil) ↓ Galactonic acid, turanose (in bulk soil) ↑ Myristic acid, glutamic acid, thymidine, proline (in microbes) ↓ Thymidine (in microbes) The metabolome of bulk soil was more sensitive to temperature than those of microbes when comparing different soils.	Coucheney et al. 2008
Arabidopsis thaliana	GC-MS, PEM polar metabolites	↑ Several sugars, leucine, valine, tyrosine, uracil, quinic acid, xylytol	Kaplan et al. 2004
Agrostis stolonifera	GC-MS, PEM lipids	↑ Lipid insaturation	Larkindale and Huang 2004
Drosophila spp.	^{1}H NMR, PEM polar metabolites	↑ Leucine, valine, tyrosine	Malmendal et al. 2006
Belgica antarctica	GC-MS, PEM polar metabolites	↓ Serine	Michaud et al. 2008
Schizosaccharomyces pombe	LS-MS, PEM polar and semi-polar metabolites	↑ Some aminoacids, threhalosa, glycerophosphoethanolamina, arabitol, ribulose, ophthalmic acid Many changes in secondary metabolites such as ↓ urea cycle intermediates and ↑ acetylated compounds	Pluskal et al. 2010
Arabidopsis spp.	GC-MS, PEM polar metabolites	↑ Sucrose, maltose, glucose	Rizhsky et al. 2004
Oncorhynchus mykiss	^{1}H NMR, MF	Different metabolomic fingerprinting	Turner et al. 2007
Oncorhynchus mykiss	^{1}H NMR, PEM polar metabolites	↑ Antithermal stress protein pathways ↓ ATP, glycogen	Viant et al. 2003
Folsomia candida	^{1}H NMR, PEM polar metabolites	↓ Arginine, Lysine, Leucine, Phenylalanine, Tyrosine (after 7hr heat exposure).	Waagner et al. 2010
Oryza sativa	Capillary electrophoresis-MS, PEM polar metabolites	↑ Sucrose, Pyruvate/Oxalacetate-derived aminoacids ↓ Sugar phosphates and organic acids involved in glycolisis/gluconeogenesis and the tricarboxylic acid cycle (TCA)	Yamakawa et al. 2010
DROUGHT			
Zea mays	HPLC-MS/MS, PEM polar metabolites	↑ Threonine, GABA, 6-benzylaminopurine, praline, tryptofan, leucine	Alvarez et al. 2008

Pisum sativum	^{1}H NMR, PEM polar metabolites	↑ Proline, valine, threonine, homoserine, myoinositol, GABA,	Charlon et al. 2008
Vitis vinifera	GC-MS, PEM polar metabolites	↑ Glucose, maltose, proline	Cramer et al. 2007
Lolium perenne	GC-MS, PEM polar and semipolar metabolites	↑ Glucose, raffinose, fructose, trehalose, maltose ↓ Fatty acids	Foito et al. (2009)
Oryza sativa	**^{1}H NMR, polar and non-polar metabolites**	↑ Glucosa, glutamate, glutamine	Fumagalli et al. 2009
Stagonosphera nodorum	GC-MS, PEM polar and semipolar metabolites	↑ Glycerol, arabitol ↓ Several aminoacids	Lowe et al. 2008
Arabidopsis sp.	^{1}H NMR, HPLC-UV, PEM polar metabolites	↑ Proline, tyrosine, malate, GABA	Lugan et al. 2009
Belgica antartica	GC-MS, PEM polar metabolites	↑ Glycerol, arythritol ↓ Serine	Michaud et al. 2008.
Lupinus albus	^{13}C NMR, PEM polar metabolites	↑ Sucrose, glucose, proline	Pinheiro et al. 2004
Arabidopsis spp.	GC-MS, PEM polar metabolites	↑ Sucrose, maltose, glucosa, proline	Rizhsky et al. 2004
Solanum sp.	GC-MS, PEM polar metabolites	↑ Alanina, GABA, □-alamina, homoserine, isoleucine, proline, serine, valine ↓ Glutamina, glycine, cysteine	Semel et al. 2007
NATURAL GRADIENTS			
Differents climate and soil, *Pseudotsuga menxiesii*	GC-MS, PEM polar metabolites	Carbohydrate and lignine synthesis pathways were the most affected when comparing individuals of different sites	Robinson et al. 2007
Different level of soil degradation. Soil worms	**^{1}H NMR, polar and non-polar metabolites**	Levels of glucose, maltose, alanine and triacilglycerides are potential biomarkers of worm stress due to soil quality decrease	Rochfort et al. 2009
Different soils, *Populus tremuloides* (litter)	UPLC-MS, MF	Different metabolic fingerprinting in different soils	Wallenstein et al. 2010
NUTRIENT STRESS			
N, P, S, Fe stress, *Chlamydomonas reinhardtii*	GC-MS, PEM polar metabolites	↓ Some aa, soluble sugars, but differents depending on the nutrient that induces the stress.	Bölling and Fiehn 2005
P stress, *Phaseolus vulgaris* (roots)	GC-MS, PEM polar metabolites	↑ Polyols and sugars	Hernández et al. 2007
N and P stress *Arabidopsis* spp	**HPLC-DAD, FT-MS, Electrophoresis, polar and non-polar metabolites**	Changes in glucosinolates pathway	Hirai et al. 2004 and 2005
N and S stress, *Triticum aesticum*	^{1}H NMR, PEM aminoacids	↑ Glutamine	Howarth et al. 2008
P stress, *Hordeum vulgare*	HPLC-MS, CG-MS, PEM polar and semipolar metabolites	↑ Ammonium metabolism ↓ Sugar metabolism	Huang et al. 2008
S stress, *Arabidopsis* spp.	HPLC-DAD, HPLC-MS, GC-MS, PEM polar metabolites	↑ Purines, allantoine ↓ Several lipids	Nikiforova et al. 2005a
N stress, *Solanum lycopersicum*	GC-MS, PEM polar metabolites	↑ Several sugars and phosphoesters ↓ Several aminoacids and organis acids	Urbanczyk-Wochniak and Fernie 2005
N stress, *Eisenia veneta* (1)*, Lumbricus terrestris* (2)	^{1}H NMR, PEM polar metabolites	↑ Glutamate, citrate, isoleucine, aspartate ↓ Lysine, threonine	Warne et al. 2001
SALT STRESS			
Pseudomonas aeruginosa	^{1}H NMR, extracelular PEM polar metabolites	↑ Tetrahalose, glycine, betaine, valine, choline	Behrends et al. 2010
Mytilus edulis	FT-IR, MF polar	Salinity changes metabolic	Bussell et al.

	metabolites	fingerprinting	2008
Vitis vinifera	GC-MS, PEM polar metabolites		Cramer et al. 2007
Oriza sativa	**^{1}H NMR, polar and non-polar metabolites**	↑ Glucose, glutamate, glutamine, valine, lactose, threonine	Fumagalli et al. 2009
Limanium latifolia	HPLC, GC-MS, ^{1}H NMR, PEM polar metabolites	↑ Proline, inorganic sals, succinate, hexoses	Gagneul et al. 2007
Lycopersicum esculentum	FT-IR spectrometry, MF	Different metabolome fingerprinting at different salinity levels	Johnson et al. 2003
Arabidopsis thaliana	HPLC, GC-MS, PEM polar metabolites	↑ Lignin biosynthesis and methylation, sucrose catabolism	Kim et al. 2007
Lycopersicum esculentum	FT-IR spectrometry,MF	Different metabolome fingerprinting at different salinity levels	Smith et al. 2003
Hordeum vulgare	GC-MS, PEM polar metabolites	↑ Hexose phosphates, TCA cycle intermediates, GABA, proline, putrescine, several aminoacids	Widodo et al. 2009
ATMOSPHERIC CHANGES			
N deposition, *Calluna vulgaris*, *Gallium saxatile*	FT-IR, MF	Changes in metabolome at different levels of N deposition	Gidman et al. 2005 and 2006
↑ [CO_2], *Betula pendula*	HPLC, PEM polar metabolites (Phenolics)	↑ phenolics	Huttunen et al. 2008
↑ [CO_2], *Arabidopsis thaliana* (different genotypes)	GC-MS, PEM polar metabolites	Global changes in polar metabolome due to different ecotypes and also to different levels of [CO_2]	Li et al. 2006
ORGANIC POLLUTANTS			
↑ (Paraquat, pyrenophorol, mesotrione, norflurazon), *Lemma minor*	^{1}H NMR, MF	Different changes in the metabolome fingerprinting observed as consequence to different xenobiotics.	Aliferis et al. 2009
↑ Glyophosphate, *Aporrectodea caliginosa*	^{31}P NMR and ^{1}H NMR, PEM polar metabolites	↓ Phosphalombricine, lombricine	Bon et al. 2006
↑ 3-fluoro-4-nitrophenol, *Eisenia veneta*	^{1}H NMR, PEM polar metabolites	↑ Acetate, malonate	Bundy et al. 2001
↑ (4-fluoroaniline (1), 3,5-difluoro aniline (2), 2-fluoro-4-methylanyline 3)) *Eisenia veneta*	^{13}C NMR and ^{1}H NMR, HPLC-DAD, PEM polar metabolites	↓ Mallose (1), 2 hexhyl-5-ethyl-3-furanosulfonate (2,3)	Bundy et al. 2002
↑ 17-a-ethynylestradiol, *Pimepheles promelas*	**^{1}H NMR, polar and non-polar metabolites**	↑ Creatine, glycogen, glucose, lactate	Ekman et al. 2008
↑ Atrazine (1), fluoranthene (2), *Lumbricus rubellus*	^{1}H NMR, PEM polar metabolites	↑ Fumarate (1), cytidine triphosphate (2)	Guo et al. 2009
↑ Diethanolamine (DEA) *Calanus finmarchicus*	^{1}H NMR, PEM polar metabolites	↓ choline, phosphocholine, glycerophosphocholine, taurine, sarcosine, alanine, arginine, leucine, glutamine, metionine, threosine.	Hansen et al. 2010
↑ Pyrene, *Lumbricus rubellus*	**^{1}H NMR, GC-MS polar and non-polar metabolites**	↑ Alanine, leucine, valine, isoleucine, lysine, tyrosine, methionine ↓ Tetradecanoic acid, hexadecanoic acid, octadecanoic acid	Jones et al. 2008a
↑ Chlorpyritos, *Mytillus*	^{1}H NMR, PEM polar metabolites	↑ Acetilcholine	Jones et al. 2008b

galloprovincialis			
↑ Prometryn, *Scenedesmus vacuolatus*	**GC-MS, polar and non-polar metabolites**	↓ Catabolic respiratory metabolism	Kluender et al. 2009
↑ (DDT, endosulfan), *Eisenia fetida*	^{1}H NMR, GC-MS PEM polar metabolites	↑ Alanine	Mckelvie et al. 2009
↑ Estrogens, *Oncorhynchus mykiss*	**^{1}H NMR, polar and non-polar metabolites**	↑ Vitellogenin, glyceryl ↓ Alanine, cholesterol	Samuelsson et al. 2006
↑ (Glucosinalate, sulcotrione, AE944, furamsulfuron, benfuresate, glyphosate) *Arabidopsis thaliana*	GC-MS, PEM polar metabolites	Some metabolites changed in different way depending on the herbicide applied; these metamolites were: glucose, fructose, metionine, phenylalamine, isoleucine, valine, lysine, tyrosine, glycine, glutamine, gliceric acid	Trenkamp et al. 2009
↑ (Atrazine, lindane), *Mytilus edulis*	^{1}H NMR, PEM polar metabolites	↑ Alanine ↓ betaine, homarine, taurine (lindane) ↑ Leucine, isoleucine (atrazine)	Tuffnail et al. 2009
↑ Dinoseb, *Oryzias latipes*	^{31}P NMR and ^{1}H NMR, HPLC-DAD, PEM polar metabolites	↑ Ortophosphate ↓ ATP, phosphocreatine	Viant et al. 2006a
↑ (Dinoseb, diazinen, esfenvalerate), *Oryzias latipes*	^{1}H NMR, PM polar metabolites	↓ ATP, phosphocreatine	Viant et al. 2006b
↑ 3-trifluoro-methyl-aniline, *Eisenia veneta*	^{1}H NMR, PEM polar metabolites	↑ Alanine, glycine, asparagine, glucose, citrate, succinate	Warne et al. 2000
↑ Dibenzanthracene, *Gasterosteus aculeatus*	^{1}H NMR, PEM polar metabolites	↑ Malonate, glutamine, alamine ↓ Taurine	Williams et al. 2009
TRACE ELEMENTS			
Cd, *Silene cucubalus*	^{1}H NMR, PEM polar metabolites	↑ Malic acid and acetate ↓ Glutamine	Bailey et al. 2003
Diverse heavy metals, *Lumbricus rubellus*	^{1}H NMR, PEM polar metabolites	↓ Histidine, Methylhistidine	Bundy et al. 2004
Zn, *Lumbricus rubellus*	^{1}H NMR, PEM polar metabolites	↑ Triptofan, uracil, scyllo-inositol, AMP, ADP	Bundy et al. 2007
Cu, *Lumbricus rubellus*	**^{1}H NMR, polar and non-polar metabolites**	↓ Glucose, manose, 3.methylhistidine	Bundy et al. 2008
Cu, *Eisenia andrei, Lumbricus rubella*	^{1}H NMR, PEM polar metabolites	↓ Histidine	Gibb et al. 1997
Cd, *Clethrionomys glareolus* kidney	^{1}H NMR, PEM polar metabolites	↑ Lactate and fatty acids ↓ Glutamine	Griffin et al. 2000
As^{3+}, *Clethrionomys glareolus*	**^{1}H NMR, polar and non-polar metabolites**	Changes in lipid and glutamate metabolisms	Griffin et al. 2001
Cd, *Lumbricus rubellus*	^{1}H NMR, PEM polar metabolites	↑ Nicotinic acid ↓ Succinate	Guo et al. 2009
Cd, *Myodos glareolus, Apodemus sylvaticus*	**^{1}H NMR, polar and non-polar metabolites**	↑ Lactate ↓ Glucose, leucine, isoleucine	Jones et al. 2007
Ni, *Mytilus galloprovincialis*	^{1}H NMR, PEM polar metabolites	Changes in respiratory catabolism	Jones et al. 2008b
^{131}Cs, *Arabidopsis thaliana*	^{131}CsNMR, ^{31}PNMR, ^{13}CNMR, PEM polar metabolites	↑ Amino acids	Le Lay et al. 2006
Cu, *Rattus norvegicus*	**^{1}H NMR, polar and non-polar metabolites**	↑ Citrate, lactate ↓ Glutamine, taurine	Lei et al. 2008

B, *Hordeum vulgare*	GC-MS, PEM polar metabolites	↑ Glycerine, putresceine, valine, fumaric acid, maleic acid, glucose, fructose	Roessner et al. 2006
Cd, *Arabidopsis thaliana*	HPLC-MS/MS, PEM polar metabolites	↑ Production of proteins phytochelans	Sarry et al. 2006
Cd, *Arabidopsis thaliana*	**GC-MS, polar and non-polar metabolites**	↑ Several sugars and aminoacids, □-tocoferol, campesterol, □-sitosterol, isoflavone	Sun et al. 2010
Cu, *Daphnia magna*	FT-MS, PEM polar metabolites	↑ N-acetilspermidine	Taylor et al. 2009
RADIATION STRESS			
↓ Visible,	GC-MS, ^{13}C NMR, PEM polar metabolites	↓ Sugars and tricarboxylic acids	Bathellier et al. 2009
↑ UV, *Medicago trunculata*	**GC-MS, HPLC-DAD, polar and non-polar metabolites**	↑ a-glicerophosphate ↓ leucine	Broeckling et al. 2005
↑ UV, *Arabidopsis thaliana*	GC-MS, HPLC-MS/MS, PEM polar metabolites	↓ Phenylpropanoids ↑ kaempfenrol-glycoside, quercitin glycoside	Lake et al. 2009
TROPOSPHERIC OZONE			
↑ [O_3], *Oryza sativa*	**Electrokinetic-C, polar and non-polar metabolites**	↑ Glutamine, GABA	Cho et al. 2008
↑ [O_3], *Betula pendula*	**GC-MS, HPLC-DAD, polar and non-polar metabolites**	↑ phenolics	Kontunen-Soppela et al. 2007
↑ [O_3], *Betula pendula*	**GC-MS, HPLC-DAD, polar and non-polar metabolites**	↑ phenolics	Ossipov et al. 2008

PEM = Partial ecometabolomic study. MF = Metabolic fingerprinting study.
Polar fraction: Aminoacids and organic acids (citric, malate,..), mono, di and tri sacharides, inositol, sucrose, phenolics.
Lypophilic (non polar) fraction: Fatty acids and their derivates, hydrocarbones, alkaloids, flavolon aglycones, triterpenoids, steroids.
HPLC = High pressure liquid chromatography. UHPLC =Ultra high pressure liquid chromatography
MS = Mass spectroscopy.
FT-IR = Fouries transformation-Infrared Spectroscopy.
^{1}H NMR = Nuclear magnetic resonance of ^{1}H.
^{13}C NMR = Nuclear magnetic resonance of ^{13}C.
^{31}P NMR = Nuclear magnetic resonance of ^{31}P
GC = Gas chromatography.
UPLC = Ultra-performance liquid chromatography.

Biotic interactions between two or more species

Plant-fungus

Few studies have applied ecometabolomic techniques to study changes in the plant metabolome during fungal infection that can be a host-pathogen or a mutualistic relationship. Some partial ecometabolomic studies of polar metabolites have shown that plant defensive responses involve the induction of diverse polar secondary metabolites such as p- and m-coumaric acid, inositol, caffeic acid, indolic derivates, phenylpropanoids and flavonoids and some non-polar metabolites such as phosphatidyl glycerol, aromatic compounds and fatty acids (Bednarek et al. 2005; Allwood et al. 2006; Strack et al. 2006; Jobic et al. 2007; Cao et al. 2008;

Hamzehzarghani et al. 2008; Muth et al. 2009; Abdel-Farid et al. 2009; Lima et al. 2010) and also the decrease of other metabolites such as GABA, fructose and sucrose involved in plant growth and primary metabolism (Jobic et al. 2007; Andel-Farid et al. 2009) (Table 2). Similarly, higher contents of some amino and organic acids, carbohydrates and mainly of phenolic compounds have been observed to be constitutive defenses in fungus-resistent subspecies of *Vitis vinifera* when compared with susceptible subspecies of *Vitis vinifera* (Figuereido et al. 2008; Ali et al. 2009). These results impply a rerouting of carbon and energy from primary to secundary metabolm and suggest the posible change in the plant nutricional quality for animals feeding with the posible impact on other ecological relationships. The other component of the relationship, the fungus, also changes its metabolism when it infests a plant. For example, Parker et al. (2009) has suggested that the fungus *Magnaporthe grisacea* deploys common metabolic pathways of host plant species *Brachipodium sistachyon* to suppress plant defences and colonize plant tissues. In this vein Jobic et al. (2007) found that glycerol was only produced for the hyphal of the fungus *Sclerontinia sclerotiorum* when it grew into infected tissues of *Helianthus annuus*. Glycerol can be synthesized by fungus to enhance fungal growth and to protect fungal cells. Metabolites released from dying plant tissues could stimulate its synthesis. These ecometabolomic studies show not only the metabolites induced in plants by fungal infection (Hamzehzarghani et al. 2005 and 2008; Muth et al. 2009; Abdel-Farid et al. 2009; Lima et al. 2010) but also shows that the fungus can use plant photosyntate that thereafter causes hyphal growth, altogether suggesting that the fungus has developed a common metabolic re-programme strategy in the plant host (Parker et al. 2009). Another interesting finding reported by four different studies is that fungal infection induces the emission of new volatile organic compounds (VOC) with a composition that varies depending on the species of infecting fungus (Prithiviraj et al. 2004; Vikram et al. 2004; Lui et al. 2005; Moaleniyan et al. 2007) (Table 2). VOC has been shown to have direct and indirect roles in protecting plants against herbivory (Llusia & Peñuelas 2001; Peñuelas & Llusia, 2004), as well as in contributing to the plant's defence strategies against thermal damage (Copolovici et al. 2005; Peñuelas et al. 2005). Thus, these ecometabolomic studies of plant emissions due to fungus infection suggest a role of defense coordination among plant organs and/or among different individual plants at community level.

Fungus can also establish symbiotic relationships with plants to enhance the nutrient and water absorption capacity. Akiyama et al. (2005) using HPLC-MS and ^{1}H NMR ecometabolomic studies of root exudates found that Lotus japonicus exudates sesquiterpene lactones, stimulating the hyphal branching of the symbiotical fungus *Giaspora margarita*. Cao et al. (2008) studied the symbiotic relationship between *Lolium perenne* and the endophytic fungus *Neotyphodium lolii* using mass spectrometry. The ecometabolomic study showed that plants with fungus symbionts

contain some fungal compounds such as peramine, mannitol and other metabolites in their metabolome as a result of their relationship with the fungus. Some of these metabolites have been identified as plant-endophyte symbiotic metabolism regulation, but its role is not already stablished. All hypothesyes suggest a possible role in the improvement in the resistance to biotic or abiotic factors such as herbivores or drought (Cao et al. 2008). Thus the current ecometabolomic studies in plant-fungus symbiotic relationships are promising for a better comprehension of the metabolic mechanisms underlying in the symbiosis success in field conditions.

Plant-bacteria and Plant-virus

Similar to plant-fungus, the relationship between plants and microbes can be either a host-pathogen relationship or a mutualistic relationship. Shifts in metabolome fingerprinting have been described in host plant-pathogen bacteria relationships (Allwood et al. 2010). Some partial ecometabolomic studies of polar metabolites have shown that bacterial infection induces chemical defensive mechanisms involving an increased synthesis of diverse metabolites such as threonine, sinapoyl-malate, caffeoyl-malate, γ-aminobutic acid, rutin or phenylpropanoids depending on the species (Barsch et al. 2006; Jahangir et al. 2008; López-Gresa et al. 2010) (Table 2).

Pathogen bacteria can change plant metabolism. In this way, studies of polar and non-polar metabolites have reported that some primary plant metabolites increased their concentration under microbe infection but also that some secondary metabolite pathways were also stimulated by microbe infection, eg. terpenoid indole alkaloids and phenylpropanoids (Choi et al. 2004) and flavonoids (Cevallos-Cevallos et al. 2009) (Table 2). Ecophysiological studies using metabolomic techniques have also shown that they are able to detect differences in plant metabolism shift depending on the virulence (Simoh et al. 2008) or the nutritional requeriments (André et al. 2005) of the microbe strain. Ecometabolomic studies can also help to discern what environmental conditions are more or less favourable for the success of the symbiontic relationships. Barsch et al. (2006) have observed that the host plant *Medicago sativa* reacts against nitrogen-fixation-deficient bacteroids with a decrease of organic acid synthesis and an early induction of their senescence.

The symbiotic relationships between some plant taxons, such as the Fabaceae, with certain anaerobic bacteria are of great ecological and farming importance. The few metabolic studies available on this topic have observed that plants induced nodule formation by increasing the synthesis and nodule accumulation of particular metabolites such as asparagine, glutamate, putrescine, mannitol, threonic acid or gluconic acid (Desbrosses et al. 2005).

Studies to improve the ecological tools for environmental restoration are also an emerging area. In this field, Narasimhan et al. (2003) in a rhizosphere study using a HPLC-MS partial ecometabolomic analysis of rhizosphere secondary metabolites has reported that phenylpropanoid-utilizing microbes are able to enhance soil polychlorinated biphenyls (PCBs) depletion. This is useful in soil phytoremediation for a successful selection of rizhosphere microbes that degrade PCBs, which are very toxic organic pollutants.

The repercussions in the metabolome of viral infections in plants in the field have been little studied. Ecometabolomics of polar metabolites suggest that plants respond similarly to bacteria infection, increasing the synthesis of organic acids and terpenoids (Choi et al. 2006; López-Gresa et al. 2010).

Plant-animal

Plant insect interactions are one of the most widely studied topics in ecology. Ecometabolomics offers the possibility of a more in-depth study of the biochemical aspects of this interaction and of its ecological implications. Some partial ecometabolomic studies of polar metabolites have found a great chemical variation in the metabolites used by plants to defend themselves against insect attacks (Widarto et al. 2006; Jones et al. 2006; Poëssel et al. 2006; Jansen et al. 2009; Leiss et al. 2009a and 2009b; Kuzina et al. 2009; Minezhad et al. 2010) (Table 2). These studies have demonstrated that some hormones, acylsugars and, mainly, different secondary metabolites such as chlorogenic acid, saponins, phenolics, terpenes, alkaloids, terpenes and glucosinolates are the most general plant chemical defenses against insect herbivores. These results further confirm that a great variability of chemicals are used by plants as chemical deterrents and also the different capacity of chemical resistence among different plant species and also among different subsp. of the same species (Mirneshad et al. 2010). These results also suggest that the consequences at community level of plant-insect interaction can vary depending on the taxonomy of the plant and insect as a result of the variability of the changes in palatability, stoichiometry and chemical signals. Insect attack not only affects soluble metabolites but also the molecular composition of volatile emissions from plants (Peñuelas et al. 1995; Ozawa et al. 2000; Gaquerel et al. 2009) (Table 2). Terpenes are induced and emitted in response to internal (genetic and biochemical) and external (ecological) factor both biotic and abiotic and play an important role in plant communication among plants and with animals has been widely observed (Peñuelas and Llusia 2001; Peñuelas et al. 2005). Metabolome studies in specialist insects *Pieris brassicae* feeding on the leaves of *Brassica oleracea* have shown that insect growth was not affected and that the insect metabolized some flavonoids of *B*. *oleracea* (Ferreres et al. 2007) and accumulated certain other flavonoids (Jansen et al. 2009) suggesting a

possible defense mechanism against predators. Ecometabolomic studies have also allowed observing changes in hormone concentrations of Pseudotsuga menziesii in response to the insect, Megastigmus spermotrophus attack (Chiwocha et al. 2006). The conclusion of all these results is that ecometabolomics is a useful tool to discover unexpected bioactive compounds involved in ecological interactions between plants and their herbivores.

Arany et al. (2008) in an ecometabolomic study of polar and non-polar metabolites found that *Arabidopsis thaliana* shows that aliphatic glucosinolates and total glucosinolates affect the generalist herbivore *Spodoptera exigua* but not the specialist herbivore *Plutella xylostella.* This study demonstrates different strategies of plant defence against generalist and specialist herbivores although future studies are necessary to advance in this topic. In the research line of plant-herbivore relationships, Yang and Bernards (2007) have studied the metabolome shift after leaf cutting to investigate the metabolic pathways involved in suberification. Some recent studies suggest that ecometabolomic studies can be also useful to study the molecular interaction between mammals and plants (Tucker et al. 2010).

Plants synthesize complex sets of substances that interact with other plants and with animals. Ecometabolomic studies can play a key role in advancing the knowledge of the mechanisms of action of these substances on organisms. An example of this possible role comes from the information provided by several partial ecometabolomic studies that have observed increases in the contents of metabolites in terrestrial plants linked to chemical defensive and/or antistress mechanisms in response to methyl jasmonate (Liang et al. 2006a and 2006b; Hendrawati et al. 2006). Methyl jasmonate has also proved to enhance the monoterpene emission by 20-30% in *Quercus ilex* leaves (Filella et al. 2006) suggesting that plants change their metabolism not only to enhance a direct defensive response but also to enhance signalling to attract natural enemies of herbivores. However, we must highlight that in the most studies the animal was an insect an there are a lack of studies with vertebrate herbivores.

Competition

Peiris et al. (2008) by using GC-MS partial ecometabolomics of polar metabolites have studied the mycelial tissues of three competing fungus species during wood decomposition. They observed that the synthesis of 2-methyl-2,3-dihydroxypropinoic and pyridoxine acids can be involved in defensive mechanisms activated in response to direct contact between the mycelia of the different fungus species studied, thus showing the importance of exudated metabolites as resources for chemical defence in direct competition for space and sources. Recently, Paul et al. (2009) have studied the inter-specific competition and allelophatic effects between two diatom species, observing different metabolites in monocultures than those observed in a co-culturing

set up. This indicates either transformation or uptake of released metabolites by the competing species. The effects of species richness on plant metabolomes were also studied in five herbs; two tall-growing herbs (*Medicago* x *varia* and *Knautia arvensis*) and three small-growing herbs (*Lotus corniculatus*, *Bellis perennis* and *Leontodon autumnalis*) competing in communities of different species composition and richness (Scherling et al. 2010) (Table 2). They found different metabolome shifts in plant species growing in such different communities. All these results suggest new lines of research in the frame of competition in natural ecosystems by studying the ecometabolomic relationships between competitors.

Other biotic relationships

A functional assessment of antagonistic microbial communities in soil requires in-depth knowledge of the mechanisms involved in these interactions. Metabolome studies provide an adequate tool for this purpose. Haesler et al. (2008) found the metabolite polyketide cycloheximide to be the molecule responsible for the antagonistic effect of the actinobacterial genus *Kitasatospora* against the oomycetous root pathogen *Phytophthora citricola*.

Other important biotic relationships such as animal-microbe have been rarely studied by partial ecometabolomic methods (Solanky et al. 2005) (Table 2).

Table 2. Ecometabolomic studies that focused on biotic interaction effects on organism metabolism. The studies which aimed to analyze the entire metabolites spectrum (both polar and non-polar metabolites) are highlighted in bold type.

Species	Analytical techniques and study type	Main results	Reference
PLANT-FUNGUS			
Brassica rapa, Leptosphaeria maculans, Aspergillus niger, Fusarium oxysporum	^{1}H NMR, PEM polar metabolites	↑ Flavonoids, phenylpropanoids in infested plants	Abdel-Farid et al. 2009
Lotus japonicus, Gigaspora marginata	IR, HPLC-MS, ^{1}H NMR, PEM polar metabolites	↑ Strigolatones in root plant exudates are related to hyphal branching in arbuscular mycorrhizal fungi	Akiyama et al. 2005
Brachypodium distachyon, Magnaporthe grisea	FT-IR MF MS/MS, PEM non-polar metabolites and polar fatty acids	↓ Phosphatidyl glycerol in infested plants	Allwood et al. 2006
Lolium perenne, Neotypodium lolii	Linear ion trap-MS, PEM polar metabolites	Detection of fungus metabolites in infested plants (Manitol, cyclic oligopeptides)	Cao et al. 2008
Vitis vinifera, Uncinula nector, Plasmopara viticola	^{1}H NMR, PEM polar metabolites	↑ Inositol, caffeic acid in infested plants	Figueiredo et al. 2008
Triticum aestivum, Fusarium	**GC-MS, polar and non-polar**	↑ Metahidroxycinnamic acid, myo-inositol, glucose, malonic acid,	Hamzehzarghani et al. 2005

graminearum	**metabolites**	several fatty acids, malonic acid were related to fungus infection resistance in plats	
Triticum aestivum, Fusarium graminearum	**GC-MS, polar and non-polar metabolites**	↑↓ Many metabolic shifts emphasizing in: fatty acids, organic acids and phenolics	Hamzehzarghani et al. 2008
Helianthus annuus, Sclerotinia sclerottorum	^{13}C NMR, ^{31}P NMR, PEM polar metabolites	↓ Sugars and aminoacids in plant and fungus during infection Glycerol was exclusively produced in infected plant tissues	Jobic et al. 2007
Triticum aestivum, Mycosphaerella graminicola	^{1}H NMR, PEM polar metabolites	↑ aminoacids in infested plants	Keon et al. 2007
Vitis vinifera, Phaeomoniella spp, Fomitiporia spp.	^{1}H NMR, PEM polar metabolites	↑ Phenolics, methanol, alanine, γ-aminobutyric acid (defence mechanisms). ↓ Carbohidrates	Lima et al. 2010
Solanum tuberosum,Phytophthora infestans, Pythium ultimum, Botrytis cinerea	GC-MS, PEM volatile metabolites	↑ Increases of emission and emission of new volatiles in infested Plants with changes depending of the fungus species	Lui et al. 2005
Alnus incana, *Frankia* spp.	^{15}N NMR, ^{31}P NMR PEM polar metabolites	↑ Alanine, glutamine, citruline, arginine, c-aminobutyric acid	Lundberg and Lundquist 2004
Glycine max, Phytophora sojae	**GC-MS, polar and non-polar metabolites**	↑ lactic acid, salicylic acid ↓ sugars, aa	McGarvey and Pocs 2006
Magnifera indica, Lasiodiplodia theobromae, Colletotrichum gloeosporiodes	GC-MS, PEM volatile metabolites	↑ Increases of emission and emission of new volatiles in infested plants that changes depending of the fungus species	Moalemiyan et al. 2007
Lupinus angustifolius, Colletotrichum lupini	HPLC-DAD, HPLC-MS, PEM phenolics	↑ Isoflavones aglycones in infested plants	Muth et al. 2009
Triticum aestivum, Fusarium graminearum	**GC-MS, polar and non-polar metabolites**	↑ Putrescine, inositol, inositol phosphate, several aminoacids in infected plants	Paranidharan et al. 2008
Brachypodium sistachyon, Magnaporthe grisea	**GC-MS, polar and non-polar metabolites**	↑ Mannitol and glycerol production in fungus with plant photosyntate are the cause to conduct hyphal grown suggesting that fungal deploys a common metabolic re-programmin strategy in host species	Parker et al. 2009
Allium cepa, Erwinia carotovor a ssp. *Carotova, Fusarium oxysporum, Botrytis alli*	GC-MS, PEM volatile metabolites	↑ Increases of emission and emission of new volatiles in infested plants that changes depending of the fungus species	Prithiviraj et al. 2004
Triticum aestivum, Stagonospora nocorum	^{1}H NMR, PEM polar metabolites	tetrahalose is necessary for sporulation during infection	Solomon et al. 2005
Medicago truncatula, Glomus intraradices	GC-MS, HPLC-MS, HPLC-DAD, PEM polar metabolites	↑ isoflavones, saponins, apocarotenoids	Strack et al. 2006
Malus domestica, Botrytis cinerea, Mucor piriformis, Penicillum expansum, Monilinia spp.	GC-MS, PEM volatile metabolites	↑ Increases of emission and emission of new volatiles in infested plants that changes depending of the fungus species	Vikram et al. 2004
PLANT-MICROBE			
Arabidopsis thaliana, Pseudomonas syringae (Pst)	FT-IR, MF, polar metabolites	Evidendes that infection produces metabolic changes in both plant and microorganism	Allwood et al. 2010

Medicago sativa, Sinorh*izobium meliloti*	^{1}H NMR, PEM polar metabolites	Plant react to N-fixation-deficient bacteroids by decreasing organic acid synthesis and by inducing early induction of senescence	Barsch et al. 2006
Citrus sinensis, *Candidatus liberibacter*	**Capillary electrophoresis-DAD, Polar and non-polar metabolites**	↑ Hesperidin, naringenin, quercitin and other 3 non identified compounds	Cevallos-Cevallos et al. 2009
Catharanthus rosea, phytoplasma	**^{1}H NMR, HPLC-MS, polar and non-polar metabolites**	↑ Terpene indole alkaloids	Choi et al. 2004
Arabidopsis thaliana, Rhodococcus fascians	GC-MS, PEM polar metabolites	↑ Aminoacids, sugars in infested plants were induced by cytokines secreted by *R. fascians* ↓ Defensive pathways in infested plants	Depuydt et al. 2009
Medicago trunculata, Lotus japonicus, Rhizobium spp.	HPLC-DAD, GC-MS, PEM polar	↑ Octadecanoic acid, asparagine, glutamate, homoserine, cysteine, putresceine, mannitol, threonic acid, gluconic acid and glycerol in nodules than in the rest of plant	Desbrosses et al. 2005
Brassica rapa, Staphyloccocus aureus, Escherichia coli, Salonella typhimurium, Shigella flexneri	^{1}H NMR, PEM polar metabolites	↑ GABA in plants infected by Gram+ ↑ Sinapoyl-malate, caffeoyl-malate acid, histidine in plants infected by Gram-	Jahangir et al. 2008
Solanum lycospericum, Pseudomanas syringae	^{1}H NMR, HPLC-MS, PEM polar metabolites	↑ Rutin and phenylpropanoids	López-Gresa et al. 2010
Arabidopsis spp., phenyl-propanoids utilizing microbes	HPLC-MS, PEM polar metabolites of rhizosphere	Detected changes in phenylptopanoids that allows information of microbes that are able to degrade polychlorinated biphenyls	Narasimhan et al. 2003
PLANT-VIRUS			
Nicotiana tabacum, tobacco mosaic virus	^{1}H NMR, PEM polar metabolites	↑ Sesqui- and di-terpenoids, □-linolenic acid analogues, 5-caffeoylquinic acid	Choi et al. 2006
Solanum lycospericum, Citrus exocortis virioid	^{1}H NMR, HPLC-MS, PEM polar metabolites	↑ Glycosilated gentisic acid	López-Gresa et al. 2010
PLANT-ANIMAL (herbivory, including simulated wound stress)			
Pseudotsuga menziesii, Megastigmus spermotherophus	HPLC-MS-MS, PEM volatile metabolites	↑ Acid abcisic metabolism in unpolinized plants buy not in polinized plants and some temporal variations in the rest of hormones.	Chiwocha et al. 2007
Brassica oleracea, *Pieris brassicae*	HPLC-DAD, HPLC-MS/MS, PEM polar metabolites	Evidences that carterpillar metabolize some plant secondary compounds and accumulate others as possible mechanism of defense	Ferreres et al. 2007
Nicotiana attenuate, *Manduca sexta*	GC-MS, PEM volatile metabolites	↑ Terpenoids. hexenylesters ↓ Short chain alcohols	Gaquerel et al. 2009
Brassica oleracea, *Pieris rapae*	UPLC-MS, PEM polar metabolites	Evidences that carterpillar metabolize some plant secondary compounds and accumulate others as possible mechanism of defense	Jansen et al. 2009
Arabidopsis thalian, and specialists and generalist herbivore insects	HPLC-MS, PEM secondary metabolism	Changes in methylation patterns sinapoyl malate and in the ratios between thioether versus methylsulfinyl glucosinolates.	Jones et al. 2006
Barnarea vulgaris, *Phyllotreta nemorum*	HPLC-MS, PEM polar and semipolar metabolites	↑ Saponins	Kuzina et al.2009

Senecio jacobaea, Senecio aquaticus, Frankiniella occidentalis	^{1}H NMR, PEM polar metabolites	↑ Pyrrolizine alkaloids, jacobine, jaconine, kaempferol glucoside	Leiss et al. 2009a
Dendranthema grandofolia, *Frankiniella occidentalis*	^{1}H NMR, PEM polar metabolites	↑ Chlorogenic acid, feruloyl quinic acid	Leiss et al. 2009b
Lycopersicom spp., *Solanum* spp., *Frankliniella occidentalis*	^{1}H NMR, PEM polar and semipolar metabolites	↑ Acylsugars	Mirnezhad et al. 2009
Arabidopsis thaliana, *Spodoptera exigua* (generalist), *Plutella xylostella* (specialist)	**^{1}H NMR, PEM polar and non polar metabolites**	Several metabolites were different among different Arabidopsis populations and glucosinolates concentration decreased the generalist but not the specialist insect growth	Arany et al. 2008
Phaseolus lunatus, *Spodoptera exigua*, *Mythimna separata*, *Tetranychus urticae* (spider)	GC-MS, PEM volatile metabolites	Different volatile signalling pathway in the insect attack than in spider attack	Ozawa et al. 2000
Prunas persica, Myzus persicae	^{1}H NMR, PEM polar and semipolar metabolites	↑ Phenolics, cyanogenic compounds	Poëssel et al. 2006
Brassica oleracea, *Pieris rapae*	**^{1}H NMR, HPLC-MS, PEM polar and non polar metabolites**	↑ pinoresinol	Schroeder et al. 2006
Eucalyptus sp. Marsupial and insect folivorous	^{1}H NMR, PEM apolar metabolites	↑ flavones	Tucker et al. 2010
Brassica rapa, *Plutella xylostella, Spodoptera exigua*	^{1}H NMR, PEM polar metabolites	↑ Glucose, Feruloyl, sinapoyl malate, gluconapin, sucrose, threonine	Widarto et al. 2006
Solanum tuberosum, wound stress	**GC-MS, polar and non-polar metabolites**	↑ Organic acids, sugars, aminoacids, phenylpropanoids and suberin aliphatic monomers	Yang and Bernards 2007
INFOCHEMICALS			
Methyl jasmonate	^{1}H NMR, PEM polar metabolites	↑ Flavonoids, fumaric acid, singirin, tryptophan, valine, threonine, valine ↓ Malic acid, ferulyolmalate, glutamine, several sugars	Hendrawati et al. 2006
Caenorhabditis elegans	^{1}H NMR, GC-MS, HPLC-MS, PEM exudate metabolites	Exudates include 36 common metabolites including sugars, aminoacids and organic acids. These metabolites attract bacteries	Kaplan et al. 2009
Methyl jasmonate, *Brassica rapa*	^{1}H NMR, HPLC-MS, PM polar metabolites	↑ Phenylpropanoids	Liang et al. 2006a
Methyl jasmonate, *Brassica rapa*	**^{1}H NMR, HPLC-MS, polar and non-polar metabolites**	↑ Hydroxycinnamate, glucosinolates	Liang et al. 2006b
Methyl jasmonate and it precursores, *Zea mays, Cotesia kariyai*	GC-MS, PEM volatile metabolites	Methyl jasmonate induces attraction of insect by ↑ hexenyl acetate iit the precursor of methyl jasmonate attracted insets by ↑ -pinene and menthol	Ozawa et al. 2008
COMPETITION			
Skeletonema costatum, Thalassiosira weissflogii	UPLC-MS, PEM polar exhudate metabolites	Several metabolites detected in monocultures were not found in co-culturing set up, indicating either a transformation or uptake of released metabolites by competing species	Paul et al. 2009
Stereum hirsutum, *Coprinus*	TLC, GC-MS, PEM polar	↑ 2-methyl-2,3-	Peiris et al. 2008

disseminatus, Coprinus micaceus	exhudate metabolites	dihydroxypropionic acid, pyridoxine involved in defensive mechanisms in direct contact between mycelia of different fungus species	
OTHER RELATIONSHIPS			
Phytophhora citricota (fungus), *Kitasatospora* spp. (bacterium)	^{1}H NMR, FT-MS, PEM polar and semi-polar metabolites	Polyketide cycloheximide was found to be secreted by actinobacteria and vas strong antibiosis effect against fungus.	Haesler et al. 2008
Medicago x varia, Knautia arvensis, Lotus corniculatus, Bellis perennis, Leontodon autumnalis (effect of biodiversity)	GC-MS, LC-FT-MS, PEM polar and semi-polar metabolites	Species richness produce different metabolomic shifts in the studied species. Changes are different depending of the specie.	Scherling et al. 2010
Salmo salar (animal), *Aeromanas Samonicida* (bacterium)	^{1}H NMR, PEM polar metabolites	↑ Acetylcholine, phosphotidylcholine, methylamine pathway ↓ Betaine, cholesterol, □- □-carbohidrates	Solanky et al. 2005
ANIMAL BEHAVIOR			
Migration, *Schistocerca gregaria*	^{1}H NMR, PEM polar metabolites	↑ Putresceine	Lenz et al. 2001
Migration, *Schistocerca gregaria*	^{1}H NMR, PEM polar metabolites	↑ L-dopa analogue	Miller et al. 2008
Caenorhadditis elegans	^{1}H NMR, HPLC-MS, PEM exudate metabolites	↑ Ascarosides regulated sexual synchronism between male and female	Pungaliya et al. 2009
Caenorhadditis elegans	^{1}H NMR, GC-MS, PEM exudate metabolites	↑ Ascarosides increased mating and development	Srinivasan et al. 2008

PEM = Partial ecometabolomic study. MF = Metabolic fingerprinting study.
Polar fraction: Aminoacids and organic acids (citric, malate,..), mono, di and tri sacharides, inositol, sucrose, phenolics.
Lypophylic (non polar) fraction: Fatty acids and their derivates, hydrocarbones, alkaloids, flavolon aglycones, triterpenoids, steroids.
HPLC = High pressure liquid chromatography.
MS = Mass spectroscopy. TLC = Thin Layer Chromatography
FT-IR = Fouries transformation-Infrared Spectroscopy.
^{1}H NMR = Nuclear magnetic resonance of ^{1}H.
^{13}C NMR = Nuclear magnetic resonance of ^{13}C.
^{31}P NMR = Nuclear magnetic resonance of ^{31}P
GC = Gas chromatography.
UPLC = Ultra-performance liquid chromatography.

Animal behaviour

Animal processes such as migrations, mutualistic associations or reproductive strategies are species-specific traits associated to physiological and metabolic changes. In spite of this, some of these phenomena have not yet been studied using ecometabolomic approaches. *Schistocerca gregaria,* a desert locust that forms great migratory swarms has been studied using a ^{1}H NMR partial ecometabolomics analysis of polar metabolites. This study has shown that some metabolites like putrescine are linked to solitary behaviour (Lenz et al. 2001), while others such as the alkaloid L-dopa analogue are linked to gregarious behaviour (Miller et al. 2008). These studies should provide significant advances in knowledge about and predictions of the future behaviour of such populations. This is of great importance for plague management. Another

promising study has been conducted in the exudates of the nematode, *Caenorhadditis elegans*, with ^{1}H NMR and has proved that ascarosides are the substances responsible for mating and sexual reproductive synchronization between males and females of this species (Srinivasan et al. 2008; Pungaliya et al. 2009). However, there is al lack of studies on other taxonomic groups than insects to reach a more global knowledge on this topic.

Interactions between abiotic and biotic factors

Few studies have investigated the effects of abiotic factors on biotic relationships. Among them we highlight ecometabolomic studies of polar metabolites that have investigated the effects of N availability on plant-fungus infection relationships (Rasmussen et al. 2008), showing that N availability affects both plant metabolite concentrations and fungus infection (Table 3). Recently, Larrainzar et al. (2009) studying N_2-fixation under drought observed a decrease in amino acids and sugar concentration and in the N_2-fixation activity in roots of *Medicago trunculata*. Prior to this, Rosenblum et al. (2005) used a ^{1}H NMR partial ecometabolic analysis to study the polar metabolites in red abalone, Haliotis rufenses, during infection by the rickettsia *Candidants xenohaliotis californiensis* under different levels of food sources. They concluded that food levels and other abiotic factors such as water temperature are responsible for disease development Likewise the *Haliotis* spp. metabolome shift response induced by *Rickettsia* spp included decreases in aminoacids and carbohydrates and increases in taurine, glycine, betaine and homarine similarly at different temperatures (Rosenblum et al. 2006) (Table 3).

Table 3. Ecometabolomic studies involving two or more ecological relationship effects on organism metabolism.

Factors and species	Analytical techniques, study type	Main results	Reference
Volatile emissions of pant (*Zea mays*)-attacked by herbivore (carterpillars to attract parasit wasps , natural enemies of the herbivores	GC-MS, TP volatile compounds	23 volatile compounds were identified as response to carterpillar regurgitant injection in different *Zea mays* genotypes and the species specific emissions varied among *Z. mays* phenotypes	Degen et al. 2004
Drought effects on plant (*Medicago trunculata*)-N_2 fixers.	GC-MS, PEM polar metabolites	Drought reduced N_2 fixation rates and aminoacid and carbohydrates concentration	Larrainzar et al. 2009
N availability effect on Plant (*Lolium perenne*)-fungus	GC-MS, PEM polar and non-polar primary metabolites,	The effects of fungus on plant metabolome is great and depends of N supply levels	Rasmussen et al. 2008

(*Neotyphodium lolii*) relationships	flavonoids and anthocyanins		
Effects of water, temperature and food availability on animal (*Haliotis refrescens*)-microbe (*Candidatus xenohaliotis californiensis*) relationships	^{1}H NMR, PEM polar metabolites	Glucose:homarine concentration ratio in foot muscle results the metabolic marker for differentiating *Haliotis* individuals only infected than those both infected and also food limited	Rosenblum et al. 2005
Effects of temperature on animal (*Haliotis* spp.)-microbe (*Rickettsia* spp.) relationships	^{1}H NMR, PEM polar metabolites	Infection increased taurine, glycine, betaine and homarine at all temperatures studied	Rosenblum et al. 2006

PEM = Partial ecometabolomic study. MF = Metabolic fingerprinting study.
Polar fraction: Aminoacids and organic acids (citric, malate,..), mono, di and tri sacharides, inositol, sucrose, phenolics.
Lypophylic (non polar) fraction: Fatty acids and their derivates, hydrocarbones, alkaloids, flavolon aglycones, triterpenoids, steroids.
HPLC = High pressure liquid chromatography.
MS = Mass spectroscopy. TLC = Thin Layer Chromatography
FT-IR = Fouries transformation-Infrared Spectroscopy.
^{1}H NMR = Nuclear magnetic resonance of ^{1}H.
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GC = Gas chromatography.
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Metabolomics coupled with other omics approaches

Metabolomics allows the determination of the phenotype response directly (Fiehn et al. 2000) whereas transcriptomics and proteomics analyze the way genome expression translates to biological response thus allowing to detect the genes that are been expressed in one determined moment (Colebatch et al. 2004; Fridman and Pichersky 2005; Saito and Matsuda 2010). The simultaneous use of metabolomics with genomics, transcriptomics and/or proteomics provides a global overview from trancription to final metabolic products that allows reaching better knowledge of the regulatory networks of metabolic pathways than the omics studies used without metabolomics analysis. For instance there are many metabolomics pathways regulated at post-transcriptional level (Kaplan et al. 2007). The integrated omic studies are specially promising for ecological studies since they make possible to discern between genotype dependent and independent response to environmental changes for multiple characters at once (Pena et al. 2010; Weinkoops et al. 2008). This provides an overview of the short, medium and long-term responses to environemental changes and their integrative relationships. Metabolomic databases can be combined with data sets from other "omic" technologies (Weckwerth 2008) to enhance data value and permit a system-wide analysis from genome to phenome (just as the genome and proteome signify all of an organism's genes and proteins, the phenome represents the total sum of its phenotypic traits).

Some past studies have begun to demonstrate these arguments. Metabolomics and transcriptomics can be combined and analyzed mathematically together. In this way, the

metabolites and genes regulated by the same mechanisms cluster together (Table 4). For example, integrated experiments in transcriptomic and ecometabolomic studies have been successfully conducted in *Arabidopsis* at different levels of sulphur supply to elucidate gene-to-gene and metabolite-to-gene networks (Hirai et al. 2004 and 2005; Nikiforova et al. 2004 and 2005b; Fukushima et al. 2009). These studies allow the detection of general and specific responses to different nutrient deficiencies and the identification of gene function with the added value of the improvement in the production of useful compounds in plants. Furthermore, some of these studies have provided novel knowledge among metabolic pathways linked to physiological endpoint involved in plant homeostasis and responses to nutrient stress (Nikiforova et al 2004 and 2005b). Similarly coupling metabolomic with other omic approaches has allowed the improvement of our knowledge of the metabolic process and their regulation in response to other environmental factors such as salinity (Brosché et al. 2005; Gong et al. 2005), drought (Vasquez-Robinet et al. 2008), oxidative stress (Baxter et al. 2007), symbiontic N_2 fixation (Hernández et al. 2009; Sánchez et al. 2010), and plant-herbivore (Kant et al. 2004) and plant-microbe (Ward et al. 2010) interactions (Table 4). Similar holistic results can be expected by combining ecometabolomics with proteomics (Weckworth 2008). For more detailed and specific information see Macel et al. (2010) that provides detailed information about the possibilities of integration of metabolomics with other omics approaches.

Table 4. Ecometabolomic studies coupled to other omics studies.

Factors and species	Analytical techniques, study type	Main results	Reference
Arabidopsis sp under S stress	Metabolomics (GC-MS, PEM polar metabolites) and transcriptomics	↓ carbon and aa pathways and overall vision of antioxidative metabolism strategy	Baxter et al. 2007
Different *Populus* sp. under drought	Metabolomics (GC-MS, PEM polar metabolites), genomic and transcriptomic	Drought increased aa contents. The most drought adapted species did not present different genes per se than the other species but the regulation of gene expression might be different	Brosché et al. 2005
Arabidopsis thaliana and *Thellungiella halophile* under salt stress	Metabolomics (GC-MS, polar and apolar metabolites) and transcriptomic	Identification of genes and metabolites used for two species in response to salt stress	Gong et al. 2005
Phaseolus vulgaris (plant), *Rhizobium tropici* (simbyont) under different phosphorus availability	Metabolomics (GC-MS, PEM polar metabolites), transcriptomics	Different levels of P affects the gene expression of plant In P deficient nodules aa decreased while organic and polihydroxy acids increased	Hernández et al. 2009
Arabidopsis thaliana, N and S stress	Metabolomics (FT-ICR-MS, polar and non polar metabolites)	General change of N and S metabolism detected in different plant organs	Hirai et al. 2004

	and transcriptomics		
Arabidopsis sp under S stress	Metabolomics (FT-ICR-MS, HPLC-DAD, PEM polar metabolites) and transcriptomics	↑ Anthocyanidin synthesis	Hirai et al. 2005
Lycopersicon esculentum (plat) and *Tetranychus urticae* (animal)	Metabolomics ((GC-MS, PEM volatile metabolites), transcriptomics	↑ genes of biosynthesis of mono- and diterpenes and genes of phospholipid metabolism ↑ emissions on monoterpenes that increased the olfactory presence of predators	Kant et al. 2004
Arabidopsis thaliana under S stress	Metabolomics (GC-MS, PEM polar metabolites) and transcriptomics	Elucidate the response gene-metabolite network from the transcript and metabolomic profile using mathematical algorithms	Nikiforova et al. 2004 and 2005b
Lotus japonicus, salt stress studied in different experiments with different experimental conditions	Metabolomic (GC-MS, PEM polar metabolites), transcriptomics	Large fraction of the transcriptional and metabolomic responses to salt stress were not reproducible between experiments	Sanchez et al. 2010
Solanum tuberosum subsp. *andigena* and *solanum* subsp. *tuberosum*, drought stress	Metabolomics (GC-MS, PEM polar metabolites), transcriptomics	Different gene expression between two species ↑ proline, trehalose, GABA	Vasquez-Robinet et al. 2008
Arabidopsis thaliana, Pseudomonas syringqae	Metabolomics (^{1}H NMR, fingerprinting, GC-MS PEM apolar metabolites), transcriptomics	↑ aa, glucosinolates, phenolics Pathogen bacteria were able to superimpose to defense plant suppression The study enables to distinguish metabolic pathways that are transcriptionally activated from those that are post-transcriptionally activated	Ward et al. 2010

PEM = Partial ecometabolomic study. MF = Metabolic fingerprinting study.
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Further applications in ecology

Ecometabolomic studies may provide new perspectives and insights to many ecological topics. In an attempt to highlight the important role that metabolome studies could have in ecology, here we propose the application of metabolome studies to some critical questions, currently under debate in ecology (Figure 1).

Stoichiometry, growth rate and other ecological hypotheses

Organisms are the products of chemical reactions, and their growth depends on the availability of various elements, especially carbon, nitrogen and phosphorus. In this context, ecometabolomic

studies also provide an opportunity to make direct advances related to the ecological hypotheses that aim to study ecosystem structure and function from a chemical perspective. One those hypotheses, the growth-rate hypothesis (GRH) (Sterner and Elser 2002) links the relative element content of organisms to their growth rate, the idea being that fast-growing organisms need relatively more P-rich RNA, which is the main component of the protein-producing ribosome, in order to support rapid protein synthesis. Consequently, ecosystem conditions that produce organic matter with low C:P and N:P ratios would be expected to result in higher adaptive growth rates, more efficient energy transfer through a food web, and increased biomass of large-bodied animals relative to that of small-bodied organisms (Sterner and Elser 2002). Further assessment of the GRH evidently requires many more studies on the effects of C:N:P ratios on the ratios of different metabolic products such as proteins and RNA, and on growth rates and body sizes in different taxa and ecosystems (Peñuelas and Sardans 2009a). A promising way to couple stoichiometry with phenotypic metabolic expression can now be provided by ecometabolomic studies. Ecometabolomics should thus help to interpret the response of different groups of organisms in allocating resources to growth, storage and defence. It may also provide the elemental and metabolic budgets for different species along gradients from low to fast growth, which would allow a better test of the links between the C:N:P ratio, growth rate and body-size spectrum. Similarly, ecometabolomics can be a promising tool in the frame of metabolic theory of ecology (MTE) (Brown et al. 2004). MTE states that body mass and body temperature together predict per capita rates of metabolism, respiration, growth and resources consumption and that these rates can be scaled up to the level of populations and communities (West et al. 1999; van der Meer 2006). Ecometabolomics will allow to evaluate the change in the metabolism allocation to different general functions (respiration, growth, storage) in response to different environmental situations and changes.

More on biotic relationships

Ecometabolomic studies remain to be used to investigate other important relationships, such as plants-higher herbivores (e.g. mammals) or herbivore-predator. Moreover, most ecometabolomic studies have focused on the metabolic shifts in only one of the members of the relationships. Future ecometabolomic studies should investigate the metabolic shifts in the two species that interact simultaneously in order to have a whole vision of the phenotypic consequences of the biotic relationship. For example, some current studies aimed to study the metabolome shifts of both plant and herbivore (insect) have observed novel and useful information demonstrating the herbivore capacity to use plant metabolites to further defense itself against predators (Jansen et al. 2009). Similar reasoning can be applied to plant-pathogen,

plant-symbiont, or plant-vertebrate interactions and in general to all key ecological relationships between two or more organisms.

The possibility of studying the metabolome traits and changes of more complex trophic relationships, such as three or more trophic levels at once, under different environmental circumstances, and if possible in field conditions, remains a future challenge for ecologists. For instance, the study of Harvey et al. (2003) using conventional analytical methods is promising and suggest that the study of complex trophic relationships can take advantage by using of modern ecometabolomic approaches. These authors by analyzing water soluble glucosinolates studied the complex interactions of four trophic levels, observing that the leaf glucosinolates of *Brassica oleraceae* and *Brassica nigra* affect the development of the hyperparasitoid as mediated through the herbivore and its primary parasitoid. *B*. *nigra* has a more than 3.5 times higher level of glucosinolates than *B*. *oleracea*. Thus there is a greater constraint in the size and survival of primary and secondary parasitoids reared from herbivores feeding on *B*. *nigra* than in those reared from herbivores feeding on *B*. *oleracea*. These results demonstrate that herbivore diet can affect the performance of interacting organisms differently across several trophic levels, and suggests that bottom-top web structure and energy fluxes can be mediated by the quality of food sources.

Some studies suggest that symbiotic fungal endophytes control insect host-parasite interaction webs (Omachi et al. 2001; Hartley and Gange 2009). Ecometabolomic studies could characterize the metabolic pathways involved in these complex relationships. Some biotic relationships are mediated by exudated metabolites; the rhizosphere is a case in point. The ecometabolomics of the rizhosphere provides another interesting perspective that also merits future study because the chemical plant and microbial exudates found there have been shown to be a key factor in the regulation of plant-microbe relationships (Micallef et al. 2009; Biedrzycki and Bais 2009) and in absorption processes (Dessureault-Rompre et al. 2007).

Other relevant future applications in the context of multiple trophic relationships involve the analysis of highly significant abiotic gradients such as water availability in terrestrial ecosystems or temperature in aquatic ecosystems. This should provide a global perspective of the phenotype responses that are the most adequate to each environmental change and of the capacity of different species to respond to environmental changes by a plastic metabolome response. In this regard, ecometabolomics may provide insight into species' stress tolerance, resistance and avoidance, giving information about species' capability of changing their lifestyle and their capacity to respond to environmental changes such as severe drought, direct competition or perturbations. Ecometabolomics provides the possibility of studying different species' capacity to response to different environmental factors at short term and to study the

effects of those changes in chemical composition onto the trophic webs. Finally, this will increase knowledge of the mechanisms underlying species composition shifts in ecosystems, in response to changes in environmental conditions.

Species competition is an important ecological field where ecometabolomic studies have a promising future because as mentioned above only few studies have been conducted and the results suggest interesting species mechanisms in interspecific competition. Ecometabolomics provides a useful approach to the understanding of the mechanisms underlying competitive relationships. For example, as we have discussed earlier, Peiris et al. (2008) in a ecometabolomic study of the competition among three different fungus species competing during wood decomposition, reported that 2-methyl-2,3-dihydroxypropionic acid and pyridoxine are synthesized by some fungi to inhibit the growth of their direct competitors. Thus, competitive advantage that could be attributed to simply a greater growth capacity of one species compared to the others is in fact linked, at least partially, to a chemical growth suppression of the competitor species. However competition is not only an interspecific question. Intraspecific competition is also important in several ecological scenarios and ecometabolomics is a novel tool to be used to discern which metabolome changes occur in individuals submitted to different levels of intraspecific competition.

Animal behaviour is fundamental in the performance of several ecosystems; mutualism, reproductive phenology and several other behavioural phenomena are species characters that affect the performance and structure of whole ecosystems. Still, the mechanisms underlying such animal behaviour are as yet not well known; which are the metabolome shifts when behaviour changes and which are the implications in organism body composition. Ecometabolomics can contribute considerably in this area.

From individuals to populations and ecosystems

Ecometabolomic applications are not only limited to the ecophysiology of organisms. Some studies upscale the use of metabolomics from individual to population and ecosystem levels (Figure 2). For example, Davey et al. (2008) have shown that metabolite fingerprinting and profiling is sufficiently sensitive to be able to identify the metabolic differences between populations of Arabidopsis petraea. They found 2-4 fold differences in many free aminoacid concentrations between different populations. Many free carbohydrate concentrations were also different, while polyhydric alcohol concentrations were not. A principal component analysis of metabolite fingerprints revealed different metabolic phenotypes for each population. At the landscape level, Gidman et al. (2006) have shown that different metabolic fingerprints measured with rapid Fourier transform-infrared spectroscopy in tissue samples of *Galium saxatile* are

correlated with a gradient of N deposition across the entire UK landscape. Ecometabolomics thus allows the investigation of complex ecological systems and provides a rapid and sensitive indicator of ecosystem health. Viant (2007) has gone a step further, using the same argument as in the origin of metabolomics. Namely, that the measurement of multiple metabolites (versus one or a small group such as occurs in classical analytical approaches) can provide a more robust assessment of the metabolic health of an organism, and hence characterizing the health of multiple species will provide a more complete assessment of ecosystem responses to environmental stressors, and even of the nature of the stressor. The ecometabolomic studies may also be used to differenciate between genetically modified and non-modified plants.

Global change

Ecometabolomic studies of the effects of environmental gradients or of manipulation experiments in the field on various species should help to assess the impacts of the different components of global change on natural ecosystems namely: climate change, atmosphere composition changes, pollution, invasiveness and loss of biodiversity, among others. Ecometobolomic studies can help to discern species'and communities' capacity for adaptation to global changes by highlighting the metabolic pathways that are inhibited or stimulated, and by coupling with transcriptomics to determine what genes are involved in adaptation-evolution mechanisms. Moreover, the knowledge of chemical body changes that can affect the performance of trophic chains, aids in understanding shifts in ecosystem structure. The effects of these global changes can only be understood by using natural environmental gradients or field manipulation experiments that allow studying the responses of species and biotic relationships such as predation, herbivorism or parasitism in conditions which closely resemble actual environments.

Ecometabolomic studies of organisms growing while subject to different levels of pollutants are especially important to understand organism responses to these pollutants. This is particularly true since the mechanism of action of toxins is frequently based on chemical interactions in the cells that affect metabolic expression. Moreover, ecometabolomic studies can help to discover the metabolic pathways of these chemotoxic compounds. In this vein, studies on the hyperaccumulation of trace elements in plants to discover genotypes that accumulate large amounts of certain trace elements are another example where ecometabolomic studies could be useful. Hyperaccumulator plants are an important tool in phytoremediation and soil restoration strategies and the use of ecometabolomic studies could facilitate understanding the mechanisms of phenotypic expressions that allow plants to become hyperaccumulators.

Invasiveness is another current ecological problem of increasing global importance. When an ecosystem is invaded by one or more invasive species some studies have reported changes in ecosystem or body composition stoichiometry (Hughes and Uowolo 2006) and in some groups of metabolites (Llusia et al. 2010; Sardans et al. 2010; Peñuelas et al. 2011). Ecometabolomic studies can help to determine what metabolic pathways and target metabolites are involved in alien success and in the resistance capacity of native species. One example of the possibilities of ecometabolomic studies use in this field is in the advance in the knowledge of the suitability of the increased competitive ability hypothesis (EICA). EICA proposes that invasive plants may still experience attack by local generalist herbivores (Müller-Schärer et al. 2004) but not by specialist herbivores. In this way, selection may favour a reduction in the expression of chemical defences which are effective against specialist herbivores, but metabolically demanding, and an increase in the concentrations of less costly qualitative defences that may be more toxic to generalist herbivores (Joshi and Vrieling 2005; Stastny et al. 2005; Peñuelas et al. 2010). Using ecometabolomic studies of native and alien plants and of generalist and specialist herbivores conducted in the field and with an accurate use of methodologies can clarify this debate by showing the plant and herbivore metabolome changes and the differences in metabolic adaptation of herbivores to plant sources. Moreover, ecometabolomics will also allow the comparison of the metabolomes of alien plant populations, in their novel habitat, without their specialist herbivores, with the metabolomes of populations of the same species growing in their natural endemic habitat with both their generalist and specialist herbivores.

The loss of biodiversity at a global scale warrants further studies to discern its causes and its consequences. In some cases, the causes are obvious and direct: loss of habitat surface or habitat fragmentation, overexploitation of the sources or direct hunting. But in other cases, the causes are still controversial. For example, decreases in the biodiversity of pollinators in Europe and in other parts of the world are a current hot topic. As far as we know, there are no ecometabolomic studies investigating plant-pollinator metabolomes. Knowledge of changes in the metabolome in the field under climatic or pollutant stress gradients, for example of pesticides, could be very revealing in the study of the causes and the mechanisms responsible for the decrease in pollinators in some areas of the world.

Challenges

A serious challenge for ecometabolomic studies is to satisfy the need to disentangle the biological relevant functions and responses shifts under environmental changes, which implies to determine and to quantify the maximum number of metabolites as possible. The exact number of metabolites remains a mystery, even in the case of micro-organisms with simple and well-

understood metabolisms. Typical non-plant eukaryotic organisms are estimated to contain from 4000 to 20000 metabolites (Fernie et al. 2004) and the plant kingdom produces 100000–200000 different metabolites (Fiehn et al. 2001), although the actual number present in any individual plant species is still unknown. Furthermore, the metabolome changes continuously, an additional challenge that is accentuated when measuring the metabolomes of several individuals from a free-living population, which will necessarily include considerable metabolic variation. There will be high levels of variation in metabolite concentrations between individuals, owing to differences in individual genetics, gender, age, organs, health status and spatial and temporal environmental changes. Simple issues such as the time since the animal last ate or the plant last received sunlight may also be determinant. The treatment of the large temporal and individual variability found in metabolomes, which may tend to mask the sources of variation that are of interest for ecologists, can be successfully approached by trying to 'film' temporal changes in metabolite levels and their turnover rates (Peñuelas and Sardans 2009b) instead of merely taking 'snapshots' of metabolite levels, and by multiplying the number of individuals sampled. The continuous development of new advances in in vivo NMR spectroscopy and imaging, proton-transfer-reaction mass spectrometry, or isotope labeling and in the treatment of large data sets in bioinformatics will be of great assistance in this line of work (Gehlenberg et al. 2010).

Another challenge to face up to is the risk that, the overwhelming '-omics-type' information reaches a field which is conceptually not properly prepared, thereby leading to the loss of an opportunity to advance ecological knowledge. Certain explanatory principles accounting for the complexity of living organisms and their populations and ecosystems, as well as of their responses to the environment, are still lacking. Ecometabolomics will need thus to focus on conceptual advancement and functional trait discovery and not just on technological development if it is to shed light on the fundamental system-biological mechanisms at work on scales ranging from the individual to the ecosystem. Certainly, the use of multitrophic interactions by ecometabolomics is a complex task requiring the coupling of field studies to metabolomic studies of the organisms involved in the study. This requires interdisciplinary approaches to relate the changes observed in filed measurements (growth, density, reproduction, predation,..) with those observed in organisms' metabolomes.

Ecometabolomic techniques have proven to have enough sensitivity for ecological studies of the metabolome response of diverse genotypes under different environmental conditions. However the lack of past experiments in the field focused on the effects of several factors on the organism metabolome limits at present the findings of metabolome studies from an ecological perspective. On the other hand, the experimental designs of future studies should aim to also disentangle the causes of the metabolome shifts. For example, Robinson et al. (2007) observed a

strong relation between the anatomical and physiological changes and the metabolic profile changes in *Pseudosuga menziesii* growing in different localities with different climate and soil traits. Although the study design did not allow a quantitative separation of the different environmental factors, it had the sensitivity to detect the environmental differences in soil type and climate.

A great drawback to dealing with all these challenges comes from the fact that ecometabolomic techniques imply the use of sophisticated and expensive equipment (GC-MS, HPLC-MC, HNMR) which are not always available in the laboratories where ecologists work. However, the present rapid improvements in analytical methods and in the ability of computer hardware and software to interpret large datasets multiply the possibilities of rapidly identifying and quantifying simultaneously more and more compounds with great facility, even for non-specialists in this field. On the other hand, the increase in interdisciplinary research will allow a progressively wider use of these molecular techniques in ecology studies. In the future we envision an increasing use of these methodologies in ecological studies. Metabolome studies coupled with transcriptomics and genomics studies, together with statistical analyses improvements and with mathematical modelling should allow a more holistic vision of the organism, population and ecosystem structure and functioning both on a space and time scale, with a better understanding of the consequences of environmental changes from individual to ecosystem level. In a step forward and unlike classical target analytical methods, ecometabolomic studies allow the characterization of the complete metabolome shift and thus help to discern the parts of the genome involved in these relationships. In this way these studies improve our knowledge of ecological consequences throughout the trophic chains, at the adaptation-selection-evolution level.

As commented in analytical techniques section, the different analytical techniques have different capacity to determine different analytical groups (polar-non polar, volatiles-non volatiles) and different sensitivity and elucidation power. On the other hand, the lack of extensive data bases to help in the molecular structure determination is increasingly been solved by the continuous enhancement of available commercial ofert of informatic programs and databases (Hall 2006; Allwood et al. 2008). This specially impacts the study of plant metabolome due to the large number of secondary metabolites. Moreover, the use of several analytical methods to analyze the same organism extracts has been successfully used in some recent studies using ^{1}HNMR and GC-MS (Srinivasan et al. 2008; Jones et al. 2009) and 1HNMR and HPLC-MS (Schroeder et al. 2006; López-Garcia et al. 2010) by thus coupling the greater sensitivity of chromatographic methods with the greater elucidation power of HNMR. At this regard the recent

HPLC-DAD-MS-SPE-NMR provide high sensitivity and elucidation power at once (Schlotterberg and Ceccarelli 2009).

If ecological metabolomics succeeds in overcoming these challenges and uses them as opportunities for advancing knowledge, we can expect to see stimulating new developments and applications in the near future in many areas of ecological sciences, including issues of stress responses, life history variation, population structure, trophic interaction, nutrient cycling, and the ecological niche. For example, the temporal and spatial characterization of the responses of individuals, populations and ecosystems to perturbations such as global change and the disentangling of evolutionary aspects of plant and animal communities both offer ecological metabolomics an immediate opportunity as a new and exciting application. In turn, ecology can provide a unique insight and a significant contribution to the study of functional metabolomics by helping to understand the ecological basis for interactions among metabolites.

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Chapter 2.

Ecometabolomics: Optimized NMR-based method.

Ecometabolomics: Optimized NMR-based method.

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Abstract

1. Metabolomics is allowing great advances in biological sciences. Recently, an increasing number of ecological studies are using a metabolomic approach to answer ecological questions (ecometabolomics). Ecometabolomics is becoming a powerful tool which allows following the responses of the metabolome of an organism environmental changes and the comparison of populations. Some Nuclear Magnetic Resonance (NMR) protocols have been published for metabolomics analyses oriented to other disciplines such as biomedicine but there is a lack of a description of a detailed protocol applied to ecological studies.

2. Here we propose a NMR-based protocol for ecometabolomic studies that provides an unbiased overview of the metabolome of an organism, including polar and nonpolar metabolites. This protocol is aimed to facilitate the analysis of many samples, as typically required in ecological studies. In addition to NMR fingerprinting, it identifies metabolites for generating metabolic profiles applying strategies of elucidation of small molecules typically used in natural-product research, and allowing the identification of secondary and unknown metabolites. We also provide a detailed description to obtain the numerical data from the ^{1}H-NMR spectra needed to perform the statistical analyses.

3. We tested and optimized this protocol by using two field plant species (*Erica multiflora* and *Quercus ilex*) sampled once per season. Both species showed high levels of polar compounds such as sugars and amino-acids during the spring, the growing season. *E. multiflora* was also experimentally submitted to drought and the NMR analyses were sensitive enough to detect some compounds related to the avoidance of water loses.

4. This protocol has been designed for ecometabolomic studies. It identifies changes in the compositions of metabolites between individuals and detects and identifies biological markers associated with environmental changes.

Key-words metabolomics · ecometabolomics · NMR · plant physiology · ecology · protocol

Introduction

Ecometabolomics

Metabolomics as a new research approach has been widely used in biomedicine (Nicholson, Lindon and Holmes 1999; Urban et al. 2010), ecotoxicology (Robertson 2005; Alam et al. 2010), animals (Deyrub et al. 2011) and plant biology (Fiehn et al. 2000; Weckwerth et al. 2004; Scott et al. 2010), and its application is increasing in ecological studies (Peñuelas and Sardans 2009a; Peñuelas and Sardans 2009b; Sardans, Peñuelas and Rivas-Ubach 2011). The metabolome is the entirety of molecules present in an organism as the final expression of its genotype at a particular moment (Fiehn 2002; Peñuelas and Sardans 2009a) and can be considered a molecular picture of biological diversity because each living species has its own metabolic profile (Gromova and Roby 2010). In ecological studies, metabolomics (ecometabolomics) has become a promising tool for following the responses of the metabolome of organisms to biotic and abiotic environmental changes (Sardans, Peñuelas and Rivas-Ubach 2011). The metabolome is the chemical phenotype of organisms and is the first to respond to internal and external stressors to maintain physiological homeostasis (Fiehn et al. 2000; Sterner and Elser 2002; Peñuelas and Sardans 2009a; Leiss et al. 2011; Sardans, Peñuelas and Rivas-Ubach 2011).

An optimized NMR-based protocol for ecometabolomics. Scope of application

Several protocols to explore the metabolome in humans and animals (Beckonert et al. 2007; Martineau et al. 2012) and in microorganisms (Smart et al. 2010; Roberts et al. 2012) have recently been published. Vascular plants have received less attention, but some methods for conducting metabolomic analyses of laboratory subjects based on Nuclear Magnetic Resonance (NMR) spectroscopy have been described (Kruger, Troncoso-Ponce and Ratcliffe 2008; Leiss et al. 2009; Gromova and Roby 2010; Kim, Choi and Verpoorte 2010; Kim and Verpoorte 2010). The field of ecology currently lacks a standard protocol for analyzing the metabolomes of wild plants, which are sampled from the field under very heterogeneous environmental conditions. The objective of ecology is to explain general patterns of structure, function, and evolution in ecosystems. This task is complicated by the large number of factors interacting concurrently and by the resulting high variability at all levels. Wild individuals within the same species present large differences in elemental composition, metabolism, phenology, and life style, much more so than do entries in a laboratory model. This implies that the number of individuals to be analyzed should be sufficiently large for a consistent statistical analysis. A protocol is thus needed to provide an overall analysis of the main metabolites in field samples, including secondary and nonpolar compounds, and which allows the detection and identification of those metabolites

that play a key role in an organism's response to environmental change. This protocol must also be reproducible and amenable to robust statistical analyses.

Among the different analytical techniques, NMR spectroscopy has the advantage of providing an unbiased overview of all the small molecules in a solution. Its analyses require minimal sample preparation, are relatively quick, non-destructive and highly reproducible and robust. ^{1}H NMR signals are directly and linearly correlated to metabolite abundance and it is the key technique for the elucidation of unknown metabolites, allowing the identification of most of the molecules detected and the differentiation between structural isomers, diastereoisomers (Pérez-Trujillo et al. 2010; Ellis et al. 2012), and even enantiomeric molecules (Pérez-Trujillo et al. 2012).

Here, we describe an optimized NMR-based protocol for ecometabolomic studies of wild plants, especially for those plants with robust structures such as sclerophyllous leaves. The samples are not part of laboratory models as described in other protocols (Leiss et al. 2009; Gromova and Roby 2010; Kim, Choi andVerpoorte 2010) but are typically taken from the field, where variability among individuals is high and a large number of samples are usually analyzed, and where, therefore, high reproducibility and robustness of method are critical. For this, special stress is also put in the automation of the NMR fingerprinting and in the use of NMR spectrometers of a high magnetic field (600 MHz or higher), which provide high resolution NMR fingerprint spectra of the cohort of samples (Leiss et al. 2009; Kim, Choi and Verpoorte 2010). Besides, the method considers polar and nonpolar metabolites and it optimizes the sample preparation methodology for their extraction (carefully described in detail in the procedures and materials section). Even so, this method may also be applied to greenhouse and laboratory studies.

The following protocol covers from sample collection and storage to the NMR spectra processing and statistical analysis (Figure 1), going through the metabolite extraction, the acquisition of NMR fingerprint data, and the identification of the detected metabolites to obtain the metabolic profile following the structural characterization of natural products (Robinette et al. 2012) that also includes the identification of the secondary and unknown metabolites, which may play a key role in an organism's response to an environmental change.

Even though this protocol has been optimized for the analysis of plant structures, these procedures can also be applied to the study of animals such as zooplankton, insects, annelids, and molluscs, among others, because the procedures have been tested for detecting the most abundant metabolites in the current extraction, making comparative analyses possible and rigorous.

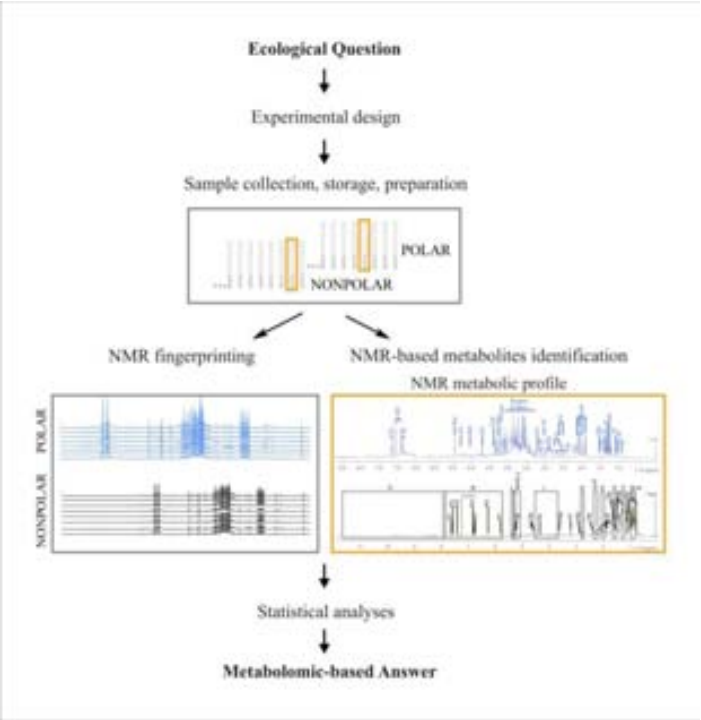

Figure 1. General NMR-based procedures for an ecometabolomic study.

Procedures and materials

This protocol was optimized in order to obtain robust results in ecometabolomics studies. For this reason we tested the grinding time of samples, the amount of sample, the sonication time, repeated extractions and the recovery (see Appendix 4 for details and the step by step procedure is also described).

The complete procedure as explained below (Figure 2) is divided into six main categories.

A. Sample collection and storage (Steps 1-3 of Figure 2).

Field fresh plant material was collected and rapidly packed, labelled, and frozen *in situ* in a container of liquid nitrogen. It prevented the degradation of metabolites (Kim and Verpoorte 2010). The physiological processes of plants vary throughout the day, so individual subjects were sampled within a short period of time and under a constant environment to reduce any effects of diurnal rhythms. The frozen plant material was lyophilized and kept frozen in plastic cans. Lyophilization (freeze-drying) avoids the hydrolysis of metabolites by maintaining enzymes in an inactive state and is thus a crucial step in ecometabolomics by keeping the metabolomes intact. Samples were ground with a ball mill (Mikrodismembrator-U, B. Braun Biotech International) at 1600 rpm for 9 min for leaves of *Erica multiflora* and for 6 min for leaves of *Quercus ilex*. Keeping the samples lyophilized and frozen and using ball mills that allow the rapid grinding of several samples independently of their nature are highly recommended procedures for ecometabolomic studies with a large number of samples, often more than 100.

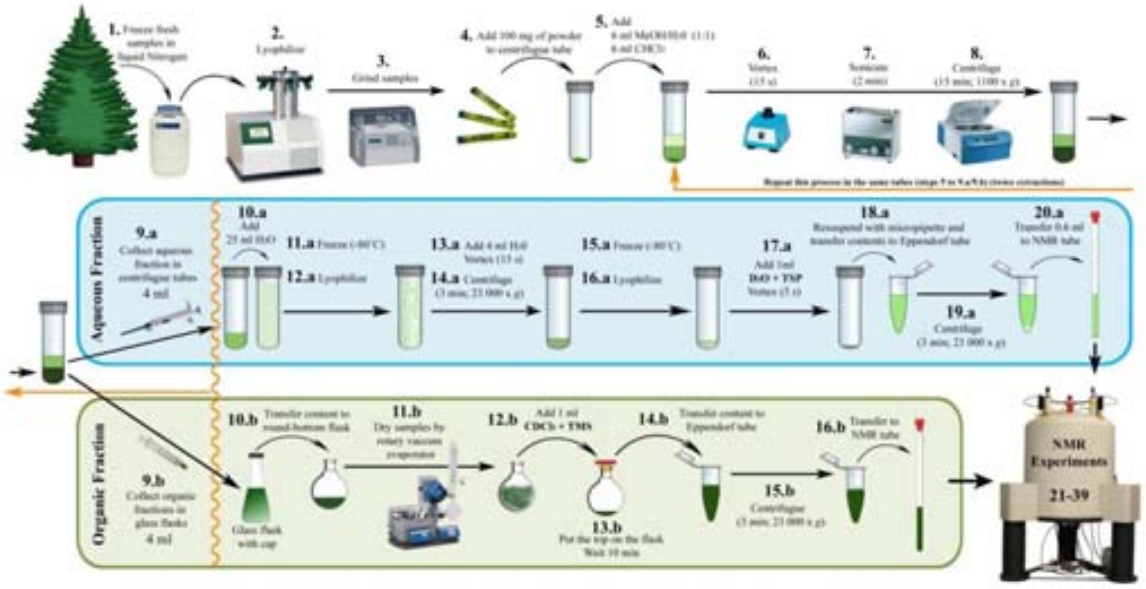

Figure 2. Experimental procedure to obtain polar and nonpolar extracts for NMR analyses.

B. Metabolite extraction and NMR sample preparation (Steps 4-20.a/16.b of Figure 2).

For each sample, one hundred mg of dried powder was added to a 50 mL centrifuge tube. 6 mL of a water-methanol (1/1) mixture and 6 mL of chloroform were added and all samples were vortexed (15 s) and sonicated (2 min). Samples were centrifuged at 1100 x *g* (15 min). The two liquid phases were collected separately.

For the aqueous extraction; for each sample, 4 mL of the aqueous extract was collected by micropipette in another centrifuge tube series. Then, the steps 5 to 10 of Figure2 were repeated in the same tubes. After the two extractions, 25 mL of water were added to each tube to reduce the methanol concentration and allowing lyophilization and were kept at -80ºC. Once frozen, samples were lyophilized with caps loosened. Four mL of water were added to each sample and were vortexed (15 s) to resuspend all the content. Samples were centrifuged at 23000 x *g* (3 min) to concentrate the content at the bottom and frozen again at -80ºC. Again, the samples were lyophilized with caps loosened. Once totally dried, 1 mL of phosphate buffer in D_2O+0.01% TSP was added to each sample and vortexed for 5 s. All the content was resuspended with micropipette and transferred to Eppendorf tubes. Eppendorf tubes were centrifuged at 23 000 x *g* (3 min) and 0.6 mL of the supernatant was transferred to NMR tubes.

Finally, the recovery of two polar metabolites (glucose and alanine) was tested for validation.

For the organic extraction; for each sample, 4 mL of the organic extraction was collected by crystal syringes in crystal jars avoiding any collection of pellet. Then, the steps 5 to 10 of

Figure2 were repeated in the same tubes. The organic fractions were placed into 25 mL round-bottom evaporation flasks and were dried in a rotary vacuum evaporator. To each flask, 1 mL of chloroform D+0.03% TMS was added and closed. After 10 minutes of waiting, their content were resuspended and transferred into Eppendorf tubes, centrifuged at 23 000 x *g* (3 min) and 0.6 mL of the supernatant was transferred to NMR tubes. The use of Eppendorf tubes for organic solvents is recommended to avoid any interaction of chloroform with plastic polymers.

C. Acquisition of NMR fingerprint data

The NMR fingerprint of a sample consists of a quantitative NMR spectrum of it. The spectra are obtained under specific identical, defined conditions. This ecometabolomic protocol is based on ^{1}H NMR, but the procedures described may be helpful when using other nuclei (Palomino-Schätzlein et al. 2011). All analyses are completely automatized to guarantee a high reproducibility and robustness. For this, an autosampler allowing a load of 60 samples is used (BACS, Bruker Biospin). A NMR spectrometer of high magnetic field generating high resolution spectra (600 MHz or higher) must be used, due to the typical high variability among samples and complexity of the spectra in ecometabolomic studies. A 600 MHz NMR spectrometer is used in order to assure high resolution spectra (Avance 600 equipped with a triple inverse 5-mm tube Z-gradient TBI probe and with a temperature control unit, Bruker Biospin). The temperature of the equipment must be previously calibrated and maintained constant for all the experiments (at 298.0 ºK). With this purpose an equilibration delay must be left once the tube is in the magnet and prior to the acquisition (2 min).

The automation must be configured (ICONNMR software application, Bruker Biospin) to allow the automatic control of processes such as insertion/ejection of the sample into the magnet, waiting delay, automatic locking onto the signal of the deuterated solvent, tuning and/or adjusting the tune frequency to the Larmor frequency of the nuclei measured, homogenization of the magnetic field, adjustment of the receiver gain of the sample, and the execution of the experiment, which includes the acquisition of the FID, its Fourier transformation, and the preprocessing of the spectrum. The automation of the aforementioned processes improves the reproducibility of the analysis, reducing errors from human source.

Samples of the polar and nonpolar fractions are analysed and compared separately. However, within each set of samples, it is recommended to randomize them (e.g. randomize treatments, populations etc.). Polar samples are analyzed using a standard quantitative 90º pulse-acquisition ^{1}H NMR experiment with solvent suppression. The standard water presaturation experiment, a conventional composite 90° ^{1}H pulse sequence with suppression of the residual water signal (Bax 1985), or a 90° ^{1}H NOE enhanced pulse sequence commonly

termed 1D NOESY-presat (Nicholson et al. 1995) are adequate (Table 1). Nonpolar samples are analyzed with a standard 90° pulse-acquisition ^{1}H NMR experiment. Acquisition parameters and processing parameters must be set up adequately (carefully detailed in the Appendix 4). All spectra must then be visually inspected, and those that are poorly phased or baseline corrected must be corrected manually. Finally, the spectra must be calibrated using the signal of the internal calibrating reference added to the sample.

D. NMR-based metabolite identification. NMR metabolic profile

The NMR metabolic profile of a sample is obtained when each peak of the NMR spectrum is assigned to its corresponding metabolite. The profile gives the NMR signals a biomolecular meaning. This analysis is usually performed on a single representative sample. The differences observed among samples are mainly due to differences in metabolite concentrations, however, qualitative differences may occur. When the assignment of a specific peak is not possible (for example, when peaks overlap or when signals are of low intensity) the analysis of another sample can be helpful. A visual inspection of all spectra of the fingerprinting can help to find a better sample for the elucidation of a specific signal.

The assignment of the ^{1}H NMR signals is conducted following two approaches. First, by comparison of the resonance frequencies (chemical shifts, □) and line shapes (multiplicity and coupling constants, *J*) of the spectrum to bibliographical data and NMR spectral databases (Table S1 of Appendix 4). Second, by the structural elucidation of the mixture (sample) through the performance of a suite of 2D NMR experiments (Table 1) and the concerted analyses of the data obtained. Basic NMR strategies followed for the structural characterization of natural products are applied for the elucidation of complex mixtures of small biological molecules (metabolites), instead of to isolated molecules as in the case of natural products research (Robinette et al. 2012). Briefly, protons connected by three to five chemical bonds are identified using 2D ^{1}H-NMR homonuclear COSY and TOCSY correlations. ^{1}H-^{1}H NOESY experiments determine connections between different parts of a same molecule, and heteronuclear ^{1}H-^{13}C HSQC and HMBC methods identify the carbon skeleton of a molecule. This approach, the structural elucidation of the mixture by NMR spectroscopy, is particularly helpful for the identification of secondary metabolites, since less published NMR data of them is available (Table 1). These experiments are time-consuming, but they only need to be performed once and just for one sample. 1D-selective ^{1}H experiments can be complementary to the 2D experiments, depending on the problem requiring elucidation. They are less time-consuming than the 2D correlations but retain maximum resolution and are used to get specific information of a chosen NMR signal of the 1D ^{1}H spectrum. The spectrum is much simpler to analyse and only shows the correlation information for the

selected peak. 1D-selective ^{1}H experiments are valuable tools for elucidating and confirming problematic molecules (Ellis et al. 2012). NMR experiments are performed at the same experimental temperature used for the NMR fingerprint spectra (298.0 ºK) and using the same spectrometer (detailed in the Appendix 4). However, to use the same NMR spectrometer is not necessary, since NMR data are fully comparable independently of the spectrometer used. The use of cryoprobes will also increase the sensitivity and considerably reduce the experimental time needed.

Table 1. Most common NMR experiments for identification of metabolites. Standard versions and a brief description of their application for structural elucidation problems are indicated.

Experiment	**Version (pulse sequence[a])**	**Description[b]**
1D ^{1}H	· Conventional pulse-acquisition (zg) · With solvent presaturation[c] (zgpr or zgcppr), also 1D NOESY with presaturation (noesypr1d)	Standard experiment routinely used for fingerprinting, identification of metabolites, and determining chemical shifts (d) and coupling-constant (J) values. Also used for quantification.
2D ^{1}H-^{1}H COSY	· Gradient selection (cosygpqf) (Aue, Bartholdi and Ernst 1976; Nagayama et al. 1980) · With solvent presaturation (cosypgqfpr)	Homonuclear Correlation Spectroscopy based on ^{1}H-^{1}H scalar coupling. Routinely used for the identification of metabolites, it correlates spin systems separated through chemical bonds.
2D ^{1}H-^{1}H TOCSY	· Conventional (mlevph)(Braunschweiler and Ernst 1983; Bax 1985) · With solvent presaturation (mlevphpr) · Selective 1D mode (selmlgp.2)(Bax 1985; Kessler et al. 1986; Stonehouse et al. 1994; Stott et al. 1995)	Total Correlation Spectroscopy. Based on homonuclear ^{1}H-^{1}H scalar coupling. It correlates spin subsystems within the same molecule.
2D ^{1}H-^{1}H NOESY	· Conventional (noesygpph) (Jeener et al. 1979; Wagner and Berger 1996) · With solvent presaturation (noesygpphpr) · Selective 1D mode (selnogp) (Kessler et al. 1986; Stonehouse et al. 1994; Stott et al. 1995)	Nuclear Overhauser Effect Spectroscopy. Based on homonuclear ^{1}H-^{1}H through-space interactions. Routinely used for the identification of metabolites, it provides information about which protons are close together in space (≤ 4Å).
2D ^{1}H-^{13}C HSQC	· Conventional using adiabatic ^{13}C pulses (hsqcetgpsisp) (Palmer et al. 1991; Kay, Keifer and Saarinen 1992; Schleucher et al. 1994)	Heteronuclear Single Quantum Correlation. Based on heteronuclear one-bond ^{1}H-^{13}C scalar coupling. Routinely used for the identification of metabolites, it correlates protons to their directly bonded carbon atom.
2D ^{1}H-^{13}C HMBC	· Conventional using low-pass J-filter (hmbcgplpndqf) (Bax and Summers 1986; Bax and Marion 1988)	Heteronuclear Multiple Bond Correlation. Based on heteronuclear long-range ^{1}H-^{13}C scalar coupling. Routinely used for the identification of metabolites, it correlates protons to carbon atoms separated by multiple (usually 2,3) bonds.

[a] According to Bruker nomenclature.

[b] Extensive and updated description of the vast library of NMR experiments and their differents versions is collected in the NMR Guide and Enciclopedia of Bruker.

[c] Experiments for suppression of the signal of the residual water.

Each specific ecometabolomic study and assignment problem will require the performance of some or all of the experiments indicated in Table 1. The version of the experiment with water-signal presaturation is recommended for polar samples. In this protocol, we describe the most common and useful NMR experiments that provide structural information, but many other NMR experiments are available in the spectrometric libraries that can be useful for the elucidation of specific problems.

E. Spectra processing

The NMR data from fingerprint spectra were adequately processed before conducting the statistical analyses. The bucketing process consists in obtaining the integral numeric value of the selected regions of the spectra (buckets) directly correlated with the molar concentration by their relationship to the initial concentration of the internal standard (TMS or TSP). In our protocol for ecometabolomics, we used a variable-size bucketing that is highly recommended over regular-size bucketing (Leiss et al. 2009; Gromova and Roby 2010; Kim, Choi and Verpoorte 2010) for reducing the number of variables for statistical analyses. First, a pattern for each kind of spectrum (polar and nonpolar) was created. The pattern is determined from identifying exactly where an NMR signal (peak) begins and ends for all peaks in the spectrum, and then the bucketing process can be executed based on this pattern. All empty areas (without peaks) of spectra can also be introduced into the pattern to detect any qualitative differences between samples. We used the variable-size bucketing option of AMIX (Bruker Biospin, Rheinstetten, Germany), scaling the buckets relative to the internal standard (TMS or TSP), although other programmes can be used. The output was a data set containing the integral values for each ^{1}H-NMR spectral peak accounted for in the described pattern.

The bucketed data sets from the NMR fingerprint spectra can be analysed directly (without a previous assignment of the metabolites), because rapidly classifying samples according to their origin or their ecological or ecophysiological relevance is sufficient (Sardans, Peñuelas and Rivas-Ubach 2011) (Figures 3 and S1). This last approach does not attempt to identify all metabolites but provides the metabolomic signature of the organism and allows detection of any shift or anomaly in its metabolism (Figures 3 and S1).

Statistical analyses of metabolomic profiles can be performed by two main ways when the ^{1}H-NMR spectra have been treated by variable-size bucketing. (i) All ^{1}H-NMR spectral buckets can be used as individual variables. Here the result is a data set where the number of variables is equivalent to the number of buckets in the bucketing process. (ii) The buckets corresponding to

the same molecular compound can be added up. The final number of variables in the data set is reduced and the statistical results are easier to interpret. For our analyses we used this method adding up the buckets corresponding to same compound.

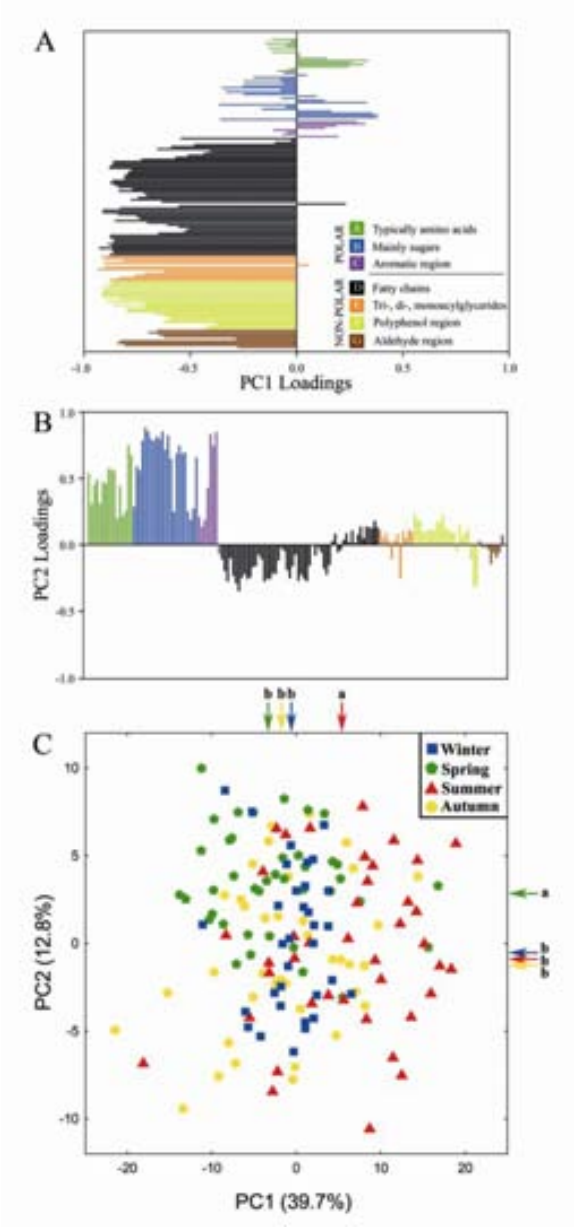

Figure 3. PCA plots conducted from ^{1}H NMR fingerprinting data from *Quercus ilex* leaves. **A)** panel of loadings of PC1 and **B)** panel of loadings of PC2. Loadings of the different spectral regions are represented by different colors as indicated. **C)** Panel of individuals categorized by season. Arrows outside the plot indicate the mean PC score for each season. The statistically significant differences between seasons are indicated by lowercase letters ($p < 0.05$).

F. Statistical data analysis

PCA (Principal Component Analysis) and PLS-DA (Partial Least Squares - Discriminant Analysis) are the most common multivariate ordination analysis (MOA) used in metabolomic studies (Ramadan et al. 2006; Leiss et al. 2009; Ebbels and Cavill 2009; Kim, Choi and Verpoorte 2010; Rivas-Ubach et al. 2012). MOA do not provide any measure of significance and it is limited to show the relation of cases with the used variables. PCs loadings of cases provided by MOA can be used to detect any significant difference of the investigated treatments by statistical inference

such as t-student test or ANOVAs (See Appendix 4 for more details). To get measures of statistical significance in multifactorial designs, MANOVA (Multivarite Analisis of Variance) and PERMANOVA (Permutational MANOVA) are the most suitable options for metabolomic studies (Jonhson et al. 2007; Anderson, Gorley and Clarke 2008). PERMANOVA is a modern statistical multivariate method used when the data for all metabolites are non-normal or when a better accommodation of random effects and interaction terms is needed (Anderson, Gorley and Clarke 2008; Rivas-Ubach et al. 2012).

Additionally, O-PLS (Orthogonal PLS) and GAs (Genetic Algorithms) are other statistical methods also used in metabolomic analyses (Ramadan et al. 2006; Ebbels and Cavill 2009).

Results and discussion

Optimization of the procedure

Different steps of the metabolite extraction procedure were tested in order to optimize the time and obtain reliable results for the statistical analyses.

Ball mills: Most metabolomic studies have used liquid nitrogen for grinding plant samples in mortars (Leiss et al. 2009, Gromova and Roby 2010; Kim, Choi and Verpoorte 2010). In our protocol, we preferred a ball mill, for three main reasons. First, the sampled plant materials frequently resist hand-grinding in mortars with liquid nitrogen due to the large proportion of cuticles and lignin (slerophyllous leaves, needles, wood, roots, among others), making the grinding difficult and time consuming. Second, lyophilization helps to maintain the metabolomes of sampled organisms intact for a long period of time (Kim, Choi and Verpoorte 2010), and grinding in mortars is laborious once the water is removed. Third, ecological studies often require a large number of samples, and ball mills allow a more rapid grinding of several samples than do mortars and produce also a homogeneous powder, with minimal variability in particle size between samples. Liquid nitrogen and mortars, though, may also be used with soft tissues by applying some modifications to the first steps of sample processing (detailed in Appendix 4).

Sonication time: After testing different times of sonication our experiments showed that the optimum extraction of metabolites was obtained with two minutes of sonication (Step 7 of Figure 2) (Table S2 and Figures S2 and S3 of Appendix 4). The use of chloroform during extraction will dissolve cellular membranes and thereby reduce the time of sonication, being then an interesting step when processing different batches of samples in the same day. In other NMR protocols of

polar metabolites (whithout chloroform extraction), sonication for 15-20 min has been recommended (Kim, Choi and Verpoorte 2010). Additionally, longer times for sonication reduce the signal strength of metabolites in ^{1}H NMR spectra, perhaps due to the heating of samples and the formation of metabolite artefacts (t'Kindt et al. 2008).

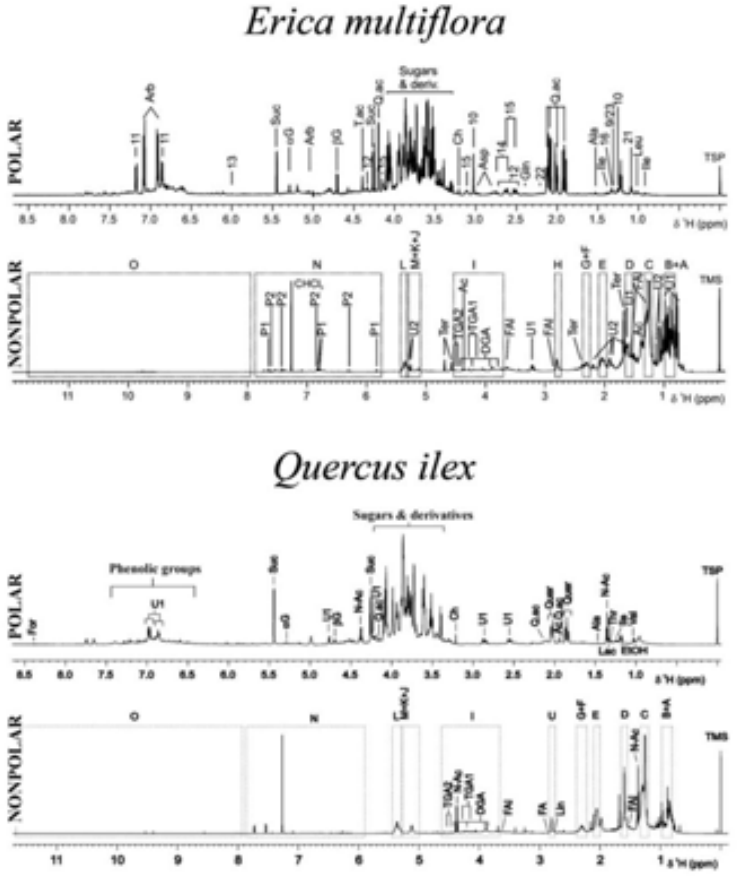

Figure 4. Typical ^{1}H NMR metabolic-profile spectra of polar and nonpolar extracts of *E. multiflora* and *Q. ilex* leaves. **Polar metabolites:** α-glucose (αG); β-glucose (βG); sucrose (Suc); alanine (Ala); asparagine (Asp); glutamine (Gln); leucine (Leu); isoleucine (Ile); threonine (Thr); valine (Val); 6-deoxypyranose (10); 4-hydroxyphenylacetate (11); malate (12); maleate (13); citrate (14); 3-amino-4-hydroxybutyrate (15); N-acetyl group (N-Ac, 16); quinic acid (Q.ac); tartaric acid (T.ac); arbutin (Arb); choline (Ch); 1,2-propanediol (21); 22, γ-hydroxybutyrate (22); lactate (23); quercitol (Quer); formate (For); catechin derivative (U1). **Nonpolar metabolites** (assigned signals/regions indicated with letters): A, C, D, and F, fatty acid spectral regions; B, linoleyl fatty acid region; E and L, unsaturated fatty acid regions; G, free fatty acid region; H, polyunsaturated fatty acid region; I, diacylglycerid and triacylglycerid region; J, triacylglycerid 2 region; K, triacylglycerid 1 region; M, 1,2 diacylglycerid region; N, polyphenol region; O, aldehyde group region; Ac, acetyl group; DGA, 1,2-diacylglycerid; FAI, fatty alcohols; Lin, linolenyl chain; P1, polyphenol derivative of *p*-coumaric acid 1; P2, polyphenol derivative of *p*-coumaric acid 2; Ter, terpene compound 1; TGA1, triacylglycerid 1; TGA2, triacylglycerid 2; U1, unknown compound 1; U2, unknown compound 2 (data from *Erica multiflora* modified from Rivas-Ubach et al. 2012).

Repeated extractions: The importance of repeated extractions has been discussed in protocols based on LC-MS analyses (t'Kindt et al. 2008), but there is a lack of metabolomic studies showing the differences between different extractions. In our protocol we tested one, two and three extractions (see Appendix 4) and our tests showed that two extraction procedures were the optimum since the NMR samples presented the maximum concentration of metabolites in the extracts (Table S3 of Appendix 4). A first extraction from *E. multiflora* and *Q. ilex* leaves yielded 78.5% and 85.6% metabolites respectively, relative to a second extraction, although the differences were not statistically significant (ANOVA test of the global concentration of metabolites; p=0.32 for *E. multiflora* and p=0.26 for *Q. ilex*). Three extractions from these species showed no differences to two extractions (ANOVA test of the global concentration of metabolites; p=0.97 for *E. multiflora* and p=0.89 for *Q. ilex*); more than 98% of metabolites from the third extraction had already been extracted after the second extraction (see Appendix 4).

Recovery: The recovery is an important factor to take into account when comparing different groups of samples by numerical data and not all metabolomic protocols took it into account. In our protocol it was tested for two polar metabolites; glucose and alanine. Our results showed a recovery of 92.8% for alanine and of 86.4% for glucose (Figures S4 and S5 of Appendix 4).

The analyzed samples were also used to determine the reproducibility of the method. For 12 independent extractions of the same sample powder, we obtained a mean NMR signal of 0.0913±0.0026 for alanine and 0.3266±0.0021 for glucose (mean±SE). These results indicate that larger ^{1}H-NMR spectral signals provide better reproducibility. Alanine usually gives very low signals in the ^{1}H-NMR spectra of plants because its concentration in plants is very low compared to that of sugars. This high reproducibility greatly decreases methodological errors.

Anticipated results with wild plants.

The ^{1}H NMR metabolic profiles (polar and nonpolar) of the leaves of *E. Multiflora* and *Q. ilex* are shown in Figure 4. A typical ^{1}H NMR metabolic profile of a polar extract from a wild plant in ecometabolomics shows the presence of primary metabolites, such as sugars, amino acids, organic acids, hydroxyacids, alcohols, and nucleic acids, as well as secondary metabolites characteristic of the particular species or family. These molecules can be completely elucidated and identified as discrete molecules (Fan 1996; Fan and Lane 2008). Nonpolar extracts contain fatty molecules (such as free fatty acids; fatty alcohols; and mono-, di-, and triglyceraldehydes) and nonfatty molecules (such as polyphenols and terpenes). Nonfatty molecules, as with polar molecules, can be completely elucidated as discrete molecules. Fatty molecules, however, are qualitatively analysed as a group, identifying and quantifying the presence of mono- and

polyunsaturated fatty chains; mono-, di-, or triglyceraldehydes; free fatty alcohols; and/or free fatty acids (Gunstone 1995; Vlahov 1999; Engelke 2007; Fan and Lane 2008).

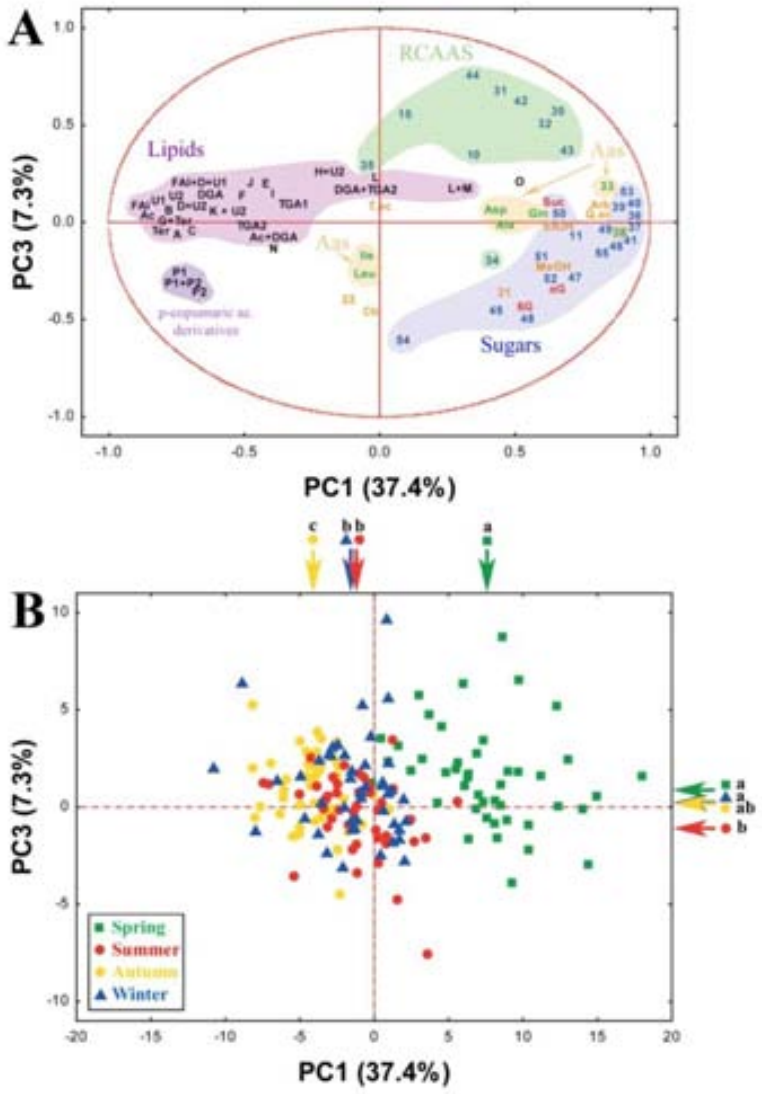

Figure 5. Plots of the first principal component (PC1) versus the third principal component (PC3) resulting from PCA conducted through the metabolomic profiles of *E. multiflora* leaves. (**A**) Panel of metabolomic variables. Variables are indicated by different colours: blue, polar metabolites from primary metabolism; red, glucose and sucrose; orange, polar metabolites from secondary metabolism; black, nonpolar compounds. Different metabolic families are separated by clusters in different colours: blue, sugars; yellow, amino acids; green, compounds related to amino acids and sugar metabolism in plants (RCAAS); violet, lipids. Variable labels are described in Figure 4 and variables from 30 to 55 represent overlapped signals (Table S4 of Appendix 4). (**B**) Panel of samples categorized by season. Seasons are indicated by different colors (red, summer; yellow, autumn; blue, winter; and green, spring). Arrows outside the plots indicate the mean PC score for each season. The statistically significant differences between seasons are indicated by lowercase letters ($p < 0.05$) (Adapted from Rivas-Ubach et al. 2012).

A multivariate analysis of the foliar metabolic fingerprint of *E. multiflora* throughout the seasons of the year is represented in Figure 5, The principal component analysis (PCA) resulted in a first principal component (PC1) separating the foliar metabolome in the different seasons demonstrating the sensitivity of NMR to detect seasonal metabolic shifts. The leaves of spring (the growing season in Mediterranean climate) presented the highest concentrations of polar metabolites, such as alanine, glutamine, asparagine, threonine, α-glucose, β-glucose, and sucrose. In contrast, they had the lowest concentrations of lipids and secondary metabolites, such as terpene compound 1 and derivatives of *p*-coumaric acid (the results were in more detail in Rivas-Ubach et al. 2012).

Additionaly, *E. multiflora* plants were experimentally stressed by conditions of drought throughout the year. A PCA was performed with only those variables presenting significant differences between control and droughted plants in summer (Figure 6), even though differences among all seasons were detected (results explained in detail in Rivas-Ubach et al. 2012). Mainly the foliar metabolomes of droughted plants presented higher concentrations of quinic acid, tartaric acid, lipids and terpenes ahowing that our protocol was also sensitive enough to detect shifts in the metabolomes as a response to climatic changes (Rivas-Ubach et al. 2012). The increases of these compounds in accordance with the known increase of oxidative stress in plants that have endured drought (Peñuelas et al. 2004).

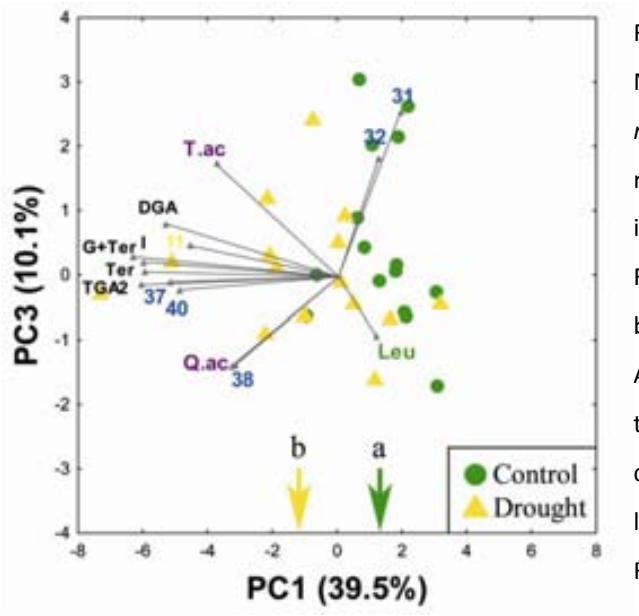

Figure 6. Plots of the PCAs conducted on the ^{1}H NMR metabolomic variables of the *Erica multiflora* analyses that presented different responses to experimental climatic treatments in summer. Variable labels are described in Figure 4 and Table S4. Treatment is indicated by color: green, control; yellow, drought. Arrows indicate the mean PC score for each treatment. The statistically significant differences are indicated by arrows with lowercase letters ($p < 0.05$) (Adapted from Rivas-Ubach et al. 2012).

A NMR fingerprinting data analyses were performed for *Q. ilex* (Figure 3). Data were classified in the different regions (Figure S1 of Appendix 4). The results revealed, as in the case of *E. multiflora*, higher concentrations of polar compounds such as sugars and amino acids in spring

leaves. Also, leaves of the summer (the warmest season) presented lower concentrations of nonpolar compounds than the rest of the seasons in agreement with other experimental warming studies on plants (Livonen et al. 2004), chlorophyta (*Fuschino* et al. 2011), and zooplankton (Gladyshev et al. 2011).

Conclusions

Here we presented a new optimized NMR-based protocol for application of metabolomics to field ecology. It has been specially developed to reduce the experimental errors and to be applied to a large number of samples, as often required in ecology. It thus allows performing more accurate statistical analyses. It has demonstrated to be sensitive enough to detect the differences in metabolomes of plants across different seasons and among different experimental climatic treatments. This protocol has been designed for studying the metabolome of wild plants but can also be used with animals and it is effective both for targeted and untargeted studies. It will help to increase the knowledge in the shifts of the wild organism's metabolomes across environmental gradients and it will allow making a step forward in the understanding of the role of metabolism driving the ecosystem structure and function.

Acknowledgements

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Chapter 3.

Strong relationship between elemental stoichiometry and metabolome in plants

Strong relationship between elemental sotichiometry and metabolome in plants.

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Abstract

Shifts in the elemental stoichiometry of organisms in response to their ontogeny and to changing environmental conditions should be related to metabolomic changes since elements operate mostly as part of molecular compounds. Here we show this relationship in leaves of *Erica multiflora* throughout their seasonal development and in response to moderate experimental field conditions of drought and warming. The N:P ratio in leaves decreased in the metabolically active growing seasons, coinciding with an increase in the content of primary metabolites. These results support the growth rate hypothesis that states that rapidly growing organisms present low N:P ratios due to the increase in allocation of P to RNA. The foliar N:K and P:K ratios were lower in summer and in the drought treatment, in accordance with the role of K in osmotic protection, and coincided with the increase of compounds related to the avoidance of water stress. These results provide strong evidence of the relationship between the changes in foliar C:N:P:K stoichiometry and the changes in the leaf's metabolome during plant growth and environmental stress. These results thus establish a first step in the understanding of the relationships between stoichiometry and an organism's lifestyle.

Keywords Metabolomics · stoichiometry · N:P · growth rate hypothesis · climate change

Introduction

The ratios of C:N:P concentrations in the environment and biomass have statistically significant relationships with traits of an organism's lifestyle and even seem to influence the structure and function of ecosystems (Güsewell 2004; Sardans et al. 2011). The growth rate hypothesis (GRH), one of the central paradigms of ecological stoichiometry (Elser et al. 1996: Elser et al. 2010), proposes that growing organisms must increase their allocation of P to RNA to meet the elevated demands for the synthesis of proteins required for growth. Low ratios of environmental N:P and C:P favor species with very high rates of growth (Main et al. 1997), which may induce shifts in species communities (Smith et al. 1983; Conde Porcuna et al. 2002). The GRH has strong experimental support in freshwater ecosystems (Sardans et al. 2011), with a few exceptions related to the allocation of nutrients to functions other than growth (Frost et al. 2008; Færøvig and Hessen 2003). In terrestrial ecosystems, however, the direct application of the GRH frequently fails or is incompletely accomplished (Sardans et al. 2011; Matzek and Vitousek 2009). Apart from investing N and P in growth, terrestrial plants can invest important amounts of these nutrients to other functions, such as storage, defense and mechanisms of stress avoidance. The phenotypic responses in these other basic organismic functions should therefore be considered when assessing the relationships of C:N:P ratios with an organism's metabolome and lifestyle and with the structure and function of ecosystems (Sardans et al. 2011; Peñuelas and Sardans 2009a and 2009b). Our goal was to consider the first step of such relationships, i.e. to link stoichiometry to the metabolome.

The metabolome is the entirety of small molecules present in an organism as the final expression of its genotype (Fiehn 2002) and can be considered as the organism's chemical phenotype (Peñuelas and Sardans 2009a). Metabolomics has recently been applied to physiological and ecological studies to assess the physiological status and functions of organisms, including their energetic and oxidative states; functions of growth, defense, storage and reproduction; and mechanisms of stress avoidance and health (Shulaev et al. 2008; Sardans et al. 2011; Weckwerth 2003; Graham et al. 2009).

Global climatic change and the marked variation in ontogeny-seasonality throughout the year in most regions of the world should affect the elemental content, stoichiometry and metabolome of organisms (*3*), but most metabolomic studies have not considered these effects (Sardans et al. 2012a and 2012b). We hypothesized that stoichiometric and metabolomic studies of plants in different ontogenetic stages or exposed to different environmental conditions should thus reveal an organism's flexibility to modulate its stoichiometry and metabolome for maintaining an optimal fitness under different conditions. We hypothesized that seasonal

differences in the metabolome should be similar to the shifts in individuals growing under varying conditions of temperature and availability of water.

We conducted a stoichiometric and metabolomic study of the plant *Erica multiflora*, a Mediterranean shrub, during different ontogenetic periods and submitted to field conditions of moderate warming (0.9 ºC) and drought (19% reduction of soil moisture). We thus tested our hypothesis that seasonal and climatic changes would force organisms to adjust both their C:N:P:K biomass stoichiometry and their metabolome in an inter-related way to maintain optimal performance under each specific condition. Elemental stoichiometry should determine an organism's capacity to build molecules and thus to shape metabolomic responses, so an organism's metabolomic adjustments should determine the C:N:P:K biomass stoichiometry, and the seasonal and climatic changes in environmental ratios of C:N:P:K availability should influence an organism's metabolomic responses.

Materials & Methods

Study site & experimental design

The study was conducted in Garraf Natural Park on the central coast of Catalonia (41˚ 18' N, 1˚49' E), which has a Mediterranean climate. Nine plots were established in March 1999: three as controls and six submitted to a treatment of climatic change, three of which involved nocturnal warming, and three represented conditions of drought. The warming treatment increased the temperature on average 0.9ºC during the night. The drought treatment reduced rainfall during spring and autumn. Soil moisture decreased an average of 19% in these drought plots (Appendix 3: Materials & Methods). For details see Peñuelas et al. 2004 and Beier et al. 2004.

Sampling and processing leaves

Sampling was conducted once per season from summer 2009 to spring 2010. Five plants in each plot were randomly marked as study objects. A homogeneous fraction of youngest-cohort, well-developed leaves from each individual in each season was frozen *in situ* in liquid nitrogen. The youngest leaves were thus spring leaves, and the oldest leaves were winter leaves. Frozen leaves were lyophilized and ground with a ball-mill grinder (Appendix 3: Materials & Methods).

Chemical analyses

Concentrations of C and N were determined by combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy). For the analyses of

P and K, samples were first digested in acid (Sardans et al. 2010) in a high-pressure microwave and then analyzed by an *Optima 2300RL* ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (The Perkin-Elmer Corporation, Norwalk, USA) (Appendix 3: Materials & Methods).

Plant-extraction procedures for nuclear magnetic resonance (NMR) analyses

Extracts with water-methanol (1:1) and chloroform were obtained. Briefly, 200 mg of powdered leaf material was introduced into a centrifuge tube. Six mL of water-methanol (1:1) and 6 mL of chloroform were added to each tube (Lin et al. 2007). Samples were vortexed for 20 seconds and then sonicated for 1 minute at room temperature (Choi et al. 2004). All tubes were centrifuged at 3000 rpm for 30 minutes. Four mL of each fraction (aqueous and organic) were collected independently into jars. This procedure was repeated twice. Aqueous samples, previously re-dissolved in water (<15% methanol), were lyophilized. Organic samples were placed in a round-bottom evaporation flask and dried in a rotary vacuum evaporator. Finally, 1 mL of KH_2PO_4-NaOD buffered D_2O (pH=6.0) was added to the dried aqueous fractions, and 1 mL of chloroform-d was added to the dried organic fractions. All contents were transferred into Eppendorf tubes and centrifuged for 3 min at 6000 rpm and 2 min at 10 000 rpm, and the supernatants were transferred into NMR sample tubes (Appendix 3: Materials & Methods).

NMR experiments

Samples were scanned through high-resolution 1D ^{1}H NMR spectroscopy generating polar and non-polar metabolic profiles (spectra) (Figure 1, Figures S2 and S3, and Table S4 of Appendix 3) using a Bruker AVANCE 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) at a field strength of 14.1 T (600.13 MHz ^{1}H frequency). The probe temperature was set to 298.0 K. Sample handling, automation and acquisition were controlled using TOPSPIN 2.1 software (Bruker Biospin, Rheinstetten, Germany). For both kinds of samples, a standard ^{1}H 90° pulse sequence was used, and the residual water resonance was suppressed in the samples extracted with water-methanol. Following the introduction of the probe, samples were allowed to equilibrate (1 min). Each spectrum was acquired into 32k data points over a spectral width of 16 ppm, as the sum of 128 transients and with a relaxation delay of 2 s. The total experimental time was ca. 8 min per sample (Appendix 3: Materials & Methods).

1D and 2D NMR experiments for the identification of the metabolites were performed in a Bruker AVANCE 500 spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a high-sensitivity, cryogenically cooled, triple-resonance, TCI probehead at a field strength of 11.7 T (500.13 MHz ^{1}H frequency). The probe temperature was set to 298.0 K. The software used was

TOPSPIN 1.3 from Bruker Biospin. ^{1}H (500.13 Hz) and ^{13}C (125.76 MHz) NMR experiments were performed on the control samples. 1D ^{1}H selective TOCSY (Total Correlation Spectroscopy) experiments, as well as 2D experiments, such as ^{1}H-^{1}H-COSY (Correlated Spectroscopy), ^{1}H-^{1}H TOCSY, ^{1}H-^{13}C HSQC (Heteronuclear Single Quantum Correlation) and ^{1}H-^{13}C HMBC (Heteronuclear Multiple Bond Correlation), allowed the identification of the metabolites. All 1D and 2D experiments were performed directly in the samples prepared for the metabolomic study and used standard Bruker pulse sequences and routine conditions (Appendix 3: Materials & Methods).

Data analysis

1D ^{1}H NMR spectra were used for statistical analyses. All ^{1}H-NMR spectra were treated by TOPSPIN 1.3 (Bruker Biospin, Rheinstetten, Germany). Bucketing was conducted with AMIX (Bruker Biospin, Rheinstetten, Germany) to obtain the integral values for spectral peaks (for the bucketing details see *Appendix 3: Materials & Methods*). All ^{1}H-NMR signals corresponding to the same molecular compound, molecular family or with the same molecules overlapped were summed to reduce the final number of variables. The overlapped signals were given a different number code (Figure 1).

We tested the normality of each variable in each season by Kolmogorov-Smirnov tests. All variables followed normal distributions. The differences in the ^{1}H-NMR spectral peaks and in elemental stoichiometric variables among seasons and/or climatic treatments (control, drought and warming) were analyzed using mixed models with individuals and plots as random independent variables, with individuals nested within plots and with climatic treatments and seasons as fixed independent categorical variables (Table S1 of Appendix 3). We thereafter performed a mixed model for repeated measures of the stoichiometric and metabolomic variables. The MANOVA model included individual plants and plots as random factors, and climatic treatment, season and stoichiometric and metabolomic concentrations (under an unstructured correlation structure) and their interactions as fixed effects. When an interaction between effects was detected, Bonferroni's multiple comparisons were performed. SSPS 19.0 (SPSS Inc. Chicago, USA) was used to conduct the mixed model. To test for the differences among seasons and climatic treatments in nutrient concentrations, stoichiometry and the metabolome, to better accommodate random effects and interaction terms, we also conducted permutational multivariate ANOVAs (PERMANOVA) (Anderson et al. 2008) using the Euclidean distance, with "season" (spring, summer, autumn, winter), and "treatment" (control. drought, warming) as fixed factors, and "block" and "individuals" nested in "block" as random factors. When PERMANOVA analyses were significant, we subsequently ran univariate permutational ANOVAs on the

concentrations and ratios of nutrients and metabolites using the Euclidean distance. These univariate analyses allowed us to detect the variables causing the differences in nutrient and metabolomic composition among seasons and treatments. All these PERMANOVA analyses were conducted with the software PERMANOVA+ for PRIMER v.6 (Anderson et al. 2008).

Multivariate ordination analyses (PCAs based on correlations) were also performed to detect patterns of sample ordination in the metabolomic and stoichiometric variables. Additional PCAs for each season, including only the variables that presented differences among climatic treatments, were performed to identify the main patterns of those treatment-induced changes (Figure 4). Finally, discriminant analyses were conducted to identify the capacity of the PC1 axes of Figure 2 and the N, P and N:P variables to separate plants of different seasons. Statistica v8.0 (Statsoft, Tulsa, USA) was used to perform ANOVAs, post-hoc tests, PCAs, Kolmogorov-Smirnov tests and discriminant analyses.

Results

Seasonal stoichiometric and metabolomic changes.

The foliar concentrations of C, N, P and K and their respective ratios (C:N, C:P, C:K, N:P, N:K and K:P) changed with the seasons (Mixed Model Analyses). The lowest N:P, C:P and C:N ratios were found in spring, whereas summer leaves showed the highest K:P and the lowest N:K concentration ratios (Table S1 of Appendix 3). Almost all the elucidated polar and non-polar compounds (Figure 1) showed significant seasonal differences in concentration (Mixed Model Analyses; $p<0.05$) (Table S1 of Appendix 3). Spring leaves had the highest concentrations of polar metabolites, such as alanine, glutamine, asparagine, threonine, α-glucose, β-glucose and sucrose. In contrast, spring leaves had the lowest concentrations of lipids and secondary metabolites, such as terpene compound 1 and derivatives of p-coumaric acid.

MANOVA analysis showed a significant interaction between stoichiometric and metabolomic variables and seasons ($F_{3,13484}=71.6$, $p<0.0001$). Thus, different distributions of global metabolomic and stoichiometric values were observed among seasons. PERMANOVA analysis also showed significant global differences on metabolomic and stoichiometric variables among seasons (pseudo-$F_{3,68}= 29.3$, $p = 0.01$, Table S2 of Appendix 3).

The seasonal principal component analysis (PCA) with all the stoichiometric and metabolomic data resulted in a PC1 separating the foliar stoichiometry and metabolome in the different seasons (Figure 2). The PC3 separated the foliar stoichiometry and metabolome in summer from those in the other seasons (Figure 2 and Table S3 of Appendix 3). Also, the PC3 was directly related by the effects of the climatic treatments, because droughted plants presented

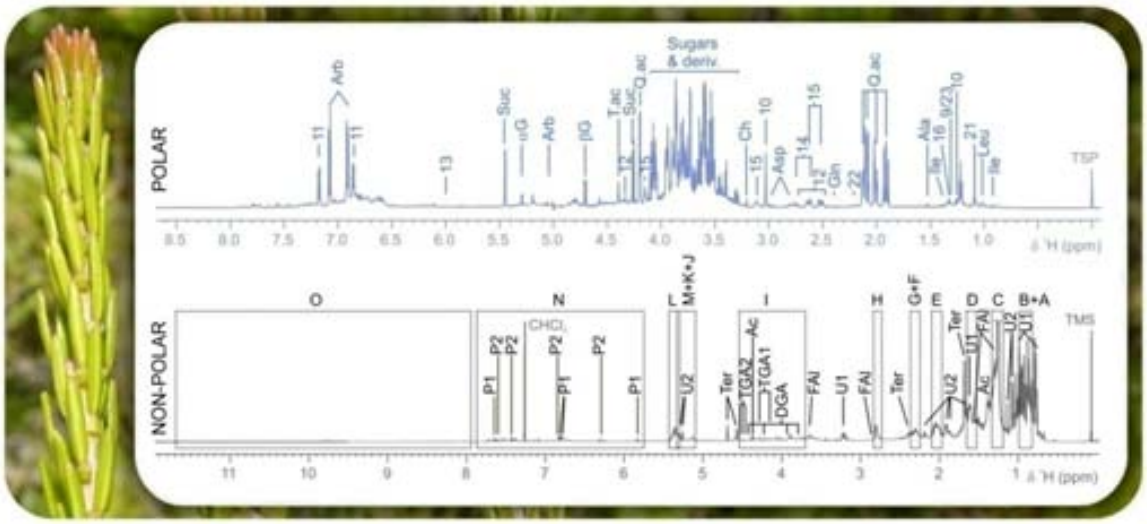

Figure 1. Typical ^{1}H NMR spectra of polar (water/methanol) and non-polar (chloroform) extracts of E. multiflora leaves. Assignments of signals to metabolites are indicated in blue in the polar profile. A different number has been assigned to each metabolite and overlapped signals [**αG (1):** α-glucose, **βG (2):** β-glucose, **Suc (3):** Sucrose, **Ala (4):** Alanine, **Asp (5):** Asparagine, **Gln (6):** Glutamine, **Leu (7):** Leucine, **Ile (8):** Isoleucine, **Thr** (**9**)**:** Threonine, **10:** 6-deoxypyranose, **11:** 4-hydroxyphenylacetate, **12:** Malate, **13:** Maleate, **14:** Citrate, **15:** 3-amino-4-hydroxybutyrate, **16:** N-Acetyl group, **Q.ac (17):** Quinic acid, **T.ac (18):** Tartaric acid, **Arb (19):** Arbutin, **Ch (20):** Choline, **21:** 1,2-propanediol, **22:** γ-hydroxybutyrate, **23:** Lactate and **30** to **55:** Overlapped signals: **30:** 11+15, **31:** 5+11, **32:** 5+11+13, **33:** 6+21, **34:** 12+Unk., **35:** 15+22, **36:** 16+19, **37:** 3+16+19, **38:** 6+16, **39:** 1+2+3+16+18, **40:** 1+2+3+5+16+18, **41:** 1+2+3+16, **42:** 11+14, **43:** 9+14+22, **44:** 13+14, **45:** 1+2+9, **46:** 1+2+3+18, **47:** 1+2+3+18+19, **48:** 1+2+3+18+19+20, **49:** 1+2+3+9+18+21, **50:** 1+2+3+Unk., **51:** 1+2+18, **52:** 1+2+9+18, **53:** 1+2+6+18, **54:** 2+20, **55:** 2+3+18+19]. Assignments of signals to metabolites are indicated in grey in the non-polar profile. A different letter/s code has been assigned to each non-polar region or metabolites [**A, C, D, F:** Fatty acid spectrum regions, **B:** Linoleyl fatty acid region, **E, L:** Unsaturated fatty acid regions, **G:** Free fatty acids region, **H:** Polyunsaturated fatty acids region, **I:** Diacylglicerid and triacylglicerid region, **J:** Triacylgricerid 2 region, **K:** Triacylglicerid 1 region, **M:** 1,2 Diacylglicerid region, **N:** Polyphenols region, **O:** Aldehydes group region, **Ac:** Acetyl group, **DGA:** 1,2-Diacylglicerid, **TGA1:** Triacylglicerid 1, **TGA2:** Triacylglicerid 2, **FAI:** Fatty alcohols, **U1:** Unknown compound 1, **U2:** Unknown compound 2, **Ter:** Terpene compound 1, **P1:** polyphenol derivated 1 of p-coumaric acid, **P2:** polyphenol derivated 2 of p-coumaric acid].

the highest foliar K:P and the lowest foliar N:K concentration ratios in autumn, winter and summer.

In the additional PCA of only the metabolomic data (Figure S1 of Appendix 3), the PC1 scores were significantly correlated with the foliar concentrations of N and P and with the N:P ratio (Figure 3). Discriminant analyses of those relationships also showed significant differences

among seasons in all cases: PC1 vs N (Wilks' Lambda=0.35, $F_{(3,172)}$=108.6, p=0.000), PC1 vs P (Wilks' Lambda=0.24, $F_{(3,172)}$=183.6, p=0.000) and PC1 vs N:P (Wilks' Lamda=0.61, $F_{(3,172)}$=37.4, p=0.000).

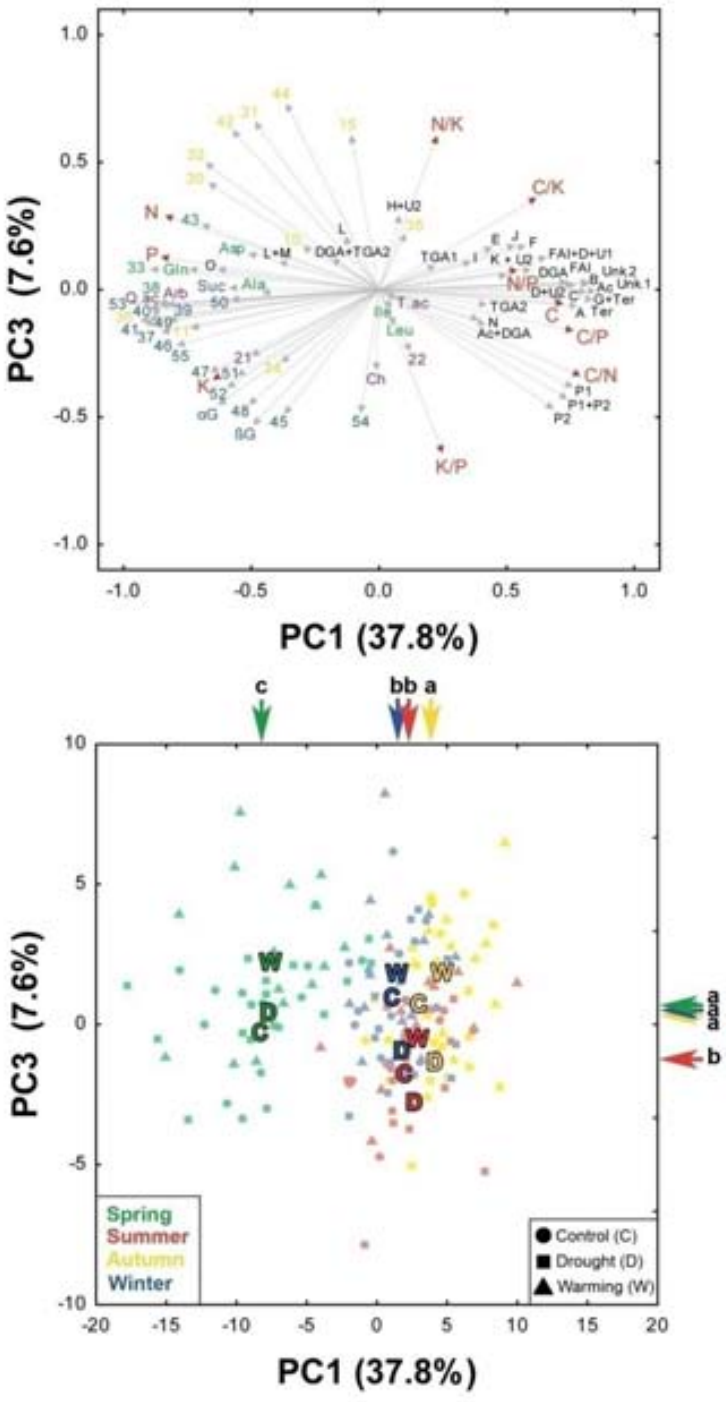

Figure 2. Biplots of PC3 versus PC1 loadings and scores resulting from PCA conducted with the elemental stoichiometric and ^{1}H-NMR metabolomic variables in E. multiflora leaves using PC1 and PC3 axes. **(a)**. Panel of stoichiometric and metabolomic variables. C:N:P:K ratios are represented in red. Different metabolic families are grouped by different colors (Blue = Sugars, green = amino acids, yellow = compounds related to amino acids and sugars plant metabolism (RCAAS), violet = secondary polar metabolites, black = non-polar metabolites) [assignments are described in Fig. 1] **(b)**. Panel of cases represented by seasons and climatic treatments. Seasons are indicated with different colors (Red = summer, yellow = autumn, blue = winter and green = spring). Treatments are indicated with different geometric figures (circles = controls, squares = droughted plants and triangles = warmed plants). Arrows outside the plots indicate the mean PC score for each season. The different letters of the cases plot represent the mean of PC1 and PC3 scores for each treatment within each season (C = control, D = drought, W = warming). The statistically significant differences between seasons were detected by Bonferroni post-hoc tests and are indicated by different letters ($p < 0.05$).

Climatic stoichiometric and metabolomic changes.

In the experimental plots simulating climatic change, the leaves of droughted plants had the highest K:P and the lowest C:K and N:K concentration ratios, while leaves of the warming plants showed the lowest C:P and N:P concentration ratios (Table S1 of Appendix 3). The metabolomic profiles of leaves in the drought plots had the highest concentrations of polyphenolic compounds (region N of Figure 1 and Figure S2 of Appendix 3), quinic acid, tartaric acid and choline, while the profiles of leaves in the warming plots had the highest concentrations of fatty acids and compounds related to the amino acids and sugars of plant metabolism (RCAAS) (from molecule 10 to 16 of Table S4 of Appendix 3).

The MANOVA analysis showed a significant interaction between stoichiometric and metabolomic variables and treatments ($F_{2,13484}$=5.14, p=0.01). Different distributions of global metabolomic and stoichiometry values were thus observed among climatic treatments. In the PERMANOVA analysis, the different climatic treatment also showed marginally significant global differences on metabolomic and stoichiometric variables (pseudo-$F_{2,68}$=1.90, p=0.10, Table S2 of Appendix 3) .

The means of the PC3 scores for droughted plants in winter and autumn and the means for all treatments in summer presented similar values (Figure 2), indicating a similar elemental stoichiometry and metabolome in individuals in summer and under drought. Additional PCAs, including only the variables that presented significant differences among climatic treatments within each season, were performed to identify the main patterns of those changes (Figure 4 and Tables S5 and S6 of Appendix 3). In general, warmed plants were distinct from control and droughted plants in the coldest seasons (winter and autumn), while both warmed and droughted plants differed from control plants in the warmest seasons (summer and spring). Leaves of droughted plants tended to have more quinic acid, tartaric acid and choline than control and warmed plants in all seasons, and they also had the highest K and K:P ratios and the lowest N:K and C:K ratios (Figure 4a, b, c). Warmed plants presented the highest foliar concentrations of RCAAS, such as malate, citrate and 3-amino-4-hydroxybtuanoic acid, in all seasons. In autumn, droughted plants had higher foliar concentrations of polyphenolic compounds than control and warmed plants, while control plants presented the highest concentrations of N and P and the lowest C:N, C:P and N:P ratios (Figure 4d). These trends also occurred in summer when control leaves had the lowest C:P values (Figure 4c). One terpene compound had its highest concentrations in leaves of droughted and warmed plants in autumn and summer (Figure 4c, d).

Discussion

The ecometabolomic seasonal analyses showed the highest primary metabolic activity in spring (the growing season) when the concentrations of sugars and amino acids directly linked to growth were highest (Figure 2 and Table S1 of Appendix 3). This increase in the concentration of primary metabolites coincided with an increase of N and P concentrations in leaves, with a proportionally higher increase in P than in N content leading to lower N:P and C:P content ratios. These results are in agreement with the GRH. The highest concentrations of sugars and amino acids and a lower N:P content ratio were thus associated with the high metabolic activity and rate of growth of the spring season and coincided with the lowest concentrations of non-polar metabolites (Figure 2 and 3 and Table S1 of Appendix 3). The favorable climatic conditions of spring, with high availability of soil water, enhance photosynthetic rates and the uptake of N and P. Higher levels of these elements allow more synthesis of amino acids and proteins (more N), which requires more synthesis of RNA (more N and especially more P) (Figure 3). These decreasing N:P ratios during the growing season have also been found in other terrestrial plants (Ågren 2004; Méndez and Karlsson 2005) and in animals (Carrillo et al. 2001; Pilati and Vanni 2007). Moreover, under these favorable conditions for growth, the C assimilated is allocated more to growth and energy supply (more primary metabolism) than to anti-stress or defensive mechanisms (less secondary metabolism). In fact, C:N, C:P and C:K ratios were also positively correlated with non-polar metabolites, such as fatty acids, terpenoids, polyphenols and other C-rich components (Figure 2).

Summer leaves had higher concentrations of sugars that likely remained from the high accumulation during the spring and/or were due to the increase in cellular osmotic potentials. On the other hand, summer leaves had the highest K:P and the lowest N:K and C:K ratios (Figure 2 and Table S1 of Appendix 3). K is involved in the plant-water relationship (Babita et al. 2010) through plant osmotic control (Babita et al. 2010; Sngakkara et al. 2000; Laus et al. 2011) and improvement in stomatal function (Khosravifar et al. 2008). The present ecometabolomic study also demonstrates a shift in the metabolome of *E. multiflora* in response to the treatment of moderate climatic change (Figure 2 and 4 and Tables S1 and S5 of Appendix 3), which follows a very conservative projection of the forthcoming decades (IPCC 2007). Warming increased the level of fatty acids relative to the drought treatment and the control (Figure 4), which agrees with other experimental studies of warming (Larkindale and Huang 2004). RCAAS also tended to increase under the warming treatment compared to the control (Tables S1 and S5 of Appendix 3), in agreement with other studies in plants (Rizhsky et al. 2004) and also in *Drosophila* (Malmendal et al. 2006). Interestingly enough, warming plants showed low concentrations of P, directly

related to high C:P and N:P ratios (Figure 4, and Tables S1 and S5 of Appendix 3). This response of P in plants under warming is still unclear and warrants further study (Sardans et al. 2012b).

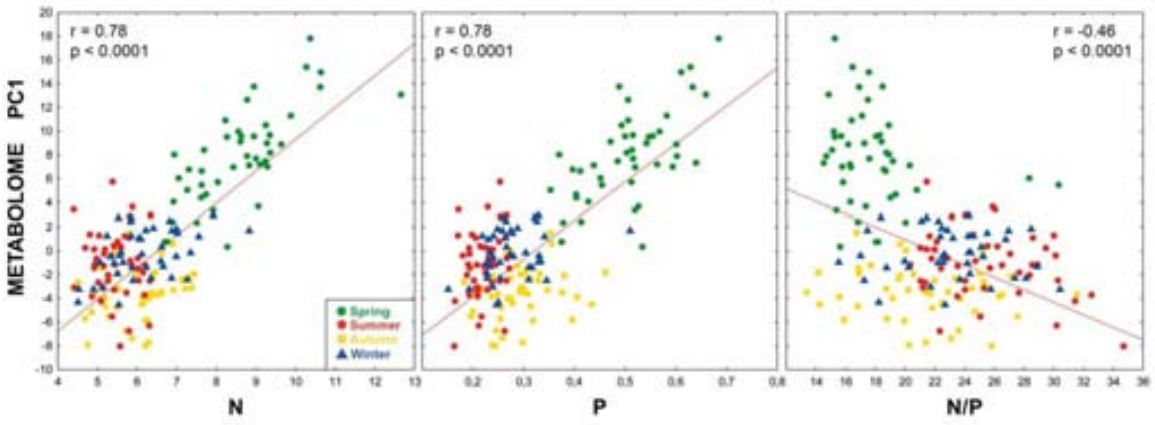

Figure 3. Relationships of the PC1 scores of a PCA analysis conducted only with the metabolomic data (Figure S1 of Appendix 3) with the foliar concentrations of N and P and N:P ratio . Seasons are represented in different color (Red = summer, yellow = autumn, blue = winter and green = spring).

As expected in a water-limited Mediterranean ecosystem, the drought treatment had considerable effects on the foliar stoichiometry and metabolome due to the increase of oxidative stress under drought conditions (Munné-Bosch and Peñuelas 2004); Peñuelas et al. 200; Dat et al. 2000; Price et al. 1989). In autumn and spring, the leaves of droughted plants had higher concentrations of compounds with antioxidant function, such as some polyphenolic compounds, quinic acid and tartaric acid, than the control plants (Figure 4, and Tables S1 and S5 of Appendix 3). Quinic acid is a precursor of the shikimic-acid pathway, a common metabolic pathway in the biosynthesis of aromatic amino acids such as tyrosine and phenylalanine (Draths et al. 1999) that are precursors of flavonoids (Harborne 1988). Their antioxidant capacity results from their high reactivity as hydrogen or electron donors (Rice-Evans et al. 1997) and from the role of some (eg. flavonoids) in altering the kinetics of peroxidation (Arora et al. 2000). In summer, the drought leaves also had higher concentrations of choline, which is involved in osmotic protection (McNeil et al. 2001). These results are supported by some studies that found high concentrations of aromatic amino acids in plants under drought stress (Alvarez et al. 2008; Lugan et al. 2009). These metabolomic differences were accompanied by an increase in K content, resulting in low C:K and N:K ratios and high K:P ratios that were also observed in summer (Figure 2 and 4), the drought season, and seem related to the improvement in the control of water use (Sangakkara et al. 2000; Khosravifar et al. 2008). The mean of the droughted plants of winter and autumn in Figure 2 were located together with the means of all treatments in summer in the PC3 versus PC1

biplot, demonstrating a similar stoichiometry and metabolome in individuals grown in summer with individuals grown under drought.

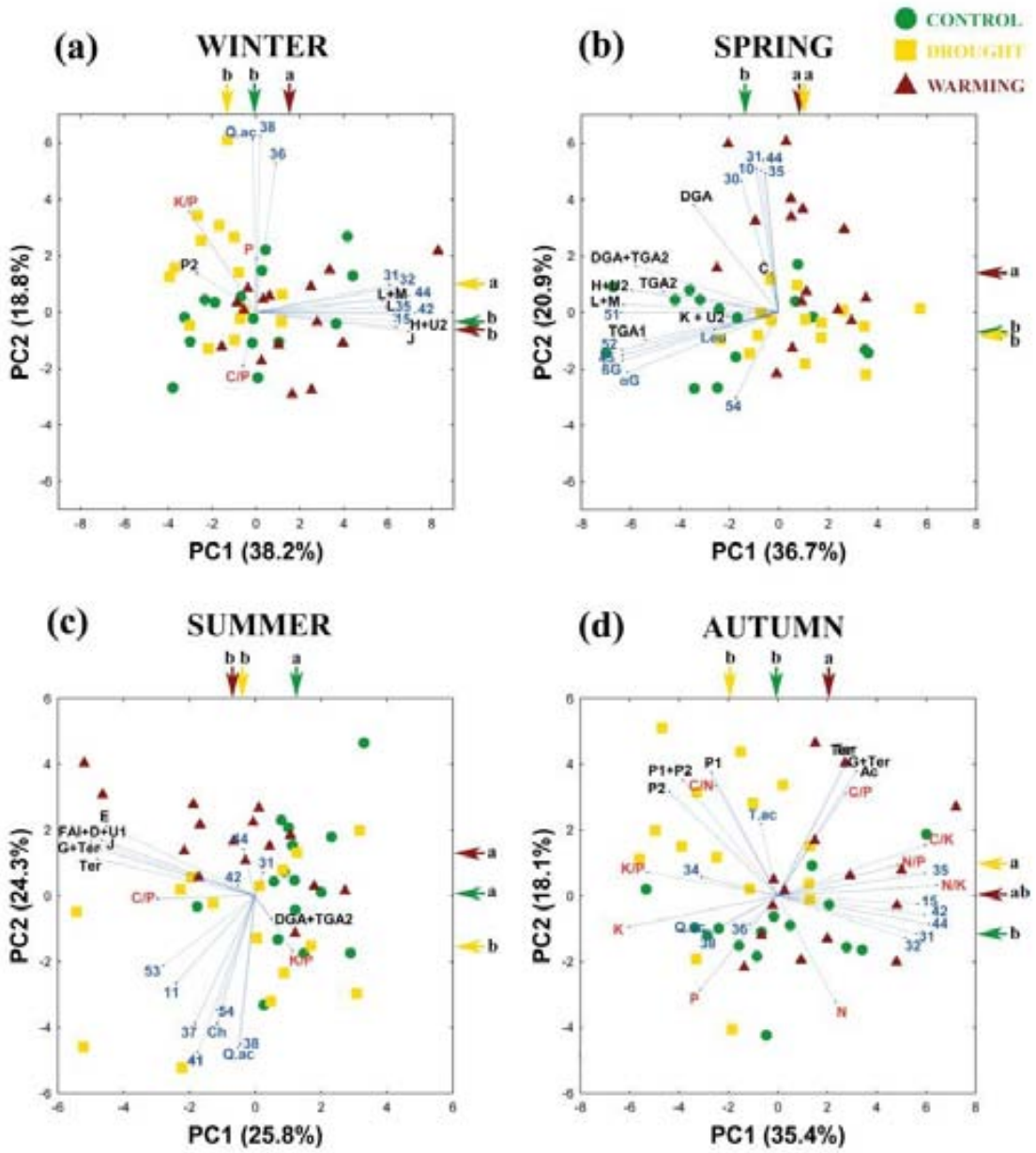

Figure 4. Plots of the PCAs conducted with the ^{1}H-NMR metabolomic and the stoichiometric variables that presented different responses to climatic treatments in each season of the study. C:N:P:K ratios are represented in red. Organic and water-soluble fractions are separated by color (black = non-polar, blue = polar). Variable labels are indicated in detail in Figure 1. Cases are represented by treatment (green = Control, yellow = Drought and red = Warming). Arrows outside the plots indicate the mean PC score for each treatment. The statistically significant differences were tested by Bonferroni post-hoc tests and are indicated by different letters ($p < 0.05$).

Some studies have failed or have not fully corroborated the GRH in terrestrial ecosystems. Matzek & Vitousek 2009 found no link in trees between N:P and protein:RNA ratios. Terrestrial plants and animals invest large amounts of N and P to mechanical structures (wood and skeleton), reproduction, storage, defense and mechanisms of stress avoidance, which exceed the amounts invested in growth, thus making the relationship between N:P and protein:RNA ratios less prominent. Our C:N:P:K analyses were based on whole-leaf contents (including structural elements), while the metabolomics were based only on the extractable aqueous and non-aqueous cellular components. The most abundant foliar structural compounds of plants (mainly cellulose and lignin) however, have no N and P. The global changes in foliar N and P can thus be directly compared with the shifts in foliar metabolism, since most of the N and P of leaves are extracted in the polar and non-polar extracts. In any case, the link between metabolome and stoichiometry may have been even stronger had we analyzed only the N and P contained in the extractable fractions. This result, though, would not have been comparable to most studies of ecological stoichiometry where all the N and P contents of the tissues are usually analyzed.

In conclusion, the results show that the N:P and C:P content ratios decreased in the growing season, supporting the growth rate hypothesis, and that these changes were related to shifts in the metabolome of the plants, with high concentrations of sugars and amino acids. The results also show that the study of shifts in the stoichiometry of terrestrial plants should also consider other elements, for example K and its elemental ratios C:K, N:K or K:P, that may vary from metabolomic shifts in response to environmental changes such as drought. All these results support our hypothesis of a strong relationship between stoichiometry and the metabolome. By coupling stoichiometry and ecometabolomics, these results improve our understanding of developmentally and environmentally linked shifts in C:N:P:K contents and of how these contents can change to achieve an optimum allocation for growth and other functions, such as storage, defense, reproduction or resistance to stress. This coupling enhances our knowledge of the first step of stoichiometry's influence on the lifestyle of organisms and the structure, function and evolution of ecosystems (Sardans et al. 2012a; Peñuelas and Sardans 2009a and 2009b).

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Chapter 4.

Drought stress enhances folivory by shifting foliar metabolomes in *Quercus ilex* trees.

Drought stress enhances folivory by shifting foliar metabolomes in *Quercus ilex* trees.

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Abstract.

Plants respond to external environmental conditions such as drought or to the seasonal changes by shifting the foliar C:N:P:K stoichiometry and metabolome. Rates of folivory are usually associated with the water status of plants and/or concentrations of foliar nutrients, especially N, but studies have focused mainly on the concentrations of foliar nutrients or on particular foliar-specific metabolic families. Emerging ecometabolomic techniques allow the study of metabolomes, the total set of metabolites in an organism at a specific moment. This study attempts to integrate stoichiometric and metabolomic techniques to understand the responses of *Quercus ilex* throughout seasons and under moderate experimental conditions of drought and how these responses affect rates of folivory. Foliar K concentrations increased in summer, the driest Mediterranean season. This increase was expected because K acts as an osmoprotectant under water stress, which consequently leads to higher foliar K:P and lower C:K and N:K ratios. These changes in summer were also accompanied by higher concentrations of phenolic compounds related to antioxidant functions. Moreover, trees exposed to a moderate drought in the field had higher foliar concentrations of total sugars and polyphenolic compounds (flavonoids). These compounds were mainly associated with the avoidance of water stress: sugars that provide osmotic protection, and flavonoids that act as antioxidants. Folivory increased in the trees of the droughted plots. Our data suggest that the increase of sugars and flavonoids in *Q. ilex* leaves in droughted trees can lead to an increase in herbivorous attack, which implies an indirect relationship between increased drought and rate of folivory produced by metabolomic shifts. The present study represents an advance in understanding the potential cascade effects of drought at different trophic levels and their possible implications in the structure, function and evolution of ecosystems.

Keywords metabolomics · stoichiometry · drought · folivory · ecology

Introduction

Drought, one of the most important effects of climate change, is predicted to increase in the coming decades in several regions of the world, including the Mediterranean basin (IPCC, 2007). Drought is a potential driver of changes in the elemental C:N:P:K stoichiometries of different plant organs and ecosystems (Rivas-Ubach et al. 2012, Sardans et al. 2012a, Sardans et al. 2012b). These shifts in stoichiometry may thus affect ecological processes and ultimately the structure and function of ecosystems (Elser et al. 1996, Sardans et al. 2012c, Sterner & Elser, 2002). N and P have been the most studied elements in the context of ecological stoichiometry (Sterner & Elser, 2002), but several studies have demonstrated the central role that foliar K concentrations play under conditions of drought (Ingram & Bartels, 1996, Sardans et al. 2012a, Wang et al. 2013). For example, a study of seasonal stoichiometry in plants in Mediterranean climates reported that foliar K concentrations tended to increase under both natural summer and experimental drought (Sardans et al. 2013b). These changes were larger than the changes in foliar N and P concentrations (Sardans et al. 2012b). The study of foliar K concentrations in the context of ecological stoichiometry both seasonally and under drought conditions requires more attention (Sardans et al. 2012a).

The relationships between water status in plants and herbivory have been reviewed and highlight the importance of the status of soil water in the resistance of trees against herbivorous attack (Rouault et al. 2006). Also, some studies have observed that herbivores enhanced their activity in response to increased concentrations of soluble N in the foliage (Larsson, 1989, Larsson & Bjorkman, 1993, Rouault et al. 2006), while other studies have observed that foliar nutritional quality is affected by drought, which indirectly stimulates insect folivory (Rouault et al. 2006, White, 1984). The large variation in C:N:P biomass stoichiometries in plants, both in time and space, may thus be a significant factor, among others, in the selection of foliage with high nutritional content by herbivores (Gusewell & Koerselman, 2002, Lindroth et al. 2002, Oleksyn et al. 2002, Raubenheimer & Simpson, 2003, Sardans et al. 2012c). Additionally, the rates of folivory are more directly related to the proportion of foliar structural compounds (high C:N ratios) than to foliar N concentrations, with high proportions of these structural compounds usually being avoiding (Choong et al. 1992, Williams et al. 1998). Thus, C:N ratios has proven to play a key role in folivory (Ji et al. 2011) through the effects of structural compounds or N concentrations.

Most elements, such as C, N and P, do not mainly act by themselves but as components of molecular compounds (Peñuelas & Sardans, 2009a) such as lignin and cellulose in lignified structures or various compounds that defend against herbivorous attack (Bennett & Wallsgrove, 1994, Kessler & Baldwin, 2001). Furthermore, changes in plant stoichiometry may influence the

coevolution of insects and the defensive chemical compounds of plants (Raubenheimer & Simpson, 2003). The relationship between plant stoichiometry and metabolism and herbivorous attack, however, is still unclear and warrants further study (Raubenheimer & Simpson, 2003).

The molecular responses of plants to herbivores have also been recently reviewed (Ali & Agrawal, 2012), references there in). Plants respond to herbivory by producing chemical defenses such as alkaloids, terpenes and phenolics (Bennett & Wallsgrove, 1994, Kessler & Baldwin, 2001). Plants can even alter the concentrations of primary metabolites in response to tissue predation or infection (Ehness et al. 1997, Sardans et al. 2013a, Widarto et al. 2006). The study of the responses of plants to herbivorous predation has generally focused on the identification of single compounds or families of metabolites (Sardans et al. 2013a). The application of the new metabolomic techniques in ecology and plant physiology (ecometabolomics) allows the study of the complete metabolome of organisms, the total set of metabolites in an organism at a specific moment (Fiehn, 2002), and its response to abiotic and biotic environmental shifts (Peñuelas & Sardans, 2009b, Peñuelas et al. 2013, Rivas-Ubach et al. 2013, Sardans & Peñuelas, 2012, Sardans et al. 2011a). Recent ecometabolomic studies have reported metabolomic changes in plants throughout Mediterranean seasons (Rivas-Ubach et al. 2012), in plants exposed to a biotic stress such as herbivorous predation (Jansen et al. 2009, Leiss et al. 2009) or an abiotic stress such as drought (Charlton et al. 2008, Lugan et al. 2009, Rivas-Ubach et al. 2012, Sardans et al. 2011a). The number of studies, however, has been insufficient for understanding the general trends of metabolomic shifts in plants grown under stress, and more so under interacting stresses. The link between shifts in foliar stoichiometries and shifts in foliar metabolomes throughout seasons and under experimental drought in the field has been recently demonstrated (Rivas-Ubach et al. 2012), so the nutritional quality of the food for herbivores may thus also shift.

The rates of folivory can thus be influenced directly by food quality and indirectly by plant water status in what could be a cascade effect of drought. We hypothesize that shifts in both foliar elemental stoichiometry and metabolomics produced by drought would influence the characteristics of herbivores, such as feeding, growth and/or reproduction. By affecting the quality of the food available to herbivores, drought-induced shifts in foliar chemical composition may lead to long-term cascades that could alter trophic webs.

Once per season, we sampled leaves of *Quercus ilex* trees from a mature forest in Catalonia (northeastern Iberian Peninsula) exposed to conditions of moderate experimental drought similar to those projected for the coming decades. The metabolomes and elemental compositions of all samples were analyzed. The rates of folivory were also determined for each sampled tree. Our goal was to understand the stoichiometric and metabolomic responses of *Q. ilex*, the most dominant tree in the forests of the Mediterranean basin, to seasonal changes and

to experimental conditions of drought. We also wanted to understand how the elemental and metabolic concentrations of plants may affect the selection of food by herbivores. For that, the *Q. ilex* forest was used to test our hypothesis that drought, simulated in experimental plots, could alter folivorous activity by altering the foliar metabolome. Our results could thus help to predict the impact of climate change on trophic webs (Peñuelas & Sardans, 2009a).

Material & Methods

Study site

This study was performed in a natural *Q. ilex* forest in the Prades Mountains in southern Catalonia (see Ogaya & Peñuelas, 2007 for details) (41º 21' N, 1º 2' E). All sampled plots faced south-southeast on a 25% slope at 930 m above sea level. The climate of the region is mesic-Mediterranean with a marked summer drought for three months (Figure S1A of Appendix 4). The vegetation consists of a forest dominated by *Q. ilex*, followed by *Phillyrea latyfolia* and *Arbutus unedo*, among others.

Experimental design

Four plots of mature *Q. ilex* forest (15 × 10 m) were established 15 m apart in March 1999 (Ogaya et al. 2003). Two randomly assigned plots received drought treatment, and the other two served as control plots. The drought treatment consisted of covering approximately 30% of the soil surface with 1 × 14 m PVC strips oriented down the slope and 0.5-0.8 m above the soil surface . A ditch 0.8-1 m in depth was dug along the entire top edge of the treatment plots to intercept upslope runoff. All water intercepted by the strips was channeled to the bottom edge of the drought plots. Soil moisture, air humidity, precipitation and air and soil temperatures, were monitored every 30 min. The drought treatment resulted in an average annual reduction of 18% in relative soil moisture (Figures S1B and S1C of Appendix 4; see Barbeta et al. 2013 for details).

Sampling of leaves.

Five adult *Q. ilex* individuals 25-50 cm in diameter were randomly selected from each plot as study cases (n = 5 × 4 = 20). Leaves were sampled once each season: February in winter, May in spring, August in summer and November in autumn. A small branch exposed to the sun was removed from each tree with a pole, and a sample of the youngest well-developed leaves was frozen in liquid nitrogen for the stoichiometric and metabolomic analyses. The remaining leaves

were stored in bags at 6-8 ºC for determining water content and for photographic analysis of the extent of consumption by herbivores.

Calculation of the proportion of leaves consumed by herbivores.

Fifteen randomly selected leaves stored at 6-8ºC from each tree were placed on a flat white surface and photographed with a Nikon D80 camera with a Nikkor AF-S 18-135/3.5-5.6 G DX lens to calculate the percentage of folivory. The area of the leaves consumed was calculated using Adobe Photoshop CS2 (Adobe Systems Incorporated, San Jose, California, USA). The assigned value of consumed area for each tree was the mean of its 15 leaves analyzed. The values of folivory for each tree were then standardized by the total foliar biomass of *Q. ilex* in its plot. All values were thereafter transformed for normality (arcsin(square root(percentage))).

Foliar processing for elemental and metabolomic analyses.

The processing of the leaves is described in detail in Rivas-Ubach et al. 2013. Briefly, leaves frozen in liquid nitrogen were lyophilized and stored in plastic cans at -20 ºC. Samples were ground with a ball mill at 1600 rpm for 6 min (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany), producing a fine powder that was then stored at -80 ºC until the extraction of the metabolites.

Elemental analysis

For the C and N analyses, 1.4 mg of the powder from each sample was analyzed. The C and N concentrations were determined by elemental analysis using combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).

Macroelements (P and K) were extracted by acid digestion in a microwave reaction system under high pressure and temperature (Sardans et al. 2010). Briefly, 250 mg of leaf powder were placed in a Teflon tube with 5 mL of nitric acid and 2 mL of H_2O_2. A **MARSXpress** microwave reaction system (CEM, Mattheus, USA) was used for the acid digestions (see the Chemical analyses section of the Appendix 4 for details). The digested material was transferred to 50-ml flasks and resuspended in Milli-Q water to a final volume of 50 mL. After digestion, the P and K concentrations were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (Perkin-Elmer Corporation, Norwalk, USA).

Extraction of metabolites for analysis by nuclear magnetic resonance (NMR).

The extraction of foliar metabolites for NMR analysis is described in detail in Rivas-Ubach et al. 2013. First, two sets of 50-mL centrifuge tubes were labeled: set A for the extraction of

metabolites and set B for lyophilization. A set of crystal jars for the organic fractions was also labeled. The tubes of set A received 200 mg of powdered leaf material of each sample. Six mL of water/methanol (1:1) and 6 mL of chloroform were added to each tube. The samples were vortexed for 15 s and then sonicated for 2 min at room temperature. All tubes were centrifuged at 1 100 × g for 15 min. Four mL of each fraction (aqueous and organic) were collected independently; aqueous fractions were transferred to the centrifuge tubes of set B, and organic fractions were transferred to the crystal jars. This procedure was repeated twice for two extractions of the same sample. The aqueous samples, previously resuspended in water to reduce the proportion of methanol (<15% methanol), were lyophilized, then 4 mL of water were added to each tube, which was vortexed and centrifuged at 23 000 x g for 3 min. The samples were frozen at -80 ºC and lyophilized again. The organic fractions were transferred to round-bottomed evaporation flasks and dried in a rotary vacuum evaporator. Finally, 1 mL of KH_2PO_4-NaOD-buffered D_2O + 0.01% TSP (trimethylsilyl propionic acid sodium salt) (pH 6.0) was added to the dried aqueous fractions, and 1 mL of chloroform-D + 0.01% TMS (tetramethylsilyl) was added to the dried organic fractions. TSP and TMS were used as internal standards. All solutions were resuspended with a micropipette, transferred to 2-mL centrifuge tubes and centrifuged at 23 000 x *g* for 3 min. The supernatants were transferred to NMR sample tubes.

Extraction of metabolites for liquid chromatography-mass spectrometry (LC-MS) analysis.

The extraction of metabolites followed the protocol of t'Kindt et al. 2008 with minor modifications. Two sets of Eppendorf tubes were labeled: set A for extractions and set B for the extracts from set A.

The tubes of set A received 100 mg of sample powder of each sample, then 1 mL of MeOH/H_2O (80:20) was added to each tube. The tubes were vortexed for 15 min, sonicated for 5 min at room temperature and then centrifuged at 23 000 x *g* for 5 min. After centrifugation, 0.6 mL of the supernatant from each tube was transferred to the corresponding Eppendorf tubes of set B. This procedure was repeated for two extractions of the same sample. The tubes of set B were centrifuged at 23 000 x *g* for 5 min. The supernatants were collected by crystal syringes, filtered through 0.22 µm pore microfilters and transferred to a labeled set of HPLC vials. The vials were stored at -80 ºC until the LC-MS analysis.

LC-MS analysis.

LC-MS chromatograms were obtained using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Dionex,Waltham, Massachusetts, USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA)

equipped with an HESI II (heated electrospray ionization) source. Chromatography was performed on a reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3µ particle size; Thermo Scientific, Waltham, Massachusetts, USA) at 30 ºC. The mobile phases consisted of acetonitrile (A) and water (0.1% acetic acid) (B). Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath prior to use. At a flow rate of 0.3 mL per minute, the elution gradient began at 10% A (90% B) and was maintained for 5 min, then to 10% B (90% A) for the next 20 min. The initial proportions (10% A; 90% B) were gradually recovered over the next 5 min, and the column was then washed and stabilized for 5 min before injecting the next sample. The injection volume of the samples was 5 µL. All samples were injected twice, once with the HESI operating in negative ionization mode (-H) and once in positive ionization mode (+H). The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60 000). The resolution and sensitivity of the spectrometer were monitored by injecting a standard of caffeine after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analyzed during the sequence.

NMR analysis.

NMR experiments were performed on a Bruker AVANCE 600 spectrometer working at a magnetic field of 14.1 T (^{1}H and ^{13}C NMR frequencies of 600.13 and 150.13 MHz respectively) and equipped with an automatic sample changer, a multinuclear triple resonance TBI probe and a temperature control unit (BrukerBiospin, Rheinstetten, Germany). The temperature into the probehead was previously calibrated and maintained constant for all the experiments at 298.0 K. With this purpose and for all analyses an equilibration delay (2 min) is left once the tube is into the magnet and prior to the shimming process. All NMR sample handling, automation, acquisition and processing were controlled using TopSpin 3.1 software (BrukerBiospin, Rheinstetten, Germany). Spectra were referenced to TSP (^{1}H and ^{13}C at δ 0.00 ppm) in the case of polar samples and to the signal of the residual solvent, $CHCl_3$, (^{1}H at δ 7.26 ppm and ^{13}C at δ 77.16 ppm) in the case of nonpolar samples.

^{1}H NMR fingerprinting

All extract samples were analyzed by high resolution one dimensional (1D) ^{1}H NMR spectroscopy following the conditions described at Rivas-Ubach et al. 2013 and using standard pulse-acquisition 1D ^{1}H-NMR experiments. In the case of the water/methanol extract samples proton spectra were acquired with suppression of the residual water resonance. The water resonance signal was presaturated at a power level of 55 dB, corresponding to an effective field

of 30 Hz during a relaxation delay of 2s. Each experiment was acquired as a set at 32 k data points, over a spectral width of 16 ppm, as the sum of 128 transients and with and acquisition time of 1.7 s. The resulting interferograms (FID) were Fourier transformed and the spectra obtained were phased and baseline corrected. The FID of polar samples were multiply by an exponential apodization function equivalent to 0.2 Hz line broadening prior to the Fourier transform. The experimental time was of *ca.*8 min per sample.

NMR metabolite identification

Standard 2D NMR experiments (^{1}H-^{1}H correlated spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC)), and 1D selective ^{1}H TOCSY experiments were performed in representative polar and nonpolar samples for the identification of the metabolites. For the water–methanol extract samples, 2D experiments were carried out with standard presaturation of the residual water peak during the relaxation delay. Experiments were acquired using standard Bruker pulse sequences and routine conditions. The procedure followed was that described at (Rivas-Ubach et al. 2013). All elucidated metabolites were further confirmed by reported literature data (Tables S1 and S2 of Appendix 4 for references).

Processing of LC-MS chromatograms.

The raw data files from the spectrometer were processed by MZmine 2.10 (Pluskal et al. 2010). Chromatograms were baseline corrected, deconvoluted, aligned and filtered before the numerical database was exported in CSV format (see Table S3 of Appendix 4 for details). Metabolites were assigned by exact mass and retention time from the measurements of the standards in the MS and MS^{n} modes of the spectrometer (see Table S4 of Appendix 4 for details). The different assigned variables corresponding to the same molecular compounds were summed. The numerical values of the variables extracted from the LC-MC chromatograms correspond to the absolute peak areas of the chromatograms detected by the spectrometer. The area is directly proportional to the concentration of the variable, so we use the term *concentration* in this article when referring to changes in the amount of metabolites among factors (treatment, season).

NMR bucketing.

The processing of ^{1}H NMR spectra is detailed in Rivas-Ubach et al. 2013. Briefly, before the exportation of the ^{1}H NMR numerical databases, all spectra were phased, baseline corrected and referenced to the resonance of the internal standard (TSP for polar and TMS for nonpolar samples) at δ 0.00 ppm with TOPSPIN 3.1. A variable-size bucketing was thus applied to all ^{1}H

NMR spectra with AMIX software (Bruker Biospin, Rheinstetten, Germany), scaling the buckets relative to the internal standard (TMS or TSP). The output was a data set containing the integral values for each assigned ^{1}H NMR spectral peak in the described pattern. The buckets corresponding to the same molecular compound were summed.

Statistical analyses

To test for differences between seasons and drought treatments in foliar elemental stoichiometry and metabolome, the LC-MS and NMR metabolomic fingerprints of the *Q. ilex* leaves were subjected to PERMANOVA analysis (Anderson et al. 2008) using the Euclidean distance, with season (spring, summer, autumn and winter) and climatic treatment (control and drought) as fixed factors and folivory as a covariable. The number of permutations was set at 999. The PERMANOVA analysis was conducted with PERMANOVA+ for PRIMER v.6 software (Anderson et al. 2008).

Additionally, to understand how the foliar stoichiometry and metabolome of *Q. ilex* shifted with the factors studied (season, climatic treatment), the foliar stoichiometric and metabolomic fingerprints were also subjected to principal component analysis (PCA). The seasonal PCA included the fingerprints of all seasons combined (Figure 1). Fingerprints from spring and summer leaves were additionally submitted to separate PCAs (Figure 2), because these seasons have higher levels of folivory in the Mediterranean basin. The PCAs were performed by the *pca* function of the *mixOmics* package of R (R Development Core Team, 2011). The score coordinates of the variables were subjected to one-way ANOVAs to find statistical differences among groups (See Supporting Information of Rivas-Ubach et al. 2013). A Kolmogorov-Smirnov (KS) test was performed on each variable to test for normality. All assigned and identified metabolites were normally distributed, and any unidentified metabolomic variable that was not normally distributed was removed from the data set. The KS tests were performed with the *ks.test* function of the *tuncogof* package of R (R Development Core Team, 2011).

Folivory begins in spring and early summer, and trees do not produce new leaves in summer, so the sampled leaves had accumulated signs of folivory by the middle of the summer. An additional PCA was thus conducted with summer data excluding the proportions of herbivorous consumption (Figure S4 of Appendix 4). The PC1 scores of this PCA, including the entire elemental, stoichiometric and metabolomic variation of the case trees in summer, were plotted against the degree of herbivorous consumption (Figure 3). Folivory was analyzed with a general linear model (GLM) as a function of the climatic treatment and the metabolomic variation (PC1 scores). Another GLM analyzed the total foliar sugar and phenolic concentrations as functions of climatic treatment and season. Statistica v8.0 (StatSoft, Tulsa, USA) was used for

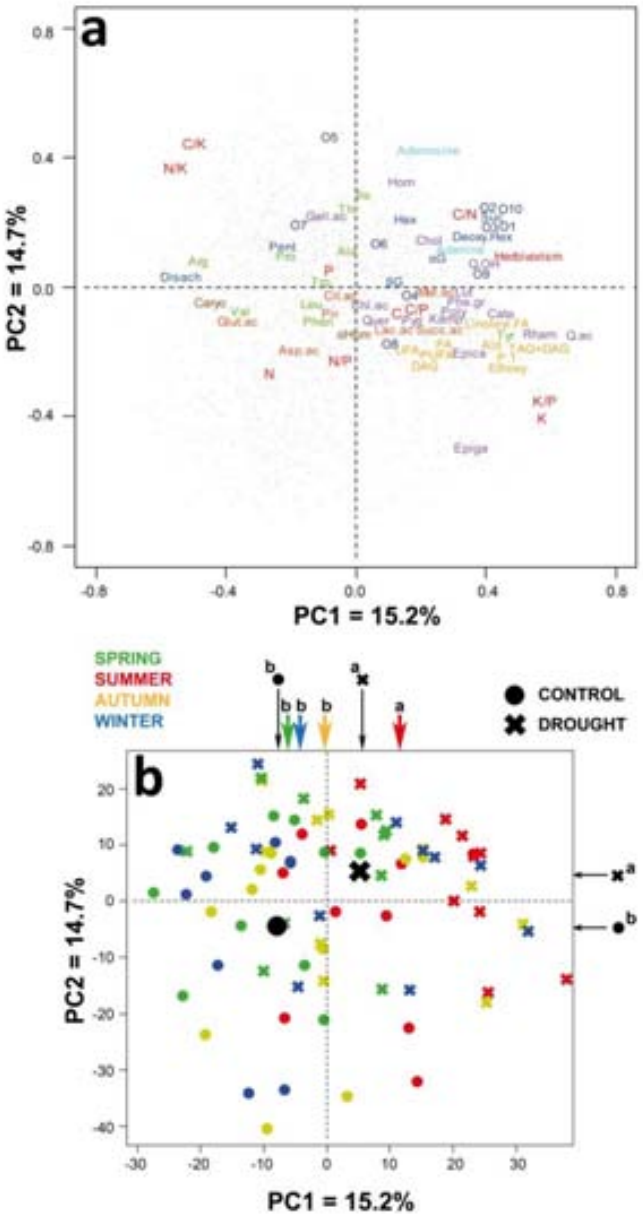

Figure 1. PC1 versus PC2 of the PCA of the elemental, stoichiometric and metabolomic variables in *Q. ilex* leaves. Panel A shows the stoichiometric and metabolomic variables. C:N:P:K ratios and folivory are shown in red. Colors indicate different metabolomic families: blue, sugars; green, amino acids; orange, RCAAS; cyan, nucleotides; violet, phenolics; light orange, nonpolar metabolites; dark blue, overlapped NMR signals and brown, terpenes. A number has been assigned to each metabolite forming part of the overlapped NMR signals: sucrose (Suc; 1), α-glucose (αG; 2), β-glucose (βG; 3), deoxy-hexose (Deoxy-Hex), hexose (Hex), pentose (Pent), disaccharide (Disacch), aspartic acid (Asp.ac), lactic acid (Lac.ac; 11), succinic acid (Succ.ac), citric acid (Cit.ac; 4), pyruvate (Pir), malic acid (Mal.ac), gallic acid (Gall.ac), alanine (Ala; 5), isoleucine (Ile; 6), threonine (Thr), valine (Val; 7), leucine (Leu), phenylalanine (Phen), proline (Pro), arginine (Arg), tryptophan (Trp), tyrosine (Tyr), quercitol (Q.OH; 8), quinic acid (Q.ac; 9), choline (Chol; 10), catechin (Cate), epicatechin (Epica), epigallocatechin (Epiga), homoorientin (Hom), quercetin (Quer), rhamnetin (Rham), kampferol (Kamp), luteolin (Lut), chlorogenic acid (Chlo.ac), N-acetyl group (12), polyphenol (Poly; 13), phenolic group (Phe.gr), pyridoxine (Pyri), caryophyllene (Caryo), α-humulene (αHum), fatty acids (FA), unsaturated fatty acids (UFA), polyunsaturated fatty acids (PUFA), diacylglycerides (DGA), triacylglyceride 1 (TGA1), Triacylglyceride 2 (TGA2) aldehyde group (Ald), acetyl group (Acetyl), linoleyl fatty acid (Linoleyl FA), polyphenol derived 1 (P.1), overlapped NMR signals (O1 - O10): O1, 5+10+2+1; O2, 10+2+1; O3, 10+3+1; O4, 4+13; O5, 6+7; O6, 11+unknown, O7, 11+12, O8, 8+9; O9, 8+9+2+3+1 and O10, 8+1. Unassigned metabolites are represented by small grey dots. Panel B shows the samples categorized by season and drought treatment. Seasons are indicated by different colors (green, spring; red, summer; yellow, autumn and blue, winter). Climatic treatment is indicated by geometric figures: circles, controls; triangles, drought. The black symbols indicate the mean PC1 vs. PC2 scores for the treatments (control trees, circle and droughted trees, cross). Arrows outside the plot indicate the mean PC for each season (colored arrows) or treatment (black arrows). The statistically significant differences between seasons were detected by Bonferroni post-hoc tests and are indicated by letters (p<0.05).

the one-way ANOVAs and the post-hoc tests of the score coordinates of the PCAs. GLM analyses were performed with the *lme* function of the *nlme* package of R (R Development Core Team, 2011).

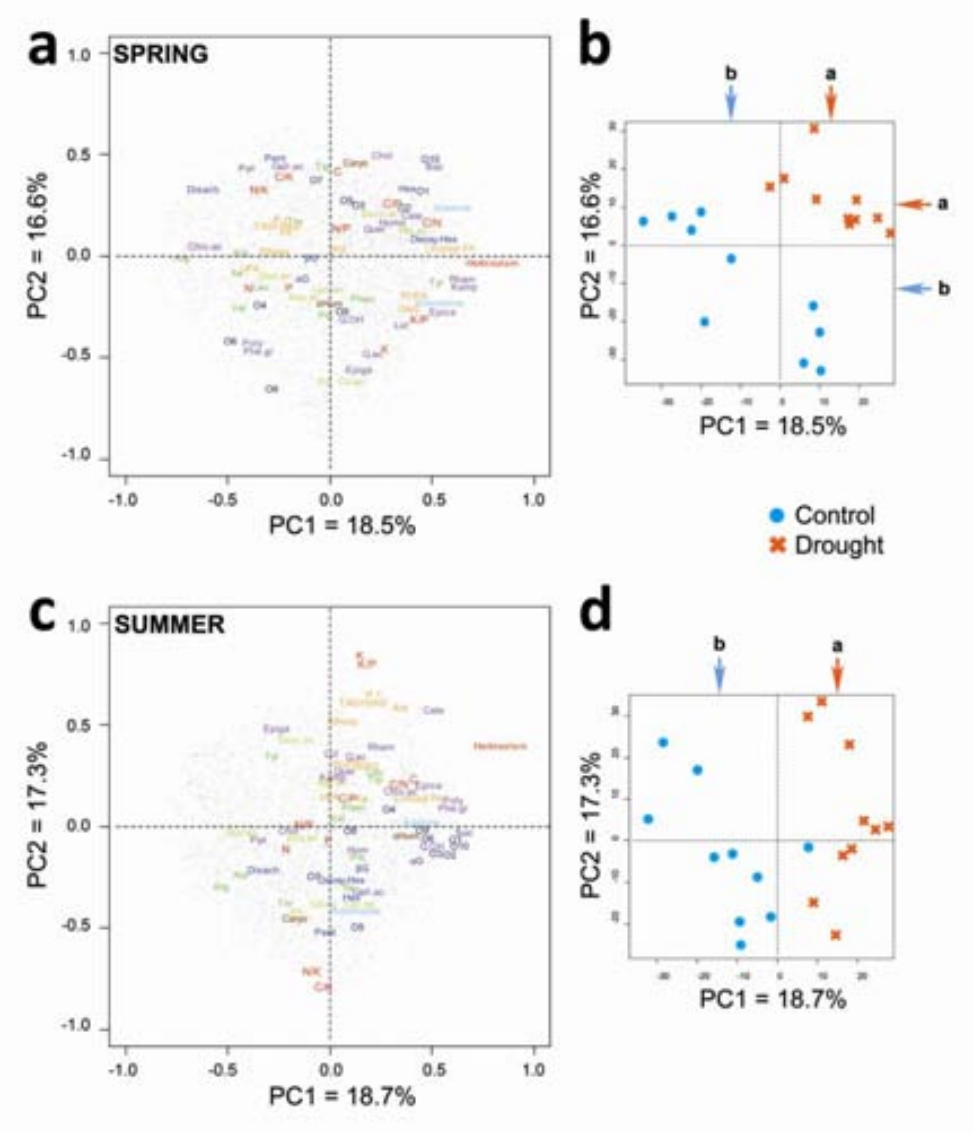

Figure 2. Plots of the PCAs of the metabolomic and stoichiometric variables for spring and summer. (A) variable plot for spring, (B) case plot for spring, (C) variable plot for summer and (D) case plot for summer. C:N:P:K ratios and herbivory are shown in red. Colors indicate different metabolomic families: blue, sugars; green, amino acids; orange, RCAAS; cyan, nucleotides; violet, phenolics; light orange, nonpolar metabolites; dark blue, overlapped NMR signals and brown, terpenes. Unassigned metabolites are represented by small grey dots. The variables are described in Figure 1. Control trees are indicated by circles and blue colors, and droughted trees are indicated by crosses and orange colors. Arrows outside the plots indicate the mean PC for each treatment. The statistically significant differences between seasons were detected by Bonferroni post-hoc tests and are indicated by letters (p<0.05).

Results

Elemental, stoichiometric and metabolomic shifts across seasons and experimental drought treatments.

The PERMANOVA analysis performed with all elemental, stoichiometric and metabolomic data (assigned and unassigned metabolites) indicated that the different seasons, drought treatments and degrees of folivory had different foliar chemistries and metabolisms (folivory: pseudo-F = 2.4832, p<0.001; season: pseudo-F = 2.4749, p<0.001 and treatment: pseudo-F = 3.1031, p<0.001).

The PCA conducted with all seasons, including all the elemental, stoichiometric and metabolomic data (Figure 1), indicated that more than 50% of the total variance was already gathered by the first four PCs (PC1 = 15.2%, PC2 = 14.7%, PC3 = 14.1% and PC4 = 12.7%). Differences between seasons were markedly explained by PC1 (p<0.001), PC3 (p<0.05) and PC4 (p<0.001). Post-hoc analysis of the score coordinates of the different case trees showed that the stoichiometries and metabolomes of summer leaves differed significantly from those of the other seasons on the PC1 axis. Droughted trees differed from control trees in PC1 (p<0.001), PC2 (p<0.05) and PC4 (p<0.001).

The C, N, P and K foliar concentrations and the ratios C:N, C:P, C:K, N:K and K:P, but not N:P, changed with the seasons (Figure 1). The highest K:P and K/N concentration ratios occurred in summer, coinciding with the highest foliar K concentrations. Foliar N and P concentrations were highest in autumn, thus resulting in the lowest C:N and C:P ratios (See Table S5 of Appendix 4 and Sardans et al. 2013b for more details). Most assigned and identified metabolites in the leaves of *Q. ilex* shifted across different seasons (Table S5 of Appendix 4). The main changes were between summer and spring, two of the most critical seasons in the Mediterranean basin because spring is the period of growth, and summer is the driest season with the lowest water availability. Summer leaves generally had higher concentrations of polyphenolics and sucrose, whereas spring leaves had higher concentrations of amino acids, some related compounds of amino acids and sugar metabolism (RCAAS) and some sugars such as pentoses and disaccharides, products directly related to growth. The effects of experimental drought were also detected on the seasonal PCA plot (Figure 1; Table S6 of Appendix 4). In the case plots of the PCA, droughted trees tended to be distributed in the same direction as the summer trees; summer is the driest season in Mediterranean climates (Figure S1A of Appendix 4).

Total concentrations of amino acids, sugars and polyphenolics.

The concentrations of the assigned and identified variables of the different metabolite families (amino acids, sugars and phenolics) were summed. The integral values of ^{1}H NMR spectra relative to the internal standard were standardized to sum with the peak area of the LC-MS chromatograms. Factorial ANOVAs indicated that trees presented higher concentrations of foliar amino acids in spring and winter (Figure 4A). The concentrations of sugars were higher in winter than in the other seasons due to the increased concentrations in the droughted trees (Figure 4B). Trees had higher concentrations of total phenolics in summer, and the drought treatments increased the phenolic concentrations significantly in summer and winter (Figure 4C). The drought treatment affected the trees in all seasons, increasing the concentrations of total sugars, but the concentrations in summer were not significantly different from those of the control trees (Figure 4B).

The GLM analysis with *total sugars* as a dependent variable, *season* and *climatic treatment* as fixed independent variables and *individuals* as a random variable showed statistical significance for both climatic treatment (p<0.001) and season (p<0.05). The GLM analysis with the same independent variables but with *total phenolics* as a dependent variable also showed statistical significance in both climatic treatment (p<0.05) and season (p<0.05).

Folivory and drought.

The PCAs conducted to investigate the relationships of folivory with drought in spring and summer also indicated a difference between control and droughted trees in the multidimensional space (Figure 2). The first four PCs explained 51% of the total variability in spring. PC1 and PC2 explained the difference between control and droughted trees, with 18.5% and 16.6%, respectively, of the total variance (Figure 2A). One-way ANOVAs on the PC score coordinates showed significant differences for both PCs (p<0.05) (Figure 2B). PC3 (16%) and PC4 (14.7%) did not differentiate control from droughted trees (p>0.05). The four first PCs explained more than 66% of the total variance in summer (Figure 2C), but only PC1 (18.7%) significantly separated control from droughted trees (p<0.001) (Figure 2D).

The GLM conducted with the summer data, including the accumulated proportions of folivory and with folivory as a dependent variable and climatic treatment and PC1 scores of the PCA (without the folivory variable, Figure S4 of Appendix 4) as independent variables (p<0.001; R^2=0.60), indicated no significant effect of the *PC1 scores* (p=0.53) but significant effects of the *drought treatment* (p<0.05). These results imply that the existence of a significant relationship between folivory and the PC1 score coordinates of the PCA is mainly due to the fixed effect of drought (Figure 3).

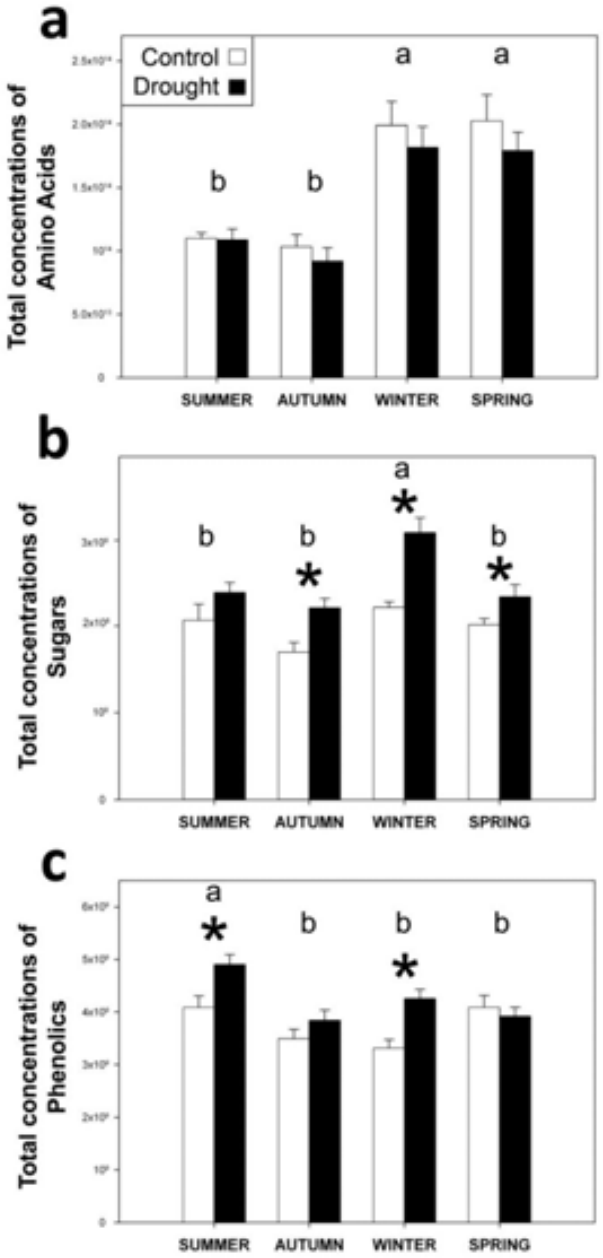

Figure 3. PC1 scores of a PCA analysis, excluding the degree of folivory, of summer metabolomic and stoichiometric data (Figure S1) versus the proportion of foliar consumption. Grey circles represent control trees, and grey crosses represent droughted trees. The black circle represents the mean of control trees ±SE, and the black cross represents the mean of droughted trees ±SE.

Discussion

Seasonality and drought

As expected, the foliar metabolome of *Q. ilex* varied with season (PERMANOVA $p<0.001$; Figure 1). PC1 of the seasonal PCA identified significant stoichiometric and metabolomic shifts between summer and the other seasons, although the differences between seasons were not as marked as those of an earlier study with the Mediterranean shrub *Erica multiflora* (Rivas-Ubach et al. 2012). Trees in spring, the Mediterranean growing season, had high concentrations of amino acids, some RCAAS and some sugars such as pentoses and disaccharides, in accordance

with the earlier study (Rivas-Ubach et al. 2012), even though the total concentration of amino acids did not differ from the levels in winter (Figure 4A), the coldest season in the Mediterranean basin. Foliar N:P ratios were not lowest in spring as expected from the growth rate hypothesis (Elser et al. 1996, Rivas-Ubach et al. 2012), which proposes that organisms with high rates of growth require high levels of P (low N:P ratios) to meet the demands of protein synthesis (Elser et al. 2003) Our results are nevertheless in accordance with other studies in plants. The high foliar concentrations of amino acids in winter trees might be due to accumulation under cold stress. Some amino acids such as proline and glycine are able to buffer the NADP+/NADPH ratio in plants (Xu et al. 2013) consequent to the low photosynthetic activity in winter to provide reducing agents for the generation of ATP in mitochondria (Hare and Cress, 1997, Xu et al. 2013). On the other hand, Matzek and Vitousek, 2009 found no significant relationships between the N:P ratio and increased growth in 14 species of pines and concluded that terrestrial plants must invest in other fundamental functions other than growth, such as storage and defense that often require different investments in N and P (Herms and Mattson, 1992, Peñuelas and Estiarte, 1998).

The concentrations of the various sugar species detected were distributed differentially among the seasons (Figure 1), but droughted trees in winter had the highest concentrations of total sugars (Figure 4B), which may also be related to the synthesis of sugars in response to cold stress, allocation from other organs (wood, lignotubers) to leaves or further preparation for the growth season (Grimaud et al. 2013, Xu et al. 2013). *Q. ilex* is an evergreen tree with slow growth; they present large lignotubers, swollen woody structures at the base of the stem (James, 1984) that are able to store essential nutrients and metabolites such as carbohydrates to ensure rapid growth after severe stress (Canadell and Zedler, 1995). Our stoichiometric and metabolomic data suggest that terrestrial trees, with large woody structures such as trunks and/or lignotubers, are able to store large amounts of various nutrients, allowing resistance to severe environmental disturbances by supplying essential resources to photosynthetic organs (Canadell and Zedler, 1995, Galiano et al. 2012). Terrestrial trees are consequently able to buffer the metabolomic changes in foliar ontogeny that may occur in abnormal seasons.

The largest stoichiometric shift between seasons appeared to be related to the responses to drought stress (Figure 1). Foliar concentrations of K were higher in trees in summer, the driest season. The resulting higher foliar K:P and lower N:K and C:K concentration ratios (Sardans et al. 2013b) were thus in agreement with the findings of other Mediterranean seasonal studies (Sardans et al. 2012a, Sardans et al. 2011b). K participates in the water economy of plants (Babita et al. 2010) through osmotic control (Babita et al. 2010, Laus et al. 2011) and improves the functioning of foliar stomata (Khosravifar et al. 2008). These results demonstrate the important

role of K in the ecology of terrestrial plants (Cakmak, 2005, Sardans et al. 2012a, Sardans et al. 2013b). Moreover, these shifts in K concentrations in summer leaves were accompanied by higher concentrations of sucrose, which can also act as an osmolyte that, together with K, can help to avoid the loss of water through osmotic control (Ingram and Bartels, 1996).

PC1 and PC2 of the seasonal PCA also showed interesting differences between the trees in the drought plots and those in the control plots (Figure 1; PERMANOVA $p<0.001$). Droughted trees tended to have the same pattern as summer trees on a PC1 vs. PC2 plot, in accordance with a previous study (Rivas-Ubach et al. 2012), thus indicating a certain foliar elemental-metabolomic response to drought independent of plant ontogeny. Summer and droughted trees in all seasons tended to have higher foliar concentrations of flavonoids (Figure 4C), the largest group of naturally occurring polyphenols (Strack et al. 1994), but the flavonoid composition differed between summer and the drought treatment. Summer trees had higher concentrations of quinic acid, catechin and luteolin, among other polyphenolics, whereas droughted trees had higher concentrations of catechin, quercitol, homoorientin and quercetin (Figure 1; Table S6 of Appendix 4). These differences should correlate with the differential effects of drought treatment in the different seasons (Figure 4C). Many biological functions have been ascribed to flavonoids, especially the role of antioxidant (Burda and Oleszek, 2001, Lee et al. 2003). Their antioxidant activity is mainly due to their role as electron donors (RiceEvans et al. 1997) and their ability to alter the kinetics of peroxidation (Arora et al. 2000). As expected in a water-limited Mediterranean ecosystem, oxidative stress in plants tends to increase under conditions of drought (Dat et al. 2000, Munne-Bosch and Peñuelas, 2004, Peñuelas et al. 2004, Price et al. 1989). Quercitol has been associated with the avoidance of osmotic stress during drought in *Quercus* species (Passarinho et al. 2006, Spiess et al. 2012). Our study found no significant differences ($p=0.12$) between choline levels in droughted and control trees but did identify a tendency toward higher concentrations in stressed plants. Choline is also involved in osmotic protection (McNeil et al. 2001). Drought will likely have a significant impact on the metabolomes of Mediterranean plants, because dry periods will become more frequent and intense in the coming decades, as predicted by the projections of climate change (IPCC, 2007).

Droughted trees also presented higher concentrations of total soluble sugars in their leaves (Figure 4C), supporting the premise that sugars act as osmolytes to prevent the loss of water (Ingram and Bartels, 1996). Control trees in summer did not have significantly lower foliar concentrations of sugars relative to droughted trees (Figure 4B), even though droughted trees had a tendency toward higher concentrations. The small differences in foliar sugar concentrations between control and droughted leaves may be mainly due to the naturally dry

summers of the Mediterranean basin that may have partially increased the foliar sugar concentrations of the control trees.

Folivory

The highest herbivorous activity by insects occurs mainly in spring and early summer in the Mediterranean basin (Bonal et al. 2010, Powell and Logan, 2005), and the leaves of trees have accumulated most signs of folivory by the middle of the summer. Interestingly, folivorous activity was higher in the droughted trees in both spring and summer (Figures 2 and 3). GLM analyses for the summer season indicated that the relationship between folivory and PC1 score coordinates of the PCA for foliar stoichiometry and metabolomics (Figure S4) was mainly due to the fixed effect of drought, because folivory was not correlated with PC1 within groups (control, drought) (p>0.05) (Figure 3). Our results thus suggest that the stoichiometric and metabolomic shifts were mainly caused by the experimental drought, which subsequently indirectly increased the degree of folivory.

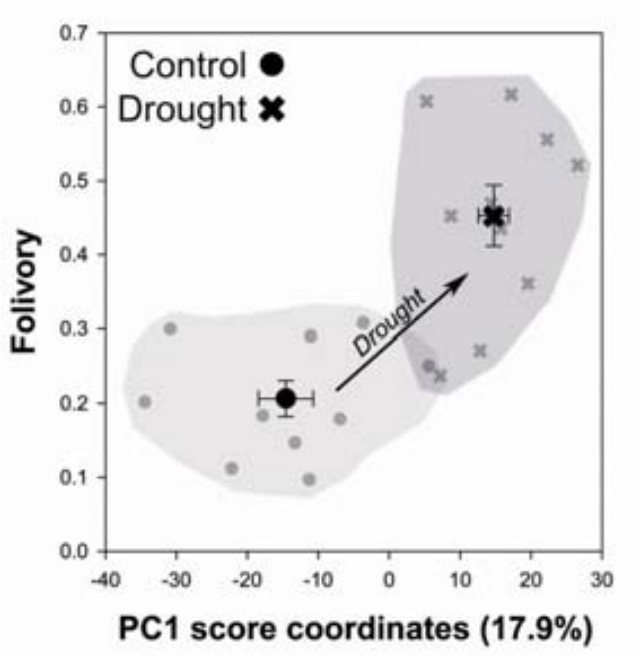

Figure 4. Bar graphs of the total foliar concentrations of amino acids (A), sugars (B) and phenolics (C) ±SE. White bars represent control plots, and black bars represent droughted plots. The statistically significant differences between seasons and treatments were detected by Bonferroni post-hoc tests (p<0.05). Seasonal statistical differences are indicated by different letters, and climatic statistical differences are indicated by asterisks. *Concentration* of metabolites of Y axis is explained in detail in Material & Methods section.

Our stoichiometric results identified no significant relationships between foliar N concentrations and degree of folivory, as reported in other studies (Larsson, 1989, Larsson and Bjorkman, 1993, Rouault et al. 2006), indicating that folivory is not influenced only by foliar N concentrations (Choong et al. 1992, Williams et al. 1998). As discussed above, droughted trees

tended to have higher concentrations of sugars and polyphenols (flavonoids) than did the controls (Figure 4). Flavonoids such as quercetin have proven to act as phagostimulants in herbivorous insects (Diaz Napal et al. 2010, Kosonen et al. 2012).The higher concentrations of sugars and some flavonoids found in the foliage of droughted *Q. ilex* trees appeared to increase herbivorous activity (Figure 3). Despite the scarcity of supporting evidence, our results also indicated a higher folivorous activity in droughted trees, supporting the premise of the high palatability of some flavonoids not directly related to defense but that act as antioxidants (Diaz Napal et al. 2010, Kosonen et al. 2012). Also, droughted leaves had higher concentrations of sugars (Figure 4), which could thus provide a source of rapid energy for herbivores. Not all the assigned polyphenols, however, provide only antioxidant protection in plants. Several studies have reported that polyphenolics serve as a chemical defense against predation by herbivores (Berg, 2003, Kosonen et al. 2012, Lokvam and Kursar, 2005, Rani and Pratyusha, 2013, Treutter, 2006). Kosonen et al. 2012 reported that some polyphenolics could be toxic to specialist herbivores but could increase the palatability of plants for generalist herbivores andsuggests that climate change is able to reduce the damage caused by specialist herbivores and increase that caused by generalists. Phenolic acids could thus potentially be used for pest management (Rani and Pratyusha, 2013). Mammals are also affected by polyphenols in plants. For example, Berg, 2003 reported that elevated concentrations of catechin negatively affected the consumption of plants by collared lemmings. In our study, nearly all the assigned polyphenols were associated with drought. Most of the assigned flavonoids have antioxidant activity, but droughted trees also had significantly higher foliar concentration of catechin (Figure 2A and 2C), thus supporting the idea that plant-induced defenses are not only produced by drought but also by a higher degree of herbivorous attack.

We have demonstrated that seasonal drought influences the stoichiometry and metabolome of plants and that experimental drought indirectly affects folivorous activity by shifting the metabolism of plants (Figure 3). The more severe and frequent droughts predicted for the Mediterranean basin (IPCC, 2007) may thus have an indirect impact on trophic webs by shifting the amount of herbivory through foliar stoichiometric and metabolomic shifts (Figure 3). This impact may lead to more intensive and extensive outbreaks of pests and to possible changes in the distribution of food between specialist and generalist herbivores (Kosonen et al. 2012).

Conclusions and final remarks.

· Foliar N:P ratios of *Q. ilex* did not significantly change between seasons as expected from the growth rate hypothesis. Foliar K concentrations, however, did change, demonstrating the importance of K in natural summer droughts. The lack of significant variation in N and P concentrations in *Q. ilex* may be due to the buffering effect of lignotubers in this woody species.

· Drought stress increased the concentrations of sugars and polyphenolic compounds with antioxidant function in the leaves of *Q. ilex*. Our results suggest that these metabolomic shifts stimulate foliar consumption by herbivores, although folivory may also induce higher concentrations of defensive polyphenolic compounds.

· Our data indicated that summer and droughted trees could avoid or respond to water stress by different means, although both had higher foliar concentrations of flavonoids. Summer trees tended to use more K as an osmolyte than did drought-stressed trees; K is more easily translocated than are other metabolites such as sugars. These results may indicate that this tree species is adapted to the natural periods of summer drought.

· Coupling stoichiometric with metabolomic techniques have proven to be useful for identifying the molecular responses of plants to stresses such as drought, for understanding the mechanisms and functions that underlie the responses of plants to stress and for interpreting the implications of drought on trophic webs.

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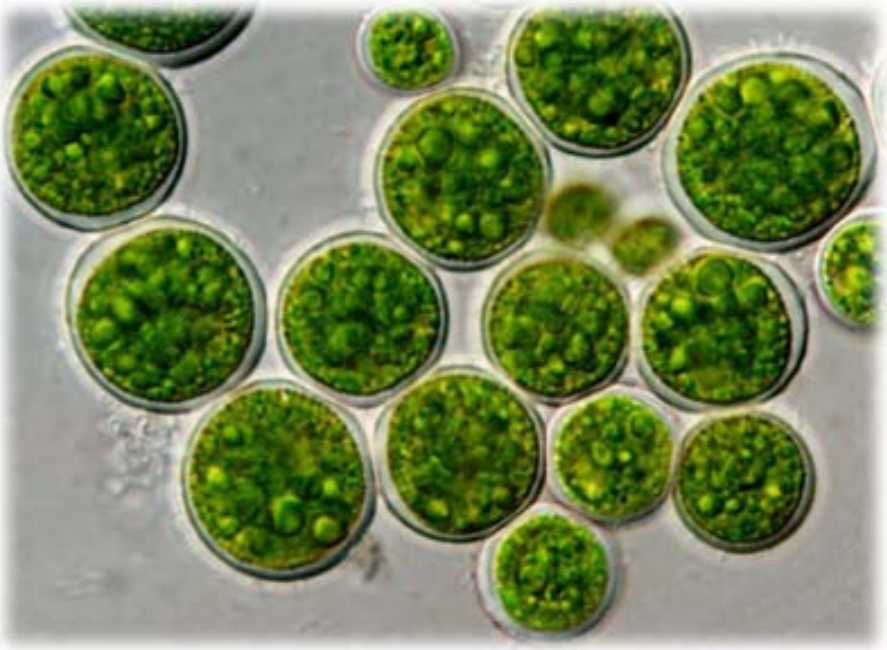
Chapter 5.

Coping with P and Fe limitations: A metabolomic study of *Synechocystis sp*. PCC 6803.



Coping with P and Fe limitations:

A metabolomic study of *Synechocystis sp.* PCC 6803.

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Abstract

Phosphorus (P) and iron (Fe) are key elements for cyanobacterial growth. P is essential in the transcription and translation processes given that rRNA is the most P-rich biomolecule in organisms. Fe plays an important role in the photosynthetic electron transport chains. The limitation of primary production by these essential nutrients has important implications for ecosystem structure and function. We integrated stoichiometric and metabolomic techniques to study the whole metabolome response to the different nutrient availability (P and Fe limitation) of the cyanobacterium, *Synechocystis sp.* PCC 6803. *Synechocystis* growing under conditions of complete nutrient supply had higher concentrations of protein, chlorophyll *a*, RNA, N, P, and C and lower protein:RNA, C:N, and C:P ratios than cultures growing under P and Fe limited conditions. These shifts coincided with the higher concentrations of amino acids and nucleotides and higher growth rates. Moreover, we found that nutrient-limited cultures shifted their metabolism from aerobic processes to anaerobic pathways, thereby producing more lactic acid. Our results suggest that cyanobacteria growing under P of Fe nutrient limitation activate the anaerobic pathway producing lactic acid to maintain the internal redox homeostasis ($NADH/NAD^+$ ratio) to avoid internal oxidation. The present study represents an advance in understanding the metabolic strategies that cyanobacteria use to cope with P and Fe limitation and helps to understand the possible implications of P and Fe limitation in the structure and function of aquatic ecosystems.

Keywords metabolomics · stoichiometry · Synechocystis · nutrient limitation · phosphorus · Iron

Introduction

Ecological stoichiometry is a framework used to understand organism life styles and roles in ecosystem structure and function through following how elements (i.e., carbon, nitrogen, phosphorus) and ratios of such elements influence the interaction between organisms and the environment (Elser et al. 1996; Sterner and Elser 2002). C, N, and P are bioessential elements and their uptake from the environment is necessary for all organisms. The first studies of ecological stoichiometry began in aquatic marine ecosystems when Redflied published a classic paper proposing the constant phytoplankton C:N:P ratios of 106:16:1 (Redfield 1963). Even though, several other studies demonstrated later that these C:N:P values are not fixed across all oceans and continental waters (Sardans et al. 2012a) and organisms often vary their C:N:P ratios due mainly to the nutrient availability in the habitat and the relative balance of light and nutrients in the case of autotrophs (Sterner et al. 1998). C:N:P biomass ratios play an important role in several ecological processes in both aquatic and terrestrial ecosystems, such as decomposition of litter (d'Annunzio et al. 2008; Guesewell and Gessner 2009), capacity of N_2 fixation (Sañudo-Wilhelmy et al. 2001), plant-herbivore-predator relationships (Ngai and Jefferies 2004; Tibbets and Molles 2005; Kagata and Ohgushi 2006), ecosystem-specific composition and diversity (Roem and Berendse 2000; Venterink et al. 2003; Güsewell et al. 2005) and the capacity to adapt to environmental stresses (Woods et al. 2003; Sardans andPeñuelas 2007; Sardans et al. 2008). Even so, most elements, such as C, N and P, do not act by themselves but as components of molecular compounds (Peñuelas and Sardans 2009a). For example, N is a central component of many biomolecules such as proteins, chlorophylls, and nucleic acids (DNA and RNA) (Bernhard 2012), while P is mainly a component of nucleic acids, particularly in P-rich rRNA with a N:P ratio approximately of 6:1 in bacteriapresenting the lowest N:P ratios of any other subcellular structure (Elser et al. 1996). The link between the shifts in organism stoichiometry with the shifts in metabolism has been recently demonstrated by coupling stoichiometric and ecometabolomic techniques in terrestrial plants (Rivas-Ubach et al. 2012). Ecometabolomics is a new emerging analytical technique in ecology that allows analyzing the metabolome of organisms, the total set of metabolites present in an organism at a specific moment (Fiehn 2002) and its response to abiotic and biotic environmental shifts (Peñuelas and Sardans 2009b; Sardans et al. 2011; Peñuelas et al. 2013a; Rivas-Ubach et al. 2013a).

The multiple factors that influence P availability, such as climate or edaphic characteristics, make it difficult to achieve a clear picture of the relative importance of P limitation in the biosphere (Elser et al. 2007). However, there is some evidence that P is the main limiting nutrient in freshwater ecosystems (Schindler and Eby 1997) and exerts a primary control of oceanic production (Tyrrell 1999). Usually, nutrient-limitation in cyanobacteria induces changes in

metabolism increasing catabolism and decreasing anabolism including the overall protein synthesis (Aldehni et al. 2003) and growth (Schwarz and Forchhammer 2005) followed by a degradation of the intracellular membranes and accumulation of cellular inclusions (Allen 1984). Although P-limitation induces the activation of several genomic sequences to synthesize different enzymes related to P acquisition (Grillo and Gibson 1979; Ray et al. 1991; Wagner et al. 1995), in many cases this response is not sufficient to reverse P limitation. The slow growth of cyanobacteria produced by P starvation potentially impacts significantly the upper levels of trophic webs by both the reduced food quality (high C:P ratios) for grazers with a high P demand and the reduced biomass of cyanobacteria (low food availability) (Sterner 1993; Sterner et al. 1998). Higher P availability for autotrophs allows the existence of larger trophic webs (Sterner et al. 1998), although at certain low levels of N:P ratios, N can also limit the production capacity of ecosystems affecting the species richness (Sardans et al. 2012a).

P imbalance with N (high P availability) or P (low P availability) is projected to increase in several areas around the world affecting both aquatic and terrestrial ecosystems (Vitousek et al. 1997; Peñuelas et al. 2012; Peñuelas et al. 2013b). N inputs in aquatic ecosystems increase the environmental N:P ratios (Elser et al. 2009). These inputs of nitrogen in terrestrial ecosystems increase the leaching of P and consequently reduce its availability (Tilman and Lehman 2001). This reduction of available P in soils may produce further reduction of P inputs in waters and consequently limit phytoplankton growth. An increased N deposition in certain ecosystems where N availability was usually limiting, has led to a shift from N to P limitation (Vitousek et al. 2010). This projected P availability reduction may thereafter lead to changes in the ecosystem structure and function throughout the effects on trophic webs mentioned above (Sterner 1993; Sterner et al. 1998; Sardans et al. 2012a).

Apart from C, N and P, Fe is also a crucial element especially for photosynthetic organisms as cyanobacteria. Fertilization of natural ecosystems with Fe results in rapid growth of diatoms, causing in many cases blooms (Assmy et al. 2007; Boyd et al. 2007). Fe is present in many enzymes necessary for basic physiological processes such as respiration, nitrogen reduction, pigment and DNA synthesis, reduction of reactive oxygen species or fatty acid metabolism and is especially important in photosynthesis (Behrenfeld and Milligan 2013; Twining and Baines 2013). In thylakoid membranes of obligate autotrophs, Fe plays a crucial role in photosynthetic electron transport chain (PETC). Many molecular complexes of the PETC are Fe-rich: photosystem I (PS I), photsystem II (PS II), and the cytochrome b6/f complex contain 12, 3 and 5 Fe atoms respectively (Ferreira and Straus 1994). In aqueous well-oxygenated environments, Fe^{2+} is rapidly oxidized forming insoluble hydroxides and precipitates as Fe^{3+} which is difficult for primary producers to take up (Ferreira and Straus 1994; Behrenfeld and Milligan 2013). Even though Fe is the fourth

most abundant element in the Earth's crust (Bibby et al. 2001), the high contents of this element in the PETC and the low availability of Fe^{2+} make Fe a key limiting nutrient for phytoplankton photosynthesis and growth, and structuring plankton communities. Altogether, these studies are placing Fe in the forefront of oceanographic studies (Martin et al. 1994; Behrenfeld et al. 1996; Moore et al. 2002; Boyd et al. 2007; Timmermans and van der Wagt 2010). In lakes, Fe is also at the forefront of limnological studies since projected future acidification of several lakes, especially of boreal countries, mainly due climate change as well as changes in forest harvest practices (Christensen et al. 2006; Moldan et al. 2013) may decrease the Fe^{2+}/Fe^{3+} ratios, thus making Fe even less available for phytoplankton and affecting its growth.

Iron deficiency induces several responses at different levels in cyanobacteria. Reductions in cell size and growth rate are most common visual symptoms of Fe starvation (Timmermans and van der Wagt 2010). At the cellular level, Fe stress produces changes on the thylakoid architecture firstly by the replacement of cytochrome C553 and partially ferredoxin with plastocyanin and flavodoxin respectively (no Fe-dependentents) (Ferreira and Straus 1994) followed by an overall decrease of the photosynthetic capacity (Behrenfeld and Milligan 2013). These changes in thylakoids and photosynthesis indirectly regulate the carbon fixation capacity in Calvin cycle (Allen et al. 2008) and consequently the growth of cells.

Although the effects of iron stress in cyanobacteria have been studied since 1930s (Grant 1931; Hart 1934) and P represents one of the key elements for life (Sterner and Elser 2002), the effects of P and Fe limitation on the metabolome of cells and other biomolecular groups are mostly unknown and metabolomic techniques have not been used to study the Fe and P starvation effects as far as we know. We chose to apply metabolomics to cyanobacteria to tackle this question. The present study aims to provide a better understanding of the metabolic strategies that cyanobacteria use to cope with P and Fe limitations, two key elements for phytoplankton growth. We cultured *Synechocystis sp.* PCC6803*,* a non-N_2-fixing freshwater cyanobacterium, under complete nutrient, P-limited, and Fe-limited conditions. All samples were subjected to elemental and metabolomic analyses and DNA, RNA and protein quantification. This study, by coupling stoichiometric with metabolomic techniques in cyanobacteria, helps to understand the metabolic mechanisms and functions that underlie the cyanobacterial responses to nutrient stress and make a step forward in the interpretation of the possible implications of nutrient starvation on aquatic ecosystem structure and function.

Materials & Methods

Media preparation for growth of *Synechocystis* sp. PCC 6803.

Three different media for culturing *Synechocystis sp*. PCC 6803 were prepared: Complete (C), Phosphorus limited (P-lim) and Iron limited (Fe-lim). BG-11 media was prepared with the following modifications (Allen 1968; Stanier et al. 1971). Briefly, the basic media for all treatments consisted of a mixture of calcium chloride (10 mL/L), magnesium sulfate (10 mL/L), citric acid (1 mL/L), sodium carbonate (1 mL/L) and sodium EDTA (1 mL/L). After that, each treatment received different proportions of trace elements, potassium phosphate, potassium chloride, and iron chloride. Complete and P-Lim media received 10 mL/L of trace elements mixture. Fe-Lim received also 10 mL/L of trace elements mixture without ferric chloride (See Table S1 of Appendix 5 for trace elements mixture). For P-Lim media, 0.2 mL/L of postassium phosphate and 0.88 mL/L of potassium chloride were added and 1 mL/L of potassium phosphate was added for Complete and Fe-Lim. For the Fe-lim media, 3.33 mL/L of iron chloride was added and 10 mL/L were added to Complete and P-Lim. All media were thus adjusted to a pH = 8.0 by NaOH solution.

Culturing and harvesting of *Synechocystis* sp. PCC 6803.

Initial cultures for inoculation: Five initial 2 L trace metal clean polycarbonate bottles with cultures of *Synechocystis sp*. PCC 6803 were grown to exponential growth under the different nutrient conditions. We cultured 1 bottle of complete (IC) (N:P = 100), 2 bottles under P limitation (N:P = 1000) (IP.a) and (N:P= 900) (IP.b)) and 2 bottles under Fe limitation (1/10 of Fe with respect to Complete (IFe.a) and 1/2 of Fe with respect to Complete (IFe.b)).

Inoculation of Synechocystis sp. PCC 6803*:* For each treatment (Complete, P-Lim and Fe-Lim), six trace metal clean polycarbonate bottles of 2 L were prepared with the corresponding BG-11 media; 1.8 L of Complete medium was added to Complete bottles and 1.5 L of P and Fe limited media were added to P and Fe limited bottles respectively. After that, *Synechocystis sp.* PCC6803 was inoculated to each bottle. For each Complete culture bottle; 200 mL of IC was added, for each Phoshorus limited culture bottle; 260 mL of cyanobacteria IPa and 100 mL of IPb were added and for each Iron limited culture bottle; 260 mL of cyanobacteria IFea and 40 mL of IFeb were added.

Growth of Synechocystis sp. PCC 6803: The cultures of *Synechocystis* were maintained at room

temperature (24ºC) under continuous aeration with 0.2 m filtered air and illumination (150 µmol photons m^{-2} s^{-2} irradiance). Different treatments had different growth rates and cultures were harvested at the exponential phase of growth. The concentration of chlorophyll *a* was monitored through time for all cultures and used to graph the growth rates and detect the optimum harvesting time. Complete cultures were harvested after 6 days of growth, Fe-limited after 9 days of growth, and P-limited after 12 days of growth. Always, sterile syringes were used for harvesting cells.

Harvesting for quantification of chlorophyll a: The final yield of *Synechocystis sp*. PCC 6803 for each treatment was assessed via chlorophyll *a* measurements. Chlorophyll *a* was measured on 2 mL of culture harvested via centrifugation at 21,000 x g for 10 minutes at 4°C. Supernatant of all tubes was poured and cell pellets were extracted with 1 mL of 100% methanol and vortexed at max speed. After that, cells were incubated for 5 minutes in the dark at room temperature (24ºC). After centrifugation (21,000 x g; 5 min; 4ºC) to collect cell debris, the supernatant was poured into a polystyrene cuvette to measure the absorbance of cell extracts at 665 nm. Methanol (100%) was used as blank. Chlorophyll *a* concentration (g mL^{-1}) was calculated as follows (Tandeau de Marsac and Houmard, 1988):

$$Chl_a[ug \cdot mL^{-1}] = 13.9 \cdot Abs_{665nm}$$

Harvesting for Carbon, Nitrogen and Phosphorus determination: approximately 30 mL of cells for each measurement were harvested at 5,000 x g for 10 minutes at 4°C and washed with 0.85% NaCl to remove excess media and frozen at -80ºC until analyses.

Harvesting for RNA and Protein quantification: Two sterile 2 mL tubes per sample and analysis were labeled (3 analyses (DNA, RNA and Protein) × 2 tubes per sample = 6 tubes per sample). 20 mL of each culture were placedinto a 50 mL tubes. Samples were centrifuged at 5,000 *x g* for 10 minutes at 4ºC. Supernatants were removed and cells were distributed to the 2 mL tubes. Samples were again centrifuged at 14,000 *x g* for 5 min at 4ºC and supernatants were poured. Samples were quickly frozen by dry ice and methanol. After that, samples were kept at -80ºC until analyses.

Harvesting for metabolomic analyses: All the remaining biomass was placed into 200 mL tubes. All tubes were centrifuged at 10,000 *x g* for 10 minutes, the supernatants were poured and cells

were resuspended and transferred into 50 mL centrifuge tubes. Tubes were centrifuged at 5,000 *x g* for 5 minutes at 4ºC and supernatants were poured again. Tubes were frozen and lyophilized. Samples were kept at -80ºC until metabolite extractions.

Elemental analyses.

Samples were dried at 60°C for 24 to 72 hours before measurement of C, N and P. C and N was measured on 1-3 mg dry weight samples using a Costech Elemental Analyzer coupled to a Finnigan DeltaPlus Isotope Ratio Mass Spectrometer (EA-IRMS; Thermo-Finnigan MAT 253, West Palm Beach, FL, USA). C and N contents were calculated via comparison with a tomato leaves standard (NIST SRM 1573a).

Phosphorus was measured on 0.5-1.2 dry weight samples following the APHA Standards (APHA, 2005).

Biological macromolecule (RNA and protein) composition analyses.

RNA was extracted from cells using the FastRNA® Pro Blue Kit (MP Biomedicals, Solon, OH, USA) following the manufacturer's protocol with the following exception. A second chloroform extraction was performed to further purify nucleic acids and samples were precipitated overnight with isopropanol at -20°C. After precipitation, nucleic acids were pelleted via centrifugation at 16,000 x g for 15 minutes at 4°C, washed with 75% ethanol, and air dried for 5 minutes. Nucleic acid pellets were resuspended in 100 µL of nuclease-free H_2O and RNA integrity (intact 23S and 16S rRNA bands) ascertained via gel electrophoresis. To remove gDNA, samples were treated with the RTS DNase (MO BIO Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. DNased RNA was purified via the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA, USA) and eluted in 14 µL of nuclease free water. RNA concentration of 100 to 200 fold diluted samples was measured using the Qubit® RNA Assay Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Purified RNA from three replicates per treatment (12 samples) was randomly selected for transcriptomic analysis as described below.

Protein was extracted from cells using a protocol modified from Gao et al. (2009). Briefly, cells were resuspended in 500 µL of 10 mM HEPES-NaOH (pH 7.2) amended with 1X Protease Inhibitor Cocktail (Promega, Madison, WI, USA). Resuspended cells were transferred to Lysing Matrix B tubes (MP Biomedicals, Solon, OH, USA) and homogenized for 45 s at a speed of 6.0 m/s in a FastPrep®-24 homogenizer. Samples were centrifuged at 5,000 x g for 5 minutes at 4°C to pellet cellular debris and lysing beads. 250-300 µL of supernatant was transferred to another tube and the protein concentration of 40 fold diluted samples measured using the Qubit® Protein Assay Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions.

Metabolite extraction from *Synechocystis sp*. PCC 6803 cells.

Metabolite extraction for NMR analyses: The metabolite extraction for the NMR analyses was performed following Rivas-Ubach et al. 2013a with extraction of only the polar and semi-polar metabolites. First, two sets of 50-mL centrifuge tubes were labeled: set A for the extraction of metabolites and set B for lyophilization. The tubes of set A received 30 mg of lyophilized culture from each treatment replicate. Six mL of water/methanol (1:1) was added to each tube. The samples were vortexed for 15 s and then sonicated for 2 min at room temperature. All tubes were centrifuged at 1,100 × g for 15 min. Four mL were collected and transferred to the centrifuge tubes of set B. This procedure was repeated twice for two extractions of the same sample. Samples of set B were resuspended in water to reduce the proportion of methanol (<15% methanol). Set B was lyophilized and 4 mL of water were added to each tube. Samples were then vortexed, centrifuged at 23,000 x g for 3 min, frozen at -80 ºC and lyophilized again. Finally, 1 mL of KH_2PO_4-NaOD-buffered D_2O + 0.01% TSP (trimethylsilyl propionic acid sodium salt) (pH 6.0) was added to the tubes. TSP was used as internal standard. All solutions were resuspended with a micropipette, transferred to 2 mL centrifuge tubes and centrifuged at 23,000 x *g* for 3 min. The supernatants were transferred to NMR sample tubes.

Metabolite extraction for LC-MS analyses: The extraction of metabolites followed the protocol of t'Kindt et al. 2008 with minor modifications. Two sets of 2 mL centrifugue tubes tubes were labeled: set A for extractions and set B for the extracts from set A. Set A received 30 mg of lyophilized cells of each sample, then 1 mL of MeOH/H_2O (80:20) was added to each tube. Set A was vortexed for 15 min, sonicated for 5 min at room temperature and centrifuged at 23,000 x *g*for 5 min. After centrifugation, 0.6 mL of the supernatant from each tube was transferred to the corresponding Eppendorf tubes of set B. This procedure was repeated again to the same tubes for two extractions of the same sample. The tubes of set B containing two extraction procedures were thus centrifuged at 23,000 x *g* for 5 min. The supernatants were collected by crystal syringes, filtered through 0.22 µm pore microfilters and transferred to a labeled set of HPLC vials. The vials were stored at -80 ºC until the LC-MS analysis.

Metabolomic measurements of cellular extracts:

NMR-based metabolomics: NMR experiments were performed on a Bruker AVANCE 600 spectrometer (BrukerBiospin, Rheinstetten, Germany) equipped with an automatic sample changer, a multinuclear triple resonance TBI probe, a temperature control unit and working at a

magnetic field of 14.1 T (^{1}H and ^{13}C NMR frequencies of 600.13 and 150.13 MHz respectively). The temperature into the probe head was previously calibrated and maintained constant for all the experiments at 298.0 K; for this purpose an equilibration delay (2 min) is left once the tube is into the magnet and prior to the shimming process. All NMR sample handling, automation, acquisition and processing were controlled using TopSpin 3.1 software (BrukerBiospin, Rheinstetten, Germany). Spectra were referenced to the internal reference TSP (^{1}H and ^{13}C at δ 0.00 ppm). For the NMR analyses the protocol of Rivas-Ubach et al. 2013a was followed.

^{1}H NMR fingerprinting: All extract samples were analyzed through standard pulse-acquisition one dimensional (1D) ^{1}H-NMR experiments with suppression of the residual water resonance. The water resonance signal was presaturated at a power level of 55 dB, corresponding to an effective field of 30 Hz during a relaxation delay of 2s. Each experiment was acquired as a set at 32 k data points, over a spectral width of 16 ppm, as the sum of 128 transients and with an acquisition time of 1.7 s. The resulting interferograms (FID) were Fourier transformed and the spectra obtained were phased and baseline corrected. The FID of polar samples were multiplied by an exponential apodization function equivalent to 0.2 Hz line broadening prior to the Fourier transform. The experimental time was of *ca.*8 min per sample.

NMR metabolite identification: Standard 2D NMR experiments (^{1}H-^{1}H correlated spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC)) and 1D selective ^{1}H TOCSY experiments were performed in representative extract samples for the identification of the metabolites. Experiments were acquired with standard presaturation of the residual water peak during the relaxation delay using standard Bruker pulse sequences and routine conditions. All elucidated metabolites were further confirmed by reported literature data (Breitmaier et al. 1979; Walker et al. 1982; Bolinger et al. 1984; Iles et al. 1985; Brown et al. 1989; Fan 1996; Fan and Lane 2008); See Table S2 of Appendix 5 for identified metabolites with NMR).

LC-MS analysis: LC-MS chromatograms were obtained using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific/Dionex RSLC, Dionex,Waltham, Massachusetts, USA) coupled to an LTQ Orbitrap XL high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) equipped with an HESI II (heated electrospray ionization) source. A reversed-phase C18 Hypersil gold column (150 × 2.1 mm, 3µ particle size; Thermo Scientific, Waltham, Massachusetts, USA) was used for chromatography at 30 ºC. Both mobile phases were filtered and degassed for 10 min in an ultrasonic bath prior to use and consisted of acetonitrile (A)

and water (0.1% acetic acid) (B). The injection volume of the samples was 5 µL. At a flow rate of 0.3 mL per minute, the elution gradient began at 10% A (90% B) and was maintained for 5 min, then to 10% B (90% A) for the next 20 min. The initial proportions (10% A; 90% B) were gradually recovered over the next 5 min, and the column was then washed and stabilized for 5 min before injecting the next sample. The Orbitrap mass spectrometer was operated in FTMS (Fourier Transform Mass Spectrometry) full-scan mode with a mass range of 50-1000 m/z and high-mass resolution (60000). The resolution and sensitivity of the spectrometer were monitored by injecting a standard of caffeine after every10 samples, and the resolution was further monitored with lock masses (phthalates). All samples were injected twice, once with the HESI operating in positive ionization mode (+H) and once in negative ionization mode (-H). Blank samples were also analyzed during the sequence.

Metabolomic data processing:

NMR bucketing: The processing of ^{1}H NMR spectra is detailed in Rivas-Ubach et al. 2013a. First, all ^{1}H NMR spectra were phased, baseline corrected and referenced to the resonance of the internal standard (TSP) at δ 0.00 ppm with TOPSPIN 3.1. A variable-size bucketing was thus applied to all ^{1}H NMR spectra with AMIX software (Bruker Biospin, Rheinstetten, Germany), scaling the buckets relative to the internal standard (TSP). The output was a data set containing the integral values for each assigned ^{1}H NMR spectral peak in the described pattern. The buckets corresponding to the same molecular compound were summed.

LC-MS chromatograms: The raw data files from the spectrometer were processed by MZmine 2.10 (Pluskal et al. 2010). Chromatograms were baseline corrected, deconvoluted, aligned, filtered and finally the numerical database was exported in CSV format (see Table S3 of Appendix 5 for details). Metabolites were assigned by exact mass and retention time from the measurements of the standards in the spectrometer (see Table S4 of Appendix 5 for details). The different assigned variables corresponding to the same molecular compounds were summed. The numerical values of the variables extracted from the LC-MS chromatograms correspond to the absolute peak areas of the chromatograms detected by the spectrometer. The area is directly proportional to the concentration of the variable, so we use the term *concentration* in this article when referring to changes in the amount of metabolites among treatments.

Statistical analyses

To test for differences between treatments in cyanobacteria, the LC-MS and NMR metabolomic fingerprints were subjected to PERMANOVA analysis (Anderson et al. 2008) using the Bray Curtis distance, with treatment as fixed factor.

Additionally, to understand how the cellular stoichiometry and the metabolome of *Synechocystis* shifted with the treatments, the elemental stoichiometry and metabolomic fingerprints were also subjected to principal component analysis (PCA). The score coordinates of the samples were subjected to one-way ANOVAs to find statistical differences among groups (See Supporting Information of Rivas-Ubach et al. 2013a). A Kolmogorov-Smirnov (KS) test was performed on each variable to test for normality. All assigned and identified metabolites were normally distributed, and any unidentified metabolomic variable that was not normally distributed was removed from the data set. The PERMANOVA, PCAs and KS tests were performed with R (R Development Core Team 2011), *adonis* function of *vegan* package was used for PERMANOVA analyses setting the permutations at 1000, PCAs were performed by *pca* function of miXomics package and the *ks.test* function of the *tuncogof* package was used for KS tests. Statistica v8.0 (StatSoft, Tulsa, OK, USA) was used for the one-way ANOVAs and the post-hoc tests of the individual variables and the score coordinates of the PCAs.

Results

Elemental, stoichiometric and metabolomic shifts of nutrient starved cyanobacteria.

The PERMANOVA analysis performed with metabolomic, macromolecules and stoichiometric data indicated that the different treatments grouped by biological macromolecule compositions, stoichiometries, and metabolomes (whole model: $p < 0.001$, $F = 12.071$, $R^2 = 0.62$; Complete vs. Fe-Lim: $p<0.001$, $F = 16.076$, $R^2 = 0.62$; Complete vs. P-Lim: $p<0.001$, $F = 5.94$, $R^2 = 0.37$).

The PCAs of Complete vs Fe-Lim and Complete vs P-Lim cyanobacteria including all the elemental, stoichiometric and metabolomic data (Figure 1), indicated that almost 70% of the total variance was already gathered by the first four PCs in both Fe-Lim (66.8%) and P-Lim (68.8%). One-way ANOVAs of the score coordinates of cases showed differences PC1 values between treatments (Complete vs Fe-Lim: $p<0.001$ and $F = 97.73$; Complete vs P-Lim $p<0.001$ and $F = 117.71$).

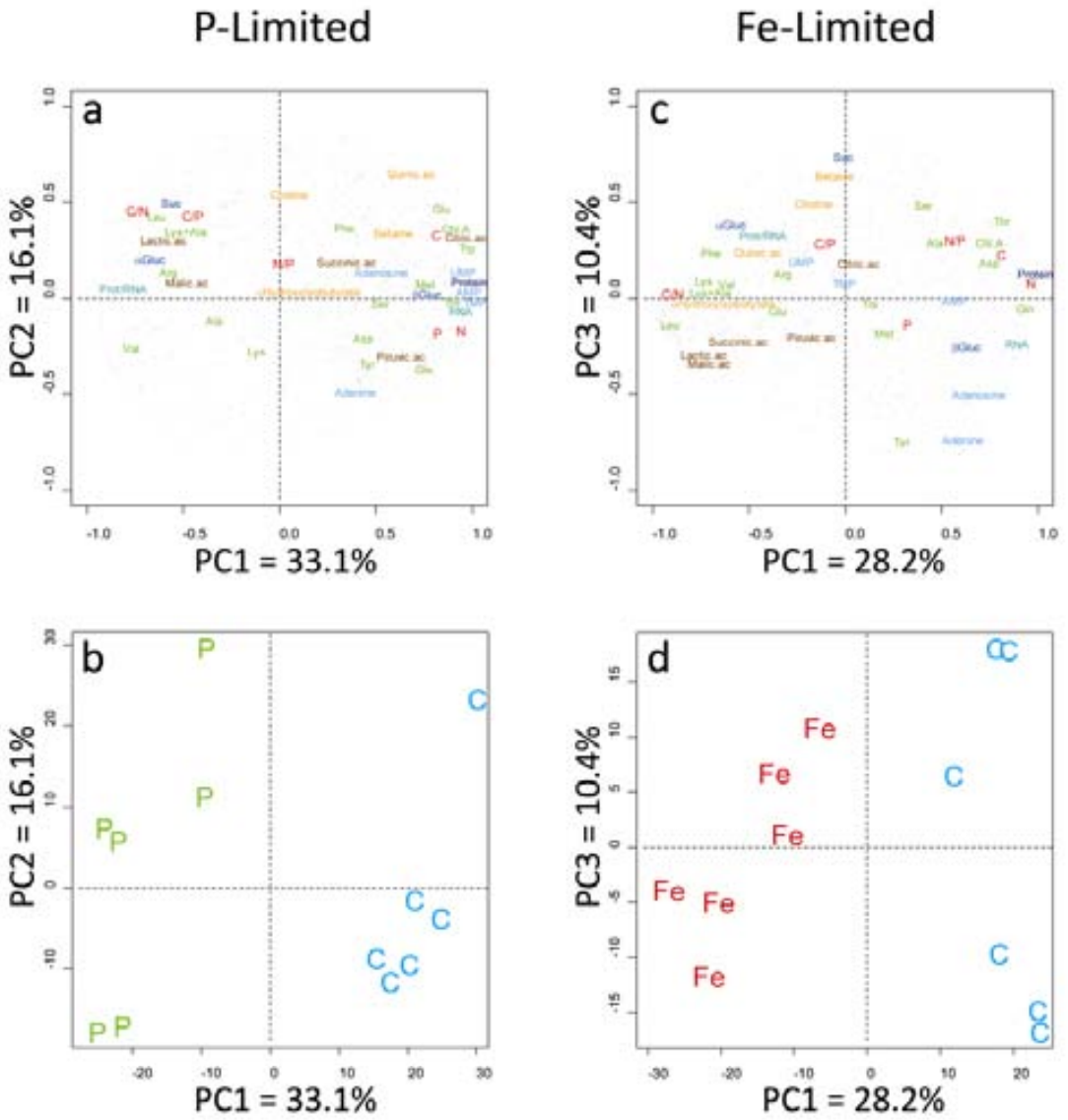

Figure 1. PC1 versus PC2 of the PCA of the elemental, stoichiometric and metabolomic variables in *Synechocystis sp.* PCC 6803. Panels **a** and **c** show the stoichiometric and metabolomic variables of Complete vs. P-Lim and Complete vs. Fe-Lim respectively. C:N:P ratios shown in red. Colors indicate different metabolomic families: blue: carbohydrates; darkblue: protein; green: amino acids; cyan: nucleotides; brown: organic acids; turquoise: RNA and Prot/RNA ratio; orange: other secondary metabolites. Abbreviations have been used for metabolites: chlorophyll *a* (Chl.A); sucrose (Suc), α-glucose (αG), β-glucose (βG), aspartic acid (Asp), lactic acid (Lac), succinic acid (Succinic.ac), citric acid (Citric.ac), pyruvate (Pyruvic.ac), malic acid (Malic.ac), alanine (Ala), threonine (Thr), valine (Val), leucine (Leu), phenylalanine (Phe), arginine (Arg), tryptophan (Trp), tyrosine (Tyr), glutamine (Gln), glutamate (Glu), methionine (Met), lysine (Lys), serine (Ser), and quinic acid (Quinic.ac). Unassigned metabolites are represented by small grey dots. Panels **b** and **d** show the samples categorized by treatment (blue: Complete; green: P-Lim; red: Fe-Lim) of Complete vs. P-Lim and Complete vs. Fe-Lim respectively.

The C, N, and P concentrations and the C:N ratio differed among treatments (Figure 1; Table S5 of Appendix 5). Complete cultures had the highest concentrations of C, N, and P and the lowest C:N even though P concentrations in complete cultures did not vary significantly from the P-Lim treatment (Table S5 of Appendix 5). C:P and N:P ratios did not vary significantly between treatments (p>0.05), although the complete treatment presented the lowest C:P ratios. Clearly, complete cultures had also the highest concentrations of RNA and Protein (p<0.001) and the lowest Protein:RNA ratio (p<0.01) (Figure1; Table S5 of Appendix 5).

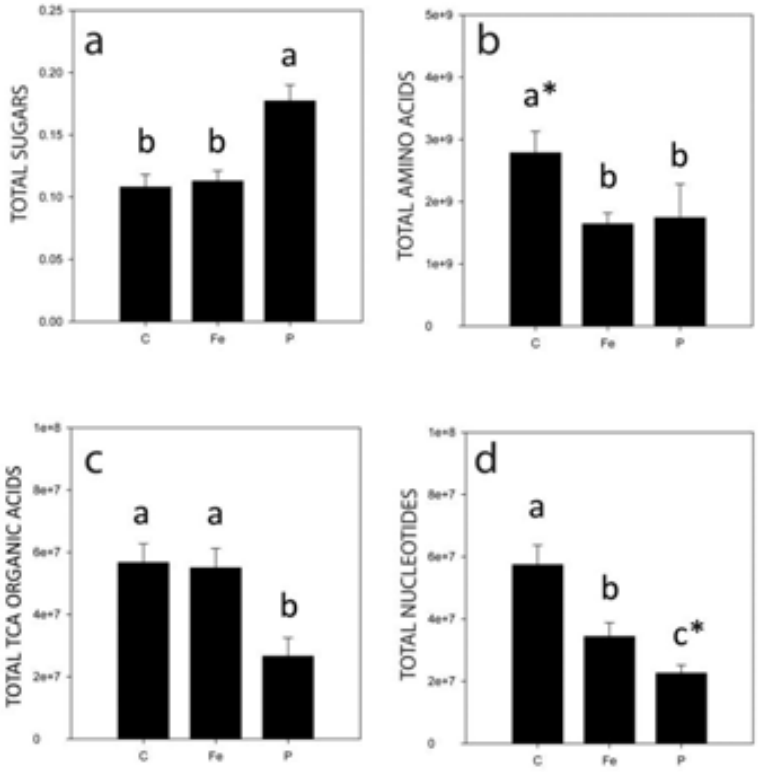

Figure 2. Bar graphs of the total cellularconcentrations of carbohydrates (**a**), amino acids (**b**), organic acids (**c**), and nucleotides, nucleosides, and nucleobases (Nucleotides) (**d**) ±SE (n=5). Treatments are indicated by different letters; Complete (C), Fe-Lim (Fe) and P-Lim (P). The statistically significant differences between treatments were detected by LSD-Fisher post-hoc tests (p<0.05). Statistical differences between treatments are indicated by different letters. Asterisks represent marginally statistical difference (p<0.1), for total amino acid concentrations; Complete vs. Fe-Lim (p = 0.053) and Complete vs. P-Lim (p = 0.073), for total nucleotide concentrations; Fe-Lim vs. P-Lim (p = 0.096).Y axis represents the c*oncentration* of total metabolites (explained in detail in Material & Methods section).

space defined by the PCAs (Figure 1), the concentrations of the assigned and identified variables of the different metabolite families (sugars, amino acids, organic acids of the trycarboxylic acid cycle (TCA cycle) and nucleotides, nucleosides and nucleobases (Nucleotides)) were summed

(Figure 2). The integral values of ^{1}H NMR spectra relative to the internal standard were standardized to sum with the peak area of the LC-MS chromatograms. One-way ANOVAs indicated that P-Lim cyanobacteria presented higher concentrations of carbohydrates (p<0.001) (Figure 2a). Complete cultures had the highest concentrations of amino acids even though the differences were only marginally significant (Complete vs Fe-Lim: p=0.053; Complete vs P-Lim: p=0.075) (Figure 2b). P-Lim cells showed the lowest concentrations of organic acids of the TCA cycle (p<0.01) (Figure 2c). The concentrations of nucleotides varied between all treatments. Complete cultures presented the highest concentrations of nucleotides that were significantly different from Fe-Lim (p<0.001) and P-Lim (p<0.01) (Figure 2d).

Discussion

Our analyses showed a clear shift in the stoichiometry and metabolome of *Synechocystis sp*. PCC 6803growing under P or Fe limitation (Figure 1; PERMANOVA p < 0.001). The relationship between the shifts in the stoichiometry of organisms with the shifts in their metabolomes has been recently demonstrated in plants (Rivas-Ubach et al. 2012). In this study, there were also several clear relationships among molecular compounds and the stoichiometry of *Synechocystis*. Complete cultures showed higher concentrations of N, RNA, and protein than Fe and P limited cyanobacteria. Proteins are N-rich macromolecules and in photosynthetic organisms almost 50% of the cellular N is in RubisCO (Spreitzer and Salvucci 2002). Moreover, metabolomic analyses showed that complete cultures also had the highest concentrations of total amino acids (Figure 2b), the molecular subunits of proteins, coinciding with past studies in plants (Rivas-Ubach et al. 2013a; Rivas-Ubach et al. 2013b). Even so, the changes in total concentrations of amino acids between complete and nutrient starved cyanobacteria were marginally significant (Figure 2b). The biosynthesis pathways of amino acids are quite complex since not all amino acids are synthesized from the same pathway and the different treatments showed different concentrations of the detected amino acid species showing no clear patterns (Figure 1). The P-richest macromolecular compounds in cells are DNA and RNA, especially rRNA that is necessary for translation of mRNA into proteins (Mathews et al. 2000). Our stoichiometric results showed that complete cultures had the highest concentrations of P and also of nucleobases (Adenine), nucleosides (Adenosine) and nucleotides (AMP, UMP and TMP), the molecular subunits of RNA, even though the concentrations of UMP and TMP did not changed significantly between complete and Fe-Lim (Figure 1; Table S5 of Appendix 5).

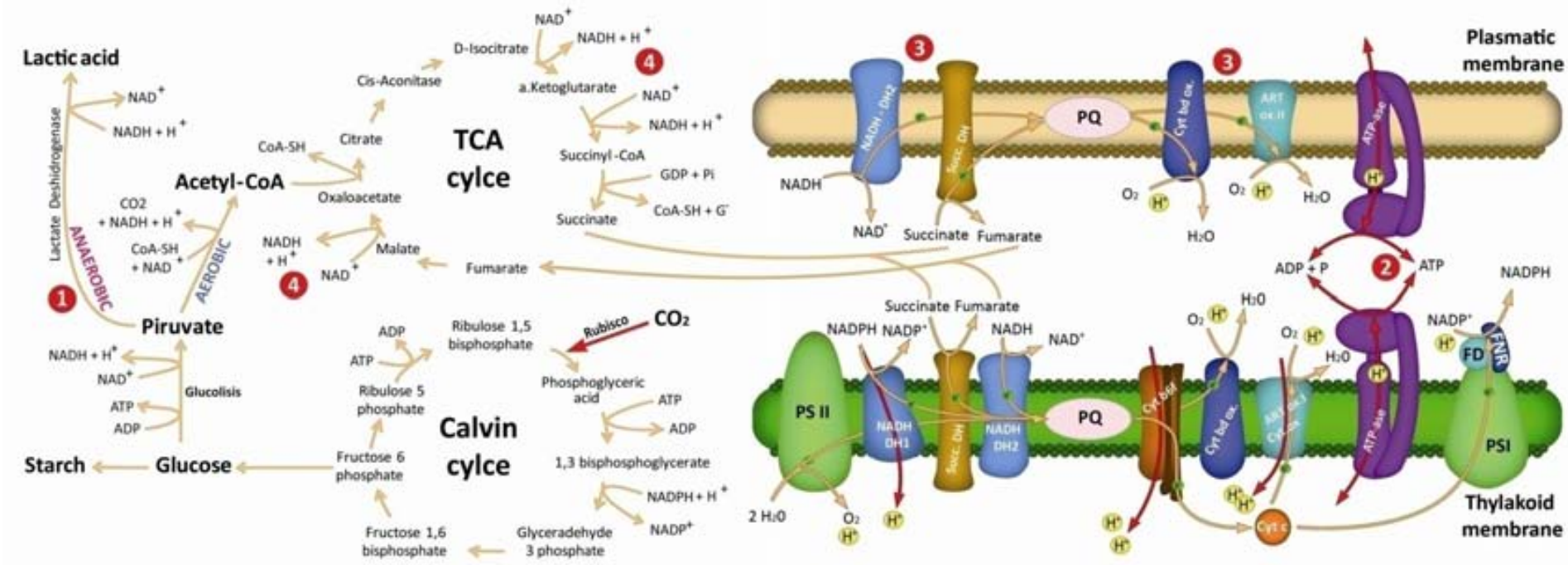

Figure 3**.** Schematic representation of both respiratory and photosynthetic electron transport chain (RETC and PETC respectively) and their connections to the metabolism of carbohydrates and energy production. Different numbers in red circles indicate different critical points of the figure discussed in the main article. RETC and PETC are modified from Schultze et al. 2009.

Phosphorus and Fe limited *Synechocystis* showed some common shifts in the stoichiometry and metabolism. Complete cultures had the highest concentraton of RNA and Protein and the lowest Protein:RNA ratios. As expected in the frame of the growth rate hypothesis (GRH) (Elser et al. 1996), high protein contents are required in rapidly growing organisms and such high protein content are supported by higher levels of RNA, especially rRNA, for translation processes. The GRH tries to explain the growth rate of organisms through the N:P biomass ratios (Elser et al. 2000; Rivas-Ubach et al. 2012) without taking into account other factors such as nutrient deprivation stresses. For example it has been suggested that GRH cannot be directly related to N:P biomass ratios in nutrient-poor aquatic ecosystems as a consequence of different patterns ofallocation to protein, RNA and other molecular compounds (Vrede et al. 2004). Our study shows that the lowest N:P ratio did not correspond to rapid growth of *Synechocystis* growing under complete media (Figure 1) as would be expected. It is explained mainly by the considerable reduction of N uptake of cells under P and Fe limitation (Figure 1: Table S5 of Appendix 5). Moreover, metabolomic analyses showed a reduction of amino acids involved in the N uptake as Glutamine under Fe and P limitation (Figure 1; Table S5 of Appendix 5) (Osanai et al. 2013). The difference of N:P biomass ratios between P-Lim and Complete is lower than the difference between Fe-Lim and Complete (Figure 1). It can be explained by the considerable reduction of P uptake of P-Lim cyanobacteria respect to Fe-Lim cyanobacteria (Table S5 of Appendix 5). On the other hand, Complete presented the lowest C:N and C:P ratios although they had also the highest C concentrations (highest CO_2 fixation) showing the high proportions of N and P respect to C necessary for high growth rates. Our results showed that deprivation of P and Fe in *Synechocystis* resulted in a reduction of N:P biomass ratio due the decreases in N uptake but it is not directly related to increased growth rate of cells. It is in accordance with past studies suggesting that other factors than the N:P biomass ratios should be taken into account when extrapolating the GRH to organisms (Matzek and Vitousek 2009; Rivas-Ubach et al. 2013b).

P- and Fe-limited *Synechocystis* used different metabolic strategies to cope with nutrient starvation although both treatments appear to shift the metabolic pathways in *Synechocystis* from aerobic to fermentative pathways. Both Fe and P nutrient deprivation caused increases of lactic acid in cells (Figure 1). Lactic acid is produced from the fermentation of pyruvate via the enzyme lactate dehydrogenase (Xue and Yeung 1995) (Figure 3 (1)). Redox homeostasis in cells is required for sustained metabolism and growth in organisms (Vemuri et al. 2007). The anaerobic metabolic route allows oxidize the NADH to NAD+ produced in the Calvin cycle and glycolysis of glucose (Xue and Yeung 1995) helping thus cyanobacteria to maintain the $NADH/NAD^+$ ratio and the redox homeostasis avoiding the formation of the dangerous superoxide radical anions ($O_2^-\cdot$) (Beckman et al. 1990). The Fe-limited cultures cannot support all the protein complexes of the

respiratory electron transport chain (RETC) necessary for a rapid growth since all these complexes contain several Fe-S centers (Mathews et al. 2000). In those bacteria, most of the energy as ATP provided from the PETC by using an alternative iron economy pathway, that requires less iron, producing an electrochemical gradient by pumping protons (H^+) into the intra-membrane space and consequently obtaining ATP by ATP-synthases (Behrenfeld and Milligan 2013) (Figure 3 (2)). Nevertheless, the iron limitation also reduces the amounts of phycobilisomes, membranes and protein complexes of PETC (Sherman and Sherman 1983; Sandmann 1985) making the photosynthetic processes slower. The shift of aerobic to anaerobic metabolism of P-limited may be explained by the reduction of protein complexes in the RETC since cells need high concentrations of RNA to support protein synthesis and RNA is low in P-limited cells (Figure 3 (3)). As cyanobacteria grow, CO_2 fixation is required in the Calvin cycle to synthesize fructose-6-phosphate and glucose (Buchanan et al. 2000). The slower anaerobic metabolism of P-limited cells may explain the accumulation of pyruvate and sugars that can be mobilized to storage. Since NADH cellular production is mainly done in glycolysis and TCA cycle (Vemuri et al. 2007), our results suggest that both Fe-limited and P-limited cultures need to shift partially the aerobic to anaerobic metabolism since they cannot compensate the high NADH generation during the TCA cycle by the RETC and maintain the NADH/NAD^+ cellular ratio (Figure 3 (4)). This partial shift in metabolism of both P- and Fe-limited cultures contribute to slower growth rates since the efficiency of ATP production by anaerobic respiration (2 ATP produced in the glycolysis) is much poorer than the aerobic respiration (38 ATP per moleculue of glucose) (Mathews et al. 2000). The high contents of malic acid in both P-limited and Fe-limited cells, being more significant in Fe-Limited cells, may be explained by anapleurotic reactions where oxaloacetate is reduced to malate by NADH allowing also decreasing the concentrations of reducing agents in cells maintaining the internal homeostasis (Mathews et al. 2000; Owen et al. 2002).

The recent global change drivers such as the increasing N inputs that are able to decrease the P availability for cyanobacteria or the acidification of boreal lakes that could increase the oxidation of Fe^{2+} to Fe^{3+} hindering its uptake, are important factors that may produce a significant reduction of phytoplankton growth. This decrease of primary producers in aquatic ecosystems may lead thereafter to a change in trophic webs and biodiversity (Elser et al. 1996; Sterner and Elser 2002; Güsewell et al. 2005; Sardans et al. 2012a) and a further decrease in fixation of atmoshperic CO_2 (Sardans et al. 2012b). Since phytoplankton is an important sink of C and it is of special interest in global change studies, fertilizations with P and Fe demonstrated to increase phytoplankton growth and CO_2 fixation (Vadeboncoeur et al. 2001; Boyd et al. 2007). The present study shows that metabolomes of P and Fe limited cyanobacteria shifted respect to those

growing under complete nutrient requeriments showing a clear shift in metabolism from aerobic to anaerobic fermentation enhancing acid lactic production and slowing down cell growth. This study also makes a step forward in the understanding the metabolic mechanisms that phytoplankton uses to cope with P and Fe limitations, potential drivers of shifts in species composition and biodiversity of communities in aquatic ecosystems (Sterner et al. 1998) and of controls of ecosystem production and CO_2 sequestration (Boyd et al. 2007).

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General conclusions.

1. Metabolomics techniques are sensitive enough to detect the shifts of the metabolomes of organisms under different environmental conditions. It thus can be applied to ecological studies to make a step forward in the understanding of structure, function and evolution of organisms and ecosystems.

2. The different environmental conditions of the different seasons of the year in Mediterranean climate make plants to shift their metabolomes, especially in the most critical seasons; summer and spring.

3. Since most bioelements as C, N and P do not actuate as themselves in organisms but as molecular compounds, there is a relationship between the shifts of foliar C:N:P:K and the shifts of metabolomes.

4. Mediterranean plants under drought conditions tend to present higher levels of foliar K to prevent water losses. This trend is most common in summer drought than in experimental induced droughts. These shifts in foliar K sotichiometry were also accompanied by higher levels of phenolic compounds with antioxidant function and other osmoregulants such as sugars or choline.

5. *Erica multiflora* shrub presented higher foliar concentrations of sugars, amino acids and other intermediate compounds related to growth and energetic metabolism in spring, the growing season. These increments of compounds of the primary metabolism were accompanied by lower foliar N:P ratios as expected in the frame of the growth rate hypothesis.

6. The growth rate hypothesis seems to be fulfilled in shrubs but not totally in big trees with large wood structures that act as a reservoir of nutrients such as lignotubers. There are several factors to take into account in terrestrial organisms that make the relationship between low N:P body ratios and growth rate less immediate as in aquatic ecosystems as the skeleton, the storage necessity, the defense compounds or other mechanisms of stress avoidance.

7. Induced shifts of foliar metabolomes of plants produced by drought stimulate the folivory activity. It could have an indirect impact on trophic webs by shifting the amount of herbivory through foliar stoichiometric and metabolomic shifts.

8. The P and Fe limited cyanobacteria showed alterated metabolomes showing a clear shift in metabolism from aerobic to anaerobic fermentation enhancing acid lactic production and slowing down cell growth. The reduced growth of phytoplankton may drive to shift the species composition and biodiversity of communities in aquatic ecosystems.

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Aquesta tesi podria arribar a tenir un sisè capítol només amb els agraïments. Han sigut uns anys difícils, probablement he afrontat els reptes més complicats que se m'han creuat mai pel camí, com ara la tesi, però tot i així han estat uns anys plens d'il·lusió i experiències que no podrien haver sigut millors sense tota la gent que m'envolta i m'ha donat suport.

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APPENDIX

APPENDIX 1

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Table S1. Results of the studies on the effects of atmospheric [CO_2] increases on biomass C:N:P. Some N:P, C:N, and C:P ratios were estimated using the reported C, N, and P concentrations (articles marked with two asterisks **).

Factor and species and/or communities studied	CO_2 enrichment	Main results	Reference
Pinus taeda	FACE experiment	- C:N litter	Allen et al. 2000
Soil study of agricultural lands	FACE experiment	- C:N microbial biomass	Anderson et al. 2011
Carex sp. grassland	Chambers	- C:N litter	Arnone and Hirschel 1997
Triticum aestivum	Glasshouse	- C:N grain	Bai et al. 2003
Spartiuna patens, Scirpus olneyi	Fiel based mesocosm	↑ C:N litter (*S. olneyi)* - C:N Litter (*S. patens)*	Ball and Drake, 1997
Raphanus sativus	Phytotron	↑ C:N roots - C:N shoots ↓ [P] roots - [P] shoots - N:P roots ** ↑ N:P shoots **	Barnes et al,. 1992
Emiliana huxleyi (plankton)	Enrichment by bubbling CO_2	↑ C:N plankton biomass ↑ C:P plankton biomass - N:P plankton biomass	Bellerby et al. 2008
Erica arborea	Natural CO_2 spring	↓ [N] leaves (when N is limiting)	Bettarini et al. 1995
Review study about insect-plant interactions	Review study	↓ [N] leaves	Bezemer and Jones, 1998
Larrea tridentate, Ambriosia dumosa, Lycium andersonii, Lycium pallidum	FACE experiment	↑ C:N leaves (*L. andersonni* and *L. pallidum*) - C:N leaf litter (all species)	Billings et al. 2003
Soil study of a *Pinus taeda* forest	FACE experiment	↑ C:N mineralized organic matter - C:N bulk soil organic matter	Billings et al. 2005
Lepidium latifolim	Glasshouse	↑ C:N aboveground biomass ↓ [P] aboveground biomass ↓ N:P aboveground biomass **	Blank and Derner, 2004 (*)
Rumex obtusifolius	Chambers	↑ C:N leaves	Brooks and Whittaker, 1998 (*)
Soil study	Chambers	- C:N plant roots - C:N organic matter	Brown et al. 2007
Populus tremuloides	Chambers	↓ [P] plant biomass ↓ [N] plant biomass	Brown, 1991
Soil study under *Pinus sylvestris* seedlings	Chambers	↑ C:N lower horizons - C:N upper horizons	Carnol et al. 2002
Populus sp.	Chambers	↑ C:N all aboveground organs	Ceulemans et al. 1995
Raphanus sativus	Chambers	↓ [N] leaves	Chu et al. 1992
Avena fatua, Plantago erecta	Microcosms	- C:N shoots and roots (both species)	Chu et al. 1996
Lindera benzoin	Chambers	↓ [N] leaves and stems	Cipollini et al. 1993
Abutilon theophrasti, Amaranthus retroflexus	Chambers	↓ [N] leaves (both species) ↓ [N] roots and stems (*A. theophrasti* and not clear for *A. retroflexus)*	Coleman and Bazzaz, 1992
Abutilon theophrasti, Amaranthus retroflexus	Chambers	↓ [N] plant biomass (all species)	Coleman et al. 1993
Betula pendula	Microcosms	↑ C:N leaf litter	Cotrufo and Ineson 1996
Betula pendula, Picea sitchensis	Microcosms	↑ C:N root litter (both species)	Cotrufo and Ineson, 1995
Fraxinus excelsior, Betula pubescens, Acer pseudoplatanus, Picea sitchensis	Microcosms	↑ C:N leaf litter (all species)	Cotrufo et al. 1994
Review of 75 published studies on terrestrial plants previous to 1998	Review study	↓ [N] aboveground biomass	Cotrufo et al. 1998
Quercus pubescens	Natural CO_2 spring	↓ [P] leaf litter - [N] leaf litter	Cotrufo et al. 1999

Castanea sativa	Chambers	↑ C:N litter	Coûteaux et al. 1991
Castanea sativa	Chambers	↑ C:N litter	Coûteaux et al. 1996
Populus grandidentata	Chambers	↓ [N] leaves	Curtis et al. 1992
Populus x euramericana	Chambers	↓ [N] leaves	Curtis et al. 1995
Lolium perenne	FACE experiment	↓ [N] aboveground biomass	Daepp et al. 2000
Lolium perenne	FACE experiment	↓ [N] aboveground biomass and roots	Daepp et al. 2001
Spartina patens, Scirpus olneyi	Chambers	↑ [N] shoots (*S. patens*) - [N] roots (*S. patens*) ⩛ [N] shoots (*S. olneyi)* ↓ [N] roots (*S. olneyi)*	Dakora and Drake, 2000
Quercus ilex, Phillyrea angustifolia, Pistacia lentiscus	Chambers	↑ C:N leaf litter (all species)	De Angelis et al. 2000
Trifolium repens, *Lolium perenne* and soil study	FACE experiment	↑ C:N aboveground biomass (*L. perenne*) - C:N aboveground biomass (*T. repens*) ⩚ C:N soils	de Graaff et al. 2004 (*)
Triticum aestivum	Chambers	↓ [N] leaves, aboveground biomass	Del Pozo et al. 2007
Soil study (Luvisol)	FACE experiment	↑ C:N organic matter	Dorodnikov et al. 2011
Sciprus olneyi	Chambers	↓ [N] plant tissues	Drake, 1992
Euclayptus tetrodonta, Eucalyptus miniata	Chambers	↓ [N] leaves (*E.tetrodonta*) - [N] leaves (*E miniata*)	Duff et al. 1994
Castanea sativa	Chambers	↓ [N] aboveground biomass and roots	El Kohen et al. 1992
Acer saccharum, Tsuga canadensis	Chambers	↑ C:N leaves (both species)	Eller et al. 2011 (*)
Betula pendula	Chambers	⩚ C:N litter ⩛ [N] leaves	Esmeijer-Liu et al. 2009
Betula pendula	FACE experiment	↓ C:N litter ↑ C:P litter ↑ N:P litter	Ferreira et al. 2010
Plankton communities	Review study	↑ C:N phytoplankton biomass ↑ C:P phytoplankton biomass	Finkel et al. 2010
Pinus taeda	FACE experiment	↑ C:N needles ⩛ N:P needles	Finzi et al. 2004
Cercis canadensis, Cornus florida,Acer rubrum, Liquidambar styraciflua, Pinus taeda	FACE experiment	↓ [N] leaves (*C. canadensis, C. caflorida, A. rubrum, L. styraciflua*) - [N] leaves (*P. taeda*) ↓ [N] litter (*C. florida, A. rubrum, L.styraciflua*) - [N] litter (*C. canadensis, P. taeda*) ⩛ [P] leaves (*C. canadensis, C. caflorida, L.styraciflua*) - [P] leaves (*A. rubrum, P. taeda)* ⩚ [P] litter (all species) ↓ N:P litter (all species) ** ↓ N:P leaves (*P. taeda*) ** - N:P leaves (*C. canadensis, C florida, A. rubrum, L. styraciflua*) **	Finzi et al. 2001
Lolium multiflorum, Avena fatua	Chambers	↑ C:N roots (*L. multiflorum*) ↓ C:N aboveground biomass (*A. fatua)*	Franck et al. 1997 (*)
Synechococcus, Prochlorococcus (marine cyanobacteria)	Enrichment by bubbling CO_2	↓ C:N biomass (*Synechococcus*) ↑ C:P biomass (*Synechococcus*) ↑ N:P biomass (*Synechococcus*) - C:N biomass (*Prochlorococcus*) ⩚ C:P biomass (*Prochlorococcus*) ⩚ N:P biomass (*Prochlorococcus*)	Fu et al. 2007
Heterosigma akashiwo, Prorocentrum minimum (marine cyanobacteria)	Enrichment by bubbling CO_2	↑ C:N biomass (*H.akashiwo*) ↑ C:P biomass (*H.akashiwo*) - C:N biomass (*P.minimum*) - C:P biomass (*P.minimum*)	Fu et al. 2008

		- N:P biomass (*H. akashiwo* and *P.minimum)*	
Quercus cerris, Quercus pubescens	Natural CO_2 spring	- C:N litter (both species)	Gahrooee, 1998
Abutilon theophrasti, Ambrosia artemisiifolia, Amaranthus retroflexus, Chenopodium album, Setaria faberii	Chambers	↓ [N] leaves (all species)	Garbutt et al. 1990
Data from several studies of terrestrial ecosystems previous to 2000	Review study	↑ C:N general trend in plant biomass No conclusive results regarding C:P	Gifford et al. 2000
Soil organic matter study	Chambers	↑ C:N soil organic matter	Gill et al. 2002
Litter soil study *Solanum dimidiatum, Riochloa ischaemum*	Chambers	↑ C:N litter soil organic matter	Gill et al. 2006
Lolium perenne, Agrostis capillaries, Festuca ovina	Chambers	↑ C:N leaves and aboveground biomass	Gorissen and Cotrufo, 2000
Pinus ponderosa	Chambers	- C:N leaves and aboveground biomass	Griffin et al. 1996a
Pinus taeda, Pinus ponderosa	Glasshouses	↑ C:N leaves (both species)	Griffin et al. 1996b
Lolium perenne, Trifolium repens	FACE experiment	↓ [N] aboveground biomass (both species)	Hartwig et al. 2000
Lolium perenne, Trifolium repens	FACE experiment	↓ [N] aboveground biomass (both species) - [N] roots (both species)	Hartwig et al. 2002
Seven subtropical grasses	Microcosm experiment	↑ C:N leaves - C:P leaves (six species) ↑ C:P leaves (*Alloteropsis semialata*)	Hattas et al. 2005 (*)
Fagus sylvatica, Picea abies	Chambers	↓ [N] leaves (*F. sylvatica*) ↓ [N] wood and shoot (*P. abies*)	Hattenschwiler et al. 1999
Phytolacca americana	Chambers	↑ C:N seeds	He et al. 2005
Sphagnum magellanicum, Eriophorum angustifolium	Glasshouses	↓ [N] aboveground biomass (both species) ↓ [P] aboveground biomass (both species) ↓ N:P aboveground biomass **	Heijmans et al. 2002
Glycine max, Sorghum bicolor	Chambers	↑ C:N aboveground biomass(both species)	Henning et al. 1996
Different plant species from Tropical, temperate grassland and alpine *Carex curvula* ecosystems	Chambers	- C:N leaf litter, Tropical and Temperate ecosystems ↑ C:N litter *(C. curvula,* alpine ecosystem)	Hirschel et al. 1997
Tritricum sp.	Glasshouses	↓ [N] stems and leaves	Hocking and Meyer, 1991
Betula pendula, Alnus glutinosa, Fagus sylvatica	FACE experiment	↓ [N] leaves (all species)	Hoosbeek et al. 2011
Avena fatua, Bromus hordeaceus, Lolium multiflorum, Plantago erecta, Vulpia microstachys	Chambers	↑ C:N leaves (*A. fatua, B. hordeaceus, P. erecta, V. microstachys*) - C:N leaves (*L. multiflorum*) ↑ C:N shoots (*B. hordeaceus, L. multiflorum P. erecta, V. microstachys)* - C:N shoots (*A. fatua)* ↑ C:N plant biomass (all species)	Hungate et al. 1996
Avena barbata, Bromus hordaceaus, Nassela pulchra, Lotus wrangelianus	Chambers	↑ C:N roots (all species)	Hungate et al. 1997
Two Mediterranean grasslands	Chambers	- [N] litter	Hungate et al. 1997
Trichodesmium (cyanobacteria from Atlantic and Pacific oceans)	Enrichment by bubbling CO_2	↑ N:P cyanobacteria (Atlantic and Pacific) ↓ C:N cyanobacteria (Pacific) - C:N cyanobacteria (Atlantic)	Hutchins et al. 2007
Bromus rubens	Chambers	↑ C:N seeds	Huxman et al. 1998 (*)
Labisia pumila	Chambers	↑ C:N leaves	Ibrahim and Jaafar, 2011a (*)
Emiliana huxleyi (*plankton)*	Enrichment by bubbling CO_2	- C:N plankton biomass	Iglesias-Rodriguez et al. 2008
Avena fatua, Bromus hordeaceus, Lolium multiflorum, Plantago erecta, Vulpia microstachys, Lasthenia californica	Chambers	↑ C:N roots (all species)	Jackson and Reynolds, 1996
Grassland with *Andropogon gerardii* and *Sorhastrym nutans* as dominant vegetation	Chambers	↑ C:N litter - C:N roots	Jastrow et al. 2000
Sphagnum fuscum, Sphagnum magellanicum, Sphagnum angustifolim, Sphagnum warnstorfii	Chambers	- N:P aboveground biomass (all species)	Jauhiainen et al. 1998 (*)

Artemisia tridentata	Chambers	↑ C:N leaves	Johnson and Lincoln, 1991
Trifolium repens	Chambers	↑ C:N leaves and roots	Johnson and McNicol, 2010 (*)
Pinus ponderosa	Chambers	↓ [N] plant biomass	Johnson et al. 1995
Soil study with *Pinus ponderosa* vegetation	Chambers	↑ C:N soil (Ap and Bw horizons)	Johnson et al. 2000
Soil microbial study	Chambers	- C:N soil microbial biomass	Johnson et al. 2002
Querucs geminate, Quercus myrtifolia	Chambers	↓ [N] leaves (both species) - [P] leaves (both species) - N:P leaves (both species) ** - [N] stems and roots (both species) - [P] stems and roots (both species) - N:P stems and roots (both species) **	Johnson et al. 2003
Liquidambar styraciflua	FACE experiment	↓ [N] leaves and litter - [N] roots - N:P leaves, litter and roots **	Johnson et al. 2004
Pinus ponderosa	Chambers	↑ C:N aboveground plant biomass	Johnson et al. 2006
Lolium perenne, Trifolium repens	FACE experiment	- [N] roots (both species)	Jongen et al. 1995
Pinus ponderoa	Chambers	↓ [N] leaves ↓ [P] leaves ↑ N:P leaves **	Jonhson et al. 1997
Phaseolus vulgaris	Greenhouses	↑ C:N leaves	Joutei et al. 2003
Salix myrsinifolia	Chambers	↓ [N] leaves	Julkunentiitto et al. 1993
Medicago sativa, Trifolium repens, Lotus corniculatus	-	- C:N leaves (all species)	Karowe and Migliaccio, 2011
Adropogon gerardii and soil study	Chambers	- C:N aboveground biomass ↑ C:N roots and soils	Kassem et al. 2008 (*)
Betula pendula	Chambers	↑ C:N leaf litter ↓ C:P leaf litter	Kasurinen et al. 2006
Populus tremuloides, Acer saccharum, Salix alba	Chambers	↑ C:N leaves (all species)	Kelly et al. 2010 (*)
Pinus densiflora	Chambers	↑ C:N leaves - C:N roots	Kim et al. 2011
Populus tremuloides	Chambers	- [N] litter	King et al. 2001
Populus tremuloides, Acer saccharum	Chambers	- C:N fine roots (both species)	King et al. 2005
Twelve hardwood species	FACE experiment	↑ C:N leaves	Knepp et al. 2005
Populus tremula x Populus alba hibrid	Glasshouses	↑ C:N leaves - C:N roots and stems	Kruse et al. 2003
Microbial soil study	FACE experiment	↑ C:N microbial biomass	Lagomarsino et al. 2006
Calluna vulgaris, Deschampsia flexuosa, mosses	FACE experiment	↑ C:N leaves (all species)	Larsen et al. 2011 (*)
Eucalyptus tereticornis	Glasshouses	↑ C:N leaves	Lawler et al. 1997 (*)
Thrichodesmium sp. (cyanobacteria)	Enrichment by bubbling CO_2	↑ C:N cyanobacteria	Levitan et al. 2007
Lycopersicon esculentum	Chambers	↓ C:N leaves and stems - C:N roots	Li et al. 2007
Triticum aestivum	Field manipulation	↑ C:N litter	Liao et al. 2002
Soils of *Pinus taeda* forest study	FACE experiment	- C:N organic soils ↑ C:N mineral soils	Lichter et al. 2008
Soil study in a *Pinus echinata* and *Pinus virginata* forest	FACE experiment	↑ C:N 0-15 cm depth Mineral soil - C:N 15-30 cm depth Mineral soil - C:N soil organic matter	Lichter et al. 2005
Populus tremuloides, Quercus rubra, Acer saccharum	Experimental rooms	↓ [N] leaves (*P. tremuloides, A. saccharum)* - [N] leaves (*Q. rubra*)	Lindroth et al. 1993
Betula papyrifera	Experimental rooms	↑ C:N leaves	Lindroth et al. 1995
Populus tremuloides	Glasshouses	↓ [N] leaves	Lindroth et al. 2001
Populus tremuloides	FACE experiment	↓ [N] leaves	Lindroth et al. 2002
Review study on forests	Review study	↑ C:N leaves	Lindroth, 2010

Ten plant species from tropical forest	Chambers	↑ C:N leaves	Lovelock et al. 1998 (*)
Metadata of 104 published studies on terrestrial plants previous to 2006	Review study	↑ C:N litter, soil, roots, aboveground biomass	Luo et al. 2006
Pinus sylvestris	Chambers	↓ [N] needles ↓ [P] needles ↓ N:P needles (not always significant)	Luomala et al. 2005
Danthonia richardsonii	Glasshouses	↓ [N] leaves	Lutze and Gifford, 1998
Danthomia richardsonii	Microcosms	↑ C:N senesced leaves, litter and roots	Lutze and Grifford, 2000 Lutze et al. 2000
Oryza sativa, Triticum aestivum	FACE experiment	↑ C:N plant biomass (both species)	Ma et al,. 2007 (*)
Juglans regia	Chambers	↑ C:N plant biomass	Maillard et al. 1999
Sinapis arvensis	FACE experiment	↑ C:N litter	Marhan et al. 2010
Scirpus olneyi, Spartina patens and soil study	Chambers	↑ C:N stems (*S. olneyi*) - [N] roots (*S. olneyi*) ↑ C:N stems (*S. patens*) ↓ [N] soil	Matamala and Drake, 1999
Fine-root study in *Pinus taeda* forest	FACE experiment	- C:N fine roots	Matamala and Schlesinger, 2000
Spartina densiflora	Greenhouses	↓ C:N leaves and roots	Mateos-Naranjo et al. 2010 (*)
Populus deltoides	Glasshouses	↓ [N] aboveground biomass	McDonald et al. 2002
Acer rubrum	FACE experiment	↑ C:N leaves	McElrone et al. 2005 (*)
Tritricum aestivum	Chambers	↓ [N] shoots	Mckee and Woodward, 1994
Festuca rubra	Chambers	↑ C:N leaves	Mevi-Schütz et al. 2003 (*)
Grassland with 88% biomass of *Bouteloua gracilis, Stipa comate, Pascopyrum smithii*	Chambers	- C:N roots	Milchunas et al. 2005
Sphagnum peatland	FACE experiment	↑ C:N leaves, litter ↑ C:P leaves - C:P litter	Milla et al. 2006
Vitis vinifera	Chambers	↑ C:N leaves	Moutinho-Pereira et al. 2009 (*)
Dry grassland community	Chambers	↑ C:N roots	Nagy et al. 2008
Oryza sativa	Chambers	↓ [N] leaves	Nakano et al. 1997
Agrostis capillaris	Chambers	- [N] leaves	Newbery et al. 1995
Soil microbial study of calcareous grassland	Greenhouses	- C:N microbial biomass	Nikalus, 1998
Soil microbial study of alpine grassland	-	- [N] microbial biomass	Niklaus and Körner, 1996
Calcareus grassland dominated by *Bromus erectus* (>98% no legume)	Greenhouses	↑ C:N aboveground (no legumes) - [P] aboveground (no legumes) - N:P aboveground (no legumes) ** - C:N aboveground (legumes) ↑ N:P aboveground (legumes) **	Niklaus et al. 1998
Soil microbial study	Greenhouses	- C:N microbial biomass	Niklaus et al. 2001
Liriodendron tulipifera	Chambers	↓ [N] leaves and roots ↓ [N] stems	Norby and Oneill, 1991
Quercus alba	Chambers	↓ [N] stems and roots ↑ C:N litter ↑ C:P litter	Norby et al. 1986a
Querucs alba	Chambers	↓ [N] roots, stems and leaves - [N] leaf litter	Norby et al. 1986b
Acer rubrum, Acer saccharum	Chambers	↓ [N] leaves and litter (both species)	Norby et al. 2000
Meta-analysis of data from senesced leaves in several field experiments	Review study	↑ C:N litter leaves	Norby et al. 2001
Solidago rigida, *Achillea millefolium*, *Amorpha canescens, Lespedeza capitata*, *Lupinus perennis*.	FACE experiment	↑ C:N leaves (all species) ↑ C:P leaves (all species) - N:P leaves (all species)	Novotny et al. 2007
Nephrolepsis exaltata	Chambers	↑ [N] leaves	Nowak et al. 2002

		- [P] leaves ↓ N:P leaves **	
Pseudotzuga menziessii	Chambers	↑ C:N leaves	Olszyk et al,. 2003 (*)
Trifolium repens, Trifolium pretense, Lolium perenne, Festuca pratensis	-	↓ [N] aboveground biomass (most species) - [P] aboveground (all species)	Overdieck, 1993
Andropogon gerardii, Sorghastrum nutans, Poa pratensis	Chambers	↓ [N] aboveground biomass (all species) Inconclusive results of [P]	Owensby et al. 1993
Austrodanthonia caespitosa, Austrodanthonia carphoides, Austrostipa mollis, Themeda triandra	FACE experiment	↑ C:N aboveground biomass (all species)	Pendall et al. 2011 (*)
14 plant species	Herbarium study	↑ C:N leaves	Peñuelas and Matamala, 1990
13 plant species	Herbarium study	↓ [P] leaves	Peñuelas and Matamala, 1993
12 plant species	Herbarium study	↑ C:N leaves	Peñuelas and Estiarte, 1997
Citrus aurantium	Chambers	↓ [N] leaves	Peñuelas et al. 1997
Erica arborea, Juniperus communis, Myrtus communis	Field gradient. Distances from natural CO_2 spring	↑ C:N leaves (*M. communis*) - C:N leaves (*E. arborea, J. communis*) ↑ [P] leaves (*E. arborea*) ↓ [P] leaves (*M. communis*) - [P] leaves (*J. communis*)	Peñuelas et al. 2001
Pinus sylvestris	Chambers	↓ [N] leaves	Pérez-Soba et al. 1994
Picea abies	Phytotron	↑ C:N needles ↓ [P] needles ↓ N:P needles ** ↓ [N] roots ↓ [P] roots ↑ N:P roots **	Pfirrmann et al. 1996
27 different plant species including, crop, herbaceous and woody species	Glasshouse and chambers	↑ C:N leaves; average of 27 species, in all cases	Poorter et al. 1997
Populus x euramericana	Chambers	↑ C:N leaves, stems and roots	Pregitzer et al. 1995
Commelina benghalensis	Chambers	↑ C:N aboveground biomass - C:N roots ↑ C:N plant biomass	Price et al. 2009
Zea mays	Chambers	↑ C:N leaves	Prins et al. 2011
Glycine max, Sorghum bicolor	Chambers	↑ C:N leaves (*S.bicolor*) ↑ C:N stems (*S.bicolor*) ↑ C:N roots (*G.max*) ↓ C:N roots (*S.bicolor*) - C:N leaves and stems (*G.max*)	Prior et al. 2004
Populus sp. and soil microbial study	-	- C:N leaf litter - C:N microbial biomass	Randlett et al. 1996
Ricinus communis	Chambers	↑ C:N leaves	Rao et al. 2009
Glycine max, Sorghum bicolor	Chambers	↓ [N] leaves (*G. max*) - [N] leaves (*S. bicolour*)	Reeves et al. 1994
Sixteen perennial grassland species	FACE experiment	↓ [N] aboveground biomass (average of C3 and legume plants) - [N] belowground biomass (average of C3 and legume plants) ↓ [N] aboveground and belowground biomass (average of forbs) - [N] aboveground and belowground biomass (average of C4 plants)	Reich et al. 2001
Plankton community	Mesocosm enclosures	↑ C:N plankton	Riebesell et al. 2007
Betula pendula	Chambers	↑ C:N leaves	Riikonen et al. 2005
Festuca vivipara	Chambers	↑ C:N aboveground biomass - C:N roots	Robinson et al. 1997 (*)
Trtricum sp, Gossypum sp.	-	↓ [N] leaves (both species)	Rogers et al. 1993

		↑ [P] leaves (both species) ↓ N:P leaves (both species) **	
Soil study; Rygrass/White clover turves of an endoaquept soil	Chambers	- [N] soil	Ross et al. 1995
Betula papyrifera, Pinus strobus	Experimental rooms	↓ [N] leaves (*B. papyrifera*) - [N] leaves (*P. strobus*)	Roth and Lindroth, 1994
Populus tremuloides, Betula payrifera, Quercus rubra, Acer saccharum	Experimental rooms	↓ [N] leaves (*P. tremuloides, B. papyrifera, A. saccharum*) - [N] leaves (*Q. rubra*)	Roth and Lindroth, 1995
Oryza sativa	Chambers	⇣ [N] leaves	Rowlandbamford et al. 1991
Pinus palustris	Chambers	↓ [N] leaves, stems and roots	Runion et al. 1999
Medicago sativa	Chambers	↑ C:N leaves	Sanz-Sáez et al. 2010 (*)
12 plant species	Chambers	↑ C:N leaves, most of the species	Schädler et al. 2007 (*)
Lolium perenne, Trifolium repens	Chambers	↓ [N] shoots (*L. perenne*) ↓ [N] roots (*T .repens*) - [N] roots (*L. perenne*) - [N] shoots (*T. repens*)	Schenk et al. 1995
Soil study of *Pinus taeda* forest	FACE experiment	- C:N forest floor and mineral soil	Schlesinger and Lichter, 2001
Triticum dicoccoides	FACE experiment	↑ C:N grain	Sinha et al. 2009
Holcus lanatus, Pennisetum clandestinum	Chambers	↑ C:N litter (both species)	Sowerby et al. 2000
Bromus erectus	Greenhouses	↑ C:N seeds	Steinger et al. 2000 (*)
Data providing of several studies of terrestrial ecosystems previous to 2003	Review study	↑ C:N leaves	Stiling and Cornelissen, 2007
Quercus myrtifolia, Quercus geminanta	Chambers	↓ [N] leaves (both species)	Stiling et al. 1999
Solanum dulcamara, Amaranthus viridis	Chambers	↑ C:N leaves (*S. dulcamara*) - C:N leaves (*A. viridis*)	Sudderth et al. 2005 (*)
Gossypum sp.	Chambers	↑ C:N phloem	Sun et al. 2009
Lycopersicum esculentum	Chambers	↑ C:N leaves	Sun et al. 2010
Citrus sinensis, Citrus aurantium	Glasshouse	- C:N roots and leaves (*C. sinensis*) ↓ C:N leaves (C. aurantium) - C:N roots (*C. aurantium*)	Syvertsen and Graham, 1999
Citrus volkameriana, Citrus sinensis	Greenhouse	↓ [N] leaves (both species) ↓ [P] leaves (both species)	Syvertsen et al. 2000
Gliricida sepium	Chambers	↓ [N] aboveground biomass	Thomas et al. 1991
Alnus hirsuta	Phytotron	↑ C:N litter ↓ [N] plant biomass	Tobita et al. 2005
Quercus ilex	Chambers	↑ C:N leaves - C:N stems and roots	Tognetti and Johnson, 1999a (*)
Quercus virginata	Chambers	↑ C:N plant biomass	Tognetti and Johnson, 1999b (*)
Erica arborea, Myrtus communis and Juniperus communis	Field gradient. Distances from natural CO_2 spring	↑ C:N leaves (*M.communis, J.communis*) - C:N leaves (*E.arborea*)	Tognetti and Peñuelas, 2003
Glycine max, Sorghum bicolor	Chambers	↑ C:N grain (both species) ↑ C:N plant biomass (*S.bicolor)*	Torbert et al. 2004
Green algae, Diatoms and Cyanobacteria study	Chambers	↑ C:N biomass (green algae, diatoms) - C:N biomass (cyanobacteria) ↑ C:P biomass (green algae, diatoms) ⇡ C:P biomass (cyanobacteria)	Urabe and Waki, 2009
Scenedesmus acutus	Chambers	↑ C:P biomass (*S. acutus*)	Urabe et al. 2003
Soil study of grassland	FACE experiment	- C:N soil	Van Groeningen et al. 2002
Oryza sp., Tritricum sp.	FACE experiment	↑ C:N litter (both species)	Viswanath and Purakayastha, 2010
Alnus glutinosa	Chambers	- [N] leaves	Vogel et al. 1995
Alnus glutinosa	Chambers	↑ C:N leaves and plant biomass	Vogel et al. 1997

		↗ C:N stems and roots	
Populus deltoides	Greenhouses	- C:N leaves	Wait et al. 1999
Populus pseudo-simonii, Betula platyphylla, Quercus mongolica	Chambers	↑ C:N leaves (all species)	Wang et al. 2009
Larrea tridentate, Lycium pallidum, Lycium andersonii	FACE experiment	↑ C:N litter (all species)	Weatherly et al. 2003
Pinus taeda	Chambers	↓ [N] leaves	Williams et al. 1994
Soil microbial study dominated by *Andropogon gerardii* and *Sorghastrum nutans*	Chambers	- C:N soil	Williams et al. 2000
Ficus insipida, Virola surinamensis	Chambers	↑ C:N leaves (both species)	Winter et al. 2000
Calluna vulgaris	Chambers	↑ C:N leaves ↑ [N] stems ↓ [P] leaves and stems	Woodin et al. 1992
Triticum aestivum	Chambers	↓ [N] grain ↓ [P] grain ↑ N:P grain **	Wu et al. 2004
Oryza sativa	FACE experiment	↑ C:N leaves	Xie et al. 2004
Caragana intermedia	Chambers	↑ C:N leaves, stems and roots	Xu et al. 2007
Populus tremuloides	Chambers	↓ [N] aboveground and belowground biomass	Zak et al. 2000
Trifolium repens	FACE experiment	↓ [N] aboveground biomass	Zanetti et al. 1996
Oryza sativa, Echinochloa crusgalli	FACE experiment	↑ C:N aboveground biomass (*O. sativa*) - C:N aboveground biomass (*E. crusgalli*)	Zeng et al. 2011 (*)
Populus cathayana	Chambers	↑ C:N leaves	Zhao et al. 2011 (*)
Onobrychis viciaefolia	Chambers	↑ C:N leaves, stems, aboveground biomass and plant biomass - C:N roots	Zhou et al. 2009
Bromus tectorum	Chambers	↑ C:N leaves	Ziska et al. 2005

↑ or ↓ Significant ($P < 0.05$) ↗ or ↘ Marginally significant trend ($P < 0.1$) - No significant effects

FACE-Free Air CO_2 Enrichment

Table S2. Results of the studies on the effects of eutrophication on the C:N:P stoichiometry of organisms and ecosystems.

Species and/or communities studies and pollutant sources	Study site	Main results	Reference
Atmospheric N deposition			
Sphagnum	Field	↓ C:N biomass	Aerts et al. 1992
Sub-artic shrublands	Field	↑ N:P water	Arbuckle and Downing, 2001
North American forest (Colorado)	Field	↑ N:P leaves ↓ C:N leaves	Baron et al. 2000
Natural and seminatural European terrestrial and freshwater ecosystems	Review	↓ C:N biomass and soils	Bobbink et al. 1998
North American forest (Apalaches)	Field	↓ C:N soil	Boggs et al. 2005
Sphagnum ecosystems	Field	↑ N:P biomass	Bragazza et al. 2004
Calluna vulgaris	Field	↑ N:P biomass	Britton and Fisher, 2007
Temperate forest	Field	↓ C:N soil	Brookshire et al. 2007
Quercus rubra and *Prunus serotina* forest	Field	↓ C:N stems and soil	Castro et al. 2007
Fagus sylvatica	Field	↑ N:P leaves	Duquesnay et al. 2000
Lakes	Field	↑ N:P whole ecosystem including water	Elser et al. 2009a
Lakes	Metaanalyses of published data	↑ N:P water	Elser et al. 2009b
Pinus sp and *Quercus* sp forest (California)	Field	↑ N:P leaves ↓ C:N leaves	Fenn et al. 1996
Noth American and Europe forests and water ecosystems	Metaanalyses of field data	↓ C:N ground water leaches ↑ N:P leaves	Fenn et al. 1998
Pinus hartwegii forest (Mexico)	Field	↑ N:P leaves ↓ C:N leaves	Fenn et al. 1999
German forest and ghrasslands	Field	↑ N:P biomass ↑ N:K biomass	Franzaring et al. 2010
North American Forest (Main)	Field	↓ C:N soil	Goodale and Aber, 2001
Lakes	Metaanalyses of field data	↑ N:P water	Hessen et al. 2009
Lakes	Field	↑ N:P water	Jassby et al. 1994
Dune ecosystems (United Kingdom)	Field	↑ C:N soil	Jones et al. 2004
Lake	Field	↓ C:N sediments	Li et al. 2008
Lakes	Field	↑ N:P water and organisms	Liess et al. 2009
Picea sp.	Field	↓ C:N soil	McNulty et al. 1991
Temperate forest	Metaanalyses of field data	↓ C:N soil	Nave et al. 2009
Populus tremula forest	Field	↓ C:N leaves	Nikula et al. 2010
Picea sp. forest (North America)	Field	↓ C:N leaves and soil	Pardo et al. 2007
Abies alba forest (France)	Field	↓ C:N soil	Pinto et al. 2007
Calluna vulgaris shrubland	Field	↓ C:N leaves	Power and Collins, 2010
Pinus sylvestris forest (Germany)	Field	↑ N:P needles	Prietzel and Stetter, 2010

Pinus sylvestris forests (Germany)	Field	↓ C:N soil	Prietzel et al. 2006
California dry grasslands	Field	↓ C:N soil	Rao et al. 2009
Quercus robur and *Quercus petrea* forest	Field	↑ N:P leaves	Thomas and Büttner, 1998
Acer saccharum forest	Field	↓ C:N soil	Watmough, 2010
North American forest (Colorado)	Field	↑ N:P leaves	Williams et al. 1996
Mosses	Field	↓ C:N leaves	Wilson et al. 2009
Soil fertilization			
Livestock excreta overfertilization	Field	↓ N:P soil	Cech et al. 2008
Croplands with intense fertilization	Field study	↓ C:P water ↓ N:P water	Frost et al. 2009
Pig slurry eutrophication effects Catalonia, NE (Spain)	Metaanalysis study	↓ N:P soil	Peñuelas et al. 2009
Croplands with intense fertilization	Field	↓ C:P water	Pilati et al. 2009
Pig slurry over fertilization effects (Spain)	Field	↓ C:N soil	Plaza et al. 2003
Water eutrophication			
Lakes	Field	↑ N:P water in cropland areas ↓ N:P in pasture areas	Arbuckle and Downing, 2001
Urban and crop waste loadings in a Estuary (Brazil)	Field	↓ C:N algal	Barrera-Alba et al. 2009
Urban and crop waste loadings in estuary (Turkey)	Field	↓ N:P water	Bizsel and Uslu, 2000
Urban and crop waste loadings in estuary (USA)	Field	↑ N:P water	Capriulo et al. 2002
Urban and crop waste loadings in estuary (China)	Field	↑ N:P water	Chai et al. 2006
Loadings from 2 estuaries in Chesapeake Bay	Field	↑ N:P water from Choptank river ↓ N:P water from Patuxent River	Fisher et al. 2006
Great river loadings	Review	↑ N:P water	Gächter et al. 2004
Urban and crop waste loadings in estuary (China)	Field	↑ N:P water	Harrison et al. 2008
Urban and crop waste loadings in coastal waters (Baltic sea)	Field	↓ C:N algal	Hillebrand and Sommer, 2000
Loadings from Rhine to sea (periode 1950-1985)	Field	↑ N:P water	Jickells, 1998
Increase of grazing and land use on *Sphagnum* sp, *Carex* sp and *Juncus* sp vegetation in lowlands of Black sea coast.	Field	↓ C:N vegetation	Kaffke, 2010
Urban and crop waste loadings in Florida everglades	Field	↓ C:N organisms ↓ C:P organisms	King and Richardson, 2007
Crop waste loadings in coastal ocean waters (Trinidad Tobago)	Field	↓ C:N water	Lapointe et al. 2010
Urban and crop waste loadings in estuary (China)	Field	↑ N:P waters ↑ N:Si waters	Li et al. 2010b
Urban and crop waste loadings in water reservoirs USA and (Spain)	Field	↓ C:N water	Marcé et al. 2008
Urban and crop waste loadings in estuary (France)	Field	↓ C:N sediments ↓ C:P sediments	Mesnage et al. 2002
Urban and crop waste loadings in estuary (Argentina)	Field	↑ N:P water	Nagy et al. 2002
Urban and crop waste loadings in some estuaries (England)	Field	↑ N:P waters	Nedwell et al. 2002
Urban and crop waste loadings in North sea from Belgium to Gemany (1977-2000)	Field	↑ N:P waters	Radach and Pätsch, 2007
Urban and crop waste loadings in	Field	↓ C:P soils	Reddy et al. 1999

Florida everglades			
Sewage treatment waste loadings in lakes Wales)	Field	↓ C:N sediments ↓ C:P sediments ↓ N:P sediments	Rees et al. 1991
Sewage treatment waste loadings in coastal waters (Brazil)	Field	↑ N:P water	Santos et al. 2008
Urban and crop waste loadings in a lake (Guatemala)	Field	↓ C:N sediments	Rosenmeier et al. 2004
Urban and crop waste loadings in estuary (Mexico)	Field	↓ C:P sediments ↓ N:P sediments	Ruiz-Fernández et al. 2007
Loadings from Changjiang river (China)	Field	↑ N:P waters	Shen and Liu, 2009
Urban and crop waste loadings in lakes (China)	Field	↓ C:N sediments	Shuncai and Chen, 2000
Great river loadings	Review	↑ N:P water	Turner et al. 2003
Mississippi River loadings in Gulf of Mexico	Field	↑ N:P water	Turner et al. 2006
N rich crop waste loadings in water streams	Field	↓ C:N organisms	Von Schiller et al. 2007
Urban and crop waste loadings in a water reservoir (Brazil)	Field	↑ N:P waters	Von Sperling and Souza, 2007
Sewage treatment waste loadings in water reservoirs (Brazil)	Field	↑ N:P water	Von Sperling et al. 2008
Urban waste loadings in coastal ocean waters (China)	Field	↓ C:N waters ↑ N:P waters	Wei and Huang, 2010
Urban and crop waste loadings in estuary (China)	Field	↑ N:P water	Yin and Harrison, 2007
Loadings from Pearl river (China)	Field	↑ N:P waters	Yin et al. 2004
Nitrogen fertilization experiments (realistic N deposition)			
Pinus edulis, Juniperus monospermum	Field fertilization	↑ N:P leaves (both species)	Allen et al. 2010 (*)
Carex curvula	Field fertilization	- N:P litter	Arnone and Hirschel, 1997
Pseudoscleropodium purum, Rhytiadelphus squarrosus	Field fertilization	↑ N:P aboveground biomass (*P. purum*) - N:P aboveground biomass (*R. squarrosus*)	Arróniz-Crespo et al. 2008 (*)
Quercus petraea	Greenhouse experiment	↑ N:P leaves, roots	Berger and Glatzel, 2001
Salicornia virginica	Field fertilization	↑ N:P aboveground biomass	Boyer et al. 2001 (*)
Sphagnum sp.	Greenhouse experiment	↑ N:P aboveground biomass	Breeuwer et al. 2009
Populus balsamifera, Picea glauca, soil study	Field fertilization	↓ C:N roots, soil microbial	Brenner et al. 2005
Calluna vulgaris	Field fertilization	↑ N:P aboveground biomass	Britton et al. 2008 (*)
Calamagrostis epigejos, Carex arenaria	Greenhouse experiment	↑ N:P roots (both species) ↑ N:P shoots (*C. arenaria*) ↑ N:P shoots (*C. epigejos*)	Can den Berg et al. 2005
Soil study (*Fagus grandiflora, Tsuga canadensis, Acer saccharum, Quercus rubra, Betla alleghaniensis*)	Field fertilization	- C:N soils (all species forests)	Christenson et al. 2009 (*)
Tree epiphylls, mosses and soils study	Field fertilization	- C:N tree epiphylls, mosses and soils ↓ C:N forest floor	Cusack et al. 2009
Molinia caerulea	Field fertilization and Greenhouse experiment	↑ [N] aboveground biomass (in 2006, field) - [N] aboveground biomass (in 2008, field) ↑ [N] aboveground biomass (greenhouse) - [P] aboveground biomass (in both 2008 and 2009, field) ↓ [P] aboveground biomass (greenhouse)	Falk et al. 2010
Pinus massoniana, Schima superba, Cryptocarya chinensis	Field fertilization	↓ C:N leaves (all species)	Fang et al. 2009 (*)
Soil and litter study (3 different ecosystems: *Acer saccharum-Tilia*	Field fertilization	- C:N litter, soils (all ecosystems)	Gallo et al. 2005 (*)

Americana, Acer saccharum-Quercus rubra, Quercus vetulina-Quercus alba)			
Sphagnum sp.	Field fertilization	↑ N:P aboveground biomass	Gerdol et al. 2007 (*)
Sphagnum sp.	Field fertilization	↑ N:P aboveground biomass	Gunnarsson and Rydin, 2000
Cladonia portentosa	Field fertilization	↑ N:P lichen biomass	Hogan et al. 2010
Soil study (*Pinus sylvestris*)	Field fertilization	↓ C:N organic soils - C:N mineral soils	Högbom et al. 2001 (*)
Labisia pumila	Greenhouse experiment	↓ C:N leaves	Ibrahim and Jaafar, 2011b (*)
Alectoria sarmentosa, Platismatia glauca	Field fertilization	↑ N:P lichen biomass (both species)	Johansson et al. 2010
Blepharocalyx salicifolius, Caryocar brasiliense, Qualea parviflora, Schefflera macrocarpa, Ouratea hexasperma	Field fertilization	- [N] leaves (*C. brasiliense, Q. parviflora, B. salicifolius, Q. hexasperma*) ↓ [N] leaves (*S. macrocarpa*) - [N] litter (*Q. parviflora, S. macrocarpa*) ↑ [N] litter (*C. brasiliense, B. salicifolius, O. hexasperma)* - [P] leaves (*C. brasiliense, Q. parviflora, B. salicifolius, Q. hexasperma*) ↓ [P] leaves (*S. macrocarpa*) - [P] litter (*C. brasiliense, Q. parviflora, B. salicifolius, Q. hexasperma*) ↓ [P] litter (*S. macrocarpa*)	Kozovitis et al. 2007
Acer mono, Betula platyphylla, Fraxinus mondschurica, Pinus koraiensis, Populus davidiana, Populus koreana	Field fertilization	↓ C:N leaves, litter (all species)	Li et al. 2010a (*)
Soil study (*Quercus rubra, Quercus prinus, Carya sp.*)	Field fertilization	- C:N mineral soils	Lovett et al. 2011 (*)
Acer rubrum, Liriodendron tulipifera, Prunus serotina	Field fertilization	↓ N:P leaves (*A. rubrum*) - N:P leaves (*L. tulipifera, P. serotina*)	May et al. 2005 (*)
Soil study (*Quercus velutina, Quercus alba, Pinus resinosa*)	Field fertilization	↓ C:N dissolved organic matter	McDowell et al. 1998
Soil study (*Quercus velutina, Quercus rubra, Pinus resinosa*)	Field fertilization	↓ C:N dissolved organic matter	McDowell et al. 2004 (*)
Fagus sylvatica	Field fertilization	↑ [N] leaves ↑ [N] litter - [P] leaves, litter	Meiwes et al. 1998
Avena sp.	Field fertilization	↑ N:P leaves, litter	Menge and Field, 2007
Pinus massoniana (disturbed forests and rehabilited forests)	Field fertilization	- C:N litter (disturbed forests) ↓ C:N litter (rehabilited forests) ↓ C:P litter (disturbed forests) ↓ C:P litter (rehabilited forests) ↓ N:P litter (disturbed and rehabilited forests)	Mo et al. 2007
Soil study (*Picea abies, Pinus sylvestris*)	Field fertilization	- C:N organic and mineral soils	Moldan et al. 2006
Solidago rigida, *Achillea millefolium*, *Amorpha canescens, Lespedeza capitata*, *Lupinus perennis*.	Field fertilization	↓ C:N leaves (all species) - C:P leaves (all species) - N:P leaves (all species)	Novotny et al. 2007
Phleum pratense	Field fertilization	↓ C:N aboveground biomass	Pan et al. 2010 (*)
Calluna vulgaris, Vaccinium myrtillus	Field fertilization	↓ C:N litter	Pilkington et al. 2005a
Calluna vulgaris	Microcosm experiment	↓ C:N litter	Pilkington et al. 2005b
Pinus sylvestris	Field fertilization	↑ N:P forest floor	Prietzel and Stetter, 2010
Pinus sylvestris	Field fertilization	↓ C:N forest floor - C:P forest floor	Prietzel et al. 2006
Soil study (*Pinus sylvestris*)	Field fertilization	↓ C:N organic soil	Sjöberg & Persson, 1998 (*)
Festuca ovina, Agrostis capillaris, Galium saxatile and soil study of the grassland	Field fertilization	- N:P aboveground biomass (all species) ↑ C:N O/A soil horizons	Stevens et al. 2006

Sphagnum fallax, Betula pubescens, Molinia caerulea	Microcosm experiment	↓ C:N aboveground biomass (*S. fallax*) ↑ N:P aboveground biomass (*S. fallax*) ↑ N:P leaves (*B. pubescens*) - N:P leaves (*M. caerulea*)	Tomassen et al. 2003 (*)
Molinia caerulea, Betula pubescens, Eriophorum vaginatum, Calluna vulgaris, Cladonia portentosa and soil study	Field fertilization	↓ C:N organic soil ↑ C:P organic soil ↑ N:P organic soil, leaves (*B. pubescens, E. vaginatum, C. vulgaris, C. portentosa*) - N:P leaves (*M. caerulea*)	Tomassen et al. 2004 (*)
18 grassland species (3 legumes, 5 Herbs, 9 Grasses)	Microcosm experiment	↑ N:P aboveground biomass (Herbs, grasses) - N:P aboveground biomass (Legumes)	van der Heijden et al. 2008
Calluna vulgaris	Field fertilization	↑ N:P aboveground biomass	von Oheimb et al. 2010 (*)
Two ecosystems soil study (Chaparral: *Adenostoma fasciculatum-Ceanthus greggii*, Coastal sage scrub: *Artemisia californica-Salvia mellifera*)	Field fertilization	- C:N humus soils (both ecosystems) - N:P humus soils (both ecosystems) ↓ C:N mineral soil (Chaparral) - N:P mineral soil (Chaparral) ↑ C:N mineral soil (Coastal sage scrub) ⩛ N:P mineral soil (Coastal sage scrub) ⩚ C:N aboveground biomass (Chaparral) ↓ C:N aboveground biomass (Coastal sage scrub)	Vourlitis et al. 2007
Two ecosystems (Chaparral: *Adenostoma fasciculatum-Ceanthus greggii*, Coastal sage scrub: *Artemisia californica-Salvia mellifera*)	Field fertilization	↓ C:N litter (Chaparral) - C:N litter (Coastal sage scrub) ↓ C:N aboveground biomass (Chaparral and Coastal sage scrub)	Vourlitis et al. 2009 (*)
Soil study (2 forest types: hardwood and pine)	Field fertilization	- C:N humus soil (both forests)	Wallenstein et al. 2006 (*)
Soil study (*Graffenrieda emarginata*)	Fiel fertilization	↓ C:N organic soil - C:N mineral soil	Wullaert et al. 2010 (*)
Soil study (*Calluna* grassland)	Field fertilization	↓ C:N soil peat	Yesmin et al. 1996
Populus cathayana	Microcosm experiment	↓ C:N leaves, stems, roots	Zhao et al. 2011 (*)

↑ or ↓ Significant ($P < 0.05$) ⩚ or ⩛ Marginally significant trend ($P < 0.1$) - No significant effects

Table S3. Results of the studies on the effects of warming and drought on biomass C:N:P stoichiometry.

Factor and species	Study site	Main results	Reference
Warming			
Sphagnum fuscum, *Empetrum hermaphroditum, Andromeda polifolia*, *Betula nana*, *Vaccinium uliginosum*, *Calamagrostis lapponica* and *Rubus chamaemorus*.	Field manipulation in artic ecosystem	- [N] leaves (*S.fuscum, A.polifolia, C.lapponica*) ↓ [N] (*E.hermaphroditum, B.nana, R.chamaemorus*, V.uliginosum) - N:P leaves (all species)	Aerts et al. 2009
Schizachyrium scopariums, Sporobolus asper, Dichanthelium oligosnathes, Aster ericoides, Ambriosia psilostachya	Field manipulation	↑ C:N leaves (*S.scopariums, S.asper, D.oligosnathes, A.ericoides)* - C:N leaves (*A.psilostachya*) - C:N litter (all species)	An et al. 2005
Deschampsia antarctica, *Colobanthus quitensis*	Field manipulation	↑ C:N aboveground (both species) ↑ C:N litter (*C.quitensis*) - C:N litter (*D.antarctica*)	Day et al. 2008
Calluna vulgaris, Deschampsia flexuosa and Mosses	Field manipulation	- C:N leaves, stems (*C.vulgaris*) - C:N leaves, plant biomass (*D.flexuosa*) ↑ [N] litter (*D.flexuosa*) - [N] litter (*C.vulgaris*) - C:N Mosses aboveground biomass	Larsen et al. 2011
Globularia alypum, *Dorycnium pentaphyllum*, *Erica multiflora*	Field manipulation	↑ C:N leaves (*G.alypum*) - C:N leaves (*E.multiflora,D.pentaphyllum*) ↓ C:N stems (*E.multiflora, G.alypum*) - C:N stems (*D.pentaphyllum*) - C:N litter (*E.multiflora, G.alypum*) ↑ N:P leaves (*D.pentaphyllum*) - N:P leaves (*E.multiflora)* ↓ N:P leaves (*G.alypum*) - N:P stems (all species) ↑ N:P litter (*E.multiflora, G.alypum*)	Sardans et al. 2008a
Alaska dry and moist tundra species	Field manipulation	↓ C:N leaves (moist tundra species) ↓↓ C:N leaves (dry tundra species)	Welker et al. 2005
Drought			
Pinus halepensis	Chamber	↓ C:N needles, twigs - C:N roots	Inclan et al. 2005
Calluna vulgaris, Deschampsia flexuosa and Mosses	Field manipulation	↑ C:N leaves (*D.flexuosa*) - C:N plant biomass (*D.flexuosa*) - C:N leaves, stems (*C.vulgaris*) - [N] litter (both species) ↑ C:N Mosses aboveground biomass	Larsen et al. 2011
Populus cathayana	Greenhouse	↓ C:N leaves, stems, roots	Lu et al. 2009a
Microbial soil study	Field manipulation	↑ C:N microbes biomass	Matías et al. 2010
Hypnum cupressiforme	Field manipulation	↑↓ C:N aboveground biomass - C:P aboveground biomass ↓ N:P aboveground biomass	Sardans and Peñuelas, 2008
Quercus ilex, *Phillyrea latifolia* and *Arbutus unedo*	Field manipulation	- C:N leaves, wood (all species) ↓ C:N roots, litter (*Q.ilex*) - C:N litter (*P.latifolia, A.unedo*) - C:P leaves (all species) ↑ C:P wood, roots (*Q.ilex*) - C:P wood, litter (*P.latifolia, A.unedo*) ↓ C:P litter (*Q.ilex*) - N:P leaves (all species)	Sardans et al. 2008b

		↑ N:P wood, roots (*Q.ilex*) - N:P wood (*P.latifolia, A.unedo*) - N:P litter (all species)	
Globularia alypum, *Dorycnium penthaphyllum*, *Erica multiflora*	Field manipulation	↑ C:N leaves (*E.multiflora, G.alypum*) - C:N leaves, stems (*D.pentaphyllum*) ↓ C:N stems (*E.multiflora, G.alypum*) - C:N litter (*E.multiflora, G.alypum*) ↓ N:P leaves (*D.pentaphyllum*) - N:P leaves, litter (*E.multiflora, G.alypum*) ↑ N:P stems (*E.multiflora*) - N:P stems (*G.alypum, D.pentaphyllum*)	Sardans et al. 2008a

↑ or ↓ Significant ($P < 0.05$) ⇡ or ⇣ Marginally significant trend ($P < 0.1$) - No significant effects

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APPENDIX 2

Rivas-Ubach et al., Methods in Ecology and Evolution (2012) 4: 464-47.

SOME PREVIOUS CONSIDERATIONS

The sensitivity and resolution of the equipment are important for identifying metabolites by NMR spectroscopy. Good signal intensities and high resolution, especially for crowded regions of the spectra, are required. The preparation of samples for NMR fingerprinting is critical. Extraction of the metabolites must be optimal, avoiding any loss of material during the different steps of the preparation that could lead to an erroneous variability among samples. These procedures have been designed and developed to fulfil the requirements of both comparable ^{1}H NMR fingerprinting and statistical analyses with a minimum of experimental error.

MATERIALS

Reagents used to develop this protocol

· Methanol (UV-IR-HPLC-HPLC isocratic) PAI-ACS (Panreac Quimica S.L.)
· Trichloromethane stabilized with ethanol (UV-IR-HPLC-HPLC preparative) PAI (Panreac Quimica S. L.).
· Chloroform D, 99.8% D + 0.03% TMS (v/v) (CortecNet)
· Chloroform D, 99.8% D (CortecNet)
· Deuterium oxide 99.87% (CortecNet)
· Deuterium oxide 99.9% D + 0.05% TSP (w/w) (Sigma-Aldrich)
· Sodium deuteroxide (40% w/w solution in D_2O) 99.5% D (CortecNet)
· Potassium dihydrogen phosphate (Sigma-Aldrich)
· Potassium hydrogen phosphate (Sigma-Aldrich)
· Ethanol (Panreac Quimica, S.L.)
· Liquid nitrogen.

Reagent Setup

Polar extracts. Prepare phosphate buffer solution (pH 6.0) (containing 0.01% TSP) with D_2O, D_2O (0.03%TSP), potassium dihydrogen phosphate, potassium hydrogen phosphate and sodium deuteroxide . Enough buffer solution for all the samples in the study should be prepared at the same time to minimize variability between samples.

Nonpolar extracts. Use $CDCl_3$ (containing 0.03% TMS). The concentration of the TMS can be adjusted, if necessary, by dissolving with regular $CDCl_3$ without TMS.

Equiment used to develop this protocol

· *Upright-UPUL580* freezer (Dairei Europe A/S)
· *P Selecta 209* stove (JP Selecta S.A.)
· pH meter (Crison Instruments S.A.)
· Lyophilizer *Cryodos* (Telstar S.A.) equipped with a *Varian DS 102* pump (Varian)
· *Mikrodismembrator-U* Ball Mill (B. Braun Biotech International)
· Vortex *RX3* (Velp Scientifica SRL)
· Sonicator *Transonic 570/H* (Elma Gmbh & CokG)
· *Beckman J2-21* Refrigerated Centrifuge (Beckman Coulter)
· Rotary vacuum evaporator *Labo Rota B300* (Resona Technics)
· *5 mm borosilicate NMR sample tubes, 7" length* and of a quality adequate for the NMR spectrometer used (CortecNet)
· Avance 600 NMR spectrometer equipped with a triple inverse 5-mm tube TBI probe with Z-gradient and a temperature control unit (BCU-X) (Bruker Biospin), or similar (e.g. Agilent Technologies, Jeol). The high sensitivity of a cryoprobe is helpful for the identification of metabolites, and the experimental time could be considerably reduced for heteronuclear correlations.
· Autosampler BACS60 for tube NMR (Bruker Biospin), or similar
· NMR software package for data acquisition and processing Topspin (Bruker Biospin), or similar
· AMIX software for the analysis of NMR data using statistical methods (Bruker Biospin)
· STATISTICA v.9.0 (StatSoft Inc.), SPSS (IBM), or R (R Development Core Team) and/or PERMANOVA+ for PRIMER (Anderson, Gorley & Clarke 2008) to perform the statistical multivariate analyses

METHODS

Sample preparation and metabolite extraction

Gringing time with ball miller: For example, freeze-dried *Fagus sylvatica* leaves (deciduous) can be ground in four minutes, while *Quercus ilex* and *Erica multiflora* leaves (evergreen) required more time, 9 minutes for E. multiflora and 6 minutes for Q. ilex. Plant roots often need longer times for a complete grinding (15-20 min). Powdered samples can be then kept in hermetic cans in desiccators or frozen (-80ºC or -20ºC) until the extraction of metabolites.

Mortar under liquid nitrogen: Hand-grinding under liquid nitrogen can be used by following this protocol but modifying some steps.

1. Collect fresh plant material, pack and label rapidly and freeze it in situ in a container of liquid nitrogen.
2. Keep the samples frozen at -80ºC to maintain the metabolomes as intact as possible.
3. Hand-grind the samples under liquid nitrogen.
4. Keep the sample powder in tubes and maintain again at -80ºC.
5. Lyophilize the powder of samples to maintain the metabolomes intact during all the following steps.
6. Step 4 of Figure 2 and follow the incoming steps of protocol.

Amount of sample: Samples should not be saturated for NMR fingerprinting. Between 50-100 mg of powder should be enough to obtain an adequate ^{1}H NMR spectrum of the extract relatively quickly in the NMR spectrometer (8-10 min). Sample sizes in ecometabolomics are large, so we recommend using equal sample weights to avoid having to later correct for differences.

Sonication: After testing different times of sonication (0, 2, 5, 12 and 20 min), our results showed that the optimum extraction of metabolites was obtained with two minutes of sonication (Step 7 of **Fig. 2**) (**Table S2** and **Figures S2, S3**) . The use of chloroform during extraction will dissolve cellular membranes and thereby reduce the time of sonication. Longer times for sonication reduce the signal strength of metabolites in ^{1}H NMR spectra, perhaps due to the heating of samples and the formation of metabolite artefacts (t'Kindt et al. 2008). In other NMR protocols of polar metabolites where chloroform extraction is not performed, sonication for 10 min has been recommended (Kim, Choi & Verpoorte 2010).

Repeated extractions: The importance of repeated extractions has been discussed in protocols based on LC-MS metabolomic analyses (von Roepenack-Lahaye et al. 2004; Nikiforova et al. 2005; Böttcher et al. 2007; t'Kindt et al. 2008). For our protocol, the optimal number of repeated extractions to get maximal NMR data was tested by analysing metabolomic fingerprinting data, and the results indicated that two extractions were sufficient to maximize the concentration of metabolites in the extracts (**Table S2**). A first extraction from *E. multiflora* and *Q. ilex* leaves yielded 78.5% and 85.6% metabolites, respectively, relative to a second extraction, although the differences were not statistically significant (one-way ANOVA test of the global concentration of metabolites; p=0.32 for *E. multiflora* (n=6) and p=0.26 for *Q. ilex* (n=6)). No differences were found between one and two extractions from leaves of the deciduous tree *F. sylvatica* (one-way ANOVA test of the global concentration of metabolites; p=0.996 (n=6)), so one extraction was enough. Three extractions from these species showed no differences to two extractions (one-way ANOVA test of the global concentration of metabolites; p=0.97 for *E. multiflora* (n=6), p=0.89 for *Q. ilex* (n=6), and p=0.97 for *F. sylvatica* (n=6)); more than 98% of metabolites from the third extraction had already been extracted after the second extraction.

Six millilitres of each extractant ($MeOH/H_2O$, Cl_3CH) for 50-100 mg of dry material are recommended for each extraction step (Step 5 of **Fig. 2**). This volume is sufficient to avoid the formation of a pellet during the first collection of 4 mL of both polar and nonpolar fractions (Steps 9.a and 9.b of **Fig. 2**) and to avoid saturation of the samples. The same volume of solvent has been used in previous studies (Choi et al. 2004).

Recovery: The recovery was tested for two polar compounds, an amino acid (alanine) and a sugar (glucose), of the primary metabolism of all organisms throughout two extractions. A recovery of 92.8% was obtained for alanine and of 86.4% for glucose (**Figures S4 and S5**).

Statistical analyses, some considerations:

PCA (Principal Component Analysis) can be used as a classification technique clarifying the relations among variables and cases. To do that, variables and cases are plotted in the space generated by the PCs (Principal component axes).The projection of the cases can be conducted in 2 or 3 axes. Usually two axes are represented in the PCA to study the classification of the cases on the factor-plane (in our case we plotted PC1 vs PC2 in Fig.3 and PC1 vs PC3 in Fig.5). The PCs are ordered by the explained variability of the cases, being the PC1 the component that explains always the largest variability. The sum of variabilities of all PCs is always the 100% of all variability generated by the variables (PC1 + PC2 + PC3 + … + PCn-1 = 100%).

Variables plot: The variables are projected in a plot described by 2 or 3 PCs (as explained above, two PCs representation is the most common plot), each variable present a coordinates (factor coordinates of variables) that are the correlations of the variables and the factor axes. The further a point is from the central point of the plot, the larger is the correlation of the variable with the PC axis. The correlation among variables and PCs can thus be identified and it provides the information of which variables explain the variability of each PC.

Cases plot: The cases are projected onto the factor-plane generated by the PCs selected (usually PC1 vs PC2). This projection is in narrow relation with the variables plot since cases are projected with a specific coordinates (factor coordinates of cases) on the factor-plane determined by the strench of each variable. This graph is useful to classify the cases in the studied categories (treatments).

Statistical inference on PCs axis: As explained in the main text, multivariate ordination analyses do not provide any measure of significance and the graphs are limited to show the visual relation of cases with the used variables. The PCs loadings of cases plots can be used to detect any significant difference of the investigated categories by statistical inference such as t-student test if we want to compare two groups, or ANOVAs if we have two or more groups. In order to do it, first, the normality of the coordinates projected of each group on each PC must to be tested. ANOVAs test can be applied only if they present normality distributions. In our analyses, we took the coordinates of each case of each PC and they were compared by ANOVA tests to detect any statistically significant difference on the distribution of cases on the factor-plane generated by the selected PCs. It allowed to confirm which groups (looking at the cases plot) presented higher concentrations of certain compounds (looking at the variables plot) than others. The analyses with three or more groups must be tested by a Post-Hoc test such as Bonferroni or LSD-fisher to detect which groups differ from the others.

Another way to detect which studied groups contain higher concentrations of specific compounds than others is by performing ANOVA test for each metabolite, but it does not allow to explain general trends as multivariate ordination analyses do.

PLS-DA (Partial Least Squares – Discriminat Analysis) is quite similar to PCA with the difference that PLS-DA considers previously the different studied groups (Control, Treatment 1, Treatment 2…) and the PCs explain the variance between marked groups, not cases as in PCAs. In this case, the PCs are ordered by the variance explained by the groups, being the PC1 the factor that explains more variation among different groups.

Step by step procedure

The complete procedure is divided into six main categories: **A**. Sample collection and storage (Steps 1-3 of **Fig. 2**), **B**. Metabolite extraction and NMR sample preparation (Steps 4-20.a/16.b of **Fig. 2**), **C.** Fingerprint NMR data acquisition (Steps 21-29 of **Fig. 2**), **D**. NMR experiments for metabolite identification (Steps 30-32 of **Fig. 2**), **E**. Spectra processing (Steps 33-34 of **Fig. 2**) and **F**. Statistical data analysis (Step 35 of **Fig.2**).

A. **SAMPLE COLLECTION AND STORAGE**

1. Collect the fresh plant materials and rapidly pack, label, and freeze them *in situ* in a container of liquid nitrogen.
2. Lyophilize the frozen plant material (3 d).
3. Grind the samples with a ball mill. (3-10 min: total time depends on the sample).

B. **METABOLITE EXTRACTION AND NMR SAMPLE PREPARATION**

4. Add 50-100mg of dried powder to a 50 ml centrifuge tube.
5. Add 6 ml of a water-methanol (1/1) mixture and 6 ml of chloroform.
6. Mix by vortexing (15 s).
7. Sonicate (2 min).
8. Centrifuge at 11000 x *g* (15 min).

   Two liquid phases are obtained (aqueous and organic). The two phases (extracts) will be collected separately.

i) Aqueous extraction.

9. a. Collect 4 ml of the aqueous extract in centrifuge tubes.

REPEAT STEPS 5-10 IN THE SAME TUBES.

10. a. Add 25 ml of water to reduce the proportion of methanol.
11. a. Freeze samples at -80ºC.

12. a. Lyophilize samples with caps loosened (3 d, total time depends on the pump's working pressure).

13. a. Add 4 ml of water and mix by vortexing (15 s).

14. a. Centrifuge at 23000 x *g* (3 min).

15. a. Freeze samples at -80ºC.

16. a. Lyophilize samples with caps loosened (8-12 hours, total time depends on the pump's working pressure).

17. a. Add 1 ml of phosphate buffer in D_2O + 0.01% TSP to obtain a solution with a final pH=6.0 and vortex for 5 s.

18. a. Resuspend with micropipette and transfer the contents to Eppendorf tubes.

19. a. Centrifuge at 23000 x *g* (3min).

20. a. Transfer 0.6 ml of the supernatant to NMR sample tubes.

ii) <u>Organic extraction.</u>

9.b. Collect 4 ml of the organic fraction in crystal jars. Use calibrated crystal syringes to execute all steps with the organic fractions.

REPEAT STEPS 5-10 IN THE SAME TUBES.

10. b. Place organic fraction into a 25 ml round-bottom evaporation flask.

11. b. Dry samples in a rotary vacuum evaporator.

12. b. Add 1 ml of chloroform D containing 0.03% TMS.

13. b. Put the top on the round-bottom flasks, mix, and wait for 10 min.

14. b. Mix the flasks and transfer the contents into Eppendorf tubes.

15. b. Centrifuge at 23000 x *g* (3min)

16. b. Transfer 0.6 ml of supernatant to NMR sample tubes.

C. **<u>ACQUISITION OF NMR FINGERPRINT DATA</u>**

21. Set the temperature of the spectrometer to 300.0 K (it must be maintained constant for the entire study).

22. Insert a representative sample into the NMR magnet (polar or nonpolar sample) and allow the sample sufficient time (2-4 min) to reach and maintain the configured temperature.

23. Enter the parameter set of the experiment to be performed (previously saved under a specific name, see EQUIPMENT SETUP).

24. Tune and match the probe for ^1H nuclei.

25. Lock the sample to the deuterated solvent used (D_2O or $CDCl_3$).

26. Optimize the homogeneity of the magnetic field ("shimming"). Most equipment allows automatic optimization.

27. Determine the 90° ^{1}H pulse length at the configured power level, and save it in the parameter set.

28. For polar samples, adjust the frequency offset for an optimal suppression of the water signal (use the go-setup mode or equivalent in non-Bruker spectrometers) and save it in the parameter set. For nonpolar samples, go to Step 29.

29. Load the samples in the autosampler and select the experiment to be performed (previously set up) (use ICONNMR for Bruker Biospin spectrometers):

(A). Polar samples: quantitative 1D 90° pulse ^{1}H NMR experiment with water suppression (see EQUIPMENT SETUP). Typical acquisition parameter values are: spectral width of 20 ppm, 32 768 time-domain data points, relaxation delay of 2.0 s, acquisition time of 1.7 s, power level for the presaturation during the relaxation delay of ~55 dB, and a total of 128 transients. For the processing, zero-fill the FID to 65 536 data points and multiply by an exponential apodization function equivalent to 0.2-0.6 Hz line broadening. Fourier transform and correct the phase and baseline automatically. Finally, the spectrum must be calibrated to the internal reference (TSP singlet at δ_H 0.00 ppm); the calibration can also be done automatically for the complete set of spectra using the AMIX software. The acquisition time becomes ~8 min per sample and 10-12 min per sample for the entire analysis.

(B). Nonpolar samples: quantitative conventional 1D 90° pulse ^{1}H experiment. Typical parameter values are 128 transients, 32 768 time-domain data points, relaxation delay of 2.0 s, spectral width of 20 ppm, and acquisition time of 1.7 s. The processing is analogous to that of the polar samples, but the exponential apodization is generally not needed. The spectra are calibrated to the TMS singlet (δ_H 0.00 ppm) or to the residual $CHCl_3$ peak (7.24 ppm). The experimental time per sample is analogous to that in (A).

D. **NMR EXPERIMENTS FOR METABOLITE IDENTIFICATION**

These experiments must be performed in a representative sample of each set (polar and nonpolar).

30. Repeat Steps 21-28 of the procedure in section C. In Step 24, tune the probe also for ^{13}C nuclei.

31. Assign to the different experiments to perform consecutive experiment numbers. Choose experiment depending on the elucidation/assignment problem to resolve and use standard conditions. For polar samples, select the version with water presaturation. The most commonly used experiments are shown in **Table 1**.

32. Acquire and process the experiments. Use the option for multiple acquisitions.

E. **SPECTRA PROCESSING**

33. Create a peak pattern for each sample type (organic and aqueous).

34. Transform the NMR spectra to data sets for a more suitable statistical analysis (bucketing process). The output file can be saved in various formats (e.g. *.xls, *.ods, *.txt). The *.xls format is recommended for large data sets.

F. **STATISTICAL DATA ANALYSIS**

35. Perform the statistical analyses (e.g. PCAs, FAs, PLS-DA, ANOVAs, PERMANOVAs).

Estimated time invested in applying this protocol

The total time invested for all processes will depend on the number of samples. For studies with a large number of samples (> 60), we recommend processing smaller series of samples on different days.

Sample preparation and metabolite extraction

The number of samples that can be prepared simultaneously depends mainly on the number of tubes that fit into the centrifuge (fourteen tubes in our case). For the preparation of 42 samples simultaneously (three centrifuges each with 14 tubes):

Step 1, field work; **Step 2**, 2-3 d for lyophilization (drying more than 50 samples simultaneously is usually possible); **Step 3**, grinding samples, 4 h (3-10 min per sample; the exact time will depend on the characteristics of the sample); **Steps 4-9.a/9.b**, 3 h;

Aqueous extraction: Steps 10.a-12.a, 3 d for lyophilization; **Steps 13.a-14.a**, 1 h; **Steps 15.a-16.a**, 1 d for lyophilization; **Steps 17.a-20.a**, 3 h;

Organic extraction: Steps 10.b-16.b, 5 h

NMR experiments

For the metabolic fingerprinting, a maximum of 140 samples (polar or nonpolar) can be analysed per day. The duration of the experiments for the elucidation of metabolites will vary notably depending on the types of experiments performed, the concentrations of the samples, the strength of the NMR magnetic field, and whether or not cryogenically cooled probes are used (Rivas-Ubach et al. 2012).

SOME POSSIBLE PROBLEMS AND POTENTIAL SOLUTIONS

Step	Problem	Possible reason	Solution
Preparation of phosphate buffer in D_2O	Presence of shifts in the δ among samples	Small differences of pH among samples	Work at a pH value similar to the natural average pH of your set of samples. For instance, if the natural average pH is 4.5, then prepare a buffer solution for this or a very proximate pH value. Prepare enough volume of buffer solution for the whole study. This will avoid differences in pH between samples.
12.a	Possible wastage of sample during lyophilization	Low pressures may drag some sample powder out of tubes	Do not fill the tubes to their maximum volume capacity. Use tubes of 50 mL volume or larger. For small tubes, reduce the amount of sample and extractants.
17.a-18.a	Pellet difficult to resuspend	Large amounts of metabolites are difficult to resuspend with only 1 mL of D_2O	If you cannot resuspend all contents in 1 mL, you should reduce the initial amount of dry material. We recommend to do a first trial extraction Normally, 50-100 mg of dry material do not present problems.
21	Appearance of precipitates in the D_2O sample tubes	Keeping samples under low temperatures before the NMR measurements may induce the appearance of precipitates	As commented, we recommend executing Steps 17.a-21 of **Figure 2** on the same day. If that is not possible, the samples can be kept at 4ºC or frozen before the NMR measurements. We then recommend ultrasonicating the samples for 10-15 min and mixing them as well as possible.

Table S1. The following table shows some relevant bibliography regarding the identification of metabolites at NMR spectra.

Author	Year of publication	Title	Type of publication
Berger, S. & Sicker, D.	2009	Classics in Spectroscopy.	Book
Breitmaier, Haas & Voelter	1979	Atlas of carbon-13 NMR data.	Book
Cui, Q. et al.	2008	Metabolite identification via the Madison metabolomics consortium database.	Article & Database
Fan, T.W.M. & Lane, A.N.	2008	Structure-based profiling of metabolites and isotopomers by NMR.	Review article
Fan, W.M.T.	1996	Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures.	Review article
Gunstone, F.D.	1995	The lipid handbook.	Book
Lundberg, P et al.	2005	MDL - The magnetic resonance metabolomics database.	Article & Database
Sacchi, R., Addeo, F. & Paolillo, L.	1997	^{1}H and ^{13}C NMR of virgin olive oil. An overview.	Article
Ulrich, L.E. & Zhulin, I.B.	2007	MiST: a microbial signal transduction database.	Article & Database
Vlahov, G.	1999	Application of NMR to the study of olive oils.	Review article
Wishart, D.S. et al.	2009	HMDB: a knowledgebase for the human metabolome.	Article & Database
Wishart, D.S. et al.	2007	HMDB: the human metabolome database.	Article & Database

Table S2. Integral values of spectra referred to internal standards (TSP or TMS) of polar and nonpolar metabolites extracted from *Erica multiflora* foliar samples submitted to different sonication times (0, 2, 5, 12, and 20 minutes). The integral value corresponds to a mean of four individuals submitted to the same sonication time. Means of all different sonication times for every bucket have been used to determine the positive or negative difference of the integral means for each sonication time.

POLAR METABOLITES (Water)											
Bucket number	**Integral values referred to internal standard TSP of polar metabolites extracted from *Erica multiflora* samples submitted to different sonication times (minutes).**						**Difference of the integral value with respect to the mean of all sonication times for polar metabolites.**				
	0	**2**	**5**	**12**	**20**	**MEAN**	**0**	**2**	**5**	**12**	**20**
1	0.158	0.170	0.157	0.136	0.151	**0.154**	0.004	0.016	0.003	-0.019	-0.004
2	0.535	0.548	0.517	0.474	0.506	**0.516**	0.019	0.032	0.000	-0.042	-0.010
3	0.468	0.479	0.475	0.456	0.463	**0.468**	0.000	0.010	0.006	-0.012	-0.005
4	0.380	0.385	0.383	0.382	0.370	**0.380**	-0.001	0.005	0.003	0.002	-0.010
5	0.379	0.394	0.366	0.344	0.355	**0.368**	0.012	0.026	-0.002	-0.024	-0.013
6	0.251	0.220	0.252	0.237	0.215	**0.235**	0.016	-0.015	0.017	0.002	-0.021
7	0.403	0.341	0.404	0.397	0.326	**0.374**	0.029	-0.033	0.030	0.023	-0.048
8	0.562	0.538	0.560	0.555	0.513	**0.546**	0.016	-0.007	0.015	0.009	-0.032
9	0.304	0.304	0.314	0.316	0.307	**0.309**	-0.005	-0.005	0.005	0.007	-0.002
10	0.193	0.194	0.198	0.200	0.194	**0.196**	-0.003	-0.002	0.002	0.005	-0.002
11	0.513	0.481	0.812	0.793	0.807	**0.681**	-0.168	-0.200	0.131	0.112	0.126
12	0.107	0.106	0.158	0.135	0.120	**0.125**	-0.018	-0.019	0.033	0.010	-0.005
13	0.087	0.087	0.085	0.093	0.085	**0.087**	-0.001	0.000	-0.003	0.006	-0.003
14	0.037	0.038	0.036	0.036	0.035	**0.037**	0.001	0.002	-0.001	0.000	-0.001
15	0.046	0.046	0.043	0.042	0.042	**0.044**	0.002	0.002	-0.001	-0.001	-0.002
16	0.314	0.308	0.291	0.280	0.282	**0.295**	0.019	0.013	-0.004	-0.015	-0.013
17	0.154	0.157	0.140	0.130	0.137	**0.144**	0.010	0.013	-0.004	-0.013	-0.006
18	0.329	0.345	0.293	0.248	0.293	**0.302**	0.027	0.043	-0.009	-0.053	-0.008
19	0.538	0.557	0.531	0.508	0.519	**0.530**	0.007	0.027	0.000	-0.023	-0.012
20	1.032	1.052	1.050	1.032	1.014	**1.036**	-0.004	0.016	0.014	-0.004	-0.022
21	0.532	0.545	0.563	0.555	0.534	**0.546**	-0.014	-0.001	0.017	0.010	-0.012
22	0.792	0.812	0.799	0.775	0.777	**0.791**	0.001	0.021	0.008	-0.016	-0.014
23	1.304	1.332	1.351	1.335	1.291	**1.323**	-0.018	0.010	0.028	0.012	-0.032
24	1.392	1.427	1.420	1.393	1.373	**1.401**	-0.008	0.026	0.019	-0.008	-0.028
25	1.977	2.012	2.013	1.997	1.952	**1.990**	-0.013	0.022	0.023	0.007	-0.039
26	0.789	0.801	0.843	0.860	0.793	**0.817**	-0.028	-0.017	0.026	0.043	-0.024
27	0.401	0.401	0.389	0.407	0.384	**0.396**	0.004	0.005	-0.008	0.011	-0.012
28	0.087	0.090	0.072	0.088	0.082	**0.084**	0.003	0.006	-0.012	0.004	-0.002
29	0.673	0.698	0.651	0.673	0.647	**0.668**	0.005	0.029	-0.017	0.005	-0.021
30	0.741	0.763	0.752	0.767	0.730	**0.751**	-0.010	0.013	0.001	0.017	-0.021
31	0.163	0.171	0.150	0.156	0.155	**0.159**	0.004	0.011	-0.009	-0.003	-0.004
32	0.299	0.309	0.297	0.299	0.289	**0.299**	0.001	0.010	-0.001	0.001	-0.010
33	0.172	0.180	0.197	0.173	0.170	**0.178**	-0.006	0.002	0.019	-0.006	-0.008
34	0.050	0.054	0.042	0.049	0.046	**0.048**	0.002	0.006	-0.007	0.001	-0.002
35	0.285	0.323	0.172	0.213	0.240	**0.247**	0.039	0.076	-0.075	-0.033	-0.007
36	0.767	0.805	0.779	0.781	0.776	**0.782**	-0.015	0.024	-0.002	-0.001	-0.006
37	0.417	0.439	0.391	0.405	0.398	**0.410**	0.007	0.029	-0.019	-0.005	-0.012
38	0.295	0.326	0.222	0.240	0.270	**0.271**	0.025	0.055	-0.048	-0.031	0.000
39	0.102	0.110	0.083	0.085	0.096	**0.095**	0.007	0.015	-0.012	-0.010	0.001
40	0.275	0.285	0.250	0.253	0.261	**0.265**	0.011	0.020	-0.015	-0.012	-0.004
41	0.441	0.449	0.420	0.417	0.425	**0.430**	0.010	0.019	-0.011	-0.014	-0.005
42	0.327	0.336	0.321	0.322	0.320	**0.325**	0.002	0.011	-0.004	-0.003	-0.005
43	0.168	0.175	0.163	0.163	0.163	**0.166**	0.001	0.009	-0.004	-0.003	-0.003
44	0.291	0.306	0.269	0.277	0.280	**0.285**	0.006	0.022	-0.015	-0.008	-0.005
45	0.696	0.721	0.651	0.657	0.665	**0.678**	0.018	0.043	-0.027	-0.021	-0.013
46	0.868	0.751	0.806	0.771	0.829	**0.805**	0.063	-0.054	0.001	-0.034	0.024
47	0.570	0.601	0.591	0.584	0.572	**0.584**	-0.013	0.018	0.007	0.000	-0.012
48	0.517	0.536	0.494	0.492	0.500	**0.508**	0.009	0.028	-0.014	-0.016	-0.008
49	0.799	0.817	0.775	0.772	0.774	**0.787**	0.012	0.030	-0.012	-0.015	-0.014
50	0.816	0.836	0.802	0.803	0.796	**0.811**	0.006	0.025	-0.008	-0.008	-0.015
51	1.201	1.249	1.160	1.166	1.167	**1.189**	0.013	0.061	-0.028	-0.023	-0.022

52	1.890	1.953	1.915	1.899	1.877	**1.907**	-0.017	0.046	0.008	-0.007	-0.029
53	2.398	2.466	2.423	2.416	2.362	**2.413**	-0.015	0.053	0.010	0.003	-0.051
54	2.386	2.449	2.405	2.394	2.349	**2.397**	-0.011	0.052	0.009	-0.002	-0.048
55	1.705	1.766	1.738	1.734	1.697	**1.728**	-0.023	0.038	0.010	0.006	-0.031
56	4.001	4.140	3.980	4.001	3.873	**3.999**	0.002	0.141	-0.019	0.002	-0.126
57	3.291	3.350	3.403	3.367	3.283	**3.339**	-0.048	0.011	0.064	0.028	-0.055
58	1.615	1.664	1.708	1.676	1.611	**1.655**	-0.040	0.009	0.053	0.021	-0.044
59	1.534	1.562	1.604	1.585	1.552	**1.567**	-0.034	-0.006	0.037	0.018	-0.015
60	2.177	2.232	2.231	2.226	2.150	**2.203**	-0.026	0.029	0.028	0.023	-0.053
61	0.988	1.016	1.008	1.015	0.961	**0.997**	-0.009	0.018	0.010	0.017	-0.037
62	0.667	0.669	0.729	0.711	0.693	**0.694**	-0.026	-0.025	0.035	0.017	-0.001
63	0.530	0.510	0.556	0.555	0.568	**0.544**	-0.014	-0.034	0.012	0.011	0.024
64	0.566	0.556	0.561	0.567	0.584	**0.567**	-0.001	-0.011	-0.006	0.000	0.017
65	0.848	0.859	0.882	0.863	0.849	**0.860**	-0.012	-0.001	0.021	0.003	-0.011
66	3.144	3.254	3.214	3.204	3.129	**3.189**	-0.045	0.065	0.025	0.015	-0.060
67	0.900	0.934	0.924	0.923	0.890	**0.914**	-0.014	0.019	0.010	0.009	-0.024
68	1.495	1.511	1.476	1.491	1.432	**1.481**	0.014	0.030	-0.005	0.011	-0.049
69	0.841	0.864	0.862	0.845	0.830	**0.849**	-0.008	0.016	0.014	-0.003	-0.018
70	1.023	1.027	1.037	1.022	0.999	**1.022**	0.001	0.005	0.016	0.001	-0.023
71	1.311	1.347	1.316	1.309	1.275	**1.312**	0.000	0.035	0.004	-0.002	-0.037
72	1.469	1.523	1.526	1.515	1.462	**1.499**	-0.030	0.024	0.027	0.016	-0.037
73	1.680	1.730	1.662	1.669	1.625	**1.673**	0.007	0.057	-0.011	-0.004	-0.048
74	1.910	1.958	2.051	2.017	1.950	**1.977**	-0.067	-0.020	0.073	0.040	-0.027
75	2.168	2.221	2.198	2.220	2.142	**2.190**	-0.022	0.031	0.009	0.030	-0.048
76	2.208	2.293	2.200	2.191	2.146	**2.208**	0.001	0.086	-0.008	-0.017	-0.061
77	2.948	3.046	3.191	3.138	3.018	**3.068**	-0.120	-0.022	0.123	0.070	-0.051
78	1.079	1.111	1.141	1.143	1.084	**1.112**	-0.033	0.000	0.030	0.031	-0.028
79	1.939	1.992	2.095	2.068	1.976	**2.014**	-0.075	-0.022	0.081	0.054	-0.038
80	6.267	6.421	6.515	6.473	6.219	**6.379**	-0.113	0.042	0.136	0.094	-0.160
81	0.697	0.716	0.665	0.674	0.659	**0.682**	0.015	0.034	-0.017	-0.008	-0.024
82	1.841	1.874	1.912	1.895	1.823	**1.869**	-0.028	0.005	0.043	0.026	-0.047
83	0.622	0.638	0.604	0.615	0.584	**0.613**	0.009	0.026	-0.009	0.002	-0.028
84	0.866	0.885	0.959	0.925	0.881	**0.903**	-0.038	-0.018	0.056	0.022	-0.022
85	0.487	0.501	0.457	0.486	0.452	**0.476**	0.011	0.025	-0.020	0.009	-0.025
86	0.438	0.450	0.542	0.507	0.477	**0.483**	-0.045	-0.033	0.059	0.025	-0.006
87	0.421	0.431	0.467	0.455	0.433	**0.441**	-0.020	-0.011	0.026	0.014	-0.009
88	0.632	0.645	0.666	0.664	0.628	**0.647**	-0.015	-0.002	0.019	0.016	-0.019
89	0.465	0.474	0.469	0.489	0.439	**0.467**	-0.002	0.007	0.002	0.022	-0.028
90	0.465	0.470	0.516	0.505	0.470	**0.485**	-0.020	-0.016	0.030	0.020	-0.015
91	2.229	2.290	2.331	2.315	2.205	**2.274**	-0.045	0.016	0.057	0.041	-0.069
92	0.683	0.712	0.730	0.726	0.689	**0.708**	-0.026	0.004	0.022	0.018	-0.019
93	0.581	0.602	0.633	0.626	0.584	**0.605**	-0.024	-0.003	0.028	0.021	-0.021
94	0.495	0.488	0.693	0.704	0.681	**0.612**	-0.118	-0.125	0.081	0.092	0.069
95	0.614	0.623	0.796	0.741	0.725	**0.700**	-0.086	-0.076	0.097	0.041	0.025
96	0.201	0.209	0.202	0.207	0.192	**0.203**	-0.001	0.007	0.000	0.005	-0.010
97	0.382	0.386	0.416	0.414	0.361	**0.392**	-0.010	-0.006	0.024	0.023	-0.030
98	0.329	0.331	0.333	0.335	0.318	**0.329**	0.000	0.002	0.004	0.006	-0.011
99	0.304	0.305	0.315	0.313	0.295	**0.306**	-0.002	-0.001	0.009	0.006	-0.012
100	0.747	0.734	0.760	0.736	0.729	**0.741**	0.006	-0.007	0.019	-0.005	-0.012
101	0.067	0.068	0.067	0.078	0.053	**0.067**	0.000	0.001	0.000	0.012	-0.013
102	0.254	0.256	0.248	0.265	0.232	**0.251**	0.003	0.005	-0.003	0.014	-0.019
103	0.204	0.207	0.210	0.215	0.194	**0.206**	-0.002	0.001	0.004	0.009	-0.012
104	0.297	0.296	0.299	0.302	0.284	**0.296**	0.001	0.000	0.004	0.006	-0.011
105	1.016	1.061	1.071	1.063	1.021	**1.046**	-0.031	0.015	0.025	0.016	-0.025
106	1.185	1.201	0.985	1.167	1.022	**1.112**	0.073	0.089	-0.127	0.055	-0.090
107	2.252	2.310	2.178	2.289	2.185	**2.243**	0.010	0.067	-0.065	0.046	-0.058
108	5.649	5.788	5.732	5.741	5.579	**5.698**	-0.049	0.090	0.034	0.043	-0.119
109	1.224	1.263	1.330	1.227	1.210	**1.251**	-0.027	0.012	0.080	-0.024	-0.041
110	5.181	5.302	5.284	5.254	5.108	**5.226**	-0.045	0.076	0.058	0.028	-0.118
111	1.030	1.073	1.118	1.063	1.044	**1.066**	-0.036	0.008	0.053	-0.003	-0.022
							∑	∑	∑	∑	∑
							-1.234	**1.351**	**1.426**	**0.890**	**-2.433**

NONPOLAR METABOLITES (Chloroform)

Bucket number	**Integral values referred to internal standard TMS of nonpolar metabolites extracted from *Erica multiflora* samples submitted to different sonication times (minutes).**	**Difference of the integral value with respect to the mean of all sonication times for nonpolar metabolites.**

	0	2	5	12	20	MEAN	0	2	5	12	20
1	0.163	0.228	0.174	0.196	0.193	**0.191**	-0.028	0.037	-0.017	0.005	0.002
2	0.109	0.158	0.118	0.131	0.131	**0.129**	-0.020	0.029	-0.011	0.001	0.002
3	0.103	0.150	0.103	0.116	0.113	**0.117**	-0.014	0.033	-0.014	-0.001	-0.004
4	0.141	0.188	0.146	0.156	0.154	**0.157**	-0.016	0.031	-0.011	-0.001	-0.003
5	0.321	0.432	0.320	0.337	0.339	**0.350**	-0.029	0.082	-0.030	-0.013	-0.011
6	1.267	1.426	1.246	1.240	1.252	**1.286**	-0.019	0.140	-0.040	-0.046	-0.034
7	1.145	1.240	1.066	1.015	1.021	**1.098**	0.048	0.142	-0.031	-0.082	-0.076
8	2.719	2.888	2.623	2.559	2.588	**2.675**	0.044	0.212	-0.052	-0.117	-0.088
9	0.842	0.932	0.830	0.768	0.824	**0.839**	0.003	0.093	-0.010	-0.072	-0.015
10	0.374	0.453	0.377	0.355	0.391	**0.390**	-0.016	0.063	-0.013	-0.035	0.001
11	0.328	0.405	0.339	0.336	0.355	**0.353**	-0.024	0.053	-0.014	-0.017	0.002
12	1.184	1.419	1.217	1.175	1.309	**1.261**	-0.077	0.158	-0.043	-0.086	0.048
13	0.564	0.780	0.603	0.598	0.736	**0.656**	-0.092	0.123	-0.053	-0.058	0.080
14	0.810	0.949	0.829	0.797	0.873	**0.852**	-0.041	0.097	-0.022	-0.055	0.021
15	0.353	0.417	0.346	0.332	0.342	**0.358**	-0.005	0.059	-0.012	-0.026	-0.016
16	0.372	0.411	0.357	0.358	0.339	**0.367**	0.005	0.044	-0.010	-0.010	-0.029
17	0.839	0.975	0.893	0.846	0.875	**0.885**	-0.047	0.090	0.007	-0.040	-0.010
18	1.049	1.235	1.155	1.137	1.159	**1.147**	-0.098	0.088	0.008	-0.010	0.012
19	0.494	0.611	0.534	0.504	0.531	**0.535**	-0.041	0.076	-0.001	-0.030	-0.004
20	0.585	0.653	0.572	0.556	0.550	**0.583**	0.002	0.070	-0.012	-0.027	-0.033
21	0.927	1.016	0.910	0.860	0.880	**0.918**	0.008	0.097	-0.008	-0.059	-0.038
22	1.874	2.007	1.759	1.664	1.687	**1.798**	0.076	0.209	-0.039	-0.134	-0.112
23	1.494	1.570	1.441	1.381	1.420	**1.461**	0.033	0.109	-0.020	-0.080	-0.041
24	1.208	1.263	1.143	1.093	1.116	**1.164**	0.043	0.098	-0.022	-0.072	-0.048
25	1.316	1.375	1.262	1.232	1.257	**1.288**	0.028	0.087	-0.026	-0.057	-0.032
26	0.298	0.314	0.284	0.275	0.282	**0.291**	0.007	0.024	-0.007	-0.015	-0.009
27	1.619	1.696	1.525	1.439	1.466	**1.549**	0.070	0.147	-0.024	-0.110	-0.083
28	3.253	3.434	3.190	3.077	3.170	**3.225**	0.029	0.209	-0.035	-0.148	-0.055
29	1.512	1.595	1.501	1.468	1.504	**1.516**	-0.004	0.079	-0.015	-0.048	-0.012
30	1.721	1.814	1.682	1.623	1.684	**1.705**	0.016	0.110	-0.023	-0.082	-0.021
31	0.604	0.649	0.596	0.569	0.601	**0.604**	0.000	0.045	-0.008	-0.035	-0.003
32	0.263	0.295	0.260	0.252	0.268	**0.268**	-0.004	0.027	-0.007	-0.016	0.001
33	1.091	1.172	1.068	1.045	1.082	**1.092**	-0.001	0.081	-0.023	-0.047	-0.010
34	1.102	1.158	1.013	0.930	0.971	**1.035**	0.067	0.123	-0.021	-0.104	-0.064
35	0.617	0.664	0.598	0.557	0.583	**0.604**	0.013	0.060	-0.006	-0.047	-0.021
36	0.683	0.761	0.683	0.647	0.662	**0.687**	-0.004	0.074	-0.004	-0.040	-0.025
37	0.143	0.171	0.145	0.140	0.145	**0.149**	-0.006	0.022	-0.004	-0.008	-0.004
38	0.754	0.944	0.789	0.790	0.833	**0.822**	-0.068	0.122	-0.033	-0.032	0.011
39	0.625	0.779	0.637	0.645	0.692	**0.675**	-0.051	0.103	-0.039	-0.031	0.017
40	10.561	13.203	12.358	12.660	12.868	**12.330**	-1.769	0.873	0.028	0.330	0.538
41	1.141	1.366	1.240	1.219	1.285	**1.250**	-0.109	0.116	-0.011	-0.031	0.034
42	3.101	3.482	3.189	3.086	3.166	**3.205**	-0.104	0.277	-0.016	-0.119	-0.039
43	0.952	1.058	0.967	0.927	0.971	**0.975**	-0.023	0.083	-0.008	-0.048	-0.004
44	0.819	0.897	0.833	0.793	0.812	**0.831**	-0.012	0.067	0.002	-0.038	-0.019
45	0.559	0.607	0.574	0.535	0.558	**0.567**	-0.007	0.040	0.007	-0.032	-0.008
46	0.643	0.685	0.706	0.717	0.688	**0.688**	-0.044	-0.003	0.018	0.029	0.000
47	0.568	0.604	0.568	0.555	0.559	**0.571**	-0.003	0.033	-0.003	-0.016	-0.012
48	0.497	0.528	0.633	0.644	0.613	**0.583**	-0.086	-0.055	0.050	0.061	0.030
49	2.529	2.623	2.572	2.486	2.525	**2.547**	-0.018	0.076	0.025	-0.061	-0.022
50	0.400	0.410	0.387	0.365	0.374	**0.387**	0.013	0.023	0.000	-0.022	-0.013
51	0.364	0.371	0.360	0.337	0.346	**0.356**	0.008	0.016	0.004	-0.018	-0.010
52	0.506	0.529	0.498	0.464	0.479	**0.495**	0.011	0.034	0.003	-0.032	-0.016
53	0.980	1.035	0.965	0.921	0.948	**0.970**	0.011	0.065	-0.005	-0.049	-0.022
54	0.563	0.595	0.552	0.526	0.544	**0.556**	0.007	0.039	-0.004	-0.030	-0.012
55	0.185	0.196	0.176	0.167	0.174	**0.180**	0.006	0.016	-0.004	-0.012	-0.006
56	0.591	0.616	0.568	0.538	0.550	**0.572**	0.018	0.044	-0.005	-0.035	-0.022
57	0.198	0.201	0.185	0.176	0.180	**0.188**	0.010	0.013	-0.003	-0.012	-0.008
58	0.829	0.865	0.796	0.756	0.764	**0.802**	0.027	0.063	-0.006	-0.046	-0.038
59	0.484	0.496	0.468	0.442	0.452	**0.468**	0.016	0.027	0.000	-0.026	-0.017
60	0.497	0.527	0.480	0.452	0.462	**0.484**	0.013	0.044	-0.003	-0.032	-0.022
61	0.446	0.470	0.434	0.411	0.423	**0.437**	0.009	0.033	-0.003	-0.026	-0.014
62	0.567	0.610	0.557	0.527	0.538	**0.560**	0.007	0.051	-0.003	-0.033	-0.022
63	2.934	3.082	2.817	2.711	2.742	**2.857**	0.077	0.225	-0.040	-0.146	-0.115
64	1.162	1.232	1.161	1.070	1.083	**1.142**	0.020	0.090	0.019	-0.072	-0.058
65	0.431	0.453	0.427	0.396	0.399	**0.421**	0.010	0.032	0.005	-0.026	-0.022
66	1.605	1.663	1.563	1.472	1.486	**1.558**	0.047	0.105	0.006	-0.086	-0.072
67	3.772	3.880	3.654	3.489	3.562	**3.671**	0.100	0.209	-0.017	-0.182	-0.110
68	0.438	0.427	0.428	0.383	0.395	**0.414**	0.024	0.013	0.014	-0.031	-0.019

69	0.122	0.117	0.122	0.107	0.108	**0.115**	0.007	0.002	0.007	-0.008	-0.007
70	0.148	0.143	0.147	0.130	0.130	**0.140**	0.008	0.003	0.007	-0.010	-0.010
71	0.263	0.251	0.264	0.232	0.235	**0.249**	0.014	0.002	0.015	-0.017	-0.014
72	0.341	0.335	0.340	0.306	0.310	**0.326**	0.015	0.009	0.013	-0.020	-0.017
73	0.430	0.438	0.417	0.385	0.389	**0.412**	0.018	0.026	0.005	-0.027	-0.023
74	0.276	0.284	0.265	0.247	0.252	**0.265**	0.011	0.020	0.000	-0.018	-0.013
75	0.471	0.482	0.447	0.430	0.430	**0.452**	0.019	0.031	-0.005	-0.022	-0.022
76	0.370	0.376	0.356	0.330	0.338	**0.354**	0.016	0.022	0.002	-0.024	-0.016
77	0.524	0.534	0.494	0.454	0.469	**0.495**	0.029	0.039	-0.001	-0.041	-0.026
78	0.243	0.245	0.239	0.221	0.227	**0.235**	0.008	0.010	0.004	-0.014	-0.008
79	3.189	3.272	3.093	2.941	2.999	**3.099**	0.091	0.173	-0.006	-0.158	-0.100
80	0.191	0.179	0.195	0.189	0.193	**0.189**	0.001	-0.010	0.006	-0.001	0.003
81	0.107	0.107	0.106	0.102	0.103	**0.105**	0.002	0.002	0.001	-0.003	-0.002
82	0.475	0.598	0.194	0.219	0.209	**0.339**	0.136	0.259	-0.145	-0.120	-0.130
83	0.202	0.202	0.199	0.180	0.180	**0.193**	0.010	0.010	0.006	-0.013	-0.012
84	0.768	0.848	0.823	0.793	0.796	**0.805**	-0.038	0.043	0.017	-0.013	-0.010
85	0.864	0.907	0.903	0.851	0.867	**0.878**	-0.014	0.028	0.025	-0.027	-0.012
86	0.035	0.037	0.041	0.036	0.036	**0.037**	-0.002	0.000	0.004	-0.001	-0.001
87	0.047	0.050	0.053	0.047	0.047	**0.049**	-0.001	0.001	0.004	-0.002	-0.002
88	0.031	0.033	0.035	0.031	0.032	**0.033**	-0.001	0.000	0.003	-0.001	-0.001
89	0.031	0.034	0.036	0.031	0.032	**0.033**	-0.001	0.001	0.004	-0.001	-0.001
90	0.017	0.020	0.022	0.020	0.020	**0.020**	-0.003	0.000	0.003	0.000	0.000
91	0.023	0.027	0.028	0.026	0.026	**0.026**	-0.003	0.001	0.002	0.000	0.000
92	0.020	0.025	0.026	0.023	0.023	**0.023**	-0.003	0.002	0.002	0.000	-0.001
93	0.470	0.530	0.486	0.458	0.457	**0.480**	-0.010	0.050	0.006	-0.023	-0.023
94	0.074	0.084	0.081	0.070	0.071	**0.076**	-0.002	0.008	0.005	-0.006	-0.005
95	0.013	0.021	0.020	0.018	0.018	**0.018**	-0.005	0.003	0.002	0.000	0.000
96	0.012	0.019	0.017	0.016	0.016	**0.016**	-0.004	0.003	0.001	0.000	0.000
97	0.004	0.007	0.007	0.007	0.007	**0.006**	-0.002	0.001	0.001	0.000	0.000
98	0.139	0.161	0.146	0.144	0.145	**0.147**	-0.008	0.014	-0.001	-0.003	-0.002
99	0.241	0.256	0.228	0.222	0.223	**0.234**	0.007	0.022	-0.005	-0.012	-0.011
100	0.126	0.135	0.122	0.110	0.114	**0.121**	0.004	0.014	0.000	-0.011	-0.007
101	0.033	0.033	0.028	0.026	0.027	**0.029**	0.003	0.003	-0.001	-0.003	-0.002
102	0.256	0.275	0.248	0.228	0.233	**0.248**	0.008	0.027	0.000	-0.020	-0.015
103	0.030	0.035	0.034	0.030	0.031	**0.032**	-0.002	0.003	0.002	-0.002	-0.001
104	0.031	0.040	0.040	0.035	0.035	**0.036**	-0.005	0.004	0.004	-0.001	-0.001
105	0.024	0.034	0.033	0.031	0.030	**0.030**	-0.006	0.003	0.002	0.000	0.000
106	0.027	0.037	0.035	0.033	0.033	**0.033**	-0.006	0.004	0.002	0.000	0.000
107	0.004	0.008	0.007	0.007	0.007	**0.007**	-0.002	0.001	0.001	0.000	0.000
108	0.022	0.033	0.032	0.029	0.028	**0.029**	-0.007	0.005	0.003	0.000	-0.001
109	0.029	0.087	0.042	0.055	0.056	**0.054**	-0.025	0.033	-0.012	0.002	0.002
110	0.124	0.148	0.132	0.124	0.120	**0.130**	-0.006	0.018	0.003	-0.005	-0.009
111	0.029	0.035	0.032	0.030	0.029	**0.031**	-0.002	0.004	0.001	-0.001	-0.002
112	0.012	0.016	0.014	0.013	0.012	**0.013**	-0.001	0.003	0.001	-0.001	-0.001
113	0.041	0.053	0.048	0.047	0.047	**0.047**	-0.006	0.005	0.001	0.000	0.000
114	0.039	0.049	0.045	0.044	0.044	**0.044**	-0.005	0.005	0.001	0.000	0.000
115	0.055	0.062	0.056	0.055	0.054	**0.056**	-0.001	0.006	0.000	-0.002	-0.002
116	0.075	0.081	0.075	0.072	0.072	**0.075**	0.000	0.006	0.000	-0.003	-0.003
117	0.071	0.077	0.070	0.069	0.068	**0.071**	0.000	0.006	-0.001	-0.002	-0.003
118	0.050	0.057	0.050	0.048	0.049	**0.051**	-0.001	0.006	0.000	-0.003	-0.002
119	0.037	0.043	0.038	0.037	0.037	**0.038**	-0.002	0.004	0.000	-0.001	-0.002
120	0.021	0.025	0.023	0.022	0.022	**0.023**	-0.002	0.003	0.000	-0.001	-0.001
121	0.020	0.025	0.023	0.022	0.021	**0.022**	-0.003	0.003	0.001	0.000	-0.001
122	0.021	0.028	0.026	0.025	0.025	**0.025**	-0.003	0.003	0.001	0.000	0.000
123	0.047	0.066	0.061	0.058	0.059	**0.058**	-0.011	0.008	0.002	0.000	0.001
124	0.107	0.169	0.150	0.150	0.148	**0.145**	-0.038	0.024	0.005	0.005	0.003
125	0.014	0.023	0.019	0.019	0.018	**0.019**	-0.004	0.004	0.001	0.000	-0.001
126	0.016	0.023	0.021	0.020	0.020	**0.020**	-0.004	0.003	0.001	0.000	0.000
127	0.018	0.025	0.023	0.022	0.022	**0.022**	-0.004	0.003	0.001	0.000	0.000
128	0.012	0.019	0.017	0.016	0.016	**0.016**	-0.004	0.003	0.001	0.000	0.000
129	0.021	0.030	0.025	0.024	0.023	**0.025**	-0.003	0.006	0.000	-0.001	-0.002
130	0.022	0.032	0.023	0.020	0.019	**0.023**	-0.001	0.009	-0.001	-0.003	-0.004
131	0.025	0.037	0.026	0.024	0.022	**0.027**	-0.001	0.010	-0.001	-0.003	-0.005
132	0.025	0.036	0.026	0.024	0.023	**0.027**	-0.002	0.009	-0.001	-0.003	-0.004
133	0.015	0.025	0.022	0.021	0.019	**0.021**	-0.005	0.004	0.002	0.001	-0.002
134	0.045	0.064	0.057	0.051	0.043	**0.052**	-0.007	0.012	0.005	-0.001	-0.009
135	0.019	0.031	0.027	0.024	0.017	**0.024**	-0.004	0.007	0.004	0.000	-0.006
136	0.022	0.035	0.031	0.027	0.023	**0.028**	-0.006	0.007	0.003	0.000	-0.005
137	0.051	0.086	0.098	0.099	0.082	**0.083**	-0.032	0.003	0.015	0.016	-0.001

138	0.058	0.071	0.134	0.149	0.124	**0.107**	-0.049	-0.036	0.027	0.042	0.017
139	0.055	0.064	0.126	0.143	0.119	**0.101**	-0.047	-0.037	0.025	0.041	0.017
140	0.011	0.016	0.015	0.015	0.014	**0.014**	-0.003	0.002	0.001	0.001	-0.001
141	0.021	0.027	0.047	0.050	0.044	**0.038**	-0.017	-0.011	0.009	0.012	0.006
142	0.004	0.008	0.006	0.006	0.005	**0.006**	-0.002	0.002	0.001	0.000	0.000
143	0.008	0.013	0.012	0.011	0.011	**0.011**	-0.003	0.002	0.001	0.000	0.000
144	0.006	0.013	0.012	0.011	0.011	**0.011**	-0.004	0.003	0.001	0.001	0.000
145	0.009	0.015	0.014	0.014	0.014	**0.013**	-0.004	0.002	0.001	0.001	0.001
146	0.009	0.015	0.014	0.014	0.013	**0.013**	-0.004	0.002	0.001	0.001	0.000
147	0.030	0.039	0.036	0.036	0.036	**0.036**	-0.006	0.004	0.001	0.001	0.001
148	0.023	0.030	0.027	0.026	0.026	**0.026**	-0.004	0.003	0.001	0.000	0.000
149	0.025	0.034	0.030	0.030	0.030	**0.030**	-0.005	0.004	0.001	0.000	0.000
150	0.015	0.021	0.019	0.018	0.018	**0.018**	-0.003	0.003	0.001	0.000	0.000
151	0.544	0.596	0.557	0.545	0.556	**0.560**	-0.016	0.036	-0.003	-0.014	-0.003
152	0.520	0.556	0.518	0.506	0.517	**0.524**	-0.004	0.033	-0.005	-0.017	-0.006
153	0.362	0.405	0.366	0.362	0.370	**0.373**	-0.010	0.032	-0.007	-0.011	-0.003
154	0.059	0.078	0.074	0.071	0.071	**0.071**	-0.012	0.008	0.003	0.001	0.000
155	0.252	0.278	0.239	0.222	0.226	**0.243**	0.008	0.035	-0.004	-0.021	-0.018
156	0.129	0.150	0.135	0.123	0.125	**0.132**	-0.004	0.017	0.003	-0.009	-0.007
157	0.206	0.239	0.221	0.217	0.216	**0.220**	-0.014	0.019	0.001	-0.003	-0.004
158	0.090	0.100	0.094	0.093	0.093	**0.094**	-0.004	0.006	0.000	-0.001	-0.001
159	0.062	0.069	0.063	0.062	0.063	**0.064**	-0.002	0.005	-0.001	-0.002	-0.001
160	0.065	0.073	0.066	0.064	0.066	**0.067**	-0.001	0.006	-0.001	-0.003	-0.001
161	0.069	0.078	0.071	0.071	0.071	**0.072**	-0.003	0.006	-0.001	-0.001	-0.001
162	0.060	0.071	0.066	0.064	0.065	**0.065**	-0.005	0.006	0.000	-0.001	-0.001
163	0.018	0.024	0.022	0.021	0.021	**0.021**	-0.004	0.003	0.001	0.000	0.000
164	0.002	0.017	0.015	0.014	0.013	**0.012**	-0.010	0.005	0.002	0.002	0.001
165	0.015	0.024	0.023	0.023	0.023	**0.022**	-0.007	0.003	0.001	0.001	0.001
166	0.015	0.024	0.023	0.023	0.023	**0.022**	-0.007	0.003	0.001	0.001	0.001
167	-0.003	0.000	0.000	0.000	0.000	**-0.001**	-0.002	0.001	0.001	0.000	0.000
168	-0.004	0.000	0.000	0.000	0.000	**-0.001**	-0.003	0.001	0.000	0.001	0.001
169	-0.024	0.000	-0.004	-0.004	-0.003	**-0.007**	-0.017	0.007	0.003	0.003	0.004
170	-0.002	0.001	0.000	0.000	0.000	**0.000**	-0.002	0.001	0.000	0.000	0.000
171	0.027	0.045	0.041	0.044	0.044	**0.040**	-0.013	0.005	0.001	0.003	0.004
172	0.008	0.012	0.013	0.010	0.011	**0.011**	-0.003	0.001	0.002	-0.001	0.000
173	0.022	0.027	0.025	0.025	0.025	**0.025**	-0.002	0.002	0.000	0.000	0.000
174	0.047	0.054	0.053	0.051	0.053	**0.051**	-0.004	0.003	0.001	0.000	0.001
175	0.056	0.061	0.060	0.058	0.061	**0.059**	-0.004	0.002	0.001	-0.001	0.001
176	0.081	0.088	0.086	0.083	0.086	**0.085**	-0.004	0.003	0.001	-0.002	0.001
177	0.076	0.082	0.083	0.081	0.083	**0.081**	-0.005	0.001	0.002	0.000	0.002
178	0.056	0.062	0.061	0.059	0.061	**0.060**	-0.004	0.002	0.001	-0.001	0.001
179	0.023	0.038	0.011	0.019	0.016	**0.021**	0.001	0.017	-0.011	-0.002	-0.005
180	0.001	0.007	0.004	0.003	0.006	**0.004**	-0.004	0.003	0.000	-0.001	0.002
181	0.007	0.013	0.012	0.011	0.014	**0.011**	-0.004	0.002	0.000	0.000	0.002
182	0.048	0.054	0.045	0.047	0.046	**0.048**	-0.001	0.006	-0.003	-0.001	-0.002
183	0.085	0.106	0.071	0.079	0.076	**0.084**	0.001	0.023	-0.012	-0.004	-0.007
184	0.114	0.141	0.186	0.198	0.158	**0.159**	-0.046	-0.018	0.027	0.039	-0.002
185	0.006	0.014	0.010	0.011	0.008	**0.010**	-0.004	0.004	0.000	0.001	-0.002
186	0.043	0.054	0.050	0.050	0.049	**0.049**	-0.006	0.005	0.000	0.001	0.000
187	0.032	0.037	0.037	0.037	0.037	**0.036**	-0.004	0.001	0.001	0.001	0.001
188	0.047	0.053	0.050	0.049	0.049	**0.050**	-0.002	0.003	0.000	-0.001	0.000
189	0.064	0.078	0.137	0.151	0.122	**0.110**	-0.046	-0.033	0.026	0.041	0.011
190	-0.001	0.000	0.000	0.000	0.000	**0.000**	-0.001	0.000	0.000	0.000	0.000
191	-0.001	0.000	0.000	0.000	0.000	**0.000**	-0.001	0.000	0.000	0.000	0.000
192	-0.001	0.000	0.000	0.000	0.000	**0.000**	-0.001	0.000	0.000	0.000	0.000
193	-0.001	0.000	0.000	0.000	0.000	**0.000**	-0.001	0.000	0.000	0.000	0.000
194	-0.001	-0.001	-0.001	-0.001	-0.001	**-0.001**	0.000	0.000	0.000	0.000	0.000
195	0.002	0.002	0.002	0.002	0.002	**0.002**	0.000	0.000	0.000	0.000	0.000
196	-0.001	0.000	0.000	-0.001	0.000	**-0.001**	0.000	0.000	0.000	0.000	0.000
197	0.006	0.005	0.005	0.005	0.005	**0.005**	0.001	0.000	0.000	0.000	0.000
198	0.004	0.003	0.004	0.003	0.003	**0.003**	0.001	0.000	0.000	0.000	0.000
199	0.007	0.008	0.010	0.010	0.010	**0.009**	-0.002	-0.001	0.001	0.001	0.001
							∑	∑	∑	∑	∑
							-2.285	**7.460**	**-0.559**	**-3.257**	**-1.360**

Table S3. Integral values of spectra referred to internal standards (TSP or TMS) of polar metabolites extracted from *Erica multiflora, Quercus ilex*, and *Fagus sylvatica* foliar samples submitted to different numbers of extractions (1, 2, or 3). The integral value corresponds to a mean of six individuals. The integral values for each bucket were compared (1 extraction vs. 2 extractions, and 2 extractions vs. 3 extractions). ANOVA tests of the entire polar metabolome were performed in these comparisons.

Erica multiflora					
	Number of Extractions			**Comparisons**	
	1	**2**	**3**	**1 vs. 2 (%)**	**2 vs. 3 (%)**
1	1.150	1.506	1.512	76.386	99.559
2	1.710	2.123	2.155	80.563	98.536
3	1.380	1.994	1.988	69.217	100.300
4	0.945	1.271	1.294	74.355	98.286
5	2.037	2.577	2.611	79.046	98.734
6	1.175	1.488	1.468	78.966	101.350
7	1.509	1.966	1.997	76.745	98.468
8	2.786	3.490	3.520	79.815	99.141
9	2.761	3.474	3.412	79.493	101.795
10	3.810	4.737	4.769	80.421	99.339
11	2.005	2.456	2.490	81.629	98.635
12	2.009	2.323	2.349	86.464	98.926
13	1.172	1.541	1.527	76.053	100.953
14	1.579	2.005	2.080	78.751	96.400
15	1.291	1.667	1.694	77.434	98.371
16	0.461	0.646	0.680	71.330	95.004
17	0.660	0.916	0.926	72.080	98.970
18	0.204	0.339	0.336	60.171	100.964
19	0.225	0.338	0.346	66.616	97.636
20	0.423	0.584	0.642	72.416	90.988
21	0.310	0.415	0.403	74.755	103.151
22	0.289	0.401	0.392	72.228	102.138
23	0.408	0.586	0.595	69.664	98.451
24	0.874	1.200	1.230	72.861	97.530
25	0.447	0.686	0.698	65.175	98.292
26	0.811	1.106	1.125	73.345	98.274
27	0.601	0.848	0.843	70.875	100.664
28	0.779	1.056	1.082	73.727	97.588
29	0.825	1.106	1.219	74.607	90.741
30	1.220	1.653	1.710	73.847	96.632
31	5.070	6.473	6.514	78.325	99.364
32	2.963	3.818	3.922	77.608	97.347
33	2.226	2.931	2.943	75.936	99.592
34	13.892	17.380	17.318	79.930	100.353
35	2.191	2.517	2.684	87.034	93.771
36	2.763	3.367	3.422	82.051	98.410
37	1.751	2.098	2.125	83.479	98.689
38	0.936	1.136	1.157	82.395	98.214
39	1.342	1.688	1.741	79.472	96.967
40	4.580	5.622	5.765	81.466	97.524
41	2.564	3.233	3.255	79.291	99.343
42	1.117	1.398	1.483	79.919	94.228
43	3.782	4.741	4.892	79.766	96.915
44	4.828	5.880	5.798	82.108	101.419
45	2.285	2.779	2.789	82.225	99.609
46	10.094	12.346	12.894	81.758	95.752
47	8.851	10.681	9.988	82.866	106.940
48	7.741	9.488	9.781	81.584	97.006
49	0.572	0.645	0.711	88.754	90.765
50	1.549	1.777	1.758	87.205	101.046
51	0.880	1.044	1.152	84.265	90.680
52	4.466	5.447	5.568	81.995	97.816
53	0.241	0.304	0.315	79.156	96.495
54	1.514	1.784	1.820	84.877	97.986
55	0.440	0.502	0.510	87.653	98.395

56	0.343	0.363	0.378	94.613	95.882
57	0.346	0.417	0.403	83.014	103.575
58	0.339	0.408	0.410	83.126	99.448
59	0.342	0.444	0.442	76.975	100.420
60	1.369	1.686	1.783	81.201	94.522
			Average %	**78.485**	**98.238**
			p_value	**0.32**	**0.97**

Quercus ilex

	Number of Extractions			**Comparisons**	
	1	**2**	**3**	**1 vs. 2 (%)**	**2 vs. 3 (%)**
1	0.407	0.431	0.432	94.361	99.779
2	1.967	1.941	1.975	101.349	98.265
3	0.644	0.703	0.710	91.629	98.917
4	0.633	0.689	0.685	91.852	100.602
5	1.165	1.392	1.385	83.672	100.472
6	0.743	1.030	1.084	72.099	95.011
7	1.267	1.572	1.584	80.632	99.224
8	2.174	2.631	2.622	82.634	100.352
9	1.217	1.643	1.649	74.073	99.666
10	2.090	2.504	2.492	83.479	100.471
11	1.383	1.505	1.513	91.910	99.439
12	0.986	0.968	0.978	101.887	98.888
13	0.423	0.262	0.268	161.551	97.594
14	0.186	0.142	0.155	131.316	91.489
15	0.340	0.305	0.355	111.620	85.939
16	0.295	0.307	0.300	96.025	102.446
17	0.264	0.278	0.279	94.980	99.746
18	0.185	0.179	0.182	103.562	98.123
19	0.375	0.356	0.368	105.483	96.589
20	0.333	0.307	0.349	108.746	87.939
21	0.577	0.610	0.612	94.584	99.606
22	1.525	1.915	1.895	79.656	101.019
23	0.519	0.590	0.595	87.986	99.124
24	0.633	0.725	0.711	87.300	102.021
25	0.585	0.687	0.698	85.096	98.394
26	0.420	0.483	0.492	86.921	98.217
27	1.368	1.467	1.498	93.218	97.928
28	2.054	2.489	2.522	82.519	98.700
29	1.384	1.838	1.811	75.340	101.490
30	0.785	1.049	1.098	74.799	95.513
31	0.682	0.798	0.800	85.434	99.719
32	7.909	9.899	9.922	79.894	99.775
33	1.002	1.354	1.383	73.997	97.913
34	1.081	1.255	1.268	86.148	98.967
35	1.155	1.239	1.249	93.166	99.260
36	5.722	7.330	7.311	78.057	100.265
37	3.211	3.942	3.973	81.458	99.226
38	3.287	4.165	4.016	78.915	103.713
39	2.732	3.401	3.383	80.306	100.540
40	2.321	2.929	2.910	79.247	100.637
41	1.636	2.211	2.283	73.990	96.852
42	2.433	3.016	3.083	80.684	97.810
43	8.711	10.872	10.948	80.124	99.307
44	4.386	7.016	7.102	62.517	98.788
45	1.709	2.244	2.302	76.170	97.476
46	3.440	4.841	7.850	71.059	61.667
47	3.029	4.039	4.083	74.989	98.925
48	1.201	1.485	1.510	80.881	98.357
49	3.941	4.868	4.981	80.956	97.735
50	1.541	2.204	2.282	69.902	96.570
51	0.784	1.278	1.283	61.397	99.562
52	1.506	2.082	2.083	72.342	99.918
53	1.410	1.650	1.641	85.480	100.582
54	1.208	1.918	1.921	62.976	99.883
55	0.263	0.319	0.321	82.476	99.485
56	0.212	0.273	0.295	77.546	92.438

57	0.200	0.287	0.285	69.663	100.613
58	0.169	0.233	0.241	72.606	96.721
59	2.282	2.526	2.053	90.317	123.058
			Average %	**85.644**	**98.283**
			p_value	**0.26**	**0.89**

Fagus sylvatica

	Number of Extractions			**Comparisons**	
	1	**2**	**3**	**1 vs. 2 (%)**	**2 vs. 3 (%)**
1	1.647	1.720	1.691	95.715	101.715
2	2.290	2.283	2.301	100.323	99.201
3	2.726	2.676	2.734	101.895	97.863
4	5.022	5.011	5.034	100.209	99.550
5	0.274	0.218	0.251	125.987	86.662
6	1.764	1.649	1.751	106.931	94.177
7	1.431	1.246	1.245	114.852	100.042
8	1.144	1.011	1.150	113.169	87.878
9	0.403	0.349	0.414	115.610	84.339
10	0.170	0.177	0.178	95.978	99.250
11	0.316	0.325	0.335	97.093	97.082
12	0.187	0.193	0.192	97.024	100.413
13	0.411	0.414	0.420	99.389	98.454
14	0.858	0.860	0.862	99.755	99.802
15	0.572	0.594	0.580	96.377	102.340
16	0.589	0.617	0.610	95.437	101.168
17	0.182	0.194	0.195	93.474	99.597
18	0.528	0.552	0.540	95.627	102.180
19	0.561	0.592	0.583	94.818	101.528
20	1.221	1.245	1.260	98.029	98.854
21	2.365	2.428	2.430	97.418	99.898
22	1.476	1.540	1.543	95.814	99.837
23	0.623	0.655	0.643	95.077	101.859
24	0.459	0.500	0.516	91.923	96.910
25	2.770	2.753	2.775	100.611	99.206
26	8.807	8.981	8.871	98.065	101.248
27	2.049	2.027	2.035	101.045	99.619
28	2.156	2.143	2.168	100.564	98.850
29	1.346	1.352	1.365	99.546	99.044
30	1.159	1.175	1.187	98.586	99.049
31	7.784	7.947	7.935	97.944	100.151
32	1.592	1.574	1.592	101.144	98.867
33	2.070	2.117	2.216	97.767	95.563
34	2.796	2.820	2.843	99.157	99.174
35	3.941	3.999	4.001	98.547	99.956
36	17.941	18.055	18.055	99.370	100.003
37	1.908	1.778	1.955	107.355	90.930
38	3.978	3.939	3.942	100.993	99.916
39	2.768	2.699	2.751	102.538	98.108
40	2.006	1.968	2.016	101.925	97.627
41	2.563	2.590	2.610	98.977	99.219
42	1.166	1.121	1.178	103.978	95.131
43	2.030	1.914	2.057	106.103	93.047
44	0.511	0.444	0.549	115.169	80.940
45	0.388	0.334	0.368	116.214	90.543
46	3.321	3.337	3.349	99.535	99.647
			Average %	**101.371**	**97.531**
			p_value	**0.996**	**0.97**

Table S4. Number assignation for the polar compounds elucidated and the overlapped ^{1}H-NMR signals.

Elucidated compounds			Overlapped compounds		
Given reference number	Compound		Given Overlapped reference number	Reference numbers of overlapped compounds	Compounds overlapped
1	α-glucose (αG)		30	11+15	4-hydroxyphenylacetate (11), 3-amino-4-hydroxybutyrate (15),
2	β-glucose (βG)		31	5+11	asparagine (Asp), 4-hydroxyphenylacetate (11),
3	sucrose (Suc)		32	5+11+13	asparagine (Asp), 4-hydroxyphenylacetate (11), maleate (13),
4	alanine (Ala)		33	6+21	glutamine (Gln), 1,2-propanediol (21),
5	asparagine (Asp)		34	12+unknown	Malate, unknown,
6	glutamine (Gln)		35	15+22	3-amino-4-hydroxybutyrate (15), γ-hydroxybutyrate (22),
7	leucine (Leu)		36	16+19	N-acetyl group (N-Ac), arbutin (Arb),
8	isoleucine (Ile)		37	3+16+19	sucrose (Suc), N-acetyl group (N-Ac), arbutin (Arb),
9	threonine (Thr)		38	6+16	glutamine (Gln), N-acetyl group (N-Ac),
10	6-deoxypyranose		39	1+2+3+16+18	α-glucose (αG), β-glucose (βG), sucrose (Suc), N-acetyl group (N-Ac),
11	4-hydroxyphenylacetate		40	1+2+3+5+16+18	α-glucose (αG), β-glucose (βG), sucrose (Suc), asparagine (Asp), N-acetyl group (N-Ac), tartaric acid (T.ac),
12	malate		41	1+2+3+16	α-glucose (αG), β-glucose (βG), sucrose (Suc), N-acetyl group (N-Ac),
13	maleate		42	11+14	4-hydroxyphenylacetate (11), citrate (14),

14	citrate		43	9+14+22	threonine (Thr), citrate (14), γ-hydroxybutyrate (22),
15	3-amino-4-hydroxybutyrate		44	13+14	maleate (13), citrate (14),
16	N-acetyl group (N-Ac)		45	1+2+9	α-glucose (αG), β-glucose (βG), threonine (Thr),
17	quinic acid (Q.ac)		46	1+2+3+18	α-glucose (αG), β-glucose (βG), sucrose (Suc), tartaric acid (T.ac),
18	tartaric acid (T.ac)		47	1+2+3+18+19	α-glucose (αG), β-glucose (βG), sucrose (Suc), tartaric acid (T.ac), arbutin (Arb),
19	arbutin (Arb)		48	1+2+3+18+19+20	α-glucose (αG), β-glucose (βG), sucrose (Suc), tartaric acid (T.ac), arbutin (Arb), choline (Ch),
20	choline (Ch)		49	1+2+3+9+18+21	α-glucose (αG), β-glucose (βG), sucrose (Suc), threonine (Thr), tartaric acid (T.ac), 1,2-propanediol (21),
21	1,2-propanediol		50	1+2+3+unknown	α-glucose (αG), β-glucose (βG), sucrose (Suc), unknown
22	γ-hydroxybutyrate		51	1+2+18	α-glucose (αG), β-glucose (βG), tartaric acid (T.ac),
23	lactate		52	1+2+9+18	α-glucose (αG), β-glucose (βG), threonine (Thr), tartaric acid (T.ac),
24	valine (Val)		53	1+2+6+18	α-glucose (αG), β-glucose (βG), glutamine (Gln), tartaric acid (T.ac),
25	quercitol (Quer)		54	2+20	β-glucose (βG), choline (Ch),
26	formate (For)		55	2+3+18+19.	β-glucose (βG), sucrose (Suc), tartaric acid (T.ac), arbutin (Arb),

Figure S1. Example of the ^{1}H NMR spectra of the polar and nonpolar extracts of *Erica multiflora* leaves. The main families of compounds observed in the different regions of the spectra are indicated by: **A**, typically amino acids; **B**, mainly sugars; **C**, aromatic region; **D**, fatty chains; **E**, typically tri-, di-, and monoacylglycerides; **F**, polyphenol region; **G**, aldehyde region.

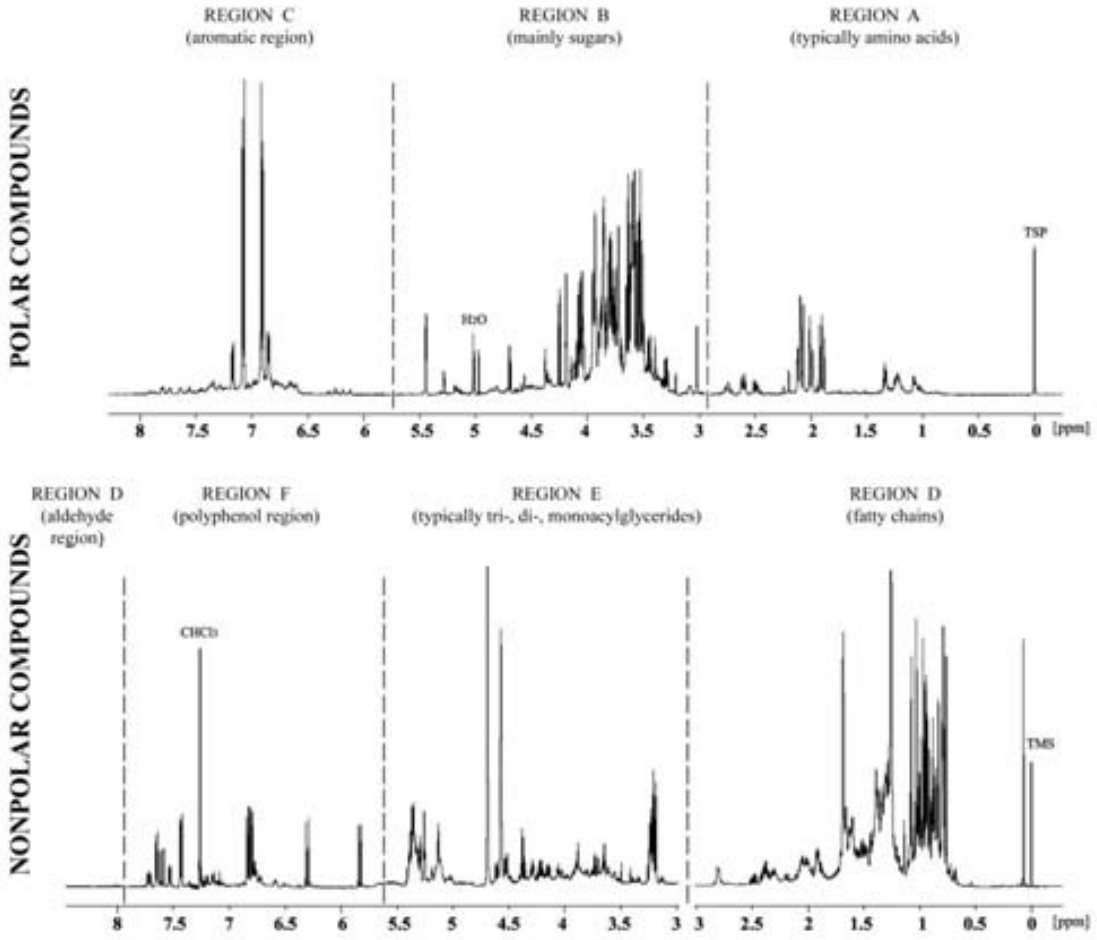


Figure S2. One-way ANOVA of the entire polar metabolome of *Erica multiflora* submitted to different sonication times (0, 2, 5, 12, and 20 minutes). The ANOVA test was performed by using the integral values extracting the mean of all different groups.

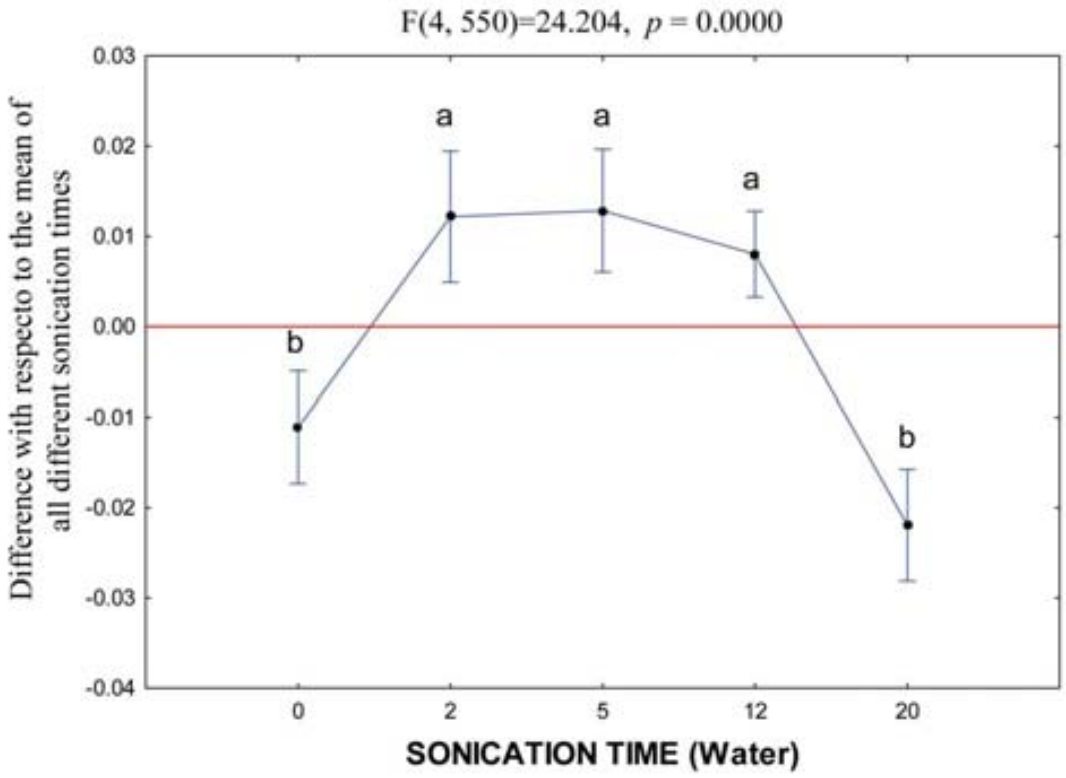


Figure S3. One-way ANOVA of the entire nonpolar metabolome of *Erica multiflora* submitted to different sonication times (0, 2, 5, 12, and 20 minutes). The ANOVA test was performed by using the integral values extracting the mean of all different groups.

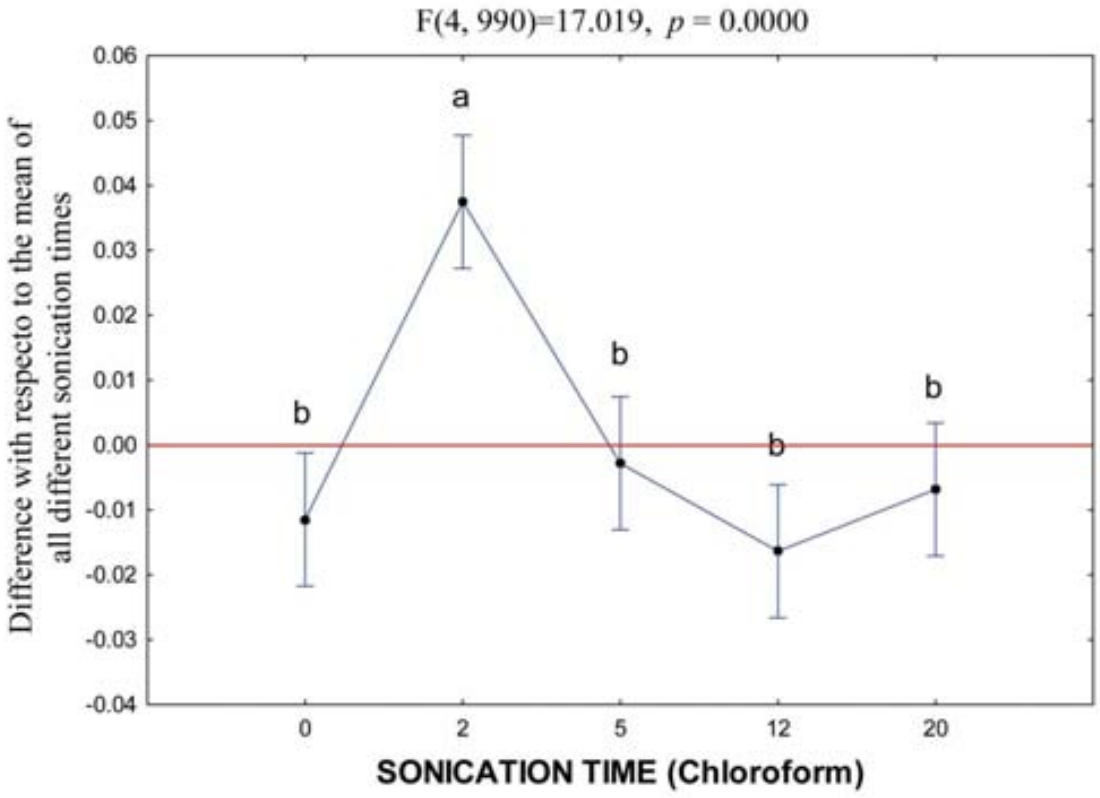


Figure S4. Recovery test for alanine in *Erica multiflora* foliar samples. We used three pure alanine NMR samples, six samples from the *E. multiflora* polar extract with pure alanine, and 12 samples from the *E. multiflora* polar extract only.

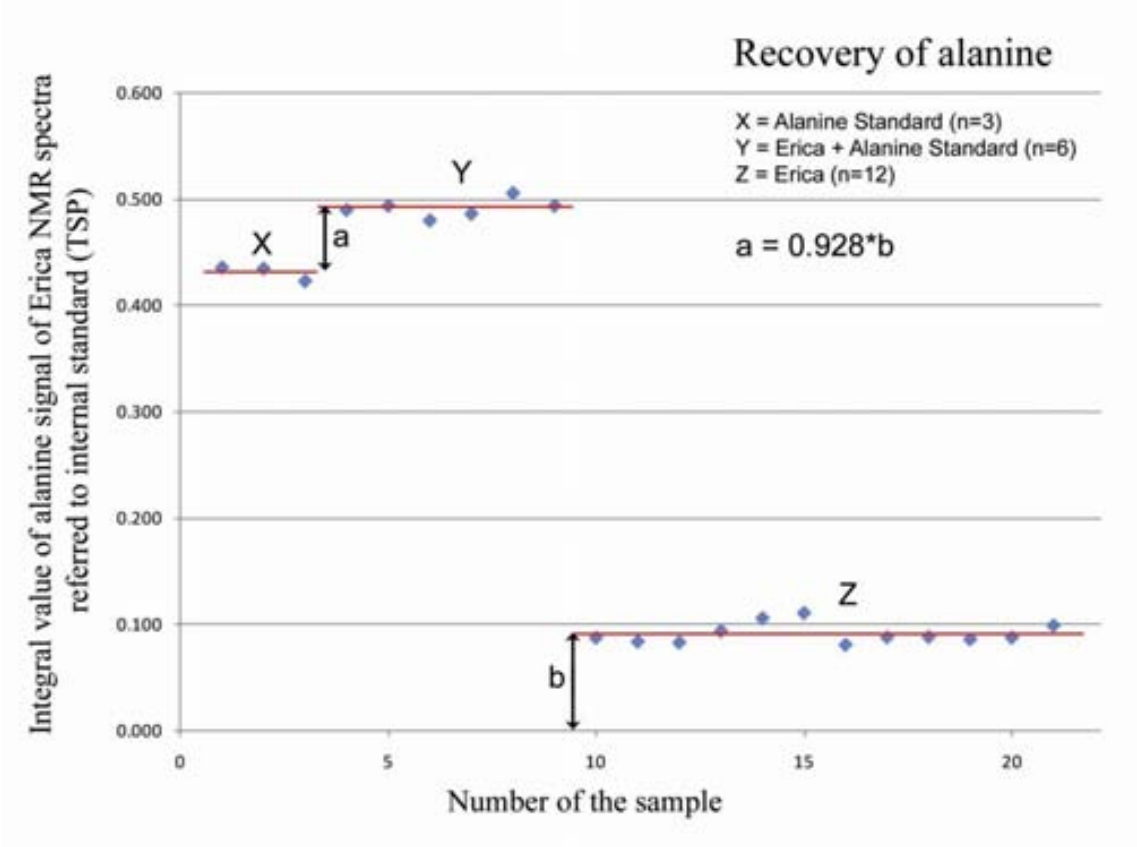
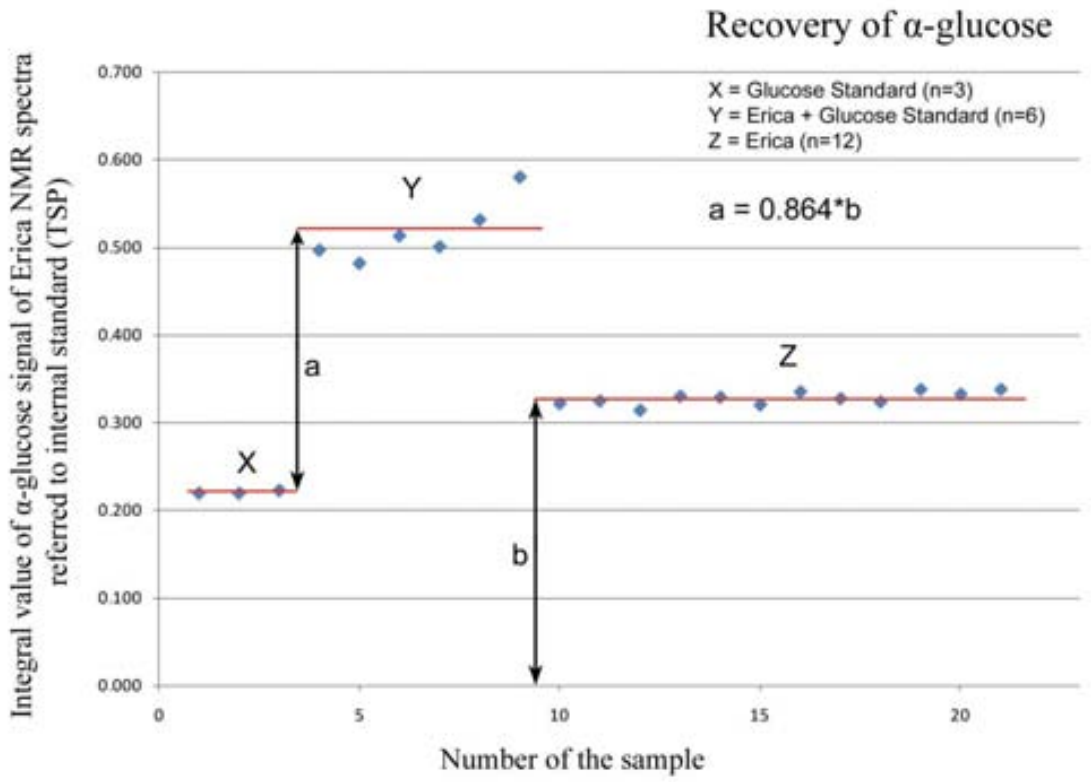


Figure S5. Recovery test for glucose in *Erica multiflora* foliar samples. We used three pure glucose NMR samples, six samples from the *E. multiflora* polar extract with pure glucose, and 12 samples from the *E. multiflora* polar extract only.



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APPENDIX 3

Rivas-Ubach et al., Proceedings of the National Academy of Sciences USA (2012) 109: 4181-4186.

Materials & Methods

Study site & experimental design

The study was conducted in the Garraf Natural Park located in the Catalonian central coast (41˚ 18' N, 1˚49' E). Climate is Mediterranean with a summer drought pronounced for 3 months. The average annual rainfall is between 550 and 600 mm. The maximum rainfall usually occurs in autumn season between September and December. Winter average temperatures are around 7˚C and warmest summers present an average temperature between 22˚C and 24˚C.

Nine plots were established in March 1999. Six plots present a climatic change treatment, three of them a night-time warming and the other three drought. Warming treatment increases temperature during the night by covering the vegetation with a reflective cover. These covers are automatically retracted when it rains thus avoiding water exclusion. Drought treatment reduces rainfall input during spring and autumn (Mediterranean rainy seasons). This treatment is achieved by covering automatically the plot vegetation with a transparent plastic canopy during rain events and removing it when rain stops. For details see (*33, 43* of the manuscript references list).

Sampling and processing of leaves

Samplig was conducted once per season from spring to winter (2009-2010). Five individuals in each plot were marked randomly as study subjects. A homogeneous fraction of youngest leaves from each individual was frozen *in situ* into a liquid nitrogen. Frozen leaves were lyophilized. Dried samples were ground with a Braun Mikrodismembrator-U (B. *Braun* Biotech International, Melsungen, Germany) at 1600rpm for 8 minutes. All sample powders were then maintained at -20˚C until NMR extract preparation.

Leaves powder (200 mg) was introduced into a centrifuge tube. Six mL of 50% water-methanol mixture and 6 mL of Chloroform were added to each tube (*45* of the manuscript references list). Samples were mixed during 15 seconds by vortex and then 1 minute sonicated at room temperature. All tubes were centrifuged at 3.000 rpm during 30 minutes. Four mL of each fraction (aqueous (polar phase) and organic (non-polar phase)) were collected independently into jars. This procedure was repeated twice obtaining 8 mL of aqueous and organic soluble fractions (AF and OF respectively) for each sample. Organic fractions were collected by crystal syringes.

One hundred mL of water were added to AF samples in order to reduce the methanol percentage to 5% approximately. Samples were frozen at -80 ˚C and lyophilized. OF samples were placed separately in a round-bottom evaporation flask and dried in a rotary vacuum evaporator.

For the NMR analyses, 1 ml of KH_2PO_4-NaOD buffer in D_2O + 0.01% TSP (trimethyl silane propionic acid sodim salt) was added to AF dried samples obtaining a solution with final pH=6.0.

One mL of Chloroform D + 0.01% TMS (tetramethylsilyl) was added to OF dried samples. TSP and TMS were used as internal standards. All content was transferred into Eppendorfs and centrifuged 3 min at 6.000 rpm and 2 min at 10.000 rpm. For each sample, 0.6 mL of supernatant was transferred into NMR sample tubes.

NMR experiments

NMR data acquisition for the metabolomic study was conducted through high-resolution ^{1}H NMR spectroscopy measurements. ^{1}H NMR spectra were acquired and processed, based on described protocols with little modifications (*1-3*). A Bruker AVANCE 600 spectrometer fitted with an automatic sample changer and a multinuclear triple resonance (TBI) probe (Bruker Biospin, Rheinstetten, Germany) and working at a field strength of 14.1 T (600.13 MHz frequency of ^{1}H) was used. The probe temperature was maintained at 298.0 K. Sample handling, automation, acquisition and processing were controlled using TOPSPIN 2.1 software (Bruker Biospin, Rheinstetten, Germany).

Samples were allowed to equilibrate (1 min) in the magnet before the acquisition started. In the case of the D_2O samples (polar phase), a conventional composite 90° ^{1}H pulse sequence with suppression of the residual water signal was used (*4-6*). The water resonance was presaturated using a power level of 55 dB during a relaxation delay (RD) of 2 s. Spectra were collected in the time domain as free induction decays (FIDs) across a spectral width of 9615 Hz and during an acquisition time (AQS) of 1.7 s. A total of 128 transients were acquired into 32k data points. Each FID was zero-filled to 64k data points and multiplied by an exponential apodization function equivalent to a 0.2 Hz line broadening, prior to Fourier transformation. The frequency-domain spectra were automatically phase and baseline corrected, and manually referenced to the TSP residual resonance at δ_H 0.00 ppm.

In the case of the $CDCl_3$ samples (non-polar phase), a standard 90° ^{1}H pulse sequence was used. A total of 128 transients were acquired into 32k data points across a spectral width of 9591 Hz and during an AQS of 1.7 s and a RD of 2 s. As for the D_2O samples, FIDs were zero-filled to 64k data points and an exponential apodization function equivalent to a 0.2 Hz line broadening was applied to the FID prior to Fourier transformation. Spectra were then automatically phase and baseline corrected, and manually referenced to the TMS residual resonance at δ_H 0.00 ppm. In all cases (D_2O and $CDCl_3$), when the automatic phase correction was not accurate, spectra were manually phase corrected.

Typical polar and non-polar 1D ^{1}H NMR spectra profiles of *Erica Multiflora* leaves are presented in Figure 1 of the manuscript (also in detail in Figures S2 and S3 and Table S4).

NMR Elucidation

For spectral resonance assignment purposes, 1D and 2D NMR experiments were performed in selected samples of the water/methanol and chloroform extracts. A Bruker AVANCE 500 spectrometer (Bruker Biospin, Rheinstetten, Germany) equipped with a 5 mm high-sensitivity cryogenically cooled triple-resonance TCI probe with Z-gradients, operating at a field strength of 11.7 T (500.13 MHz ^{1}H and 125.76 MHz ^{13}C resonance frequencies), was used to perform the experiments. The probe temperature was maintained at 298.0 K. Sample acquisition and processing were controlled using TOPSPIN 1.3 software (Bruker Biospin, Rheinstetten, Germany).

1D ^{1}H NMR experiment and 2D ^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{1}H total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC) and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC), were acquired using standard Bruker pulse sequences and routine conditions (3). In the case of the water/methanol extract samples, 2D experiments were carried out with standard presaturation of the residual water peak. Spectra were referenced to TSP (^{1}H and ^{13}C at δ_H 0.00 ppm) in the case of the D_2O samples and to the residual $CHCl_3$ (δ_H 7.260 ppm and 77.00 ppm for ^{1}H and ^{13}C respectively) in the chloroform samples.

The concerted use of 2D ^{1}H-^{1}HCOSYand ^{1}H-^{13}C HSQC/HMBC experiments allowed the assignment of most of the peaks in the polar and non-polar 1D ^{1}H NMR spectra profiles. When possible, assignments were confirmed with described data (bibliography and NMR spectral data bases).

^{1}H NMR spectral resonance assignments, structural elucidated metabolites and an example of a ^{1}H NMR spectrum with the main peaks assigned of the water/methanol extracts are shown in Table S4 and Figure S3. The analogue information for the chloroform extracts are collected in Table S4 and Figure S2.

Chemical analyses

C and N analyses: For each sample, 1.4 g of powder were weighted and introduced into a tin microcapsule. C and N concentrations were determined by elemental analysis using combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).

P and K analyses: The P and K analyses determination were performed using acid digestion into microwave with high pressure and temperature control. Briefly, 250 mg of leaf powder were weighted into a Teflon tube and 5mL of Nitric acid and 2mL of H_2O_2 were added (7). A **MARSXpress** microwave (CEM, Mattheus, USA) was used to perform the acid digestions; temperature was increased until 130˚ with a 10min ramp, samples were maintained at this temperature for 5min, then a 10min ramp increases temperature to 200˚C and samples were

maintained during 20min. To finish digestions temperature was increased to 220˚C with a 5min ramp and maintained for 20min more.

All the digested contents were put into 50 mL flasks and dissolved with Milli-Q water until 50mL. After digestions, the P and K were determined by *Optima 2300RL* ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) (The Perkin-Elmer Corporation, Norwalk, USA).

Buketing procedure

A peak pattern for each sample type (polar and non-polar) was created; the pattern consists to identify exactly where a peak begins and where it ends for all existing spectra peaks. After that, the "*variable size bucket table*" option of AMIX software (Bruker Biospin, Rheinstetten, Germany) was executed for the spectra obtaining all peak integral values scaled respect internal standards (*Bucketing*).

Figure S1. PCA plot from ^{1}H-NMR metabolomic analyses of *E. multiflora* leaves using PC1 and PC3 axes. **(a)**. Panel of metabolomic variables. Different metabolic families are grouped by different colors (Blue = Sugars, green = amino acids, yellow = compounds related to amino acids and sugars plant metabolism (RCAAS), violet = secondary polar metabolites, black = non-polar metabolites) [assignments are described in Fig. 1 of the manuscript] **(b)**. Panel of cases represented by seasons. Seasons are represented by different colors (Red = summer, yellow = autumn, blue = winter and green = spring).

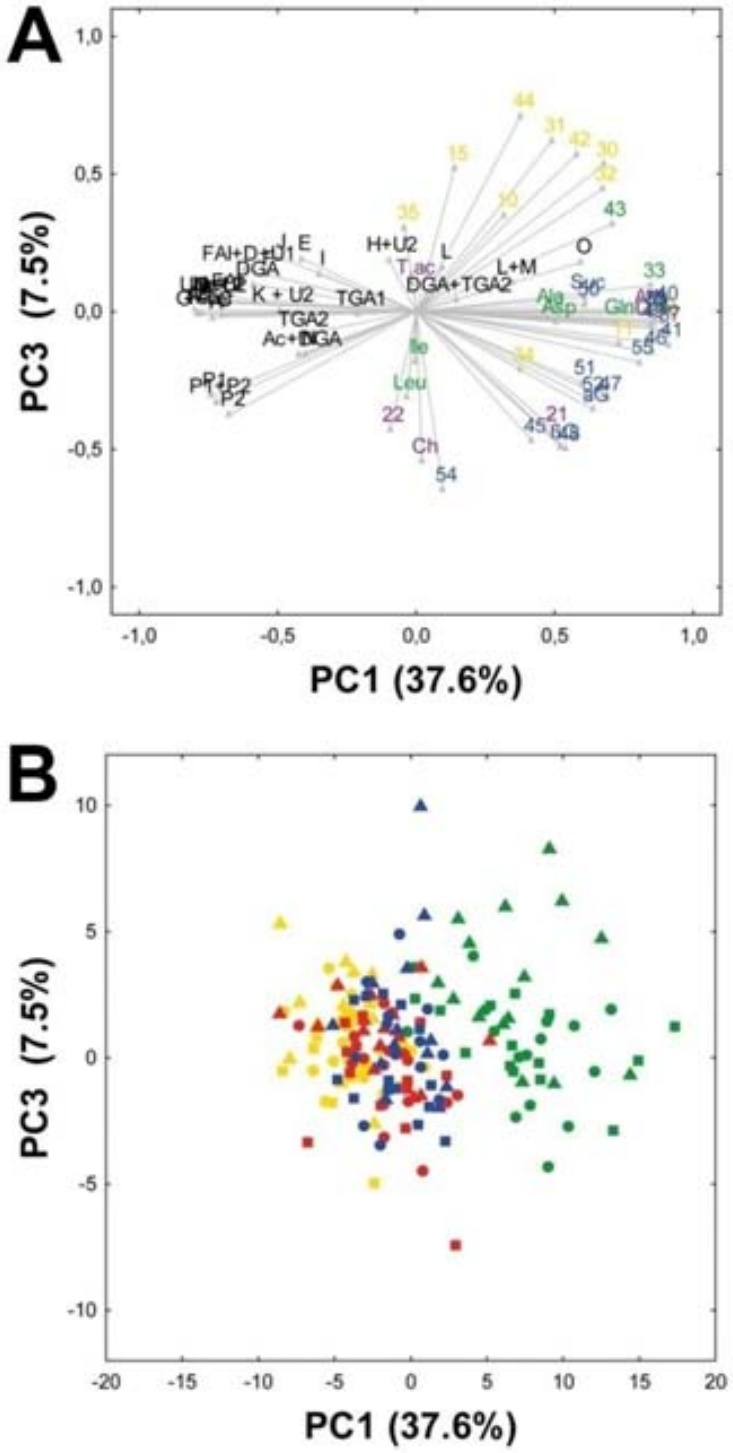


Figure S2. Example of a 1D ^{1}H NMR spectrum of the chloroform extract sample of *Erica multiflora* leaves. Assignments of the main peaks are indicated. Sample dissolved in $CDCl_3$ and referenced to the residual solvent peak ($CHCl_3$). Spectrum acquired at 600 MHz magnetic field and at 298.0 K.

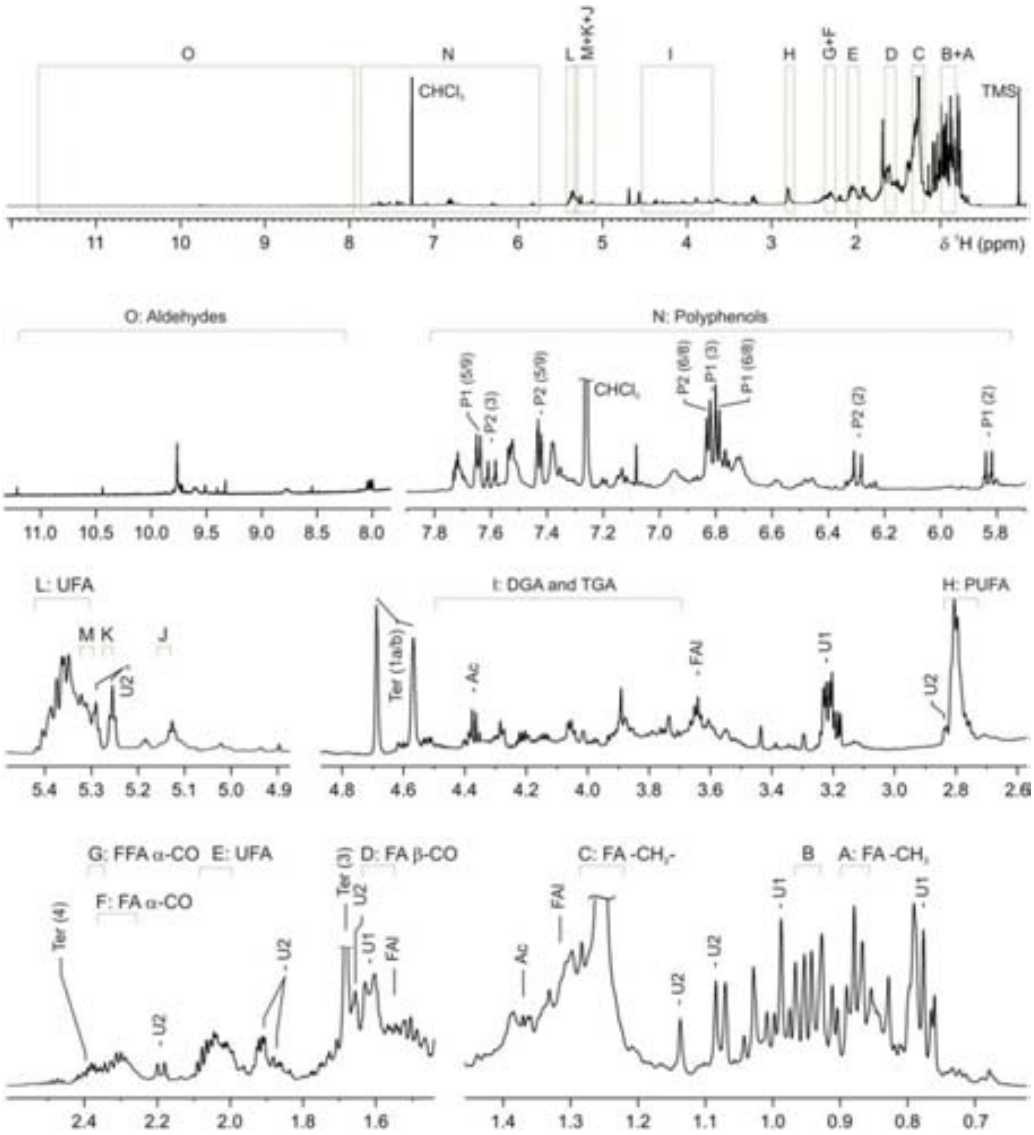


Figure S3. Example of a 1D ^{1}H NMR spectrum of the H_2O/MeOH (1:1) extract of *Erica multiflora* leaves with the assignment of the main peaks. Sample dissolved in D_2O (pH 6.0) and referenced to TSP. Spectrum acquired at 600 MHz magnetic field and at 298.0 K.

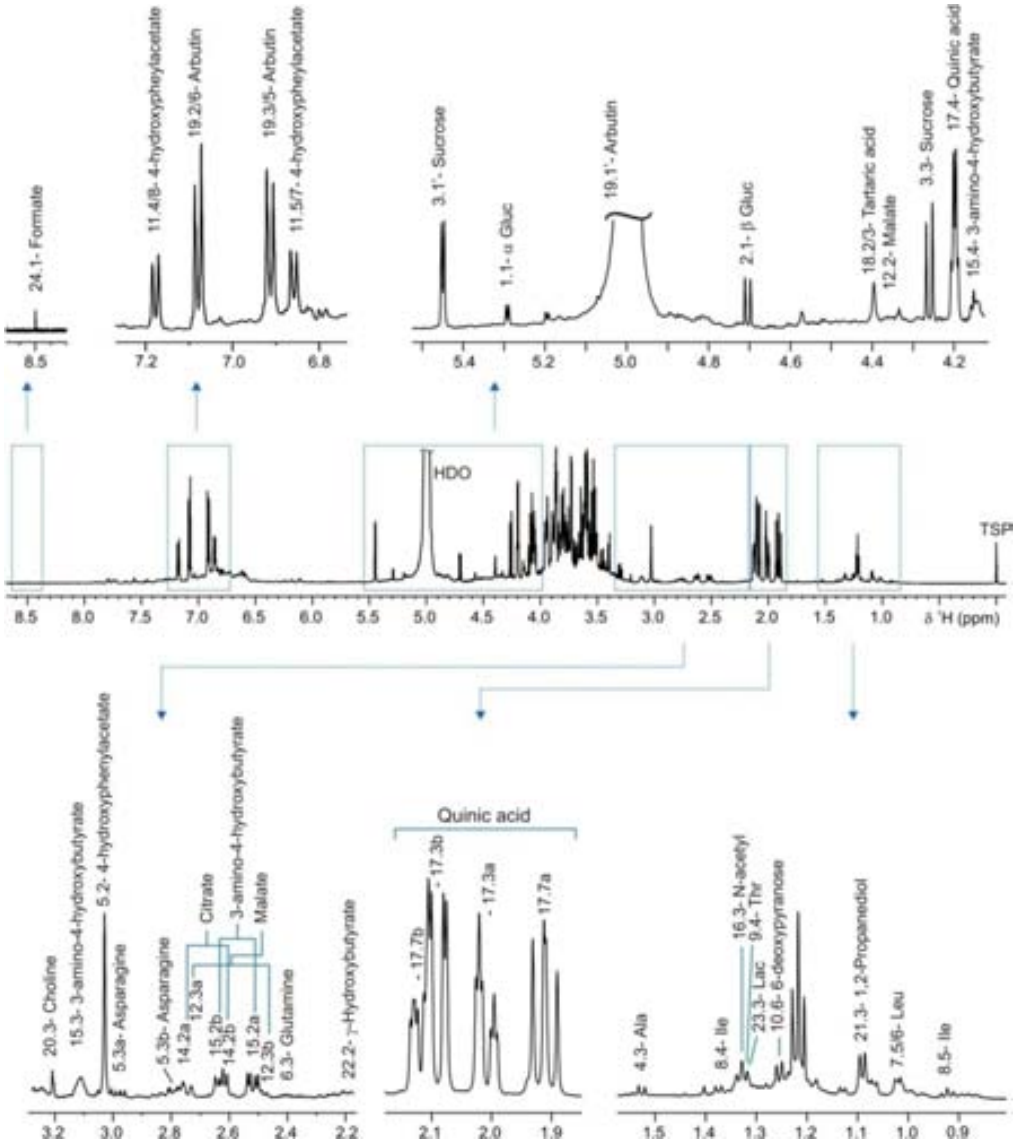


Table S1. Multivariate ANOVA (MANOVA) model. Individual plants and plots as random factors and climatic treatments, seasons, C:N:P:K ratios, metabolomic variables (under an unstructured correlation structure) and their interactions as fixed effects. Data of metabolomic variables are the mean integral value in mM with respect to TSP or TMS concentrations (0.01%).

	Var = plant + plot (plant) + treatment + season + treatment*season																						
	SEASON											TREATMENT									TREATMENT*SEASON		
	Winter		Spring		Summer		Autumn					Control		Drought		Warming							
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Df(n1,n2)	F	p	Mean	SE	Mean	SE	Mean	SE	Df(n1,n2)	F	p	Df(n1,n2)	F	P
POLAR METABOLITES																							
αG (1)	0.33	0.02	0.64	0.04	0.54	0.04	0.22	0.02	3,132	50.6	<0.0001	0,49	0,04	0,42	0,03	0,4	0,03	2,42	2.01	0.15			Not significant
ßG (2)	2.02	0.06	2.59	0.13	2.60	0.15	1.49	0.06	3,132	28.6	<0.0001	2,33	0,13	2,15	0,11	2,07	0,09	2,42	1.26	0.29			Not significant
Suc (3)	8.92	0.23	9.93	0.32	9.47	0.21	8.05	0.19	3,132	13.9	<0.0001	9,04	0,24	9,06	0,25	9,23	0,21	2,42	0.105	0.90			Not significant
Ala (4)	0.10	0.00	0.12	0.00	0.11	0.01	0.08	0.00	3,132	22.8	<0.0001	0,1	0	0,1	0	0,11	0	2,42	1.57	0.22			Not significant
Asp (5)	1.23	0.06	1.29	0.06	0.73	0.04	0.86	0.05	3,132	27.7	<0.0001	1,03	0,05	1,02	0,06	1	0,06	2,42	0.150	0.86			Not significant
Gln (6)	0.20	0.01	0.29	0.01	0.13	0.01	0.16	0.01	3,132	61.7	<0.0001	0,2	0,01	0,2	0,01	0,18	0,01	2,42	1.15	0.33			Not significant
Leu (7)	0.41	0.01	0.38	0.01	0.38	0.01	0.43	0.01	3,126	10.6	<0.0001	0,42	0,01	0,38	0,01	0,4	0,01	2,42	1.35	0.27	6,126	2.62	0.020
Ile (8)	0.35	0.01	0.31	0.01	0.34	0.01	0.31	0.01	3,132	5.93	0.001	0,32	0,01	0,32	0,01	0,33	0,01	2,42	0.113	0.89			Not significant

10	0.79	0.04	1.02	0.04	1.11	0.04	0.77	0.02	3,126	35.7	<0.0001	0,83	0,03	0,9	0,03	1,05	0,05	2,42	6.43	0.004	6,126	3.49	0.003
11	12.39	0.44	16.81	0.47	12.67	0.38	11.35	0.37	3,132	68.4	<0.0001	12,84	0,49	14,13	0,41	13,04	0,45	2,42	1.79	0.18			Not significant
15	0.87	0.04	0.77	0.05	0.64	0.04	0.68	0.04	3,132	12.9	<0.0001	0,68	0,04	0,64	0,03	0,88	0,05	2,42	4.52	0.017			Not significant
Q.ac (17)	7.60	0.31	25.02	1.39	10.58	0.44	7.85	0.30	3,132	157	<0.0001	12,54	1,06	14,61	1,36	11,52	1	2,42	4.32	0.020			Not significant
T.ac (18)	0.85	0.05	0.77	0.03	0.93	0.05	0.75	0.04	3,132	10.5	<0.0001	0,73	0,04	0,92	0,03	0,84	0,04	2,42	2.70	0.079			Not significant
Arb (19)	11.87	0.39	18.30	0.62	13.32	0.31	11.04	0.34	3,132	106	<0.0001	13,59	0,53	13,6	0,55	13,9	0,52	2,42	0.125	0.88			Not significant
Ch (20)	0.63	0.04	0.42	0.02	0.38	0.03	0.48	0.03	3,132	14.9	<0.0001	0,48	0,03	0,52	0,03	0,42	0,03	2,42	2.80	0.073			Not significant
21	2.60	0.08	2.90	0.08	2.25	0.10	2.41	0.07	3,132	18.4	<0.0001	2,51	0,08	2,61	0,09	2,49	0,07	2,42	0.303	0.74			Not significant
22	0.44	0.03	0.30	0.02	0.36	0.02	0.32	0.02	3,132	8.10	<0.0001	0,34	0,02	0,36	0,02	0,36	0,02	2,42	0.314	0.73			Not significant
30	0.73	0.04	1.05	0.04	0.76	0.03	0.57	0.02	3,132	69.7	<0.0001	0,73	0,03	0,75	0,03	0,86	0,04	2,42	2.37	0.11			Not significant
31	0.56	0.04	0.70	0.04	0.40	0.03	0.41	0.03	3,132	68.0	<0.0001	0,5	0,03	0,42	0,02	0,61	0,04	2,42	3.52	0.039			Not significant
32	0.30	0.02	0.42	0.02	0.19	0.01	0.18	0.01	3,132	95.8	<0.0001	0,26	0,02	0,23	0,02	0,31	0,02	2,42	3.31	0.046			Not significant
33	0.27	0.02	2.68	0.16	0.34	0.02	0.25	0.01	3,132	233	<0.0001	0,91	0,16	0,89	0,16	0,87	0,15	2,42	0.064	0.94			Not significant
34	1.30	0.07	1.92	0.07	1.78	0.08	1.47	0.06	3,126	20.0	<0.0001	1,61	0,08	1,69	0,06	1,59	0,07	2,42	0.501	0.61	6,126	2.20	0.047
35	0.51	0.02	0.49	0.02	0.67	0.02	0.49	0.02	3,132	29.9	<0.0001	0,5	0,02	0,51	0,02	0,61	0,02	2,42	5.81	0.006			Not significant
36	0.58	0.02	1.25	0.06	0.76	0.02	0.51	0.01	3,132	126	<0.0001	0,78	0,05	0,81	0,05	0,75	0,04	2,42	0.823	0.446			Not significant
37	6.19	0.17	11.58	0.50	7.34	0.17	5.20	0.13	3,132	118	<0.0001	7,61	0,4	8,05	0,46	7,22	0,34	2,42	2.08	0.14			Not significant
38	2.60	0.11	9.40	0.52	3.83	0.17	2.75	0.11	3,132	166	<0.0001	4,58	0,41	5,32	0,52	4,19	0,39	2,42	3.92	0.028			Not significant

39	1.94	0.06	2.99	0.10	2.16	0.04	1.86	0.05	3,132	91.9	<0.0001	2,22	0,08	2,26	0,09	2,26	0,08	2,42	0.124	0.88			Not significant
40	6.24	0.14	10.19	0.38	7.30	0.15	5.52	0.12	3,132	106	<0.0001	7,2	0,28	7,43	0,33	7,41	0,31	2,42	0.317	0.73			Not significant
41	5.27	0.17	10.30	0.43	5.24	0.15	4.70	0.15	3,132	143	<0.0001	6,5	0,39	6,78	0,41	5,96	0,3	2,42	2.21	0.12			Not significant
42	1.33	0.06	1.67	0.08	0.94	0.05	0.95	0.06	3,132	77.6	<0.0001	1,16	0,06	1,05	0,06	1,45	0,07	2,42	5.62	0.007			Not significant
43	1.71	0.07	2.21	0.07	1.58	0.06	1.29	0.05	3,132	69.8	<0.0001	1,67	0,08	1,63	0,06	1,79	0,07	2,42	0.862	0.43			Not significant
44	1.56	0.09	1.78	0.11	1.19	0.08	1.19	0.08	3,132	37.6	<0.0001	1,37	0,08	1,15	0,06	1,77	0,1	2,42	5.92	0.005			Not significant
45	2.79	0.06	3.02	0.10	3.32	0.12	2.29	0.07	3,126	27.6	<0.0001	2,88	0,11	2,86	0,1	2,82	0,08	2,42	0.091	0.91	6,126	2.50	0.025
46	9.25	0.25	13.26	0.45	9.91	0.22	8.40	0.23	3,132	70.0	<0.0001	10,33	0,4	10,28	0,38	10,12	0,3	2,42	0.062	0.94			Not significant
47	2.55	0.05	2.98	0.08	2.81	0.07	2.34	0.05	3,132	22.5	<0.0001	2,67	0,07	2,7	0,07	2,66	0,05	2,42	0.082	0.92			Not significant
48	1.55	0.04	1.72	0.04	1.66	0.05	1.42	0.04	3,132	12.5	<0.0001	1,58	0,04	1,63	0,04	1,56	0,03	2,42	0.677	0.51			Not significant
49	8.35	0.24	11.70	0.40	9.27	0.22	7.24	0.19	3,132	70.9	<0.0001	9,24	0,35	9,09	0,34	9,2	0,29	2,42	0.018	0.98			Not significant
50	15.01	0.42	16.71	0.51	16.22	0.40	13.19	0.32	3,132	19.0	<0.0001	15,18	0,41	15,13	0,42	15,67	0,38	2,42	0.329	0.72			Not significant
51	1.88	0.05	2.17	0.09	2.18	0.08	1.40	0.04	3,132	42.5	<0.0001	1,96	0,08	1,86	0,07	1,92	0,07	2,42	0.326	0.72			Not significant
52	2.61	0.07	3.13	0.13	2.99	0.11	1.92	0.05	3,126	46.5	<0.0001	2,72	0,12	2,66	0,1	2,63	0,09	2,42	0.133	0.88	6,126	3.17	0.006
53	4.82	0.14	7.00	0.24	5.32	0.11	4.44	0.12	3,132	72.7	<0.0001	5,4	0,2	5,4	0,21	5,45	0,17	2,42	0.031	0.97			Not significant
54	0.17	0.01	0.15	0.01	0.17	0.01	0.14	0.01	3,132	2.56	0.058	0,16	0,01	0,18	0,01	0,14	0,01	2,42	3.56	0.038			Not significant
55	3.09	0.08	4.08	0.14	3.42	0.08	2.81	0.07	3,132	50.6	<0.0001	3,38	0,11	3,36	0,11	3,36	0,09	2,42	0.001	0.99			Not significant

NON-POLAR METABOLITES (Spectra region or Metabolite)

A	21.10	0.96	14.18	0.24	25.16	0.76	22.41	0.68	3,132	46.9	<0.0001	19,77	0,63	20,24	0,76	20,85	0,79	2,42	0.402	0.67		Not significant
Ac	34.82	1.70	15.68	0.58	36.91	1.55	35.50	1.30	3,132	63.3	<0.0001	26,68	1,16	31,2	1,6	31,82	1,59	2,42	3.87	0.029		Not significant
Ac+DGA	0.72	0.06	0.54	0.03	0.90	0.09	1.65	0.22	3,132	16.0	<0.0001	0,88	0,08	0,91	0,09	0,89	0,09	2,42	0.516	0.60		Not significant
B	42.81	2.02	21.91	0.62	44.64	1.75	41.88	1.47	3,132	53.3	<0.0001	33,91	1,29	38,22	1,74	38,31	1,72	2,42	2.04	0.14		Not significant
C	80.04	3.70	59.25	1.24	99.21	3.75	93.17	3.10	3,132	36.0	<0.0001	79,4	2,42	78,95	3,3	85,76	3,48	2,42	1.39	0.26		Not significant
D+U2	9.34	0.46	5.84	0.13	10.46	0.38	9.49	0.33	3,132	38.5	<0.0001	8,13	0,27	8,65	0,36	8,95	0,36	2,42	1.07	0.35		Not significant
DGA	2.30	0.11	1.88	0.04	2.60	0.09	2.81	0.10	3,132	23.4	<0.0001	2,21	0,07	2,36	0,09	2,5	0,09	2,42	2.33	0.11		Not significant
DGA+TGA2	0.14	0.01	0.15	0.01	0.11	0.01	0.15	0.01	3,132	5.39	0.002	0,14	0,01	0,13	0,01	0,13	0,01	2,42	2.02	0.15		Not significant
E	19.45	0.84	18.10	0.39	25.12	1.11	22.60	0.99	3,132	15.4	<0.0001	19,83	0,61	20,19	0,7	23,29	1,04	2,42	4.88	0.012		Not significant
F	6.98	0.35	5.26	0.24	6.79	0.32	6.71	0.24	3,132	7.93	<0.0001	5,94	0,15	6,05	0,19	6,67	0,28	2,42	1.50	0.24		Not significant
FAI	30.11	1.48	19.82	0.39	33.91	1.15	30.26	1.04	3,132	33.8	<0.0001	26,56	0,83	28,01	1,07	28,97	1,12	2,42	0.909	0.41		Not significant
FAI+D+U1	13.81	0.63	10.41	0.23	17.16	0.64	15.70	0.65	3,132	32.7	<0.0001	13,01	0,45	13,88	0,55	15,24	0,65	2,42	3.43	0.042		Not significant
G+Ter	8.35	0.41	3.23	0.20	7.64	0.39	7.54	0.27	3,132	54.9	<0.0001	5,8	0,26	6,75	0,35	6,89	0,4	2,42	2.87	0.069		Not significant
H+U2	3.72	0.14	3.63	0.09	3.37	0.12	3.91	0.12	3,126	4.26	0.007	3,64	0,11	3,47	0,09	3,74	0,1	2,42	0.162	0.21	6,126	2.72	0.016
I	2.61	0.13	2.57	0.06	2.98	0.13	2.98	0.11	3,132	4.87	0.003	2,55	0,07	2,71	0,09	2,94	0,1	2,42	2.39	0.10		Not significant
J	1.71	0.08	1.50	0.04	2.43	0.10	2.26	0.12	3,132	30.4	<0.0001	1,84	0,08	1,87	0,09	2,18	0,11	2,42	3.74	0.032		Not significant
K + U2	0.96	0.05	0.79	0.02	0.92	0.03	1.05	0.03	3,132	9.71	<0.0001	0,89	0,02	0,93	0,03	0,91	0,03	2,42	0.164	0.85		Not significant
L	2.56	0.10	2.76	0.06	2.65	0.08	2.64	0.08	3,132	1.42	0.24	2,66	0,07	2,53	0,06	2,69	0,06	2,42	1.36	0.27		Not significant

L+M	1.43	0.05	1.92	0.05	1.62	0.05	1.60	0.05	3,126	22.5	<0.0001	1,68	0,05	1,57	0,05	1,65	0,04	2,42	1.62	0.21	6,126	2.36	0.034
N	0.75	0.04	0.62	0.03	0.96	0.07	1.70	0.19	3,132	20.7	<0.0001	0,91	0,08	1,01	0,08	1,08	0,15	2,42	0.796	0.45			Not significant
O	0.08	0.00	0.17	0.01	0.11	0.01	0.09	0.01	3,132	68.5	<0.0001	0,11	0,01	0,11	0,01	0,12	0,01	2,42	0.468	0.63			Not significant
P1	1.54	0.06	0.85	0.03	1.87	0.07	1.77	0.05	3,132	76.9	<0.0001	1,45	0,06	1,58	0,08	1,42	0,06	2,42	2.24	0.12			Not significant
P1+P2	1.61	0.06	0.95	0.03	1.95	0.07	1.82	0.06	3,132	68.0	<0.0001	1,53	0,06	1,69	0,08	1,47	0,06	2,42	4.06	0.024			Not significant
P2	1.44	0.06	0.87	0.03	1.69	0.07	1.78	0.07	3,132	57.1	<0.0001	1,42	0,06	1,56	0,08	1,32	0,06	2,42	4.73	0.014			Not significant
Terpene 1	37.93	1.69	17.07	0.64	40.42	1.80	37.36	1.28	3,132	68.4	<0.0001	28,54	1,24	34,44	1,73	34,13	1,71	2,42	4.86	0.013			Not significant
TGA1	1.39	0.07	1.24	0.03	1.23	0.05	1.35	0.05	3,132	2.59	0.056	1,26	0,03	1,27	0,04	1,28	0,04	2,42	0.085	0.92			Not significant
TGA2	0.48	0.02	0.42	0.01	0.48	0.02	0.55	0.02	3,132	8.58	<0.0001	0,47	0,01	0,48	0,02	0,47	0,02	2,42	0.020	0.98			Not significant
Unk.1	75.34	3.63	39.19	1.12	80.54	3.12	76.14	2.72	3,132	54.1	<0.0001	60,85	2,4	68,21	3,15	69,17	3,1	2,42	2.05	0.14			Not significant
Unk.2	40.40	1.87	25.17	0.54	42.64	1.57	39.00	1.30	3,132	33.3	<0.0001	33,75	0,93	36,58	1,35	37,34	1,54	2,42	1.40	0.26			Not significant
ELEMENTS AND RATIOS																							
C:N	97.49	2.31	67.40	1.48	107.24	1.50	99.99	2.09	3,132	156	<0.0001	90,65	2,39	97,12	2,99	91,55	2,27	2,42	1.94	0.16			Not significant
N:P	23.27	0.52	17.66	0.45	25.36	0.53	21.23	0.61	3,126	51.0	<0.0001	20,89	0,52	21,45	0,61	22,97	0,61	2,42	3.52	0.039	6,126	2.87	0.012
C:P	2260.06	69.38	1201.68	49.41	2709.50	57.9	2110.09	67.43	3,126	167	<0.0001	1880,63	77,15	2120,67	97,53	2151,83	92,58	2,42	3.38	0.044	6,126	2.55	0.023
C:K	182.03	8.32	105.50	5.93	162.79	7.47	166.42	8.66	3,126	29.3	<0.0001	152,91	7,41	138,29	7	161,91	7,23	2,42	2.55	0.090	6,126	2.21	0.046
K:P	13.31	0.60	11.76	0.32	17.73	0.62	13.82	0.64	3,126	31.8	<0.0001	12,89	0,53	16,09	0,64	13,57	0,4	2,42	10.7	<0.0001	6,126	3.26	0.005
N:K	0.38	0.02	0.30	0.01	0.30	0.01	0.34	0.02	3,126	10.2	<0.0001	0,34	0,01	0,29	0,01	0,35	0,01	2,42	3.52	0.039	6,126	3.39	0.004

N ($mg.g^{-1}$)	6.16	0.14	8.56	0.18	5.42	0.08	5.96	0.12	3,132	187	<0.0001	6,65	0,19	6,33	0,21	6,59	0,2	2,42	0.824	0.446			Not significant
P ($mg.g^{-1}$)	0.27	0.01	0.50	0.02	0.22	0.00	0.29	0.01	3,132	178	<0.0001	0,34	0,02	0,32	0,02	0,31	0,02	2,42	1.23	0.30			Not significant
K ($mg.g^{-1}$)	3.54	0.17	5.82	0.22	3.87	0.17	4.00	0.22	3,126	42.8	<0.0001	4,25	0,19	4,78	0,21	4,01	0,19	2,42	3.34	0.045	6,126	2.39	0.032
C ($mg.g^{-1}$)	585.95	1.36	565.58	1.22	576.75	1.29	585.61	1.12	3,132	77.5	<0.0001	577,67	1,37	578,94	1,6	578,59	1,66	2,42	0.286	0.75			Not significant

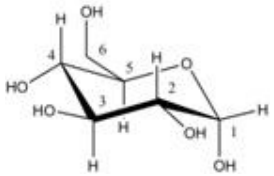
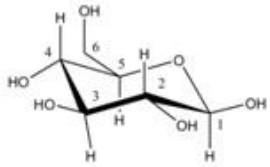
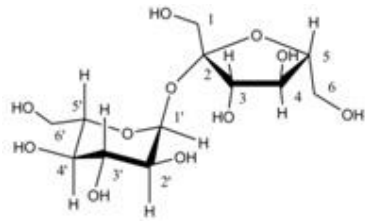
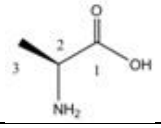
Table S2. Statistic values of MANOVA and PERMANOVA. MANOVA included individual plants and plots as random factors and climatic treatments, seasons, C:N:P:K ratios, metabolomic variables (under an unstructured correlation structure) and their interactions as fixed effects. PERMANOVA was performed using the Euclidean distance, with season (spring, summer, autumn and winter) and treatments (control, drought and warming) as fixed factors and block and individuals nested in block as random factors.

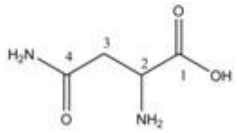
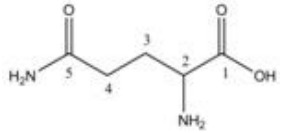
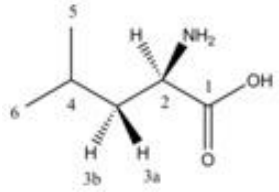
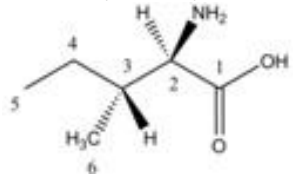
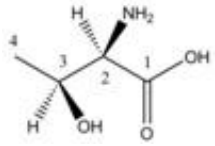
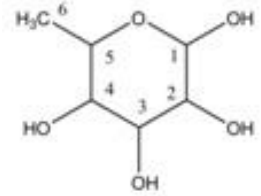
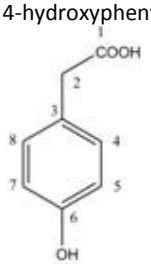
MANOVA					
Fixed Factors	df	F-value	P-value		
Intersection	1	4994	0.0001		
Season	3	99.7	0.0001		
Climatic treatments	2	5.14	0.01		
Variables	78	2907	0.0001		
Season * climatic treatments	6	0.81	0.56		
Season*variables	234	71.6	0.0001		
Climatic treatment*variables	156	4.92	0.0001		
Season*climatic treatments*variables	468	0.61	0.99		
Residual	13484				
Total	14432				
PERMANOVA					
Factors	df	SS	MS	Pseudo-F	P-value
Bloc	2	556.29	278.14	3.69	0.002
Climatic treatment	2	396.28	198.14	1.90	0.10
Season	3	4949.4	1664.8	29.3	0.001
In(Bloc)	12	907.12	75.59	1.94	0.002
Bloc*Climatic treatment	4	418.64	104.66	1.66	0.01
Bloc*season	6	341.48	56.91	1.62	0.007
Climatic treatment*season	6	314.08	52.35	1.40	0.10
In(Bloc)*climatic treatment	24	1523.7	63.49	1.63	0.001
In(Bloc)*season	36	1260.7	35.02	0.91	0.81
Bloc*climatic treatment*season	12	447.5	37.29	0.96	0.57
Residual	68	2643.2	38.87		
Total	175	14000			

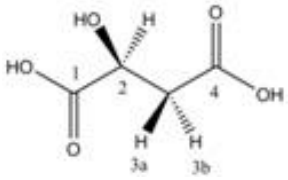
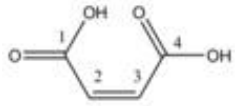
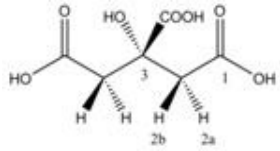
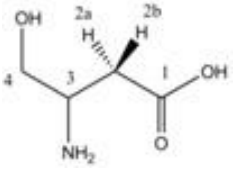
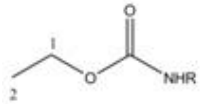
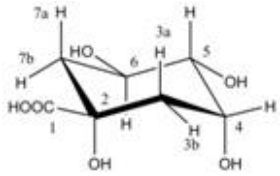
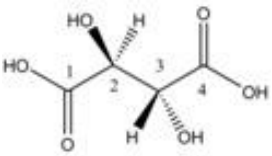
Table S3. One-way ANOVAs of the first four factor scores coordinates from the PCA including all metabolites and ratios (Figure 2) with respect the year seasons (spring, summer, autumn and winter). Different letters indicate significant statistical differences between seasons ($p<0.05$, post-hoc Bonferroni test).

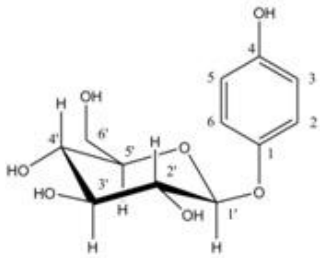
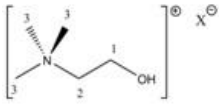
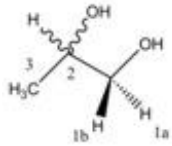
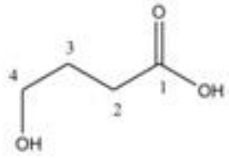
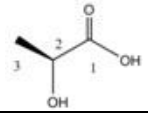
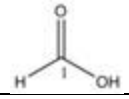
	AUTUMN			SPRING			SUMMER			WINTER				
	mean	SE		mean	SE		mean	SE		mean	SE		$F_{(172)}$	p
PC1	3.94	0.33	a	-7.84	0.60	c	2.06	0.39	b	1.84	0.45	b	137.3795	0.000000
PC2	1.20	0.58	a	-0.04	0.46	ab	-1.50	0.60	b	0.33	0.62	ab	3.9448	0.009369
PC3	0.80	0.30	a	1.13	0.31	a	-2.33	0.30	b	0.41	0.35	a	25.2095	0.000000
PC4	-1.26	0.29	b	-0.79	0.30	b	0.58	0.34	a	1.47	0.32	a	15.7779	0.000000

Table S4. NMR assignments of the major metabolites in samples of H_2O/MeOH (1:1) and chloroform extracts. For samples dissolved in D_2O (pH 6.0), and ^{1}H and ^{13}C NMR chemical shifts referenced to TSP. Samples dissolved in $CDCl_3$, ^{1}H and ^{13}C NMR chemical shifts referenced to the residual solvent peak.

POLAR METABOLITES							
Metabolite				NMR chemical shift (δ)			Assignment
Id.	Name	Atom id.		^{1}H		^{13}C	a, b, c
		spect.	molec.	(ppm)	m,[d] *J* (Hz)[e]	(ppm)	
1	α-glucose (1-3) 	1.1	1	5,291	d; 3,9	94,71	a [COSY, TOCSY, HSQC, HMBC], b, c
		1.2	2	3,594	m*	73,80	
		1.3	3	3,760	m*	75,45	
		1.4	4	3,463	m*	72,21	
		1.5	5	3,867	m*	74,10	
		1.6	6	3,943	m*	63,00	
2	β-glucose (1-3) 	2.1	1	4,703	d; 8,06	98,50	a [COSY, TOCSY, HSQC, HMBC], b, c
		2.2	2	3,300	dd; 9,1; 8,3	76,60	
		2.3	3	3,572	m*	78,06	
		2.4	4	3,449	m*	72,21	
		2.5	5	3,526	m*	78,37	
		2.6	6	3,810	m*	63,10	
3	Sucrose (1, 2, 4) 	3.1	1	3,728	s	63,95	a [COSY, TOCSY, HSQC, HMBC], b, c
		3.2	2	-	-	106,14	
		3.3	3	4,257	d; 8,84	79,01	
		3.4	4	4,084	t; 8,69	76,64	
		3.5	5	3,943	m	83,96	
		3.6	6	3,864	d, 3,4	65,15	
		3.1'	1'	5,450	d; 3,81	94,86	
		3.2'	2'	3,609	m	73,50	
		3.3'	3'	3,811	t; 9,6	75,10	
		3.4'	4'	3,520	t; 9,5	71,97	
		3.5'	5'	3,881	m	74,96	
		3.6'	6'	3,858	d; 3,3	62,72	
4	Alanine (1, 2, 4-6) 	4.1	1	-	-	178,87	a [COSY, HSQC, HMBC], b, c
		4.2	2	3,842	*	53,31	
		4.3	3	1,525	d; 7,2	17,10	

5	Asparagine (1, 2, 7) 	5.1	1	-	*	*	a [COSY], b, c
		5.2	2	3,962	*	*	
		5.3a	3a	2,980	dd; 17,8; 9,0	*	
		5.3b	3b	2,790	dd; 17,8; 3,6	*	
6	Glutamine (1, 2, 7) 	6.1	1	-	*	*	a [COSY], b, c
		6.2	2	3,804	m	*	
		6.3	3	2,135	m	*	
		6.4	4	2,392	m	*	
7	Leucine (1, 2, 4, 6, 7) 	7.1	1	-	-	**	a [COSY, HSQC, HMBC], b, c
		7.2	2	*/**	dd*/**	51,67	
		7.3a	3a	*/**	m*/**	42,77	
		7.3b	3b	*/**	m*/**	42,77	
		7.4	4	*/**	m*/**	**	
		7.5	5	1,013	d	24,14	
		7.6	6	1,017	d	24,75	
8	Isoleucine (1, 2, 4, 6) 	8.1	1	-	-	*/**	a [COSY, HSQC], b, c
		8.2	2	*/**	d*/**	*/**	
		8.3	3	*/**	m*/**	*/**	
		8.4	4	1,363	m	23,85	
		8.5	5	0,922	t; 7,3	**	
		8.6	6	*/**	d*/**	*/**	
9	Threonine (1, 2, 4, 7) 	9.1	1	-	-		a [COSY, HSQC], b, c
		9.2	2	*	*	*/**	
		9.3	3	4,470	m	66,32	
		9.4	4	1,323	d; 6,40	20,61	
10	6-deoxy pyranose 	10.1	1	5,162	s (broad)	104,13	a [COSY, TOCSY, HSQC, HMBC]
		10.2	2	4,120	s (broad)	73,02	
		10.3	3	3,762	m*	72,67	
		10.4	4	3,454	m*	73,83	
		10.5	5	3,945	m*	72,00	
		10.6	6	1,255	d	19,21	
11	4-hydroxyphenylacetate (2, 8) 	11.1	1	-	-	181,08	a [COSY, TOCSY, HSQC, HMBC], b, c
		11.2	2	3,029	s	42,70	
		11.3	3	-	-	131,23	
		11.4	4	7,170	d; 8,1	134,18	
		11.5	5	6,860	d; 8,1	117,67	
		11.6	6	-	-	156,59	
		11.7	7	6,860	d; 8,1	117,67	

		11.8	8	7,170	d; 8,1	134,18	
12	Malate (1, 2, 5, 7) 	12.1	1	-	-	179,28	a [COSY, TOCSY, HSQC, HMBC], b, c
		12.2	2	4,357	m	72,71	
		12.3a	3a	2,740	dd*	44,80	
		12.3b	3b	2,492	dd*	44,80	
		12.4	4	-	-	181,06	
13	Maleate (9) 	13.1	1	-	*	*	a [COSY, HSQC], b, c
		13.2	2	6,005	s	128,80	
		13.3	3	6,005	s	128,80	
		13.4	4	-	*	*	
14	Citrate (1, 2, 6) 	14.1	1	-	*	*	a [COSY, HSQC], b, c
		14.2a	2a	2,752	d	47,80	
		14.2b	2b	2,601	d	47,80	
		14.3	3	-		*	
15	3-amino-4-hydroxybutyrate 	15.1	1	-	-	182,72	a [COSY, HSQC, HMBC]
		15.2a	2a	2,520	dd, 15,7; 6,2	39,14	
		15.2b	2b	2,630	dd; 15,7; 8,8	39,14	
		15.3	3	3,111	m	50,91	
		15.4	4	4,140	m	75,90	
16	N-acetyl group 	16.1	1	-	*	*	a [COSY]
		16.2	2	4,360	q; 7,17	*	
		16.3	3	1,328	t; 7,17	*	
17	Quinic acid (10, 11) 	17.1	1	-	-	183,85	a [COSY, TOCSY, HSQC, HMBC], b, c
		17.2	2	-	-	79,39	
		17.3a	3a	2,008	dt; 14,86; 3,09	39,93	
		17.3b	3b	2,090	dd; 14,86; 3,09	39,99	
		17.4	4	4,197	dt; 3,6; 3,09	73,02	
		17.5	5	3,597	dd; 9,09; 3,6	77,85	
		17.6	6	4,060	m	69,48	
		17.7a	7a	1,910	dd; 13,2; 11,5	43,44	
		17.7b	7b	2,118	ddd; 13,2; 4,3; 2,8	43,37	
18	Tartaric acid (12) 	18.1	1	-	-	179,73	a [COSY, HSQC, HMBC], b
		18.2	2	4,395	s (broad)	78,79	
		18.3	3	4,395	s (broad)	78,79	
		18.4	4	-	-	179,73	
19	Arbutin (13)	19.1	1	-	-	152,72	a [COSY, TOCSY, HSQC, HMBC], b, c
		19.2	2	7,078	d; 9,0	121,00	
		19.3	3	6,912	d; 9,0	118,82	

		19.4	4	-	-	153,29	
		19.5	5	6,912	d; 9,0	118,82	
		19.6	6	7,078	d; 9,0	121,00	
		19.1'	1'	5,013	d*	103,92	
		19.2'	2'	3,564	m*	75,61	
		19.3'	3'	3,600	m*	78,10	
		19.4'	4'	3,527	m*	72,00	
		19.5'	5'	3,620	m*	78,21	
		19.6a'	6a '	3,943	dd*	63,20	
		19.6b'	6b '	3,792	dd*	63,20	
20	Choline (2, 14) 	20.1	1	4,077	m	69,87	a [COSY, HSQC, HMBC], b, c
		20.2	2	3,523	m	58,30	
		20.3	3	3,206	s	56,52	
21	1,2-propanediol (2) 	21.1a	1a	3,489	m	73,98	a [COSY, HSQC, HMBC], b, c
		21.1b	1b	3,289	m	73,98	
		21.2	2	3,350	m	71,33	
		21.3	3	1,040	d; 6,2	19,35	
22	γ-hydroxybutyrate (15) 	22.1	1	-	*	*	a [COSY, TOCSY], b
		22.2	2	2,214	m	*	
		22.3	3	1,831	m	*	
		22.4	4	3,972	m	*	
23	Lactate (1, 2, 6) 	23.1	1	-	*	*	a [COSY], b, c
		23.2	2	4,160	q*	*	
		23.3	3	1,320	d	*	
24	Formate (1, 2, 8, 16) 	24.1	1	8,487	s	*	a, b, c

* Overlapped signal.
** Not enough signal.
[a] From 1D ^{1}H NMR chemical shift and 2D ^{1}H-^{1}H and ^{1}H-^{13}C NMR couplings.
[b] Comparison with ^{1}H and ^{13}C NMR data from references indicated.
[c] Comparison with ^{1}H and ^{13}C NMR data from the *Biological Magnetic Resonance Data Bank.* (http://www.bmrb.wisc.edu/).[17]
[d] Multiplicity: singlet (s), doublet (d), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), ddd (doublet of doublet of doublets), m (multiplet).
[e] 1H-1H coupling constant.

NON-POLAR METABOLITES

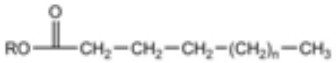
NMR Peak/Spectral Region						**Assignment(17-24)**
δ ^{1}H	**m, *J***	**δ ^{13}C**		**id.**		**metabolite (atom id.)/group**
(ppm)	(Hz)	(ppm)		spect.		
0,773	m	15,48				U1[a]
0,864-0,897	t	14,05		A		-CH3 ; FA[b] chains

0,934-0,963	t	14,28		B		-CH3 ; Linoleyl FA chains
0,985	*	28,12				U1
1,080	*	*				U2[c]
1,133	*	*				U2
1,238-1,265	br	29,05-29,90		C		-CH2 ; FA chains
1,329	m	25,40				HO-CH2-CH2-CH2- ; FAl[d]
1,368	t; 7,2	13,60				CH3-CH2-OR; acetyl group
1,569	m	32,52				HO-CH2-CH2- ; FAl
1,578-1,637	m	24,86		D		-CO-CH2-CH2- ; FA chains
1,608	*	38,50				U1
1,633	*	*				U2
1,683	s	18,80				terpene 1 (3)
1,893	*	23,40				U2
1,919	*	23,40				U2
1,928	m	29,66				terpene 1 (5)
1,991-2,062	m	27,20		E		=CH-CH2- ; UFA[e] chains
2,184	d; 11,5	52,68				U2
2,283-2,346	t	34,26		F		-CO-CH2- ; FA chains
2,330-2,364	t	33,92		G		-CO-CH2- ; FFA[f]
2,384	m	48,06				terpene 1 (4)
2,759-2,826	m	25,67		H		=CH-CH2-CH= ; PUFA[g] chains
2,824	*	41,09				U2
3,215	m	79,10				U1
3,640	t	63,02				HO-CH2- ; FAl
4,369	q; 7,2	61,63				CH3-CH2-OR; acetyl group
3,740	dd	68,53		I		-CH2OH; 1,2-DGA[h]
3,889	dd	68,53		I		-CH2OH; 1,2-DGA
4,153	dd	62,07		I		-CH2O-; TGA-1[i]
4,210	dd	62,70		I		-CH2O-; 1,2-DGA
4,290	dd	62,07		I		-CH2O-; TGA-1
4,389	dd	62,70		I		-CH2O-; 1,2-DGA
4,442	dd	61,58		I		-CH2O-; TGA-2[j]
4,493	dd	61,58		I		-CH2O-; TGA-2
4,567	br	109,34				terpene 1 (1b)
4,687	br	109,34				terpene 1 (1a)
5,131	*	72,08		J		-CHO-; TGA-2
5,251	t; 3,6	125,60				U2
5,264	*	68,85		K		-CHO-; TGA-1
5,287	t; 3,6	122,50				U2
5,291-5,428	m	127,70-132,00		L		-CH= ; UFA chains
5,310	*	70,18		M		-CHO-; 1,2-DAG
5,828	d; 12,8	117,84		N		PCAD-1[k](2)
6,293	d; 16,0	116,28		N		PCAD-2[l](2)
6,797	d; 8,6	114,97		N		PCAD-1 (6/8)
6,813	d; 12,8	142,58		N		PCAD-1 (3)
6,833	d; 8,5	115,80		N		PCAD-2 (6/8)
7,431	d; 8,5	129,91		N		PCAD-2 (5/9)
7,598	d; 16,0	143,85		N		PCAD-2 (3)
7,647	d; 8,6	132,25		N		PCAD-1 (5/9)
		124,73				terpene 1 (6)

-		150,71				terpene 1 (2)
-		165,80				PCAD-1 (1)
-		166,86				PCAD-2 (1)
-		127,05				PCAD-1 (4)
-		127,05				PCAD-2 (4)
-		156,35				PCAD-1 (7)
-		157,40				PCAD-2 (7)

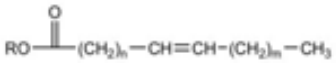
Fatty Acid (FA) chain

saturated

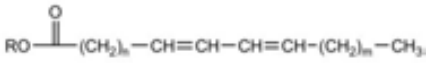


unsaturated (UFA)

monounsaturated (MUFA)

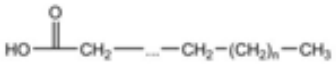


polyunsaturated (PUFA)

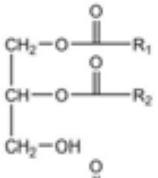


Fatty Alcohol (FAl)

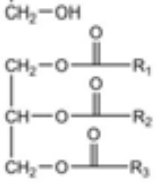
Free Fatty Acid (FFA)



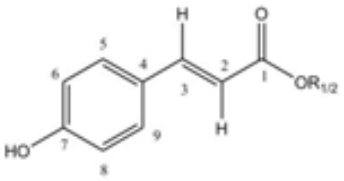
1,2-Diacylglycerol (1,2-DAG)



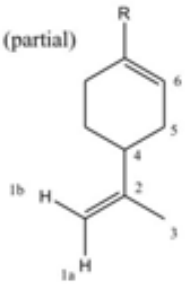
Triacylglycerol (TAG)



p-Coumaric Acid Derivative 1/2 (P1/2)



Terpene 1 (partial)



* Overlapped signal. a.Unknown 1; b.FA: Fatty Acid; c.Unknown 2; d.FAl: Fatty Alcohol; e.UFA: Unsaturated Fatty Acid; f.FFA: Free Fatty Acid; g.PUFA:Polyunsaturated Fatty Acid; h.1,2-DGA: 1,2-Diacylglycerol; i.TGA-1: Triacylglycerol 1; j.TGA-2: Triacylglycerol 2; k.PCAD-1: p-Coumaric Acid Derivative 1; l.PCAD-2: p-Coumaric Acid Derivative 2.

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SI Table 5. One-way ANOVAs of all elucidated metabolites extracted from *Erica* spectra for climatic treatments (Control, Drought, Warming) within seasons (Winter, Spring, Summer, Autumn). Data are the mean integral value in mM with respect to TSP or TMS concentrations (0.01%).

AUTUMN								
CLIMATIC TREATMENT								
CONTROL			DROUGHT			WARMING		
Mean	SE	Mean	SE	Mean	SE	F(42)	p	
POLAR METABOLITES								
αG (1)	0.22	0.03	0.25	0.02	0.19	0.03	0.962819	0.390081
ßG (2)	1.45	0.11	1.60	0.11	1.43	0.10	0.804440	0.454110
Suc (3)	8.39	0.41	7.91	0.30	7.84	0.27	0.795402	0.458081
Ala (4)	0.08	0.01	0.08	0.00	0.09	0.01	0.546303	0.583146
Asp (5)	0.88	0.10	0.88	0.09	0.81	0.10	0.186579	0.830477
Gln (6)	0.17	0.01	0.17	0.01	0.13	0.01	2.374220	0.105469
Leu (7)	0.44	0.01	0.41	0.02	0.45	0.02	0.968004	0.388153
Ile (8)	0.31	0.02	0.31	0.01	0.29	0.02	0.348076	0.708063
10	0.71	0.03	0.79	0.05	0.80	0.03	1.524696	0.229492
11	10.78	0.78	12.31	0.46	10.95	0.58	1.831829	0.172683
15	0.68	0.09	0.55	0.06	0.81	0.08	2.911937	0.065418
Q.ac (17)	7.64	0.49	8.97	0.46	6.94	0.51	4.516007	0.016732
T.ac (18)	0.66	0.08	0.88	0.05	0.72	0.06	3.096055	0.055685
Arb (19)	11.08	0.82	10.86	0.48	11.19	0.45	0.074165	0.928640
Ch (20)	0.47	0.05	0.53	0.06	0.43	0.05	0.915149	0.408293
21	2.37	0.14	2.52	0.14	2.34	0.09	0.562076	0.574253
22	0.30	0.04	0.33	0.04	0.34	0.04	0.190500	0.827256
30	0.54	0.03	0.56	0.03	0.61	0.04	1.179770	0.317327
31	0.41	0.05	0.32	0.02	0.49	0.05	4.090809	0.023812
32	0.18	0.02	0.15	0.01	0.21	0.02	3.035448	0.058710
33	0.25	0.02	0.25	0.02	0.24	0.02	0.087666	0.916234
34	1.43	0.11	1.72	0.11	1.28	0.08	4.890125	0.012325
35	0.48	0.03	0.44	0.02	0.55	0.03	3.762271	0.031405
36	0.49	0.03	0.55	0.02	0.48	0.02	2.860774	0.068427
37	5.23	0.29	5.53	0.19	4.86	0.17	2.312337	0.111507
38	2.69	0.19	3.16	0.18	2.39	0.18	4.628696	0.015253
39	1.85	0.11	1.87	0.08	1.84	0.07	0.025320	0.975013
40	5.55	0.29	5.54	0.13	5.47	0.17	0.045728	0.955350
41	4.68	0.35	4.97	0.24	4.44	0.12	1.075723	0.350261
42	0.96	0.11	0.76	0.06	1.14	0.10	4.274439	0.020431
43	1.27	0.11	1.23	0.07	1.36	0.08	0.589845	0.558940
44	1.21	0.14	0.90	0.08	1.45	0.14	5.025303	0.011048
45	2.18	0.13	2.40	0.13	2.28	0.11	0.742478	0.482076
46	8.24	0.53	8.49	0.36	8.46	0.28	0.119369	0.887780
47	2.34	0.11	2.36	0.09	2.32	0.08	0.063168	0.938875
48	1.39	0.07	1.50	0.08	1.37	0.05	1.271017	0.291115
49	7.26	0.48	7.19	0.24	7.27	0.25	0.018297	0.981877
50	13.56	0.71	12.96	0.48	13.04	0.46	0.334316	0.717715
51	1.38	0.08	1.41	0.06	1.41	0.06	0.058011	0.943715
52	1.83	0.11	2.02	0.08	1.89	0.07	1.074761	0.350582
53	4.52	0.29	4.37	0.14	4.44	0.15	0.129684	0.878723
54	0.13	0.02	0.16	0.01	0.13	0.01	0.769274	0.469767
55	2.81	0.18	2.81	0.11	2.80	0.10	0.001297	0.998704
NON-POLAR SPECTRA REGIONS OR METABOLITES								
A	21.44	1.14	22.67	1.10	23.11	1.33	0.525048	0.595359
Ac	31.01	1.73	36.93	1.97	38.55	2.61	3.443508	0.041226

Ac+DGA	1.32	0.21	1.44	0.23	2.19	0.57	1.593887	0.215177
B	38.26	2.23	43.98	2.26	43.41	2.99	1.570102	0.219989
C	89.01	4.75	91.04	4.37	99.45	6.66	1.067841	0.352897
D+U2	8.86	0.53	9.67	0.50	9.93	0.67	0.946308	0.396291
DGA	2.53	0.14	2.94	0.14	2.96	0.20	2.310824	0.111659
DGA+TGA2	0.14	0.01	0.14	0.01	0.15	0.01	0.007076	0.992950
E	20.98	1.65	22.31	1.09	24.51	2.21	1.084632	0.347305
F	6.33	0.36	6.68	0.34	7.13	0.52	0.940376	0.398547
FAI	28.23	1.63	30.89	1.53	31.67	2.17	1.003477	0.375221
FAI+D+U1	14.70	1.06	15.73	0.81	16.66	1.42	0.758778	0.474548
G+Ter	6.58	0.29	8.00	0.41	8.03	0.57	3.605545	0.035885
H+U2	3.77	0.22	3.95	0.19	4.01	0.25	0.314633	0.731762
I	2.76	0.17	3.05	0.14	3.13	0.23	1.158346	0.323833
J	2.16	0.20	2.18	0.13	2.43	0.26	0.534695	0.589783
K + U2	1.01	0.05	1.07	0.05	1.07	0.07	0.433351	0.651199
L	2.61	0.16	2.63	0.12	2.67	0.13	0.039223	0.961572
L+M	1.60	0.09	1.61	0.08	1.59	0.07	0.027936	0.972468
N	1.40	0.18	1.57	0.21	2.13	0.50	1.363426	0.266870
O	0.10	0.01	0.08	0.01	0.08	0.01	1.186918	0.315187
P1	1.66	0.09	1.95	0.10	1.69	0.08	3.223952	0.049826
P1+P2	1.72	0.09	2.05	0.12	1.69	0.08	4.211384	0.021532
P2	1.67	0.11	2.02	0.13	1.63	0.10	3.679042	0.033706
Terpene 1	32.85	1.67	40.21	1.97	39.01	2.58	3.523057	0.038507
TGA1	1.28	0.08	1.38	0.07	1.38	0.09	0.485710	0.618673
TGA2	0.52	0.04	0.56	0.03	0.55	0.04	0.403882	0.670288
Unk.1	70.02	4.38	79.50	4.09	78.89	5.47	1.284464	0.287448
Unk.2	35.88	1.88	40.36	1.94	40.76	2.76	1.476341	0.240086
ELEMENTS AND C:N:P:K RATIOS								
C:N	96.88	2.98	107.07	4.47	96.02	2.60	3.18471	0.051552
N:P	19.55	0.90	20.26	0.87	23.89	1.05	6.04556	0.004927
C:P	1878.94	81.63	2162.61	131.47	2288.71	112.36	3.61315	0.035653
C:K	166.49	16.59	135.54	12.20	197.23	12.14	4.99500	0.011322
K:P	12.41	0.93	17.17	1.19	11.87	0.56	9.81460	0.000318
N:K	0.34	0.03	0.26	0.02	0.41	0.02	7.36858	0.001807
N ($mg.g^{-1}$)	6.12	0.18	5.60	0.23	6.17	0.18	2.60545	0.085772
P ($mg.g^{-1}$)	0.32	0.01	0.28	0.01	0.27	0.02	3.27819	0.047540
K ($mg.g^{-1}$)	4.02	0.39	4.84	0.43	3.13	0.19	5.92095	0.005429
C ($mg.g^{-1}$)	585.09	1.12	585.29	2.21	586.44	2.38	0.13426	0.874731
SPRING								
CLIMATIC TREATMENT								
CONTROL			DROUGHT			WARMING		
Mean	SE	Mean	SE	Mean	SE	F(42)	p	
POLAR METABOLITES								
αG (1)	0.80	0.07	0.56	0.05	0.55	0.06	5.25853	0.009161
ßG (2)	3.14	0.26	2.34	0.20	2.29	0.17	5.11746	0.010258
Suc (3)	9.91	0.61	10.06	0.66	9.82	0.41	0.04446	0.956557
Ala (4)	0.12	0.01	0.12	0.01	0.12	0.01	0.03133	0.969174
Asp (5)	1.33	0.09	1.20	0.14	1.35	0.09	0.57406	0.567591
Gln (6)	0.31	0.02	0.28	0.03	0.28	0.02	0.56587	0.572134
Leu (7)	0.41	0.02	0.38	0.02	0.34	0.02	3.15882	0.052725
Ile (8)	0.32	0.02	0.30	0.02	0.30	0.01	0.39286	0.677580
10	0.86	0.04	0.91	0.04	1.28	0.09	14.25425	0.000019
11	16.87	0.71	17.34	0.90	16.23	0.86	0.44772	0.642098
15	0.72	0.08	0.67	0.06	0.90	0.11	1.90075	0.162092
Q.ac (17)	24.51	1.58	27.71	3.42	22.84	1.75	1.06622	0.353443
T.ac (18)	0.71	0.05	0.82	0.03	0.78	0.05	1.83503	0.172175
Arb (19)	18.19	0.88	18.34	1.22	18.38	1.15	0.00800	0.992029
Ch (20)	0.47	0.04	0.41	0.04	0.37	0.03	1.59916	0.214125
21	3.02	0.13	2.88	0.17	2.81	0.13	0.56373	0.573331

22	0.30	0.03	0.32	0.05	0.28	0.03	0.22554	0.799048
30	0.97	0.07	0.99	0.06	1.18	0.07	2.96852	0.062250
31	0.66	0.07	0.59	0.06	0.84	0.09	3.08445	0.056251
32	0.39	0.04	0.37	0.04	0.48	0.04	2.12375	0.132246
33	2.75	0.25	2.70	0.30	2.58	0.28	0.09994	0.905104
34	2.02	0.14	1.78	0.08	1.96	0.14	1.06665	0.353297
35	0.45	0.02	0.46	0.02	0.57	0.04	5.86788	0.005659
36	1.31	0.09	1.24	0.13	1.20	0.09	0.29684	0.744707
37	11.88	0.66	12.18	1.17	10.67	0.64	0.87292	0.425172
38	9.22	0.59	10.38	1.29	8.61	0.69	0.97444	0.385773
39	2.98	0.14	3.07	0.20	2.92	0.19	0.15403	0.857732
40	9.89	0.48	10.26	0.83	10.42	0.66	0.16038	0.852343
41	10.79	0.57	10.89	0.96	9.21	0.59	1.68343	0.198027
42	1.59	0.14	1.54	0.11	1.88	0.16	1.87252	0.166345
43	2.35	0.13	2.09	0.09	2.18	0.13	1.24266	0.299009
44	1.70	0.17	1.52	0.12	2.14	0.25	2.86993	0.067878
45	3.43	0.21	2.87	0.16	2.76	0.13	4.61591	0.015414
46	14.08	0.75	13.30	0.90	12.38	0.69	1.17927	0.317476
47	3.09	0.15	3.00	0.16	2.86	0.11	0.72670	0.489482
48	1.82	0.08	1.71	0.06	1.62	0.04	2.42658	0.100628
49	12.30	0.64	11.71	0.78	11.09	0.64	0.76037	0.473819
50	17.03	0.94	16.72	1.02	16.38	0.72	0.12940	0.878974
51	2.44	0.16	1.99	0.13	2.08	0.14	2.82914	0.070360
52	3.59	0.23	2.95	0.19	2.85	0.19	3.80464	0.030298
53	7.13	0.35	7.16	0.49	6.70	0.39	0.39609	0.675435
54	0.17	0.01	0.17	0.02	0.12	0.01	4.50208	0.016925
55	4.22	0.23	4.08	0.27	3.95	0.21	0.31972	0.728102
NON-POLAR SPECTRA REGIONS OR METABOLITES								
A	14.55	0.43	14.07	0.41	13.93	0.42	0.59596	0.555628
Ac	14.77	0.74	15.94	1.11	16.34	1.16	0.63993	0.532395
Ac+DGA	0.53	0.04	0.51	0.06	0.56	0.06	0.19409	0.824321
B	21.43	0.88	22.39	1.04	21.90	1.31	0.19209	0.825953
C	62.86	1.81	54.63	2.11	60.25	2.03	4.47514	0.017304
D+U2	5.97	0.22	5.81	0.20	5.74	0.26	0.26859	0.765758
DGA	1.88	0.08	1.76	0.05	2.00	0.08	2.60679	0.085670
DGA+TGA2	0.16	0.01	0.13	0.01	0.15	0.01	3.51806	0.038672
E	18.77	0.70	17.19	0.41	18.35	0.81	1.51994	0.230513
F	5.90	0.69	4.77	0.11	5.11	0.19	1.93892	0.156521
FAI	20.24	0.69	19.57	0.57	19.64	0.80	0.29031	0.749520
FAI+D+U1	10.05	0.31	10.26	0.32	10.91	0.54	1.22653	0.303599
G+Ter	3.38	0.50	3.14	0.23	3.19	0.26	0.13245	0.876309
H+U2	4.01	0.20	3.33	0.10	3.54	0.12	5.68954	0.006508
I	2.61	0.12	2.42	0.09	2.68	0.09	1.85631	0.168840
J	1.43	0.07	1.44	0.06	1.61	0.09	1.83359	0.172403
K + U2	0.80	0.03	0.83	0.04	0.73	0.02	2.73998	0.076123
L	2.88	0.10	2.62	0.07	2.78	0.12	1.75637	0.185116
L+M	2.13	0.08	1.79	0.07	1.85	0.06	6.97615	0.002421
N	0.56	0.04	0.69	0.07	0.62	0.06	1.24383	0.298679
O	0.17	0.01	0.16	0.01	0.18	0.01	1.87642	0.165751
P1	0.94	0.06	0.81	0.04	0.82	0.04	2.16056	0.127901
P1+P2	0.99	0.05	0.94	0.04	0.91	0.05	1.02185	0.368701
P2	0.92	0.07	0.83	0.05	0.85	0.04	0.64680	0.528855
Terpene 1	15.88	0.78	17.55	1.23	17.77	1.27	0.86483	0.428488
TGA1	1.35	0.06	1.21	0.05	1.15	0.04	4.24847	0.020877
TGA2	0.48	0.03	0.38	0.02	0.40	0.01	5.33426	0.008623
Unk.1	38.24	1.60	39.78	1.91	39.56	2.33	0.17967	0.836184
Unk.2	26.29	0.91	24.90	0.71	24.32	1.15	1.16238	0.322598
ELEMENTS AND C:N:P:K RATIOS								
C:N	66.89	2.04	67.59	2.97	67.73	2.77	0.02959	0.970864
N:P	18.66	1.17	16.80	0.38	17.53	0.50	1.48786	0.237517
C:P	1266.69	115.56	1140.53	62.38	1197.83	72.16	0.53317	0.590661

C:K	121.52	15.70	97.92	5.60	97.07	5.10	1.90250	0.161832
K:P	10.95	0.49	11.88	0.59	12.44	0.56	1.88693	0.164160
N:K	0.31	0.01	0.29	0.01	0.29	0.01	1.12559	0.334053
N (mg.g-1)	8.56	0.24	8.57	0.32	8.56	0.39	0.00096	0.999044
P (mg.g-1)	0.48	0.03	0.52	0.03	0.50	0.03	0.35620	0.702431
K (mg.g-1)	5.32	0.40	6.06	0.35	6.09	0.38	1.31864	0.278344
C (mg.g-1)	565.84	2.21	566.26	2.21	564.64	2.07	0.15043	0.860796
SUMMER								
CLIMATIC TREATMENT								
CONTROL			DROUGHT			WARMING		
Mean	SE	Mean	SE	Mean	SE	F(42)	p	
POLAR METABOLITES								
αG (1)	0.55	0.06	0.52	0.09	0.55	0.06	0.054481	0.947043
ßG (2)	2.64	0.23	2.60	0.33	2.55	0.21	0.031729	0.968793
Suc (3)	9.09	0.41	9.58	0.39	9.73	0.28	0.842453	0.437800
Ala (4)	0.11	0.01	0.10	0.01	0.13	0.01	1.420362	0.252994
Asp (5)	0.78	0.07	0.80	0.09	0.61	0.06	1.965448	0.152768
Gln (6)	0.13	0.01	0.15	0.01	0.11	0.01	1.940438	0.156304
Leu (7)	0.40	0.01	0.35	0.02	0.39	0.02	2.399256	0.103125
Ile (8)	0.33	0.02	0.34	0.02	0.35	0.02	0.307310	0.737062
10	1.01	0.08	1.10	0.07	1.21	0.08	1.922749	0.158856
11	11.74	0.71	13.84	0.59	12.43	0.58	2.916944	0.065131
15	0.61	0.05	0.60	0.06	0.72	0.07	1.201328	0.310919
Q.ac (17)	10.20	0.74	12.52	0.78	9.01	0.45	7.003090	0.002373
T.ac (18)	0.80	0.08	1.02	0.07	0.98	0.08	2.314468	0.111293
Arb (19)	12.80	0.58	13.64	0.57	13.52	0.46	0.706704	0.499038
Ch (20)	0.41	0.05	0.49	0.06	0.23	0.04	6.622834	0.003162
21	2.20	0.17	2.47	0.20	2.08	0.10	1.506910	0.233331
22	0.36	0.03	0.36	0.04	0.36	0.03	0.004755	0.995256
30	0.72	0.04	0.76	0.04	0.81	0.05	1.097795	0.342987
31	0.42	0.06	0.31	0.03	0.46	0.05	2.820985	0.070868
32	0.20	0.02	0.15	0.01	0.20	0.03	1.684405	0.197847
33	0.32	0.03	0.34	0.05	0.35	0.04	0.144627	0.865774
34	1.80	0.14	1.86	0.15	1.69	0.11	0.413655	0.663893
35	0.62	0.03	0.66	0.04	0.72	0.05	1.442061	0.247906
36	0.76	0.05	0.79	0.04	0.74	0.04	0.380464	0.685876
37	6.99	0.31	7.93	0.31	7.10	0.24	3.182890	0.051634
38	3.68	0.28	4.57	0.31	3.24	0.18	6.523695	0.003410
39	2.05	0.07	2.21	0.08	2.23	0.06	2.133045	0.131134
40	6.98	0.29	7.68	0.28	7.24	0.19	1.897388	0.162592
41	5.11	0.28	5.74	0.31	4.87	0.14	3.111797	0.054927
42	0.92	0.08	0.80	0.07	1.10	0.11	2.980564	0.061597
43	1.46	0.09	1.59	0.10	1.71	0.10	1.658429	0.202665
44	1.18	0.14	0.92	0.09	1.47	0.14	4.914950	0.012079
45	3.25	0.20	3.35	0.28	3.35	0.16	0.074652	0.928189
46	9.64	0.38	10.19	0.47	9.90	0.30	0.493220	0.614150
47	2.74	0.13	2.91	0.15	2.79	0.07	0.507574	0.605599
48	1.63	0.08	1.72	0.11	1.63	0.06	0.429837	0.653445
49	8.86	0.38	9.31	0.43	9.64	0.33	1.074911	0.350531
50	15.39	0.74	16.30	0.73	16.96	0.56	1.321738	0.277535
51	2.13	0.12	2.20	0.16	2.22	0.12	0.115832	0.890909
52	2.87	0.16	3.02	0.24	3.09	0.15	0.349659	0.706961
53	5.00	0.20	5.39	0.21	5.58	0.14	2.474047	0.096440
54	0.18	0.02	0.19	0.02	0.14	0.01	2.578747	0.087836
55	3.32	0.13	3.49	0.17	3.45	0.10	0.397168	0.674719
NON-POLAR SPECTRA REGIONS OR METABOLITES								
A	24.49	0.72	25.11	1.65	25.88	1.44	0.27512	0.760837
Ac	33.05	1.46	39.33	3.43	38.36	2.67	1.63300	0.207501

Ac+DGA	1.01	0.18	0.99	0.16	0.71	0.09	1.32391	0.276967
B	40.66	1.75	46.80	3.94	46.46	2.92	1.31611	0.279006
C	94.10	3.90	100.38	7.65	103.14	7.48	0.49613	0.612405
D+U2	9.82	0.37	10.73	0.88	10.82	0.62	0.71789	0.493665
DGA	2.41	0.10	2.72	0.14	2.66	0.20	1.16311	0.322373
DGA+TGA2	0.13	0.01	0.11	0.01	0.09	0.01	2.93199	0.064276
E	21.79	0.97	24.09	1.80	29.50	2.28	5.01939	0.011101
F	6.11	0.28	6.67	0.48	7.60	0.77	1.91465	0.160039
FAI	32.33	1.22	34.40	2.56	35.01	2.02	0.48922	0.616550
FAI+D+U1	15.35	0.64	17.25	1.30	18.87	1.17	2.69423	0.079270
G+Ter	6.42	0.36	8.12	0.66	8.39	0.85	2.64488	0.082818
H+U2	3.12	0.16	3.46	0.22	3.52	0.22	1.14251	0.328731
I	2.61	0.12	3.10	0.24	3.22	0.25	2.28110	0.114691
J	2.17	0.11	2.37	0.20	2.75	0.18	3.08676	0.056138
K + U2	0.88	0.05	0.95	0.08	0.93	0.04	0.40466	0.669773
L	2.68	0.12	2.63	0.16	2.65	0.14	0.03103	0.969467
L+M	1.56	0.06	1.62	0.10	1.68	0.10	0.50631	0.606343
N	1.01	0.15	1.04	0.14	0.83	0.09	0.84585	0.436369
O	0.11	0.01	0.12	0.01	0.11	0.01	0.15473	0.857133
P1	1.79	0.09	2.01	0.13	1.80	0.12	1.15425	0.325091
P1+P2	1.90	0.09	2.08	0.13	1.87	0.13	0.89839	0.414904
P2	1.67	0.08	1.84	0.13	1.58	0.12	1.32187	0.277500
Terpene 1	34.71	1.88	43.79	3.59	42.77	3.26	2.73789	0.076264
TGA1	1.16	0.04	1.27	0.11	1.27	0.09	0.55956	0.575662
TGA2	0.43	0.01	0.53	0.05	0.49	0.04	1.81081	0.176055
Unk.1	73.18	3.15	84.06	7.12	84.38	5.04	1.41719	0.253745
Unk.2	38.61	1.47	43.78	3.09	45.54	3.10	1.82770	0.173340
ELEMENTS AND C:N:P:K RATIOS								
C:N	104.76	2.38	110.68	3.39	106.28	1.66	1.42625	0.251601
N:P	24.28	0.55	25.16	0.97	26.64	1.10	1.74474	0.187113
C:P	2535.09	60.99	2759.19	88.47	2834.20	129.21	2.57276	0.088305
C:K	159.67	11.10	149.72	13.09	178.98	14.16	1.34176	0.272358
K:P	16.91	1.13	19.66	1.10	16.63	0.89	2.58626	0.087250
N:K	0.31	0.02	0.27	0.03	0.33	0.03	1.39983	0.257908
N ($mg.g^{-1}$)	5.54	0.13	5.30	0.16	5.43	0.10	0.81835	0.448067
P ($mg.g^{-1}$)	0.23	0.01	0.21	0.01	0.21	0.01	1.99244	0.149045
K ($mg.g^{-1}$)	3.87	0.28	4.22	0.30	3.52	0.29	1.46982	0.241553
C ($mg.g^{-1}$)	575.92	1.93	579.02	2.51	575.32	2.24	0.78300	0.463589

WINTER

CLIMATIC TREATMENT							
CONTROL		DROUGHT		WARMING			
Mean	SE	Mean	SE	Mean	SE	F(42)	p
POLAR METABOLITES							
αG (1)	0.35	0.03	0.33	0.03	0.30	0.03	0.74601	0.480433
ßG (2)	2.04	0.09	2.05	0.11	1.98	0.11	0.12062	0.886677
Suc (3)	8.76	0.34	8.61	0.40	9.39	0.46	1.05284	0.357973
Ala (4)	0.09	0.01	0.09	0.01	0.11	0.01	2.29020	0.113754
Asp (5)	1.21	0.12	1.20	0.10	1.28	0.09	0.20340	0.816754
Gln (6)	0.19	0.01	0.20	0.01	0.21	0.02	0.23691	0.790112
Leu (7)	0.43	0.02	0.39	0.02	0.43	0.03	0.99870	0.376935
Ile (8)	0.35	0.03	0.34	0.02	0.37	0.02	0.48934	0.616485
10	0.71	0.04	0.81	0.05	0.86	0.08	1.60794	0.212386
11	11.48	0.82	13.12	0.56	12.57	0.86	1.21432	0.307122
15	0.77	0.06	0.74	0.05	1.09	0.08	9.77961	0.000326
Q.ac (17)	7.21	0.45	8.78	0.62	6.82	0.40	4.34088	0.019335
T.ac (18)	0.72	0.08	0.95	0.06	0.89	0.12	1.67446	0.199677
Arb (19)	11.73	0.69	11.56	0.68	12.32	0.71	0.33772	0.715314
Ch (20)	0.60	0.08	0.63	0.06	0.66	0.07	0.19436	0.824095
21	2.50	0.13	2.55	0.15	2.74	0.15	0.77772	0.465958

22	0.41	0.05	0.45	0.04	0.45	0.05	0.21723	0.805641
30	0.70	0.07	0.69	0.03	0.81	0.09	0.84165	0.438140
31	0.56	0.07	0.47	0.03	0.66	0.06	2.53124	0.091636
32	0.30	0.03	0.26	0.02	0.36	0.02	3.86315	0.028835
33	0.30	0.06	0.22	0.02	0.30	0.04	1.11451	0.337584
34	1.15	0.09	1.38	0.13	1.38	0.15	0.99962	0.376607
35	0.47	0.03	0.47	0.03	0.59	0.05	3.79493	0.030548
36	0.54	0.03	0.65	0.03	0.56	0.03	4.19843	0.021765
37	6.08	0.25	6.41	0.31	6.07	0.34	0.40372	0.670395
38	2.48	0.17	3.01	0.22	2.32	0.17	3.72344	0.032458
39	1.93	0.10	1.89	0.10	2.00	0.10	0.30989	0.735192
40	6.18	0.25	6.18	0.21	6.36	0.26	0.18328	0.833199
41	5.15	0.28	5.45	0.31	5.22	0.29	0.28946	0.750150
42	1.24	0.10	1.09	0.07	1.66	0.10	10.22921	0.000240
43	1.61	0.10	1.62	0.08	1.89	0.18	1.55543	0.223015
44	1.46	0.14	1.25	0.10	1.98	0.17	7.31405	0.001882
45	2.67	0.09	2.80	0.12	2.89	0.12	1.00596	0.374332
46	9.01	0.42	9.10	0.43	9.64	0.47	0.61986	0.542868
47	2.50	0.08	2.50	0.09	2.65	0.10	0.88986	0.418313
48	1.48	0.06	1.56	0.07	1.60	0.06	0.95233	0.394015
49	8.25	0.39	8.14	0.42	8.66	0.46	0.42193	0.658527
50	14.59	0.55	14.41	0.68	16.03	0.88	1.53416	0.227477
51	1.83	0.07	1.86	0.08	1.95	0.09	0.54264	0.585231
52	2.53	0.10	2.63	0.15	2.67	0.14	0.33199	0.719359
53	4.79	0.24	4.66	0.22	5.01	0.27	0.53694	0.588493
54	0.15	0.02	0.18	0.02	0.17	0.02	0.36092	0.699175
55	3.06	0.13	3.04	0.13	3.18	0.14	0.31216	0.733547
NON-POLAR SPECTRA REGIONS OR METABOLITES								
A	21.46	2.16	19.9	1.03	21.95	1.66	0.40391	0.670269
Ac	32.48	3.19	35.01	2.54	36.95	3.13	0.56970	0.570003
Ac+DGA	0.73	0.10	0.74	0.09	0.69	0.11	0.05514	0.946423
B	40.76	3.78	42.49	2.98	45.17	3.84	0.39043	0.679195
C	81.91	8.21	73.41	4.72	84.81	5.85	0.84958	0.434812
D+U2	9.08	0.91	8.86	0.58	10.08	0.86	0.66637	0.518915
DGA	2.32	0.23	2.08	0.14	2.49	0.17	1.18466	0.315861
DGA+TGA2	0.15	0.02	0.13	0.01	0.13	0.01	0.53516	0.589516
E	19.28	1.57	17.54	0.94	21.54	1.63	2.01420	0.146113
F	7.14	0.75	6.42	0.40	7.38	0.64	0.66349	0.520364
FAI	29.56	3.08	28.76	1.88	32.01	2.69	0.42676	0.655416
FAI+D+U1	13.29	1.17	12.78	0.76	15.36	1.22	1.63241	0.207616
G+Ter	8.05	0.79	8.39	0.64	8.62	0.72	0.16436	0.848979
H+U2	3.94	0.28	3.21	0.15	4.01	0.24	3.74665	0.031824
I	2.56	0.28	2.42	0.20	2.86	0.21	0.92105	0.405990
J	1.7	0.15	1.48	0.11	1.94	0.15	2.69142	0.079468
K + U2	0.97	0.08	0.91	0.06	1	0.10	0.31792	0.729396
L	2.68	0.21	2.26	0.13	2.72	0.13	2.53712	0.091156
L+M	1.52	0.11	1.28	0.07	1.5	0.07	2.43436	0.099928
N	0.73	0.08	0.75	0.06	0.77	0.09	0.07480	0.928057
O	0.08	0.01	0.08	0.01	0.08	0.01	0.02236	0.977905
P1	1.58	0.13	1.59	0.08	1.45	0.08	0.54033	0.586549
P1+P2	1.65	0.13	1.71	0.10	1.48	0.09	1.20468	0.309936
P2	1.5	0.11	1.57	0.10	1.24	0.07	3.21748	0.050107
Terpene 1	35.22	3.10	38.82	2.75	39.76	3.01	0.65694	0.523682
TGA1	1.48	0.16	1.29	0.09	1.42	0.11	0.62412	0.540626
TGA2	0.48	0.04	0.47	0.03	0.47	0.03	0.05113	0.950213
Unk.1	71.43	6.77	73.96	5.03	80.65	7.09	0.56064	0.575059
Unk.2	39.64	3.78	39.66	2.62	41.92	3.41	0.15786	0.854473
ELEMENTS AND C:N:P:K RATIOS								
C:N	92.7	3.84	101.88	4.79	97.9	3.06	1.35047	0.270137
N:P	21.7	0.85	24.13	1.14	23.99	0.52	2.42202	0.101040
C:P	2004.38	102.12	2432.91	146.04	2342.89	79.62	4.02045	0.025258

C:K	196.91	16.37	167.95	16.20	181.24	9.64	1.01155	0.372344
K:P	10.72	0.72	16.01	1.29	13.19	0.47	8.80428	0.000641
N:K	0.43	0.04	0.34	0.04	0.37	0.02	2.16995	0.126817
N (mg.g-1)	6.46	0.27	5.93	0.26	6.09	0.20	1.20008	0.311286
P (mg.g-1)	0.3	0.02	0.25	0.01	0.26	0.01	4.68534	0.014562
K (mg.g-1)	3.21	0.22	4.04	0.42	3.37	0.17	2.32640	0.110104
C (mg.g-1)	583.91	1.61	586.39	2.99	587.53	2.33	0.60357	0.551530

Table S6. One-way ANOVAs of the first four factor score coordinates from the PCA including metabolites and ratios presenting differences between climatic treatments (Figure 3).

Different letters indicate significant statistical differences between climatic treatments within each season ($p<0.05$, post-hoc LSD-Fisher test).

		CLIMATIC TREATMENT										
		CONTROL			DROUGHT			WARMING				
		Mean	SE		Mean	SE		Mean	SE		$F_{(42)}$	p
Winter	PC1	-0.08	0.67	b	-1.51	0.40	b	1.59	0.64	a	7.1351	0.002150
	PC2	-0.36	0.40	b	0.94	0.52	a	-0.58	0.38	b	3.5388	0.037991
	PC3	1.12	0.43	a	-0.52	0.41	b	-0.60	0.34	b	6.0021	0.005096
	PC4	0.48	0.32		-0.56	0.31		0.07	0.28		2.9929	0.060932
Spring	PC1	-1.77	0.83	b	1.06	0.55	a	0.71	0.44	a	5.9647	0.005247
	PC2	-0.80	0.34	b	-0.83	0.24	b	1.63	0.64	a	10.187	0.000247
	PC3	0.41	0.47		-0.23	0.44		-0.18	0.38		0.6955	0.504468
	PC4	0.24	0.29		0.28	0.28		-0.52	0.32		2.3173	0.111005
Summer	PC1	1.19	0.31	a	-0.46	0.67	b	-0.73	0.57	b	9.2776	0.000460
	PC2	0.04	0.51	a	-1.41	0.56	b	1.37	0.34	a	5.0589	0.010752
	PC3	0.05	0.46		0.70	0.33		-0.74	0.49		2.0930	0.135988
	PC4	0.10	0.29		-0.21	0.30		0.11	0.33		0.8809	0.421905
Autumn	PC1	-0.10	0.75	b	-1.98	0.60	b	2.08	0.64	a	3.7330	0.032194
	PC2	-1.22	0.35	b	1.08	0.61	a	0.15	0.55	ab	8.4497	0.000824
	PC3	-0.45	0.50		0.80	0.36		-0.35	0.56		2.7931	0.072631
	PC4	-0.39	0.45		0.39	0.44		-0.01	0.35		0.3593	0.700265

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APPENDIX 4

Rivas-Ubach et al., (2013) Submitted in New Phytologist.

Materials & Methods

Chemical analyses

C and N analyses: For each sample, 1.4 mg of powder were analyzed. C and N concentrations were determined by elemental analysis using combustion coupled to gas chromatography with a CHNS-O Elemental Analyser (EuroVector, Milan, Italy).

P and K analyses: The P and K analyses were performed using acid digestion in a microwave reaction system under high pressure and temperature. Briefly, 250 mg of leaf powder were transferred to a Teflon tube, and 5 mL of nitric acid and 2 mL of H_2O_2 were added (7). A MARSXpress microwave reaction system (CEM, Mattheus, USA) was used to perform the acid digestions. The temperature was increased to 130 ºC with a 10-min ramp, samples were maintained at this temperature for 5 min, then the temperature was increased to 200 ºC with a 10 min ramp where the samples were maintained for 20 min. To end the digestions, the temperature was increased to 220 ºC with a 5 min ramp and maintained for a further 20 min.

All digested contents were transferred to 50-mL flasks and resuspended in Milli-Q water to a final volume of 50 mL. After digestion, the P and K concentrations were determined by ICP-OES (Optic Emission Spectrometry with Inductively Coupled Plasma) in an *Optima 2300RL* spectrometer (The Perkin-Elmer Corporation, Norwalk, USA).

NMR Elucidation.

The polar and nonpolar metabolic profiles of *Quercus ilex* leaf are shown in Figures S2a and S2b respectively and in Tables S1 and S2.

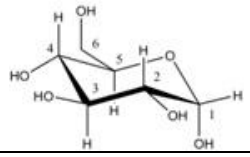
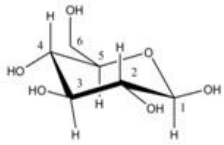
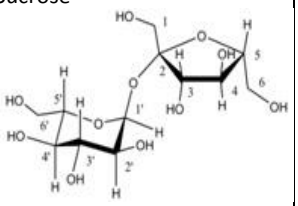
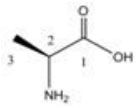
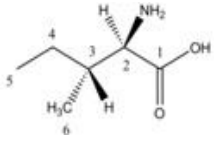
The detailed ^{1}H NMR metabolic profile of the polar extract is shown in Figures S3. The signals in the region between 3.2 and 5.9 ppm correspond mainly to sugars. Among them, α-glucose and β-glucose, with anomeric proton resonances at 5.29 ppm (d, J = 3.8 Hz) and at 4.70 ppm (d, J = 8.2 Hz) respectively, were identified. The disaccharide sucrose was also observed, with its characteristic anomeric proton doublet of the glucose unit at 5.45 ppm (d, J = 3.8 Hz). The proton singlet at 3.19 ppm is correlated via HSQC to the carbon signal at 53.8 ppm, corresponding to choline which is an important osmolyte. In the aliphatic region between 2.2 and 1.7 ppm, the secondary metabolites quinic acid and quercitol were identified, showing very intense signals. Among amino acids, valine, alanine, isoleucine, threonine and lysine were identified. Some organic acids such as formic acid (sharp singlet at 8.50 ppm), citric acid, acetic acid and lactic acid were identified as well. Finally, signals at the 6.5 - 7.4 ppm region, area mainly of aromatic

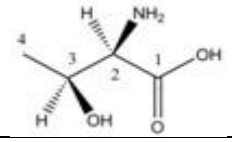
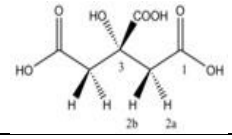
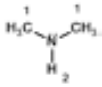
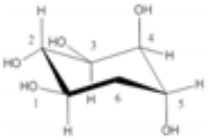
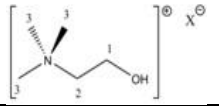
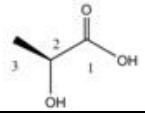
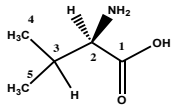
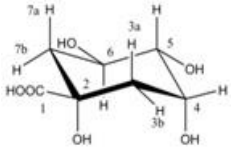
compounds, were also observed. All the identifications were based on the ^{1}H and ^{13}C NMR complete or partial assignment of the molecules based on 1D and 2D NMR experiments and on the comparison with reported data. For the complete description see Table S1.

Figure S2b shows the ^{1}H NMR metabolic profile of the nonpolar extract samples. Saturated and unsaturated fatty acid chains were identified, which come from free fatty acids, fatty alcohols, diacylglycerols and triacylglycerols. Also, *p*-coumaric acid derivatives were observed in the aromatic region. The complete description of nonpolar metabolites is shown in Table S2.

Results

Table S1. NMR assignments of the metabolites identified in polar samples. Samples dissolved in D_2O (pH 6.0) and ^{1}H and ^{13}C NMR chemical shifts referenced to TSP.

Polar Metabolites							Bibliographical References
Metabolite		NMR chemical shift (δ)			Assignment		
Name	Atom id.	^{1}H		^{13}C	a, b, c	Id.	
	molec.	(ppm)	m,[d] *J* (Hz)[e]	(ppm)			
α-Glucose 	1	5.29	d; 3,8	92.6	a [COSY, TOCSY, HSQC, HMBC], b, c	**2**	(Fan & Lane, 2008, Fan, 1996, Walker et al. 1982)
	2	-	-	-			
	3	3.68	m*	73.0			
	4	3.45	m*	70.5			
	5	3.88	m*	72.8			
	6	3.95	m*	61.2			
β-Glucose 	1	4.70	d; 8,2	96.4	a [COSY, TOCSY, HSQC, HMBC], b, c	**3**	(Fan & Lane, 2008, Fan, 1996, Walker et al. 1982)
	2	3.29	dd; 9,3; 8,3	73.9			
	3	3.59	m*	77.0			
	4	*	*	*			
	5	3.53	m*	76.2			
	6	3.81	m*	61.5			
Sucrose 	1	3.73	s	61.6	a [COSY, TOCSY, HSQC, HMBC], b, c	**1**	(Breitmaier et al. 1979, Fan & Lane, 2008, Fan, 1996)
	2	*	*	*			
	3	4.26	d; 8,8	76.8			
	4	4.08	t; 8,6	74.4			
	5	3.94	m	81.8			
	6	3.87	d, 3,4	62.9			
	1'	5.45	d; 3,8	92.7			
	2'	3.61	m	71.5			
	3'	3.82	t; 9,5	72.8			
	4'	3.51	t; 9,4	69.7			
	5'	3.88	m	72.8			
	6'	3.86	d; 3,4	60.5			
Alanine 	1	-	-	178.0	a [COSY, HSQC, HMBC], b, c	**5**	(Breitmaier et al. 1979, Fan & Lane, 2008, Fan, 1996, Iles et al. 1985, London, 1988)
	2	3.84	*	52.5			
	3	1.53	d; 7,3	16.5			
Isoleucine 	1	-	-	*/**	a [COSY, HSQC], b, c	**6**	(Breitmaier et al. 1979, Fan & Lane, 2008, Fan, 1996, Iles et al. 1985)
	2	*	d*/**	*/**			
	3	1.99	m*/**	*/**			
	4a	1.25	m	16.9			
	4b	1.48	m	16.9			
	5	0.93	t; 7,3	26.5			
	6	1.02	d;8,1	23.2			
Threonine	1	-	-		a [COSY, HSQC], b, c		(Bolinger et al. 1984, Breitmaier et
	2	3.58	*	*/**			
	3	4.25	m	66.3			

	4	1.32	d; 6,40	20.6			al. 1979, Fan & Lane, 2008, Fan, 1996)
Citrate 	1 2a 2b 3	- 2.58 2.55 -	* d* d*	* 45.6 45.6 *	a [COSY, HSQC], b, c	**4**	(Fan & Lane, 2008, Fan, 1996, Iles et al. 1985)
Dimethylamine 	1 2	2.74 2.74	s s	36.0 36.0	a [HSQC], b,c		(Fan & Lane, 2008, Fan, 1996)
Quercitol 	1 2 3 4 5 6a 6b	3.77 3.57 3.74 3.96 4.04 2.00 1.82	 t;9,5 t* q* dt;13,9;3,4 td;13,9;3,4	68.8 74.3 70.8 72.2 68.3 33.2 33.2	a [COSY, TOCSY, HSQC, HMBC], b, c	**8**	(McCasland et al. 1968)
Choline 	1 2 3	3.99 3.52 3.19	m m s	69.6 58.0 53.8	a [COSY, HSQC, HMBC], b, c	**10**	(Fan, 1996, Govindaraju et al. 2000)
Lactate 	1 2 3	- 4.20 1.37	* q* d*	* 70.3 17.0	a [COSY,HSQC], b, c	**11**	(Fan & Lane, 2008, Fan, 1996, Iles et al. 1985)
Valine 	1 2 3 4 5	- 3.61 2.28 1.01 1.05	- d* m d* d*	* * 33.4 17.6 *	a [COSY], b, c	**7**	(Fan & Lane, 2008, Fan, 1996)
Quinic acid 	1 2 3a 3b 4 5 6 7a 7b	- - 2.00 2.07 4.18 3.58 4.04 1.90 2.10	- - dt; ** dd; ** dt; ** dd; ** m dd; ** ddd; ***	* 77.5 37.3 37.3 70.4 63.9 63.1 41.0 41.0	a [COSY, TOCSY, HSQC, HMBC], b, c	**9**	(Corse & Lundin, 1970, Duarte et al. 2006)
Formate 	1	8.50	s	*	a,b,c	**10**	(Brown et al. 1989, Fan & Lane, 2008, Fan, 1996, Nicholson et al. 1985)
Lysine	1 2 3a 3b 4a 4b 5a 5b 6	- * 1.87 * * * 1.75 * 3.04	- * m * * * m * t;7,5	* * 32.15 32.15 * * 29.11 29.11 41.78	a [COSY, TOCSY, HSQC, HMBC], b, c	**11**	(Castro & Manetti, 2007, Castro et al. 2008, Fan & Lane, 2008, Fan, 1996, Manetti et al. 2006, Walker et al. 1982)

*** Overlapped signal.**
**** Not enough signal.**
a From 1D ^{1}H NMR chemical shift and 2D ^{1}H-^{1}H and ^{1}H-^{13}C NMR couplings.
b Comparison with ^{1}H and ^{13}C NMR data from references indicated.
c Comparison with ^{1}H and ^{13}C NMR data from the Biological Magnetic Resonance Data Bank. (http://www.bmrb.wisc.edu/).17
d Multiplicity: singlet (s), doublet (d), t (triplet), q (quartet), dd (doublet of doublets), dt (doublet of triplets), ddd (doublet of doublet of doublets), m (multiplet).
e ^{1}H-^{1}H coupling constant.

Table S2. NMR assignments of the main peaks of nonpolar samples. Samples dissolved in $CDCl_3$ and ^{1}H and ^{13}C NMR chemical shifts referenced to the residual solvent peak.

NMR Peak/Spectral Region				Assignment	Bibliographical References
$\delta^{1}H$ (ppm)	**m, *J*** (Hz)	**$\delta^{13}C$** (ppm)	**id.** spect.	**metabolite (atom id.)/group**	(Gunstone, 1995, Lie Ken Jie & Lam, 1995, Llusia et al. 2008, Nemethy & Calvin, 1982, Sacchi et al. 1997, The AOCS Lipid Library, Ulrich & Zhulin, 2007, Vlahov, 1999)
0.86-0.89	t	14.1	A	-CH3 ; FA[a] chains	
0.93-0.96	t	14.4	B	-CH3 ; linoleyl FA chains	
1.24-1.27	br	29.1-29.9	C	-CH2 ; FA chains	
1.37	t; 7,2	13.8		CH3-CH2-OR; Ethoxy group	
1.57	m	33.5		HO-CH2-CH2- ; FAl[b]	
1.58-1.64	m	25.0	D	-CO-CH2-CH2- ; FA chains	
2.02-2.10	m	27.0	E	=CH-CH2- ; UFA[c] chains	
2.24-2.37	t	34.3	F	-CO-CH2- ; FA chains	
2.33-2.36	t	33.9	G	-CO-CH2- ; FFA[d]	
2.76	*	25.6		=CH-CH2-CH=; linoleyl and/or linolenyl chain	
2.78-2.83	m	25.7	H	=CH-CH2-CH= ; PUFA[e] chains	
3.63	t	63.1		HO-CH2-; FAl	
4.37	q; 7,2	61.7		CH3-CH2-OR; Ethoxy group	
3.75	dd	68.4	I	-CH2OH; 1,2-DAG[f]	
3.89	dd	68.4	I	-CH2OH; 1,2-DAG	
4.15	dd	62.1	I	-CH2O-; TAG[g]	
4.21	dd	62.7	I	-CH2O-; 1,2-DAG	
4.29	dd	62.1	I	-CH2O-; TAG	
4.40	dd	62.7	I	-CH2O-; 1,2-DAG	
5.26	*	68.9	J	-CHO-; TAG	
5.29-5.42	m	127.7-132.0	K	-CH= ; UFA chains	
5.31	*	70.2	L	-CHO-; 1,2-DAG	
6.29	d; 16,0	116.3	M	PCAD[h](2)	
6.83	d; 8,5	115.8	M	PCAD(6/8)	
7.43	d; 8,5	129.9	M	PCAD(5/9)	
7.60	d; 16,0	143.6	M	PCAD(3)	
-		166.9		PCAD(1)	
-		127.1		PCAD(4)	
-		157.4		PCAD(7)	

a. FA: fatty acid; b. FAl: fatty alcohol; c. UFA: unsaturated fatty acid; d. FFA: free fatty acid; e. PUFA:polyunsaturated fatty acid; f. 1,2-DAG: 1,2-diacylglycerol; g. TAG: triacylglycerol; h. PCAD: *p*-coumaric acid derivative
* Overlapped signal.

Table S3. LC-MS chromatogram processing. Chromatograms obtained by LC-MS and processed by MzMine 2.0 (Pluskal et al. 2010). The following table summarizes the different processes and parameters applied to foliar *Q. ilex* chromatograms.

		(+H) Chromatograms		(-H) Chromatograms
1	**Baseline correction**			
	Chromatogram type	TIC		TIC
	MS level	1		1
	Smoothing	10E7		10E7
	Asymmetry	0.001		0.001
2	**Mass detection** (Exact Mass)			
	Noise level	5×10^5		4×10^5
3	**Chromatogram builder**			
	Min time span	0.05		0.05
	Min height	25000		25000
	m/z tolerance	0.002		0.002
4	**Smoothing**			
	Filter width	5		5
5	**Chromatogram deconvolution (Local minimum search)**			
	Chromatographic threshold	65%		65%
	Search minimum in RT range (min)	0.1		0.1
	Minimum relative height	5.0%		5.0%
	Minimum absolute height	30000		30000
	Min ratio of peak top/edge	2		2
	Peak duration range	0.0-2.0		0.0-2.0
6	**Chromatogram alignment (join alignment)**			
	m/z tolerance	0.001		0.001
	weight for m/z	80		80
	RT tolerance	0.15		0.2
	Weight for RT	20		20
7	**Gap filling (Peak Finder)**			
	Intensity tolerance	20%		20%
	m/z tolerance	0.001		0.001
	Retention time tolerance	0.1		0.1
	RT correction	marked		marked
8	**Filtering**			
	Minimum peaks in a row	8		8
	Ions excluded from database	<75 83.05 102.05 114.09 227.17 607.29 Between 0.0 and 1 min Between 28.5 and 30 min		<85 119.035 223.082 391.196 159.25 186.186 Between 0.0 and 1,1 min Between 27.0 and 30 min

Table S4. Metabolite assignment by LC-MS. The assignment of the metabolites was based on the standards. The following table summarizes the retention time (RT) and mass (m/z) of the assigned metabolites in both positive and negative ionization modes.

Mode	**Compound**	**RT (min)**	**m/z**
Negative	Catechin	3.44	289.0718
Negative	Chlorogenic acid	3.11	353.0873
Negative	Citric acid	1.77	191.0196
Negative	Deoxy-hexose	1.42	163.0618
Negative	Disaccharide	1.43	341.108
Negative	Epicatechin	4.93 - 5.2	289.0713
Negative	Epigallocatechin	1.54 - 2.64	305.0667
Negative	Gallic acid	1.55 - 1.83	169.0147
Negative	Hexose	1.44	179.056
Negative	Homoorientin	9.45	447.0923
Negative	Kampferol	14.82	285.0404
Negative	Lactic acid	1.52; 1.75	89.0245
Negative	Malic acid	1.51; 1.78	133.0143
Negative	Pentose	1.43	149.0456
Negative	Pyruvate	1.65	87.0089
Negative	Quercetin	13.72	301.0355
Negative	Quinic acid	1.47	191.056
Negative	Rhamnetin	15.98	315.0509
Negative	Sodium salicylate	10.51	137.0245
Negative	Succinic acid	1.74; 1.78	117.0194
Positive	Adenine	1.42; 1.77	136.0614
Positive	Adenosine	1.49; 1.75	268.1038
Positive	α-humulene	20.27	205.1949
Positive	Alanine	1.43	90.054
Positive	Arginine	1.34	175.119
Positive	Aspartic acid	1.5	134.044
Positive	Caryophyllene	21.46	221.1899
Positive	Catechin	3.44	291.0863
Positive	Chlorogenic acid	3.11	355.084
Positive	Epigallocatechin	1.54 - 2.64	307.0812
Positive	Glutamic acid	1.41	148.0604
Positive	Glutamine	1.46	147.076
Positive	Kampferol	14.82	287.0552
Positive	Leucine	1.76	132.101
Positive	Luteolin	13.68	287.0551
Positive	Phenylalanine	1.91	166.086
Positive	Proline	1.49	116.07
Positive	Pyridoxine	1.38	170.0812
Positive	Quercetin	13.72	303.0498
Positive	Rhamnetin	15.95	317.0653
Positive	Tryptophan	2.49	205.097
Positive	Tyrosine	1.54 - 1.77	182.081
Positive	Valine	1.53	118.086

Table S5. Data for one-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q. ilex* leaves for seasons (summer, autumn, winter, spring). Integral mean values (mM relative to initial TSP or TMS concentrations (0.01%)) are given for the RMN variables, marked by asterisks (*). Integral mean values of deconvoluted total intensities are given for the LC-MS variables, marked by crosses (†). Different letters beside the values indicate significant seasonal differences detected by LSD post-hoc tests ($p < 0.05$).

	SEASON									
Variable	SUMMER		AUTUMN		WINTER		SPRING		*F*	*p*
C:N	42.29829	a	39.16987	b	42.18622	a	43.55859	a	3.58477	0.017607
N:P	13.62614		12.90552		13.58659		12.65678		1.11242	0.349497
C:P	571.9214	a	502.6113	b	570.9746	a	550.5556	ab	2.84708	0.043165
C:K	77.1155	b	84.7965	b	116.2141	a	109.6029	a	9.68552	0.000018
K:P	7.869561	a	6.490965	b	5.374841	b	5.393000	b	6.07256	0.000932
N:K	1.856670	d	2.202410	cd	2.785448	ab	2.533329	bc	6.69686	0.000458
N (mg/g dry weight)	11.88250	b	12.97053	a	11.96500	b	11.59750	b	4.61285	0.005130
P (mg/g dry weight)	0.885445	b	1.033817	a	0.892095	b	0.930850	b	4.13922	0.009028
K (mg/g dry weight)	6.862471	a	6.437835	a	4.656478	b	4.922463	b	8.64768	0.000053
C (mg/g dry weight)	496.7660	b	501.5189	a	501.2745	a	501.2185	a	2.54490	0.062412
aG *	6.017010		5.627649		5.823781		6.496155		1.04830	0.376300
BG *	4.150334	b	4.488954	ab	4.179343	b	5.279338	a	3.53452	0.018712
Suc *	8.975502	a	5.342097	b	7.530463	a	4.695855	b	10.77758	0.000006
Deoxy-Hex †	11312267	a	10177827	ab	9503434	bc	8283399	c	5.24351	0.002438
Hex †	83095558	b	67722637	b	122136094	a	79844162	b	8.52596	0.000061
Pent †	6887232	b	7031891	b	8693196	a	6937933	b	3.27374	0.025675
Disacch †	14132326	c	22054521	b	26564959	a	30164246	a	26.29805	0.000000
Ile *	0.800511	ab	0.606389	b	0.931038	a	0.727391	b	3.71506	0.015041
Thr *	0.437575	ab	0.325627	b	0.441336	a	0.376471	ab	4.00130	0.010654
Ala †	3567865	a	2011520	d	2927839	ab	2775083	b	6.76460	0.000424
Val †	7.486852E+13	b	7.411317E+13	b	1.522798E+14	a	1.600834E+14	a	23.08100	0.000000
Leu †	10421698	b	8477753	b	23590504	a	11044350	b	36.63706	0.000000
Phen †	9931861	b	16308864	b	30909308	a	26103989	a	16.71035	0.000000
Pro †	3865724	c	5529905	c	10163191	b	14756314	a	13.30101	0.000000
Arg †	1761996		2530282		3002304		2594525		1.27475	0.289214
Trp †	7733855	b	1136389	b	94926108	a	11048345	b	63.73468	0.000000
Tyr †	2071404	a	1457088	b	2042432	a	628666	c	12.04537	0.000002
Adenine †	5498695	b	4228099	b	9963002	a	4429600	b	3.56145	0.018111
Adenosine †	18420795	a	10891533	b	10847325	b	11418633	b	2.98152	0.036640
Choline †	1.540490	a	1.043819	b	1.295577	ab	1.471539	ab	1.64612	0.185925
Quercitol *	27.25285		24.22621		24.74710		25.69583		0.78888	0.503864
Polyphenol *	15.39138	a	11.97778	b	15.74066	a	14.14173	ab	2.95328	0.037923
Phenolic group *	21.28824	a	16.30085	b	22.17597	a	19.24353	ab	3.08414	0.032335
Quinic acid †	1.531992E+09	a	1.290526E+09	b	1.113824E+09	c	1.116493E+09	c	10.83631	0.000005
Catechin †	1.953379E+09	a	1.541045E+09	b	1.696778E+09	b	1.957675E+09	a	5.56307	0.001679
Chlorogenic acid †	86723.69		82417.51		93842.30		88329.01		0.05330	0.983657
Epicatechin †	3887947		2982179		2703103		2438935		1.03349	0.382744
Epigallocatechin †	42592593		47130408		36217531		35222684		1.53078	0.213484
Gallic acid †	3790056		3212265		3511846		3789569		0.81244	0.490959
Homoorientin †	63611951		55919822		55675633		57063388		0.82253	0.485512
Kampferol †	25974094		22547824		22040560		26668875		1.05755	0.372323
Luteolin †	140290.3	a	47045.9	b	73569.5	b	112639.2	a	4.36493	0.006892
Pyridoxine †	697662.3	a	172048.3	b	343072.7	b	206236.2	b	5.93158	0.001096
Quercetin †	8599422		8508000		9478483		8926141		0.30910	0.818735
Rhamnetin †	5939953		4893556		5395996		4495051		0.56062	0.642668
Asp.ac †	5210008	c	7241072	b	7503356	b	9116571	a	12.35343	0.000001
Lac.ac †	6369585		6621801		6110383		6424977		0.45129	0.717134
Succ.ac †	16570697	a	12678641	b	15682285	a	17165800	a	3.85842	0.012653
Cit.ac †	158063505	b	223779176	a	135408263	b	147593431	b	14.86171	0.000000
Glut.ac †	30798422	b	41275713	ab	33262197	b	50578093	a	5.15844	0.002694

Pir †	10597297	b	14067267	a	11211737	b	9607654	b	6.99700	0.000326
Mal.ac †	288704758	ab	266570535	b	228916618	c	301694902	a	7.49909	0.000186
α-humulene	1506341	b	2605868	b	9315889	a	2894857	b	5.20789	0.002542
Caryophyllene	31458.1	b	262900.0	ab	385043.0	a	304277.0	a	2.96437	0.037414
Overlapping1 *	43.43188	a	31.36382	b	38.04842	a	30.10467	b	5.55420	0.001683
Overlapping2 *	11.12391		9.68886		10.23706		9.25318		1.47215	0.228837
Overlapping3 *	12.12945	a	9.24635	d	11.22037	abc	10.07431	cd	3.73908	0.014556
Overlapping4 *	3.051565	a	2.270426	c	3.490667	b	2.933038	a	7.78369	0.000134
Overlapping5 *	0.432328	ab	0.314493	b	0.485281	a	0.424704	ab	2.35644	0.078432
Overlapping6 *	2.263595	ab	1.949481	b	2.497122	a	2.061469	ab	2.08579	0.109099
Overlapping7 *	0.406019	b	0.400394	b	0.555244	a	0.502439	ab	3.21761	0.027410
Overlapping8 *	15.11812		13.89496		13.14657		15.23863		1.00354	0.395996
Overlapping9 *	19.20380		16.80289		17.58093		18.05957		0.95513	0.418413
Overlapping10 *	12.41667	a	8.81738	bc	11.00373	abc	8.87950	bc	5.00243	0.003215
UFA *	22.74433	b	27.29784	a	17.95084	c	23.60898	b	6.57121	0.000522
TAG+DAG *	2.546622	a	1.237474	b	1.187863	b	1.532120	b	13.31292	0.000000
PUFA *	8.631549	a	9.566383	a	5.288433	b	9.476447	a	18.50884	0.000000
P *	3.755380	a	2.835052	b	1.839125	c	2.868086	b	8.76008	0.000046
Linoleyl FA *	7.683955	a	7.735614	a	5.941504	b	7.695507	a	4.10045	0.009415
FA *	116.2851	ab	129.7729	a	106.1379	b	107.6017	b	3.21462	0.027510
DAG *	13.18280	a	14.18169	a	8.40722	b	14.29089	a	15.86459	0.000000
Ald *	0.432162	a	0.399634	a	0.268933	b	0.318003	b	6.75223	0.000425
Acetyl *	6.316552	a	4.948125	b	4.383811	b	5.384305	ab	4.51887	0.005705
Herbivory	0.329726	a	0.265019	a	0.368654	a	0.284742	a	1.89654	0.137305

Table S6. Data for one-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q. ilex* leaves for treatments (control, drought) with data from all seasons. Integral mean values (mM relative to initial TSP or TMS concentrations (0.01%)) are given for the NMR variables, marked by asterisks (*). Integral mean values of deconvoluted total intensities are given for the LC-MS variables, marked by crosses (†).Different letters beside the values indicate significant seasonal differences detected by LSD post-hoc tests ($p < 0.05$) between treatments.

	(ALL YEAR) TREATMENT					
	CONTROL		DROUGHT		*F*	*p*
C:N	40.84257	b	42.85607	a	4.04553	0.047788
N:P	13.48905		12.89829		1.62914	0.205659
C:P	548.2077		551.0343		0.02034	0.886955
C:K	98.65126		95.48032		0.20098	0.655192
K:P	6.124151		6.438726		0.35378	0.553726
N:K	2.429144		2.261255		0.94057	0.335168
N (mg/g dry weight)	12.32400		11.85590		2.58445	0.112011
P (mg/g dry weight)	0.927450		0.941341		0.15366	0.696148
K (mg/g dry weight)	5.549705		5.875869		0.58808	0.445507
C (mg/g dry weight)	498.1220	b	502.2862	a	8.66700	0.004282
aG *	5.437045	b	6.568781	a	10.93840	0.001433
BG *	4.295323		4.760449		2.53508	0.115439
Suc *	4.840646	b	8.510523	a	40.47144	0.000000
Deoxy-Hex †	9279617	b	10363487	a	3.35992	0.070667
Hex †	77861033	b	99328335	a	5.82383	0.018188
Pent †	7345000		7440337		0.03515	0.851776
Disacch †	23872520		22599121		0.44361	0.507375
Ile *	0.699775	b	0.838697	a	3.67636	0.058898
Thr *	0.408277		0.383679		0.72229	0.398030
Ala †	3017774		2639069		1.99605	0.161739
Val †	1.229186E+14		1.086165E+14		1.14476	0.287990
Leu †	12616352		14296263		0.91341	0.342202
Phen †	18206920	b	23602431	a	3.46258	0.066590
Pro †	8482520		8755692		0.02748	0.868762
Arg †	3120388	a	1806060	b	8.83947	0.003934
Trp †	23320247		34947376		1.28109	0.261209
Tyr †	1329974	b	1777840	a	3.79370	0.055091
Adenine †	3237191	b	8940312	a	17.73076	0.000068
Adenosine †	13303293		12526731		0.12016	0.729804
Choline †	1.209234		1.477316		2.43858	0.122484
Quercitol *	22.91172	b	28.14730	a	14.51730	0.000278
Polyphenol *	13.49634	b	15.21025	a	2.91242	0.091930
Phenolic group *	18.03568	b	21.60112	a	5.76095	0.018802
Quinic acid †	1.237052E+09		1.289336E+09		0.54184	0.463907
Catechin †	1.640474E+09	b	1.944040E+09	a	12.05120	0.000852
Chlorogenic acid †	88017.60		87772.54		0.00015	0.990310
Epicatechin †	2765944		3246753		0.58999	0.444773
Epigallocatechin †	43916566	a	36396699	b	2.79446	0.098650
Gallic acid †	3728851		3428421		0.98913	0.323070
Homoorientin †	53809793	b	62489855	a	4.62151	0.034713
Kampferol †	23043107		25650127		1.29770	0.258166
Luteolin †	90516.89		97517.36		0.11088	0.740054
Pyridoxine †	447097.6	a	264729.2	b	2.93551	0.090675
Quercetin †	7963006	b	9825966	a	6.09596	0.015765
Rhamnetin †	4830741		5547896		0.73926	0.392566
Asp.ac †	7682559		6842992		2.36981	0.127801
Lac.ac †	6184678		6577590		1.63022	0.205510
Succ.ac †	14838437		16300829		1.93747	0.167951
Cit.ac †	175769858	a	154931129	b	2.83074	0.096527
Glut.ac †	41941804		35880529		2.05088	0.156165

Pir †	11980111		10677113		2.74878	0.101398
Mal.ac †	267766088		275398005		0.33713	0.563188
α-humulene	3037257		5188794		1.65242	0.202483
Caryophyllene	321757.2	a	167701.9	b	2.87427	0.094046
Overlapping1 *	27.50030	b	43.97410	a	57.15315	0.000000
Overlapping2 *	8.06413	b	12.08737	a	65.29019	0.000000
Overlapping3 *	8.94536	b	12.38988	a	36.19355	0.000000
Overlapping4 *	2.811879		3.060969		1.51780	0.221654
Overlapping5 *	0.374319	b	0.454084	a	2.83613	0.096164
Overlapping6 *	2.071991		2.313843		2.04606	0.156595
Overlapping7 *	0.460949		0.471099		0.05271	0.819017
Overlapping8 *	15.03701		13.66212		1.89619	0.172445
Overlapping9 *	16.00664	b	19.81695	a	16.43988	0.000118
Overlapping10 *	7.74314	b	12.81551	a	66.89548	0.000000
UFA *	23.52921		22.27179		0.57681	0.449854
TAG+DAG *	1.601675		1.650365		0.05315	0.818272
PUFA *	8.245113		8.236293		0.00021	0.988454
P *	2.757615		2.891207		0.19493	0.660067
Linoleyl FA *	7.024638		7.503652		1.08310	0.301221
FA *	115.5635		114.3353		0.03754	0.846876
DAG *	12.46900		12.56230		0.01125	0.915819
Ald *	0.336018		0.373348		1.39237	0.241591
Acetyl *	5.241531		5.274866		0.00657	0.935597
Herbivory	0.239194	b	0.384876	a	23.07889	0.000007

Table S7. Data for one-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q. ilex* leaves for treatments (control, drought) in spring. Integral mean values (mM relative to initial TSP or TMS concentrations (0.01%)) are given for the NMR variables, marked by asterisks (*). Integral mean values of deconvoluted total intensities are given for the LC-MS variables, marked by crosses (†). Different letters beside the values indicate significant differences ($p < 0.05$) between treatments.

	(SPRING) TREATMENT					
	CONTROL		DROUGHT		*F*	*p*
C:N	42.13999		44.97719		2.88940	0.106379
N:P	12.51845		12.79511		0.12874	0.723914
C:P	524.2908		576.8204		2.08225	0.166197
C:K	106.8274		112.3785		0.15283	0.700433
K:P	5.310746		5.475253		0.05085	0.824125
N:K	2.555357		2.511301		0.01736	0.896624
N (mg/g dry weight)	11.97600		11.21900		2.69176	0.118226
P (mg/g dry weight)	0.961484		0.900215		0.89784	0.355911
K (mg/g dry weight)	5.061831		4.783095		0.20510	0.656050
C (mg/g dry weight)	499.6720		502.7650		1.12580	0.302704
aG *	6.663098		6.329213		0.28998	0.596831
BG *	5.244602		5.314074		0.01207	0.913728
Suc *	4.204184	b	5.187526	a	4.55348	0.046864
Deoxy-Hex †	7904360		8662437		0.57054	0.459815
Hex †	65242324	b	94446000	a	3.81013	0.066679
Pent †	6536739		7339127		1.02193	0.325459
Disacch †	31365468		28963024		0.58868	0.452878
Ile *	0.778965		0.675817		1.10134	0.307858
Thr *	0.383486		0.369456		0.11490	0.738561
Ala †	3080988		2469177		1.74753	0.202742
Val †	1.701922E+14		1.499746E+14		0.67852	0.420878
Leu †	11827592		10261108		1.13914	0.299941
Phen †	24766576		27441401		0.24506	0.626562
Pro †	17157745		12354883		1.15379	0.296946
Arg †	3105428		2083622		1.32647	0.264502
Trp †	8570908		13525781		1.52197	0.233188
Tyr †	523316.7		734016.0		1.77800	0.199026
Adenine †	2350238	b	6508963	a	6.36123	0.021303
Adenosine †	12515700		10321567		0.27156	0.608637
Choline †	1.030264	b	1.912814	a	5.05701	0.037287
Quercitol *	27.24363		24.14804		2.07703	0.166702
Polyphenol *	17.10965	a	11.17381	b	16.25200	0.000783
Phenolic group *	23.47285	a	15.01421	b	19.26894	0.000353
Quinic acid †	1.224914E+09	a	1.008072E+09	b	4.64068	0.045019
Catechin †	1.820540E+09		2.094809E+09		1.79155	0.197399
Chlorogenic acid †	121371.1	b	55286.9	a	5.67969	0.028387
Epicatechin †	2443714		2434155		0.00014	0.990802
Epigallocatechin †	39292016		31153353		1.42901	0.247438
Gallic acid †	3975557		3603580		0.43490	0.517944
Homoorientin †	53188447		60938330		0.82319	0.376236
Kampferol †	24826282		28511468		0.60366	0.447276
Luteolin †	135093.3		90185.1		0.78247	0.388052
Pyridoxine †	203383.2		209089.2		0.00725	0.933090
Quercetin †	7591675		10260606		2.85583	0.108288
Rhamnetin †	4580448		4409654		0.02095	0.886514
Asp.ac †	10336980	a	7896162	b	4.87660	0.040434
Lac.ac †	6650317		6199636		0.49327	0.491455
Succ.ac †	15443406		18888193		2.20263	0.155076
Cit.ac †	164267024		130919838		2.97362	0.101762
Glut.ac †	53688341		47467846		0.67814	0.421006

Pir †	10730868		8484440		1.60898	0.220795
Mal.ac †	294997176		308392627		0.77799	0.389382
α-humulene	3430045		2359669		0.64010	0.434108
Caryophyllene	222886.7		385667.2		0.95832	0.340584
Overlapping1 *	27.88787	b	32.32146	a	3.09087	0.095724
Overlapping2 *	8.745718		9.760637		2.17383	0.157652
Overlapping3 *	9.72601		10.42262		0.56357	0.462529
Overlapping4 *	3.390062	a	2.476015	b	5.72069	0.027892
Overlapping5 *	0.391762		0.457645		0.93632	0.346048
Overlapping6 *	2.629504	a	1.493434	b	13.83636	0.001568
Overlapping7 *	0.451908		0.552970		0.88695	0.358773
Overlapping8 *	19.63276	a	10.84450	b	33.38865	0.000018
Overlapping9 *	19.25143		16.86770		2.15477	0.159385
Overlapping10 *	7.60305	b	10.15596	a	8.37474	0.009672
UFA *	24.29475		22.92321		0.60755	0.445839
TAG+DAG *	1.606155		1.458085		0.71968	0.407388
PUFA *	9.361127		9.591766		0.17339	0.682036
P *	2.949732		2.786440		0.25368	0.620603
Linoleyl FA *	7.394525		7.996490		1.32084	0.265484
FA *	107.4735		107.7300		0.00156	0.968890
DAG *	14.14589		14.43590		0.13253	0.720067
Ald *	0.325804		0.310202		0.16006	0.693811
Acetyl *	5.571168		5.197442		1.14426	0.298889
Herbivory	0.231760		0.337723		2.83447	0.109524

Table S8. Data for one-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Q. ilex* leaves for treatments (control, drought) in summer. Integral mean values (mM relative to initial TSP or TMS concentrations (0.01%)) are given for the NMR variables, marked by asterisks (*). Integral mean values of deconvoluted total intensities are given for the LC-MS variables, marked by crosses (†). Different letters beside the values indicate significant differences detected by LSD post hoc tests ($p < 0.05$) between treatments.

	(SUMMER) TREATMENT					
	CONTROL		DROUGHT		*F*	*p*
C:N	41.66038		42.93619		0.34966	0.561660
N:P	13.77995		13.47232		0.09652	0.759615
C:P	569.3676		574.4752		0.01958	0.890258
C:K	81.59398		72.63693		1.07595	0.313341
K:P	7.235808		8.503313		1.55022	0.229069
N:K	1.969276		1.744063		0.86426	0.364851
N (mg/g dry weight)	11.97600		11.78900		0.09529	0.761102
P (mg/g dry weight)	0.880722		0.890169		0.02510	0.875882
K (mg/g dry weight)	6.317420		7.407521		1.84736	0.190877
C (mg/g dry weight)	494.0180	b	499.5140	a	3.65572	0.071929
aG *	5.233182	b	6.800838	a	4.70655	0.043682
BG *	4.007798		4.292870		0.33796	0.568219
Suc *	6.52583	b	11.42518	a	21.92706	0.000185
Deoxy-Hex †	11665376		10959158		0.42219	0.524053
Hex †	84067267		82123850		0.01415	0.906632
Pent †	7127894		6646569		0.32928	0.573187
Disacch †	15342556		12922096		2.77051	0.113326
Ile *	0.744576		0.856447		0.37981	0.545423
Thr *	0.500795		0.374355		2.99581	0.100586
Ala †	4158994	a	2976737	b	5.07479	0.036992
Val †	7.523658E+13		7.450046E+13		0.00411	0.949575
Leu †	8918857		11924538		1.70889	0.207583
Phen †	8742734		11120989		0.71659	0.408379
Pro †	3580016		4151432		0.53599	0.473527
Arg †	2477675	a	1046317	b	7.68947	0.012541
Trp †	5981268		9486441		1.55074	0.228994
Tyr †	2093373		2049436		0.00983	0.922129
Adenine †	2826604	a	8170786	b	4.40172	0.050284
Adenosine †	21049369		15792222		1.09683	0.308822
Choline †	1.764591		1.316388		1.54677	0.229567
Quercitol *	23.21481	b	31.29090	a	6.48586	0.020237
Polyphenol *	12.98213	b	17.80063	a	6.58725	0.019415
Phenolic group *	17.21579	b	25.36068	a	10.23488	0.004971
Quinic acid †	1.484741E+09		1.579244E+09		0.52287	0.478912
Catechin †	1.734808E+09	b	2.171950E+09	a	7.92429	0.011462
Chlorogenic acid †	43031.3	b	130416.1	a	3.01184	0.099746
Epicatechin †	2170140	b	5605754	a	3.28101	0.086807
Epigallocatechin †	47459630		37725555		1.47933	0.239589
Gallic acid †	3909556		3670557		0.11656	0.736744
Homoorientin †	61644091		65579812		0.28566	0.599558
Kampferol †	25383765		26564422		0.06162	0.806765
Luteolin †	136963.8		143616.8		0.01792	0.894986
Pyridoxine †	1005105	a	390220	b	3.44019	0.080084
Quercetin †	7823142		9375702		0.77178	0.391244
Rhamnetin †	5209374		6670531		0.47712	0.498542
Asp.ac †	5597393		4822622		1.13350	0.301104
Lac.ac †	6191870		6547300		0.27299	0.607707
Succ.ac †	17165656		15975739		0.42050	0.524873
Cit.ac †	157678450		158448559		0.00167	0.967844
Glut.ac †	37458234		24138609		2.05407	0.168942

Pir †	10913495		10281099		0.16723	0.687411
Mal.ac †	277676155		299733361		1.19513	0.288713
α-humulene	1184114		1828568		0.53673	0.473225
Caryophyllene	48176.16		14740.12		1.92285	0.182478
Overlapping1 *	33.00544	b	53.85831	a	17.70423	0.000529
Overlapping2 *	8.73067	b	13.51716	a	13.77425	0.001598
Overlapping3 *	10.08155	b	14.17735	a	9.24243	0.007042
Overlapping4 *	2.816203		3.286928		1.31184	0.267062
Overlapping5 *	0.430013		0.434643		0.00171	0.967429
Overlapping6 *	1.862727	b	2.664463	a	5.94843	0.025315
Overlapping7 *	0.438479		0.373560		0.59953	0.448808
Overlapping8 *	14.62969		15.60654		0.22186	0.643287
Overlapping9 *	16.48671	b	21.92088	a	6.17741	0.022993
Overlapping10 *	9.64605	b	15.18729	a	15.26398	0.001033
UFA *	23.61925		21.86941		0.45029	0.510719
TAG+DAG *	2.507704		2.585541		0.01841	0.893590
PUFA *	8.687640		8.575459		0.01487	0.904284
P *	3.484175		4.026586		0.55731	0.464985
Linoleyl FA *	7.363597		8.004314		0.68387	0.419085
FA *	114.9628		117.6074		0.03839	0.846865
DAG *	12.99469		13.37092		0.08809	0.770019
Ald *	0.371887	b	0.492436	a	3.42695	0.080620
Acetyl *	6.428194		6.204910		0.04476	0.834815
Herbivory	0.206635	b	0.452817	a	26.35006	0.000070

Figure S1 a) Ten-year average (1999-2009) of soil-moisture (v.v. ±SE) of control plots for each year season. b) Ten-year average (1999-2009) reduction of soil moisture of droughted plots relative to control plots (% ±SE). C) Reduction of soil moisture of droughted plots relative to control plots in 2009 (%±SE).

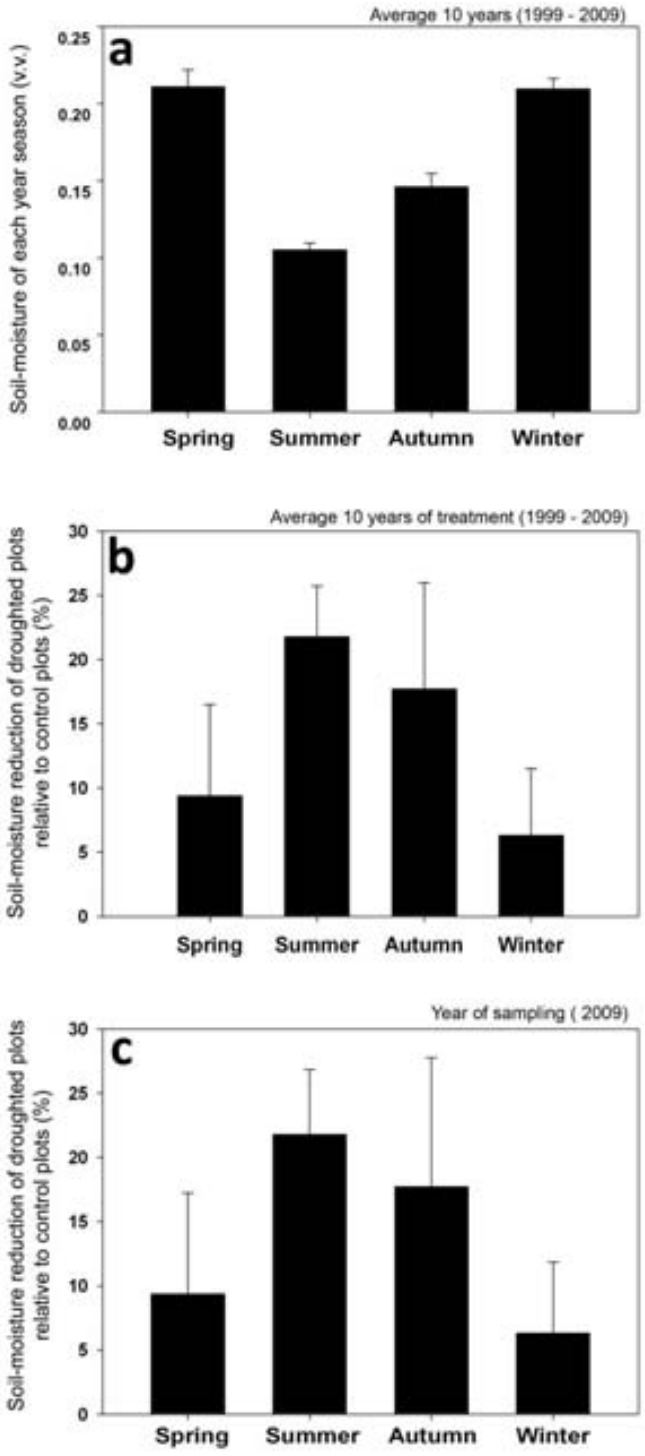


Figure S2 Example of a 1D ^{1}H NMR spectra of a polar sample (A) and of a nonpolar sample (B) of *Quercus ilex* leaf extracts. Assignments of the main peaks are indicated. Polar sample was dissolved in D_2O (pH 6.0) and referenced to TSP and nonpolar sample was dissolved in $CDCl_3$ and referenced to the residual solvent peak. Spectra acquired at a magnetic field of 600 MHz and at 298.0 K of temperature.

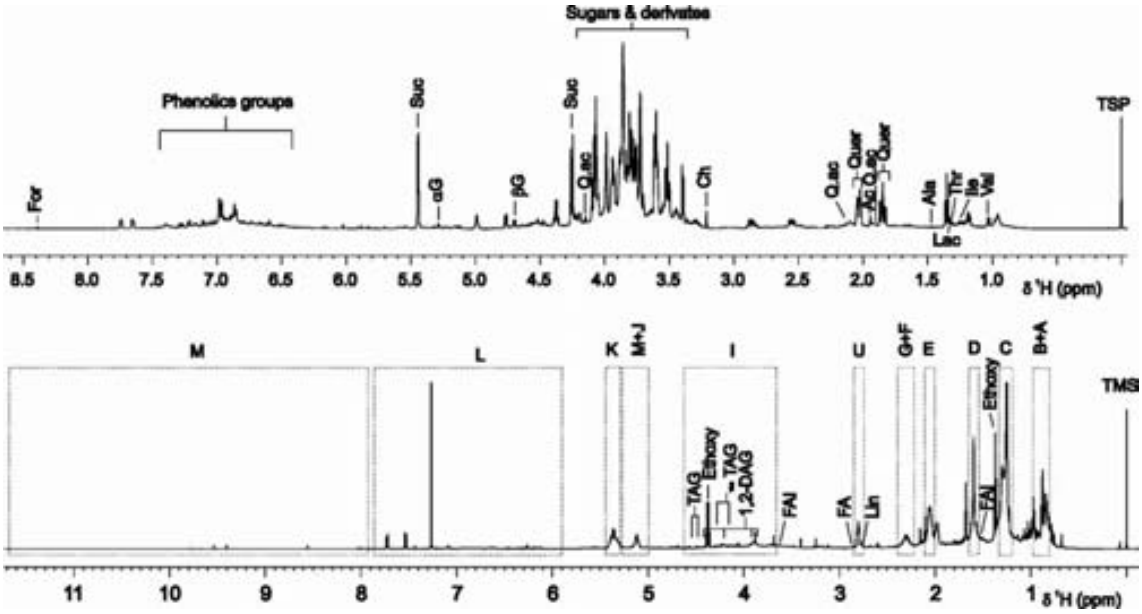


Figure S3 Detailed example of a 1D ^{1}H NMR spectrum of a polar sample of *Quercus ilex* leaf extract with the assignment of the main peaks indicated. Sample dissolved in D_2O (pH 6.0) and referenced to TSP. Spectrum acquired at 600 MHz magnetic field and at 298.0 K of temperature.

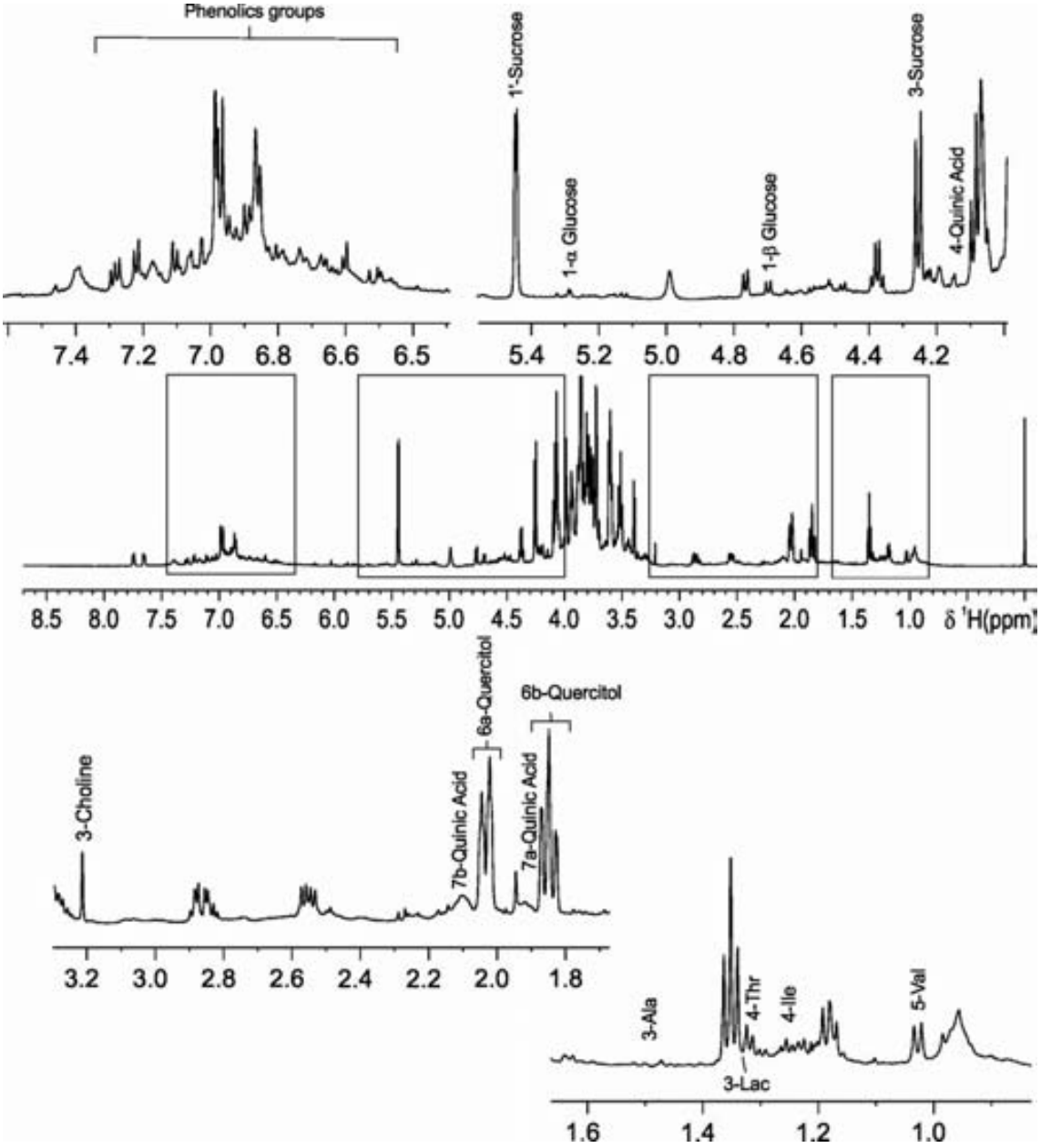
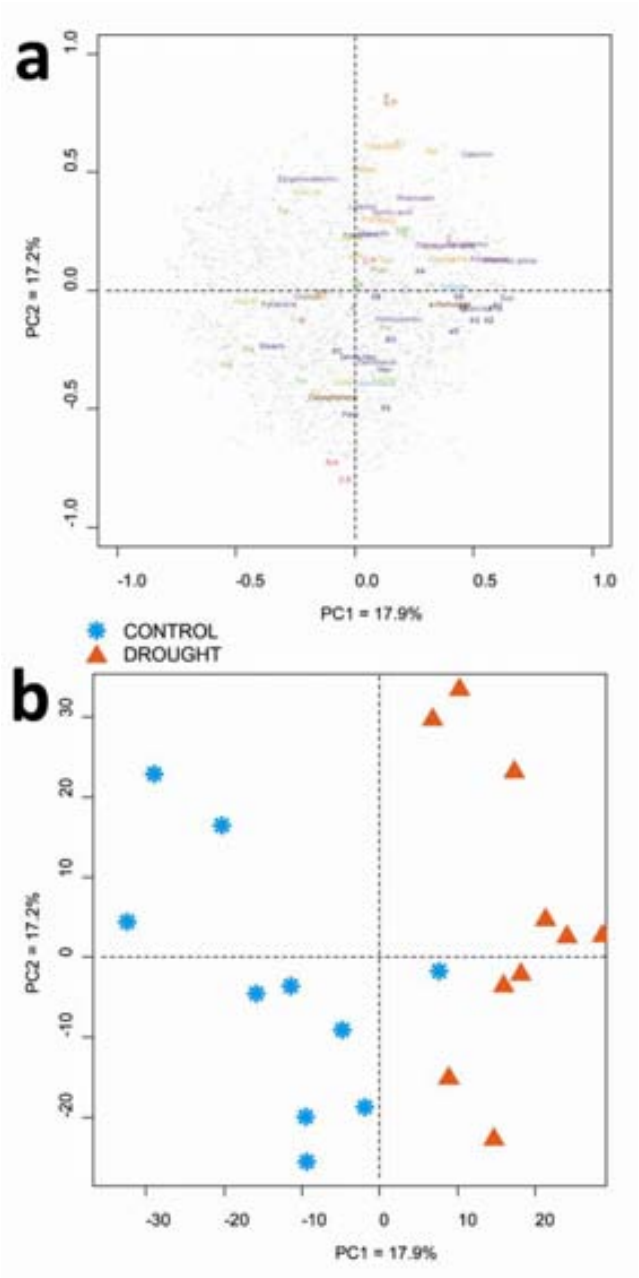


Figure S4 PCA plots of the metabolomic and stoichiometric variables, excluding folivory as a variable, of all seasons (**a**) and case trees (**b**). Colors indicate different metabolomic families: blue, sugars; green, amino acids; orange, RCAAS; cyan, nucleotides; violet, phenolics; light orange, nonpolar metabolites; dark blue, overlapped NMR signals and brown, terpenes. Unassigned metabolites are represented by small grey dots. Variable labels are described in Figure 1 of the main text. Control trees are indicated by asterisks and blue colors, and droughted trees are indicated by triangles and orange colors.



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APPENDIX 5

Rivas-Ubach et al (2013) Unpublished.

Table S1. Description of the mixture of trace elements used for the media preparation for culturing of *Synechocystis sp.* PCC 6803.

Trace element solution composition			
Compound	Formula	Quantity (g/L)	Notes
Sodium EDTA	$Na_2EDTA \cdot 2H_2O$	0.004	
Manganese Chloride	$MnCl_2 \cdot 4H_20$	$1x10^{-3}$	
Copper sulfate	$CuSO_4 \cdot 5H_20$	$1.8x10^{-4}$	
Zinc sulfate	$ZnSO_4 \cdot 7H_20$	$8.49x10^{-6}$	
Cobalt Chloride	$CoCl_2 \cdot 6H_20$	$2.2x10^{-5}$	
Sodium Molybdate	$Na_2MoO_4 \cdot 2H_20$	$1.2x10^{-5}$	
Selenium acid	H_2SeO_3	$2.2x10^{-5}$	
Sodium vanadate	Na_3VO_4	$1.6x10^{-6}$	
Potassium Chloride	$NiCl_2 \cdot 6H_20$	$1.8x10^{-6}$	
Ferric Chloride	$FeCl_3$	$4.75x10^{-6}$	Not added in Fe-Lim media

Table S2. 1H NMR spectra pattern for variable size bucketing and metabolite identification of each bucket.

Num. of bucket	1H NMR spectra pattern		Compound
	LEFT (ppm)	RIGHT (ppm)	
1	1.2052	1.1661	Ethanol
2	1.4134	1.3427	Unknown1
3	1.4538	1.4358	α-Hydroxyisobutyrate
4	1.4837	1.4735	Alanine
5	1.4976	1.4837	Alanine
6	2.038	2.0253	Glutamic acid
7	2.0481	2.0253	Glutamic acid
8	2.061	2.0481	Glutamic acid
9	2.0735	2.061	Glutamic acid
10	2.0868	2.0735	Glutamic acid
11	2.101	2.0868	Glutamic acid
12	2.1122	2.101	Glutamic acid
13	2.1187	2.1122	Glutamic acid
14	2.1258	2.1187	Glutamic acid
15	2.1319	5.1258	Glutamic acid
16	2.137	2.1319	Glutamic acid
17	2.1503	2.137	Glutamic acid
18	2.1571	2.1503	Glutamic acid
19	2.1717	2.1571	Glutamic acid
20	2.1822	2.1717	Glutamic acid
21	2.3248	2.3153	Glutamic acid
22	2.3364	2.3248	Glutamic acid
23	2.3476	2.3364	Glutamic acid
24	2.3533	2.3476	Glutamic acid
25	2.3605	2.3533	Glutamic acid
26	2.3673	2.3605	Glutamic acid
27	2.3727	2.3673	Glutamic acid
28	2.3856	2.3727	Glutamic acid
29	2.3968	2.3856	Glutamic acid
30	2.4328	2.4247	
31	2.6423	2.6315	Methionine
32	2.6549	2.6423	Methionine
33	2.6671	2.6549	Methionine
34	2.752	2.7367	
35	2.8145	2.7985	Aspartic acid
36	2.9042	2.8967	
37	3.0359	3.024	Lysine
38	3.1273	3.1144	Choline
39	3.2471	3.2363	
40	3.2536	3.2471	
41	3.2672	3.2536	
42	3.2852	3.2672	Betaine
43	3.2937	3.2852	
44	3.3086	3.2937	
45	3.3646	3.3528	
46	3.3735	3.3646	
47	3.3928	3.3735	
48	3.4115	3.3928	
49	3.4251	3.4115	
50	3.4461	3.4346	
51	3.4614	3.4461	Sucrose + others
52	3.4743	3.4614	Sucrose + others
53	3.4787	3.4743	Sucrose + others
54	3.4893	3.4787	Sucrose + others
55	3.603	3.5918	α-Glucose
56	3.6156	3.603	
57	3.6333	3.6156	
58	3.6455	3.6333	Ethanol
59	3.6577	3.6455	Ethanol

60	3.6706	3.6577	Ethanol
61	3.6788	3.6706	Ethanol
62	3.6876	3.6788	Sucrose
63	3.7052	3.6876	
64	3.7178	3.7052	
65	3.7256	3.7178	
66	3.7358	3.7256	
67	3.7419	3.7358	Sucrose
68	3.748	3.7419	
69	3.7558	3.748	Glutamic acid
70	3.7684	3.7558	Glutamic acid
71	3.7786	3.7684	Glutamic acid
72	3.7874	3.7786	Lysine
73	3.7946	3.7874	Lysine + Alanine
74	3.8092	3.7946	Lysine
75	3.8306	3.8092	Sucrose
76	3.838	3.8306	Methionine
77	3.8489	3.838	Methionine
78	3.8672	3.8489	Methionine
79	3.8808	3.8672	Methionine
80	3.9005	3.8808	
81	3.9185	3.9005	
82	4.002	3.9633	
83	4.0363	4.002	
84	4.0981	4.0639	
85	4.163	4.1243	
86	4.3837	4.3376	
87	4.4435	4.3837	Unknown1
88	4.6103	4.5865	β-Glucose
89	4.9142	4.8996	
90	4.94	4.9142	
91	4.9658	4.94	
92	4.9838	4.9658	
93	5.0646	5.0375	
94	5.1281	5.121	
95	5.1516	5.142	
96	5.2066	5.1909	α-Glucose
97	5.4242	5.4107	Sucrose
98	6.8074	6.7979	
99	7.4608	7.4485	Unknown2
100	7.4757	7.4608	Unknown2
101	7.4876	7.4757	Unknown2
102	7.5025	7.4876	Unknown2
103	7.5131	7.5025	Unknown2
104	7.6119	7.6017	Unknown2
105	7.6244	7.6119	Unknown2
106	7.6353	7.6244	Unknown2
107	7.7338	7.674	Unknown1
108	7.8401	7.798	Unknown1
109	8.2832	8.2635	AMP?
110	8.3542	8.3427	NADP?
111	8.3885	3.3783	NAD?
112	8.4656	8.4524	
113	8.8806	8.8673	AMP?

Table S3. LC-MS chromatogram processing. Chromatograms obtained by LC-MS and processed by MzMine 2.0 (Pluskal et al. 2010). The following table summarizes the different processes and parameters applied to *Synechocystis sp.* chromatograms.

		(+H) Chromatograms		(-H) Chromatograms
1	**Baseline correction**			
	Chromatogram type	TIC		TIC
	MS level	1		1
	Smoothing	10E6		10E5
	Asymmetry	0.001		0.001
2	**Mass detection** (Exact Mass)			
	Noise level	5×10^6		4×10^5
3	**Chromatogram builder**			
	Min time span	0.05		0.05
	Min height	25000		25000
	m/z tolerance	0.002		0.002
4	**Smoothing**			
	Filter width	5		5
5	**Chromatogram deconvolution (Local minimum search)**			
	Chromatographic threshold	65%		65%
	Search minimum in RT range (min)	0.05		0.1
	Minimum relative height	5.0%		5.0%
	Minimum absolute height	30000		30000
	Min ratio of peak top/edge	2		2
	Peak duration range	0.0-2.0		0.0-2.0
6	**Chromatogram alignment (join alignment)**			
	m/z tolerance	0.001		0.001
	weight for m/z	80		80
	RT tolerance	0.15		0.15
	Weight for RT	20		20
7	**Gap filling (Peak Finder)**			
	Intensity tolerance	20%		20%
	m/z tolerance	0.001		0.001
	Retention time tolerance	0.1		0.1
	RT correction	marked		marked
8	**Filtering**			
	Minimum peaks in a row	6		6
	Ions excluded from database	<75 227.17 Between 0.0 and 1 min Bewteen 4.0 and 13 min Between 27.2 and 30 min		<85 119.035 120.03 818.51 817.5 815.49 791.49 792.50 Between 0.0 and 1,1 min Between 28.0 and 30 min

Table S4. Assignment of the metabolites with LC-MS analyses was based on the standards. The following table summarizes the retention time (RT) and mass (m/z) of the assigned metabolites in both positive and negative ionization modes.

Mode	Compound	RT (min)	m/z
Negative	Glutamic acid	1.41	146.0146
Negative	Pyruvate	1.65	87.0089
Negative	Citric acid	1.77	191.0196
Negative	Lactic acid	1.52; 1.75	89.0245
Negative	Malic acid	1.51; 1.78	133.0143
Negative	Succinic acid	1.74; 1.78	117.0194
Negative	AMP	1.50 – 1.76	346.0554
Negative	UMP	1.79	323.0280
Negative	TMP	1.50 – 1.80	321.0484
Negative	Quinic acid	1.47	191.056
Positive	Adenine	1.42; 1.77	136.0614
Positive	Adenosine	1.49; 1.75	268.1038
Positive	Alanine	1.43	90.054
Positive	Arginine	1.34	175.119
Positive	Glutamine	1.46	147.076
Positive	Leucine	1.76	132.101
Positive	Phenylalanine	1.91	166.086
Positive	Tryptophan	2.49	205.097
Positive	Tyrosine	1.54 - 1.77	182.081
Positive	Valine	1.53	118.086
Positive	Methionine	1.59	150.058
Positive	Serine	1.47	106.049
Positive	Threonine	1.43	120.065
Positive	Glutamic acid	1.41	148.0604
Positive	AMP	1.45 – 1.77	348.0504

Table S5. Data for one-way ANOVAs of all stoichiometry and assigned metabolites extracted from *Synechocystis sp.* PC6803 cells. Integral mean values (mM relative to initial TSP concentrations (0.01%)) are given for the NMR variables, marked by asterisks (*). Integral mean values of deconvoluted total intensities are given for the LC-MS variables, marked by crosses (†). Different letters beside the values indicate significant differences detected by LSD post hoc tests (p< 0.05) between treatments.

TRT	Complete		Fe-Lim		P-Lim		*F*	*p*
Chl.A	7.41678667	a	6.05689333	a	4.11770333	b	8.3431	0.003666
N	10.5666667	a	5.54166667	c	6.21	b	220.8409	0.000000
C	40.6566667	a	36.015	b	34.02	b	8.3844	0.003595
P	1.81333333	a	1.44166667	a	1.03166667	b	5.9693	0.012384
C:N	3.85	c	6.50166667	a	5.48	b	84.8032	0.000000
C:P	23.4283333		28.8616667		34.4866667		1.8336	0.193901
N:P	6.08966667		4.4565		6.2135		2.0315	0.165668
Protein	269.266667	a	159.133333	b	128.8	c	55.6337	0.000000
RNA	224.831667	a	72.4366667	b	49.7633333	b	39.7503	0.000001
Prot/RNA	1.24833333	b	2.50666667	a	2.66333333	a	8.0537	0.004209
aGluc *	0.01387582	b	0.02235898	b	0.03779664	a	8.8920	0.002839
bGluc *	0.01363256	a	0.01069924	a	0.00926036	b	4.4410	0.030560
Suc *	0.08057512	b	0.07983574	b	0.1301286	a	12.9872	0.000533
Adenine †	16929606.8		6298006.04		7299874.08		2.0907	0.158160
Adenosine †	12626116.6		8035902.9		8543395.69		1.6957	0.216803
AMP †	18518716.7	a	10682108.3	b	4705264.33	c	19.0204	0.000077
UMP †	8356063.09	a	8553512.77	a	1846274.58	b	8.3403	0.003671
TMP †	980982.404	a	825410.2	a	200745.713	b	8.0452	0.004227
Ala * †	28863219	b	17694459.7	b	46617328.3	a	3.2342	0.067950
Arg †	1255707	b	1696074.49	b	2534184.97	a	3.1312	0.073043
Met *	77910081.4	a	68573911.6	a	38624697.8	b	4.4634	0.030134
Phe †	10824784	b	20032114.8	a	10225289.8	b	7.9492	0.004428
Leu †	3479684.63	b	25832625.3	a	9601174.78	b	27.0820	0.000011
Ser †	3508231		2381239.08		1997561.19		1.3712	0.283841
Thr†	5398306.14	a	1264816.85	b	1339012.32	b	21.7344	0.000037
Trp †	81658095.6	a	67431008.1	a	26813400.4	b	12.7665	0.000578
Tyr †	15701831.2		10260001.5		6316880.38		1.9041	0.183263
Val †	13455816	b	18126795.3	a	17937901.9	a	8.2380	0.003853
Lys *†	49112008.5	b	149760054	a	55315699.8	b	25.2554	0.000016
Lys+Ala *	0.0165824	a	0.02533234	a	0.02274322	b	8.0661	0.004184
Gln †	3188337.72	a	651591.303	b	1146629.38	b	16.6527	0.000155
Asp *	2230380350	a	969829225	b	1369070618	ab	2.9179	0.085039
Glu * †	258270062	a	290376077	a	154908498	b	4.7168	0.025750
Citric.ac †	50081712.5	a	44416303.7	a	20348891.1	b	7.2811	0.006168
Lactic.ac †	1012397.99	b	3499538.49	a	2463394.67	a	10.9228	0.001182
Malic.ac †	232058.55	a	772873.132	b	441953.014	a	6.7019	0.008324
Piruvic.ac †	500343.674	a	603120.57	a	200759.786	b	5.6401	0.014910
Succinic.ac †	6386492.13	b	9803627.01	a	5823588.08	b	8.5004	0.003404
Quinic.ac †	256761.541	b	632988.71	a	134031.404	b	5.1871	0.019398
Hydroxyisobutyrate *	0.01794216	b	0.09378961	a	0.01690869	b	230.5764	0.000000
Betaine *	0.09391613		0.0970999		0.07416405		1.7839	0.201834
Choline *	0.0068128		0.00759927		0.00708727		0.1540	0.858598