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Effects of ocean acidification on the levels of primary and secondary metabolites in the brown macroalga *Sargassum vulgare* at different time scales

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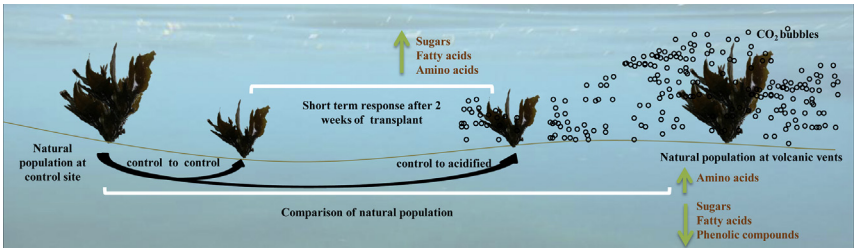
HIGHLIGHTS

- *Sargassum vulgare* growing at CO_2 vents was compared with those growing at control site.
- *S. vulgare* from control site was transplanted to CO_2 vents for 2 weeks.
- In short-term responses, *S. vulgare* showed increased level of sugars, PUFAs, and EAAs.
- Natural population at vents showed decreased sugars, PUFAs, phenols, and increased EAAs.
- Nutritional values of algae will decrease under acidification in long time scale.

GRAPHICAL ABSTRACT



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ABSTRACT

Most of the studies regarding the impact of ocean acidification on macroalgae have been carried out for short-term periods, in controlled laboratory conditions, thus hampering the possibility to scale up the effects on long-term. In the present study, the volcanic CO_2 vents off Ischia Island were used as a natural laboratory to investigate the metabolic response of the brown alga *Sargassum vulgare* to acidification at different time scales. For long-term effects, algal populations naturally growing at acidified and control sites were compared. For short-term responses, *in situ* reciprocal transplants from control to acidified site and *vice-versa* were performed. Changes in the levels of sugars, fatty acids (FAs), amino acids (AAs), antioxidants, and phenolic compounds were examined. Our main finding includes variable metabolic response of this alga at different time scales to natural acidification. The levels of sugars, FAs, and some secondary metabolites were lower in the natural population at the acidified site, whereas the majority of AAs were higher than those detected in thalli growing at control site. Moreover, in algae transplanted from control to acidified site, soluble sugars (glucose and mannose), majority of AAs, and FAs increased in comparison to control plants transplanted within the same site. The differences in

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the response of the macroalga suggest that the metabolic changes observed in transplants may be due to acclimation that supports algae to cope with acidification, thus leading to adaptation to lowered pH in long time scale.

1. Introduction

Over the last few decades, increased atmospheric carbon dioxide (CO_2) levels and temperatures resulting from anthropogenic activities have become a global concern (Meyer et al., 2014). As significant portion of this atmospheric CO_2 is continuously absorbed by the oceans, a shift in seawater carbonate chemistry is occurring resulting in a lowering of pH, a process known as ocean acidification (OA, Sabine et al., 2004). A multitude of marine organisms across different trophic levels are negatively affected by OA (Garrard et al., 2013; Harvey et al., 2014). In general, marine photoautotrophs are believed to benefit from the increased dissolved CO_2 as it is a substrate for photosynthesis. Indeed, growth of several non-calcifying seaweeds and some seagrasses are enhanced by OA conditions (Koch et al., 2013). However, the responses are species specific, even among closely related species due to variations in carbon acquisition strategies from seawater (Wu et al., 2008; Zou and Gao, 2009; Mackey et al., 2015). Moreover, benefits of additional CO_2 are compromised due to subsequent lowering of surrounding seawater pH (Britton et al., 2016). As a result, other physiological and biochemical processes of autotrophs such as reproduction, early life stage development, ion homeostasis, energy demand, nutrient uptake, and enzymatic activities are affected under OA conditions (Roleda et al., 2012; Hofmann et al., 2013; Gutow et al., 2014; Fernández et al., 2015; Nunes et al., 2015; Xu et al., 2015; Kumar et al., 2017a, b; Leal et al., 2017).

Seaweed response to OA is quite variable and depends on several factors such as carbon uptake mechanisms and developmental stage as well as the experimental approaches, laboratory scale/*in situ* or free ocean CO_2 enrichment, FOCE (Hurd et al., 2009; Koch et al., 2013; Korbee et al., 2014; Betancor et al., 2014; Celis-Plá et al., 2015; Xu et al., 2015; Cornwall and Hurd, 2015). In this context, natural CO_2 vents (*e.g.* Ischia, Vulcano, Paupa New Guinea, Methana) offer a unique opportunity to investigate the response of seaweeds to OA both in natural populations and in transplants (Porzio et al., 2011; Johnson et al., 2012; Celis-Plá et al., 2015; Kumar et al., 2017a; Porzio et al., 2017; Kumar et al., 2017b). Most studies to date have predominantly addressed either photosynthetic/carbon physiology of seaweeds or were confined to quantitative analysis of small subset of physiological and biochemical parameters (Celis-Plá et al., 2015). Although these studies provided useful insights, a wider approach would be necessary to obtain global insights in changes of metabolic processes of seaweeds under OA. A *de novo* transcriptomic analysis was recently performed on a natural population of the brown alga *Sargassum vulgare* C. Agardh growing at the volcanic CO_2 vents off Ischia Island (Kumar et al., 2017a). This study revealed that at CO_2 vents *S. vulgare* is adapted to live under acidified conditions through increased expression of genes involved in energy metabolism, photosynthetic processes, ion homeostasis, cell wall, carbon storage, and cellular signaling (Kumar et al., 2017a). Moreover, the analysis of physiological and biochemical parameters of the effects of OA on *S. vulgare* at different time scales showed that this species mitigates stress effects by reprogramming its physiology (Kumar et al., 2017b).

Seaweeds undergo metabolomic reprogramming under abiotic stress conditions (Kumar et al., 2016). Metabolites in fact are the end products of cellular regulatory processes, and represent the response to any environmental changes (Fiehn, 2002). They can also trigger gene expression thus regulating responses to stress conditions (Renberg et al., 2010). However, up to date, few studies have looked for variations in the levels of antioxidants, fatty acids (FAs), amino acids (AAs), and phenolic compounds under elevated CO_2 conditions (Figueroa et al., 2014; Chen et al., 2016; Kumar et al., 2017b). In particular, total phenolic content by colorimetric assay has been measured without quantifying the specific phenols (Celis-Plá et al., 2015; Betancor et al., 2014). Changes in the levels of algal metabolites could compromise the health status of the seaweed and its associated communities by altering algal nutritional values and prey palatability (Harley et al., 2012; Arnold et al., 2012; Poore et al., 2013; Poore et al., 2016). Indeed, marine algae are the source of polyunsaturated fatty acids (PUFA) and essential amino acids (EAAs) for many metazoans, and high CO_2/lowered pH may affect their levels with detrimental consequences on the food web (Tsuzuki et al., 1990; Rossoll et al., 2012; Torstensson et al., 2013; Leu et al., 2013; Bermúdez et al., 2015; Bermudez et al., 2016). Here, the levels of primary (carbohydrates, AAs and FAs), and secondary metabolites (phenolic compounds) in the brown alga *S. vulgare* were analyzed. The population naturally growing at the acidified site of Castello Aragonese was compared to that at a control site off Ischia Island at current pH, 8.1. To study short-term responses, these metabolites were also estimated in transplanted samples from control to acidified site and *vice-versa*.

2. Materials and methods

2.1. Study site and sample collection

The brown seaweed *S. vulgare* was collected along the Ischia Island at two locations: Castello Aragonese, acidified site (40°43.87 N, 013°57.78E) and Lacco Ameno, control site (40°45.35 N, 013°53.13E, Fig. 1). Seawater surrounding Castello Aragonese is acidified due to continuous emission of CO_2 from underwater volcanic vents. Venting activities at this site date back to nearly 2000 years (Lombardi et al., 2011), emitting mainly CO_2 (90.1–95.3%) with no detectable harmful sulfur gases. These are not thermal vents, thus seawater temperature at this site is similar to nearby control seawater (Hall-Spencer et al., 2008). *S. vulgare* is growing in the most acidified part of these vents where the pH is constantly around 6.6. The control site is Lacco Ameno, which is located almost 6 km northwest from the acidified site, where another population of *S. vulgare* is growing at current pH value (8.1). Both the sites are sheltered, share similar depth, salinity, temperature, light and other hydrodynamic and physical conditions (Kumar et al., 2017a, b).

In both locations, 9 thalli of similar size (8–10 cm frond length) were haphazardly collected by snorkeling in three different patches along a coastal stretch of about 15 m at similar depth (<1 m) to cover the natural variability of the two local populations. Samples were collected in July 2014 in both acidified and control sites, on the same day at approximately same time (between 11 am and 1 pm). Upon collection, the algae were maintained in seawater of the respective sites onboard and transported to the laboratory within an hour. The tissues were washed with filtered sea water to remove visible epiphytes and immediately stored at −80 °C until further analysis.

2.2. In situ transplant experiment

On the same day of algal collection, *in situ* reciprocal transplants were performed. Five individuals of *S. vulgare* originating from the control site (C) in Lacco Ameno were tied in a net and moved to acidified site (A) in Castello Aragonese (C-A) and *vice-versa* (A-C). In order to evaluate the stress effect due to the transplant itself, other thalli were also transplanted within their respective natural site (C-C and A-A) and were used as controls. After 2 weeks, the algal samples were

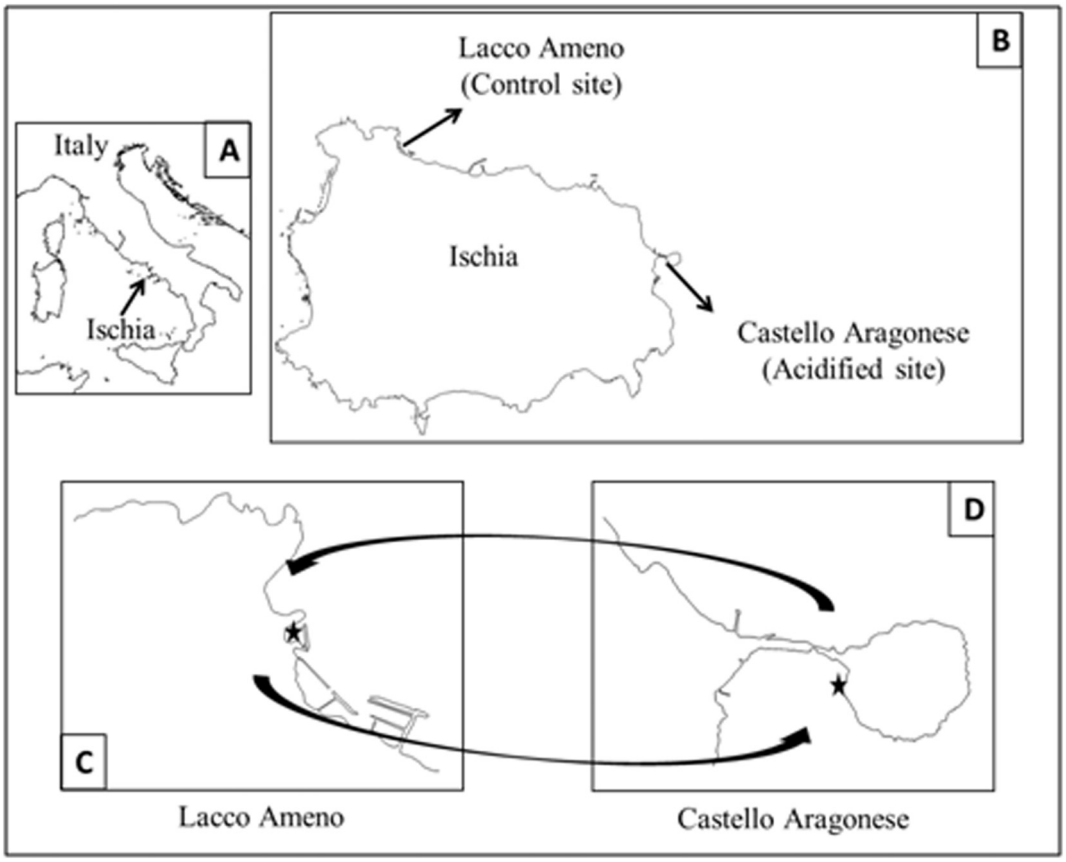

Fig. 1. Study sites: A: map of Italy, B: map of Ischia Island showing the location of Lacco Ameno and Castello Aragonese, C: close up view of Lacco Ameno, control site in the present study, Box D: close up view of Castello Aragonese, where CO_2 vents are present, acidified site in the present study. In C and D, marked points show locations of *S. vulgare*, and arrow represent *in situ* reciprocal transplant experiments.

collected, maintained onboard in water of respective sites and brought to laboratory. The tissues were washed with filtered sea water to remove the visible epiphytes and then immediately stored at −80 °C until further analysis.

2.3. Sugars determination

Sugars were extracted by homogenizing frozen algal tissue in acetonitrile/water (2 mL, 1:1, v/v) for 2 min and quantified using high-performance liquid chromatography (HPLC) according to Alasalvar et al. (2003). In particular, the extract was incubated at 55–60 °C for 15 min (stirring frequently with a glass rod to aid in dissolving the sugars) in a water bath, followed by filtration through Whatman No. 541 filter paper. Again, 20 mL of solvents were added to the remaining pulp, and the extraction was repeated three times. Finally, all supernatants were combined, brought to a final volume of 100 mL with extraction solvent, analyzed by HPLC on a 5 μm SUPELCOSIL LC-NH_2 column (250 × 4.6 mm), at a temperature of 30 °C, and eluted with acetonitrile-HPLC-grade water (75:25 v/v). Individual sugars were identified and quantified by comparing retention times and peak areas of known concentrations of standard sugars solutions.

2.4. Fatty acids determination

Fatty acids were analyzed using Gas chromatography following the methods described by Torras-Claveria et al. (2010). The dried algal tissues (0.2 g) were extracted with 50% aqueous methanol at room temperature until discoloration of the tissues occurred. As an internal standard, codeine and non adecanoic acid were used. GC/MS analysis was carried out on a Hewlett Packard 6890, MSD 5975 mass spectrometer (Hewlett Packard, Palo Alto, CA, USA), with an HP-5 MS column (30 mm × 0.25 mm × 0.25 mm). Fatty acids were identified using NIST 05 database and Golm Metabolome Database (http://gmd.mpimp-golm.mpg.de/). The levels of SFA, MUFA and PUFA are determined by summing up all FA of the respective categories divided by total content.

2.5. Amino acids determination

The frozen algal tissues were extracted in 80% (v/v) aqueous ethanol (1 mL) using MagNALyser (Roche, Vilvoorde, Belgium) (Sinha et al., 2013). The homogenate was centrifuged at 14,000 rpm for 20 min, the clear supernatant was evaporated under vacuum to dryness, and the pellet was re-suspended in 1 mL chloroform (E1). Immediately, the residue was re-extracted with 1 mL of HPLC grade water, centrifuged again, the supernatant was re-mixed with E1 and centrifuged for 10 min. The aqueous phase was isolated and filtered using 0.2 μM pore size Millipore micro filters before AAs determination. AAs were identified and quantified using a Waters Acquity UPLC-tqd system (Milford, Worcester County MA, USA), equipped with a BEH amide 2.1 × 50 column, comparing elution times and peak areas of known concentrations of individual AAs.

2.6. Phenolics determination

From algal tissues, phenolic compounds were extracted with acetone-water (250 mL, 4:1 v/v), at room temperature for 24 h on orbital shaker following Hamad et al. (2015). The extract was filtered, and, when necessary, centrifuged. The supernatant was evaporated under vacuum, the residue was then re-suspended in HPLC grade

methanol. Phenolic compounds were analyzed using Shimadzu HPLC system (SCL-10 A VP, Shimadzu Corporation, Kyoto, Japan), consisting of a diode-array detector and a Lichrosorb Si-60, 7 μm, 3 × 150 mm column. The mobile phase was water-formic acid (90:10, v/v), and acetonitrile/water/formic acid (85:10:5, v/v/v). Individual phenols were identified and quantified by comparing retention times and peak areas of known concentrations of standard compounds.

2.7. Statistical analysis

Independent sample *t*-test was performed on the mean values of the data to determine the significant differences using SPSS (v21SPSS Inc., Chicago, Illinois, USA). The analysis was made by comparing the samples from control and acidified sites (natural populations, long-term responses). For transplant experiments (short-term responses), statistical significance was measured by comparing the samples transplanted to another site with those transplanted within each site. The data were checked for the normality by visual inspection and homogeneity of variance by Levene's test. Hierarchical clustering using Pearson correlation and heat map was generated with MultiExperimental Viewer (MeV) in the TM4 software package.

3. Results

3.1. Sugar analyses

Thalli of *S. vulgare* naturally growing under acidified conditions had 19% lower levels of total soluble ($p = 0.042$) and 21% less insoluble sugars ($p = 0.023$). The similar pattern was reflected by the levels of main monosaccharides glucose, fucose and xylose (Fig. 2A, C). After transplantation of *S. vulgare* from control to acidified site, total soluble sugar increased by 33.5% ($p = 0.038$, Fig. 2B). Specifically, the levels of glucose and mannose increased almost 72% ($p = 0.0006$ and $p = 0.04$ respectively) in the algal tissues transplanted from control to acidified site (Fig. 2D). In contrast, no difference in the sugar content was observed in the samples transplanted from acidified to control site (Fig. 2B, D).

3.2. Fatty acids

A total of 12 individual FAs were identified and measured in *S. vulgare*. The total FAs profile consisted of ~58% saturated fatty acids (SFAs), ~18% monosaturated fatty acids (MUFAs) and ~24% polyunsaturated fatty acids (PUFAs) across different samples and treatments (Fig. 3). The most abundant FAs were palmitic acid, oleic acid and arachidonic acid. We did not find significant differences in total SFA, and unsaturated FA (UNFA) levels between algae naturally growing at both sites or upon short-term *in situ* reciprocal transplants. However, the level of several individual FAs within these groups showed significant variations among test conditions *e.g.* C14:0, myristic ($p = 0.005$), C20:0, arachidic ($p = 0.048$), C16:1ω-7, palmitoleic ($p = 0.001$), C18:3ω-3, linolenic ($p = 0.008$) and C20:4ω-6, arachidonic ($p = 0.048$) acids were significantly lower in the algae growing at the acidified site than control site. Similarly, transplanting thalli from control to acidified site induced an increase in the levels of FAs such as C12:0, lauric ($p = 0.011$), C14.0, myristic ($p = 0.002$), C16:0, palmitic ($p = 0.003$), C20:0, arachidic ($p = 0.005$), C16:1ω-7, palmitoleic ($p = 0.036$), C18:3ω-3, linolenic ($p = 0.0002$) and C20:4ω-6, arachidonic ($p = 0.0003$) acids in comparison to transplant within the control site. Inversely, C14:0, myristic acid ($p = 0.0009$), C18:3ω-3, linolenic ($p =$

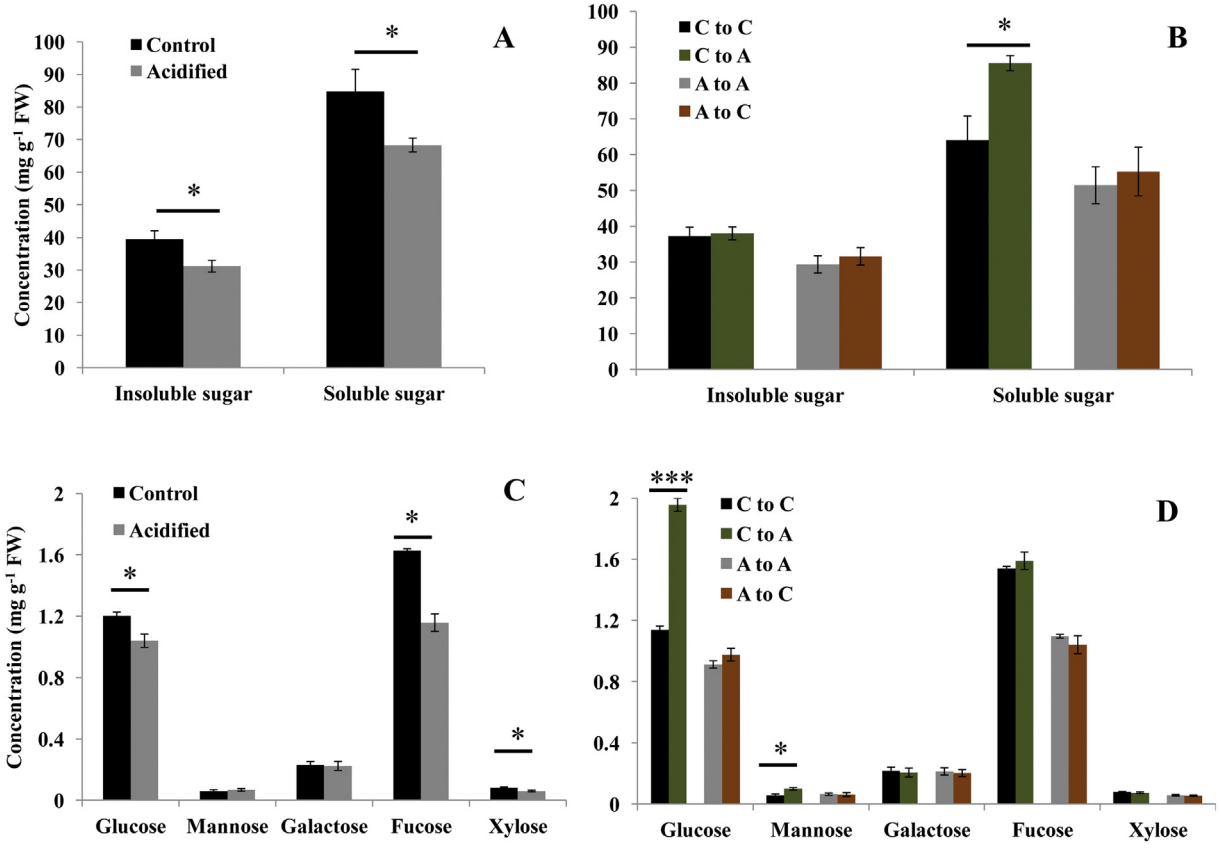

Fig. 2. Levels of sugars in *S. vulgare* naturally growing at control and acidified site (A, C); and in transplanted thalli (B, D): C to C = control to control site, C to A = control to acidified site, A to A = acidified to acidified site, A to C = acidified to control site. Values are mean ± SE, $n = 3$, * $p < 0.05$, *** $p < 0.001$.

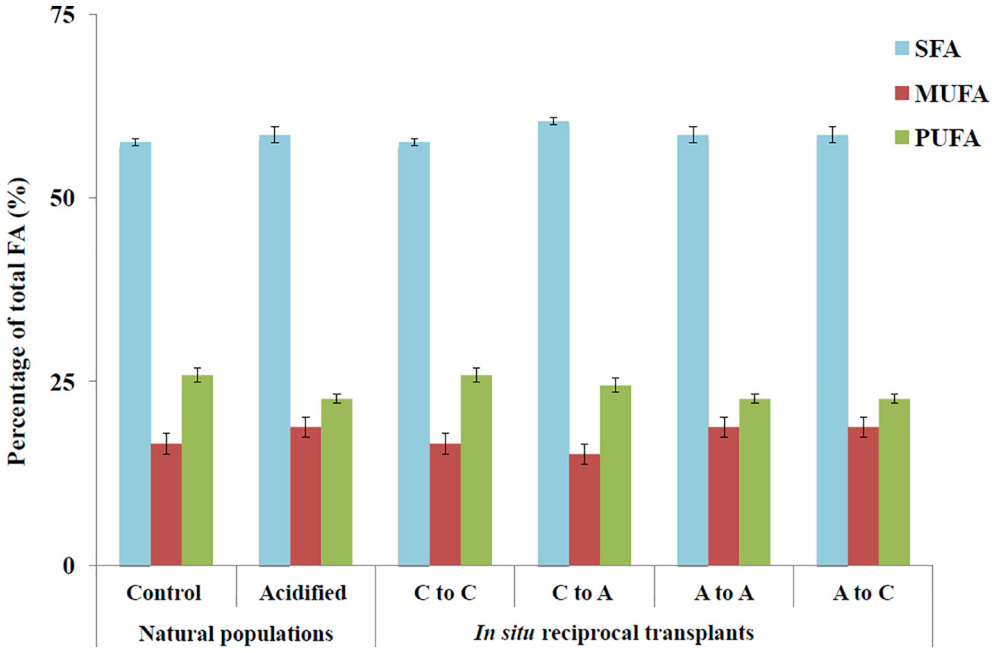

Fig. 3. Distribution of fatty acids in thalli of *S. vulgare* collected at control and acidified sites (long-term condition) and in transplants (short-term condition). C to C = control to control site, C to A = control to acidified site, A to A = acidified to acidified site, A to C = acidified to control site. Values are mean ± SE, $n = 3$.

0.0009) showed a significant increase in the samples transplanted from acidified to control site (Fig. 4).

3.3. Amino acids

In total, 17 AAs were identified and quantified in *S. vulgare* including essential and non-essential AAs. Phenylalanine was the most abundant AA under all test conditions. Even though the level of total AA in natural populations at acidified and control sites was not significantly different ($p = 0.746$), we observed significant difference in individual AAs in the thalli growing at the different sites. In particular, histidine ($p = 0.003$), threonine ($p = 0.004$), methionine ($p = 0.004$), isoleucine ($p = 0.0008$), glycine ($p = 0.042$), alanine ($p = 0.043$), and serine ($p = 0.0004$) contents were higher in the algae growing at the acidified site, while glutamate ($p = 0.00007$) and lysine ($p = 0.017$) content was lower (Fig. 5A).

Upon transplantation of the algae from control to acidified site, we found a general increase in most of the AAs including histidine ($p = 0.001$), threonine ($p = 0.004$), methionine ($p = 0.0004$), iso leucine ($p = 0.013$), proline ($p = 0.004$), tyrosine ($p = 0.001$), glycine ($p = 0.00005$), alanine ($p = 0.00005$), serine ($p = 0.0004$), glutamine ($p = 0.00005$), glutamate ($p = 0.00001$) and aspartate ($p = 0.00008$). However, when the samples were transplanted from acid to control site, only proline ($p = 0.0001$) and glutamate ($p = 0.0017$) showed a significant increase (Fig. 5B).

3.4. Phenolic compounds

In total, 17 different phenolic compounds were detected and quantified in algal tissues (Table 1). Catechin and epicatechin were the most abundant. When compared with controls, in samples from the acidified site lower levels of phenolic compounds were detected, in particular caffeic acid ($p = 0.00008$), catechin ($p = 0.002$), gallic acid ($p = 0.0001$), resorcinol ($p = 0.008$), and isoquercitrin ($p = 0.008$). Transplanting samples recorded minor changes: only ferulic acid ($p = 0.03$) and chlorogenic acid ($p = 0.007$) increased only in the samples transplanted from acidified to control site (Table 1).

3.5. Hierechical clustering

To classify metabolites content into groups according to their response, we performed a hierarchical clustering analysis (Fig. 6). Clustering resulted in the separation of metabolites into four major groups based on the difference in their expression levels in naturally acid populations and in transplanted thalli. The first cluster consisted of five metabolites including one sugar (mannose), one FA (oleic acid) and three AAs (glycine, alanine, and glutamine). The concentrations of these metabolites were always higher in the acidified condition both in natural acid population (long-term scale) and in transplants (short-term scale). The second cluster included the largest number of metabolites, in total 22, including 2 sugars (glucose, fucose), 11 FAs (lauric, palmitic, linolenic, capric, arachidic, arachidonic, palimitoleic, stearic, henocasanoic, linoleic, and myristic acids), and 9 AAs (proline, glutamate, aspartate, tyrosine, arginine, valine, phenylalanine, lysine, leucine). The concentration of these metabolites were lower in the natural population growing for long time under acidified conditions while higher in the transplanted samples from control to acidified site (short-term). The third cluster consisted of 15 metabolites, most of which were secondary metabolites except a sugar (xylose). The levels of these metabolites were not affected by pH conditions in natural population and were either unaffected or decreased in transplanted samples at acidified site. The last cluster included 5 AAs (histidine, serine, methionine, isoleucine, threonine), 1 sugar (galactose) and 3 phenolic compounds (quercitrin, apigenin, and gentisic acid). The concentration of AAs was higher in natural population under acidified condition and in transplant from control to acidified site, while the amount of sugar and phenols was higher in the algae living at the acidified site.

4. Discussion

In the present study, the changes in the levels of primary and secondary metabolites in thalli of *S. vulgare* exposed to elevated CO_2/lowered pH at the volcanic vents off Ischia Island were examined for the first time. Our main finding includes the variable metabolic response of this alga at different time scales to natural acidification. The levels of

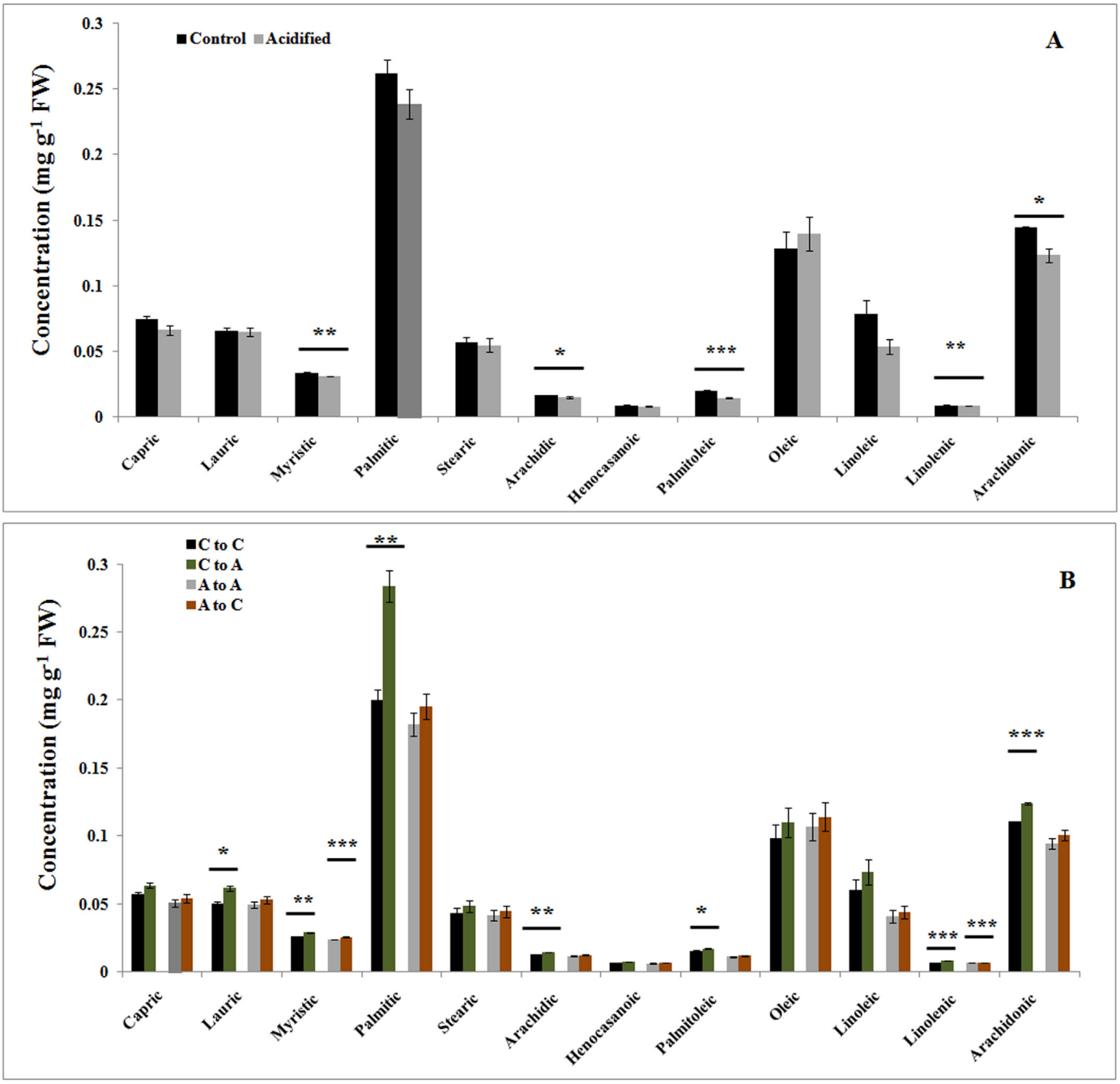

Fig. 4. Distribution of fatty acids with varying degree of saturations in thalli of *S. vulgare* naturally growing at control and acidified sites (A) and in transplants (B). C to C = control to control site, C to A = control to acidified site, A to A = acidified to acidified site, A to C = acidified to control site. Values are mean ± SE, n = 3 * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$.

soluble and insoluble sugars, FAs, and several secondary metabolites were lower in the population naturally growing at the acidified site, whereas the levels of majority of AAs were higher compared to those detected in thalli growing at the control site. Moreover, in algae transplanted from the control to the acidified site, the levels of soluble sugars, in particular glucose and mannose, the majority of AAs, and FAs increased in comparison to those transplanted in the same site. These results support our previous findings showing a variable physiological and biochemical responses of *S. vulgare* to acidification over different time scales (Kumar et al., 2017b). Indeed, the metabolic changes observed in transplants may be due to acclimatory response that supports algae to cope with acidification, thus leading to adaptation to lowered pH in long time scale.

4.1. Overview of metabolic response

Seaweeds respond to environmental stress by managing their physiological processes, especially carbon and nitrogen metabolism, which lead to alteration in metabolic networks related to sugar, AAs, and FAs (Kumar et al., 2016). An overview of the metabolic changes in *S. vulgare* after transplantation and in natural population under acidified conditions is shown in Fig. 7.

4.1.1. Response of S. vulgare to acidification through in situ reciprocal transplantation

The increase in the soluble sugar content in thalli transplanted from control to acidified site could be due to elevated photosynthesis, as we

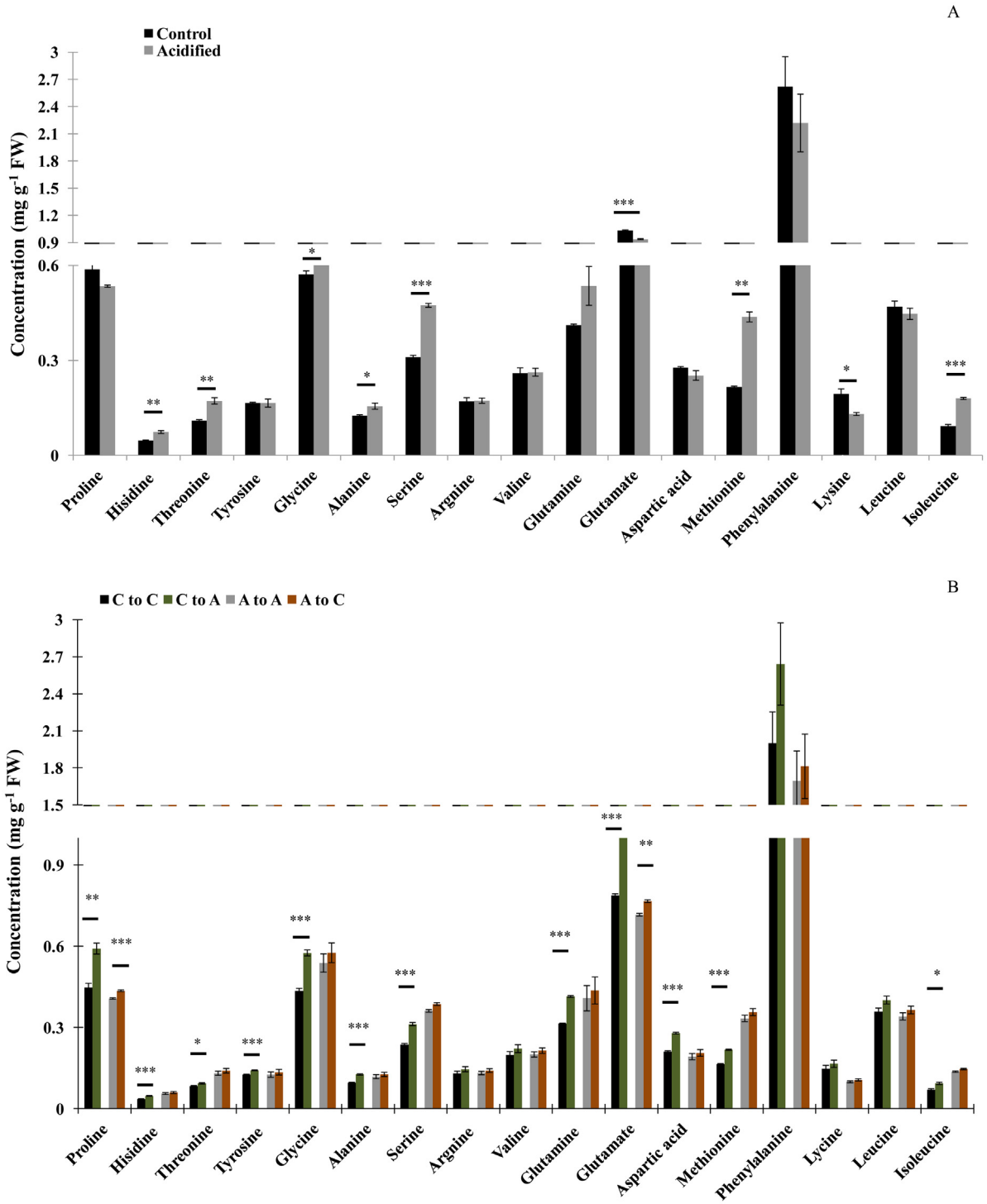

Fig. 5. Concentrations of amino acids (mg g^{-1} FW) in thalli of *S. vulgare* naturally growing at acidified and control conditions (A) and after transplants (B). C to C = control to control site, C to A = control to acidified site, A to A = acidified to acidified site, A to C = acidified to control site. $n = 5$, * $p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

have previously reported (Kumar et al., 2017b). High soluble carbohydrate concentration and maximum photosynthesis rate were also obtained in other seaweeds when grown under elevated carbon dioxide (Suárez-Álvarez et al., 2012; Figueroa et al., 2014). In addition, the increase in soluble carbohydrate alone under high CO_2 conditions has been previously described for several macroalgae including the green alga *Ulva rigida* (Gordillo et al., 2001), and red alga *Hypnea spinella* (Suárez-Álvarez et al., 2012) and brown alga *Saccharina latissima* (Olischläger et al., 2014). Besides supporting higher energy demands (Ow et al., 2015), the soluble sugars may also be involved in the formation of cell wall structures (Thanh et al., 2013). Indeed, the increased level of mannose in thalli transplanted to acidified site indicates an active allocation of carbon into the synthesis of cell wall components, alginate, and sulfated fucans.

The elevated levels of aromatic AAs including phenylalanine and tyrosine which are synthesized from phosphoenol pyruvate, an intermediate compound in glycolytic pathway, suggest that increased glucose is catabolizing to liberate energy in the algae. The increased level of aromatic AAs also indicates their participation in shikimate pathway to maintain the level of secondary metabolites in the algae. Among AAs,

Table 1
Concentration of phenolic compounds in *S. vulgare* growing naturally at acidified and control site and after reciprocal transplant, C to C = control to control site, C to A = control to acidified site, A to A = acidified to acidified site, A to C = acidified to control site. Values are mean ± SE, n = 3.

	Natural populations		Transplant			
	Control	Acidified	C to C	C to A	A to A	A to C
Caffeic acid	0.594 ± 0.010	**0.337 ± 0.012*****	0.454 ± 0.008	0.428 ± 0.007	0.258 ± 0.009	0.276 ± 0.010
Ferulic acid	0.682 ± 0.020	0.647 ± 0.009	0.521 ± 0.s015	0.491 ± 0.014	0.494 ± 0.007	**0.529 ± 0.008***
Protocatechuic acid	0.740 ± 0.014	0.695 ± 0.016	0.565 ± 0.010	0.533 ± 0.010	0.531 ± 0.012	0.568 ± 0.013
Catechin	5.063 ± 0.149	**3.163 ± 0.204****	3.867 ± 0.113	3.646 ± 0.107	2.415 ± 0.156	2.585 ± 0.166
Gallic acid	1.938 ± 0.042	**1.135 ± 0.043*****	1.480 ± 0.032	1.396 ± 0.030	0.867 ± 0.033	0.927 ± 0.035
p-Coumaric acid	0.644 ± 0.030	0.626 ± 0.070	0.492 ± 0.023	0.464 ± 0.022	0.478 ± 0.054	0.511 ± 0.057
Resorcinol	0.045 ± 0.004	**0.015 ± 0.005****	0.035 ± 0.003	0.033 ± 0.003	0.011 ± 0.004	0.012 ± 0.004
Chlorogenic acid	1.437 ± 0.099	1.404 ± 0.013	1.098 ± 0.075	1.035 ± 0.071	1.072 ± 0.010	**1.147 ± 0.011****
Syringic acid	1.297 ± 0.086	1.191 ± 0.138	0.990 ± 0.066	0.934 ± 0.062	0.909 ± 0.106	0.973 ± 0.113
Vanillic acid	1.945 ± 0.129	1.648 ± 0.141	1.485 ± 0.098	1.400 ± 0.093	1.259 ± 0.107	1.347 ± 0.115
Quercetin	0.018 ± 0.002	0.017 ± 0.001	0.014 ± 0.001	0.013 ± 0.001	0.013 ± 0.001	0.014 ± 0.001
Quercitrin	0.002 ± 0.000	0.003 ± 0.000	0.002 ± 0.000	0.001 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Epicatechin	6.074 ± 0.577	4.935 ± 0.264	4.638 ± 0.441	4.374 ± 0.416	3.769 ± 0.201	4.033 ± 0.215
Apigenin	0.157 ± 0.005	0.190 ± 0.012	0.120 ± 0.004	0.113 ± 0.004	0.145 ± 0.009	0.155 ± 0.010
Isoquercitrin	0.984 ± 0.094	**0.320 ± 0.101****	0.751 ± 0.071	0.709 ± 0.067	0.244 ± 0.077	0.261 ± 0.082
Rutin	0.724 ± 0.048	0.672 ± 0.030	0.553 ± 0.037	0.522 ± 0.034	0.513 ± 0.023	0.549 ± 0.025
Gentisic acid	1.139 ± 0.032	1.253 ± 0.100	0.870 ± 0.024	0.820 ± 0.023	0.957 ± 0.076	1.024 ± 0.082

In bold are reported the values changed compared with controls.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

also the levels of glycine, serine, proline, glutamate, aspartate and glutamine are increased indicating the up-regulation of gluconeogenesis. These AAs are major central organic nitrogenous compounds involved in the storage and transport of nitrogen and precursors of different metabolic pathways under various environmental conditions (Planchet and Limami, 2015). The increase in the level of EAAs, including histidine, threonine, methionine and isoleucine in transplanted thalli under acidified conditions, is in contrast with the recent findings, showing decreased levels of EAAs under acidified conditions (Renberg et al., 2010; Bermudez et al., 2016; Chen et al., 2016).

Regarding lipids, it is well known that they play an important role in the acclimation to dynamic environmental conditions. The oxidation product of lipids, acetyl-CoA, can be carboxylated to give malonyl-CoA that plays a key role in FA elongation. The rise in some SFAs (lauric, myristic, palmitic and arachidic acids) and UNFAs (palmitoleic, linolenic and arachidonic acids) in transplanted thalli suggest their ability to adjust membrane fluidity for acclimation at lowered pH. The possible enhanced FAs synthesis and their accumulation might represent a mechanism to produce less fluid cell membranes. These membranes might help to regulate the cellular homeostasis, being less permeable to CO_2 (Hall-Spencer et al., 2008; Harvey et al., 2014). On the other hand, the increased levels of few PUFAs (linolenic and arachidonic acids) may be necessary to maintain activity of membrane bound enzymes, particularly ion transporters, and stabilize protein complexes of photosystem II, thereby preventing damages to photosynthesis under stress conditions (Dittami et al., 2011; Kumar et al., 2016). Increase in selected PUFAs can also suggest their possible involvement in oxidative stress mediated signals of acclimation (Ritter et al., 2008; Küpper et al., 2009). Furthermore, acetyl-CoA through mevalonate pathway might increase the level of α-, β-, γ- tocopherol as it has been previously reported in *S. vulgare* transplants from control to acidified conditions (Kumar et al., 2017b).

4.1.2. Responses of S. vulgare natural populations to acidification

Our previous studies demonstrated that population of *S. vulgare* naturally growing at elevated CO_2 condition has an increased energy production and a comparable photosynthetic performance compared to thalli at current pH (Kumar et al., 2017a; Kumar et al., 2017b). The asynchrony between photosynthesis and respiration could explain the finding that the content of total soluble and insoluble sugar decreases in acidified population. In the terrestrial plants, short-term exposure to CO_2 has been reported to cause a rise in sugar content which was reversed under prolonged exposure (Levine et al., 2008). However, increased levels of glucogenic AAs including glycine, serine, glutamate, aspartate and glutamine indicated that, if necessary, these AAs can be converted into glucose through the up-regulation of gluconeogenesis. The observed higher levels of AAs are in line with the increased protein biosynthesis, as revealed by transcriptomic analysis (Kumar et al., 2017a).

An interesting result of this study is the decreased levels of caffeic acid, gallic acid, catechin, resorcinol and isoquercitrin in the acidified population, in accordance with lower content of total phenols detected in some seaweeds and seagrasses under OA scenarios (Arnold et al., 2012; Betancor et al., 2014; Kumar et al., 2017b). These compounds as well as other phenolic compounds inhibit pathogens, protecting marine plants from microbial infections and massive epiphytic colonization (Buchsbaum et al., 1990; Vergeer and Develi, 1997; Jensen et al., 1998; Audibert et al., 2010; Abad et al., 2011; Thabard et al., 2011). The decreased level of these compounds may enhance infection and disease in marine macroalgae in a future climate change scenario (Campbell et al., 2011).

4.2. Nutritional qualities and implications on trophic transfer

S. vulgare represents an important habitat forming macroalgal species along rocky coasts and provides food, shelter and nursery ground for a wide range of marine organisms (Chemello and Milazzo, 2002; Thibaut et al., 2015). Any significant OA effects on the chemical constituents of producers not only make them more vulnerable to other stressors, grazing, infection and diseases (Arnold et al., 2012), but also disturb the nutritional qualities and values at higher trophic level in the food web (Rossoll et al., 2012). *S. vulgare* is not very rich in lipids and shows FA compositions comparable to other marine macroalgae (Pereira et al., 2012). The presence of PUFAs makes them important from nutritional point of view (Silva et al., 2013). The increase in the level of individual PUFAs such as linolenic-, and arachidonic- acids in short-term transplanted thalli indicated active allocation of carbon in these fatty acids under high CO_2 conditions. On the contrary, their concentrations were significantly decreased in population growing for a long time at acidified site indicating a lower quality in terms of PUFAs. Similar decrease in PUFAs has been reported in microalgae growing for long time (>250 generations, >1 year) in culture (Bermúdez et al.,

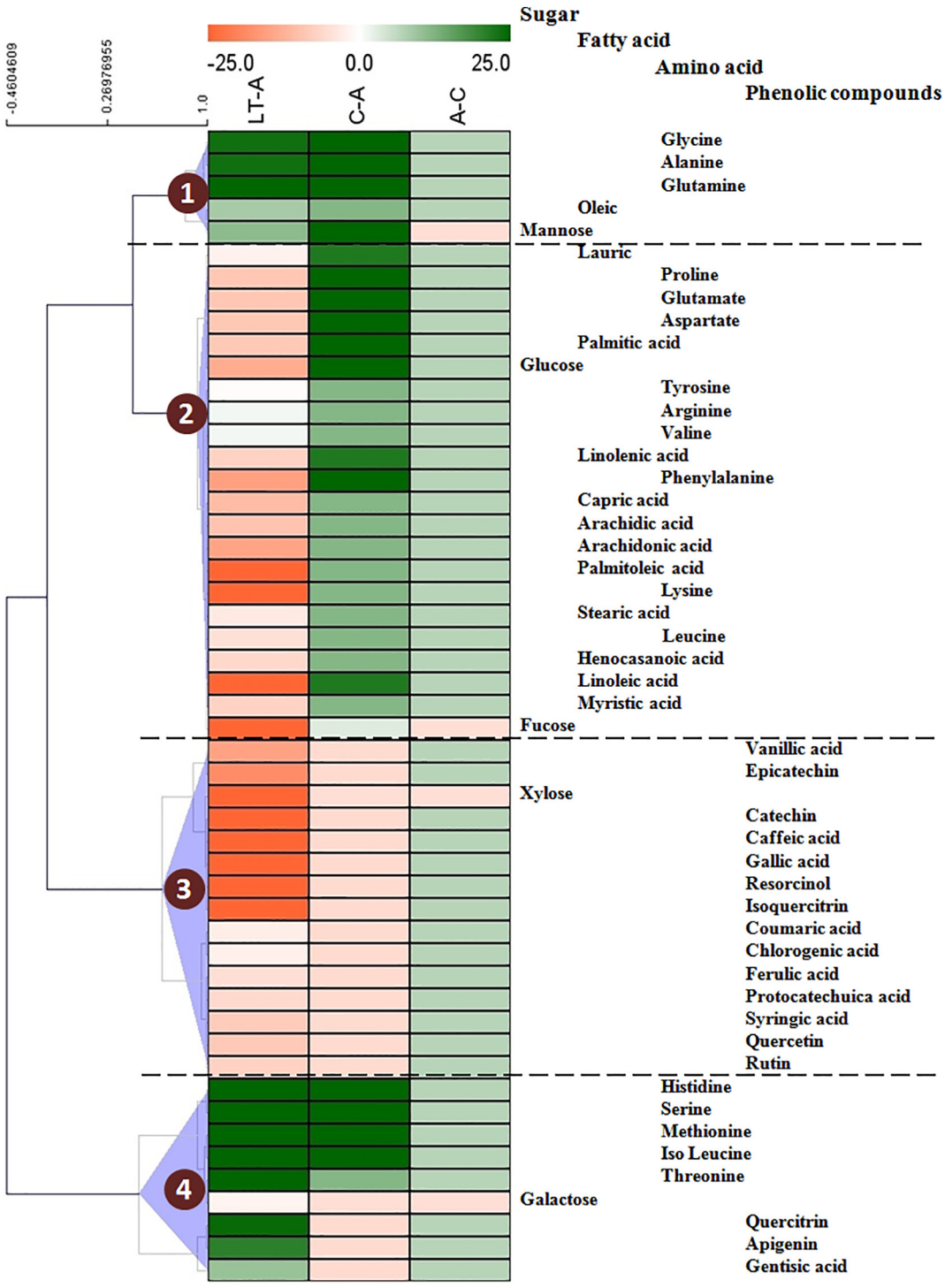

Fig. 6. Hierarchical clustering of sugars, amino acids, fatty acid and phenolic compounds in natural population (long-time scale) and in transplants (short-time scale). The changes in the metabolites were compared between conditions and converted in percentage. LT-A: comparison between natural population growing at acidified and control site, C-A: comparison between samples transplanted from control to acidified and control to control site, A-C: comparison between algae transplanted from acidified to control and acidified to acidified area.

2015). It has been shown that invertebrates feeding on low quality food have constrained growth and reproduction (Rossoll et al., 2012; Bermúdez et al., 2015). However, recent studies on brown algae-invertebrate interactions have shown variable response, indicating that changes in the algal biochemical composition might affect the species interaction (Poore et al., 2013; Gutow et al., 2014; Poore et al., 2016; Schram et al., 2017). The increase of EAAs indicates that the nutritional quality in terms of EAAs is not deteriorating under acidified conditions. Moreover, the decrease of phenolic compounds in natural population at acidified site may have implications on trophic transfer. Since the phenolic compounds acts as deterrent to the grazers, the decrease in these compounds can make *S. vulgare* more prone to grazing.

In conclusion, our findings indicate variable responses of the macroalga *S. vulgare* to natural acidification in terms of primary and secondary metabolites. While short-term responses are not deteriorating and threatening, on long time scale the macroalga will produce less sugar, FAs, including PUFAs, and phenolic compounds, which might have negative consequences on the health and the nutritional quality

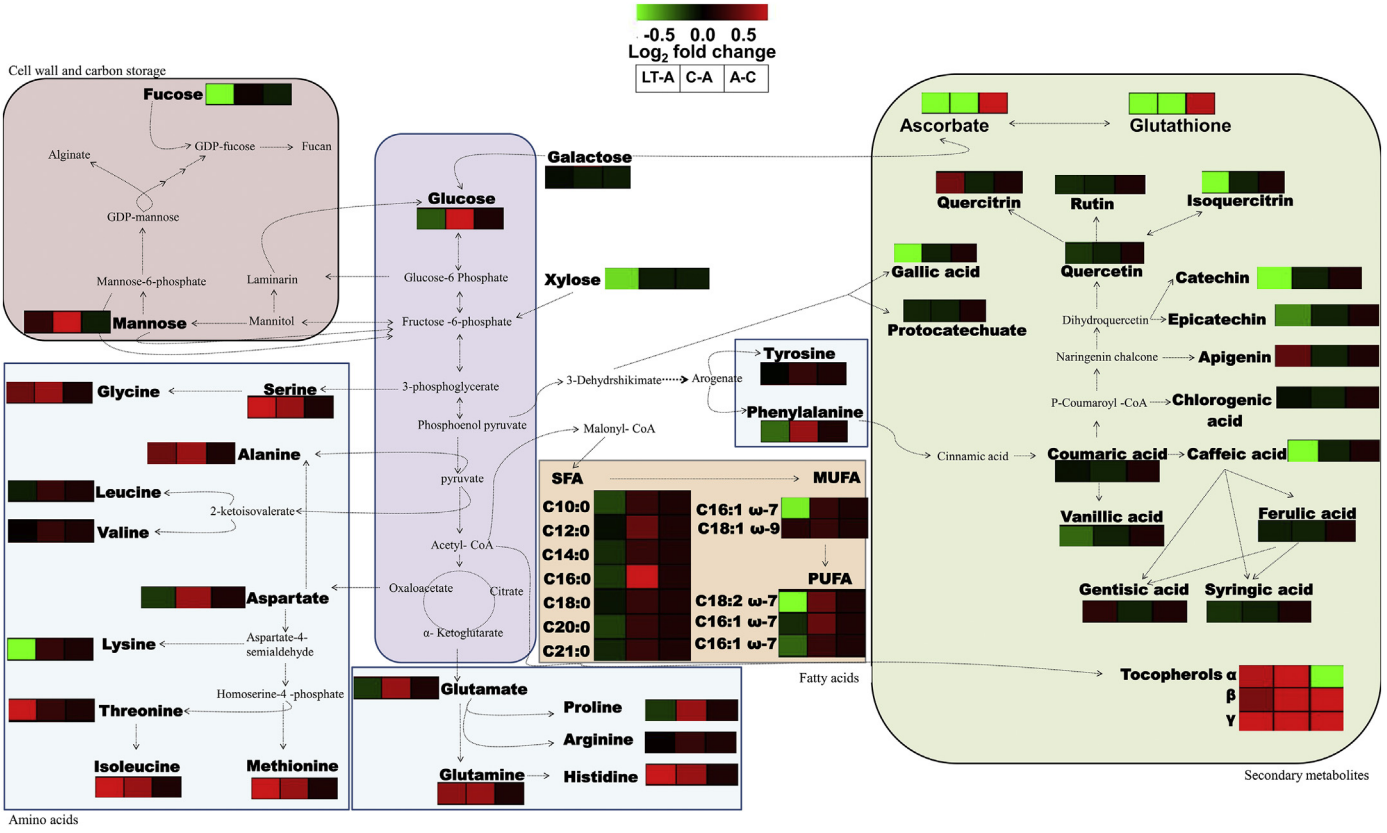

Fig. 7. An overview of the metabolites under acidified conditions. The metabolic pathways have many intermediate steps which are not shown in order to simplify the figure. The ratio of the values between thalli growing at control and acidified conditions and among transplanted algae was calculated and then the $\log_2$-fold change in this ratio was plotted. LT-A: Comparison between natural population of algae growing at acidified and control sites, C-A: comparison between samples transplanted from control to acidified and control to control site, A-C: comparison between algae transplanted from acidified to control and acidified to acidified area. The data for ascorbate, glutathione and tocopherol are redrawn from Kumar et al. (2017b).

of seaweeds and then on the coastal food web. However, further studies are needed to investigate whether lowered quality *S. vulgare* growing at acidified conditions might affect the health status of primary consumers upon feeding.

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