



Behavior of the Edible Seaweed *Sargassum fusiforme* to Copper Pollution: Short-Term Acclimation and Long-Term Adaptation

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Abstract

Aquatic agriculture in heavy-metal-polluted coastal areas faces major problems due to heavy metal transfer into aquatic organisms, leading to various unexpected changes in nutrition and primary and/or secondary metabolism. In the present study, the dual role of heavy metal copper (Cu) played in the metabolism of photosynthetic organism, the edible seaweed *Sargassum fusiforme*, was evaluated by characterization of biochemical and metabolic responses using both ¹H NMR and GC-MS techniques under acute (47 μM, 1 day) and chronic stress (8 μM, 7 days). Consequently, photosynthesis may be seriously inhibited by acute Cu exposure, resulting in decreasing levels of carbohydrates, e.g., mannitol, the main products of photosynthesis. Ascorbate may play important roles in the antioxidant system, whose content was much more seriously decreased under acute than that under chronic Cu stress. Overall, these results showed differential toxicological responses on metabolite profiles of *S. fusiforme* subjected to acute and chronic Cu exposures that allowed assessment of impact of Cu on marine organisms.

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Introduction

Over the last few decades, heavy metal pollution has become a global problem posing threat on both soil and marine ecosystems, as a result of the mass industrialization and various agricultural activities such as the intensive use of chemical fertilizers, wastewater and biosolids [1]. Many heavy metals accumulate in marine organisms, which may be subsequently transferred to human body via the food chain [2].

Marine algae, particularly seaweeds, are a food source for marine animals such as sea urchins and fishes, and are the base of many marine food webs. For several centuries, there has been a traditional use of seaweeds as food in East-Asian countries, like China, Japan and the Republic of Korea. *Sargassum fusiforme* (Sargassaceae, Phaephyceae), an endemic brown algae from the western coast of the North Pacific, is widely consumed in Japan and Korea. This alga, in great demand, is also cultivated in East-Asian countries, especially in China, where the cultivation area was 2.6% (2,482 ha) of the entire coastal area for commercial cultivation of seaweeds with a total production reached 32,000 tonnes per year (freshweight) [3].

It is reported that concentration of copper (Cu) found in standard reference material (oyster tissue) from China was above 100 μg g⁻¹ [4], indicating a serious Cu pollution in these coastal areas. Although Cu is an essential micronutrient, excessive amount can be extremely harmful to algae [5]. Seaweeds are often exposed to low concentrations of metals including Cu for long periods. In the cases of ocean outfall, they may even abruptly exposed to high levels of metals. In the study of short- and long-term response of the marine green macroalga *Ulva fasciata* to Cu excess, regulation of mRNA expression involved in redox homeostasis and antioxidant defense were different [6]. In another study, distinct changes in the antioxidant responses to acute or chronic treatment with Cu were observed in the unicellular alga *Gonyaulax polyedra*, suggesting a different oxidative status of these two types of metal stresses [7]. Thus, it seems that both micro- and macroalgae have different responsive mechanisms to short- and long-term exposures of Cu.

Chemical analysis alone is not able to provide a satisfying assessment of the environmental quality of an ecosystem due to the biotransforming of an individual pollutant by living organisms [8,9]. To gain more information regarding the health state of a particular ecosystem, it is important to monitor the response of

biota to the pollutants as well. Metabolomics characterizes and quantifies end products—the metabolites that produced by living organisms under a given set of conditions. Metabolomics has shown considerable potential as a tool for environmental toxicology [10–14]. Both GC-MS and NMR techniques have been widely used in metabolomics and metabolite profiling [15–18]. GC-MS is particularly effective in the analysis of primary metabolites, while NMR, inherently quantitative, provides universal detection for organic components without coupling to a separation technique [19,20]. Because of the complementary analytical features of NMR and MS, opportunities for leveraging both methods are being considered which will create a more comprehensive metabolic profiling [19,21–22].

It is now well known that synthesis of antioxidant and metal-chelating components and activation of antioxidant enzymes are key factors for tolerance to heavy metals and other abiotic stress in plants [23]. The toxic effect of heavy metals appears to be related to production of reactive oxygen species (ROS), which usually leads to lipid peroxidation and oxidation of some enzymes and a massive protein. To better understand oxidative stress under acute and chronic conditions, the content of malondialdehyde (MDA), which represents the level of lipid peroxidation, was measured, as well as the activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD). Additionally, activity of nitrate reductase (NR) that primarily involved in maintenance of a favorable cellular oxidation/reduction potential was also determined. In this study, we characterized the impact of Cu on the marine brown algae *S. fusiforme* using both NMR- and GC-MS-based metabolomics, which allowed identifying more analytes and created an opportunity to expand the scope of metabolomics research.

Materials and Methods

Algal material and culture conditions

S. fusiforme samples were collected from the Northeastern coast of Wenzhou, China (28.0°N, 121.2°E) in September 2012. This location is not privately-owned or protected in any way, thus no specific permissions were required, and the field studies did not involve endangered or protected species. After collection, algae were immediately transported to the laboratory in a cooler (4°C) within 2 h. Fronds were then washed with filtered natural seawater (with salinity of 27 ‰) and maintained in high-density polypropylene containers for 2 days at 20°C before Cu treatment, using a photoperiod of 12:12 h and a photon flux density of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Culture medium was aerated and changed daily.

Two experiments were carried out to evaluate the metabolic differences between responses of *S. fusiforme* to acute and chronic Cu exposures. For short-term treatment (1 day), thalli were cultivated in filtered natural seawater containing CuCl_2 (Sigma, USA) in the final concentration of 47 μM . For long-term treatment (7 days), culture medium containing CuCl_2 was prepared in the same manner as for short-term treatment but with final concentration of 8 μM . After treatment, thalli were immediately frozen in liquid nitrogen and stored at -80°C for further analysis.

In addition, the term “acute exposure” was employed for conditions of exposure to high Cu concentrations (47 μM) after 1 day in this study. On the other hand, “chronic exposure” defines the exposure to lower sub-lethal Cu concentrations (8 μM) after 7 days.

Measurement of enzyme activities and MDA content

Approximately 0.1 g algal samples (fresh weight, $n=5$) were homogenized in liquid nitrogen and extracted with 1 mL of 0.05 M potassium phosphate buffer (pH 7.0) containing 0.25% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone (PVPP). The enzyme activities in the algal tissues were detected using commercial kits (Nanjing Jiancheng Biotech., China) according to the manufacturer’s instructions. In this study, the antioxidant enzymes included SOD (EC 1.15.1.1), CAT (EC 1.11.1.6) and POD (EC 1.11.1.7). In addition, activity of nitrate NR (EC 1.7.99.4) was also determined.

Protein concentration was determined according to the method of Bradford [24] with bovine serum albumin as standard. The unit of each enzyme was defined as the activity of an enzyme per milligram of total protein (expressed in $\mu\text{mol min}^{-1}$ per mg protein, or U per mg protein).

As a measure of lipid peroxidation, MDA levels in algal tissue were estimated by measuring thiobarbituric acid reactive substances following the standard protocol using MDA detection kit (Nanjing Jiancheng Biotech., China) and were expressed as nmol per mg of protein.

NMR analysis and data processing

Polar metabolites were extracted from the algal tissues using the solvent system of methanol/water (1/1) as described previously [25,26]. NMR spectra were acquired using Bruker AV-500 spectrometer (Bruker Bio Spin, Canada), with ^1H observation frequencies of 500.18 MHz, spectral width 6,009.6 Hz, mixing time 0.1 s, and relaxation delay 0.3 s as described previously [27].

All the NMR spectra were converted to a format for pattern recognition analysis using custom-written ProMetab software based on the Matlab software package (version 7.0; The MathWorks, Natick, MA, USA) [28]. NMR spectral peaks were identified following tabulated chemical shifts [29] and using the software, Chemomx (Evaluation Version, Chemomx Inc., Canada).

GC-TOF MS analysis and data processing

Extraction and fractionation of metabolites for GC-TOF MS analysis were performed as described [30] and about 100 mg of each tissue sample was weighed accurately. After derivatization, the metabolites were analyzed by GC-TOF MS analysis that was performed using an Agilent 7890 gas chromatograph system (Agilent, CA, USA) coupled with a Pegasus 4D time-of-flight mass spectrometer (LECO Corp., MI, USA). The system utilized a DB-5MS capillary column coated with 5% diphenyl cross-linked with 95% dimethylpolysiloxane (30 m \times 250 μm inner diameter, 0.25- μm film thickness; J&W Scientific, Folsom, CA, USA). A 1 μL aliquot of the analyte was injected in splitless mode. Helium was used as the carrier gas, the front inlet purge flow was 15 mL min^{-1} , and the gas flow rate through the column was 1 mL min^{-1} . The initial temperature was kept at 80°C for 0.2 min, then raised to 190°C at a rate of 10°C min^{-1} , then to 220°C at a rate of 3°C min^{-1} and finally to 280°C at a rate of 20°C min^{-1} for 16.8 min. The injection, transfer line, and ion source temperatures were 280, 270, and 220°C, respectively. The energy was -70 eV in electron impact mode. The mass spectrometry data were acquired in full-scan mode with the m/z range of 20–600 at a rate of 10 spectra per second after a solvent delay of 480 s.

A total of 288 peaks were found in the 24 samples. Data processing was performed as following: after the missing values were imputed using k nearest neighbor method of Bioconductor (www.bioconductor.org) impute package, data was filtered in order to eliminate the noise using interquartile range, resulting in 268

peaks. The filtered data were subsequently normalized where adonitol, pentakis (trimethylsilyl) ether were used as the internal references.

Statistical analysis

Data were expressed as the mean \pm standard deviation (SD). Statistical analysis included one-way analysis of variance (ANOVA). Principal component analysis (PCA) was used to reduce the dimensionality of data and summarize the similarities and differences between multiple NMR spectra. The principal component score plots were used to visualize general clusters between various groups of samples. ANOVA was conducted on PC scores from each group to test statistical significance ($P < 0.05$) of separations.

Results and Discussion

In total, 25 metabolites were exclusively quantified by NMR (Table 1 and 2), while this number for MS was much more (288 peaks detected). Various metabolite classes were identified in NMR spectra, including amino acids, carbohydrates, and intermediates in the tricarboxylic acid (TCA) cycle. Fig. 1A shows the score plots for the acute Cu stress experiment, where PC1 and PC2 represent 76.26% and 7.03%, respectively. The acute Cu stress experiment score plots contain six distinct groups that represent the control samples and the individual treatments. The separations between the control (inverted red triangles) and exposed (green cycles) were obviously observed from the PC scores plots ($P < 0.05$), while no separation for the chronic stress (Fig. 1B).

GC-TOF MS is also a powerful analytical tool employed in metabolomics studies, which provides the detection of different metabolite peaks. The components measured by GC-TOF MS that Fig. 2 summarizes are mainly amino acids, amines, organic acids, polyols and sugars. It has been proposed that decrease in utilization of carbohydrates for growth produced by heavy metals is more pronounced than the decrease in CO₂ fixation resulting in an increased accumulation of carbohydrates [31]. However, as shown in Fig. 2, the levels of most carbohydrates, e. g., mannose, fructose, hexitol and manitol, were decreased under acute stress, so were those under chronic Cu stress, though to a lesser extent and some even with increasing levels.

Amino acid concentrations were mainly affected

The summarized results for the amino acids profiling from NMR are shown in Table 1. Generally, more than half of the amino acids exhibit different profiles in response to the acute and chronic Cu stress. Concentrations of alanine and glutamine increased (by 30% and 9%, respectively), while those of aspartate and glutamate significantly decreased (by nearly 30%), resulting in a general decrease in total amount of the predominant amino acids (alanine, glutamate, glutamine and aspartate) under acute stress. It is particularly interesting as all the predominant amino acids content increased, especially glutamine (by 338%), under chronic stress.

Branched chain and aromatic amino acids were present only in small quantities in *S. fusiforme*. Interestingly, aromatic amino acids content increased (by 23%, Table 1) under acute stress, in contrast, which decreased by 22% under chronic stress. In more detail, the content of tyrosine and tryptophan both increased by approximately 50% except that of phenylalanine, decreased by 14% under acute stress, while minor changes or a slight decrease were observed for aromatic acids under chronic stress, indicating a quite different responding pattern to these two Cu stress in *S. fusiforme*.

Aromatic amino acids are synthesized from the common precursor metabolite chorismate, which originates from the shikimate pathway as shown in Fig. 3 [32], whose importance was demonstrated by the fact that 20% of the carbon fixed by plants flows through it under normal growth conditions [33]. Presence of the shikimate pathway in macroalgae has been experimentally verified only in green and red algae, as well as in the diatom *Thalassiosira pseudonana* [34]. However, the reduction in phlorotannin content and mortality in *Fucus vesiculosus* caused by glyphosate indicated the existence of the shikimate pathway in brown algae [35]. Moreover, little is known about the influence of heavy metal stress on enzymes involved in the shikimate pathway [33], especially in algae. Surprisingly, our results indicated an enhancement of the shikimate pathway under acute but not chronic Cu stress. In pepper, due to the much more accumulated Cu in the roots than in the aerial parts, the induction of shikimate dehydrogenase (SKDH), which is the enzyme that catalyses the fourth step in the shikimate pathway, only existed in the hypocotyl [33]. It seems that the amount we used were both not high enough to inhibit the activity of this enzyme. However, influences of different concentrations of Cu on other enzymes involved in the shikimate pathway are also unclear. One hypothesis could be that more other enzymes were induced under acute than chronic Cu stress. Moreover, phenylalanine is required for the synthesis of various phenolic compounds that play important roles in non-enzymatic antioxidant defense processes. This may explain the decrease in the content of phenylalanine under both Cu stress conditions.

Different nitrogen assimilation patterns

Nitrate assimilation is an apparently simple process in photosynthetic eukaryotes, involving two transports and two reduction steps to produce ammonium in the chloroplast [36], within which NR is a key enzyme that catalyzes the first, also the rate-limiting step in the reduction of nitrate to ammonium [37]. After a short exposure to Cu (1 day), a significant decrease in the NR activation state was observed (Fig. 4I). However, after 7 days of Cu treatment, the NR activation state in thalli was found to be approximate to that in untreated thalli (Fig. 4J). The strong inhibition of NR activity by acute Cu exposure in *S. fusiforme* was entirely in agreement with results obtained with other organisms [38,39]. Furthermore, excessive Cu causes a drastic change in nitrogen metabolism affecting also other enzymes involved in nitrate reduction and amino acid metabolism and leading to diminution of total nitrogen [40]. As a result, the levels of primary amino acid products of nitrogen assimilation (glutamine and glutamate) were reduced.

During chronic Cu exposure, the total content of glutamate and glutamine, especially glutamine in *S. fusiforme* even increased comparing to the control (Table 1), meanwhile the activity of NR was little reduced (Fig. 4). Though excessive cadmium could significantly inhibit activities of glutamate dehydrogenase (GDH), glutamine synthetase (GS) and glutamine oxoglutarate aminotransferase (GOGAT) [41], through which ammonium is further incorporated into the amino acids [42,43], data concerning the inhibitory effect of excess Cu are scarce [44]. It is reported that GS appeared highly increased in the model brown alga *Ectocarpus siliculosus* exposed to 50 mg L⁻¹ of Cu after 10 days [45]. Consequently, it may suggest that GS be strongly activated in *S. fusiforme*, though whose activity was not determined in the present study, resulting in the dramatic increase in glutamine content. The ratio of glutamine to glutamate has been proposed as an indicator of nitrogen status [46], which was raised in thalli under both acute and chronic Cu stress, by approximately 55% and 200% (Table 1),

respectively, showing great variations where the latter was almost 4 times as the former.

Physiological responses

The algae were cultivated with increasing concentrations of Cu, which would trigger the synthesis of ROS that led to lipids peroxidation [47]. In general, increases in peroxidase activity are regarded as a reliable indicator of stress or potential phytotoxicity of heavy metals, the increase in peroxidase activity being a response to an increase in peroxides [48]. As a product of lipid peroxidation that accumulates greatly following heavy metal exposure, MDA is an indicator of lipid peroxidation. The algae responded similarly in the content of MDA to both acute and chronic Cu stress, resulted in 254% and 193% increase, respectively (Fig. 4A and B).

SOD has been called the cell's first line of defense against ROS that catalyzes the disproportionation of O_2^- to O_2 and H_2O_2 [49]. Generally speaking, peroxidative stress triggers higher level of MDA or lower level of SOD or both. In *S. fusiforme*, SOD activity was activated, a little increased by 34% and 28% (Fig. 4C and D), respectively, under acute and chronic Cu exposure, consistent with the result reported [47], which was also coinciding with increases in MDA content (Fig. 4A and B).

CAT is another important ROS-scavenging enzyme associated with antioxidant stress in algae, which catalyzes the production of H_2O and O_2 from the degradation of H_2O_2 in cytosol and peroxisomes [50]. It is reported that at high concentrations (above 20 μ M), Cu might be responsible for the inhibition of CAT, resulting in an insufficient ROS detoxification with enhanced H_2O_2 accumulation and lipid peroxidation [51]. Furthermore, inhibition of CAT activity may be caused by increased levels of O_2^- [52]. However, the activity of CAT declined significantly by 74% in thalli under chronic stress (Fig. 4F), while surprisingly only by 30% in that under acute stress (Fig. 4E). In addition, proper levels of Cu might lead to an increase in activity of CAT to cope with Cu stress [53]. As expected, CAT activity was activated and significantly increased in thalli under 8 μ M Cu treatment after 1 day in our study (data not shown). Thus, it is plausible that the only explanation might be that O_2^- accumulated under chronic Cu exposure was more than that under acute treatment. In other word, it is the long-term exposure, rather than the high levels, as the main reason that led to the more accumulated O_2^- . In another aspect, the results above also suggested CAT as a more sensitive antioxidant enzyme than SOD, in agreement with other studies [47].

Besides SOD, activity of POD was also up-regulated in our experiment by both treatments, where chronic stress induced more activation of POD activity than acute stress (by 118% and 47%, respectively, Fig. 4H and G).

Mannitol as the main product of photosynthesis

ROS were formed either after acute or chronic heavy metal exposure, where the former abruptly generated into high levels that exceeded the ability of the antioxidant system to cope with them, while the latter increased steadily, resulting in different levels of damage to cellular compounds [7].

Mannitol is almost universally present in brown algae, being the main product of photosynthesis instead of sucrose [54], which may also function as carbohydrate storage, translocatable assimilate, source of reducing power, osmoregulator and/or antioxidant [55]. Changes in the mannitol content of marine brown algae have been reported in many field-based studies except that of heavy metal [56]. Based on the visual inspection, mannitol is the most abundant metabolite in the NMR spectrum from tissues of *S.*

fusiforme (data not shown). The concentration of mannitol decreased strongly by 72% (Table 2) in algae under acute stress, while only by 14% (Table 2) in that under chronic stress.

A mannitol cycle has been proposed in a number of organisms, including micro and macroalgae, where the latter is essentially the same as the fungal cycle [55]. In some yeasts, Cu^{2+} supplementation activates mannitol dehydrogenase involved in the biosynthesis of mannitol, resulting in an increased mannitol production. However, little is known about the affection of Cu on these enzymes involved in the metabolism of mannitol, especially in brown algae.

At the cell membrane, Cu may interfere with cell permeability [57,58]. In the present study excess Cu treatment caused much mannitol lost in the cell of *S. fusiforme*, indicating an enormous increase in permeability to it. In another aspect, as a compatible solute, mannitol is frequently used as a scavenger of hydroxyl radicals *in vitro* [59] and *in vivo* [60]. It may be involved in the cellular ROS-scavenging system to detoxify the oxidative stress. Therefore, it seems that in the long-term adaptation to low-concentration Cu stress of this algae, no significant differences were observed in the cell permeability, resulting in a little reduction in the content of mannitol.

Malate and aspartate may play important roles

Similar to C_4 plants, malate and aspartate were accumulated as candidates for the organic store in *Fucus* spp. that has a quite close phylogenetic relationship with *Sargassum* spp., both of which belong to Fucales [61]. Additionally, C_3 - and CAM-like photosynthesis were also observed in this species [61]. This coexistence of different photosynthetic pathways may be normal in aquatic environment [62], e.g., the both C_3 and C_4 photosynthetic pathways involved in the green-tide-forming alga, *Ulva prolifera* [63–65]. Anyway, malate and aspartate may play important roles in photosynthesis. As a potent inhibitor of photosynthesis, Cu dramatically reduced the levels of malate and aspartate in acute Cu treated *S. fusiforme*, where the former was even more strongly reduced (Table 1 and 2). However, content of aspartate was surprisingly increased by nearly 30% when under chronic Cu stress (Table 1). Moreover, malate can function as a vacuolar osmolyte and may also serve as an additional sink for carbon assimilation and reducing equivalents [66].

Aspartate was found to be the most abundant amino acid, which is in line with the results previously described [67]. More than half of the content was D-aspartate, whose cellular localization was also confirmed in *S. fusiforme* [68]. It was proposed that D-aspartate may play an important role in the growth of *S. fusiforme*, as well as in both germination and growth of higher plants [68,69]. However, the two isomers of aspartate were not elucidated by the methods of our study. To gain deeper insight into the biological role, especially under Cu stress, further studies, for example, the influences of Cu on the levels of both isomers and genes involved in their metabolism, will be required.

Metabolites involved in choline metabolism

Though the quaternary ammonium compound choline, which is the major component of membrane lipids in eukaryotes [70], was not detected in the present study, its precursor o-phosphocholine was found to be decreasing in its content under Cu stress. The metabolic pathway related to choline is summarized in Fig. 5. Significant reductions to a similar extent were observed for phosphocholine, dimethylglycine and glycine in *S. fusiforme* under acute Cu stress, while a quite different metabolic pattern was observed for chronic stress, where trimethylamine showed the opposite behavior and increased 2.3-fold over the control. The

Table 1. Concentrations of amino acids and related components.

$\mu\text{mol g}^{-1}$	Acute stress			Chronic stress		
	Control	Treated	Percentage	Control	Treated	Percentage
Alanine	0.2743	0.3557±0.0427*	130%±16%	0.2096	0.2978±0.0428*	142%±20%
Glutamate	0.6674	0.4692±0.0735*	70%±11%	0.4509	0.4907±0.0888	109%±20%
Glutamine	0.1216	0.1324±0.0412	109%±34%	0.0177	0.0598±0.0240*	338%±136%
Aspartate	0.8634	0.5878±0.0763*	68%±9%	0.5140	0.6744±0.0851*	131%±17%
Tryptophan	0.0237	0.0335±0.0024*	141%±10%	0.0351	0.0369±0.0079	105%±23%
Tyrosine	0.0382	0.0626±0.0082*	164%±21%	0.0332	0.0310±0.0126	93%±38%
Phenylalanine	0.0556	0.0480±0.0072	86%±13%	0.0835	0.0655±0.0142	78%±17%
Glycine	0.2329	0.1175±0.0380*	50%±16%	0.2029	0.1211±0.0141*	60%±7%
Isoleucine	0.0325	0.0200±0.0073	62%±22%	0.0266	0.0205±0.0023*	77%±9%
Leucine	0.0238	0.0138±0.0048*	58%±20%	0.0068	0.0095±0.0041	140%±60%
Valine	0.0439	0.0458±0.0061	104%±14%	0.0241	0.0212±0.0022*	88%±9%
N,N-Dimethylglycine	0.0444	0.0226±0.0040*	51%±9%	0.0469	0.0080±0.0019*	17%±4%

The absolute concentration of components in the control condition is shown the first column of each treatment, followed by the absolute concentration of each treatment and the relative changes compared to each control. Asterisk indicates significant differences between the stress and its control condition ($P<0.05$).
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function of trimethylamine in various maritime plants may be related to the common saline habitat, possibly in osmotic regulation or in the transport of ions across membranes [71], i.e. a kind of osmolytes, which principally are sugars, polyhydric alcohols, amino acids and their derivatives, and methylamines, and all are known to be protein stabilizers. This observation suggests trimethylamine be as the preferred osmoprotectant in *S. fusiforme* under chronic Cu stress, rather than dimethylglycine.

Another most common and widely distributed compatible osmolyte proline was only detected by GC-TOF MS (Fig. 2), but not NMR. In plants, proline is synthesized mainly from glutamate [72]. They showed similar behavior that without significant accumulations in the level to chronic Cu stress (Table 1 and Fig. 2).

However, significant reductions were observed in *S. fusiforme* under acute Cu stress (Table 1 and Fig. 2). As intracellular proline levels are determined by biosynthesis, catabolism and transport between cells and different cellular compartments, we hypothesized that, dissimilar to other organisms, *S. fusiforme* may not use proline first as an osmoprotectant under Cu stress.

Ascorbate: an important antioxidant component

The concentrations of all the other detected polyols, organic acids and sugars were decreased, ranging from 20% for myo-inositol to 49% for citrate (Table 2), especially ascorbate and lactate (90% and 79%, respectively), where the former is considered as the main antioxidants in many plants. Protective

Table 2. Concentrations of organic acids, sugars, polyols and related components.

$\mu\text{mol g}^{-1}$	Acute stress			Chronic stress		
	Control	Treated	Percentage	Control	Treated	Percentage
Mannitol	19.7659	5.4364±1.2094*	28%±6%	19.9864	17.2164±1.1147*	86%±6%
Malate	0.1094	0.0275±0.0105*	25%±10%	0.0434	0.0261±0.0053*	60%±12%
myo-Inositol	0.1238	0.0986±0.0154	80%±12%	0.0803	0.0447±0.0110*	56%±14%
Citrate	0.4010	0.2056±0.0144*	51%±4%	0.2269	0.1915±0.0316	84%±14%
Xylose	0.1538	0.0997±0.0298	65%±19%	0.2121	0.1254±0.0293*	59%±14%
Succinate	0.0571	0.0302±0.0062*	53%±11%	0.0897	0.0520±0.0032*	58%±4%
Ascorbate	0.1087	0.0110±0.0061*	10%±6%	0.0720	0.0300±0.0064*	42%±9%
Lactate	0.3113	0.0652±0.0130*	21%±4%	0.0314	0.0357±0.0038	114%±12%
Trimethylamine N-oxide	0.2342	0.1628±0.0603*	70%±26%	0.2293	0.2147±0.0313	94%±14%
Trimethylamine	0.0070	0.0052±0.0011	74%±16%	0.0030	0.0069±0.0013*	230%±43%
Carnitine	0.0385	0.0297±0.0078*	77%±20%	0.0335	0.0318±0.0049	95%±15%
Acetate	0.0336	0.0215±0.0018*	64%±5%	0.0296	0.0269±0.0037	91%±13%
O-Phosphocholine	0.0347	0.0184±0.0055*	53%±16%	0.0363	0.0324±0.0051	89%±14%

The absolute concentration of components in the control condition is shown the first column of each treatment, followed by the absolute concentration of each treatment and the relative changes compared to each control. Asterisk indicates significant differences between the stress and its control condition ($P<0.05$).
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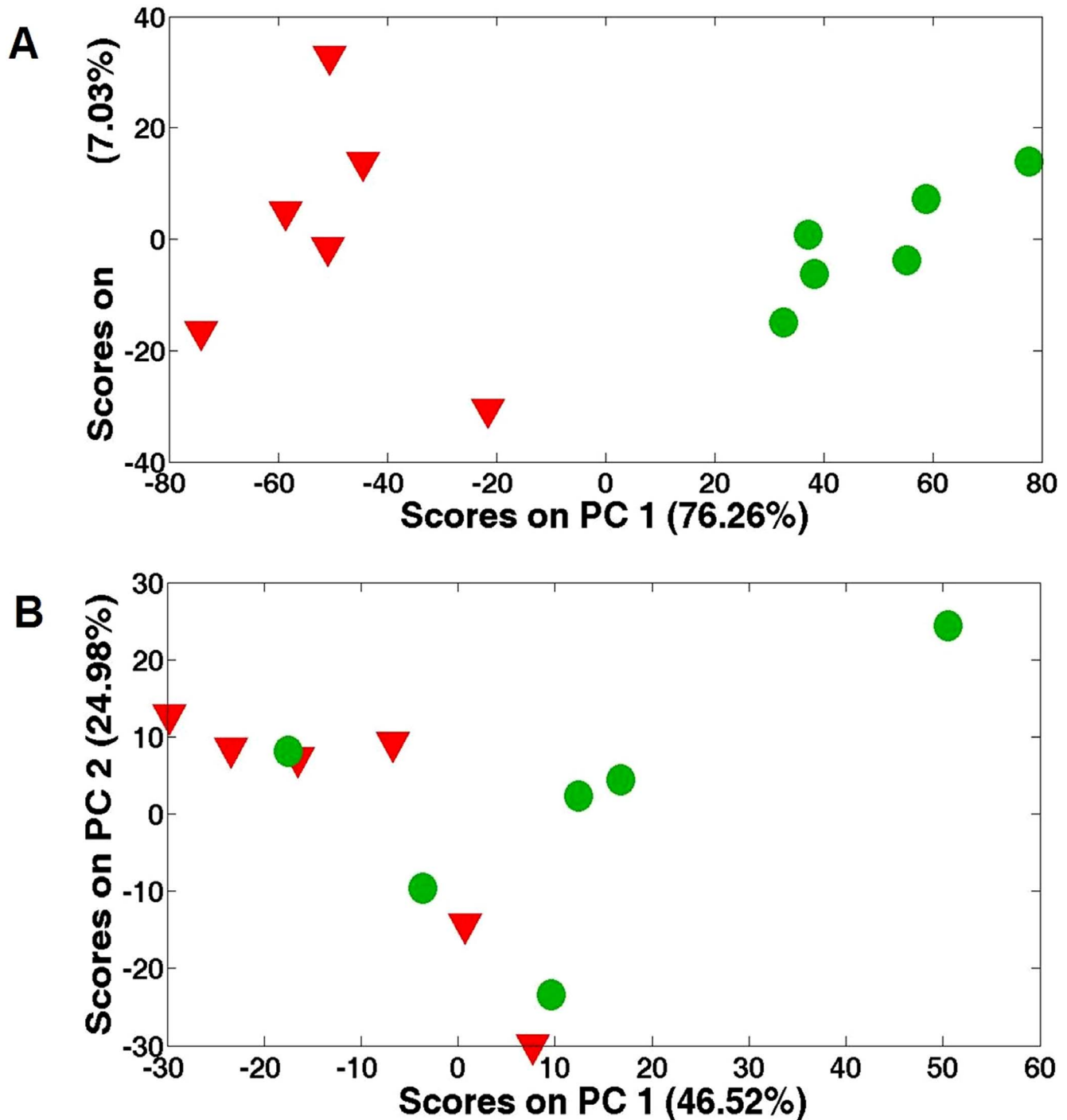


Figure 1. PCA score plots. (A) from the analysis of the 1D ^1H NMR spectra of *Sargassum fusiforme* tissue extracts from high concentration copper-treated ($47\ \mu\text{M}\ \text{Cu}^{2+}$) group after exposure for 1 day; (B) from the analysis of the 1D ^1H NMR spectra of *Sargassum fusiforme* tissue extracts from low concentration copper-treated ($8\ \mu\text{M}\ \text{Cu}^{2+}$) group after exposure for 7 days. doi:10.1371/journal.pone.0101960.g001

mechanisms in photosynthetic organisms do not only include ROS enzymes that reduce oxidative stress either with or without the aid of antioxidants but also antioxidants themselves [73].

In the brown algae *Scytosiphon lomentaria*, accumulation of ascorbate was detected in thalli from the Cu-enriched area [74]. In contrast, ascorbate content rapidly decreased and remained low in *Ulva compressa* (Chlorophyta) exposed to excess Cu [75]. Additionally, a low level of ascorbate was also observed in *U.*

compressa collected in Cu-enriched environments, indicating that short-term responses induced by excess Cu were similar to long-term responses occurring in the level of ascorbate [76]. Rapid reduction (almost 90%) of ascorbate was caused by acute Cu exposure in *S. fusiforme* in this study. Though to a relatively smaller extent, the content of ascorbate in thalli decreased by approximately 58% after chronic Cu exposure as compared to the control.

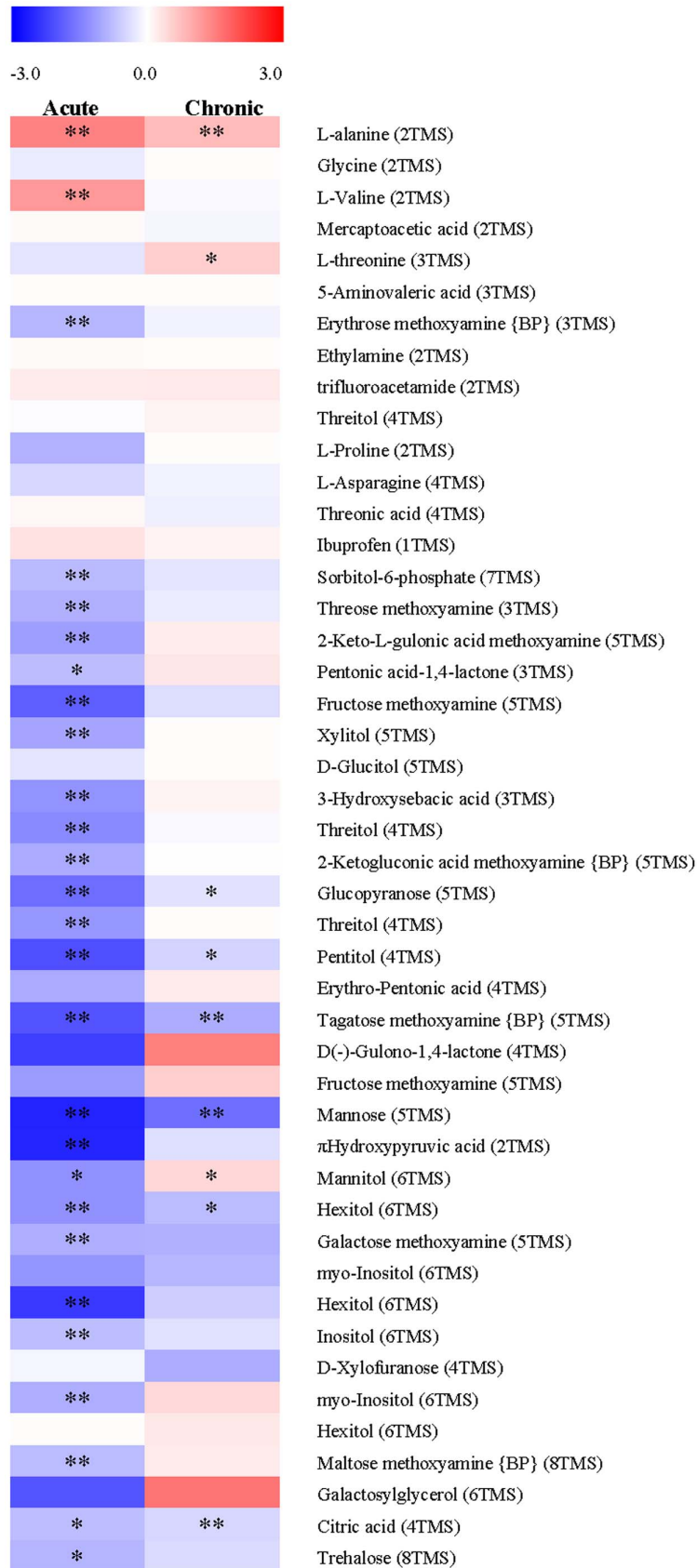


Figure 2. Heat-map of metabolic changes in *Sargassum fusiforme* under acute and chronic Cu stress. Intensity of colors represents log₂-transformed ratios of measured means (n=6) analyte's intensity to its respective mean value in the control conditions. Asterisks mark t-test P-value, where "***" marks P<0.01 and "*" marks P<0.05. doi:10.1371/journal.pone.0101960.g002

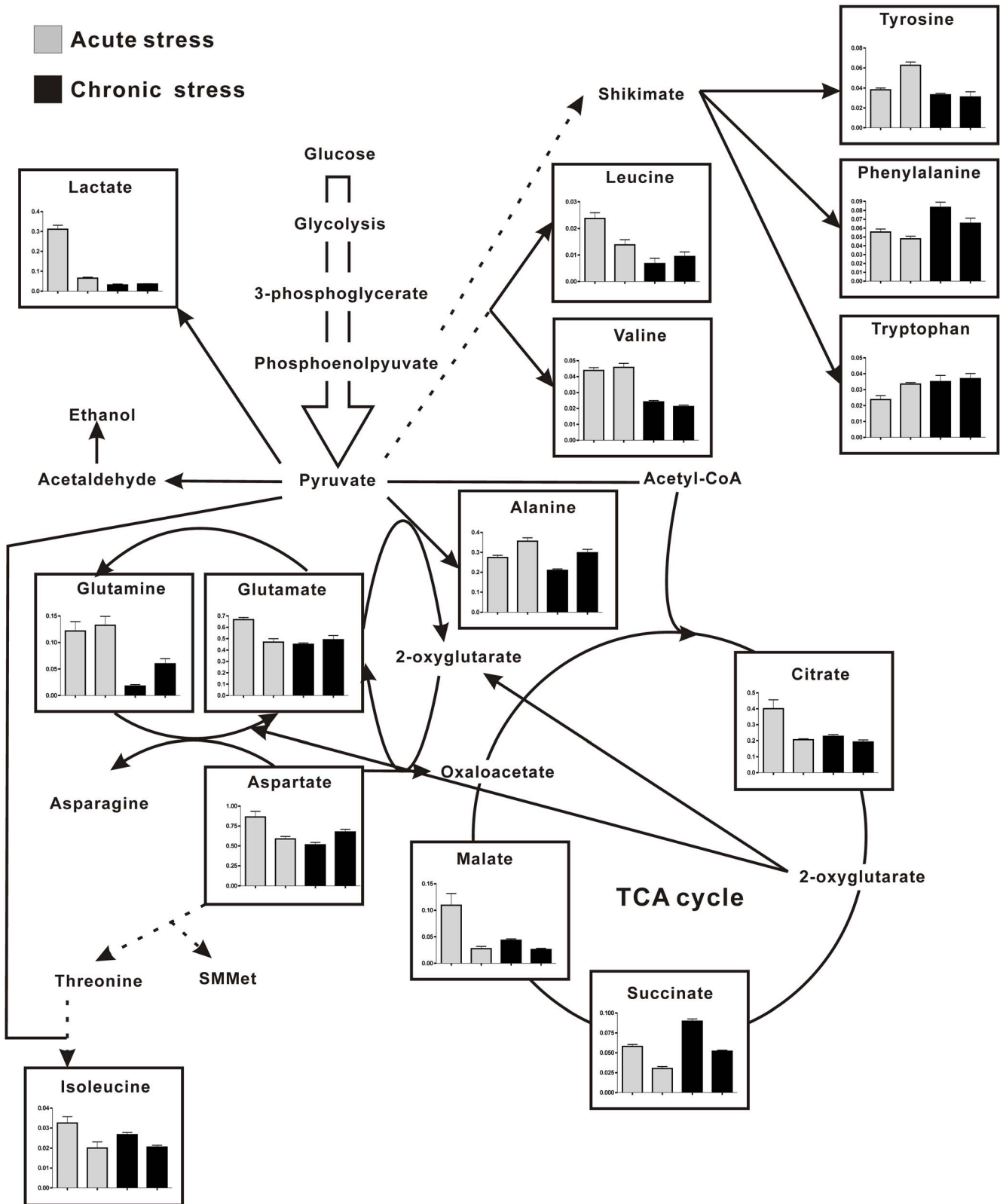


Figure 3. Pathway diagram with bar graphs representing relative metabolite abundance under acute and chronic Cu treatments. The first bar represents the control followed by the Cu treatment of the second bar. doi:10.1371/journal.pone.0101960.g003

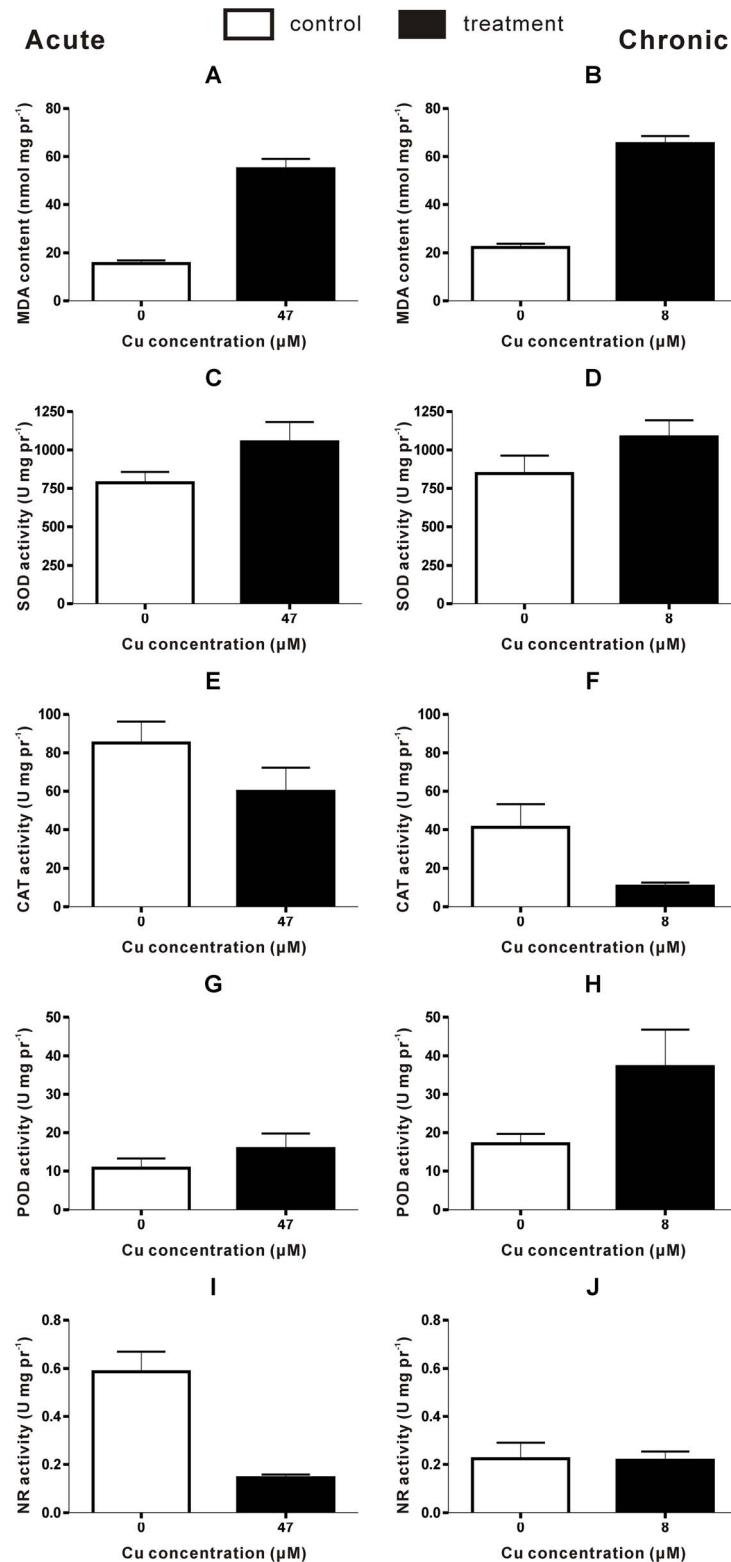


Figure 4. MDA content, antioxidant enzyme activities in *Sargassum fusiforme* under acute and chronic copper stress. Bars represent mean values of independent replicates ± 1 SD ($n=4$ or 5). doi:10.1371/journal.pone.0101960.g004

Oxidative stress can be mitigated by the synthesis of antioxidant component ascorbate which directly reduces ROS [23]. It is of particular interest as an electron donor for $\bullet\text{OH}$ radicals and also

as a substrate for ascorbate peroxidase (APX). An increase in ascorbate level was absent in *S. fusiforme*, similar to that in *U. compressa* that may be due to a direct oxidation of the newly

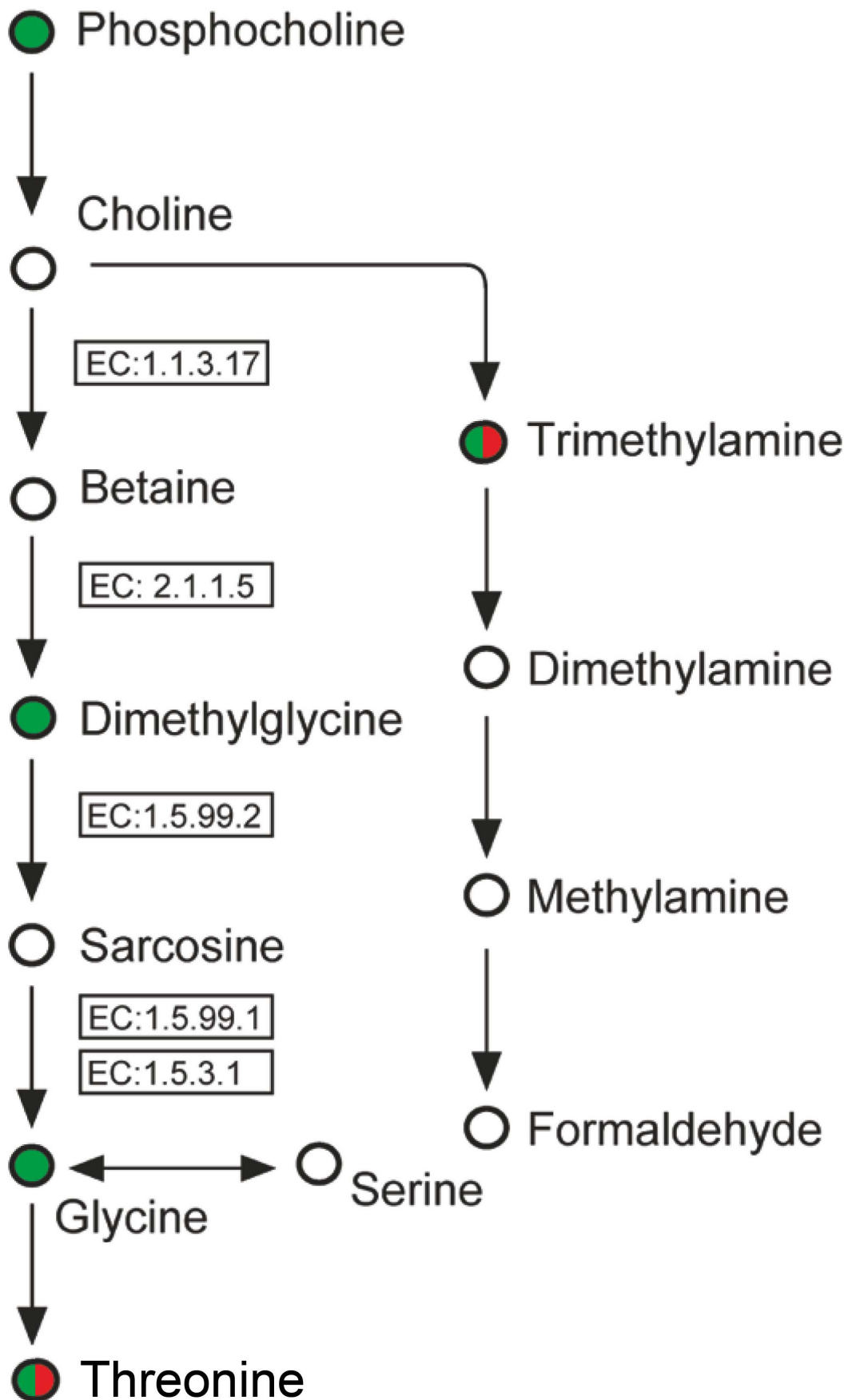


Figure 5. Schematic representation of metabolites related to choline. Identified metabolites from ^1H NMR spectroscopy are colored, increased metabolites are shown in red, and decreased metabolites are in green. The left and right half circles represent changes in level of each component under acute and chronic Cu stress, respectively. Key enzymes in choline catabolism include EC 1.1.3.17, choline oxidase; EC 2.1.1.5, betaine-homocysteine methyltransferase; EC 1.5.99.2, demethylglycine dehydrogenase; EC 1.5.3.1, sarcosine oxidase; and EC 1.5.99.1, choline oxidase. doi:10.1371/journal.pone.0101960.g005

synthesized ascorbate by Cu-induced ROS and/or to the activation of the antioxidant enzyme APX [77]. Cu^{2+} primarily triggers oxidative stress in the chloroplast, in which no active transport of ascorbate has been reported [78]. Therefore, reduced ascorbate was likely to be regenerated by the ascorbate/GSH cycle. As a result, rapid oxidation of ascorbate was provoked by acute Cu stress, which would abruptly generate high levels of ROS over a short period that usually exceed the total antioxidant capacity of algae. Furthermore, it is reported that the decrease in ascorbate availability would as a result limit not only APX but also all peroxidase activity [78]. This to some extent explained the less activation in POD activity in thalli under acute Cu exposure in this study.

Conclusions

In conclusion, we present metabolic profiles observed for *S. fusiforme* under both acute and chronic Cu exposures. In order to

identify as many metabolites as possible, which is also the goal of untargeted metabolomics experiments, ^1H NMR and GC-TOF MS were used complementally in the present study. Number of metabolites observed by MS platform was several times as that of NMR in this study, as NMR is generally considered to be less sensitive than GC-MS. These platforms would help expand our understanding of biological mechanisms related to environmental perturbations. Our results demonstrated different patterns of the marine brown algae *S. fusiforme* to acute and chronic Cu exposures in both physiological responses and regulation of metabolic pathways.

Author Contributions

Conceived and designed the experiments: HXZ QYP XFY. Performed the experiments: QYP AQZ LDL YQL LML QQW. Analyzed the data: HXZ QYP NL. Contributed reagents/materials/analysis tools: NL. Contributed to the writing of the manuscript: HXZ XFY.

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