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Abstract

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Keywords

proteins, binding, copper, microalgae, expression, marine, compare, imac, chromatography, affinity, metal, immobilised, exposed, control, CMMB

Disciplines

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The use of immobilised metal affinity chromatography (IMAC) to compare expression of copper-binding proteins in control and copper exposed marine microalgae

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Abstract:

Toxicity of metals to aquatic organisms is dependent on both external factors, such as exposure concentration and water quality parameters, and intracellular processes including specific metal-binding sites and detoxification. Current models used to predict copper toxicity in microalgae do not adequately consider these intracellular processes. This study compared the copper-binding proteins from four species of marine microalgae, *Dunaliella tertiolecta*, *Tetraselmis* sp., *Phaedactylum tricornutum* and *Ceratoneis closterium,* in controls (no added copper) and following a 72-h exposure to copper (sufficient to inhibit growth by approximately 50 %). Cells were lysed by sonication, which was optimised to obtain 54–94 % cell rupture for the different algae. Cell lysates were processed by immobilised metal affinity chromatography (IMAC) using Cu^{2+} as the bound metal (i.e. Cu-IMAC). Bound proteins were subsequently analysed by SDS-PAGE, comparing proteins recovered from algae that were exposed to copper versus untreated control cells. Individual proteins for which copper exposure resulted in changes to proteins present were excised from gels and further analysed by nano LC ESI-MS/MS; proteins were identified using the Mascot database. Proteins identified in this way included heat-shock proteins, rubisco, α- and β-tubulins and ATP synthase (β subunit). The results established that Cu-IMAC is a useful approach to identify proteins involved in copper binding in algae. This study identified several proteins that may play an active role in responses to copper toxicity in marine microalgae.

Keywords: Intracellular copper, Metalloprotein, Algae, 72-h bioassay, Sonication, SDS-PAGE

Introduction:

Copper is an essential trace nutrient that is an important component of many proteins and enzymes, however, even micronutrients become toxic at elevated concentrations [1]. Over the last century, the increasing use of copper in industrial activities (e.g. antifouling paints, algicide, fertilizer and pesticides [2]) and its presence in mining by-products, have seen an increase in the amount of copper being released into the aquatic environment [3].

Marine microalgae (in combination with other phytoplankton) are responsible for almost 50 % of the planet's photosynthesis (CO_2 fixation), as well as a number of other critical processes, such as nitrogen fixation and phosphorus metabolism [4]. Microalgae are among the most sensitive species to copper [5]. It is, therefore, important to understand how and why copper affects marine microalgae, with subsequent direct and indirect effects on higher trophic levels.

At present, metal toxicity is generally predicted by models including the gill-surface interaction model (GSIM) [6] in fish, the free ion activity model (FIAM) [7,8] and the biotic ligand model (BLM) [9,10]. These models are based on a limited understanding of the mechanisms of metal toxicity. For example, the BLM assumes that the metal-ligand interaction does not alter the biotic ligand itself (in this case, the algal cell membrane) and does not provide any insight into the nature of the ligand. Thus, such models might not always accurately predict metal toxicity in different environments. Therefore, there is a need to identify the intracellular ligands with which metals interact in aquatic organisms to better understand the mechanisms of metal toxicity and to improve the prediction of toxicity. In the cell cytosol, copper is quickly bound by chaperones and transporter proteins with an affinity for copper [11]; like other essential metals, copper is tightly regulated within the cell and is actively transported to organelles where it plays a role in cellular function (e.g. it is an important co-factor in many enzymes, including the antioxidant enzyme, superoxide dismutase [12]). Alternatively, the copper can be sequestered in granules [11,13]. Adams et al. (unpublished) showed using synchrotron data that the location of copper in an algal cell is highly correlated with that of sulfur and phosphorus. Sulfur becomes incorporated into proteins within the amino acids cysteine and methionine. Non-protein sources of sulfur include glutathione (which constitutes the highest non-protein thiol within a cell) and phytochelatins [14]. Intracellular metals may be accumulated in specific organelles within the cell, e.g. subcellular fractionation of algal cells showed that cadmium was predominately in the organelle and the heat stable protein fractions [15]. This heat stable protein fraction contains the plant defence ligands, phytochelatins, which are known to bind metals and sequester them into a less bioavailable form [13]. The fact that copper enters the cytosol first, prior to being bound by ligands and redistribution in the cell, suggests that it is appropriate to target copper-ligand binding in the cytoplasm. The aim of this study therefore was to separate and where possible identify proteins to which copper binds in algal cells.

Immobilised metal affinity chromatography (IMAC) as a technique for purification of biomolecules was first presented by Porath et al. in 1975 [16]. They proposed that the affinity of proteins for metals could be used as a purification technique. The IMAC column retains

proteins based on the interactions with the metal ions on the surface with a high specificity, allowing quick easy purification of even complex biological samples [17].

Copper (II) under the HSAB theory (hard soft acid base theory) is defined as a borderline acid [18,19] meaning that it can form strong bonds to soft bases such as sulfur as well as harder ligands such as aromatic nitrogens, other nitrogens and oxygen. Cu(II) has shown a preference for N and O binding partners [20], amongst the amino acids, histidine in particular is a major binding partner [20,21]. The binding of proteins to Cu-charged IMAC columns is therefore dominated by histidine residues. There exists a positive correlation between the amount of accessible histidines and the strength of binding. Typically one histidine is enough for weak binding to an IDA-Cu(II) complex (iminodiacetic acid, the stationary phase used in the IMAC columns in this study) [21].

IMAC can detect copper induced conformational changes in the proteins as their ability to bind to the column may change [22]. IMAC can be used to determine changes in metal-binding proteins and concentrations as a result of metal exposure. Metal-binding proteins may be altered when an over-abundance of metal is available in the cell, arising from exposure to elevated environmental metal concentrations.

IMAC (in particular Cu-IMAC) has been applied in a variety of applications including: isolating copper complexing ligands from natural waters [23-25] and identifying copper-binding ligands produced by various organisms including: marine bacteria [23,25], human hepatocytes [26], plants [27,28] and a marine cyanobacterium [29]. These studies have used IMAC to examine a variety of proteins from small peptides (< 2 kDa) [28,27,24] to larger proteins (up to ~50 kDa) [25,29]. Barnett et al. [29] investigated metal-binding proteins from a marine cyanobacterium (*Synechococcus* sp.) using different metal-IMAC columns (Ni, Fe, and Co) and found that while metal-binding proteins varied for each metal one oxidative stress protein, rubrerythrin was common to all.

In the current study, Cu-IMAC and SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) were used to identify differences in the concentrations of copper-binding proteins recovered from lysates prepared from each of four species of marine microalgae, exposed for 72 h to either no copper or to their respective IC_{50} copper concentrations. The four species (and their respective 72-h IC_{50} copper concentrations) were: *Dunaliella tertiolecta* (Butcher) (530 µg Cu/L), *Tetraselmis* sp. (50 µg Cu/L), *Ceratoneis closterium* Enrenb. (Ehrenberg, 1839b:157) (formerly known as *Cylindrotheca closterium* and *Nitzschia closterium*) (18 µg Cu/L) and *Phaeodactylum tricornutum* Bohlin (8 µg Cu/L) [30,5]. A working hypothesis was that algae that were more sensitive to copper would exhibit greater changes in expression levels of the copper binding proteins after a 72-h copper exposure than the less sensitive algae.

Experimental Methods

Algal culture and sample preparation

Axenic algal cultures were originally obtained from the CSIRO Collection of Living Microalgae, Marine and Atmospheric Research (Hobart, Australia). Algae were maintained in autoclaved f_2 media (half strength f media), except for *C. closterium* which was cultured in full strength f media [31]. Algae were kept at 21 °C, on 12:12 hour light:dark cycle at 110 µmol photons.$m^{-2}.s^{-1}$ in a controlled environment incubation chamber (Labec, Australia). Algae were transferred into fresh media weekly. Culture flasks were agitated and randomly rotated within the incubator to promote gas exchange and to allow consistent light exposure. Copper exposure bioassays were conducted in silanised glassware to minimise copper losses to the glass walls of the flasks. Test media consisted of filtered sterilised seawater (0.2 µm PES (polyethersulfone) membrane, bottle top filters, Sartorius, Australia) supplemented with 15 mg $NO_3^-.l^{-1}$ and 1.5 mg $PO_4^{3-}.l^{-1}$ (added as $NaNO_3$ and NaH_2PO_4, respectively). Copper was added as copper sulfate to each flask (except controls) to achieve the respective IC_{50} concentrations [5].

The density of algal cultures was determined by flow cytometry (LSR II, Becton Dickinson, Australia) using TruCount™ (BD Biosciences) beads as an internal reference standard. Algal cell chlorophyll a autofluorescence was measured by exciting at 488 nm and capturing the emission passing through a 685 long-pass barrier filter followed by a 695/40 band-pass filter. Data was acquired using FACSDiva software (V4.0, Becton Dickinson, Australia) and cell densities calculated as per Franklin et al. [32]. All samples were mixed by vortexing immediately prior to analysis to minimise algal settling and clumping.

Method optimisation

Several stages of the method needed to be optimised for application to marine algal cells. To liberate the proteins from the cells, cells were lysed by sonication. Sonication has been shown to be highly efficient at phytoplankton cell breakage [15,29]; it produces less metal contamination than other procedures such as freeze grinding and bead beating [29], and it avoids the addition of other chemicals such as enzymes and detergents that could also contaminate the sample. The main disadvantage of sonication is that the temperature of the sample may increase resulting in denaturation of some heat sensitive proteins. Thus, the optimal sonication parameters that allowed maximum cell disruption whilst avoiding heating and minimising detectable organelle disruption, were determined for each species.

After 72-h growth, 5-10 x 10^8 cells were concentrated by centrifugation (1000 x g, 5 min) and resuspended in 9 ml of 20 mM tris(hydroxymethyl)aminomethane-HCl salt (Tris) buffer (pH 7.5) containing a protease inhibitor (Complete™ EDTA free protease inhibitor cocktail tablets, Roche, Germany) [29]. Sample tubes (3 x 3 ml aliquots) were suspended in ice and treated with a 400 W digital sonifer (Branson) at amplitude 10% with a pulse cycle of 0.5 s ON and 1.0 s OFF for 5 x 30 s (*Tetraselmis* sp.) or 2 x 30 s (*P. tricornutum*, *D. tertiolecta* and *C. closterium*). A pause of 5 minutes between sonication treatments prevented the samples

from overheating. *Tetraselmis* sp. proved to be particularly resistant to sonication, and required a 45 min osmotic treatment with ultra-pure water prior to sonication to achieve satisfactory cell lysis. These parameters achieved cell lysis of 54±8 %, 80±10 %, 85±10 % and 94±2 % for *Tetraselmis* sp., *P. tricornutum*, *C. closterium* and *D. tertiolecta*, respectively. Cell damage was assessed by monitoring citrate synthase activity (normally contained in the mitochondrial matrix) before and after lysis [33,34]. After sonication cell lysates were recentrifuged at 4 °C (12000 x g, 5 min). The supernatant was filtered (0.45 µm, surfactant-free cellulose acetate membrane, Sartorius) prior to isolation of the copper-binding proteins and stored at 4 °C until analysis (for up to 2 h).

Optimisation of IMAC

Fast protein liquid chromatography (FPLC, Akta Explorer) and HiTrap© Chelating columns (1 or 5 ml GE Healthcare) charged with Cu^{2+} (as copper sulfate) were used to isolate copper-binding proteins from the samples. Columns were washed with at least five column volumes of 20 mM Tris buffer (pH 7.5, 0.2 µm filtered, pH adjusted with KOH). The algal lysate (coloured) was loaded using a syringe and washed with a minimum five column volumes of the Tris buffer until the eluate was clear. Bound proteins were then eluted with three to five column volumes of the elution buffer (200 mM imidazole in 20 mM Tris, pH 7.5 adjusted using HNO_3). The fraction containing the eluted proteins was collected and stored on ice for a maximum of 2 h prior to dialysis and electrophoresis. Although degradation of proteins cannot be discounted a number of steps were implemented to reduce degradation as much as possible: proteins were kept on ice; the procedure prior to IMAC separation was performed as rapidly as possible and the protease inhibitor was added prior to sonication.

Initial SDS-PAGE analysis of IMAC eluted proteins produced an unresolved smear with no distinct protein bands discernible necessitating further sample clean-up. An additional step was included in which membrane-bound proteins were removed by washing the IMAC column with detergent after the algal cell lysate had been loaded onto the column. Triton X-100 was passed through the column, and the column was subsequently washed with twenty column volumes of loading buffer (20 mM Tris pH 7.5). The remaining bound proteins were eluted from the column using the elution buffer.

The imidazole was subsequently removed by dialysis to allow quick estimation of the protein content via an absorbance measurement at 280 nm using an arbitrary extinction coefficient ($\varepsilon^{0.1\%}(280) = 1.0$). The sample was placed in 3.5 kDa molecular weight cutoff (MWCO) dialysis tubing (Snakeskin® pleated dialysis tubing, Thermo scientific) and left in 1 l of 20 mM Tris buffer pH 7.5 with stirring at 4 °C in the dark for approximately 48 h, with a buffer change after 24 h. If required, samples were concentrated (at 4 °C) using small ultrafiltration devices with a 5-kDa MWCO (Vivaspin500, GE Life Sciences) and the protein concentration estimated as above.

The cell density required for Cu-IMAC was also optimised. Unconcentrated eluted protein typically yielded <0.2 mg protein/ml (estimated by A280 nm in a quartz micro-cuvette) from 5 to 10 x 10^8 cells and was concentrated to ≥3 mg protein/ml to facilitate subsequent analysis

by SDS-PAGE. The two smaller brown pennate diatoms (*C. closterium* and *P. tricornutum*, cell volume 50-70 µm^3 [1,5]) required much higher cell numbers (~ 1 x 10^9 cells) to yield a similar amount of copper binding protein as the larger green microalgae (~ 5 x 10^8 cells) *D. tertiolecta* and *Tetraselmis* sp. (cell volume 300 ± 100 µm^3 [1,5]). This is not suprising given the disparity in cell sizes, as the green algae have more than three times the cell volume of the diatoms.

Proteins extracted from both the control and copper-exposed algae were not retained by the uncharged IMAC column (data not shown). This demonstrated that the proteins bound specifically to the immobilised copper and not to the support matrix of the column itself.

1-Dimensional SDS-PAGE

Concentrated proteins were separated using 1D SDS-PAGE (10 % SDS under reducing conditions) and visualised with Coomassie brilliant blue R-250 stain. All algal samples were prepared in duplicate; each biological replicate was run on multiple gels ($n \geq 2$) to ensure reproducibility. Gel bands of interest were excised and destained with a solution of 50% acetonitrile and 50 mM ammonium bicarbonate prior to mass spectrometry analysis. Protein bands on the stained SDS-PAGE gels were optically scanned (Gel Logic 2200 Pro, Carestream Molecular Imaging software) and the relative intensity of each band estimated using the same software. All samples loaded onto the SDS-PAGE gels contained equal quantities of protein (typically 20-30 µg).

ESI qTOF MS Analysis (Electrospray ionisation quadrupole time-of-flight mass spectrometry)

Excised gel bands were dried and then digested overnight with trypsin (150 ng) in 30 µl of 25 mM ammonium bicarbonate (pH 8). Samples were centrifuged (14,100 x g, 3 min) and supernatants were diluted to 40 µl in Electrospray Ionisation (ESI) loading buffer (2:97.9:0.1 acetonitrile:water:formic acid), injected onto a peptide trap (Michrome peptide Captrap) for pre-concentration and desalted with 0.1% formic acid, 2% acetonitrile (CH_3CN) at 8 µl/min. The peptide trap was then switched into line with the analytical column (SGE ProteCol C18, 300A, 3µm, 150 µm x 10 cm and Exigent TEMPO nanoflow). Peptides were eluted from the column using a linear solvent gradient, with steps, from water:acetonitrile 100:0 to 10:90 (+ 0.1% formic acid) at 500 nl/min over an 80 min period. The LC eluent was subject to positive ion nanoflow electrospray mass spectrometry (MS) analysis on a Q Star Elite (AB Sciex) which was operated in an information dependant acquisition mode (IDA). In IDA mode a TOF-MS (time of flight-mass spectrometry) survey scan was acquired (*m/z* 400-1,600, 0.5 s) with the three largest multiply charged ions (counts > 25) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 2 s (*m/z* 100-1,600). The data was processed using the database search program, Mascot (Matrix Science Ltd, London UK). Peak lists were searched against *Other green plants* in the SwissProt database [35]. High scores in the database search indicated a likely match, confirmed or qualified by operator inspection. Search results were generated with a

significance threshold of $p<0.01$. An overview of the entire sample preparation method can be seen in Fig. 1.

Intracellular copper analysis

Cells previously exposed for 72 h to copper at the relevant IC_{50} copper concentration were concentrated, rinsed with filtered seawater then washed for 20 minutes with an EDTA buffer (0.01 M EDTA, 0.1 M KH_2PO_4/K_2HPO_4 buffer to pH 6.0, salinity adjusted to 2.5 %). The cell suspension was centrifuged and the supernatant discarded. The remaining pellet was digested overnight in 1 ml of an 80:20 mixture of concentrated HNO_3 (TracePur, Merck) and H_2O_2 (Suprapur, Merck) at 21 ± 1 °C. Digests were then diluted to 5 % HNO_3 for copper analysis. Digestions of certified reference material DOLT-3 (dogfish liver) were also determined to ensure complete digestion. The concentrations of copper added to natural seawaters (acidified to 0.8 % HNO_3 (TracePur, Merck)) and within cells (intracellular copper, acidified to 5 % HNO_3) were measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Agilent Varian 730-ES). Copper concentrations were calculated from a matrix-matched calibration curve (clean seawater or ultra-pure water, acidified with 0.8 % or 5 % HNO_3, respectively) using serial dilution of a mixed metal standard (QCD Analysts, Eaglewood, FL, USA). A drift standard was incorporated into the analysis procedure. The detection limit for copper was typically ≤2 μg Cu/l.

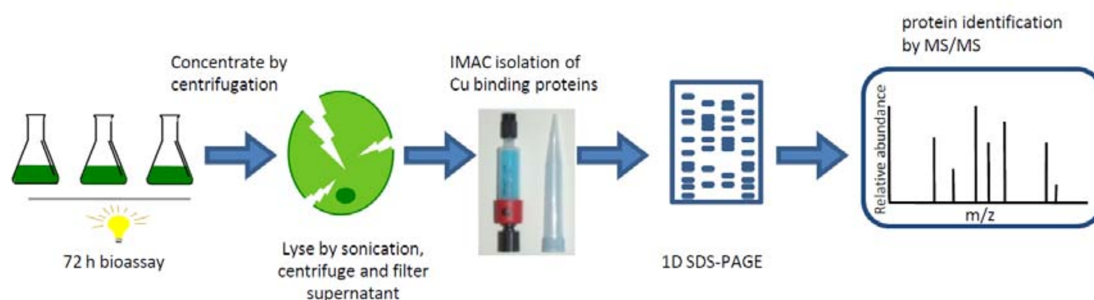

Fig. 1 Diagram representing the sample preparation steps from algal growth and copper exposure through to mass spectrometry (MS) analysis

Results and Discussion:

To our knowledge IMAC has not previously been used to examine copper-binding proteins in marine microalgae. Thus the technique to extract and isolate the proteins required considerable optimisation, as described in the Experimental methods section. Sonication conditions were optimised and effectively ruptured >80 % of cells from *D. tertiolecta*, *C. closterium* and *P. tricornutum* and about 54 % of those from *Tetraselmis* sp..

Intracellular copper concentrations

Intracellular copper concentrations were not proportional to exposure concentration nor to the sensitivity of the species (Table 1), which was similar to the findings of Levy et al. [1]. For example *D. tertiolecta* was exposed to nine times more copper than *Tetraselmis* sp. but internalised three times less copper than *Tetraselmis* sp. It should be noted that in the copper internalisation experiment *Tetraselmis* sp. exhibited less growth inhibition than expected based on the copper exposure concentration (Table 1); however this was not the case in the IMAC protein experiments.

Table 1 Exposure concentrations, intracellular copper and growth inhibition results from the intracellular copper experiment (n=3)

Copper concentrations	*D. tertiolecta*	*Tetraselmis* sp.	*P. tricornutum*	*C. closterium*
Av. exposure concentration [95% CI] (µg Cu/l)	350 [330-370]	38 [36-40]	6 [3-9]	15 [12-18]
Intracellular copper after 72 h IC_{50} copper exposure ± SE(x 10^{-16} g Cu/cell)	420 ± 60	1,200 ± 200	120 ± 50	570 ± 90
% growth inhibition after 72h copper exposure ± SE	49 ± 4	11 ± 4	46 ± 7	79 ± 4

CI = Confidence interval (s_x was calculated from linear regression and used in the formula ($t_{n-2}s_x$)
SE = Standard Error (Standard deviation/√N) where N is the number of replicates.

Analysis of isolated proteins

For each algal species, proteins in control and copper exposed cells were compared. Each species showed a different pattern of protein bands recovered by IMAC. The results from these comparisons are summarised in Table 2. The comparisons were made for individual bands and results pooled into molecular mass ranges. Individual band intensities were compared on SDS-PAGE gels between copper-exposed and unexposed control cells and bands were chosen for further analysis based on the copper exposed protein exhibiting >75% changes relative to controls in replicate gels and replicate samples. In *C. closterium,* some proteins were barely detectable following copper exposure (~75 kDa, Fig. 2), but a new band

was also detected at ~50 kDa that was absent in control cells (Fig. 2). For *Tetraselmis* sp., one band in the 40-60 kDa range was also identified as substantially less intense for copper-treated versus control cells (Fig. 2). Differences in protein recovery were also observed between control and copper-treated *D. tertiolecta* and *P. tricornutum.* In copper exposed *D. tertiolecta,* there was an increase in the intensity of protein bands in both the 60-75 and 75-85 kDa ranges, and for *P. tricornutum*, there was an increase in the 60-75 kDa range in copper-exposed cells (Table 2). However, the protein profiles produced were complex and would require further separation and/or sample clean up before individual proteins could be separated sufficiently to enable identification, thus the *D. tertiolecta* and *P. tricornutum* species were not investigated futher here.

Table 2 Copper induced changes in the levels of IMAC recoverable proteins in four marine microalgae, relative to unexposed controls

Region (kDa)	*D. tertiolecta*	*Tetraselmis sp.*	*P. tricornutum*	*C. closterium*
0-40	~	~	~	51-75 % decrease
40-60	~	76-100 % decrease	~	>100 % increase
60-75	>100 % increase	~	>100 % increase	>100 % decrease
75-85	>100 % increase	~	~	~
85-100	~	~	~	~
100-200	~	~	~	~

~ Differences in band intensity were very small in these regions

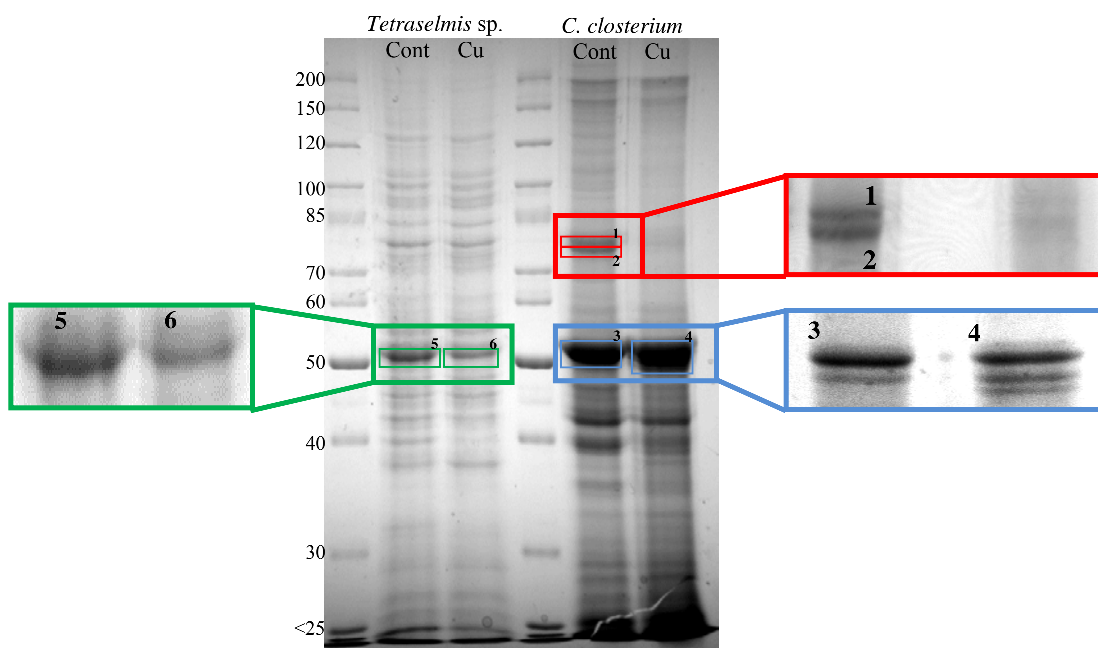

Fig. 2 SDS-PAGE showing copper-binding protein profiles of control and 72-h copper exposed cells from *Tetraselmis* sp. and *Ceratoneis closterium*. *Numbered bands* represent band clusters chosen for further analysis as the copper exposed bands exhibited changes >75 % relative to controls in replicate gels and replicate samples. *Inset* shows expanded region of band clusters noting that band cluster 3 contained 2 bands, and band cluster 4 contained 3 bands. Expanded region showing band cluster 3 and 4 comes from a separate gel with minimal protein loaded to enable resolution of these bands, there was insufficient protein for further analysis

In this study, the protein profiles produced are operationally defined, as IMAC does not capture all metal-binding proteins. Experiments were performed at the IC_{50} concentrations of each algal species, as we have assumed that cells can cope at this level of copper exposure demonstrated by growth and division still occuring at 50% the rate of control populations. Therefore, we assume that detoxification mechanisms are not completely overwhelmed at this exposure level, and any proteins produced in response to the copper would be in excess of intracellular copper. However in the event that a protein had all of its binding sites saturated with metals prior to the IMAC separation, it may not be retained by the column.

Identification of copper-affected proteins

Mass spectrometry results were analysed and the "top" matches compiled along with relevant information regarding the quality of the matches (Table 3). Protein score is -log(p) where p is the probability that the observed match is a random event. Protein scores >28 ($p < 0.01$) were considered significant. Alignments of the sequences for all species matched were performed as well as for the top three species matches and amino acid sequence homology was calculated. All results discussed in the following (except where noted) can be found in Table 3.

For *C. closterium* two bands at ~75 kDa (Fig. 2, band 1 and 2) were evident in proteins purified from control cell lysates; however, these bands were barely detectable in the corresponding protein fraction of copper exposed cell lysates. There are several possible explanations for this: the expression of the proteins may be repressed, they may have become bound to copper within the cell rendering the proteins insoluble, or binding to copper may have altered their conformation such that they are no longer able to bind to the IMAC column. In any case, the availability of these proteins for cellular activity is likely to be compromised.

As discussed above, protein band 1 examined (Table 3 and Fig. 2) from the control *C. closterium* was not able to be identified from the database, with all possible matches outside the molecular weight range of the excised band and with low protein scores. This may be because it is a newly identified protein; however, it is also possible that this band contained a mixture of proteins none of which were present in sufficient amounts to enable a match. The second *C. closterium* protein band at ~75 kDa (Table 3, band 2), which was less abundant in lysates from Cu-treated cells, showed matches for some chaperone and heat-shock proteins (all from the HSP70 family). Heat shock proteins (HSP) and chaperones are involved in the unfolding and refolding of proteins [36]. The absence, undersupply or non-functionality of this class of proteins is a possible cause of toxicity in cells as they are involved in maintaining cell homeostasis as part of the cell's defence mechanism against cellular stress [36]. HSPs respond to a variety of cellular stresses other than temperature, including metal exposure, oxygen radicals and peroxides [37]. The HSP70 family proteins identified in *C. closterium* are highly conserved and demonstrate (among eukaryotes) around a 70 % amino acid identity [38]. Although only 6 % of the amino acid sequence was matched in the search, the high level of conservation implies that the protein belongs to the HSP70 family.

Another change identified by analyses of lysates prepared from *C. closterium* cells was the appearance of an additional band at ~50 kDa in the copper exposed cells that was not present in control *C. closterium* cells (Fig. 2, band cluster 4). Resolution between bands was low when ~50 µg of protein was loaded onto the gel. Band clusters 3 and 4 (Table 3 and Fig. 2) could only be resolved when significantly less protein (~5-10 µg) was loaded onto the electrophoresis gel; however, this did not permit excision of sufficient protein from the band of interest for further analysis (Fig. 2, inset of band cluster 3 and 4). Thus the band clusters were excised as a single sample and analysed.

Table 3 Protein matches for selected proteins (chosen for further analysis as the copper exposed protein exhibited changes >75% relative to controls in replicate gels and replicate samples) bound to a Cu-IMAC column (labelled band clusters 1-6 from Fig. 2) from lysates prepared from two marine microalgal species *Ceratoneis closterium* and *Tetraselmis* sp.

Sample/ Species	Protein	# species matching protein	Homology (%)	Top species matches for the protein (max 3)	Molecular weight (Da)	Homology top 3 species (%)	# unique matches	Sequence coverage (%)	Protein score
Band cluster 1 *Ceratoneis closterium* control	Ribulose bisphosphate carboxylase large chain	1	-	*Cylindrotheca* sp.	54,001	-	2	6	104
	uncharacterized ycf88	1	-	*Odontella sinensis*	17,379	-	1	19	48
(Decrease in expression after copper exposure)	ATP synthase subunit delta, chloroplastic	1	-	*Odontella sinensis*	21,117	-	1	7	30
Band cluster 2 *Ceratoneis closterium* control	Ribulose bisphosphate carboxylase large chain	1	-	*Cylindrotheca* sp.	54,001	-	2	4	131
(Decrease in expression after copper exposure)	Chaperone protein dnaK	2	74	*Odontella sinensis*	67,128	74	2	6	112
				Thalassiosira pseudonana	65,298		2	6	104
	Heat shock 70-related protein	2	60	*Leishmania major* (fragment)	24,796	60	1	7	73
				Pyrenomonas salina	72,035		1	2	73
Band cluster 3 *Ceratoneis closterium* control	Ribulose bisphosphate carboxylase large chain	5	68	*Cylindrotheca* sp.	54,001	82	12	27	749
				Thalassiosira pseudonana	54,289		9	17	404
				Cyanidioschyzon merolae	54,099		8	16	332
(Two bands present in control cells)	ATP synthase subunit beta, chloroplastic	2	93	*Odontella sinensis*	51,477	93	2	6	134
				Thalassiosira pseudonana	51,111		2	6	134
	Glyceraldehyde-3-phosphate dehydrogenase, chloroplastic	1	-	*Chondrus crispus*	44,430	-	1	3	28
Band cluster 4 *Ceratoneis closterium* copper	Ribulose bisphosphate carboxylase large chain	8	42	*Cylindrotheca* sp.	54,001	82	12	26	1312
				Thalassiosira pseudonana	54,289		9	20	488
				Cyanidioschyzon merolae	54,099		7	14	427
(Three bands present in copper exposed cells)	ATP synthase subunit beta, chloroplastic	2	93	*Odontella sinensis*	51,477	93	1	3	101
				Thalassiosira pseudonana	51,111		1	3	101
	ATP synthase subunit alpha, mitochondrial	1	-	*Dictyostelium citrinum*	57,224	-	1	3	59

Sample/ Species	Protein	# species matching protein	Homology (%)	Top species matches for the protein (max 3)	Molecular weight	Homology top 3 species (%)	# unique matches	Sequence coverage (%)	Protein score
Band cluster 5	Ribulose bisphosphate carboxylase large chain	9	43	*Euglena viridis* (fragment)	48,328	94	5	13	315
***Tetraselmis* sp.**				*Euglena anabaena* (fragment)	48,256		6	15	209
control				*Euglena geniculata* (fragment)	48,193		6	15	209
Decrease in expression after copper exposure	Tubulin beta chain	3	87	*Cyanophora paradoxa* (beta-1)	49,792	87	3	8	165
				Ectocarpus variabilis (beta-5)	49,973		1	4	117
				Ectocarpus variabilis (beta-6)	50,114		1	4	117
	Tubulin alpha chain	4	72	*Pelvetia fastigiata* (alpha-1 chain)	49,905	74	1	4	111
				Pelvetia fastigiata (alpha-2 chain)	49,948		1	4	111
				Guillardia theta (alpha chain, nucleomorph)	49,771		1	4	90
	ATP synthase subunit beta, chloroplastic	17	60	*Cyanidium caldarium*	53,244	72	1	3	52
				Cyanidioschyzon merolae	50,382		1	3	52
				Dictyota dichotoma	52,171		1	3	52
Band cluster 6	ATP synthase subunit beta, chloroplastic	3	88	*Dictyota dichotoma*	52,171	88	7	19	262
***Tetraselmis* sp.**				*Fucus vesiculosus*	51,541		7	19	262
copper				*Pylaiella littoralis*	51,993		7	19	262
Decrease in expression after copper exposure	Tubulin beta chain	4	83	*Cyanophora paradoxa* (beta-1)	49,792	83	5	12	279
				Oomycete-like sp. strain MacKay2000 (beta-4)	50,334		4	9	191
				Ectocarpus variabilis (beta-5)	49,973		1	4	99
	Tubulin alpha chain	5	82	*Pelvetia fastigiata* (alpha-2)	49,948	88	4	15	232
				Naegleria gruberi (alpha 1/2/3)	49,810		3	10	143
				Naegleria gruberi (alpha-13)	49,782		3	10	143
	Ribulose bisphosphate carboxylase large chain	8	44	*Euglena viridis* (fragment)	48,328	94	4	7	205
				Euglena anabaena (fragment)	48,256		5	11	187
				Euglena geniculata (fragment)	48,193		5	11	187
	V-type proton ATPase subunit B	1	-	*Cyanidium caldarium*	56,398	-	1	3	59
	Mitochondrial import inner membrane translocase subunit TIM50	1	-	*Phytophtora infestans*	45,237	-	1	2	29

Protein score is -log(p) where p is the probability that the observed match is a random event. Protein scores ≥ 28 ($p < 0.01$) were considered significant

Several proteins were identified in band clusters 3 and 4 from *C. closterium* extracts at ~50 kDa (control and copper exposed, Fig. 2, band 3 and 4). The strongest match for the most abundant protein at this size was Ribulose bisphosphate carboxylase large chain (Rubisco), which is one of the most abundant proteins in the world [39], and consists of a large chain component (typically 50-55 kDa) and a small chain component (typically ~15 kDa) [40]. Rubisco is involved in carbon fixation and therefore is expected to be found in all algal cells. While this may be the higher molecular weight protein in band clusters 3 and 4, it is unlikely to be lower molecular weight protein band unique to copper-exposed *C. closterium* (Fig. 2 band 4).

Rubisco was also found in both the control and copper exposed *Tetraselmis* sp. mass spectrometry analyses of proteins extracted from the gels within bands 5 and 6 (Table 3 and Fig. 2, band 5 and 6), with less protein detected in the copper exposed cells (band 6). For *C. closterium* protein matches were to two other diatoms *Cylindrotheca* and *Thalassiosira* and for *Tetraselmis* sp. matches were to *Euglena* (unicellular flagellate protists); this likely corresponds to differences between diatoms and green algae. *Ceratoneis closterium* has also been known by the names *Cylindrotheca closterium* and *Nitzschia closterium* [41]. Therefore, the *Cylindrotheca* sp. protein matches from the Mascot databank could be from a closely-related species or indeed from the same species as that used in this study. Rubisco has been shown to decrease in both amount and enzymatic activity following copper exposure in rice plants (*Oryza sativa* L.) [42,43] and barley plants [44]. In *O. sativa* L. degradation products of the large chain subunit were observed following exposure to copper [42].

Bands 5 and 6 (Fig. 2) from *Tetraselmis* sp. had several other protein matches in addition to Rubisco. These included ATP (adenosine tri-phosphate) synthase (subunit β) and tubulin (α and β). Although the same proteins were identified in both the copper-exposed and control treatments (Table 3, bands 5 and 6), overall there was a decrease in band 6 intensity for the copper-exposed *Tetraselmis* sp. (Fig. 2, band 6).

α and β tubulins form a heterodimeric complex known as microtubules, these are important structural building blocks for structures such as flagella [45]. α and β tubulins are different in structure, however their individual structures are highly conserved amongst a variety of families [46]. When comparing the whole superfamily of tubulins (including α, β, γ, δ, ε, ζ and η) only the guanidine di- or tri-phosphate (GDP/GTP)-binding site (and several other single amino acids) are conserved. However, whilst their structures are different, tubulins have a related function [46], as an important component in spindle formation during cell division. Spindle formation and dissolution depend on the ratio between sulfhydryl groups and disulfide bonds [47]. Although the data from this experiment is unable to conclusively show whether copper has had an effect on tubulin levels within the cell, copper is redox active and would be able to oxidise sulfhydryl groups e.g. in glutathione and in tubulins, leading to impacts on microtubule assembly and subsequent inhibition of cell division [47]. ATP synthase (subunit β) was matched in both control and copper exposed cells from *C. closterium* (Fig. 2, band 3 and 4) and *Tetraselmis* sp. (Table 3, band 5 and 6). In *Tetraselmis* sp., a decrease in the overall intensity of this band was observed after copper exposure and may be a result of a decrease in the levels of one or several of the identified

proteins from this band (Fig. 2, band 6). The whole ATP synthase complex catalyses the formation of ATP, the "energy currency" of the cell [48,49]. The structure of the β subunit of ATP synthase is highly conserved. In a study by Runswick and Walker [48] the alignment between the β subunits of spinach chloroplasts, maize chloroplasts, bovine mitochondria and *Escherichia coli* had more than 50 % identity over all four species, and this increased to between 64 and 88% when comparing any two of the species. This is relatively high conservation considering the variety of species being examined. This shows that the matches for ATP synthase subunit β are quite strong matches (Table 3, band 3, 4, 5 and 6) given the high level of homology within this particular protein's evolution. Previous publications on algae, tomato plants and rat hepatoma cells have shown metal (including copper) inhibition of ATP-synthesis [50-52]. ATP is essential for energy-requiring cellular processes, thus inhibition of ATP synthesis by copper can be one important mode of metal toxicity to algae.

The most copper tolerant species in this study (*D. tertiolecta*) showed fewer changes overall in the levels of copper binding proteins following copper exposure than the more sensitive species *C. closterium*. This supports the original hypothesis that proteins bound to the IMAC column from the tolerant species would be less affected. *Tetraselmis* sp. is also reasonably tolerant (with an IC_{50} value of approximately 2-3 times greater than *C. closterium*) and it too exhibited smaller changes than *C. closterium*. However, in contrast and contradiction to the hypothesis, *P. tricornutum* has smaller changes in the copper-binding proteins detected following copper exposure than *C. closterium* despite *P. tricornutum* being more copper sensitive than the latter. This may be due to the amount of intracellular copper content where *P. tricornutum* uptakes approximately five times less copper over 72 h. Furthermore, the amount of copper internalised by the different algae differs from the order of their respective copper tolerances. When exposed to their respective IC_{50} copper concentration, *Tetraselmis* sp. internalises significantly more copper than *C. closterium* (Table 1; 1,200±200 and $570 \pm 90 \times 10^{-16}$ g Cu/cell, respectively) however both internalised more than either of the other two species.

The IMAC/SDS-PAGE method used in this study has identified a number of protein bands that may be affected by cell exposure to copper. Some of the bands contained multiple proteins so we were unable to unequivocally state that specific protein(s) were affected by copper exposure. In this study, the protein bands (bound to and recovered from IMAC) that were affected when algae were exposed to copper included heat-shock proteins, tubulins (α and β), Rubisco (large chain) and ATP synthase (β subunit). The observed changes in protein band intensity may reflect changes in protein expression, conformation, or metal complexation within the cell. Whilst the results from this study alone are not yet able to fully explain the differences in copper tolerance between these species, future work in our laboratory examining copper uptake, intracellular localisation and detoxification processes (such as phytochelatins) in marine algae may provide further insights into the mode of action of copper and will be the focus of future studies.

Conclusions

This study used Cu-IMAC and SDS-PAGE to identify changes in the recovery of copper-binding proteins from copper-exposed and untreated control cells from four species of marine microalgae. IMAC was successfully used to isolate copper binding proteins from complex marine microalgal lysates. A 72-h exposure of cells to the IC_{50} copper concentration was found to alter the levels of copper-binding proteins recovered from lysates of the four species. In some cases copper exposure led to a decrease in the amount of detectable protein, and in one case, the appearance of an additional protein not previously detected. For *P. tricornutum* and *D. tertiolecta*, although differences in proteins were detected between copper exposed and unexposed cells, the complexity of the copper-binding protein fraction meant that 1-D SDS-PAGE was unable to sufficiently resolve the protein bands to permit further investigation. In future work this could be remedied by employing 2-D electrophoresis (separating by both isoelectric point and size) to better resolve the proteins prior to subsequent analysis [53,26]. The proteins thus identified are likely to play roles in copper homeostasis and toxicity in marine microalgae.

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