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Effects of copper on growth and photosynthesis in marine diatoms: a comparison between species from two different geographical areas

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Abstract – The effects of copper on growth, photosynthesis and carbonic anhydrase activity in four marine diatoms were investigated under controlled conditions. Two species (*Amphora acutiuscula* and *Nitzschia palea*) were collected from the southeast Vietnamese coast, and the other two (*Amphora coffeaeformis* and *Entomoneis paludosa*) were native from the French Atlantic coast. Adding 1.5 and 3.0 µM of Cu to artificial seawater did not affect the growth rate, but did reduce the maximum cell density in all four diatom species. Photosynthetic parameters were determined from gross photosynthesis versus irradiance (P vs. E), and relative electron transport rate versus irradiance (rETR vs. E) curves. In *A. acutiuscula*, a similar pattern was observed for the P vs. E and rETR vs. E curves, indicating that 3.0 µM Cu significantly affected all photosynthetic parameters. In the other three species, the P vs. E and rETR vs. E curves did not show this pattern. The effect of Cu on photosynthesis depended on species, either increasing or reducing the electron transport rate in the thylakoid and oxygen production. External carbonic anhydrase activity followed a similar pattern to gross photosynthesis at growth irradiance, indicating it plays a major role in the supply of inorganic carbon to carboxylase(s).

Chlorophyll *a* fluorescence / Copper / Diatoms / Microalgae / Oximetry / Photosynthesis / Respiration

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Résumé – Effets du cuivre sur la croissance et la photosynthèse de diatomées marines : étude comparative entre les espèces d'origines géographiques différentes. Les effets du cuivre sur la croissance, la photosynthèse et l'activité anhydrase carbonique chez des espèces de la côte Sud-est du Vietnam (*Amphora acutiuscula* et *Nitzschia palea*) et de la côte atlantique française (*Amphora coffeaeformis* et *Entomoneis paludosa*) ont été mesurés. Les diatomées ont été cultivées en conditions contrôlées, dans de l'eau de mer artificielle additionnée ou non de Cu 1,5 µM ou 3.0 µM. L'ajout de Cu ne modifie pas le taux de croissance mais réduit la densité cellulaire maximale chez les quatre espèces. Chez *A. acutiuscula*, la réduction de la photosynthèse brute en fonction de l'éclairement (courbes P/E déterminées par oxymétrie) et du taux relatif de transport des électrons (courbes rETR/E déterminées par fluorimétrie modulée) indique que l'addition de Cu 3.0 µM affecte significativement les paramètres photosynthétiques. Les paramètres des courbes P/E et rETR/E montrent que les trois autres espèces ont des comportements différents selon la quantité de Cu ajouté. À l'éclairement de croissance, l'activité anhydrase carbonique externe varie de façon similaire à la photosynthèse brute, ce qui indique que cette enzyme joue un rôle majeur dans la fourniture de carbone inorganique aux carboxylases.

Cuivre / Diatomées / Fluorescence de la chlorophylle *a* / Microalgues / Oxymétrie / Photosynthèse / Respiration

INTRODUCTION

Microalgae are eukaryotic photosynthetic organisms that play a key role in primary aquatic food chains. Diatoms (Bacillariophyceae) are unicellular organisms characterized by having a silica cell wall, known as the frustule, surrounding the protoplast. They are directly exposed to water pollution, and some organisms have defence mechanisms to cope with pollutants. Metal pollution of marine coasts is usually due to human activities, and in particular, copper pollution is attributable to industrial and domestic waste, copper mine drainage, agricultural practices using copper-based pesticides, and antifouling paints (Perales-Vela *et al.*, 2007).

Copper is a required micronutrient for algae. It is a component of plastocyanine and is involved in the photosynthetic electron transport chain, mitochondrial respiration, cell wall metabolism, as well as in proteins and enzymes (Maksymiec, 1997; Raven *et al.*, 1999; Yruela, 2005). Gross *et al.* (1970) reported that *Chlorella* cultures begin to show signs of deficiency when the concentration of copper in the medium falls below 0.1 µM. This phenomenon has also been confirmed in various plant species (Maksymiec, 1997). Although metals such as copper and zinc are essential for physiological processes in plants, high concentrations of both essential and non-essential metals can also inhibit growth and induce signs of toxicity (Hall, 2002). Cid *et al.* (1995) reported growth inhibition when *Phaeodactylum tricornutum* was cultured in the presence of 0.10 mg L^{-1} Cu (1.57 µM). In the macroalga *Fucus serratus*, the presence of 0.84 µM Cu in the medium amplified the electron transport rate in the thylakoid and reduced the cell division rate, but did not affect O_2 exchanges nor the total pigment content (Nielsen & Nielsen, 2005). Parales-Vela *et al.* (2007) showed that in the green microalga *Scenedesmus incrassatulus* growth, chlorophyll and carotenoid contents, photosynthesis (electron transport rate or oxygen evolution) and respiration were all reduced to a varying degree after 6 days of exposure to 0.96 µM Cu. Chlorophyll fluorescence techniques, which have become commonplace in metal-stress research, have shown that metal stress leads to

direct inactivation of the photosystem II reaction center (Mallick & Mohn, 2003). The CO_2 concentration in seawater is limited at an alkaline pH, and the dissolved inorganic carbon is mainly present in the form of HCO_3^-. Marine microalgae have various mechanisms to concentrate inorganic carbon near the catalytic sites of ribulose bisphosphate carboxylase-oxygenase (RubisCO) (Raven & Beardall, 2003). External and internal carbonic anhydrase activities have been investigated because these enzymes play a key role in ensuring the supply of inorganic carbon to the carboxylases (Azov, 1982; Haglund *et al.*, 1992; Morant-Manceau *et al.*, 2007; Roberts *et al.*, 2007), and they can also be affected by metal toxicity (Wang *et al.*, 2005).

The present paper is an ecophysiological investigation aimed at comparing adaptation strategies to various concentrations of copper in natural seawater by measuring several physiological parameters in four marine diatoms isolated from two geographical regions, I.e. the French Atlantic (*Amphora coffeaeformis* and *Entomoneis paludosa*) and the southeast Vietnamese coasts (*Amphora acutiuscula* and *Nitzschia palea*), which show different pollution levels caused by human activities on ocean ecosystems (Halpern *et al.*, 2008). *N. palea* is considered as an indicator of polluted waters (Sylvestre *et al.*, 2001). Copper concentrations used were non-lethal and corresponded to about twice or four times those found in seawater of the Vietnamese and French Atlantic coasts. The parameters investigated during exposure to Cu were growth, photosynthesis (O_2 evolution and chlorophyll *a* fluorescence) and carbonic anhydrase activity.

METHODS

Culture conditions

All species were cultured in artificial seawater (ASW) prepared from Millipore ultrapure water according to the protocol of Harrison *et al.* (1980). The concentration of copper in the control ASW was 0.15 µM. ASW was stored in the dark at 16°C.

Amphora acutiuscula Kützing and *Nitzschia palea* (Kützing) Smith were collected and isolated in September 2005 from the Can Gio site in Southeast Vietnam. *Amphora coffeaeformis* (Agardh) Kützing and *Entomoneis paludosa* (W. Smith) Reimer were collected from the French Atlantic coast (Bourgneuf bay) by the "Laboratoire de Biologie marine" of the University of Nantes (France). The four species were axenically cultured in batch in ASW at a temperature of 16°C for the species from the French coast and 23°C for the other two species from the Vietnamese coast. In both cases a 14 h/10 h light/dark cycle was used. Cells were irradiated at a photon flux density of 300 µmol photons m^{-2} s^{-1} using cool-white fluorescent tubes (Philips TLD, 18 W). Irradiance was measured using a 4π waterproof light probe (Walz, Germany) connected to a Li-Cor 189 quantum meter (Tremblin *et al.*, 2000). All glassware was acid washed for 48 h by soaking in 1 M HCl and then rinsed three times with ultra pure water to avoid metal contamination (Arensberg *et al.*, 1995). Cells were taken from exponentially growing pre-cultures, centrifuged gently (900 g, 10 min, 4°C) and inoculated under sterile conditions into ASW in 500 mL Erlenmeyer flasks to produce an initial cell density of 10^4 cells mL^{-1}.

A stock solution of $CuCl_2$ was prepared in ultrapure water at a concentration of 10 mM, filtered through a 0.22 µm filter, and kept at 4°C.

Appropriate amounts of $CuCl_2$ stock solution were added to ASW to obtain a concentration of 1.5 or 3.0 μM added Cu. Cells in exponential growth phase were gently centrifuged and harvested for use in the experiments.

Algal growth

Cell counting using a Neubauer hemocytometer was carried out daily to measure algal growth. A logistic growth function according to Cid *et al.* (1995) was used to calculate the growth rate and the maximum cell density.

Chlorophyll *a* content

The total chlorophyll *a* (Chl *a*) content of algal samples harvested by filtering through 0.22 μm Whatman (GF/C) filters was extracted into dimethylformamide (DMF) (Mouget *et al.*, 1999). The chlorophyll contents were then measured spectrophotometrically according to Speziale *et al.* (1984).

Oxygen evolution

Oxygen evolution was determined using a Clark-type oxygen electrode included in a thermostatic measuring chamber (DW2, Hansatech Instruments Ltd., UK), which was filled with 1.5 mL of diatom suspension containing a quantity of chlorophyll ranging from 0.5 to 2.0 μg depending on species, according to Mouget *et al.* (1999). Experiments were carried out at 16°C for the species from the French coast and 23°C for those from the Vietnamese coast. The diatoms were illuminated with actinic irradiance varying from 0 to 1200 μmol photons m^{-2} s^{-1} tsupplied via an optic fiber from an Intralux cold-light source (Volpi AG, Switzerland). The respiration was measured while the diatoms were maintained in darkness. The gross photosynthesis was calculated as the net photosynthesis plus respiration, assuming that respiration was the same in light and in darkness. The experimental data were fitted to the model of Eilers & Peeters (1988) using the Sigma-plot program to plot gross photosynthesis versus irradiance (P vs E curves) and calculate three photosynthetic parameters: the light utilization coefficient, the maximum gross photosynthesis rate (P_{max}) and Talling's index or the light saturation parameter (E_k).

Chlorophyll fluorescence measurements

We used an FMS1 modulated fluorometer (Hansatech Ltd., UK) modified to be suitable for use at low Chl *a* concentrations (Rech *et al.*, 2003) to measure the chlorophyll fluorescence. The minimum (F_o) and maximum (F_m) fluorescence values of 15-min dark-adapted diatoms were measured. In preliminary tests (kinetic fluorescence protocol), we determined the times required at different actinic light exposure levels to reach steady state fluorescence. We then determined the maximum (F'_m) and steady-state (F_s) fluorescence.

The protocol for plotting the relative electron transport rate versus irradiance (rETR vs. E) described by Juneau *et al.* (2001) and by Nielsen & Nielsen (2005) was adapted for diatom suspensions. The rETR vs. E protocol

involved 11 levels of actinic light progressing from 0 to 1200 µmol photons m^{-2} s^{-1}. Fluorescence experiments were carried out at 16 or 23°C, depending on the algal species. The effective quantum yield (ϕ_{PSII}) and relative electron transport rate (rETR) were calculated using the following equations:

- $\phi_{PSII} = (F'_m - F_s)/F'_m$
- $rETR = 0.5 \times E \times \phi_{PSII}$

where E is the photosynthetic active irradiance (Beer & Björk, 2000, Maxwell & Johnson, 2000, Mouget & Tremblin, 2002). The photosynthetic parameters $_{rETR}$ (light utilization coefficient), $rETR_{max}$ (maximum relative electron transport rate) and E_{krETR} (light saturation parameter) were determined by fitting rETR vs. E curves, using the model of Eilers & Peeters (1988). The rETR was expressed in relative units.

Carbonic anhydrase activity

The carbonic anhydrase (CA) activity was determined by monitoring the time taken for the pH of the final reaction mixture to drop from 8.3 to 7.9 at 2°C. The reaction was started by rapidly injecting 3 mL of CO_2-saturated water into 3 mL of 20 mM veronal-HCl (pH 8.3) buffer containing an additional 0.48 mM of NaCl. One unit of CA activity was defined as $(T/T_s) - 1$, where T and T_s were the times taken for the pH to fall by 0.4 units for the control and the algal samples, respectively. Intact cells were used to measure the extracellular CA activity (CA_{ext}), and a crude extract of cells homogenized in liquid nitrogen to measure the total CA activity (CA_{tot}). The internal CA activity (CA_{int}) was calculated as CA_{tot} activity minus CA_{ext} activity (Dionisio-Sese & Miyachi, 1992; Morant-Manceau *et al.*, 2007).

Statistical analysis

All measurements were made on 3-5 replicates (from different cultures) and the results were expressed as means and standard errors. One-way analysis of variance (ANOVA) was used to determine significant differences in all experiments. This test can be used to compare a single factor effect in two or more groups. A statistically significant difference had to display at least a 5% level of significance ($P \leq 0.05$) using Tukey test run on Sigmastat version 3.1 software compatible with Sigmaplot 9.0.

RESULTS

Growth rate and maximum cell density

Table 1 shows the growth rate and the maximum cell density of diatoms cultured in the presence of three different concentrations of copper for five days. These growth parameters were calculated from growth curves (not shown). Growth rates of *A. coffeaeformis* and *A. acutiuscula* were significantly similar at all copper concentrations. Maximum cell densities of *A. coffeaeformis* cultured with Cu did not differ from control, but this parameter was negatively correlated

Table 1. Growth rate during the growth exponential phase and maximum cell density in *Amphora coffeaeformis*, *Amphora acutiuscula*, *Entomoneis paludosa* and *Nitzschia palea* grown in ASW (control) or in presence of $CuCl_2$ 1.5 and 3.0 µM added to ASW. Significant different data are indicated with different superscripted letters (Tukey Test, $P \leq 0.05$). Mean values ± SE (n = 3 – 5).

Species	*Growth rate (day^{-1})*			*Maximum cell density (10^3 cells mL^{-1})*		
	Control	*1.5 µM*	*3.0 µM*	*Control*	*1.5 µM*	*3.0 µM*
A. coffeaeformis	1.17 ± 0.21^{a}	1.03 ± 0.06^{a}	0.89 ± 0.14^{a}	670 ± 37^{ab}	703 ± 16^{a}	609 ± 43^{b}
A. acutiuscula	1.12 ± 0.22^{a}	1.68 ± 0.10^{a}	1.19 ± 0.42^{a}	402 ± 70^{a}	276 ± 20^{b}	262 ± 42^{b}
E. paludosa	0.83 ± 0.05^{a}	0.82 ± 0.07^{a}	1.25 ± 0.11^{b}	248 ± 13^{a}	229 ± 19^{ab}	159 ± 38^{b}
N. palea	1.24 ± 0.16^{a}	1.24 ± 0.05^{a}	1.90 ± 0.35^{b}	545 ± 40^{a}	580 ± 18^{a}	376 ± 8^{b}

to copper concentration in *A. acutiuscula*. Adding 1.5 µM Cu had no significant effect on growth rate or maximum cell density of any species, whereas exposure to 3.0 µM Cu induced a reduction in the maximum density of all diatom cultures but had a stimulating effect on the growth rates of *E. paludosa* and *N. palea* (50.6% and 53.2% greater than control respectively).

Photosynthetic responses

Oxygen evolution

Measurements of oxygen evolution in darkness (Table 2) showed that adding 1.5 µM Cu to the culture medium increased the respiration of *A. coffeaeformis* and *A. acutiuscula* (up by 22% and 38% vs. control, respectively), whereas adding 3.0 µM Cu produced no change. Unlike that of the *Amphora* species, the respiration of *E. paludosa* and *N. palea* increased only when exposed to medium to which 3.0 µM Cu had been added (by 24% and 20%, respectively).

The gross photosynthesis versus irradiance curves of diatoms grown in the presence of Cu are shown in Fig. 1. For high irradiance levels (more than 800 µmol photons $m^{-2}s^{-1}$) the control cultures of all the diatoms studied

Table 2. Respiration (µmol O_2 mg^{-1} Chl *a* h^{-1}) in *Amphora coffeaeformis, Amphora acutiuscula, Entomoneis paludosa* and *Nitzschia palea* grown in ASW (control) or in presence of $CuCl_2$ 1.5 and 3.0 µM added to ASW. Significant different data are indicated with different superscripted letters (Tukey Test, $P \leq 0.05$). Mean values ± SE (n = 3 – 5).

Species	*Control*	*1.5 µM*	*3.0 µM*
A. coffeaeformis	$39^{a} \pm 4$	$50^{b} \pm 3$	$45^{ab} \pm 2$
A. acutiuscula	$28^{a} \pm 4$	$45^{b} \pm 3$	$31^{a} \pm 1$
E. paludosa	$39^{a} \pm 5$	$47^{ab} \pm 4$	$51^{b} \pm 1$
N. palea	$60^{ab} \pm 0$	$56^{a} \pm 4$	$74^{b} \pm 8$

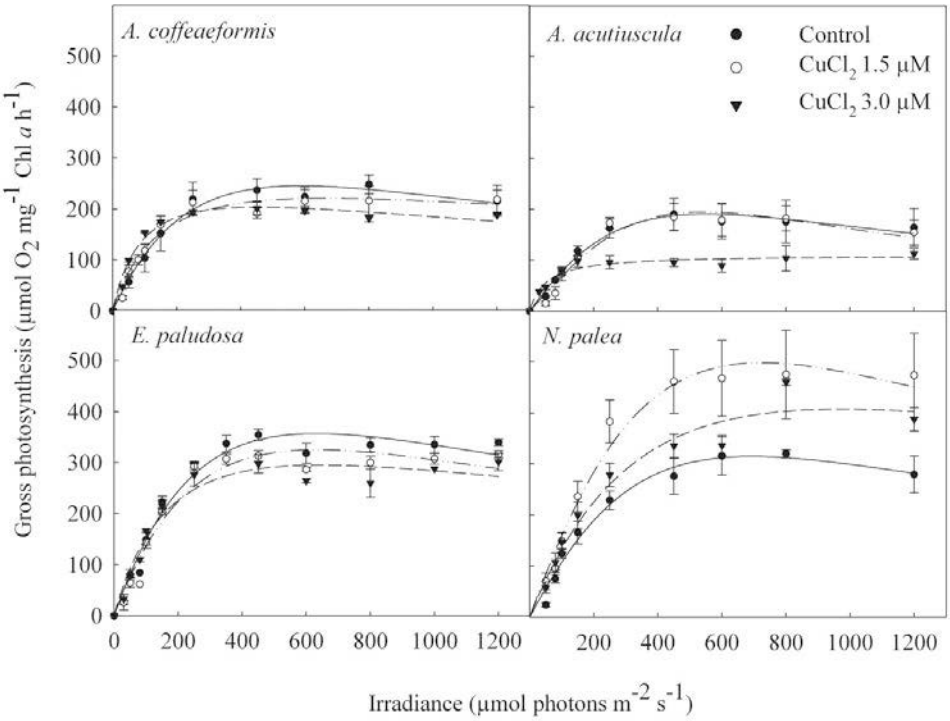

Fig. 1. Gross photosynthesis versus irradiance in *Amphora coffeaeformis*, *Amphora acutiuscula*, *Entomoneis paludosa* and *Nitzschia palea* grown in ASW (control) or in the presence of 1.5 and 3.0 µM $CuCl_2$ added to ASW. Mean values ± SE (n = 3 – 5).

displayed a slight photoinhibition phenomenon, but in the presence of Cu this phenomenon was not always observed in the species from the Vietnamese coast. The photosynthetic parameters estimated from the fitted curves are shown in Table 3. *A. coffeaeformis* and *E. paludosa* displayed the optimal photosynthesis rate under control conditions. For both these species, the higher the concentration of copper, the lower the maximum gross photosynthesis rate (Table 3). A similar trend was also observed for the values of the light saturating indice (E_k); whereas the light-utilization coefficient increased by about 50% when 3.0 µM Cu was added. *A. acutiuscula* and *N. palea* displayed differing photosynthesis responses. For *A. acutiuscula*, the rates of photosynthesis were not significantly different in the control and 1.5 µM Cu cultures, whereas P_{max} and E_k were greatly reduced by 3.0 µM Cu (down by 45% and 73%, respectively). The gross photosynthesis of *N. palea* was clearly increased by adding 1.5 µM Cu (up by 38%), and was slightly greater than control when 3.0 µM Cu was added to ASW (12%).

Chlorophyll fluorescence

The relative electron transport rate versus irradiance curves are presented in Fig. 2. When 3.0 µM Cu had been added, *A. coffeaeformis* and

N. palea showed a similar change in rETR (Fig. 2), with no significant difference from control, whereas a slight photoinhibition was observed in *A. acutiuscula* and an increase in all photosynthetic parameters in *E. paludosa* (Table 4). After adding 1.5 µM Cu to ASW, $rETR_{max}$ rose in *A. coffeaeformis* but fell in *N. palea*.

Table 3. Parameters (α, light utilization coefficient; P_{max}, maximum gross photosynthesis; E_k, affinity for light) of gross photosynthesis versus irradiance curves (Fig. 1) in *Amphora coffeaeformis, Amphora acutiuscula, Entomoneis paludosa* and *Nitzschia palea* grown in ASW (control) or in presence of $CuCl_2$ 1.5 and 3.0 µM added to ASW. Significant different data are indicated with different superscripted letters (Tukey Test, $P \leq 0.05$). Mean values ± SE (n = 3 – 5). α: µmol O_2 mg^{-1} Chl *a* h^{-1} (µmol photons m^{-2} $s^{-1})^{-1}$; P_{max}: µmol O_2 mg^{-1} Chl *a* h^{-1}; E_k: µmol photons m^{-2} s^{-1}.

Species	*α*			*P_{max}*			*E_k*		
	Control	*1.5 µM*	*3.0 µM*	*Control*	*1.5 µM*	*3.0 µM*	*Control*	*1.5 µM*	*3.0 µM*
A. coffeaeformis	1.37 ± 0.36^{a}	2.00 ± 0.21^{a}	2.96 ± 0.36^{b}	248 ± 21^{a}	222 ± 31^{a}	205 ± 6^{a}	228 ± 66^{a}	114 ± 16^{a}	72 ± 10^{b}
A. acutiuscula	0.90 ± 0.09^{a}	0.71 ± 0.05^{a}	1.79 ± 0.21^{b}	192 ± 29^{a}	199 ± 20^{a}	105 ± 15^{b}	217 ± 33^{a}	279 ± 7^{a}	59 ± 5^{b}
E. paludosa	2.04 ± 0.26^{a}	2.09 ± 0.20^{a}	2.79 ± 0.11^{a}	361 ± 10^{a}	320 ± 8^{ab}	309 ± 11^{b}	196 ± 36^{a}	158 ± 20^{ab}	121 ± 4^{b}
N. palea	0.95 ± 0.22^{a}	1.76 ± 0.18^{ab}	1.94 ± 0.28^{b}	366 ± 60^{a}	506 ± 79^{b}	411 ± 31^{ab}	414 ± 52^{a}	285 ± 21^{b}	223 ± 32^{b}

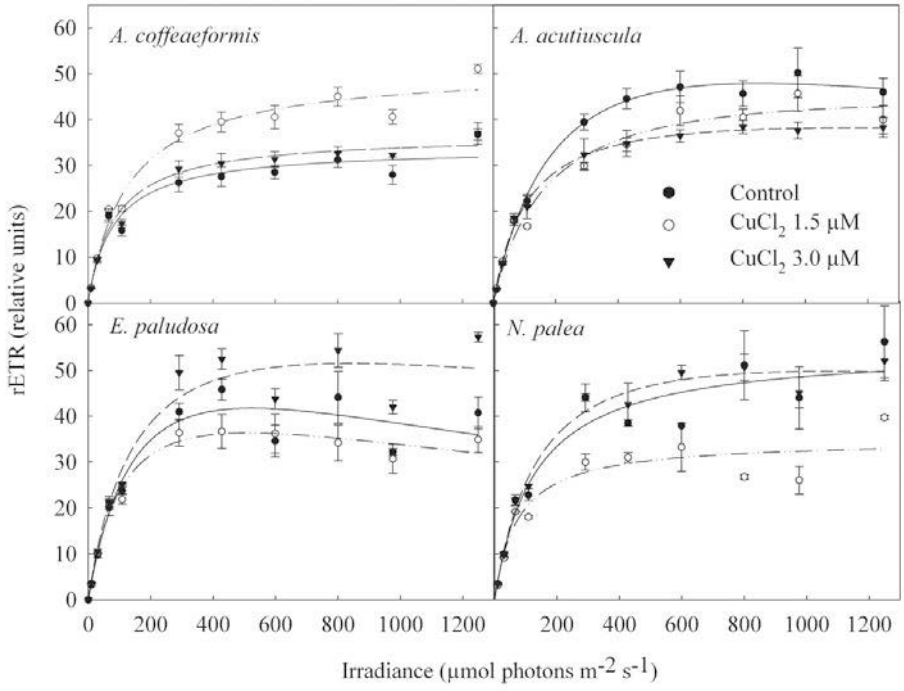

Fig. 2. Relative electron transport rate versus irradiance in *Amphora coffeaeformis*, *Amphora acutiuscula*, *Entomoneis paludosa* and *Nitzschia palea* grown in ASW (control) or in the presence of 1.5 and 3.0 µM $CuCl_2$ added to ASW. Mean values ± SE (n = 3 – 5).

A similar phenomenon was also observed for the saturating irradiance parameters in these two species. No significant difference was found in light utilization coefficient for any species in response to copper treatment (except for *A. acutiuscula* after adding 3.0 μM Cu to the medium).

Table 4. Parameters (α_{rETR}, light utilization coefficient; $rETR_{max}$, maximum relative electron transport rate; E_{krETR}, light saturation) of relative electron transport rate versus irradiance curves (Fig. 2) in *Amphora coffeaeformis, Amphora acutiuscula, Entomoneis paludosa* and *Nitzschia palea* grown in ASW (control) or in presence of added 1.5 and 3.0 μM $CuCl_2$ to ASW. Significant different data are indicated with different superscripted letters (Tukey Test, $P \leq 0.05$). Mean values ± SE (n = 3 – 5). α_{rETR}: rETR (μmol photons m^{-2} $s^{-1})^{-1}$; $rETR_{max}$: relative units; E_{krETR}: μmol photons m^{-2} s^{-1}.

Species	*rETR*			*$rETR_{max}$*			*E_{krETR}*		
	Control	*1.5 μM*	*3.0 μM*	*Control*	*1.5 μM*	*3.0 μM*	*Control*	*1.5 μM*	*3.0 μM*
Coffeaeformis	0.39 ± 0.02^{a}	0.39 ± 0.01^{a}	0.38 ± 0.02^{a}	34 ± 2^{a}	50 ± 1^{b}	39 ± 2^{a}	87 ± 6^{a}	130 ± 5^{b}	104 ± 6^{ab}
Acutiuscula	0.32 ± 0.01^{a}	0.29 ± 0.01^{a}	0.41 ± 0.03^{b}	44 ± 2^{a}	43 ± 2^{a}	36 ± 1^{b}	137 ± 3^{a}	151 ± 10^{a}	92 ± 9^{b}
Paludosa	0.39 ± 0.03^{a}	0.44 ± 0.03^{a}	0.43 ± 0.01^{a}	43 ± 2^{a}	37 ± 4^{a}	54 ± 2^{b}	113 ± 9^{ab}	87 ± 15^{a}	126 ± 8^{b}
Palea	0.41 ± 0.02^{a}	0.46 ± 0.02^{a}	0.43 ± 0.02^{a}	56 ± 7^{a}	33 ± 1^{b}	51 ± 3^{a}	141 ± 26^{a}	74 ± 5^{b}	121 ± 10^{a}

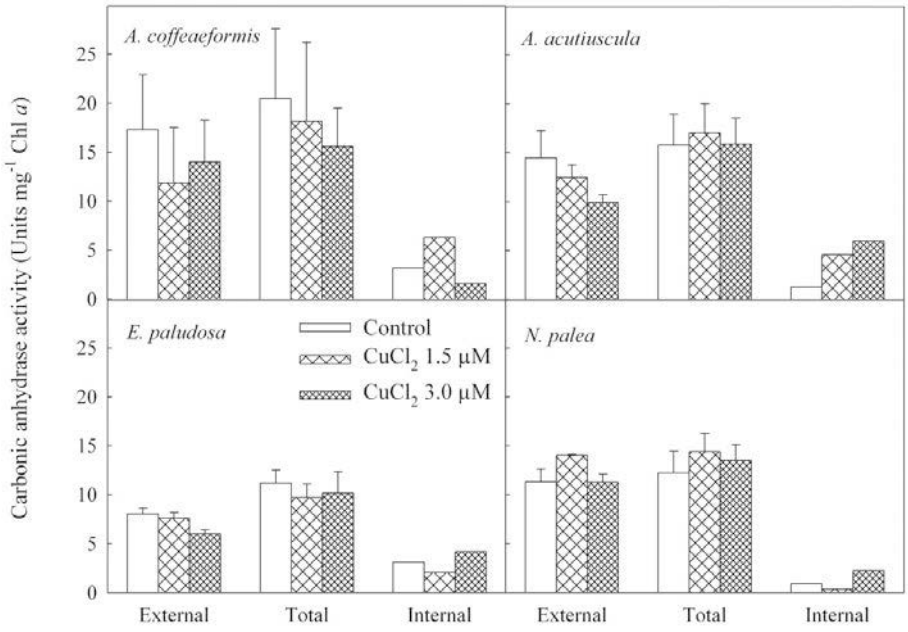

Fig. 3. External, internal and total carbonic anhydrase activities in *Amphora coffeaeformis*, *Amphora acutiuscula*, *Entomoneis paludosa* and *Nitzschia palea* grown in ASW (control) or in the presence of 1.5 and 3.0 μM $CuCl_2$ added to ASW. Mean values ± SE (n = 3 – 5).

Carbonic anhydrase activity

External and total CA activities were measured in all four diatoms, and CA_{ext} was always higher than CA_{int} (Fig. 4). The presence of copper did not affect CA_{ext} in *N. palea*, but tended to reduce this parameter in the other species. Adding 3.0 μM Cu to the medium reduced CA_{int} activity in *A. coffeaeformis*, but increased the activity of this enzyme in the other three diatoms.

DISCUSSION AND CONCLUSION

In this study, marine diatoms were grown in artificial seawater that allows most Bacillariophyceae to develop properly (Harrison *et al.*, 1980). We observed that all the four diatoms tested, especially the *Amphora* species, adhered when grown in the media supplemented with Cu. The growth rate data show that adding 1.5 μM or 3.0 μM Cu did not disrupt cell division, and that adding 3.0 μM Cu increased growth in *E. paludosa* and *N. palea*. In contrast, adding Cu to the medium reduced the maximum cell density of all four species. In *Pheaodactylum tricornutum* exposed to 1.57 μM Cu for 24 h, Cid *et al.* (1995) observed a reduction of 19% in the growth rate and of 50% in the maximum cellular density compared to control values. In *Scenedesmus obliquus*, Yan & Pan (2002) showed that 0.79 μM Cu induces 50% inhibition of the growth rate compared to control in experiments lasting 4 days (96-h EC_{50}), while Dewez *et al.* (2005) reported a 48-h EC_{50} of 15.75 μM of Cu in the same species. These studies highlight the fact that microalgae have different levels of Cu tolerance. To cope with excess Cu in the medium, *Cylindrotheca fusiformis* and *Gymnodinium sp.* extrude carbohydrates (Pistocchi *et al.*, 1997). Andrade *et al.* (2004) have shown that *Enteromorpha flexuosa* can form metal complexes that are stored in the vacuole during short-term metal stress. Because Cu is involved in various proteins and metal complexes it can affect cell mechanisms and disrupt the active sites of some enzymes (Cid *et al.*, 1995; Maksymiec, 1997). The reduction in the maximum cell density in the presence of added copper indicated that 1.5 μM or 3.0 μM, depending on species, disrupted the cell metabolism in diatoms. Moreover, some differences between experimental protocols can influence the results of toxicology tests that are based solely on growth (Moreno-Garrido *et al.*, 2000; Eisentraeger *et al.*, 2003).

Nielsen & Nielsen (2005) have shown no correlation between Cu toxicity, photosynthesis activities and growth in the macroalga *Fucus serratus* (Phaeophyceae). Our data are in agreement with this observation, except in the case of *A. coffeaeformis* at the growth irradiance (300 μmol photons m^{-2} s^{-1}). Measurements of O_2 evolution in darkness (Table 2) showed that adding Cu (1.5 μM for both *Amphora* species, and 3.0 μM for *E. paludosa* and *N. palea*) increased the respiration of the diatoms. This increase shows that diatoms require energy to cope with metal stress, and this has a detrimental effect on cell production (lower maximum cellular density). This could be related to the formation of intracellular (phytochelatins) or extracellular (carbohydrates) chelating complexes, which allows algae to tolerate some types of metal stress (Pistocchi *et al.*, 1997).

For the four diatom species grown with or without Cu, the P vs. E and rETR vs. E curves showed similar trends, with a slight photoinhibition

phenomenon observed at high irradiance levels (from about 800 μmol photons m^{-2} s^{-1}). In *A. acutiuscula* only, a similar pattern was observed in the P vs. E and rETR vs. E curves. In this species, 3.0 μM Cu significantly affected all photosynthesis parameters (Tables 3 and 4). On the other hand, Cu treatments affected gross photosynthesis in *A. coffeaeformis* and *E. paludosa*, and increased the rETR. In these two species, Cu did not affect the functioning of PSII. Nielsen & Nielsen (2005) also reported an increase in rETR in the macroalga *F. serratus* grown with 0.84 μM Cu. In contrast, in *N. palea*, 1.5 μM Cu increased gross photosynthesis, but reduced rETR. In this species, Cu could target PSII in particular. Depending on the species and the Cu treatment used, copper could affect not only the physiological process of photosynthesis, but also the ultrastructural organization of the chloroplasts without changing the chlorophyll content (data not shown). Andrade *et al.* (2004) reported some changes in cellular metabolism such as an increase in starch granules and disorganization of the thylakoid membrane in the green macroalga *Enteromorpha flexuosa* exposed to 50 μg L^{-1} Cu (0.78 μM).

Lane & Morel (2000) have shown that in vitro CA in diatom extracts can use co-factors other than Zn (Co and Cd) to catalyze the reversible conversion of CO_2 to HCO_3^-. On the other hand, in *Chlamydomonas reinhardtii* exposed to 2.0 mg L^{-1} Cu (31 μM) for 24 h, CA activity fell to 50% of control (Wang *et al.*, 2005). These authors have also observed that Cu binding to catalytic sites triggers structural changes that reduce the stability and enzymatic activity of CA. In *A. coffeaeformis*, *A. acutiuscula* and *E. paludosa*, CA_{ext} activity was slightly inhibited by the presence of Cu, whereas in *N. palea*, CA_{ext} activity was increased by exposure to 1.5 μM Cu. It is noticeable that CA_{ext} activity underwent similar changes to gross photosynthesis at growth irradiance. Moreover, CA_{ext} activity was significantly higher than CA_{int} activity in all four diatoms studied, whereas Patel & Merrett (1986) and Morant-Manceau *et al.* (2007) have shown that CA_{ext} activity in marine diatoms accounts for 36 to 67% of CA_{tot} activity. In a previous study (Morant-Manceau *et al.*, 2007), algal cultures were refreshed a day before enzymatic measurements to maintain a pH of 8.3, whereas in the study reported here the diatoms were exposed to Cu for 5 days without changing the medium. Under these conditions the pH of the medium increased from 8.7 to about 9.3. This alkaline pH resulted from a fall in the CO_2 concentration, which in turn stimulated CA_{ext} activity (Moroney *et al.*, 2001). Apart from this, CA_{tot} values for the *A. coffeaeformis* and *E. paludosa* controls were in the same range as previously reported (Morant-Manceau *et al.*, 2007).

In conclusion, these two Cu concentrations had differing impacts on growth, photosynthesis and carbonic anhydrase activities in different species. Our findings show that *N. palea*, usually found in polluted areas, has a similar behaviour to the other species. In the natural environment, only higher Cu concentrations could modify diatom assemblages. Further investigations on the production of phytochelatins and antioxidant enzymatic activities are in progress.

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