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Effects of copper and butyltin compounds on the growth, photosynthetic activity and toxin production of two HAB dinoflagellates : the planktonic *Alexandrium catenella* and the benthic *Ostreopsis* cf. *ovata*

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Abstract :

Controlled laboratory experiments were conducted to test the effects of copper (Cu^{2+}) and butyltins (BuT) on the growth, photosynthetic activity and toxin content of two HABs (Harmful Algal Blooms) dinoflagellates, the planktonic *Alexandrium catenella* and the benthic *Ostreopsis* cf. *ovata*. Microalgae were exposed to increasing concentrations of Cu^{2+} (10^{-4} to 31 nM) or BuT (0.084 to 84 nM) for seven days. When considering the growth, EC_{50} values were 0.16 (±0.09) nM and 0.03 (±0.02) nM of Cu^{2+} for *A. catenella* and *O.* cf. *ovata*, respectively. Regarding BuT, EC_{50} was 14.2 (±6) nM for *O.* cf. *ovata*, while *A. catenella* growth inhibition appeared at BuT concentrations ≥27 nM. Photosynthetic activity of the studied dinoflagellates decreased with increasing Cu and BuT concentrations. For *O.* cf. *ovata*, the response of this physiological parameter to contamination was less sensitive than the biomass. Cu exposure induced the formation of temporary cysts in both organisms that could resist adverse conditions. The ovatoxin-a and -b concentrations in *O.* cf. *ovata* cells increased significantly in the presence of Cu. Altogether, the results suggest a better tolerance of the planktonic *A. catenella* to Cu and BuT. This could result in a differentiated selection pressure exerted by these metals on phytoplankton species in highly polluted waters. The over-production of toxins in response to Cu stress could pose supplementary health and socio-economic threats in the contaminated marine ecosystems where HABs develop.

Highlights

► Growth rate and photosynthetic activity of the studied dinoflagellates decreased with increasing Cu and BuT concentrations. ► *Alexandrium catenella* was more tolerant to Cu and butyltins compared to *Ostreopsis* cf. *ovata*. ► Cu induced the formation of temporary cysts for *both species*. ► Cu induced toxin production in *Ostreopsis* cf. *ovata*.

Keywords : *Alexandrium catenella*, *Ostreopsis* cf. *ovata*, Cu, butyltin, photosynthesis, toxins

1. Introduction

Pollution of the marine environment is one of the biggest challenges facing humanity, as it can impact issues related to health, economic and environmental (Schwarzenbach et al., 2006). Metal input in marine ecosystems particularly raises concerns, since it has become one of the major pollution types ever recorded (Naser, 2013). Among this kind of contaminants, Cu and organotins have been widely used for decades in industrial activities, such as petroleum refining and the manufacture of antifouling chemical products (Alzieu et al., 1991; Wake, 2005). Tributyltin (TBT), a butyltin (BuT) compound, was designed in the 1950's as the most effective anti-fouling system available in the market for decades (Omae, 2006). However, TBT has been shown to induce imposex (female develops male sex organs such as a penis and a vas deferens) in the gastropod *Nucella lapillus* but also serious anomalies in calcification mechanisms in the oyster *Crassostrea gigas* which could lead to population decline (Bettin et al., 1996; Gibbs and Bryan, 1986, Alzieu et al. 1986). Due to its global harmful effects on marine biota at very low concentrations (a few ng L^{-1}) (Smith, 1996; Solé et al., 1998), TBT was banned from antifouling paints for boats by the International Maritime Organisation in 2008. However, despite this ban, TBT and butyltins in general are still use inland (as biocides in agriculture, catalysts, and heat stabilizers for polyvinylchloride, PVC) and continue to be released in marine coastal waters through urban and industrial sewage sludge (Díez et al., 2002; Sabah et al., 2016). TBT persists in high concentrations in some coastal waters, reaching up to 8 000 ng Sn g^{-1} of sediment and 70 ng Sn L^{-1} of seawater in some ecosystems (Abidli et al., 2015; Briant et al., 2016; Hartwell et al., 2016). TBT replacement by Cu in antifouling paint coincided with an increase in the use of Cu in industrial activities or as a biocide, and this has resulted in Cu accumulation in marine sediments and waters (Cossa et al., 2017; Zohra and Habib, 2016). The United States Environmental Protection Agency (US EPA) considered Cu as one of the greatest environmental concerns in vessel discharges (Tornero and Hanke, 2016), indicating multiple potential negative impacts of Cu on marine organisms. Hence,

phytoplankton species are able to bio-accumulate a wide range of pollutants including Cu and butyltin and can transfer them to higher trophic levels, leading to dramatic effects on many organisms (Flouty and Estephane, 2012; Fortibuoni et al., 2013).

During the last few decades, anthropization was argued to be a causative factor of harmful algal blooms (HABs) (Berdalet et al., 2016; Davidson et al., 2014). HABs have become a serious problem in many coastal marine ecosystems, since their distribution, frequency and intensity is continuously increasing (Anderson et al., 2012; Sellner et al., 2003). Among HAB dinoflagellate species, the planktonic *Alexandrium catenella* and the benthic *Ostreopsis* cf. *ovata* are characterized by high toxicity. *A. catenella* and *O.* cf. *ovata* are producers of paralytic shellfish toxins (PSTs) and ovatoxins, respectively (Amzil et al., 2012; Hallegraeff, 1993). They intoxicate marine organisms and humans through the consumption of marine mollusks or the breathing of marine aerosols and thus threaten public health and socio-economic activities (Accoroni et al., 2011; Parsons et al., 2012). However, the sensitivity of these harmful organisms to metal pollution was scarcely studied, although the Cu effects on numerous phytoplankton species have been widely investigated. Cu is essential for living organisms as it is involved in many metabolic processes, such as photosynthesis and mitochondrial respiration (Hänsch and Mendel, 2009). It also forms complexes with cellular proteins, such as plastocyanin (Jansson et al., 2003) and cytochrome c oxidase (Peiffer et al., 1990). However, signs of cellular stress and growth inhibition appear at high Cu concentrations, with the EC_{50} (concentration corresponding to 50% of the activity relative to control) values for phytoplankton being at the µM level (Perales-Vela et al., 2007; Yan and Pan, 2002). Cu impacts photosynthesis in various ways, affecting both pigment concentration and photosystem II (PSII) efficiency. In PSII, Cu inhibits the electron transport at P680 and leads to the closing of reaction centers (Cid et al., 1995; Schroders et al., 1994). It can also alter the rate of oxidoreduction, leading to an impairment of PSII electron transport (Jegerschold et al., 1995; Yruela et al., 1992). The toxicity of dissolved metals to phytoplankton is not related to their total concentration but to their free ion activity (Santore et al., 2001; Waite and Morel, 1983). This is particularly important for Cu,

which has a strong affinity for organic ligands, such as ethylene diamine tetra-acetic acid (EDTA) commonly used in culture media and which decreases Cu toxicity. However, despite the important number of studies concerning Cu effects on phytoplankton, only a few give the Cu^{2+} concentration, making comparison between studies or *in situ* values difficult to achieve. On the other hand, butyltin toxicity studies have mainly focused on invertebrates and fishes (Hanana et al., 2014; Kwok and Leung, 2005), and the lack of studies on phytoplankton has been highlighted (Petersen and Gustavson, 2000). Few studies tested the TBT effects on microalgae; for example, the chlorophyte *Tetraselmis suecica* density and chlorophyll biomass were impacted with an EC_{50} of 20 µg mL^{-1} (Yong et al., 2007). The microalgae *Nannochloropsis oculata* was more sensitive, with an EC_{50} of 0.89 nM, and it showed a decrease in pigment and protein content after TBT exposure (Sidharthan et al., 2002). However, a more precise investigation of TBT effects on photosystem activity (such as maximum quantum yield of photosynthesis) are still lacking in the literature.

Despite the large number of studies of Cu effects on phytoplankton, and to a smaller extent of butyltin, only a few considered harmful species, particularly benthic species. It has been shown that under Cu exposure, *Prorocentrum minimum* and *Alexandrium catenella*, two toxic dinoflagellates, showed an inhibition of growth, maximum quantum yield of photosynthesis (Fv/Fm) and pigment concentration (Herzi et al., 2013; Lage et al., 1994; Miao et al., 2005). Photosystem gene expression was also decreased in the Cu-treated *P. minimum* (Guo et al., 2016), indicating gene regulation action of Cu. Until now, the effect of Cu and BuT has not been investigated in *O.* cf *ovata*. It has been shown that some environmental factors, such as temperature, irradiance and nutrients, could affect the toxin content of some dinoflagellate species (Etheridge and Roesler, 2005; Pezzolesi et al., 2012). However, the observed effects on toxin content and profile could be contradictory depending on the coupled abiotic factor/microalgae species, showing complex and variable responses (Anderson et al., 2012; Laabir et al., 2013). Regarding the metallic stress effect on toxin production, very few studies have been performed on HAB species. Yessotoxin content increased in *Protoceratium reticulatum* cells treated with selenium at the pM level, but not when treated with

iron or cobalt (Mitrovic et al., 2004). Similarly, production of domoic acid by *Pseudo-nitzschia australis* increased under Cu exposure (Maldonado et al., 2002). These studies highlighted a potential metal effect on toxin production in HAB species. To our knowledge, this has never been investigated for Cu and butyltin.

The main objective of this study was to investigate the effects of Cu and BuT on the growth, photosynthetic efficiency and toxin production of the harmful dinoflagellates *A. catenella* and *O.* cf. *ovata* through controlled laboratory experiments. Moreover, cupric ion concentrations (Cu^{2+}) were calculated to compare our results with literature data and *in situ* concentrations.

2. Material and methods

2.1 Dinoflagellate cultures

Non-axenic monoclonal cultures of the benthic toxic dinoflagellate *Ostreopsis* cf. *ovata* (OOBZT14) were conducted using enriched natural seawater medium (ENSW) (Harrison et al., 1980), at stable conditions of salinity 36, temperature of 25 °C and irradiance of 100 µmol photons m^{-2} s^{-1} in a 12:12 light:dark cycle. The planktonic dinoflagellate *Alexandrium catenella* (ABZ1) was cultured under the same conditions, but at a temperature of 20 °C and salinity 35 corresponding to its optimal growth. OOBZT14 and ABZ1 strains were isolated in the Bay and the lagoon of Bizerte (Western Mediterranean Tunisia), respectively (Ben-Gharbia et al., 2016; Fertouna-Bellakhal et al., 2015).

2.2 Spiking solutions of Cu (Cu) and TBT: preparation and determination of initial and final concentrations

Cu- and TBT-supplemented stock solutions were prepared with $CuCl_2$ and $[CH_3(CH_2)_3]_3SnCl$ salts (Merck), respectively, diluted in ENSW medium. Triplicates of 250 mL sterile plastic flasks (final culture volume of 200 mL) were obtained with nominal concentrations of 15.7, 157, 504, 1575, 5039, 15 748 and 50 394 nM of Cu and 0.085, 0.27, 0.85, 2.7, 8.5, 27 and 85 nM of TBT.

2.2.1 Copper measurements

Despite the presence of EDTA (ethylene diamine tetra-acetic acid, a complexing agent facilitating the assimilation of micro-nutrients by the microalgae) in the culture medium, which forms complexes with Cu in solution, Cu could adsorb on flask walls or on cell surfaces or be taken up by the phytoplankton cells. Therefore, in order to better characterize the Cu exposure of *A. catenella* and *O.* cf. *ovata*, the dissolved concentration of Cu was measured in the flasks at the beginning (day 0) and at the end (day 7) of the incubations. For this, aliquots of the culture were filtered through 0.22 µm acetate cellulose filters and acidified with nitric acid Merck Suprapur. Analyses were carried out using ICP-MS-Q, iCAP-Q (Thermo Scientific) equipped with a high matrix interface. More details on the analytical procedure can be found in Pringault et al. (2016).

The bioavailable Cu^{2+} concentration in the ENSW culture medium was calculated using the computer program PHREEQC with the MinteqV4 database (Parkhurst and Appelo, 2013). For the laboratory experiments, the used dissolved bioavailable Cu^{2+} concentrations were 1.10^{-4} nM, 9.10^{-4} nM, 32.10^{-4} nM, 12.10^{-3} nM, 60.10^{-2} nM, 31 nM for *A. catenella* and 3.10^{-5} nM, 9.10^{-4} nM, 30.10^{-4} nM, 17.10^{-3} nM, 32.10^{-3} nM, 48.10^{-2} nM and 13nM for *O.* cf. *ovata*.

2.2.2 Butyltin measurements

During the experiment, aliquots were filtered through 0.22 µm acetate cellulose filters and acidified with nitric acid Merck Suprapur. The butyltin species, monobutyltin (MBT), dibutyltin (DBT) and tributyltin (TBT), concentrations were measured using a gas chromatograph (Focus GC Thermo Fisher Scientific®) coupled with an inductively coupled plasma mass spectrometer (ICP-MS X Series II-Thermo Fisher Scientific®). Headspace using a polydimethylsiloxane (PDMS) solid-phase microextraction (SPME) was used to preconcentrate the samples. The limit of detection was in the order of pg Sn L^{-1} for the three butyltin species (Briant et al., 2016).

The measured concentrations in the culture media spiked highlighted strong debutylation. Thus, doping initially made with TBT in solution, for concentrations of 0.084, 0.27, 0.84, 2.7, 8.4, 27 and 84 nM, led to a real initial concentration range from 0.00 to 9 nM, including the three forms of

butyltin: TBT + DBT + MBT. For practical considerations, TBT, DBT and MBT concentrations were pooled together and referred to as butyltin (BuT).

2.3 Incubation experiments

Cultures in the exponential growth phase of *A. catenella* and *O.* cf. *ovata* were used to inoculate sterile flasks each containing 200 mL of ENSW culture medium to reach a cell density of 500 to 1000 cells mL^{-1} at T_0. The contaminant gradients of Cu and butyltin were described above. Each experiment was conducted for 7 days, and 1.5 mL sub-samples were taken daily for the different measurements and observations detailed below. All treatments, including controls, were performed in triplicate.

2.4 Growth rate measurements and cell morphology observations

A 100 µL aliquot of the sub-sample was fixed with formalin to determine cell abundances in cells mL^{-1} at days 0, 1, 2, 3 and 7. Counting of fixed cells in the microplates was performed with a light microscope (Zeiss AxioVert.A1). Maximum growth rate (µmax; expressed in day^{-1}) was calculated according to Guillard (1973), from the slope of a linear regression over the entire exponential phase of growth by the least square fit of a straight line to the data after logarithmic transformation; $\mu max = [Ln(N_t) - Ln(N_0)/(T_t - T_0)]$, with N_0 and N_t, cell densities (cells mL^{-1}) at the beginning (T_0) and end (T_t) of the exponential phase, respectively. At day 7, cell morphology was observed and photomicrographies of cells were taken using a photonic microscope and a camera (ProgRes CF). Growth rate values were used to calculate the EC_{50}, the concentration at which 50% of control growth rate is reached, according to the Hill (1910) equation, using the Regtox macro for excel developed by Vindimian et al. (1983).

2.5 Photosynthetic activity measurements

The photosynthetic performance was estimated on the basis of the *in vivo* fluorescence of photosystem II (PSII) of the microalgae. Subsamples of 1.5 mL were taken at days 0, 1, 2, 3 and 7, and the fluorescence was measured with a portable Pulse Amplitude Modulation fluorometer (Aquapen C-APC100, Photon System Instruments), after 30 min of dark adaptation. The maximum quantum yield of photosystem II (Fv/Fm) was calculated following Strasser et al. (2000). Light response curves (LRC) were obtained for *O.* cf. *ovata* experiments under Cu and butyltin exposure at days 0, 3 and 7. For LRC, quantum yields (Qy) were measured in response to seven increasing light intensities (10, 20, 50, 100, 300, 500 and 1000 µmol photons m^{-2} s^{-1}) for 60 seconds in each phase. The relative electron transport rate (rETR) was obtained by multiplying Qy by the corresponding irradiance to plot the LRC as rETR against irradiance. From these curves, it was possible to model the photosynthetic parameters α (the maximum light use coefficient), Pmax (rETR max) and Ek (saturating irradiance) using the Platt et al. (1980) equation.

2.6 Cellular toxin content measurements

The toxin content and profile of *A. catenella* and *O.* cf. *ovata* cells were determined at the end of the experiment (day 7) for the control and the following nominal concentrations: 1 570 nM of Cu and 2.7 nM of butyltin. Then, 28 mL of the culture was sampled in each flask in triplicate and then centrifuged (3000× g, 8 min, 4 °C). The supernatant was carefully removed and the culture pellets were frozen at -20 °C until the extraction and toxin analyses were performed.

2.6.1 Chemical analysis of paralytic shellfish toxins (PSP) by liquid chromatography/fluorescence detection (LC/FD)

The culture pellets of *A. catenella* were suspended in 1 mL of 0.1 N acetic acid. To release the toxins, the samples were sonicated for 5 min in a water bath three times and centrifuged at 17,000× g for 10 min at 4 °C. The toxin analyses of filtered supernatants were performed using the LC/FD

PSP toxin analyses method of Van De Riet (2011). The toxins GTXs, dc-GTXs, dc-STXs and STXs were separated by reverse chromatography using a RP column (Zorbax Bonus RP, 3.5 µM, 4.6 × 150 mm) with a flow rate of 0.8 mL min^{-1}. The C-toxins were separated by a Thermo Beta Basic 8 column (5 µm, 4.6 × 250 mm) with a flow rate of 0.8 mL min^{-1}. The eluent pH and/or column temperature were optimized for the separation of some toxins (dc-GTX3/GTX5/dc-GTX-2 & C1/C2). The toxins were quantified using certified standards provided by CNRC (Halifax, Canada).

2.6.2 Chemical analysis of ovatoxins and palytoxins by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Toxin extraction from *O.* cf. *ovata* culture was carried out according to the Amzil procedure (Amzil et al., 2012). The filtered culture pellets were suspended in 1 mL of methanol/water (1/1). To release the toxins, the samples were sonicated for 40 min, while cooling in an ice bath. The treated sample was centrifuged at 3000× *g* at 4 °C for 15 min. The supernatant was filtered through a 0.2 µm filter, and the toxin profile was determined by LC-MS/MS (Brissard et al., 2014). LC-MS/MS experiments were performed using an LC system (UFLC XR, Shimadzu) coupled to a hybrid triple quadrupole/ion-trap mass spectrometer (API 4000 Qtrap, ABSCIEX). Toxins were separated on a 3 µm C18 Gemini column (150 × 2.0 mm, Phenomenex), thermostated at 22 °C with water (A) and 95% acetonitrile/water (B), both containing 2 mM ammonium formiate and 50 mM formic acid at a 0.2 mL min^{-1} flow rate. The gradient was raised from 20% to 100% B in 10 min and was held over 4 min, before dropping to the initial conditions.

Mass spectrum detection was carried out in multiple reactions monitoring (MRM) mode (positive ions). MRM experiments were established using the following source setting: curtain gas set at 30 psi, ion spray at 5000 V, a turbogas temperature of 300 °C, gas 1 and 2 set at 30 and 40 psi, respectively, and an entrance potential of 10 V. To permit the best toxin identification, each toxin was quantified with three specific transitions (Brissard et al., 2014). Because only the palytoxin

standard was available, quantitative determination of ovatoxins in extracts was carried out assuming that their molar responses were similar to palytoxin.

2.7 Statistical analysis

Data were analyzed using a one-way Anova followed by Tukey's post hoc test, in order to determine any significant differences between treatments in the growth curves and in the toxin content of the cells. Differences were considered significant at p-value < 0.05. We used Sigmaplot Software (Systat Software Inc).

3. Results

3.1 Measurements of contaminants

Fig. 1 shows these values at day 7. The nominal and measured concentrations of copper are presented in Fig. 1A on a logarithmic scale. Linear regressions were plotted for each experiment, and equations corresponded to $y = 0.67\ x^{0.95}$ and $y = 0.60\ x^{0.97}$ for the *A. catenella* and *O.* cf. *ovata* experiments, respectively. These equations indicated losses of the measured dissolved copper of 33% and 40% during the experiments of *A. catenella* and *O.* cf. *ovata*, respectively. The power index close to 1 showed a linear increase between each concentration. The computed values of the bioavailable Cu^{2+} are plotted together with the dissolved values for copper using a logarithmic scale in Fig. 1B. Equations of the linear regression indicated a ratio of 10^5 between dissolved values and computed Cu^{2+}. The power index close to 1 revealed a continuous increase of all the tested concentrations, as was expected. The nominal and measured dissolved butyltin concentrations are plotted in Fig. 1C. The measured concentrations were below expected values and showed an increase for the *A. catenella* and *O.* cf. *ovata* experiments. Butyltin speciation was also measured for tributyltin (TBT), dibutyltin (DBT) and monobutyltin (MBT) (data not shown). For both experiments, TBT was the dominant form (30% to 70%) at T_0, while DBT was the dominant form

(20% to 80%) at T_7. Successive debutylation, i.e. sequential dealkylation resulting in dibutyltin (DBT) then monobutyltin (MBT) and finally inorganic tin (TBT → DBT → MBT → Sn) was observed. For practical consideration, the three butyltin forms were pooled together and named here as butyltin (BuT).

3.2 Effects on growth

Free dissolved bioavailable Cu^{2+} values (calculated as described above) were represented for the Cu experiments, and nominal butyltin values were reported for the BuT experiments. Control cultures of *A. catenella* reached cell densities of 7000 and 10,500 cells mL^{-1} for the Cu and BuT experiments, respectively. Results showed that Copper and BuT had a significant ($p<0.05$) negative effect on the growth of both dinoflagellates. These contaminants provoked in *A. catenella* a threshold-type response (Fig. 2A and 2C). The growth curves indicated limited effects up to 12.10^{-3} nM of Cu^{2+} and 30.10^{-4} nM of BuT. The growth of this dinoflagellate was reduced significantly ($p<0.05$) from 0.032 nM of Cu^{2+} and from 27.10^{-1} nM of BuT, and was completely suppressed for Cu^{2+} concentrations of 0.6 nM and BuT concentrations of ≥27 nM for BuT.

O. cf. *ovata* responses to Cu and butyltin were different from those of *A. catenella*, with a significant ($p<0.05$) deleterious effect appearing since the first concentrations tested, 3.10^{-5} nM of Cu^{2+} and 84.10^{-3} nM of BuT (Fig. 2B and 2D). *O.* cf. *ovata* control cultures showed a lag phase of 2 days, reaching around 3600 cells mL^{-1} at day 7 for both Cu and BuT experiments. The growth of this dinoflagellate was gradually affected, with intermediate effects on the cell density from tested concentrations of 3.10^{-5} nM to 0.017 nM for Cu^{2+} and from 0.084 nM to 8.4 nM for BuT. Total growth inhibition was induced by concentrations of ≥0.48 nM for Cu^{2+} and ≥27 nM for BuT. EC_{50} calculations were derived from the curves linking growth rates to contaminant concentrations using Regtox modelization (Fig. 3). For Cu exposure, EC_{50} values were 0.16 (±0.09) nM for *A. catenella* and 0.03 (± 0.02) nM for *O.* cf. *ovata* (Fig. 3A). For BuT treatment, the EC_{50} value was 14.2 (±6)

nM for *O.* cf. *ovata* (Fig. 3B). The EC_{50} calculation for BuT treatment was not possible for *A. catenella* because of a lack of intermediate values.

3.3 Cell morphology

When exposed to 0.6 and 31 nM (Cu^{2+}), *A. catenella* cells were round-shaped, corresponding to temporary cysts (Fig. 4B), which are frequently produced in adverse environmental conditions. Fig 4C shows an uncompleted asexual ecdysis of *A. catenella* temporary cyst (Figueroa et al. 2007). *O.* cf. *ovata* exposed to concentrations of ≥0.03 nM Cu^{2+} exhibited altered (Fig 4G) thin-walled cysts with dense granular material (Fig. 4G and 4H). Butyltin concentrations of ≥8.4 nM provoked severe structural cellular damage in the two dinoflagellates. Cells lost their intracellular organelles and became lytic (Fig. 4D, 4E, 4I and 4J), leading to cell death.

3.4 Effect on photosynthetic activity

Maximum quantum yield (Fv/Fm) evolution through time and contaminant exposure is presented in Fig. 5. Fv/Fm mainly showed a threshold-type response for both organisms exposed to Cu and BuT. A significant decrease in activity was observed for concentrations above 0.60 nM (Cu^{2+}) for *A. catenella* and 0.32 nM (Cu^{2+}) for *O.* cf. *ovata* (Fig. 5A and 5B). Under BuT exposure, concentrations above 27 nM decreased the Fv/Fm value to reach only 10% of the control after 2 days of the incubation period (Fig. 5C and 5D).

Photosynthetic-irradiance (PI) curves (rETR against irradiance) were calculated for *O.* cf *ovata* under Cu and BuT exposure. Fig. 6 shows the PI curve measured after 7 days of culture in control for two distinct Cu concentrations (0.032 nM and 13 nM). Exposure to 0.032 nM (Cu^{2+}) did not significantly affect the PI curve, with a Pmax of 109 (± 15.5) and 128 (± 23) and an Ek of 148 (± 24) and 169 (± 30) µmol photons m^{-2} s^{-1}, respectively. In contrast, the treatment with 13 nM Cu^{2+} flattened the curve, with a Pmax of 34.6 (± 1.36) and an Ek of 66.1 (± 10.2) µmol photons m^{-2} s^{-1}. The initial slope of the curve (α), was also affected by the 13 nM Cu treatment and decreased from

0.74 (± 0.02) to 0.53 (±0.06). Photosynthetic parameters (α, Pmax, Ek) were determined from PI curves and plotted against contaminant concentrations at days 0, 3 and 7 (Fig. 6). There was an overall tendency for all parameters to decrease when cells were exposed to increasing concentrations of Cu and BuT. Upon Cu exposure, above 0.032 nM (Cu^{2+}), the α value decreased to 0.55 at days 3 and 7 (Fig. 6A), relative to 0.75 in the control. Similar trends were observed for BuT, with a strong decrease of α (0.2 vs 0.7 in the control) for concentrations above 27 nM of BuT (Fig. 6D). The Ek and Pmax parameters followed the same patterns: they decreased as a function of time and contaminant concentration. Moreover, the control value of Ek at day 3 was between 200 and 250 µmol photons m^{-2} s^{-1}, and decreased to 60 µmol photons m^{-2} s^{-1} and 0 µmol photons m^{-2} s^{-1} for the highest Cu and BuT exposures, respectively. The Pmax at day 3 dropped from 210 to 60 when exposed to the highest Cu concentration (Fig. 6C), and dropped from 240 to 0 at the highest BuT concentration (Fig. 6F).

3.5 Effect on toxin content

Ovatoxin-a and ovatoxin-b were detected at 3.64 (± 0.31) pg PITX equivalents $cell^{-1}$ and 1.64 (± 0.16) pg PITX equivalents $cell^{-1}$, respectively, during the Cu experiment, but ovatoxin-e and -g were below the detection limit (for both experiments) (Fig. 7). When cells were exposed to Cu at a concentration of 17.10^{-3} nM (Cu^{2+}), the toxin content significantly ($p<0.05$) increased to 8.71 (± 1.31) pg PITX equivalents $cell^{-1}$ and 4.55 (± 0.45) pg PITX equivalents $cell^{-1}$ for ovatoxin-a and ovatoxin-b, respectively (Fig. 7A). The BuT treatment (27.10^{-1} nM) had no significant effect on ovatoxin-a or ovatoxin-b (Fig. 7B).

PSTs measurements in the Cu and BuT experiments are presented in Figs 7C and 7D respectively. Toxins GTX6, C3, C4, STX, dc-STX, GTX-2, dc-GTX-2, dc-GTX-3 were not detected, whereas GTX-1 and GTX-5 were detected only in the BuT experiment. The toxins GTX-4, GTX-3, Neo-STX, C1 and C2 were detected in both experiments, with GTX-4 being the most abundant toxin at 2.00 pg $cell^{-1}$ and 2.82 pg $cell^{-1}$ in the Cu and BuT experiments, respectively. The total toxin content

was enhanced during Cu exposure (from 2.78 to 4.20 pg cell^{-1}, Fig. 7C) and decreased during BuT exposure (from 7.09 to 5.17 pg cell^{-1}, Fig. 7D). Under Cu exposure, the toxin content of GTX-3 and C1 were significantly ($p<0.05$) higher than in the control, while GTX-4 was significantly lower during BuT exposure ($p<0.05$).

4. Discussion

4.1 Contaminant measurements

The measured concentrations of dissolved Cu at the end of the experiment represented 60% to 68% of the nominal concentrations, for *A. catenella* and *O.* cf. *ovata*, respectively. This loss could be explained by the Cu adsorption on the culture flasks, but also on the organic particles present in the culture medium and the microalgae themselves (Lage et al., 1996; Franklin et al., 2002). The presence of organic and inorganic dissolved ligands in the culture medium extremely lowered the concentration of bioavailable Cu (Cu^{2+}) (computed Cu^{2+}/measured dissolved Cu = 10^{-5}), as has been shown previously (Lage et al., 1996; Sunda, 1989). It is therefore commonly accepted that the toxicity of Cu must be related to its bioavailable form, Cu^{2+} (Bruland et al., 2000). Consequently, we only considered the concentrations of the bioavailable Cu^{2+} for our interpretations. The measured dissolved BuT represented 1 to 10% of the nominal dissolved concentrations, indicating loss through adsorption and probable change to particulate forms, as shown by White et al., (1999). Speciation of OT as TBT, DBT and MBT was analyzed, and this highlighted classical debutylation of TBT as a function of time, with the dominant form being DBT instead of TBT at the end of the experiment. Dowson et al. (1993) suggested that TBT and MBT tend to go faster in the particulate form compared to DBT. The evolution of the total pool of Cu and OT during the course of the experiment is rather a complex phenomenon and depends on a wide range of parameters, such as bio-degradation, transformation, adsorption and desorption (Levy et al., 2007; White et al., 1999).

4.2 Microalgae growth

Cu and BuT inhibited the growth of both dinoflagellates. However, these microalgae responded differently to the tested contaminants. *A. catenella* showed a threshold-type response, with high cell mortality from 0.60 nM (Cu^{2+}) and 27 nM of BuT and a moderate effect below these concentrations (Figs 2A and C). In contrast, *O.* cf. *ovata* showed a progressive inhibition pattern with increasing concentrations, with significant ($p<0.05$) cell mortality rates occurring since the first concentrations tested (3.10^{-5} nM for Cu^{2+} and 0.084 nM for BuT), indicating a higher sensitivity of these dinoflagellates to these chemicals. Decreasing the cell density in relation to Cu exposure has been shown for many phytoplankton species (Brand et al., 1986; Ebenezer et al., 2014). The toxicity threshold modes of action of Cu on *A. catenella* have previously been investigated in the cells of the same species, sampled in Thau Lagoon in France (Herzi et al., 2013), and on other dinoflagellate species (Lage et al., 1994; Saifullah, 1978). Herzi et al. (2013) observed a drastic growth inhibition of *A. catenella* cells at concentrations of free dissolved Cu^{2+} above 10.8 nM, while in our experiment, significant inhibitory effects on growth were observed at very low concentrations: above 0.60 nM for *A. catenella,* and above 3.10^{-5} nM for *O.* cf. *ovata*. Contrasting *in situ* Cu contamination was reported in sediments of Bizerte and Thau Lagoons, at concentrations from 10 to 36 ppm and from 100 to 200 ppm, respectively (El Ati Hellal et al., 2011; Kawakami et al., 2008). Therefore, the higher sensitivity of *A. catenella* studied here from Bizerte Lagoon may be explained by a difference of ecotype, as it is naturally exposed to lower *in situ* Cu than the organisms from Thau. This mechanism is corroborated by the pollution-induced community tolerance, which has been shown for numerous metals, including Cu (Blanck, 2002).

To our knowledge, there is no data on the effect of Cu and BuT on *O.* cf. *ovata*. Our results showed that *O.* cf. *ovata* was clearly more sensitive to Cu than *A. catenella,* with a five-fold difference in EC_{50} (EC_{50} of 0.16 nM and 0.03 nM for *A. catenella* and *O.* cf. *ovata,* respectively). The normal concentration of dissolved Cu in seawater is from 1 to 3 nM (Biller and Bruland, 2012; Milne et al., 2010) and, as 99.9% of dissolved Cu is considered as complexed and thus not bioavailable to phytoplankton, the Cu^{2+} concentration *in situ* is lower (Bruland et al., 2000). In our study, the Cu^{2+}

dose response of *O.* cf. *ovata* suggest that bioavailable Cu^{2+} concentrations of 3.10^{-2} nM (EC50) could decrease significantly the growth of this toxic dinoflagellate in natural environment. The measured Cu^{2+} concentrations in the Atlantic Ocean is from 10^{-5} nM to 10^{-4} nM (Bruland et al., 2000; Jacquot and Moffett, 2015)". Many mechanisms allow phytoplankton to resist and detoxify toxic metals, among which the exudation of organic content to chelate and bind toxic ions (Megharaj et al., 2003; Worms et al., 2006). This was demonstrated for *A. catenella*, which secretes polysaccharides in the presence of Cu (Herzi et al., 2013). Defence mechanisms probably explain the relatively efficient resistance of *A. catenella* to Cu in comparison with *O.* cf. *ovata*. It is possible that metal availability *in situ* could play a role in the development of this HAB species.

Compared to Cu, organotin studies have mainly focused on fishes and macro-invertebrates, showing the disruption of oyster and neo-gastropod reproduction (for example Gibbs and Bryan, 1986). Organotin can also delay hatching, reduce survival and alter the morphology of fishes (Fent, 1996; Fent and Meier, 1994). On the other hand, studies targeting phytoplankton remain scarce, as mentioned by Petersen and Gustavson (2000). Organotin has been shown to be a potent toxic agent for some microorganisms by disrupting the cellular membrane of marine bacteria (Laurence et al., 1989) and by altering photosynthesis in the microalgae *Dunaliella tertiolecta* and *Skeletonema costatum* (Mooney and Patching, 1995). In our study, as observed for Cu, *O.* cf. *ovata* was more sensitive to BuT than *A. catenella*, with cell density decreasing from 0.084 nM (corresponding to 0.01 µg L^{-1}) for *O.* cf. *ovata* with an EC_{50} of 14.2 (±6) nM (corresponding to an EC_{50} of 1.42 µg L^{-1}) and from 27 nM (corresponding to 3.2 µg L^{-1}) for *A. catenella*. These results are in agreement with previous studies on the green algae *Ankistrodesmus falcatus* (EC_{50} of 5 µg L^{-1} in Wong et al., 1982) and on two diatoms *Skeletonema costatum* and *Thalassiosira pseudonana* with tribultyltin acetate, showing EC_{50} values of 0.36 µg L^{-1} and 1.28 µg L^{-1}, respectively (Walsh et al., 1985). The cell density of *Pavlova lutheri*, *Dunaliella tertiolecta* and *Skeletonema costatum* were also affected when exposed to values of 3 µg L^{-1} of TBT (as oxide) (Beaumont and Newman, 1986). Butyltin concentrations in seawater are reported from 10 to 100 ng L^{-1} (Antizar-Ladislao, 2008; Thomaidis

et al., 2007). The BuT dose response observed for the *O.* cf. *ovata* cell density in our study suggests potential inhibitory effects on this benthic dinoflagellate species in contaminated marine environments.

In our study, the benthic *O.* cf. *ovata* was more sensitive to Cu and BuT than the planktonic *A. catenella*. On the basis of an *in situ* study, Wood et al. (1983) highlighted that coastal phytoplankton took advantage of Cu-complexing agents present in coastal waters, and thus showed better tolerance to Cu in comparison to oceanic communities. However, to our knowledge, no studies were performed to compare planktonic to benthic species sensitivities to metals. Sediment is recognized to be a pollution sink with long residence time of organotins and Cu (Long et al., 1995). It has been demonstrated that toxicity of Cu and organotins is strongly correlated with binding agents and chelating molecules (White et al., 1999; Bruland et al., 2000). In addition, studies on sediment showed that interstitial water from sediment would enrich the surrounding seawater with organic ligands (Sakellari et al., 2011), while any Cu fluxing from the sediments to the water column is likely to be organically complexed (Skrabal et al., 1997). Some studies of sediment toxicity showed that metal toxicity is related to the bioavailable part and not the total metal concentration (Gillan et al., 2005; Simpson et al., 2004). Macroalgae produce exudates with Cu-binding properties. Hence, Vasconcelos and Leal (2001) showed a stimulation in the production of two chelating molecules, cysteine- and glutathione-like by the green macroalgae *Enteromorpha spp*. when it was exposed to Cu. One can suppose that the potentially important deleterious effect of Cu and organotins on microalgae in some highly contaminated ecosystems could be reduced due to the presence of chelating molecules in the benthic habitats. This could explain the apparent reduced defense mechanisms against metals observed for *O.* cf. *ovata*, which thrives in close relationships with sediment and macroalgae.

Cu has been suggested as a selecting force for organisms in planktonic communities because of the different sensitivities to this metal between phylogenetic groups (Brand et al., 1986; Pistocchi et al., 2000). The most recent studies used artificial sea water medium in their experiments, which affects

the Cu^{2+} activity in comparison to natural sea water (Metaxas and Lewis, 1991), while almost 70% of published studies do not measure the Cu introduced in the batch-cultures (Leal et al., 2016). In addition, most studies have not taken into account the bioavailable Cu^{2+}, making inter-study comparisons difficult to make. Brand et al. (1986) used similar experimental conditions to the present study, and we consequently used their EC_{50} values for comparison with our EC_{50} values obtained for *O.* cf. *ovata* and *A. catenella* contaminated with Cu (Fig. 8). We observed that *O.* cf. *ovata* sensitivity is in the range of other dinoflagellates (pCu from 10.5 to 10.64), whereas *A. catenella* appeared to be more tolerant than other dinoflagellates and diatoms. In the context of the increasing anthropization of marine environments, particularly in confined waters (e.g. harbours or lagoons), where *A. catenella* forms recurrent blooms (Fertouna-Bellakhal et al., 2015; Laanaia et al., 2013), this dinoflagellate could potentially take advantage of this higher tolerance to contaminants in impacted marine ecosystems.

4.3 Cell morphology

We observed an effect of Cu on the contaminated microalgae, with an increase in the number of round thin-walled cells (Accoroni et al. 2015) acting as temporary cysts and showing and showing for some of them morphological alterations (Fig. 4G). These cell forms were observed at Cu^{2+} concentrations of ≥0.032 nM and 0.60 nM for *O.* cf. *ovata* and *A. catenella*, respectively. A similar effect was shown for *A. catenella* (Herzi et al., 2013) and *Amphidinium cartarae* (Lage et al., 2001) when exposed to Cu. BuT induced mortality of the *A. catenella* cells, which showed severe disintegration and membrane lysis at concentrations of ≥27 nM. When *O.* cf. *ovata* was exposed to BuT concentrations of ≥8.4 nM, an increasing number of cells lost their intracellular organelles and became lytic. BuTs could impact membrane integrity because of their lipophilicity, binding or being inserted into the cellular membrane and disrupting its integrity and thus the activity of the numerous related enzymes (White et al., 1999). For example, the membrane enzymes ATPase, NADH oxidase, ß-galactosidase and alkaline phosphatase were inhibited by TBT in various bacteria, such

as *Bacillus* (Cooney and Wuertz, 1989; Tseng and Cooney, 1995). The resulting damage to the cellular membrane could lead to higher permeability inducing, for example, the release of K^+ (Laurence et al., 1989; Tobin and Cooney, 1999). The increase in permeability of the cell membrane leads to a higher diffusion of the pollutants throughout the cytoplasm, increasing their lethal effect.

4.4 Photosynthetic activity

The results showed that the maximum quantum yields of photosynthesis (Fv/Fm) for both species showed sensitivity to high Cu (≥0.60 nM) and BuT (≥ 27 nM) exposure (Fig. 5). A decrease in Fv/Fm has been well documented for phytoplankton species exposed to Cu, indicating that Cu results in the closing of the reaction centers, thus decreasing photochemistry capacity (Juneau et al., 2002; Miao et al., 2005). Cu is able to substitute the Mg^{2+} atom in the chlorophyll molecule, a process that decreases the fluorescence quantum yield (Küpper et al., 1998, 1996). Cu^{2+} ions can also affect the PSII oxidizing side by an interaction with the PSII primary electron donor, the Tyr_z component (Samson et al., 1988), and they can affect the rate of QA–QB oxidoreduction (Yruela et al., 1992; Jegerschold et al. 1995). This metal greatly influences the PSII electron transport pathway by interfering with lipid and pigment biosynthesis, and as a consequence, the general chloroplast structure becomes altered (Baron et al., 1995). A recent study by Guo et al. (2016) showed a regulation of genes implied in the photosystem maintenance of *Prorocentrum minimum* in response to the biocide Cu sulfate. Moreover, the modulation of the proteome was also observed under zinc or lead stress for the dinoflagellate *A. catenella* (Jean et al., 2017). This study found that the expression of most proteins was downregulated, including those involved in photosynthesis and the oxidative stress response.

To our knowledge, butyltin (including MBT, DBT and TBT) studies on the photosynthetic activity of phytoplankton are scarce in the literature and no study was performed on dinoflagellates, although this phytoplankton group represents up to 80% of HAB species and constitutes an

important component of the autotrophic compartment. Yong et al. (2007) showed a decrease in the photosynthetic pigment content on the chlorophycea *Tetraselmis suecica* exposed to TBT, while primary production and cell density of *Ankistrodesmus falcatus* were also lowered (Wong et al., 1982). A decrease of primary production has also been reported in the natural phytoplankton assemblage in mesocosm experiments in the presence of TBT (Arrhenius et al., 2006; Petersen and Gustavson, 1998).

When examining the effects of Cu and BuT concentrations on *A. catenella* photosynthetic activity, the evolution matched with the negative effect of these contaminants on growth at the highest tested concentrations. However, for *O.* cf. *ovata*, growth was inhibited at 3.10^{-5} nM (Cu^{2+}) and 0.084 nM of BuT, in contrast to the Fv/Fm inhibition, which occurred at much higher concentrations (0.032 nM of Cu^{2+} and 27 nM of BuT), showing a photochemistry tolerance to both contaminants. The PSII activity was considered as a good indicator of stress induced by this metal, as it is a target of Cu (Juneau et al., 2002; Protopopov et al., 2015). This is in contrast with our results, showing that growth of *O.* cf. *ovata* is a more sensitive bio-indicator both for Cu and BuT effects. This conclusion is supported by Cid et al. (1995) and Fisher et al. (1981), who found that, for the diatoms *Asterionella japonica* and *Phaeodactylum tricornutum*, growth was more sensitive to the Cu effect than the photosynthesis response. Cu and other metals can inhibit cell division mechanisms without affecting photosynthesis (Stauber and Florence, 1987). This discrepancy might be explained by the metal inhibition on methionine production, stopping cell division without consequences for photosynthesis (Davies, 1976).

We investigated the effects of Cu^{2+} and BuT, using a light response curve protocol, on PI curve parameters, such as α, Pmax and Ek of *O.* cf. *ovata*, to highlight the specific mechanism and/or structure of the photosynthetic apparatus was targeted. The decrease of the measured parameters suggested a clear disturbance effect on the rETR inside the PSII. The rETR parameters were affected at the same concentrations as Fv/Fm for both contaminants. The decrease in energy transfer from the antennae to the reaction center in the PSII was previously observed in Cu-stressed

Senedesmus obliquus cells (Mallick and Mohn, 2003). This corresponds with the clear decline of the α parameter, indicating a lowered efficiency of light capture (Ralph and Gademann, 2005), which indicates damage on the photosynthetic antenna. The decline of Ek indicates a lowering of the saturating irradiance, while the decrease of Pmax is consistent with the decline of the Fv/Fm value discussed previously. Tin had a stronger inhibitive action than Cu on α, and entirely disrupted Ek and Pmax, indicating a strong deleterious effect on the PSII general structure. Our results clearly showed that light transport through PSII was strongly affected by both contaminants in this dinoflagellate species and impacting its photosynthetic performances. Contaminants decreased photosynthetic activities, and thus the observed decrease for α values may severely impact the competition for light with other microalgae (Huisman et al., 1999).

4.5 Toxins

Toxin contents of *O.* cf. *ovata* and *A. catenella* cells in the control condition were consistent with other isolates from the Mediterranean sea (Ben-Gharbia et al., 2016; Laabir et al., 2013). Cu and BuT had contrasting effects on the toxin content of *A. catenella* and *O.* cf. *ovata*. Exposing *A. catenella* cells to 0.012 nM of Cu^{2+} increased GTX-3 and C1 paralytic shellfish toxin content. Butyltin had no clear effect on neither ovatoxin nor paralytic shellfish toxin concentrations. There was a significant increase in ovatoxin-a and -b in *O.* cf. *ovata* cells exposed to 0.017 nM of Cu^{2+}, raising the total ovatoxin content from 5.28 pg PITX equivalents $cell^{-1}$ to 13.26 pg PITX equivalents $cell^{-1}$. To our knowledge, studies of metal stress effects on phytoplankton toxin production are scarce, and no work has examined the potential effect of Cu and BuT on toxin production by dinoflagellate species. However, it was shown that a selenium-repleted condition could increase yessotoxin production in the dinoflagellate *Protoceratium reticulatum* (Mitrovic et al., 2004), whereas the domoic acid production of the diatom *Pseudo-nitzschia* increased significantly in response to Cu-stressed conditions (Maldonado et al., 2002). Here, the toxin content increase when cells were exposed to 0.017 nM of Cu^{2+} was concomitant to the cell density decrease. Future work

could analyze the chelating properties of these toxins to determine whether this production could lower the Cu stress, as was found for diatoms, with domoic acid having a potential action on trace metals (Rue and Bruland, 2001). Cellular pathways, such as gene expression and protein regulation should be studied to better understand this increase in toxin production in response to Cu^{2+} stress. Here, the reported results concerning the effect of Cu and BuT on toxin content ($pg.cell^{-1}$) of the studied microalgae are related to intracellular fraction of toxins. It will be interesting in a future work to consider the toxins released extracellularly (Pezzolesi et al., 2016) to bring information on the entire amount of toxins produced by the algae especially since the pollutants tested and particularly the butyltin causes cell lysis. Complementary experiments have to consider the estimation of the specific toxin production (µtox) of the two studied dinoflagellates grown with the pollutants (Basti et al. 2015).

5. Conclusion

This study demonstrated the higher tolerance of the planktonic *A. catenella* to Cu and butyltin compared to the benthic *O.* cf. *ovata*. However, both dinoflagellate species were negatively impacted in their physiology by the tested chemicals at specific concentrations. Calculations of EC_{50} indicated a usual Cu^{2+} sensitivity for *O.* cf. *ovata* compared to other dinoflagellates, while the sensitivity of *A. catenella* was comparable to a more tolerant group (diatoms). These differences in sensitivity from organisms of different marine compartments could suggest a contrasting habitat effect in protecting them *in situ*, through chelating properties or other unknown mechanisms. Butyltin exposure led to cell death, and Cu^{2+} was shown to induce cyst formation at the highest concentration prior to cell death. Cu^{2+} at 0.012 nM increased the ovatoxin content of *O.* cf. *ovata*, while cell density was decreased. The chelating properties of ovatoxins on metal ions should be investigated to identify a potential role in regulating ambient metal concentrations and bioavailability. The increase of intracellular ovatoxin concentrations in response to Cu^{2+} exposure

could pose a supplementary health and socio-economic threat in coastal areas with metal contamination, where HAB species develop.

Conflict of interest

There are no any conflicts of interest.

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FIGURES LEGEND

Fig. 1: Measurements and computation of different contaminant concentrations at day 7 of the experiments. A) Relationship between measured and nominal concentrations of dissolved total Cu (r = 0.99 and 0.99 for *Alexandrium catenella* and *Ostreopsis* cf. *ovata*, respectively). B) Relationship between measured dissolved total Cu and computed free bioavailable Cu^{2+} ions (the line represents the linear regression, r = 0.95 and 0.96 for *A. catenella* and *O.* cf. *ovata* respectively). C) Relationship between measured and nominal butyltin concentrations. The diagonal dashed lines indicate the 1:1 ratio for Figures 1A and 1C.

Fig. 2: Growth curves expressed in cell density (cells mL^{-1}) on a logarithmic scale. A) *Alexandrium catenella* under Cu (Cu^{2+} in nM) exposure. B) *Ostreopsis* cf. *ovata* under Cu (Cu^{2+} in nM) exposure. C) *A. catenella* under BuT exposure (nominal concentrations in nM). D) *O.* cf. *ovata* under BuT exposure (nominal concentrations in nM).

Fig. 3: Effect of contaminant exposure on growth rates (μ in day^{-1}). A) Cu (Cu^{2+} in nM) and B) BuT (nominal concentration in nM). Dashed colored lines represent Hill Modelization of the values to obtain the EC_{50} value, represented by the grey dashed lines.

Fig. 4: Pictures of the cells exposed to contaminants. A) *Alexandrium catenella* cells in good physiological state (up to 0.03 nM Cu^{2+} and 84.10^{-1} nM of BuT). B–C) Round-shaped and altered *A. catenella* cells starting from 60.10^{-2} nM Cu^{2+}. D–E) Lysed *A. catenella* cells from 27 nM of BuT. F) *Ostreopsis* cf. *ovata* cells in good physiological state (up to 17.10^{-3} nM Cu^{2+} and 84.10^{-1} nM of BuT). G–H) Round cells of *O.* cf. *ovata* from 0.03 nM (Cu^{2+}). I–J) Round-shaped and lysed cells of *O.* cf. *ovata* from 84.10^{-1} nM of BuT. Scale bar is 25 μm.

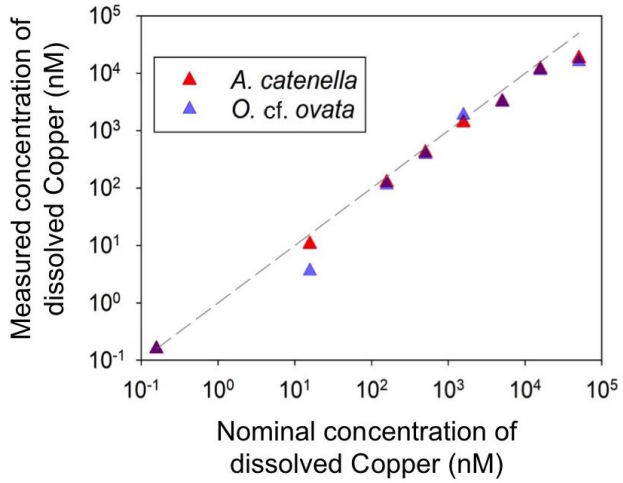
Fig. 5: Effect of contaminants on maximum quantum yield of photosystem II (Fv/Fm expressed in % of control). A) *Alexandrium catenella* under Cu (Cu^{2+} in nM) exposure. B) *Ostreopsis* cf. *ovata* under Cu (Cu^{2+} in nM) exposure. C) *A. catenella* under BuT exposure (nominal concentrations in nM). D) *O.* cf. *ovata* under BuT exposure (nominal concentrations in nM).

Fig. 6: Effects of contaminant exposure on *Ostreopsis* cf. *ovata* photosynthetic parameters (Alpha, Ek and Pmax) extracted from PI curves. Figures A, B, C under Cu (Cu^{2+} in nM) exposure and Figures D, E, F under BuT exposure (nominal concentrations in nM) using the Platt et al. (1980) equation.

Fig. 7: Contaminant exposure effects on toxin content. A–B) Toxin content of *Ostreopsis* cf. *ovata* ovatoxin a (ova-a) and ovatoxin b (ova-b); C–D) Toxin content of *Alexandrium catenella*, saxitoxins. The asterisk indicates a significant difference with control ($p<0.05$, one way ANOVA).

Fig. 8: Ranking of different EC_{50} values of phytoplankton groups and organisms exposed to Cu from the most tolerant to the most sensitive, expressed in pCu = -log [EC50 of Cu^{2+} in nM].

A



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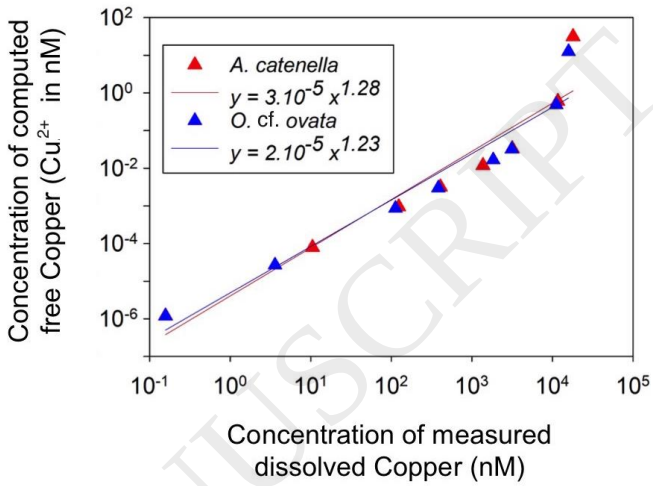

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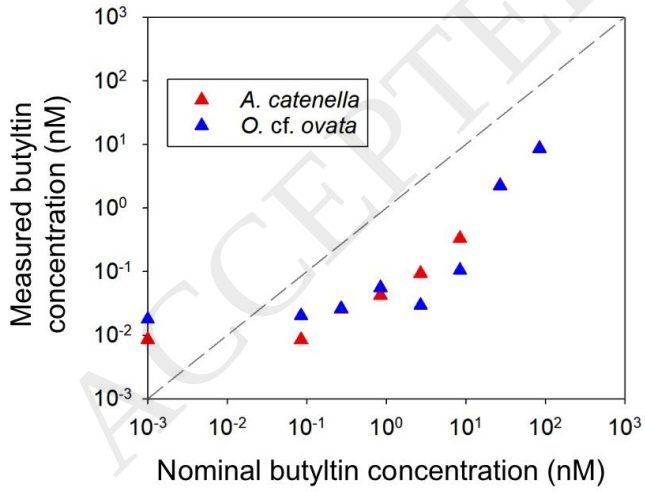
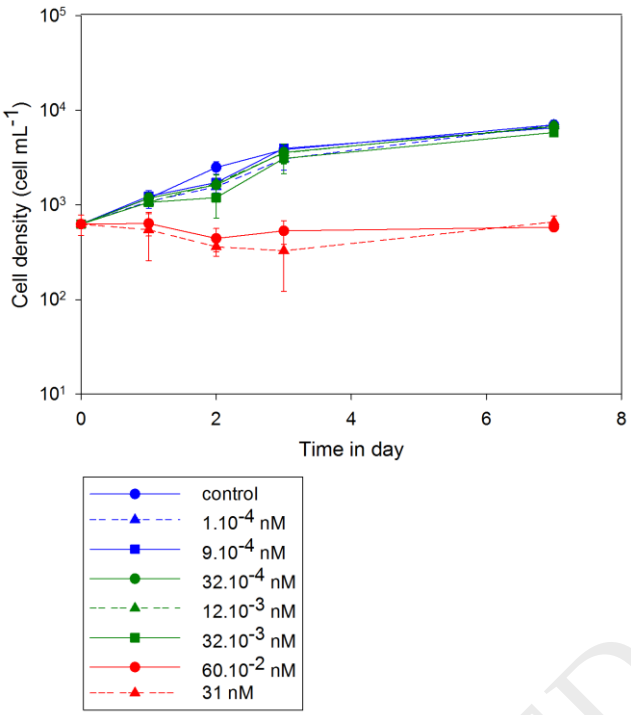


Figure 1

Figure 2

A



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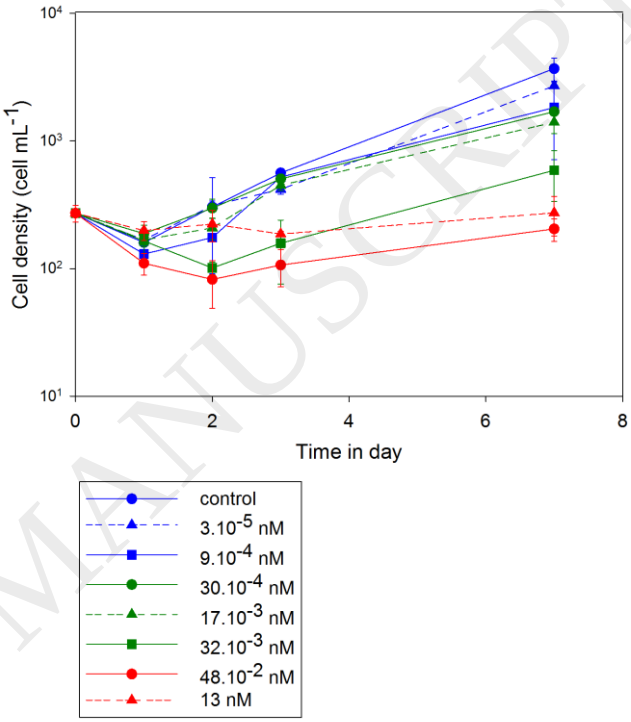

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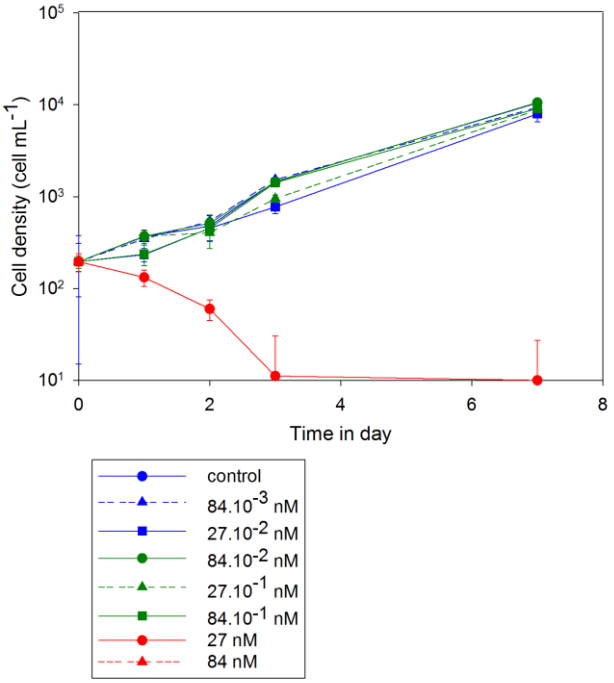

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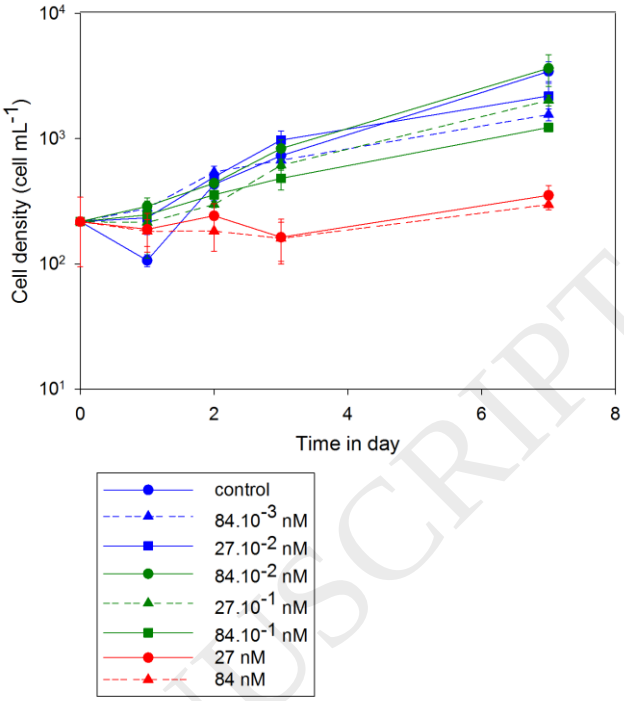
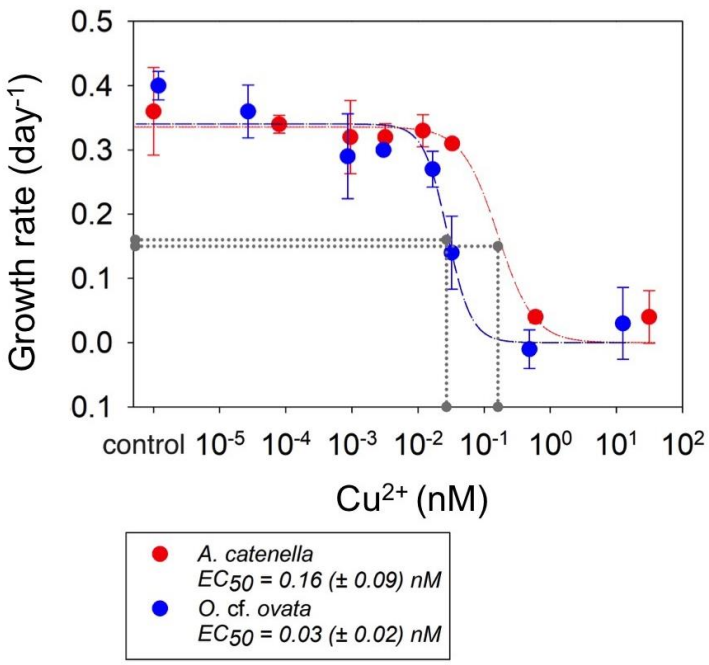


Figure 3

A

B



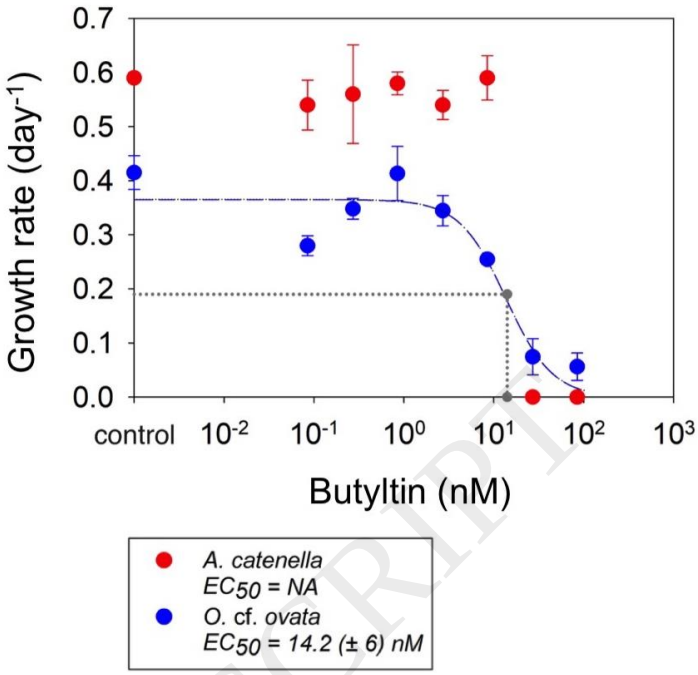
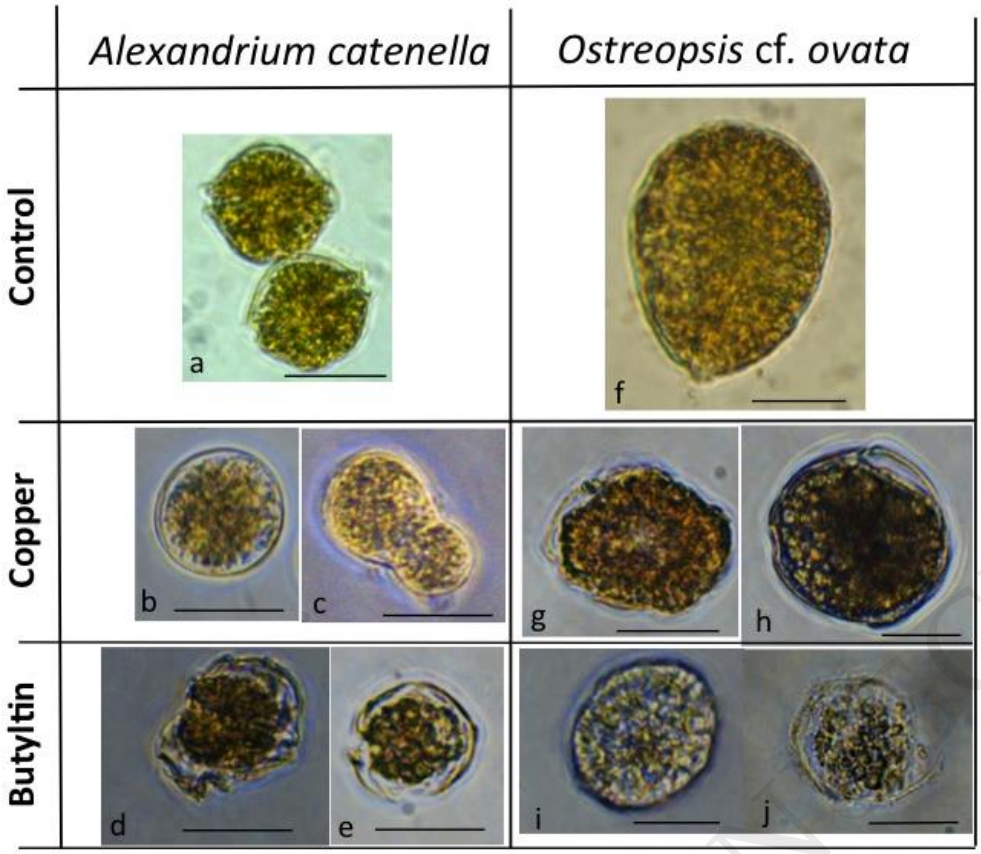


Figure 4



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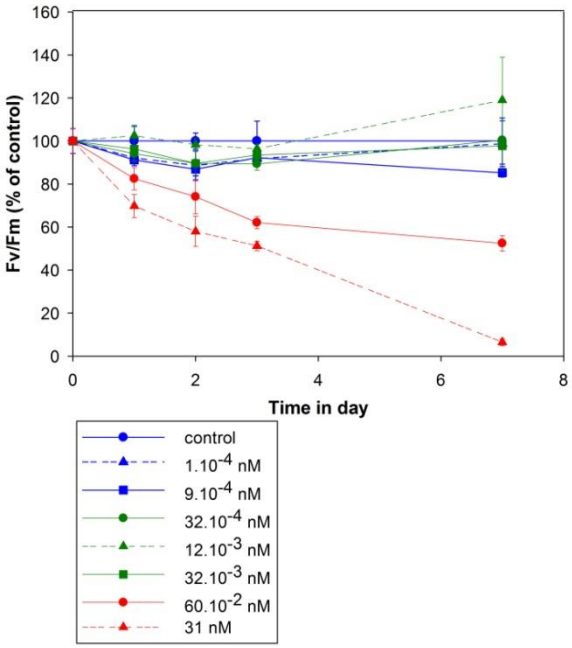

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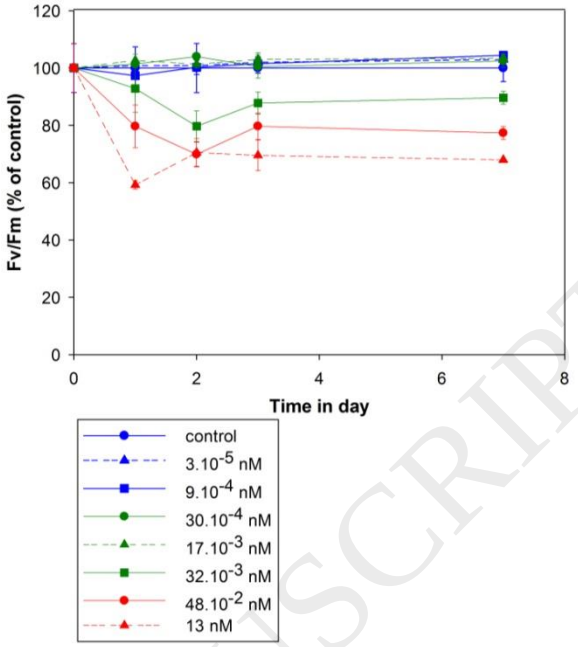

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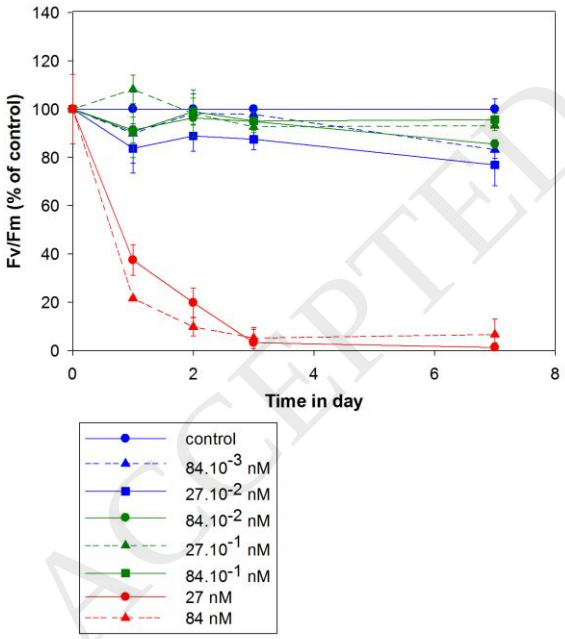

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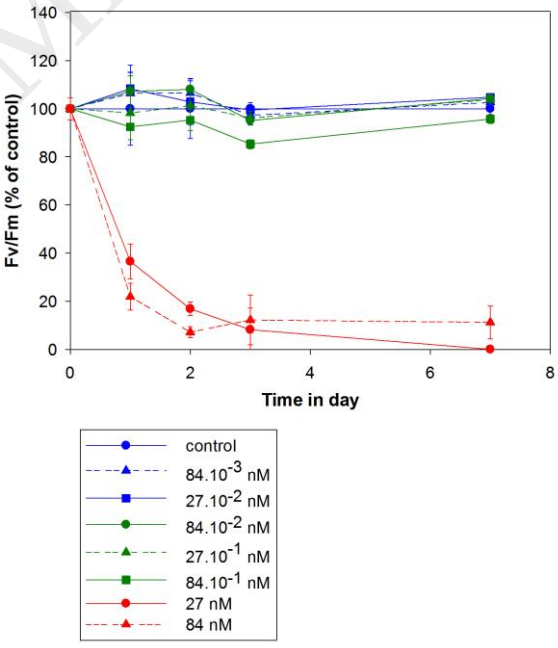

Figure 5

Figure 6

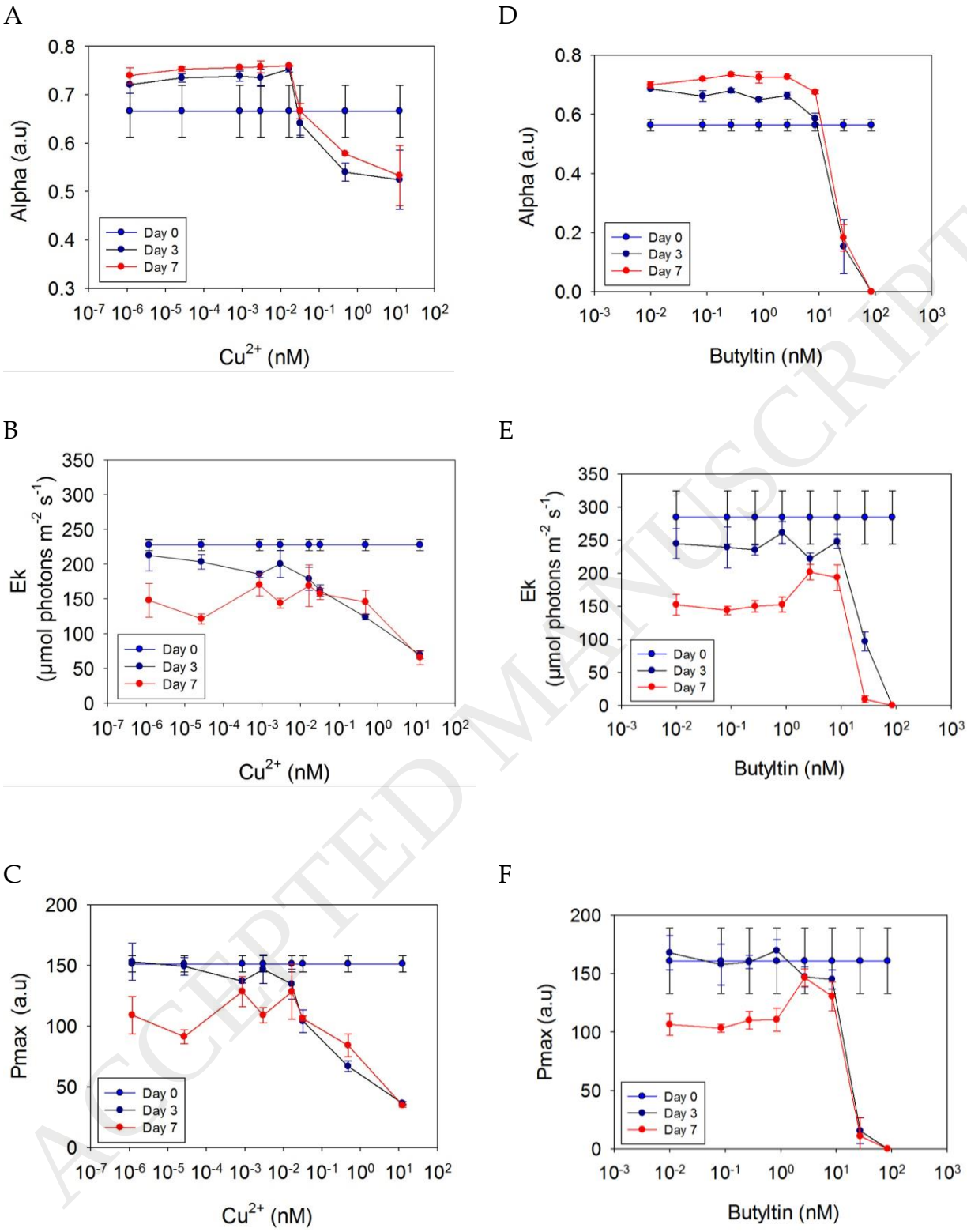

Figure 7

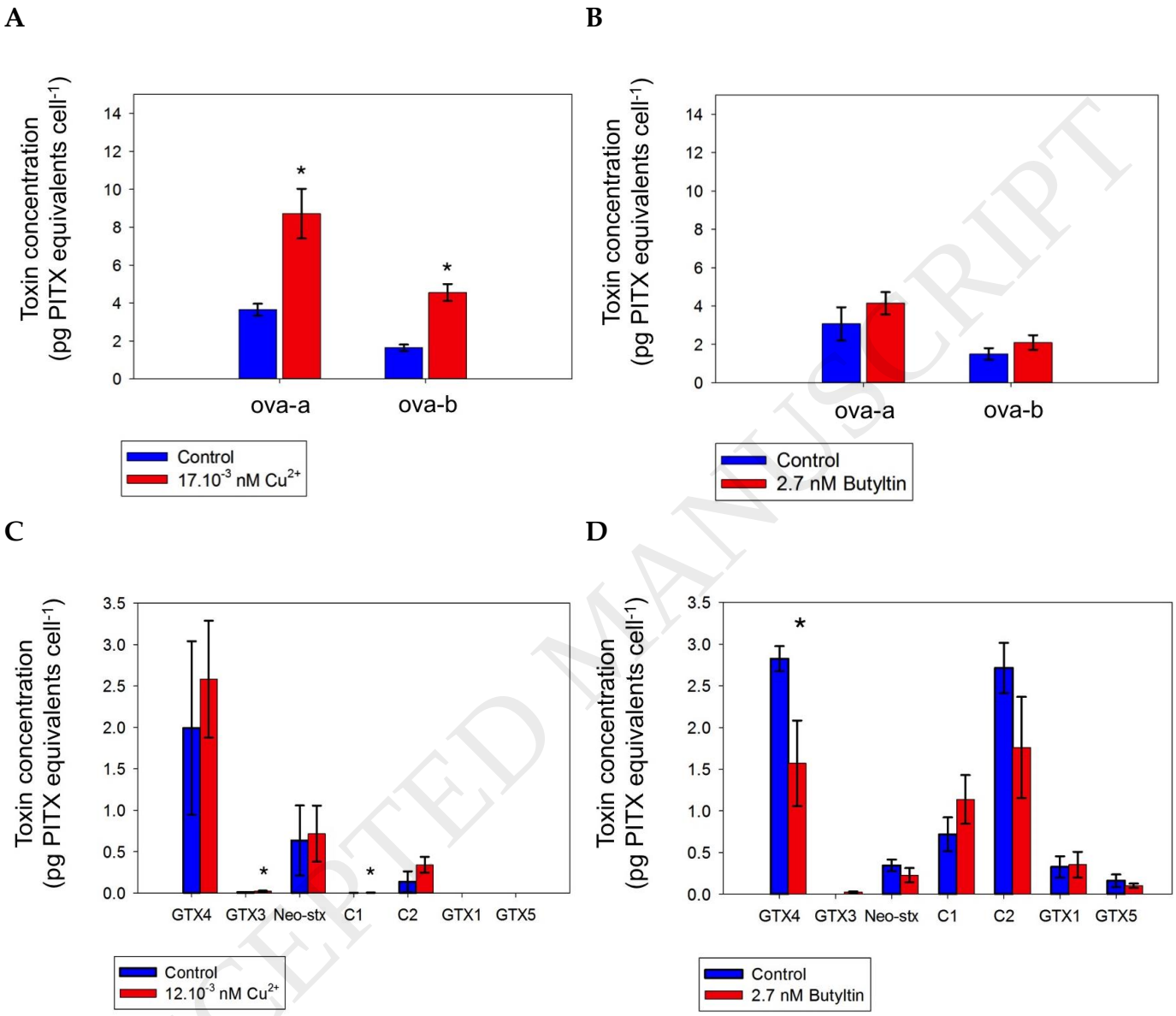

Figure 8

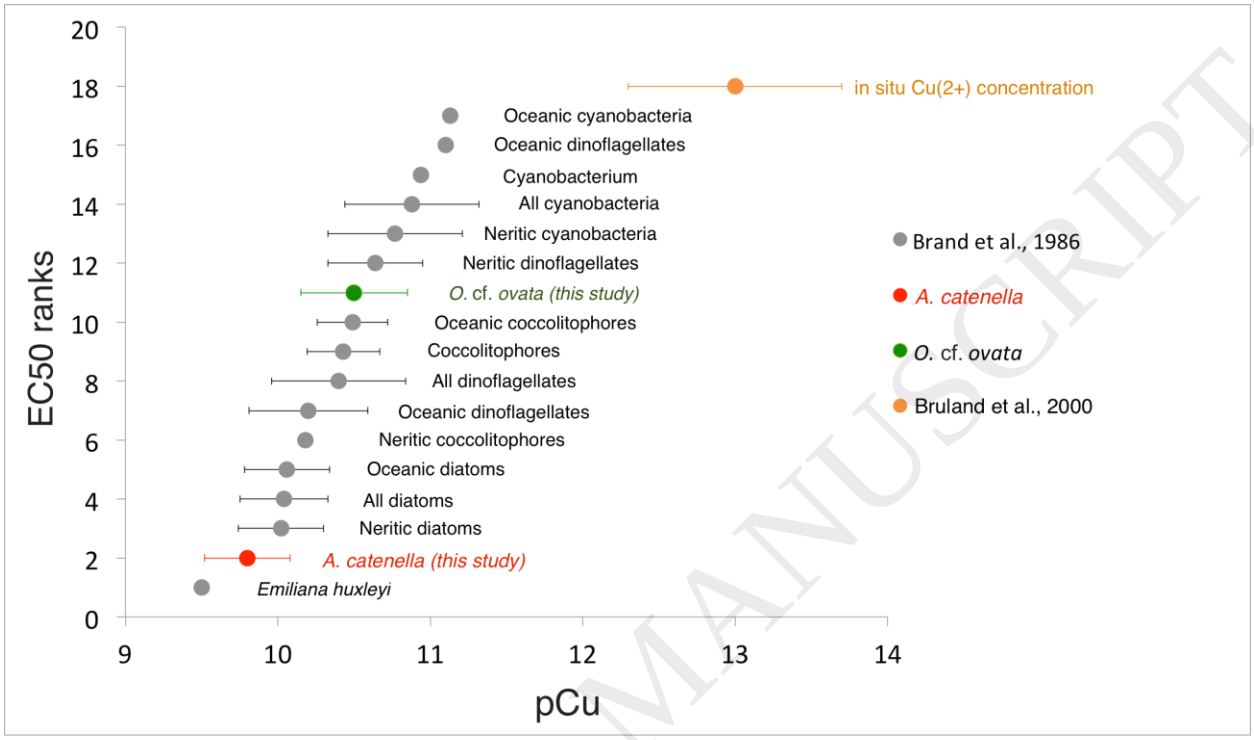

Table 1: Nominal, measured [Cu] and calculated dissolved bioavailable [Cu^{2+}] concentrations expressed in nM. Nominal and measured [butyltin] (Butyltin=([MBT]+[DBT]+[TBT]) concentrations expressed in nM. T_0 and T_7 correspond to the start and the end of the experiment, respectively.

Alexandrium catenella

	Nominal [Cu] nM	**Measured [Cu] at t=0**	**Calculated [Cu^{2+}] at t=0**	**Measured [Cu] at t=7**	**Calculated [Cu^{2+}] at t=7**		**Nominal [Butyltin] nM**	**Measured [Butyltin] at t=0**	**Measured [Butyltin] at t=7**
C1	15,7	14,3	1,41E-04	10,5	7,94E-05	C1	0,085	0,024	0,012
C2	157	142	1,43E-03	124	9,47E-04	C2	0,27	0,03	0,02
C3	504	388	4,03E-03	408	3,19E-03	C3	0,85	0,06	0,05
C4	1575	1432	1,72E-02	1377	1,18E-02	C4	2,7	0,1	0,1
C5	5039	1595	1,97E-02	3182	3,29E-02	C5	8,5	0,7	0,3
C6	15748	8503	7,02E-01	11701	5,98E-01	C6	27	2	2
C7	50394	15057	2,87E+01	18067	3,12E+01	C7	85	9	9

Ostreopsis cf. *ovata*

	Nominal [Cu] nM	**Measured [Cu] at t=0**	**Calculated [Cu^{2+}] at t=0**	**Measured [Cu] at t=7**	**Calculated [Cu^{2+}] at t=7**		**Nominal [Butyltin] nM**	**Measured [Butyltin] at t=0**	**Measured [Butyltin] at t=7**
C1	15,7	12,4	1,23E-04	3,6	2,69E-05	C1	0,085	0,028	0,021
C2	157	136	1,37E-03	113	8,62E-04	C2	0,27	0,05	0,03
C3	504	398	4,14E-03	385	3,01E-03	C3	0,85	0,03	0,06
C4	1575	1518	1,85E-02	1848	1,66E-02	C4	2,7	0,1	0,0
C5	5039	1254	1,46E-02	3143	3,23E-02	C5	8,5	0,4	0,1
C	15748	11264	3,32E+0	11168	4,84E-01	C	27	2	2

6			0				6					
C			1,50E+0		1,25E+0		C					
7	50394	20810	2	15902	1		7	85	6	6		

Table 1: Nominal, measured [Cu] and calculated dissolved bioavailable [Cu^{2+}] concentrations expressed in nM. Nominal and measured [butyltin] (Butyltin=([MBT]+[DBT]+[TBT]) concentrations expressed in nM. T_0 and T_7 correspond to the start and the end of the experiment, respectively.