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# THREE-DIMENSIONAL (3-D) FLUORESCENCE SPECTROSCOPY ANALYSIS OF THE FLUORESCENT DISSOLVED ORGANIC MATTER RELEASED BY THE MARINE TOXIC DINOFLAGELLATE *ALEXANDRIUM CATENELLA* EXPOSED TO METAL STRESS BY ZINC OR LEAD<sup>1</sup>

Faouzi Herzi<sup>2</sup>

Laboratoire de Cytologie Végétale et Phytoplanctonologie, Faculté des Sciences de Bizerte, Département des Sciences de la Vie,  
Université de Carthage, Jarzouna, Bizerte 7021, Tunisie

Laboratoire Processus de Transferts et d'Echanges dans l'Environnement (PROTEE EA 3819), Equipe Chimie Analytique et  
Processus de Transferts dans l'Environnement, Université du Sud Toulon – Var, BP 20132, La Garde Cedex 83 957, France

Natacha Jean

Laboratoire Processus de Transferts et d'Echanges dans l'Environnement (PROTEE EA 3819), Equipe Chimie Analytique et  
Processus de Transferts dans l'Environnement, Université du Sud Toulon – Var, BP 20132, La Garde Cedex 83 957, France

Asma Sakka Hlaili

Laboratoire de Cytologie Végétale et Phytoplanctonologie, Faculté des Sciences de Bizerte, Département des Sciences de la Vie,  
Université de Carthage, Jarzouna, Bizerte 7021, Tunisie

and Stéphane Mounier

Laboratoire Processus de Transferts et d'Echanges dans l'Environnement (PROTEE EA 3819), Equipe Chimie Analytique et  
Processus de Transferts dans l'Environnement, Université du Sud Toulon – Var, BP 20132, La Garde Cedex 83 957, France

We investigated the effects of zinc or lead on growth and on exudation of fluorescent dissolved organic matter (FDOM) by the marine toxic dinoflagellate *Alexandrium catenella* (Whedon & Kofoid) Balech. The species was exposed to increasing free zinc ( $1.34 \times 10^{-7}$  M– $3.98 \times 10^{-6}$  M) or lead ( $5.13 \times 10^{-9}$  M– $1.82 \times 10^{-7}$  M) concentrations. Low metal levels ( $[\text{Zn}^{2+}] = 1.34 \times 10^{-7}$  M;  $[\text{Pb}^{2+}] = 5.13 \times 10^{-9}$  M) had no effect on cell growth. Toxic effects were observed from higher metal contamination ( $[\text{Zn}^{2+}] = 3.98 \times 10^{-6}$  M;  $[\text{Pb}^{2+}] = 6.54 \times 10^{-8}$  M), as a conversion of vegetative cells into cysts. Analysis of the released FDOM by three-dimensional (3-D) fluorescence spectroscopy was achieved, using the parallel factor analysis (PARAFAC). The PARAFAC modeling revealed four components associated with two contributions: one related to the biological activity; the other linked to the organic matter decomposition in the culture medium. The C1 component combined a tryptophan peak and characteristics of humic substances, whereas the C2 component was considered as a tryptophan protein fluorophore. The two others C3 and C4 components were associated with marine organic matter production. Released fluorescent substances were induced by low ( $[\text{Zn}^{2+}] = 1.34 \times 10^{-7}$  M;  $[\text{Pb}^{2+}] = 5.13 \times 10^{-9}$  M) and

moderate ( $[\text{Zn}^{2+}] = 6.21 \times 10^{-7}$  M;  $[\text{Pb}^{2+}] = 2.64 \times 10^{-9}$  M) metal concentrations, suggesting the activation of cellular mechanisms in response to metal stress, to exudate FDOM that could complex metal cations and reduce their toxicity toward *A. catenella* cells.

**Key index words:** 3-D fluorescence spectroscopy; *Alexandrium catenella*; exudation; fluorescent dissolved organic matter; PARAFAC; trace metals

**Abbreviations:** DOC, dissolved organic carbon; DOM, dissolved organic matter; EEM, excitation–emission matrix of fluorescence; FDOM, fluorescent dissolved organic matter; HAB, harmful algal bloom; PARAFAC, parallel factor analysis

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In coastal marine ecosystems, dinoflagellates are influenced by organic pollutants coming from discharge waters and urbanization (Laabir et al. 2011). Such pollution may induce eutrophication of seawater and expansion of harmful algal blooms (HABs). Over the last 10–30 years, frequency and distribution of HABs have significantly increased in marine ecosystems (Sellner et al. 2003). Eutrophic conditions caused by anthropogenic inputs are expected to partially explain this HAB expansion, although this has yet to be directly demonstrated (Glibert et al. 2005). Among dinoflagellate groups, *Alexandrium* species are implied in HABs in various marine waters, such as the coastal waters of Chile (Cordova

and Muller 2002), the northwest Mediterranean (Vila et al. 2001, Penna et al. 2005), South Korea (Yeung et al. 2002), Tunisia (Turki and Balti 2005), Algeria (Frehi et al. 2007), and in Thau lagoon (Lilly et al. 2002). In some ecosystems perturbed by pollution like the Little Bay of Toulon (NW Mediterranean), dinoflagellates are predominant relative to other groups present in the phytoplankton community, like diatoms (Jean et al. 2005). It has been shown that Little Bay of Toulon was contaminated by trace metals, in the water column (copper: 13.5–23.8 nM; lead: 0.73–1.24 nM; zinc: 27.8–58.7 nM; Jean et al. 2012) and in the sediments (copper:  $60 \mu\text{g} \cdot \text{g}^{-1}$ ; lead:  $73 \mu\text{g} \cdot \text{g}^{-1}$ ; zinc:  $114 \mu\text{g} \cdot \text{g}^{-1}$ ; Teissier et al. 2011). In this ecosystem, the *Alexandrium* genus may be responsible for phytoplankton blooms at some periods of the year (Jean et al. 2006), suggesting its resistance to metal stress. Since the resistance to metal contamination could be a factor of *A. catenella* predominance in the phytoplankton community, behavior of this species toward trace metals should be investigated. This resistance depends on a number of factors, such as the nature of the metal tested, the environmental conditions, and the capacity of phytoplankton to overcome the metal toxicity (Pistocchi et al. 1997).

In marine ecosystems, natural organic and inorganic ligands are able to complex metal cations. This complexation leads to a competition between natural ligands present in organic matter, inorganic anions, and membrane transport proteins, for binding metal cations, which changes the bioavailability of the metal for the cells (Sunda and Hunstman 1998). As a consequence, complexation of metals by natural organic ligands decreases their toxicity in marine environments (Moffett et al. 1990). Indeed, metal toxicity toward organisms does not depend on the total metal concentrations, but essentially on the metal cation chemical speciation, which can be calculated on the basis of the free ion activity model (Sunda and Guillard 1976). Organic ligands capable of metal complexation consist of several substance classes notably including amino acids or humic substances originating from organic matter that can be produced by phytoplankton (Le Faucheur et al. 2005, Dryden et al. 2007, Herzi et al. 2013). Furthermore, organic ligands released by phytoplankton can modify trace metal speciation (Vasconcelos et al. 2002). Studies characterizing dissolved organic matter (DOM) released by phytoplankton under stress metal conditions should bring a better knowledge of the DOM implication in the metal availability for phytoplankton. This DOM can be monitored thanks to the three-dimensional (3-D) fluorescence spectroscopy technique coupled with the modeling by the parallel factor analysis (PARAFAC).

The 3-D fluorescence spectroscopy technique has been applied to several analysis domains to characterize the DOM present in terrestrial and aquatic environments. Studies on fluorescent dissolved

organic matter (FDOM) have been carried from samples collected in many types of aquatic ecosystems, ranging from rivers (Baker 2002, Nagao et al. 2003) and estuaries (Jaffé et al. 2004), to marine ecosystems (Baker and Spencer 2004). To identify fluorescent components, PARAFAC was applied as a statistical excitation–emission matrix of fluorescence (EEMs) treatment (Bro 1997). Due to PARAFAC, the FDOM can be mathematically identified, leading to the optimal separation of several fluorescent components. The PARAFAC algorithm has been successfully used to investigate the DOM origin of aquatic samples coming from estuarine (Stedmon and Markager 2005, Luciani et al. 2008), marine (Murphy et al. 2008), or freshwater ecosystems (Holbrook et al. 2006). However, few studies have used fluorescence spectroscopy coupled to PARAFAC to characterize the FDOM released as a natural by-product by phytoplankton exposed to trace metals.

The overall goal of this research was to provide insight into the relationships between the dinoflagellate *A. catenella* and trace metal contamination of coastal and marine ecosystems with regard to dinoflagellate proliferation and metal availability. The specific objectives of this work are to (i) determine the effects of increasing free zinc or lead concentrations on *A. catenella* cells grown in cultures (ii) characterize the FDOM released by *A. catenella*, based on the fluorescence results analyzed by PARAFAC modeling (iii) understand the potential relationships existing between the released FDOM and the metal toxicity toward *A. catenella*.

## MATERIALS AND METHODS

*Control and metal-contaminated cultures of Alexandrium catenella.* The strain *A. catenella* ACT03 was isolated from the Thau lagoon (France; kindly provided by the laboratory ECOSYM UMR 5119, Montpellier 2 University, CNRS, Ifremer and IRD). Cultures from this strain were maintained on enriched f/2 culture medium (Guillard and Ryther 1962), at 20°C in sterile 250 mL flasks, under 12:12 h light:dark cycle and  $135 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  of irradiance.

To study the effects of metal contamination on the growth kinetics and on the FDOM released, culture medium was amended with solutions of trace metals prepared in ultrapure water with  $\text{ZnSO}_4$ ,  $2\text{H}_2\text{O}$ , or with  $\text{PbSO}_4$ ,  $8\text{H}_2\text{O}$ , salts. For each metal tested, contaminations were performed at four total concentrations (3, 12, 25, and 60  $\mu\text{M}$ ). Contamination conditions were labeled as following:  $\text{Zn}_i$  or  $\text{Pb}_j$ , where  $i$  and  $j$  were the total metal concentrations, expressed in  $\mu\text{M}$ . Based on the MINEQL-program and on the known composition of the f/2 culture medium, the corresponding free metal concentrations of  $\text{Zn}^{2+}$  or of  $\text{Pb}^{2+}$  were calculated and reported in  $\text{pM}^{2+}$  units (Table 1). Values of  $\text{pZn}^{2+}$  or of  $\text{pPb}^{2+}$  were defined as the negative  $\text{Log}_{10}$  of the molar free  $\text{Zn}^{2+}$  or  $\text{Pb}^{2+}$  ion concentrations. In control conditions, activities of  $\text{Zn}^{2+}$  or of  $\text{Pb}^{2+}$  in the f/2 culture medium were calculated and found below 10–11 M.

*Alexandrium catenella growth kinetics.* Growth kinetics of contaminated and control *A. catenella* cultures were calculated by counting cells present in 1 mL of Lugol fixed culture

TABLE 1. Total ( $M_T$ ) and free ( $M^{2+}$ ) metal concentrations (expressed in M), and logarithmic transformations of the free metal concentrations ( $pM^{2+} = -\text{Log}_{10} [M^{2+}]$ ) used for zinc or lead contaminations of *Alexandrium catenella* ACT03 cultures.

$[M_T]$ (M)	$[Zn^{2+}]$ (M)	$pZn^{2+}$	$[Pb^{2+}]$ (M)	$pPb^{2+}$
$3 \times 10^{-6}$	$1.34 \times 10^{-7}$	6.87	$5.13 \times 10^{-9}$	8.28
$12 \times 10^{-6}$	$6.21 \times 10^{-7}$	6.20	$2.64 \times 10^{-9}$	7.57
$25 \times 10^{-6}$	$1.47 \times 10^{-6}$	5.83	$6.54 \times 10^{-8}$	7.18
$60 \times 10^{-6}$	$3.98 \times 10^{-6}$	5.40	$1.82 \times 10^{-7}$	6.73

subsamples, with an inverted microscope (Leica Microsystemes SAS, NANTERRE Cedex, France; DMI 4000B), at a magnification of  $100\times$ . Cell counts were performed in triplicates until the stationary phase (i.e., during 30 d, at 5 d intervals). Net growth rate ( $\mu$ ) was calculated using the exponential model of Landry and Hassett (1982). Decrease in cell density ( $\Delta D$ ) for zinc or lead contamination was calculated thanks to the following equation:

$$\Delta D = \frac{D_T - D_M}{D_T} \times 100$$

Where  $D_T$  was the cell density at the stationary phase of growth in the control cultures;  $D_M$  was the cell density at the stationary phase of growth in given metal-contaminated cultures. The concentration of free metal corresponding to a decrease in cell density was reported as  $\text{Log}_{10}$ .

Using the equation above, a high  $\Delta D$  value is indicative of a large decrease in cell density induced by zinc or lead contamination.

**Excitation-Emission Matrixes of fluorescence measurements.** EEMs measurements were carried out on 4 mL subsamples, first homogenized, then filtered through precombusted ( $450^\circ\text{C}$ , 4 h) glass fiber filters (Whatman, VWR International, Radnor, PA, USA, GF/F; pore size:  $0.7\ \mu\text{m}$ ). Fluorescence of the obtained filtrates was measured using a spectrofluorometer (Hitachi, Troy, MI, USA; F4500) equipped with a Xenon excitation lamp (Xc152HUS; Ushio, Cergy Pontoise Cedex, France). The slits of both excitation and emission were fixed at 5 nm. The excitation wavelengths were scan from 200 to 480 nm, and those of emission were between 200 and 600 nm, both with a 5 nm step and with a scan speed of  $2,400\ \text{nm} \cdot \text{min}^{-1}$ . The response time was fixed at 0.01 s. Before each measurement, sample was bubbled with nitrogen gas for 10 min to remove dissolved oxygen, which is considered as a possible quencher. The inner filter effect was systematically corrected by the controlled dilution approach procedure (Luciani et al. 2009) by measuring the raw filtered sample and the half diluted one. PARAFAC was programmed basing on the software MATLAB (version 6.5., Guildford, UK) and on the algorithm of Stedmon (Stedmon and Bro 2008). After elimination of principal scattering Rayleigh and Raman diffusions, the inner filter effect was corrected (Luciani et al. 2009). After these corrections, the best decomposition of the 48 EEMs ( $6\ \text{d} \times 2\ \text{metals} \times 4\ \text{concentrations}$ ) was optimized with four components (Concordia: 84.90%). Contribution of each component was divided by the cell density in the corresponding culture, so as to obtain the cell-specific fluorescence contribution, called (FCell).

**DOC measurements.** The dissolved organic carbon (DOC) concentrations were measured on 35 mL *A. catenella* subsamples, first homogenized, then filtered through precombusted ( $450^\circ\text{C}$ , 4 h) glass fiber filters (Whatman GF/F; pore size:  $0.7\ \mu\text{m}$ ). The DOC concentrations were determined in the obtained filtrates with a total organic carbon analyzer

(TOC-VCSH; Shimadzu, Nakagyo-ku, Kyoto, Japan) linked to an automatic sample injector (ASI V; Shimadzu). Each DOC measurement was carried out in triplicate. Standard deviations were always below 2%. The carbon concentration was expressed in  $\text{mg C} \cdot \text{L}^{-1}$ .

**Statistical analysis.** All statistical tests were performed using Statistica 7.1. software for Windows. The nonparametric Wilcoxon test was used to compare growth kinetics among metal-contaminated and control cultures of *A. catenella* and to determine if the effects of the metal contaminations on FDOM exudated by *A. catenella* were significant. Results of the statistical analysis were considered significant at  $P < 0.05$ .

## RESULTS

**Effects of zinc or lead on the *Alexandrium catenella* growth kinetics.** Growth kinetics of metal-contaminated *A. catenella* cultures showed quantitative perturbations in comparison with control cultures ( $P < 0.05$ ; Fig. 1). In presence of low contaminating  $Zn^{2+}$  concentrations,  $\Delta D$  was increased from 5% at  $pZn^{2+} = 6.87$  ( $Zn_3$ ) to 12% at  $pZn^{2+} = 6.20$  ( $Zn_{12}$ ; Fig. 2). In presence of higher contaminating  $Zn^{2+}$  concentrations, until  $pZn^{2+} = 5.40$  ( $Zn_{60}$ ),  $\Delta D$  increased drastically (90%), indicating that no

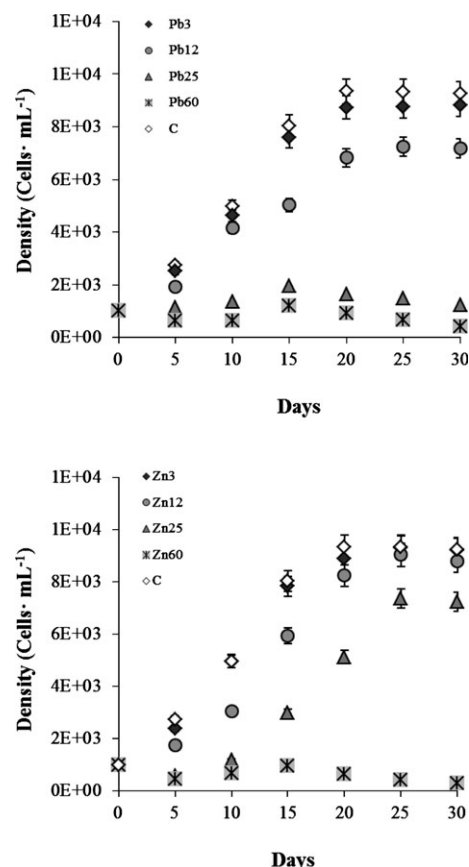


FIG. 1. Temporal evolution of the cell densities ( $\text{cells} \cdot \text{mL}^{-1}$ ) in *Alexandrium catenella* ACT03 cultures grown in the presence of excess zinc- (a) or lead- (b) contaminated conditions (total metal concentrations: 3, 12, 25, or 60  $\mu\text{M}$ ).



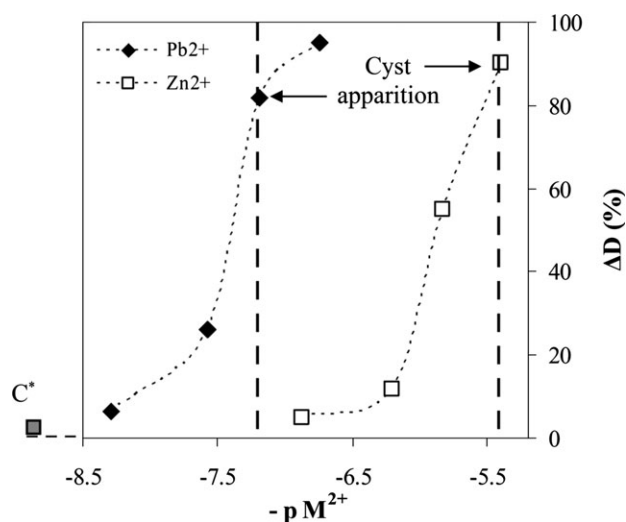


FIG. 2. Decrease in cell density of *Alexandrium catenella* ACT03 cultures at the stationary phase of growth, under zinc or lead contamination.  $\Delta D$  represents the decrease in cell density ( $C^*$ : control value represented by  $-pM^{2+} = -11.0$ , due to the presence of free  $Pb^{2+}$  and  $Zn^{2+}$  in the control f/2 medium).

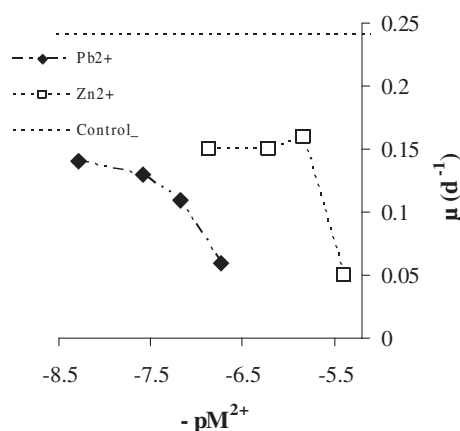


FIG. 3. Net growth rates ( $\mu$ , in  $d^{-1}$ ) of *Alexandrium catenella* ACT03 cultures, in control and in zinc- or lead-contaminated conditions.

growth was observed. In contrast, in presence of  $Pb^{2+}$ ,  $\Delta D$  was about 7% at  $pPb^{2+} = 8.28$  ( $Pb_3$ ) and reached 26% at  $pPb^{2+} = 7.57$  ( $Pb_{12}$ ). Free metal concentration reaching  $1.34 \times 10^{-7}$  M for  $Zn^{2+}$  or  $1.82 \times 10^{-7}$  M for  $Pb^{2+}$  induced respectively 5% or 95% of decrease in cell density. These results seemed to suggest that lead was more toxic for *A. catenella* than zinc.

The net growth rate ( $\mu$ ) reached  $0.24 d^{-1}$  in control cultures (Fig. 3). In the case of zinc contamination, beyond  $pZn^{2+} = 5.40$  ( $Zn_{60}$ ), the *A. catenella* growth was totally inhibited ( $\mu = 0.05 d^{-1}$ ). Between  $pZn^{2+} = 6.20$  ( $Zn_{12}$ ) and  $pZn^{2+} = 5.83$  ( $Zn_{25}$ ), growth seemed to be less affected, as attested by the slight increase in  $\mu$  ( $0.15$ – $0.16 d^{-1}$ ), keeping  $\mu$  val-

ues below those of control. Then, a regular decrease in  $\mu$  was observed from  $pZn^{2+} = 5.83$  ( $0.16 d^{-1}$ ;  $Zn_{25}$ ) to  $pZn^{2+} = 5.40$  ( $0.05 d^{-1}$ ;  $Zn_{60}$ ). In the case of lead contamination, a regular decrease in the  $\mu$  values was observed from  $pPb^{2+} = 8.28$  ( $\mu = 0.14 d^{-1}$ ;  $Pb_3$ ) to  $pPb^{2+} = 6.73$  ( $\mu = 0.06 d^{-1}$ ;  $Pb_{60}$ ).

**Fluorescent components of the EEMs.** The first component (C1) had a maximal intensity for 240 and 350 nm of excitation and emission wavelengths respectively, with a slow decrease until 420 nm (Fig. 4; C1). This component combined a tryptophan peak (maximal peak around 340 nm, in Burdige et al. 2004) and characteristics of humic/fulvic substances (Coble 1996, Patel-Sorrentino et al. 2002). It therefore represented a domain combining the fluorescent protein substance excitation wavelengths, associated with those of humic substances. The second component (C2) was centered between 250 and 315 nm for excitation wavelengths, and between 315 and 400 nm wavelengths for emission (Fig. 4; C2). However, the maximum of the peak was positioned at the couple of wavelengths: EX/EM = 280 nm/355 nm. Indeed, this component was considered as a fluorescent protein fluorophore tryptophan (type T). The third component (C3) was located around 325 nm for excitation, and 415 nm for emission (Fig. 4; C3). This component was related to humic fluorophore in environmental waters. According to Coble (1996), the existence of this fluorophore is linked to the marine environment (type C) and is widespread through the marine production (Ishii and Boyer 2012). The last component (C4) contained two well defined maximal peaks (Fig. 4; C4). The less pronounced peak was located in the high excitation wavelengths (370 nm), while the major one was excited at 300 nm of excitation and at 460 nm of emission. The maximal fluorescent intensities were associated to the same emission wavelength (460 nm). The nearest component described by Coble (1996), is the component generally associated with humic terrestrial inputs (Type A and C).

**FDOM released under zinc contamination.** In response to zinc contamination, the fluorescent contributions were lower than those obtained in control conditions, except for  $Zn_3$  and  $Zn_{12}$ , which appeared more fluorescent on day 5 and on day 30, respectively (Fig. 5). For C1 and C2 components, the fluorescent contributions increased until the conversion of vegetative cells into resistance cysts (in presence of the  $Zn^{2+}$  concentration:  $pZn^{2+} = 5.40$  ( $Zn_{60}$ )). Beyond this value, the fluorescent contribution was significantly decreased. Therefore, one can consider that the fluorescence decrease in response to strong metal stress (in presence of total concentrations: 25, 60  $\mu M$ ) was related to low *A. catenella* biological activity, rather than to metal quenching. Except for the C3 and C4 components, the maximum of fluorescent contribution was

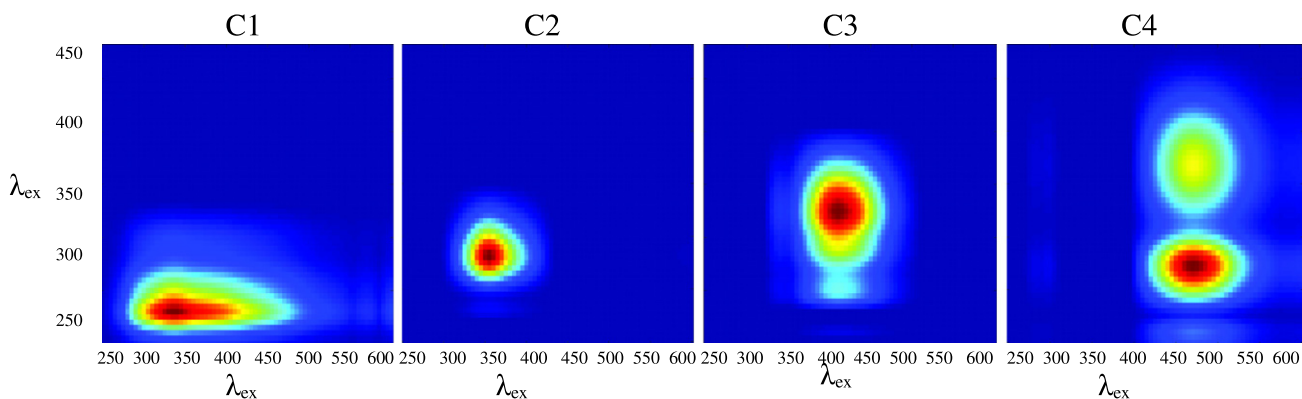


FIG. 4. Representation of the C1, C2, C3, and C4 component contributions to the fluorescent dissolved organic matter (FDOM) released by *Alexandrium catenella* ACT03 cultures exposed to the tested metal stresses. For the four graphs, abscissa represent emission wavelengths and ordinate, excitation wavelengths.

observed at the exponential phase (day 20), where the fluorescent signals generally appeared under zinc contamination of:  $pZn^{2+} = 6.87$  ( $Zn_3$ ). For the C3 and C4 components, a regular and constant increase in the fluorescent contribution was observed until the end of experiments. Besides, the C4 component seemed to be more affected by zinc contamination than the C3 component.

The specific contribution (FCell) showed two main fluorescent contributions at the beginning (day 5) and at the end of experiment (day 30), especially for  $pZn^{2+} = 5.40$  ( $Zn_{60}$ ; Fig. 6). Based on these observations, the maximum of specific fluorescence was hence observed when vegetative cells transformed into resistance cysts.

**FDOM released under lead contamination.** In response to lead contamination, the fluorescent contribution reached a maximum at the stationary phase of growth (day 25), and then decreased until the end of experiments (Fig. 5). The highest fluorescent contributions, higher than those of control, were obtained for the C1 component, under low ( $pPb^{2+} = 8.28$ ;  $Pb_3$ ) and moderate ( $pPb^{2+} = 7.57$ ;  $Pb_{12}$ ) lead contaminations. This behavior was less pronounced for the C2 component at  $pPb^{2+} = 7.18$  ( $Pb_{25}$ ). However, the C3 and C4 components showed a regular increase until the end of stationary phase of growth, reaching a peak at day 20. The C3 component was slightly affected by low concentrations, like for C1 component. A similar pattern occurred for the C4 component, with less fluorescence during the exponential phase.

The specific contribution by cell (FCell) was less pronounced than that measured for zinc contamination (Fig. 6). However, like for zinc, the maximum of fluorescence was observed at the same time than the cyst apparition.

**Comparison of the effects of zinc or lead on the FDOM released.** Between the two metals, the fluorescent contributions were stronger for lead than for zinc. These metals had different effects on the FDOM

released. The C1 and C2 components were the most affected components, however, the C4 component was less affected by lead than by zinc. Furthermore, free metal activity in solution led to two different behaviors according to the metal concentrations and the components. The first anticipated behavior was related to the fluorescence quenching. In general, this trend was observed for all growth period, through the exponential decrease in the fluorescent contribution. However, the second one was more likely, inducing an increase in fluorescent responses when free metal concentrations increased. This behavior may be due to the release of dissolved fluorescent molecules by cells, in response to metal stress.

**DOC concentrations.** Cultures contaminated by high concentrations of zinc ( $pZn^{2+} = 5.83$  ( $Zn_{25}$ );  $pZn^{2+} = 5.40$  ( $Zn_{60}$ )) released less DOC compared to those contaminated by lower levels of zinc ( $pZn^{2+} = 6.87$  ( $Zn_3$ );  $pZn^{2+} = 6.20$  ( $Zn_{12}$ ); Fig. 7). Moreover, the maximum of cell DOC produced was observed at the end exponential phase of *A. catenella* growth under 3 and 12  $\mu M$ . Similarly, DOC production seemed to be enhanced under low ( $pPb^{2+} = 8.28$ ;  $Pb_3$ ) and moderate ( $pPb^{2+} = 7.57$ ;  $Pb_{12}$ ) lead concentrations, but in a lesser way than in presence of high concentrations (25 and 60  $\mu M$ ).

## DISCUSSION

**Growth of *Alexandrium catenella* under metal contamination.** Growth kinetics of *A. catenella* grown in control f/2 culture medium showed similar patterns to those registered for other dinoflagellate species (Juhl 2005, Laabir et al. 2011). Besides, growth reduction in *A. catenella* after metal addition here observed was in agreement with results reported in previous researches carried out on other phytoplankton species (Brand et al. 1986, Lage et al. 1996). Here, we have evaluated the decrease in cell density through some variables such as the net

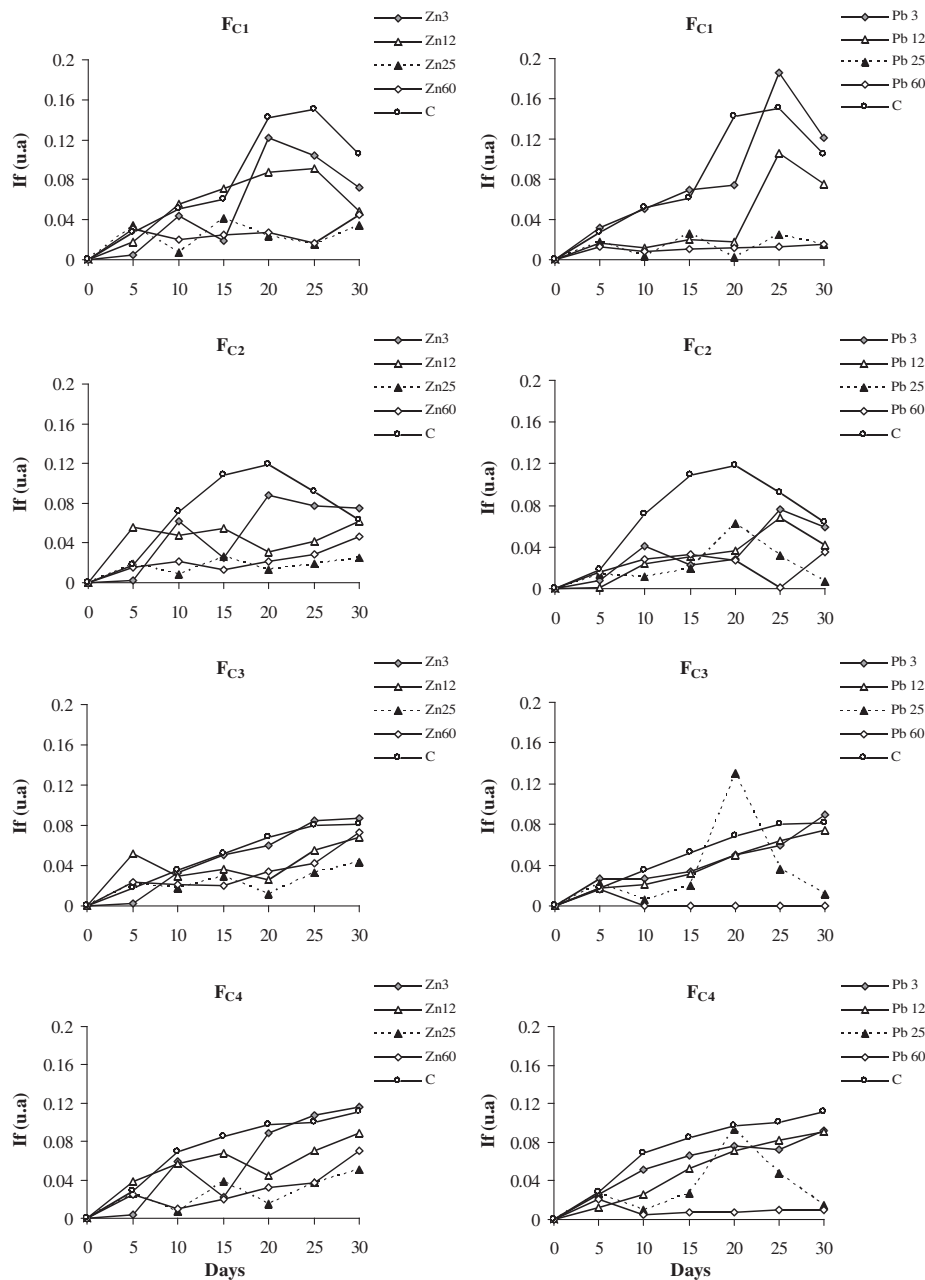


FIG. 5. Fluorescent contributions to the fluorescent dissolved organic matter (FDOM) released by *Alexandrium catenella* ACT03 cultures exposed to the tested metals. The lines represent the four components C1, C2, C3, and C4. The columns represent respectively the zinc or the lead effects on the released FDOM.

growth rate  $\mu$ , and we obtained a value of  $0.24 \text{ d}^{-1}$  in control cultures. This value was similar to that mentioned by Siu et al. (1997), which reported a net growth rate of  $0.29 \text{ d}^{-1}$  for *A. catenella* grown in vitro under  $25^\circ\text{C}$ . However, for the same strain *A. catenella* ACT03 grown between  $20^\circ\text{C}$  and  $27^\circ\text{C}$ , under salinities ranging from a salinity of 30–38, Laabir et al. (2011) obtained net growth rates comprised between  $0.4$  and  $1.0 \text{ d}^{-1}$ , values higher than those measured in this study at  $20^\circ\text{C}$  and for a salinity of 38. The cultures of *A. catenella* exposed to zinc or to lead contaminations exhibited contrasting net growth rates according to the metal level tested. For both metals, the growth rates showed lower values,

from  $0.05 \text{ d}^{-1}$  to  $0.16 \text{ d}^{-1}$ , compared to the control ( $0.24 \text{ d}^{-1}$ ). These values were relatively lower than those reported by Pistocchi et al. (1997), which observed a net growth rate between  $0.33$  and  $0.34 \text{ d}^{-1}$  for *Gymnodinium* sp. exposed to  $0.2 \text{ mg} \cdot \text{L}^{-1}$  or to  $0.5 \text{ mg} \cdot \text{L}^{-1}$  copper. In contrast, the latter authors obtained a high growth rate ( $0.92 \text{ d}^{-1}$ ) for *Cylindrotheca fusiformis* at the same metal concentrations. Furthermore, Lage et al. (1996) have shown that growth rate was more perturbed for dinoflagellates exposed to low metal concentrations. These observations suggest that dinoflagellates could be more sensitive to metal stress than diatoms. Previous studies seemed to confirm this possi-

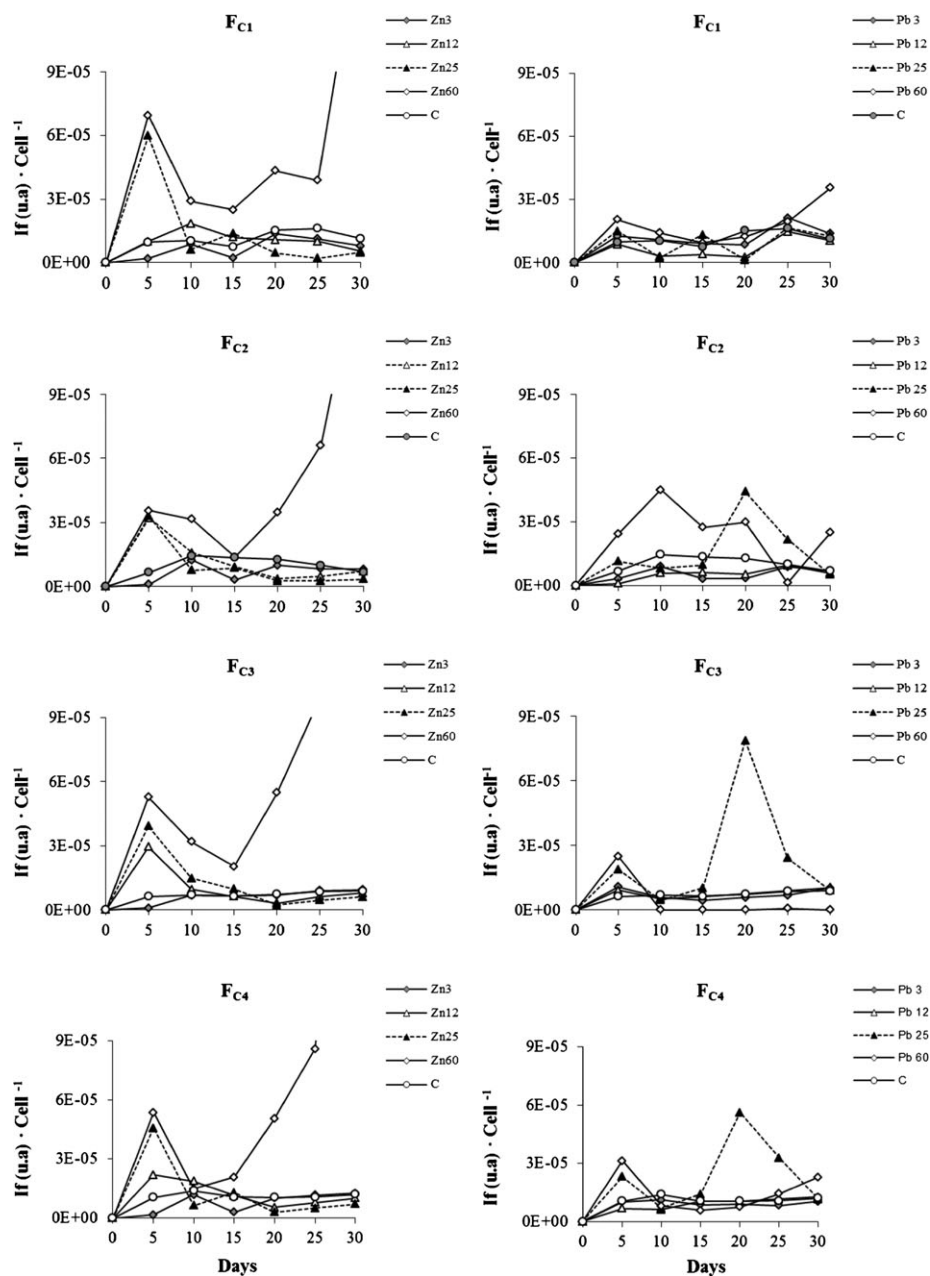


FIG. 6. Fluorescent contributions per cell to the fluorescent dissolved organic matter (FDOM) released by the *Alexandrium catenella* ACT03 cultures exposed to the tested metals. The lines represent the four components C1, C2, C3, and C4. The columns represent respectively the zinc or the lead effects on the released FDOM.

bility, reporting that, thanks to metal exclusion mechanisms, the diatom *Cylindrotheca fusiformis* was much less affected by copper stress than the dinoflagellate *Gymnodinium* sp., which accumulated a much higher percentage of this metal at the intracellular level, compared to the diatom (Pistocchi et al. 2000).

In this study, effects of increasing  $Zn^{2+}$  and  $Pb^{2+}$  concentrations on the cell growth became manifest through the observed decrease in cell density, and therefore in growth rates, suggesting that metals could act on the cell division mechanisms. Many studies about other phytoplankton species also showed that excess of these metals may reduce the

physiological growth (Gavis et al. 1981, Brand et al. 1986, Sunda 1989, Franklin et al. 2002). For *A. catenella*, at low concentration range, the effects of zinc or lead were different, indicating various adaptation mechanisms toward toxic ions. For lead, the effects on the cell density reduction and on the growth rate appeared early, at free concentrations lower ( $10^{-8}$  M) than those obtained for zinc ( $10^{-7}$  M). However, when free metal concentrations reached  $10^{-7}$  M for lead or  $10^{-6}$  M for zinc, the *A. catenella* cell density considerably decreased, and vegetative cells were converted into cysts. In previous studies, Lage et al. (1996) observed an inhibition of the *Amphidinium* genus cultivated under copper contam-



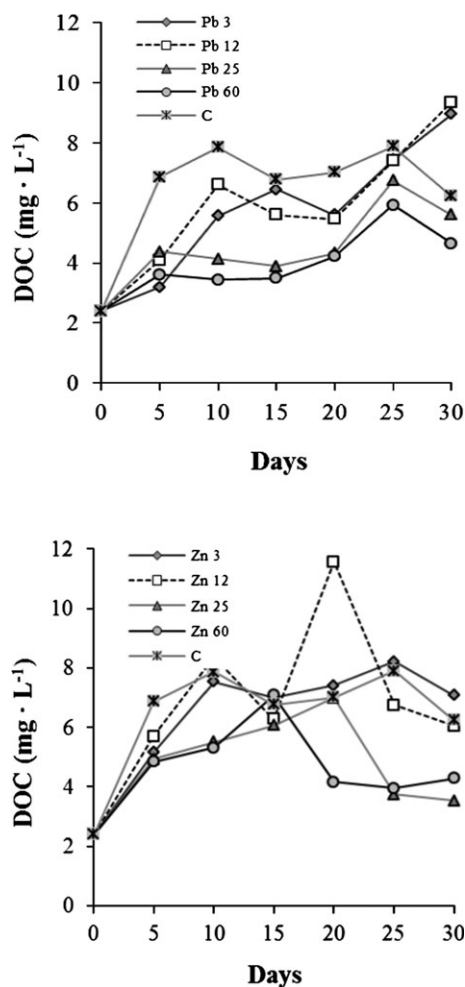


FIG. 7. Temporal evolution of the dissolved organic carbon (DOC) concentrations (expressed in  $\text{mg} \cdot \text{L}^{-1}$ ) released by the *Alexandrium catenella* ACT03 cultures in control, and in zinc- or lead-contaminated conditions (C: control; total zinc or lead concentrations: 3, 12, 25, or 60  $\mu\text{M}$ ).

ination (7.9  $\mu\text{M}$ ). Moreover, although free metal concentrations are toxic for the cells, the *A. catenella* ACT03 strain has been still able to survive in these conditions, may be developing tolerance toward free zinc or lead concentrations.

**Fluorescence responses.** Results showed an enhancement of the fluorescence contribution during the *A. catenella* growth kinetics. This increase was synchronized with the exponential cell growth for the C1, the C2, and the C3 components, but showed a slight fluctuation impacted by metal concentration, for the C4 component. It can be supposed that fluorescence contribution associated with cell density should be constant during the incubation of cultures, for low (3  $\mu\text{M}$ ) and medium (12  $\mu\text{M}$ ) concentrations of lead or zinc, which was not the case for higher contamination levels (25 and 60  $\mu\text{M}$ ; Fig. 5). Furthermore, as the DOC was correlated with cell density, it can be suggested that FDOM was due to

extracellular substances released by *A. catenella*, as products of physiological excretion. However, the DOM measured as FDOM or as DOC in the culture medium could have other origins, as the release of the cell internal contents further to the *A. catenella* lysis associated with the cell senescence, or the release of organic matter during the excystment phase as the vegetative cell walls break open and release cysts.

On the other side, the fluorescent substances were more induced by low ( $\text{pZn}^{2+} = 6.87$  ( $\text{Zn}_3$ );  $\text{pPb}^{2+} = 8.28$  ( $\text{Pb}_3$ )) or moderate metal concentrations ( $\text{pZn}^{2+} = 6.20$  ( $\text{Zn}_{12}$ );  $\text{pPb}^{2+} = 7.57$  ( $\text{Pb}_{12}$ )). This may indicate the activation of cellular mechanisms to exudates fluorescent organic matter. However, at high levels of  $\text{Zn}^{2+}$  ( $\text{pZn}^{2+} = 5.40$  ( $\text{Zn}_{60}$ )) or  $\text{Pb}^{2+}$  ( $\text{pPb}^{2+} = 6.73$  ( $\text{Pb}_{60}$ )), cells were transformed into resistance cysts and therefore released less DOC and FDOM. In these conditions, a part of the fluorescent contribution could be associated with (i) either an extensive loss of cellular matter by cysts under stress (ii) or a specific and direct production of FDOM by cysts.

As a result, the previous mentioned tolerance of *A. catenella* under metal stress could be associated with the FDOM release in contaminated conditions, since FDOM could (i) complex free metals, (ii) modify metal speciation, and (iii) thus, reduce metal toxicity. Another response to metal stress could be associated with the DOC production by *A. catenella* measured during its exponential phase and at the end of its stationary phase of growth. Indeed, the DOC showed a maximum at  $\text{pPb}^{2+} = 7.57$  ( $\text{Pb}_{12}$ ) or at  $\text{pZn}^{2+} = 6.20$  ( $\text{Zn}_{12}$ ), which could be used by cells as a response to the free metal increase. In a previous study, some authors did not observe DOM production in *Alexandrium tamarense* cultures submitted to nutrient stresses (Chen and Wangersky 1996), which could confirm that metal stress enhanced cell DOC production in comparison to other types of stress.

The variation in fluorescence was observed through the C3 and the C4 components, showing that the specific contribution increased with free metal concentration and with exposure time. These fluorophores were typical of marine humic-like material (Parlanti et al. 2002) and their occurrences were a sign of organic matter degradation. In addition, the fluorophores responsible of these two components have been recognized as typical humic components having received individual designation, like A and C (Coble 1996, Patel-Sorrentino et al. 2002). Indeed, the C3 and C4 components were produced during the cell growth, and likely associated with DOM, meaning their relationship with the marine production of organic matter (Coble 1996). Furthermore, the maximum FDOM production was measured during the exponential growth phase, suggesting that the corresponding fluorescent components occurred in the

same time that the mechanisms set up against metal stress.

The occurrence of C1 and C2 components may indicate exudation of dissolved proteins and be used as markers to estimate biological activity, as it has been demonstrated in previous studies (Mayer et al. 1999, Parlanti et al. 2002). However, the C1 component seemed to be related to the cell mechanism shown by the exudation of dissolved proteins in the medium, which could be involved in complexation of free metal ions, whereas the C2 component appeared rather linked to the *Alexandrium* growth process. Through this process, the cells could reduce the availability of free metals, and thus, their intracellular uptake. This may reflect the degree of tolerance of *A. catenella* cells toward metal stress.

#### CONCLUSIONS

This study dealt with the characterization of the FDOM released by a marine toxic dinoflagellate grown under metal contamination. The results obtained revealed that under metal stress, the *A. catenella* development is affected from moderate metal concentrations (12  $\mu\text{M}$ ), whereas cells exposed to low concentrations (3  $\mu\text{M}$ ) exhibit a less affected growth. In the presence of  $\text{Pb}^{2+}$ , a cell encystment occurred at  $10^{-7}$  M, and in the presence of  $\text{Zn}^{2+}$ , cysts began to appear at  $10^{-6}$  M. An increase in fluorescent organic molecules exudated by cells under stress metal conditions was also observed during the exponential phase of growth. This was shown by different temporal accumulation of the FDOM released by cell of *A. catenella* over the incubation time and among the different metal contaminants. Release of organic molecules should play an important role in metal speciation, and therefore could constitute a means of defense against metal stress.

The fluorescence results treated by the PARAFAC modeling gave four different components. Two components (C1 and C2) were related to the biological activity involved in the growth processes. Two other components (C3 and C4) were rather linked to the products of degradation of the organic matter coming from algal cells.

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