Development of q-PCR approaches to assess water quality: Effects of cadmium on gene expression of the diatom *Eolimna minima*.

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Abstract

This study was undertaken to develop molecular tools to assess water quality using diatoms as the biological model. Molecular approaches were designed following the development of a rapid and easy RNA extraction method suited to diatoms and the sequencing of genes involved in mitochondrial and photosystem metabolism. Secondly the impact of cadmium was evaluated at the genetic level by q-PCRs on 9 genes of interest after exposure of *Eolimna minima* diatom populations cultured in suspension under controlled laboratory conditions. Their growth kinetics and Cd bioaccumulation were followed.

Population growth rates revealed the high impact of Cd at 100 μg/L with total inhibition of growth. These results are linked to the high bioaccumulation values calculated after 14 days of exposure, 57.0 ± 6.3 μgCd/g dw and 734.1 ± 70 μgCd/g dw for exposures of 10 and 100 μgCd/L respectively.

Genetic responses revealed the impact of Cd on the mitochondrial metabolism and the chloroplast photosystem of *E. minima* exposed to 10 and 100 μgCd/L with induction of *cox1*, *12S*, *d1* and *psaA* after 7 days of exposure for the concentration of 100 μgCd/L and of *nad5*, *d1* and *psaA* after 14 days of exposure for both conditions.

This is the first reported use of q-PCR for the assessment of toxic pollution on benthic river diatoms. The results obtained presage interesting perspectives, but the techniques developed need to be optimized before the design of new water quality diagnosis tools for use on natural biofilms.

Key words

*Eolimna minima*, Diatom, Cadmium, Metal, Quantitative real time PCR.

1.Introduction
Over the past decades, the ever increasing release of agricultural, industrial and domestic waste led to a significant contamination of the environment and particularly of the aquatic compartment.

In regard to the resulting degradation of freshwater quality, various diagnostic tools have been designed. In France, in collaboration with Water Agencies and Environment Ministry services, CEMAGREF has developed several diatom indices to estimate global water quality like the “Indice de Polluosensibilité Spécifique” (IPS, Cemagref 1982) or the Biological Diatom Index (BDI, Coste et al. 2009), both routinely used for monitoring applications in several European countries. Nevertheless, the indices currently used for general water quality assessment - including the IPS and BDI - were not designed to assess specific toxic alterations (metals, synthetic organic pollutants) and are not really suitable for a sensitive diagnosis of this type of pollution. Thus, heavy metals are of particular concern because of the different toxic effects they can produce (Sauvant et al. 1997, Bucio et al. 1995).

Recent works have studied the responses of diatom communities to metal pollution, for example Morin et al. (2008a) observed structural impact at the community level and morphological abnormalities in a metal-polluted stream. Other studies assessed effects of metal-induced oxidative stress on functional descriptors such as photosynthetic (Antal et al. 2004) or antioxidant (Branco et al. 2010) enzyme activities.

However, studies assessing genetic responses of diatoms to contamination exposure are still at the early stage principally because of the lack of available diatom nucleotide sequences in genomic databases and difficulties to access genetic material. Only *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* - two marine diatom species - have as yet been entirely sequenced, and genomic information about freshwater species is still extremely scarce.

Extracting the genetic material of organisms is the first step to obtain sequences of interest. Diatoms require a particular approach for this because of their unique external structure. Diatoms are unicellular algae and their most obvious distinguishing characteristic is their siliceous cell wall called the frustule. Various approaches have been used to break the frustule, but these are often time consuming (Wawrik et al. 2002) and/or need specialized laboratory material such as a French press (Stabile et al. 1990, Davis and Palenik 2008, Hildebrand et al. 1998) or a MiniBeadBeater (Fawley and Fawley 2004). More rapid and straightforward extraction methods are needed, specifically for diatoms. The expression of many genes is involved in the response of organisms to toxicants and can be disturbed by them. Once inside the cell, metals can cause oxidative stress (Wang et al. 2004) affecting the way in which the mitochondria (Stohs and Bagchi 1995) or photosynthesis function (Knauert...
and Knauer 2008). The mitochondrial superoxide dismutase (sodMn) gene was selected because of its involvement in antioxidant defences. The mitochondrial metabolism was investigated using the cytochrome C oxidase (cox1) subunit and the NADH dehydrogenase subunit 5 (nad5) genes. In addition, the quantity of mitochondria in the cells was estimated using mitochondrial 12S ribosomal RNA. D1 protein (d1) and PsaA protein (psaA) are components of PS2 and PS1 respectively and were selected to investigate photosystem metabolism. Cytochrome P450 1A1 (cyp1A1) was selected as a biomarker for exposure to polynuclear aromatic hydrocarbons. Two genes commonly used as references in numerous genetic studies were selected as for study in our work: β-actin (act) and 18S ribosomal RNA. Consequently, the first objective of this work was to develop a diatom-specific extraction method and to shape molecular tools to select and sequence genes of interest to assess metal contamination. We used cultures of Eolimna minima, a metal-tolerant freshwater diatom commonly collected within periphytic biofilm samples in running water and especially in metal-contaminated areas (Morin et al, in press). Our attention was particularly focussed on genes involved in mitochondrial and photosystem metabolism and responses of E. minima to a gradient of cadmium contamination was then characterised using these selected molecular tools. Cultures of E. minima were exposed in the laboratory for 14 days to 10 µgCd/L or 100 µgCd/L. The moderate exposure corresponds to the concentrations reported in cadmium polluted rivers like the river Riou-Mort (South West France) (Feurtet-Mazel et al. 2003) and the highest is comparable to those reported in more highly polluted European rivers (Ivorra et al. 1999). Cell numeration, bioaccumulation and genetic responses were followed 1, 2, 7 and 14 days after contamination.

2. Materials and methods

2.1. Molecular methodological developments

2.1.1. RNA extraction method

In order to access the genetic material of diatoms, a novel RNA extraction method was developed. Designing an efficient method able to break the external silica wall of diatoms without degrading the genetic material was a prerequisite. For this purpose, cultures of the diatom Eolimna minima were grown in batches from axenic strains provided by the UTEX algal collection (Texas University, USA). The diatom cells were cultured in sterile Dauta medium (Dauta 1982) with silica added to a final concentration of 10mg/L in 3L Erlenmeyer
flasks. The cultures were maintained at between 17 and 18°C in a thermostatic room with a photon flux density of 160\mu mol.m^{-2}.s^{-1} and a 12:12 h light:dark cycle. Fresh cultures were inoculated every seven days to promote optimal growth of the diatoms until sufficient cells were obtained.

30 mL (2.10^6 cells/mL) were removed from the E. minima cultures and the cells harvested after centrifugation at 3863g for 5 min at room temperature. The cell pellet was transferred to 2 mL Eppendorf tubes and resuspended in 1 mL of diethyl pyrocarbonate (DEPC) treated water. Then, the diatoms were centrifuged again at 2419g for 5 min at room temperature and the supernatant was removed. 1 mL of Trizol (invitrogen) and 300 \mu L of glass beads (0.10 – 0.11mm-diameter, B. Braun Biotech International) were added to the cell pellet. Then the sample was vortexed 3 times for 30 seconds, and the supernatant transferred into a clean Eppendorf tube and placed in a water bath at 30°C for 5 min. After addition of 200 \mu L of Chloro RECTAPUR (VWR), the sample was vortexed for 10 s and placed in water bath at 30°C for 2 min. The tubes were centrifuged at 11 360g for 5 min at room temperature and the aqueous phase containing the genetic material was transferred into clean tubes. The end of the extraction was realized using Absolutely RNA Miniprep Kit (Stratagène) according to the manufacturer’s instructions:

550 \mu L of 75% ethanol were added to the aqueous phase, the sample was vortexed, transferred to affinity columns and centrifuged for 1 min at 11 360g at room temperature. Filtrates were removed, 600 \mu L of low-salt buffer were placed on the column which was centrifuged at 11 360g at room temperature for 2 min. 5 \mu L of DNAse 1 [1U] and 50 \mu L of activity buffer were added to the column and placed in a water bath at 37°C for 15 minutes. 600 \mu L of high-salt buffer were added to the column which was centrifuged for 1 min at 14 500 rpm. 300 \mu L of low-salt buffer were added and the column was centrifuged for 2 min at 14 500 rpm. The columns were transferred into clean tubes and 30 \mu L of elution buffer at 60°C were added and centrifuged for 1 min at 12 250g at room temperature. Total RNA was analysed on 1% (w/v) agarose gel with ethidium bromide by UV light on an illuminator.

2.1.2 Reverse transcription of RNA

The first strand of cDNA was synthesised from 14 \mu L of total RNA (3 to 5 \mu g) using the Stratascript first strand synthesis system (Agilent). After the addition of 1 \mu L of oligo(dT) [1\mu M], 1 \mu L of random primers [1\mu M], 0.8 \mu L of dNTPs [10mM] and 2 \mu L of 10x first-strand buffer the reaction was incubated for 5 min at 65°C. Then 1 \mu L of Stratascript reverse
transcriptase [1U/µL] and 0.5 µL of RNase inhibitor [0.5U] were added, the reaction was incubated for 1 h at 42°C in an Eppendorf Mastercycler. The cDNA mixture was conserved at -20°C until it was used in a real-time PCR reaction.

2.1.3. Cloning and molecular characterization of the target genes

Genetic research focussed on seven genes involved in responses to environmental contamination and/or in which expression can possibly be disturbed by environmental factors (sodMn, nad5, d1, cox 1, psaA, cyp 1A1, and 12S) and for two reference genes (18S and act). Primers were designed by performing ClustalW analysis from protein or nucleic sequences (12S and 18S) from marine diatoms and other phylogenetically related aquatic organisms available in NCBI databases. From these alignments, primer pairs about 20-25 bp long were designed in the most conserved regions for amplification of fragments between 280 and 900 bp.

PCR reactions were performed using these primers following the manufacturer’s instructions. After the addition of 1 µL of dNTP [10 mM], 3 µL of MgCl2 [25 mM], 0.2 µL of Taq [5U/µL], 10 µL of activity buffer 5X, 0.5 µL of each primer (upstream and forward primers) [100 µM], 34 µL of DEPC treated water and 1 µL of cDNA, 40 PCR cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min were carried out in an Eppendorf AG thermocycler. PCR products were analyzed on 1% (w/v) agarose gel with ethidium bromide. The ethidium bromide stained bands were revealed by UV light on an illuminator.

After a purification step using the PCR purification kit (Qiagen) according to the manufacturer’s instructions, cDNA was cloned with pGEM®-T, (PROMEGA).

Successful insertion of the fragments was checked by PCR using T7 and SP6 universal primers following the manufacturer’s instructions (40 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 1 min.). All the partial gene sequences obtained were submitted to GenBank under the accession numbers reported in Table1.

2.1.4. q-RT-PCR primer design and real time q-PCR

For each gene, specific q-RT-PCR primer pairs (table 1) were determined using the LightCycler probe design software (version 1.0, Roche).

Real time PCR reactions were performed in a LightCycler (Roche) following the manufacturer’s instructions (one cycle at 95°C for 1 min and 50 amplification cycles at 95°C for 5 s, 60°C for 5 s and 72°C for 20 s).
Each 20 µL reaction contained 1 µL of activity buffer (Syber Green I (Roche), Taq Polymerase, dNTP), 3.2 µL of MgCl$_2$ [25µM], 2 µL of the gene-specific primer pair at a final concentration of 300 nM for each primer, 12.8 µL of DEPC-treated water and 1 µL of cDNA. Specificity was determined for each reaction from the dissociation curve of the PCR product. This dissociation curve was obtained by following the SYBR Green fluorescence level during gradual heating of the PCR products from 60 to 95°C. Relative quantification of each gene expression level was normalized according to β-actin gene expression. Relative mRNA expression was generated using the 2$^{-}\Delta$CT method (Livak and Schmittgen 2001).

**2.1.5. Determination of sensitivity limit**

In order to optimize the RNA extraction method, the sensitivity limit was quantified. Ten dilutions of *E. minima* cultures were prepared to obtain theoretical numbers of cells ranging from $1.8\times10^7$ cells to 1.8 cells ($1.8\times10^7$; $9\times10^6$; $1.8\times10^6$; $9\times10^5$; $1.8\times10^5$; $1.8\times10^4$; $1.8\times10^3$; $1.8\times10^2$, 18 and 1.8 cells). Extraction and reverse transcription of RNA were performed as described previously on these different samples. The resulting cDNA was used for q-PCR with *cox1* and *act* specific primer pairs as described above.

**2.2. Experimental protocol to assess cadmium effects on *Eolimna minima***

**2.2.1. Exposure conditions**

250 mL of *E. minima* culture in suspension in modified Dauta medium were placed in 500-ml Erlenmeyers to reach a final concentration of $10^6$ cells/mL. The organisms were directly exposed to three Cd concentrations ($C_0=0$, $C_1=10\pm3.2$ and $C_2=96\pm34.2$ µgCd/L, mean values ± standard deviations over 14 days, from CdCl$_2$ stock solution, prepared from a 1000 mg vial, Merck, Germany). The cultures were maintained at between 16 and 18°C along the 14 days of exposure with a photon flux density of $59.19\pm7.75$ µmol.m$^{-2}$.s$^{-1}$ and a 12:12 h light:dark cycle. The Erlenmeyer flasks were closed with sterile cotton wool and kept on an orbital shaker (60 rpm). Triplicate flasks were collected at each of the four different sampling periods (after 1, 2, 7 and 14 days of exposure to Cd) leading to 36 different experimental conditions. Thus 7.5 mL of culture were sampled after the exposure durations assigned for water Cd analyses, intracellular and total Cd concentration analyses and cell counting. Simultaneously, three 100 mL replicates were sampled for genetic analysis and stored at -80 °C.
2.2.2. Cd analysis

Cadmium concentrations in water and in diatom cells were determined by atomic absorption spectrophotometry (AAS) using a graphite tube atomiser after filtration through a GH polypropylene membrane (0.45 µm pores, 25 mm diameter, PALL) for diatom cell Cd concentration and on a 0.45µm Teflon filter (from CAS) for Cd concentration in water. The analytical method was simultaneously validated for each sample series by analysing standard biological reference materials (TORT 2, Lobster hepatopancreas; DOLT 3 dogfish liver; NCR/CNRC, Ottawa, Canada). 3 mL of nitric acid (65% HNO$_3$) were added to reference the material before digestion in a pressurized medium (borosilicate glass tube) at 100°C for 3 h. 15 mL of ultra pure water were added and samples were stored at 4°C before analysis.

Samples used for intracellular cadmium determination within diatoms were treated first with EDTA (10 mM), a strong metal complexing ligand, to remove the metal ions adsorbed to the diatom cell walls (Behra et al. 2002). In this case, a drop of EDTA was added to each 2 mL sample and filtered after 10 min. Samples used for total cadmium determination in diatoms skipped this step. After 24h at 44°C, all samples were digested by nitric acid (3 mL of 65% HNO$_3$) in a borosilicate glass tube at 100°C for 3 h. The samples were then analysed by atomic absorption spectrophotometry as described previously.

2.2.3. Diatom cell density

1.5 mL aliquots were immediately fixed in formalin (37% formaldehyde, Prolabo, France) for counting. Each sample was counted in triplicate using a Nageotte counting chamber (Marienfeld, Germany). After 10 min ultrasonication, 200 µL of sample were placed on the counting chamber. The total number of individuals and the number of dead cells were recorded in 4 fields of the gridded area (1.25 µL each, 0.5 mm depth) under light microscopy at 400x magnification (Leitz photomicroscope). Distinction between dead and live organisms was estimated by the observation of the turgescence and colour of the chloroplast.

2.2.4. Statistical analysis

Statistical analyses were performed with R 2.12.0 using the Linear Mixed Effect model (library nlme). Homogeneity of variances was verified by Leven test and Fisher’s LSD was calculated.
To assess the effects cadmium on *E. minima* by the molecular tools described above, expression of the nine target genes sequenced in this study was examined. The expression levels of the nine genes involved in antioxidant defences, or mitochondrial or photosynthetic metabolism were investigated by quantitative real-time PCR as described previously using the q-PCR primers designed in this study.

3.Results and discussion

3.1.Methodological developments

3.1.1.Extraction method and optimization

Electrophoresis revealed the presence of large quantities of q-PCR amplification products, underlining the efficiency of this method for breaking open the silicate frustules while preserving the RNA. Moreover, the analysis of triplicates from the cadmium exposure experiment by q-PCR showed the regularity in the threshold cycle of most of the genes (apart from 18S and 12S see below). As shown in figure 1, seven of the nine genes studied showed a percentage of variation within triplicates of under 8%. This homogeneity reveals the high efficiency of the extraction method and suggests a constant basal level in RNA production from these genes in *E. minima*.

In order to optimize the extraction protocol, the sensitivity limit was determined by performing q-PCR with *act* and *cox1* q-PCR primers. Analysis of dissociation curves of the different amplification products revealed the amplification of cDNA for an initial number of cells in the range $1.8 \times 10^7$ to $1.8 \times 10^5$. Below this number no amplification was detected through lack of sufficient genetic material. As the minimal number of cells needed to obtain q-PCR amplification was determined at $1.8 \times 10^5$ cells for the diatom *E. minima*, the minimum number of cells used in subsequent q-PCR analyses was fixed at $10^7$ cells. The RNA extraction method developed in this study showed its high efficiency in the extraction of total RNA for the diatom *E. minima*. Moreover the method is simple and only requires standard laboratory equipment, which is an important advantage compared to existing methods that can require expensive and/or specialised apparatuses to extract RNA like a French pressure cell press (Hildebrand et al. 1998) or MiniBeadBeeater (Fawley and Fawley 2004). The RNA extraction method developed is also rapid compared to existing methods using a muffle furnace overnight (Wawrik et al. 2002). Moreover, the method has been successfully tested on some quite different diatom species (*Achnanthidium minutissimum* and *Nitzschia palea*) suggesting it will be applicable to a large range of diatom species.
3.1.2. Nucleotide sequence accession number and q-PCR primers

The 9 genes of interest selected in our study were successfully cloned and sequenced, accession numbers of sequenced genes and q-PCR primers are shown in table 1. cDNA sequences of the 9 genes have been deposited in the GenBank database.

3.2. Effects of cadmium on *Eolimna minima*

3.2.1. Cd water concentrations in solution

Cadmium concentration in water for the three different treatments is plotted against time in Figure 2. During the 14 days of exposure, mean Cd concentrations in water were 10±3.2 µg/L and 96±34.2 µg/L for nominal concentrations of 10 and 100 µgCd/L respectively. The cadmium concentrations in water were very stable from days 0 to 7 in both C₁ (11.5±0.6 µgCd/L) and C₂ (111.3±3.8 µgCd/L) treatments. A strong decrease occurred in cadmium concentrations on day 14 with values falling to 4.3±4 µgCd/L and 35.2±14.2 µgCd/L for treatments C₁ and C₂ respectively. This is unlikely to be explained by metal adsorption onto the surface of the experimental units, as no decrease was observed during the first seven days of exposure: bioaccumulation of cadmium by diatoms is more likely responsible for this decrease, as shown by the following results.

3.2.2. Effect of cadmium on the growth of *E. minima*

The number density, over the 14 days of direct exposure to the three different concentrations of cadmium, is plotted in Figure 3. Statistical analysis revealed that growth was not significantly different in controls and with a Cd concentration of 10.0±3.2 µg/L over the whole duration of the experiment. Besides, Gold et al. (2003) and Duong et al. (2008) observed a high proportion of small, adnate species like *Achnanthidium minutissimum*, *Encyonema minutum* and *E. minima* in stations contaminated with around 6 µg/L cadmium. This suggests the tolerance of these species to high levels of cadmium and thus their ability to develop in cadmium-contaminated conditions with around 10 µgCd/L. Nevertheless, densities in contaminated samples were on average lower than in control treatments throughout the 14 days of exposure. It could be interesting to study population kinetics over a longer period to see if, after 14 days of exposure, significant differences are observed between the C₁ treatment and the control as observed by Morin et al. (2008a) who reported differences in biofilm diatom densities between control and 10 µgCd/L-contaminated treatments only after 6 weeks of exposure.
Significant differences in cell densities were observable from day 7 between the controls and the series with the highest contamination of cadmium (2,900,000 ± 250,000 cells/mL for control versus 1,300,000 ± 500,000 cells/mL). After 14 days of exposure, the diatom cell density was 3.2 times higher than with the control treatment (4,200,000 ± 390,000 cells/mL versus 1,300,000 ± 280,000 cells/mL). Moreover, diatom growth was null with treatment C2. Our results are in accordance with those of Tourtelot (2003) who studied the acute effects of Cd on *Achnanthidium minutissimum*. For this species, referenced as pollution resistant, the rate of growth was null after 14 days of exposure to 100 µgCd/L. Similar results were observed at the community level, Morin et al. (2008b) exposed biofilms in microcosms to 10 and 100 µgCd/L for 6 weeks and observed a significant reduction in diatom density for biofilms exposed to the higher concentration compared to the control and the low cadmium contamination treatment.

### 3.2.3. Cd bioaccumulation in diatoms

Intracellular and total bioaccumulation are not given for days 1 and 2 after exposure because of the insufficient quantity of cells in the samples. Intracellular and total Cd concentration in diatoms after 7 and 14 days of exposure to the different treatments (0, 10±3.2 and 96±34.2 µgCd/L) are reported in Figure 4. Intracellular and total bioaccumulation increased significantly between days 7 and 14 for the 2 contaminated treatments. For the lower contamination concentration (C1), the intracellular and total concentration increased from 3.6±1.8 to 51.4±5.3 µgCd/g dw and from 6.4±3.4 to 57±6.3 µgCd/g dw respectively. For the higher contamination pressure (C2), there was an increase from 5.6 ± 2.7 µgCd/g dw to 430.1 ± 86.4 µgCd/g dw and from 11.4 ± 3.0 to 734.1 ± 70 µgCd/g dw respectively, this latter value is in the range of literature values (Morin et al. 2008a, Duong et al. 2008). The increase in cadmium bioaccumulation with exposure time was also shown in environmental samples by Duong et al. (2008). The cadmium content in the biofilm increased gradually for days 14 and then remained constant. This could be due to the saturation of binding sites in the biofilm leading to a limitation of Cd accumulation. In our experiment it is difficult to estimate if the saturation phase is reached after 14 days of exposure. In order to determine the kinetics of saturation and the saturation value for *E. minima*, experiments will have to cover a longer period with a constant contamination pressure.
After 7 days of exposure a significant difference occurred between the controls and the cadmium-contaminated treatments, but no such difference appeared between moderate and high contamination (C₁ and C₂) or between intracellular and total bioaccumulation. After 14 days of exposure, total bioaccumulation in the controls was significantly different from that in the cadmium-contaminated treatments (p = 0.008 and p < 0.001 respectively). Total bioaccumulation was significantly higher than intracellular bioaccumulation for contamination C₂ (430.1 ± 86.4 μgCd/g dw versus 734.1 ± 70 μgCd/g dw).

The high values of cadmium bioaccumulated and the large increase between days 7 and 14 for the higher Cd concentration could so explain the total inhibition of growth observed for this treatment.

The ratios of adsorbed to absorbed cadmium are difficult to estimate owing to the heterogeneity of the results except for exposure to 100 μgCd/L after 14 days. In order to evaluate this difference better for shorter periods and lower concentrations, sampling volumes would have to be increased. In our study however, increasing sample volumes from 2 to 40 mL still led to very low dry weight measurements (between 2 and 6 mg for day 7 and 14) increasing the incertitude of measurement and errors in bioaccumulation calculations. Therefore particular attention must be paid to the volumes tested when diatom cultures are considered.

3.2.4. Gene expression levels

In our study, two genes were selected as potential references, β-actin and 18S ribosomal RNA. Both are widely used as references in q-PCR analyses. The average percentage variation in the threshold cycle (Ct) of 18S ribosomal RNA within triplicates was 13.7% against 4.9% for β-actin (figure 1). The Ct value was defined as the number of cycles needed for the amplification signal to reach a specific detection threshold, and so is inversely correlated with the amount of cDNA template present in the PCR amplification reaction. The low stability and the high abundance of 18S rRNA underlined by our results (figure 5) shows the unsuitability of this gene as a reference. Consequently, 18S was finally not kept as a reference gene in our experiments. On the other hand, β-actin showed a high regularity and high threshold cycle values and was thus defined as reference gene in our study. 18S rRNA has also been described as a poor reference gene in other species such as buffalo (Bubalus bubalis) (Castigliego et al. 2010) or humans (Radonić et al. 2004). A recent study underlined this point; Siaut et al. (2007) reported that 18S rRNA was a very poor reference gene for Phaeodactylum tricornutum, a marine diatom. Here, we chose β-actin as reference gene.
owing to the stability of its expression, although Siaut et al. (2007) show that the β-actin level appears to be tightly correlated to light with an increase during the light period. In our study, however, the photoperiod was controlled and sampling performed at a fixed time, which avoided this source of error and allowed us to use β-actin as reference gene. Nevertheless it would be interesting to study other reference genes as proposed by Siaut et al. (2007) (H4, RPS, Cdka and TBP) for future analyses and particularly in field samples.

Expression of genetic levels during the 14 days of exposure to cadmium at 10.0±3.2 and 96.0±34.2 µg/L are shown in table 2. During the 14 days, an impact resulting in the amplification of 5 of the genes studied was observed (cox1, nad5, d1, psaA and 12S). Different genetic responses are expressed as a function of time and concentration of exposure. Differential genetic expression revealed an effect on mitochondrial and photosynthetic metabolism, observed after 7 days of exposure only at the highest contamination pressure, and for both contamination pressures on day 14.

After 7 days of exposure, cox1, d1, psaA and 12S were up-regulated for the exposure concentration of 96.0±34.2 µgCd/L. After 14 days of exposure, there was a strong up-regulation of nad5, d1 and psaA for both concentrations. Moreover, a large difference occurred between the 2 treatments: expression of nad5, d1 and psaA were 4, 12 and 19 times higher respectively for C2 than C1. We can also see that responses of d1 and psaA in the C2 treatment increased with exposure time by a factor of 4.4 and 6.4 respectively between days 7 and 14.

The disturbance in the expression of the cytochrome C oxidase (cox1) subunit and the NADH dehydrogenase subunit 5 (nad5) observed in our study suggests an impact on mitochondrial metabolism. This is in accordance with data in the literature. Indeed, after entering the cell, cadmium can inhibit electron transfer in mitochondrial respiration, and also lead to the formation of reactive oxygen species (ROS) (Wang et al. 2004, Gonzalez et al. 2006). The oxidative stress generated then brings about DNA breaks and induction of apoptotic mechanisms leading to cell death (Bagchi et al. 2000, Chan and Cheng 2003). Nevertheless, the ratio cox1/12S was constant throughout the experiment indicating that the number of mitochondria increases to maintain the level of ATP production. This increase of cox1 expression has already been reported in zebrafish Danio rerio (Gonzalez et al. 2006). Electron microscopy studies also in the zebrafish have shown extensive disturbance of the ultrastructure of the mitochondria after metal exposure to methyl mercury (Cambier et al.
2009). In future work it will be of particular interest to use electron microscopy to view *E. minima* after Cd exposure so as to be able to evaluate damage at the mitochondrial level.

Secondly, with regard to the up-regulation of *psaA* and *d1* after 7 days of exposure to 100 µgCd/L and after 14 days for the two contamination levels, it is clearly shown that the photosynthetic system of *E. minima* is strongly affected by exposure to cadmium. The impact of metals on the photosystem is well known and has been studied in numerous works. Photosystem inhibition has been reported by Sudo et al. (2008) at physiological and genetic levels with, in particular, repression of *d1* and *psaA* in rice leaves after copper stress. Hörcsik et al. (2007) studied the effect of chromium on PS2 activities in *Chlorella pyrenoidosa* cells: they showed the inhibition of PS2 by photodestruction of reaction centres in cells cultivated in the presence of chromium. At the ultrastructural level, Ouzounidou et al. (1997) showed damage to chloroplast structures with changes in the shape and thickness of the thylakoid membranes in wheat (*Triticum aestivum* L.) after exposure to 265 µgCd/L for 7 days.

Moreover, the impact at the genetic level underlined by the up-regulation of *nad5*, *d1* and *psaA* after 14 days of exposure revealed effects at a Cd concentration of 10 µg/L. This result is of particular interest because, at a more integrative level of population kinetics, this effect was not noted. Overall, the above findings stress the importance of considering molecular markers when studying pollution because of the potentially higher sensitivity and the earlier response of genomic markers to toxic pollution compared to more global endpoints like mortality or population kinetics.

No significant difference in genetic expression levels was observed for the genes *cyp1A1* or *sodMn* during the experiment, at either of the concentrations tested. Mitochondrial superoxide dismutase is known to be involved in the oxidative stress response and several studies have shown the up-regulation of this gene after metal contamination. For example Gonzalez et al. (2006, 2005) demonstrated the up-regulation of *sodMn* in the gills of zebrafish after 7 days of direct exposure to 86 ± 0.26 µgCd/L and in another study in skeletal muscle and liver after 21 and 63 days of dietary exposure to methyl mercury for concentrations of 5 to 13.5 µgHg/g dw.

In our work, this up-regulation was not observed, several hypotheses can be proposed: the sampling rate may be unsuitable and/or other enzymes of the oxidative stress response may be involved such as catalase, glutathione peroxidise or other types of superoxide dismutases (*sodFe, sodCu/Zn or sodNi*). Other authors have studied antioxidant enzyme activities, for example Branco et al. (2010) observed an increase in catalase activity compared to controls after 5 days of exposure to Cd from 100 to 300 µgCd/L on the diatom *Nitzschia palea* and in the global superoxide dismutase activity for concentrations from 200 to 300 µgCd/L. In a
field study, Bonet et al. (2010) showed a clear inhibition of glutathione-S-transferase at high metal exposure and no difference in superoxide dismutase activity between sites impacted by metals at different levels.

For future analyses it will be interesting to sequence genes coding for catalase, gluthatione peroxidise, glutathione-S-transferase and the other superoxide dismutases (sodFe, sodCu/Zn or sodNi) in order to study their responses to metal exposure together with enzymatic activities. Cytochrome P450 1A1 plays a role in the metabolism of organic compounds such as PAH or pesticides, which explains the null genetic response observed in our study after cadmium contamination. This gene has been studied and q-PCR primers have been developed for use in future analyses for diatom exposure to compounds like PAH or PCB.

4. Conclusions and perspectives

In this study, a new glass-bead RNA extraction technique for diatoms was successfully developed and optimized. Nine genes of interest were sequenced for *Eolimna minima* allowing the application of q-PCR tools to this species.

Our results underlined the toxicity of Cd towards *E. minima* population kinetics only for the highest concentration, while q-PCR analyses revealed an impact on mitochondrial metabolism and the chloroplast photosystem for both Cd exposure concentrations. Genetic expression of *nad5*, *cox1*, 12S, *d1* and *psaA* by q-PCR could thus constitute an early warning biomarker of metal pollution. Future studies should investigate sequences of genes coding for catalase or glutathion peroxidase in order to study the response to oxidative stress.

The present study is the first reported use of q-PCR on river benthic diatoms and the results obtained are extremely promising. The techniques developed were successfully tested using simplified mixtures of diatom species. Further interesting steps for the early and sensitive assessment of metal pollution would firstly involve validating the results obtained by examining sensitive vs tolerant diatom species response levels, then finding or confirming genetic biomarkers for use on natural multispecific biofilms for impact assessment of toxic pollution.

References


Ecological Indicators 9(4), 621-650.


Tourtelot (2003) Contamination expérimentale par le cadmium et le zinc de diatomées périphytiques d’eau douce, Achnanthidium minutissimum, en conditions contrôlées de laboratoire., Université Bordeaux


### Table 1. Accession numbers and specific primer pairs for the 9 *E. minima* genes used in our study.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession number</th>
<th>Primer (5’-3’)</th>
</tr>
</thead>
</table>
| sodMn     | HM 449706        | GGTAGTAGCGCTGGCTCCA \(^a\)  
               |                  | CCAGGACACCCCGCTC \(^b\)  |
| cox1      | HM 449704        | CAGTAATTCTCACTGCCCCAGC \(^a\)  
               |                  | CCGTGACCACCGCTG \(^b\)  |
| nad5      | HM 449708        | TCAACTTTGGTTTCATCATACTGGC \(^a\)  
               |                  | TTGAACTAATCTCTGTTGTTGGAAGC \(^b\)  |
| d1        | HM 449711        | ACCACCAAATACACCAGCAAC \(^a\)  
               |                  | GCGTCTTTGGATTTTCGTGAC \(^b\)  |
| psaA      | HM 449705        | CATAAAGCGGCCACCCAAAC \(^a\)  
               |                  | CTTGGATATAAATGACTCACTTAAACTCAGG \(^b\)  |
| cyp1A1    | HM 449709        | ACAGAGGAGTCTCCCATCA \(^a\)  
               |                  | CTTGGCCGAAACGCTCAG \(^b\)  |
| act       | HM 449707        | GGCTCCACAAAAACCCCAAG \(^a\)  
               |                  | GCGTACCCCTCGTAGAT \(^b\)  |
| 12s       | HM 449710        | CGCGGTAACTAAGGAGGATGC \(^a\)  
               |                  | AGTGCCTTCGCCCATCG \(^b\)  |
| 18s       | HM 449712        | CATTGTACAGGTTGAAATTCTTGG \(^a\)  
               |                  | CCCGGAACCCCCAAGT \(^b\)  |

Abbreviations: *sodMn*-mitochondrial superoxide dismutase; *cox1*-cytochrome C oxidase subunit I; *nad5*-NADH dehydrogenase subunit 5; *d1*-D1 protein; *psaA*-PsaA protein; *cyp1A1*-cytochrome P450 1A1; *act*-β-actin, *12s*-mitochondrial ribosomal RNA 12S; *18s*-ribosomal RNA 18S. \(^a\)Upstream primer. \(^b\)Forward primer.
Table 2. Differential gene expression as compared to actin from *E. minima* after 1, 2, 7 and 14 days of cadmium exposure to 10 and 100 μgCd/L by direct route.

<table>
<thead>
<tr>
<th>Functions</th>
<th>Genes</th>
<th>Cadmium contaminated experimental units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C1 (10.0 ± 3.2 μg/l)</td>
<td>C2 (96.0 ± 34.2 μg/l)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Mitochondrial metabolism</td>
<td><em>cox1</em></td>
<td>/</td>
</tr>
<tr>
<td></td>
<td><em>nad5</em></td>
<td>/</td>
</tr>
<tr>
<td></td>
<td><em>12s</em></td>
<td>/</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td><em>sodMn</em></td>
<td>/</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td><em>d1</em></td>
<td>/</td>
</tr>
<tr>
<td></td>
<td><em>psaA</em></td>
<td>/</td>
</tr>
<tr>
<td>Xenobiotic metabolisation</td>
<td><em>cyp1A1</em></td>
<td>/</td>
</tr>
</tbody>
</table>

*Significant induction and repression factors are indicated by positive and negative values, respectively compared to the control *E.minima*. / : identical to control levels.
Figure 1. Average variation of threshold cycle within triplicates in percentage over the cadmium exposure experiment for the nine genes studied.
Figure 2. Cadmium concentration in water ± standard deviation versus exposure time for the 3 treatments ($C_0=0$, $C_1=10$ and $C_2=100 \mu g/Cd/L$). Full and empty triangles represent contaminated conditions. Full squares represent control treatments.
Figure 3. Total density of *Eolimna minima* ± standard deviation versus exposure time for the 3 treatments ($C_0=0$, $C_1=10$ et $C_2=100 \mu gCd/L$), significant differences between a and b (p value <0.05). Solid and open triangles represent contaminated conditions. Solid squares represent control treatments.
Figure 4. Intracellular and total Cd bioaccumulation in *E. minima* cultures for the two exposure times and three treatments (*C*₀=0, *C*₁=10 and *C*₂=100μgCd/L) in μg Cd/g dw. Significant differences between a, b, c, d and e (p value <0.05). White bars represent control treatments, grey bars represent *C*₁ treatments and black bars represent *C*₂ treatments. Full and hatched bars represent total and intracellular cadmium bioaccumulation respectively.
Figure 5. Expression of the two potential reference genes 18S and act in all treatments. mRNA levels of each reference gene is shown in absolute Ct values during the 14 days of the experiment. Closed and open circle represent 18S and act respectively.