

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-PCR 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 *coxI*, *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

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

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

52 Recent works have studied the responses of diatom communities to metal pollution, for
53 example Morin et al. (2008a) observed structural impact at the community level and
54 morphological abnormalities in a metal-polluted stream. Other studies assessed effects of
55 metal-induced oxidative stress on functional descriptors such as photosynthetic (Antal et al.
56 2004) or antioxidant (Branco et al. 2010) enzyme activities.

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

62 Extracting the genetic material of organisms is the first step to obtain sequences of interest.
63 Diatoms require a particular approach for this because of their unique external structure.
64 Diatoms are unicellular algae and their most obvious distinguishing characteristic is their
65 siliceous cell wall called the frustule. Various approaches have been used to break the
66 frustule, but these are often time consuming (Wawrik et al. 2002) and/or need specialized
67 laboratory material such as a French press (Stabile et al. 1990, Davis and Palenik 2008,
68 Hildebrand et al. 1998) or a MiniBeadBeater (Fawley and Fawley 2004). More rapid and
69 straightforward extraction methods are needed, specifically for diatoms. The expression of
70 many genes is involved in the response of organisms to toxicants and can be disturbed by
71 them. Once inside the cell, metals can cause oxidative stress (Wang et al. 2004) affecting the
72 way in which the mitochondria (Stohs and Bagchi 1995) or photosynthesis function (Knauert

73 and Knauer 2008). The mitochondrial superoxide dismutase (*sodMn*) gene was selected
74 because of its involvement in antioxidant defences. The mitochondrial metabolism was
75 investigated using the cytochrome C oxidase (*coxI*) subunit and the NADH dehydrogenase
76 subunit 5 (*nad5*) genes. In addition, the quantity of mitochondria in the cells was estimated
77 using mitochondrial 12S ribosomal RNA. D1 protein (*d1*) and PsaA protein (*psaA*) are
78 components of PS2 and PS1 respectively and were selected to investigate photosystem
79 metabolism. Cytochrome P450 1A1 (*cyp1A1*) was selected as a biomarker for exposure to
80 polynuclear aromatic hydrocarbons. Two genes commonly used as references in numerous
81 genetic studies were selected as for study in our work: β actin (*act*) and 18S ribosomal RNA.
82 Consequently, the first objective of this work was to develop a diatom-specific extraction
83 method and to shape molecular tools to select and sequence genes of interest to assess metal
84 contamination. We used cultures of *Eolimna minima*, a metal-tolerant freshwater diatom
85 commonly collected within periphytic biofilm samples in running water and especially in
86 metal-contaminated areas (Morin et al, in press). Our attention was particularly focussed on
87 genes involved in mitochondrial and photosystem metabolism and responses of *E. minima* to
88 a gradient of cadmium contamination was then characterised using these selected molecular
89 tools. Cultures of *E. minima* were exposed in the laboratory for 14 days to 10 $\mu\text{gCd/L}$ or 100
90 $\mu\text{gCd/L}$. The moderate exposure corresponds to the concentrations reported in cadmium
91 polluted rivers like the river Riou-Mort (South West France) (Feurtet-Mazel et al. 2003) and
92 the highest is comparable to those reported in more highly polluted European rivers (Ivorra et
93 al. 1999). Cell numeration, bioaccumulation and genetic responses were followed 1, 2, 7 and
94 14 days after contamination.

95

96 **2. Materials and methods**

97

98 2.1. Molecular methodological developments

99

100 *2.1.1. RNA extraction method*

101 In order to access the genetic material of diatoms, a novel RNA extraction method was
102 developed. Designing an efficient method able to break the external silica wall of diatoms
103 without degrading the genetic material was a prerequisite. For this purpose, cultures of the
104 diatom *Eolimna minima* were grown in batches from axenic strains provided by the UTEX
105 algal collection (Texas University, USA). The diatom cells were cultured in sterile Dauta
106 medium (Dauta 1982) with silica added to a final concentration of 10mg/L in 3L Erlenmeyer

107 flasks. The cultures were maintained at between 17 and 18°C in a thermostatic room with a
108 photon flux density of $160\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 12:12 h light:dark cycle. Fresh cultures were
109 inoculated every seven days to promote optimal growth of the diatoms until sufficient cells
110 were obtained.

111
112 30 mL ($2\cdot 10^6$ cells/mL) were removed from the *E. minima* cultures and the cells harvested
113 after centrifugation at 3 863g for 5 min at room temperature. The cell pellet was transferred to
114 2 mL Eppendorf tubes and resuspended in 1 mL of diethyl pyrocarbonate (DEPC) treated
115 water. Then, the diatoms were centrifuged again at 2 419g for 5 min at room temperature and
116 the supernatant was removed. 1 mL of Trizol (Invitrogen) and 300 μL of glass beads (0.10 –
117 0.11mm-diameter, B. Braun Biotech International) were added to the cell pellet. Then the
118 sample was vortexed 3 times for 30 seconds, and the supernatant transferred into a clean
119 Eppendorf tube and placed in a water bath at 30°C for 5 min. After addition of 200 μL of
120 Chloro RECTAPUR (VWR), the sample was vortexed for 10 s and placed in water bath at
121 30°C for 2 min. The tubes were centrifuged at 11 360g for 5 min at room temperature and the
122 aqueous phase containing the genetic material was transferred into clean tubes. The end of the
123 extraction was realized using Absolutely RNA Miniprep Kit (Stratagène) according to the
124 manufacturer's instructions:

125 550 μL of 75% ethanol were added to the aqueous phase, the sample was vortexed,
126 transferred to affinity columns and centrifuged for 1 min at 11 360g at room temperature.
127 Filtrates were removed, 600 μL of low-salt buffer were placed on the column which was
128 centrifuged at 11 360g at room temperature for 2 min. 5 μL of DNase 1 [1U] and 50 μL of
129 activity buffer were added to the column and placed in a water bath at 37°C for 15 minutes.
130 600 μL of high-salt buffer were added to the column which was centrifuged for 1 min at 14
131 500 rpm. 300 μL of low-salt buffer were added and the column was centrifuged for 2 min at
132 14 500 rpm. The columns were transferred into clean tubes and 30 μL of elution buffer at
133 60°C were added and centrifuged for 1 min at 12 250g at room temperature. Total RNA was
134 analysed on 1% (w/v) agarose gel with ethidium bromide by UV light on an illuminator.

135

136 *2.1.2. Reverse transcription of RNA*

137 The first strand of cDNA was synthesised from 14 μL of total RNA (3 to 5 μg) using the
138 Stratascript first strand synthesis system (Agilent). After the addition of 1 μL of oligo(dT)
139 [1 μM], 1 μL of random primers [1 μM], 0.8 μL of dNTPs [10mM] and 2 μL of 10x first-
140 strand buffer the reaction was incubated for 5 min at 65°C. Then 1 μL of Stratascript reverse

141 transcriptase [1U/ μ L] and 0.5 μ L of RNase inhibitor [0.5U] were added, the reaction was
142 incubated for 1 h at 42°C in an Eppendorf Mastercycler. The cDNA mixture was conserved at
143 -20°C until it was used in a real-time PCR reaction.

144

145 *2.1.3. Cloning and molecular characterization of the target genes*

146 Genetic research focussed on seven genes involved in responses to environmental
147 contamination and/or in which expression can possibly be disturbed by environmental factors
148 (*sodMn*, *nad5*, *d1*, *cox 1*, *psaA*, *cyp 1A1*, and 12S) and for two reference genes (18S and *act*).
149 Primers were designed by performing ClustalW analysis from protein or nucleic sequences
150 (12S and 18S) from marine diatoms and other phylogenetically related aquatic organisms
151 available in NCBI databases. From these alignments, primer pairs about 20-25 bp long were
152 designed in the most conserved regions for amplification of fragments between 280 and 900
153 bp.

154 PCR reactions were performed using these primers following the manufacturer's instructions.
155 After the addition of 1 μ L of dNTP [10 mM], 3 μ L of MgCl₂ [25 mM], 0.2 μ L of Taq
156 [5U/ μ L], 10 μ L of activity buffer 5X, 0.5 μ L of each primer (upstream and forward primers)
157 [100 μ M], 34 μ L of DEPC treated water and 1 μ L of cDNA, 40 PCR cycles at 94°C for 1
158 min, 55°C for 1 min and 72°C for 1 min were carried out in an Eppendorf AG thermocycler.
159 PCR products were analyzed on 1% (w/v) agarose gel with ethidium bromide. The ethidium
160 bromide stained bands were revealed by UV light on an illuminator.

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

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

167

168 *2.1.4. q-RT-PCR primer design and real time q-PCR*

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

171 Real time PCR reactions were performed in a LightCycler (Roche) following the
172 manufacturer's instructions (one cycle at 95°C for 1 min and 50 amplification cycles at 95°C
173 for 5 s, 60°C for 5 s and 72°C for 20 s).

174 Each 20 μL reaction contained 1 μL of activity buffer (Syber Green I (Roche), *Taq*
175 Polymerase, dNTP), 3.2 μL of MgCl_2 [25 μM], 2 μL of the gene-specific primer pair at a final
176 concentration of 300 nM for each primer, 12.8 μL of DEPC-treated water and 1 μL of cDNA.
177 Specificity was determined for each reaction from the dissociation curve of the PCR product.
178 This dissociation curve was obtained by following the SYBR Green fluorescence level during
179 gradual heating of the PCR products from 60 to 95°C.

180 Relative quantification of each gene expression level was normalized according to β -actin
181 gene expression. Relative mRNA expression was generated using the $2^{-\Delta\text{CT}}$ method (Livak
182 and Schmittgen 2001).

183

184 *2.1.5. Determination of sensitivity limit*

185 In order to optimize the RNA extraction method, the sensitivity limit was quantified.

186 Ten dilutions of *E. minima* cultures were prepared to obtain theoretical numbers of cells
187 ranging from 1.8×10^7 cells to 1.8 cells (1.8×10^7 ; 9×10^6 ; 1.8×10^6 ; 9×10^5 ; 1.8×10^5 ; 1.8×10^4 ;
188 1.8×10^3 ; 1.8×10^2 , 18 and 1.8 cells). Extraction and reverse transcription of RNA were
189 performed as described previously on these different samples. The resulting cDNA was used
190 for q-PCR with *coxI* and *act* specific primer pairs as described above.

191

192

193 2.2. Experimental protocol to assess cadmium effects on *Eolimna minima*

194 *2.2.1. Exposure conditions*

195 250 mL of *E. minima* culture in suspension in modified Dauta medium were placed in 500-ml
196 Erlenmeyers to reach a final concentration of 10^6 cells/mL. The organisms were directly
197 exposed to three Cd concentrations ($C_0=0$, $C_1=10 \pm 3.2$ and $C_2=96 \pm 34.2$ $\mu\text{gCd/L}$, mean values
198 \pm standard deviations over 14 days, from CdCl_2 stock solution, prepared from a 1000 mg vial,
199 Merck, Germany). The cultures were maintained at between 16 and 18°C along the 14 days of
200 exposure with a photon flux density of 59.19 ± 7.75 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ and a 12:12 h light:dark
201 cycle. The Erlenmeyer flasks were closed with sterile cotton wool and kept on an orbital
202 shaker (60 rpm). Triplicate flasks were collected at each of the four different sampling periods
203 (after 1, 2, 7 and 14 days of exposure to Cd) leading to 36 different experimental conditions.
204 Thus 7.5 mL of culture were sampled after the exposure durations assigned for water Cd
205 analyses, intracellular and total Cd concentration analyses and cell counting. Simultaneously,
206 three 100 mL replicates were sampled for genetic analysis and stored at - 80 °C.

207

208 *2.2.2. Cd analysis*

209 Cadmium concentrations in water and in diatom cells were determined by atomic absorption
210 spectrophotometry (AAS) using a graphite tube atomiser after filtration through a GH
211 polypropylene membrane (0.45 µm pores, 25 mm diameter, PALL) for diatom cell Cd
212 concentration and on a 0.45µm Teflon filter (from CAS) for Cd concentration in water. The
213 analytical method was simultaneously validated for each sample series by analysing standard
214 biological reference materials (TORT 2, Lobster hepatopancreas; DOLT 3 dogfish liver;
215 NCR/CNRC, Ottawa, Canada). 3 mL of nitric acid (65% HNO₃) were added to reference the
216 material before digestion in a pressurized medium (borosilicate glass tube) at 100°C for 3 h.
217 15 mL of ultra pure water were added and samples were stored at 4°C before analysis.
218 Samples used for intracellular cadmium determination within diatoms were treated first with
219 EDTA (10 mM), a strong metal complexing ligand, to remove the metal ions adsorbed to the
220 diatom cell walls (Behra et al. 2002). In this case, a drop of EDTA was added to each 2 mL
221 sample and filtered after 10 min. Samples used for total cadmium determination in diatoms
222 skipped this step. After 24h at 44°C, all samples were digested by nitric acid (3 mL of 65%
223 HNO₃) in a borosilicate glass tube at 100°C for 3 h. The samples were then analysed by
224 atomic absorption spectrophotometry as described previously.

225

226 *2.2.3. Diatom cell density*

227 1.5 mL aliquots were immediately fixed in formalin (37% formaldehyde, Prolabo, France) for
228 counting. Each sample was counted in triplicate using a Nageotte counting chamber
229 (Marienfeld, Germany). After 10 min ultrasonication, 200 µL of sample were placed on the
230 counting chamber. The total number of individuals and the number of dead cells were
231 recorded in 4 fields of the gridded area (1.25 µL each, 0.5 mm depth) under light microscopy
232 at 400x magnification (Leitz photomicroscope). Distinction between dead and live organisms
233 was estimated by the observation of the turgescence and colour of the chloroplast.

234

235 *2.2.4. Statistical analysis*

236 Statistical analyses were performed with R 2.12.0 using the Linear Mixed Effect model
237 (library *nlme*). Homogeneity of variances was verified by Leven test and Fisher's LSD was
238 calculated.

239

240

241 To assess the effects cadmium on *E. minima* by the molecular tools described above,
242 expression of the nine target genes sequenced in this study was examined. The expression
243 levels of the nine genes involved in antioxidant defences, or mitochondrial or photosynthetic
244 metabolism were investigated by quantitative real-time PCR as described previously using the
245 q-PCR primers designed in this study.

246

247 **3.Results and discussion**

248 3.1.Methodological developments

249

3.1.1.Extraction method and optimization

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

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

274

275 *3.1.2. Nucleotide sequence accession number and q-PCR primers*

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

279

280 3.2. Effects of cadmium on *Eolimna minima*

281 *3.2.1. Cd water concentrations in solution*

282 Cadmium concentration in water for the three different treatments is plotted against time in
283 Figure 2. During the 14 days of exposure, mean Cd concentrations in water were 10 ± 3.2 $\mu\text{g/L}$
284 and 96 ± 34.2 $\mu\text{g/L}$ for nominal concentrations of 10 and 100 $\mu\text{gCd/L}$ respectively. The
285 cadmium concentrations in water were very stable from days 0 to 7 in both C₁ (11.5 ± 0.6
286 $\mu\text{gCd/L}$) and C₂ (111.3 ± 3.8 $\mu\text{gCd/L}$) treatments. A strong decrease occurred in cadmium
287 concentrations on day 14 with values falling to 4.3 ± 4 $\mu\text{gCd/L}$ and 35.2 ± 14.2 $\mu\text{gCd/L}$ for
288 treatments C₁ and C₂ respectively. This is unlikely to be explained by metal adsorption onto
289 the surface of the experimental units, as no decrease was observed during the first seven days
290 of exposure: bioaccumulation of cadmium by diatoms is more likely responsible for this
291 decrease, as shown by the following results.

292

293 *3.2.2. Effect of cadmium on the growth of *E. minima**

294 The number density, over the 14 days of direct exposure to the three different concentrations
295 of cadmium, is plotted in Figure 3. Statistical analysis revealed that growth was not
296 significantly different in controls and with a Cd concentration of 10.0 ± 3.2 $\mu\text{g/L}$ over the
297 whole duration of the experiment. Besides, Gold et al. (2003) and Duong et al. (2008)
298 observed a high proportion of small, adnate species like *Achnanthydium minutissimum*,
299 *Encyonema minutum* and *E. minima* in stations contaminated with around 6 $\mu\text{g/L}$ cadmium.
300 This suggests the tolerance of these species to high levels of cadmium and thus their ability to
301 develop in cadmium-contaminated conditions with around 10 $\mu\text{gCd/L}$. Nevertheless, densities
302 in contaminated samples were on average lower than in control treatments throughout the 14
303 days of exposure. It could be interesting to study population kinetics over a longer period to
304 see if, after 14 days of exposure, significant differences are observed between the C₁
305 treatment and the control as observed by Morin et al. (2008a) who reported differences in
306 biofilm diatom densities between control and 10 $\mu\text{gCd/L}$ -contaminated treatments only after 6
307 weeks of exposure.

308 Significant differences in cell densities were observable from day 7 between the controls and
309 the series with the highest contamination of cadmium ($2\,900\,000 \pm 250\,000$ cells/mL for
310 control versus $1\,300\,000 \pm 500\,000$ cells/mL). After 14 days of exposure, the diatom cell
311 density was 3.2 times higher than with the control treatment ($4\,200\,000 \pm 390\,000$ cells/mL
312 versus $1\,300\,000 \pm 280\,000$ cells/mL). Moreover, diatom growth was null with treatment C₂.
313 Our results are in accordance with those of Tourtelot (2003) who studied the acute effects of
314 Cd on *Achnantheidium minutissimum*. For this species, referenced as pollution resistant, the
315 rate of growth was null after 14 days of exposure to 100 µgCd/L.
316 Similar results were observed at the community level, Morin et al. (2008b) exposed biofilms in
317 microcosms to 10 and 100 µgCd/L for 6 weeks and observed a significant reduction in diatom
318 density for biofilms exposed to the higher concentration compared to the control and the low
319 cadmium contamination treatment.

320

321 *3.2.3. Cd bioaccumulation in diatoms*

322 Intracellular and total bioaccumulation are not given for days 1 and 2 after exposure because
323 of the insufficient quantity of cells in the samples. Intracellular and total Cd concentration in
324 diatoms after 7 and 14 days of exposure to the different treatments (0, 10 ± 3.2 and 96 ± 34.2
325 µgCd/L) are reported in Figure 4.

326 Intracellular and total bioaccumulation increased significantly between days 7 and 14 for the
327 2 contaminated treatments. For the lower contamination concentration (C₁), the intracellular
328 and total concentration increased from 3.6 ± 1.8 to 51.4 ± 5.3 µgCd/g dw and from 6.4 ± 3.4 to
329 57 ± 6.3 µgCd/g dw respectively. For the higher contamination pressure (C₂), there was an
330 increase from 5.6 ± 2.7 µgCd/g dw to 430.1 ± 86.4 µgCd/g dw and from 11.4 ± 3.0 to $734.1 \pm$
331 70 µgCd/g dw respectively, this latter value is in the range of literature values (Morin et al.
332 2008a, Duong et al. 2008). The increase in cadmium bioaccumulation with exposure time was
333 also shown in environmental samples by Duong et al. (2008). The cadmium content in the
334 biofilm increased gradually for days 14 and then remained constant. This could be due to the
335 saturation of binding sites in the biofilm leading to a limitation of Cd accumulation. In our
336 experiment it is difficult to estimate if the saturation phase is reached after 14 days of
337 exposure. In order to determine the kinetics of saturation and the saturation value for *E.*
338 *minima*, experiments will have to cover a longer period with a constant contamination
339 pressure.

340 After 7 days of exposure a significant difference occurred between the controls and the
341 cadmium-contaminated treatments, but no such difference appeared between moderate and
342 high contamination (C_1 and C_2) or between intracellular and total bioaccumulation.

343 After 14 days of exposure, total bioaccumulation in the controls was significantly different
344 from that in the cadmium-contaminated treatments ($p = 0.008$ and $p < 0.001$ respectively).
345 Total bioaccumulation was significantly higher than intracellular bioaccumulation for
346 contamination C_2 ($430.1 \pm 86.4 \mu\text{gCd/g dw}$ versus $734.1 \pm 70 \mu\text{gCd/g dw}$).

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

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

358

359 *3.2.4. Gene expression levels*

360 In our study, two genes were selected as potential references, β -actin and 18S ribosomal
361 RNA. Both are widely used as references in q-PCR analyses. The average percentage
362 variation in the threshold cycle (C_t) of 18S ribosomal RNA within triplicates was 13.7%
363 against 4.9% for β -actin (figure 1). The C_t value was defined as the number of cycles needed
364 for the amplification signal to reach a specific detection threshold, and so is inversely
365 correlated with the amount of cDNA template present in the PCR amplification reaction. The
366 low stability and the high abundance of 18S rRNA underlined by our results (figure 5) shows
367 the unsuitability of this gene as a reference. Consequently, 18S was finally not kept as a
368 reference gene in our experiments. On the other hand, β -actin showed a high regularity and
369 high threshold cycle values and was thus defined as reference gene in our study. 18S rRNA
370 has also been described as a poor reference gene in other species such as buffalo (*Bubalus*
371 *bubalis*) (Castigliengo et al. 2010) or humans (Radonić et al. 2004). A recent study underlined
372 this point; Siaux et al. (2007) reported that 18S rRNA was a very poor reference gene for
373 *Phaeodactylum tricornutum*, a marine diatom. Here, we chose β -actin as reference gene

374 owing to the stability of its expression, although Siaut et al. (2007) show that the β -actin level
375 appears to be tightly correlated to light with an increase during the light period. In our study,
376 however, the photoperiod was controlled and sampling performed at a fixed time, which
377 avoided this source of error and allowed us to use β -actin as reference gene. Nevertheless it
378 would be interesting to study other reference genes as proposed by Siaut et al. (2007) (*H4*,
379 *RPS*, *Cdka* and *TBP*) for future analyses and particularly in field samples.

380
381 Expression of genetic levels during the 14 days of exposure to cadmium at 10.0 ± 3.2 and
382 96.0 ± 34.2 $\mu\text{g/L}$ are shown in table 2. During the 14 days, an impact resulting in the
383 amplification of 5 of the genes studied was observed (*cox1*, *nad5*, *d1*, *psaA* and 12S).
384 Different genetic responses are expressed as a function of time and concentration of exposure.
385 Differential genetic expression revealed an effect on mitochondrial and photosynthetic
386 metabolism, observed after 7 days of exposure only at the highest contamination pressure, and
387 for both contamination pressures on day 14.

388 After 7 days of exposure, *cox1*, *d1*, *psaA* and 12S were up-regulated for the exposure
389 concentration of 96.0 ± 34.2 $\mu\text{gCd/L}$. After 14 days of exposure, there was a strong up-
390 regulation of *nad5*, *d1* and *psaA* for both concentrations. Moreover, a large difference
391 occurred between the 2 treatments: expression of *nad5*, *d1* and *psaA* were 4, 12 and 19 times
392 higher respectively for C_2 than C_1 . We can also see that responses of *d1* and *psaA* in the C_2
393 treatment increased with exposure time by a factor of 4.4 and 6.4 respectively between days 7
394 and 14.

395 The disturbance in the expression of the cytochrome C oxidase (*cox1*) subunit and the NADH
396 dehydrogenase subunit 5 (*nad5*) observed in our study suggests an impact on mitochondrial
397 metabolism. This is in accordance with data in the literature. Indeed, after entering the cell,
398 cadmium can inhibit electron transfer in mitochondrial respiration, and also lead to the
399 formation of reactive oxygen species (ROS) (Wang et al. 2004, Gonzalez et al. 2006). The
400 oxidative stress generated then brings about DNA breaks and induction of apoptotic
401 mechanisms leading to cell death (Bagchi et al. 2000, Chan and Cheng 2003). Nevertheless,
402 the ratio *cox1*/12S was constant throughout the experiment indicating that the number of
403 mitochondria increases to maintain the level of ATP production. This increase of *cox1*
404 expression has already been reported in zebrafish *Danio rerio* (Gonzalez et al. 2006). Electron
405 microscopy studies also in the zebrafish have shown extensive disturbance of the
406 ultrastructure of the mitochondria after metal exposure to methyl mercury (Cambier et al.

407 2009). In future work it will be of particular interest to use electron microscopy to view *E.*
408 *minima* after Cd exposure so as to be able to evaluate damage at the mitochondrial level.
409 Secondly, with regard to the up-regulation of *psaA* and *d1* after 7 days of exposure to
410 100 µgCd/L and after 14 days for the two contamination levels, it is clearly shown that the
411 photosynthetic system of *E. minima* is strongly affected by exposure to cadmium. The impact
412 of metals on the photosystem is well known and has been studied in numerous works.
413 Photosystem inhibition has been reported by Sudo et al. (2008) at physiological and genetic
414 levels with, in particular, repression of *d1* and *psaA* in rice leaves after copper stress. Hörcsik
415 et al. (2007) studied the effect of chromium on PS2 activities in *Chlorella pyrenoidosa* cells:
416 they showed the inhibition of PS2 by photodestruction of reaction centres in cells cultivated in
417 the presence of chromium. At the ultrastructural level, Ouzounidou et al. (1997) showed
418 damage to chloroplast structures with changes in the shape and thickness of the thylakoid
419 membranes in wheat (*Triticum aestivum* L.) after exposure to 265 µgCd/L for 7 days.
420 Moreover, the impact at the genetic level underlined by the up-regulation of *nad5*, *d1* and
421 *psaA* after 14 days of exposure revealed effects at a Cd concentration of 10 µg/L. This result
422 is of particular interest because, at a more integrative level of population kinetics, this effect
423 was not noted. Overall, the above findings stress the importance of considering molecular
424 markers when studying pollution because of the potentially higher sensitivity and the earlier
425 response of genomic markers to toxic pollution compared to more global endpoints like
426 mortality or population kinetics.
427 No significant difference in genetic expression levels was observed for the genes *cyp1A1* or
428 *sodMn* during the experiment, at either of the concentrations tested. Mitochondrial superoxide
429 dismutase is known to be involved in the oxidative stress response and several studies have
430 shown the up-regulation of this gene after metal contamination. For example Gonzalez et al.
431 (2006, 2005) demonstrated the up-regulation of *sodMn* in the gills of zebrafish after 7 days of
432 direct exposure to 86 ± 0.26 µgCd/L and in another study in skeletal muscle and liver after 21
433 and 63 days of dietary exposure to methyl mercury for concentrations of 5 to 13.5 µgHg/g dw.
434 In our work, this up-regulation was not observed, several hypotheses can be proposed: the
435 sampling rate may be unsuitable and/or other enzymes of the oxidative stress response may be
436 involved such as catalase, glutathione peroxidase or other types of superoxide dismutases
437 (*sodFe*, *sodCu/Zn* or *sodNi*). Other authors have studied antioxidant enzyme activities, for
438 example Branco et al. (2010) observed an increase in catalase activity compared to controls
439 after 5 days of exposure to Cd from 100 to 300 µgCd/L on the diatom *Nitzschia palea* and in
440 the global superoxide dismutase activity for concentrations from 200 to 300 µgCd/L. In a

441 field study, Bonet et al. (2010) showed a clear inhibition of glutathione-S-transferase at high
442 metal exposure and no difference in superoxide dismutase activity between sites impacted by
443 metals at different levels.

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

451

452 **4. Conclusions and perspectives**

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

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

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

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470 **References**

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

Gene name	Accession number	Primer (5'-3')
<i>sodMn</i>	HM 449706	GGTAGTAGGCGTGCTCCC ^a CCAGGACAACCCGCTC ^b
<i>cox1</i>	HM 449704	CAGTAATTCTCACTGCCAGC ^a CCGTGTACCCACCGTTG ^b
<i>nad5</i>	HM 449708	TCAACTTGGTTTGCATACATGGC ^a TTGAACTAATCCTGTTGTGGAAGC ^b
<i>d1</i>	HM 449711	ACCACCAAATACACCAGCAAC ^a GCGTCCTTGGATTTTCGTAGC ^b
<i>psaA</i>	HM 449705	CATAAAGCGGCACCCAAAC ^a CTTGGATATAACTGACTCATTAACTCAGG ^b
<i>cyp1A1</i>	HM 449709	ACGAAGGAGTCTCCCATCA ^a CTTGGCCCGAACGATCAG ^b
<i>act</i>	HM 449707	GGCTCCACAAAACCCCAAG ^a GGCGTACCCCTCGTAGAT ^b
<i>12s</i>	HM 449710	CGCGGTAATACGGAGGATGC ^a AGTGCCTTCGCCATCGG ^b
<i>18s</i>	HM 449712	CATTGTCAGAGGTGAAATTCTTGGA ^a CCCCGGAACCCAAAAGT ^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. ^aUpstream primer. ^bForward 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.

Functions	Genes	Cadmium contaminated experimental units							
		C1 (10.0 ± 3.2 µg/l)				C2 (96.0 ± 34.2 µg/l)			
		1	2	7	14	1	2	7	14
Mitochondrial metabolism	<i>cox1</i>	/	/	/	/	/	/	9.5	/
	<i>nad5</i>	/	/	/	2.5	/	/	/	9.5
	<i>12s</i>	/	/	/	/	/	/	15	/
Oxidative stress	<i>sodMn</i>	/	/	/	/	/	/	/	/
Photosynthesis	<i>d1</i>	/	/	/	2	/	/	5.5	24
	<i>psaA</i>	/	/	/	2.5	/	/	7.5	48
Xenobiotic metabolism	<i>cyp1A1</i>	/	/	/	/	/	/	/	/

^aSignificant 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.

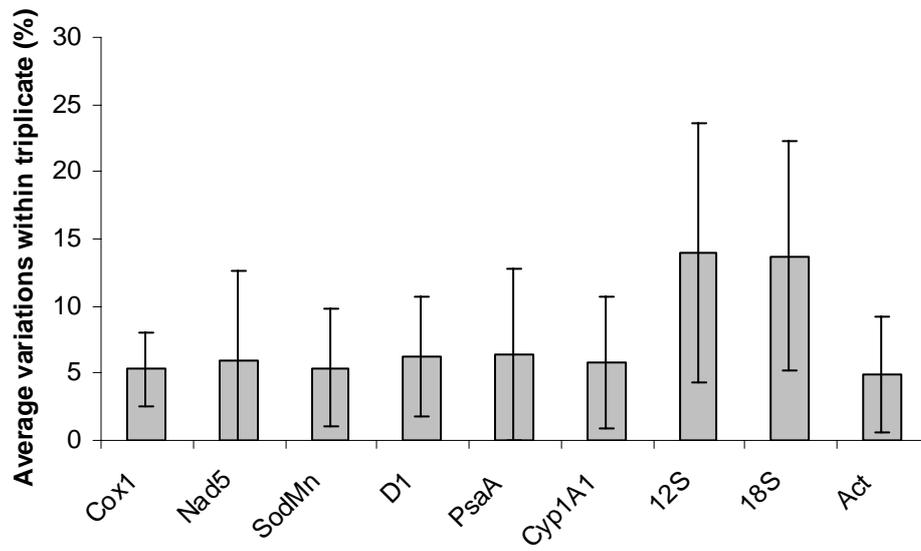


Figure 2. Cadmium concentration in water \pm standard deviation versus exposure time for the 3 treatments ($C_0=0$, $C_1=10$ and $C_2=100$ $\mu\text{gCd/L}$). Full and empty triangles represent contaminated conditions. Full squares represent control treatments.

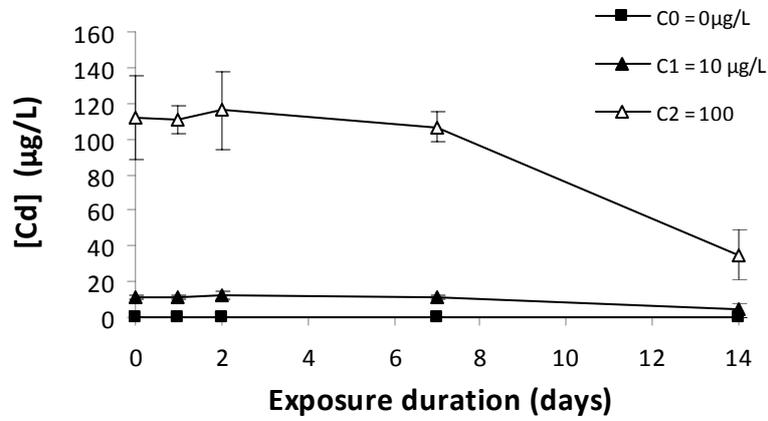


Figure 3. Total density of *Eolimna minima* ± standard deviation versus exposure time for the 3 treatments (C₀=0, C₁=10 et C₂= 100 µgCd/L), significant differences between a and b (p value <0.05). Solid and open triangles represent contaminated conditions. Solid squares represent control treatments.

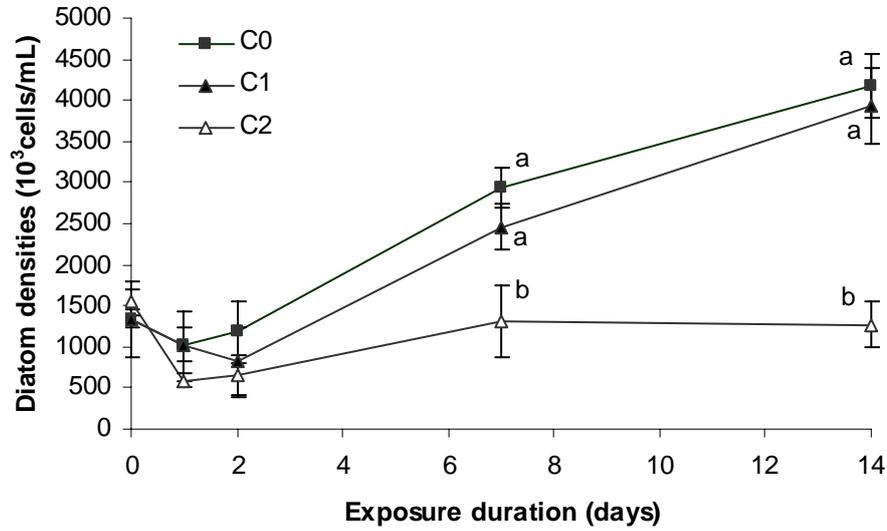


Figure 4. Intracellular and total Cd bioaccumulation in *E. minima* cultures for the two exposure times and three treatments ($C_0=0$, $C_1=10$ and $C_2=100\mu\text{gCd/L}$) in $\mu\text{g Cd/g dw}$. Significant differences between a, b, c, d and e (p value <0.05). White bars represent control treatments, grey bars represent C_1 treatments and black bars represent C_2 treatments. Full and hatched bars represent total and intracellular cadmium bioaccumulation respectively.

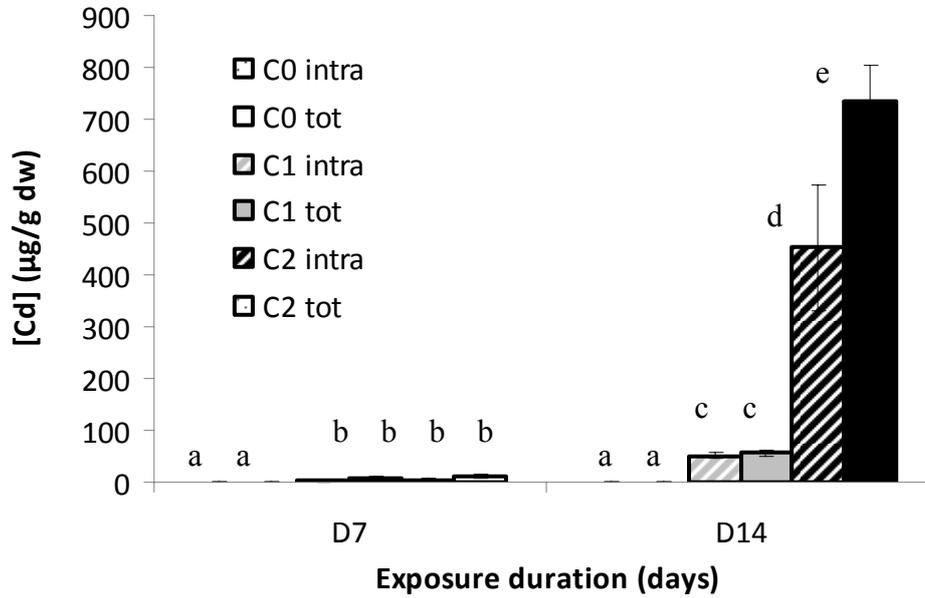


Figure 5. Expression of the two potential reference genes 18S and *act* in all treatments. mRNA levels of each reference gene is shown in absolute Ct values during the 14 days of the experiment. Closed and open circle represent 18S and *act* respectively.

