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Title:

Effect of low-dose cadmium exposure on DNA methylation in the endangered European eel

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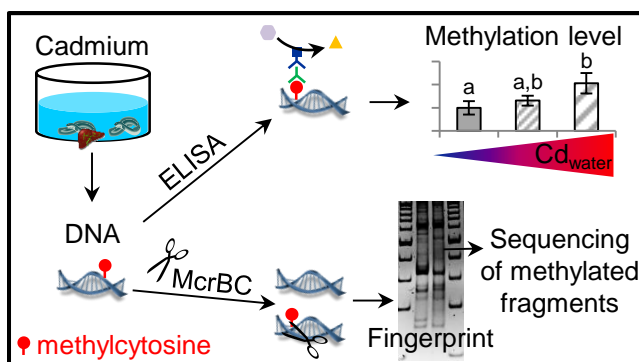
Keywords: Epigenetics, Ecotoxicology, DNA methylation, fish, cadmium

26 **Abstract**

27 There is increasing evidence that epigenetics can play a key role in the etiology of diseases
28 engendered by chronic pollutant exposure. Although epigenetics has received significant
29 attention in the field of biomedicine during the last years, epigenetics research is surprisingly
30 very limited in ecotoxicology. The aim of the present study was to investigate the possible
31 effects of low-dose cadmium exposure on the DNA methylation profile in a critically
32 endangered fish species, the European eel. Eels were exposed to environmentally realistic
33 concentrations of cadmium (0.4 and 4 $\mu\text{g}\cdot\text{L}^{-1}$) during 45 days. The global CpG methylation
34 status of eel liver was determined by means of a home-made ELISA assay. We then used a
35 methylation-sensitive arbitrarily primed PCR method to identify genes that are differentially
36 methylated between control and Cd-exposed eels. Our results show that cadmium exposure is
37 associated with DNA hypermethylation and with a decrease in total RNA synthesis. Among
38 hypermethylated sequences identified, several fragments presented high homologies with
39 genes encoding for proteins involved in intracellular trafficking, lipid biosynthesis and
40 phosphatidic acid signaling pathway. In addition, few fragments presented high homologies
41 with retrotransposon-like sequences. Our study illustrates how DNA methylation can be
42 involved in the chronic stress response to Cd in fish.

43

44 **TOC/Abstract Art**



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46

47 **1. Introduction**

48 Epigenetics is the study of changes in gene function that are mitotically and/or meiotically
49 heritable and that do not entail a change in DNA code.¹⁻³ One of the most studied epigenetic
50 processes is DNA methylation. DNA methylation, which refers to the addition of a methyl
51 group to cytosine to form the 5-methylcytosine base, is commonly associated with “gene
52 silencing”, i.e. transcriptional repression (with few exceptions; see Vandegheuchte and
53 Janssen, 2011¹). In mammals and plants, for which significant research was carried out, DNA
54 methylation was found to be involved in fundamental biological functions such as
55 development, cell differentiation and chromosome stability. In recent years, the role of
56 epigenetics in the etiology of human diseases, especially carcinogenesis, has received
57 increasing attention. Recent discoveries have shown that environmental factors, such as
58 nutrition quality or exposure to pollutants, can strongly affect the epigenetic status of
59 mammalian cells. These defects or unintended changes in the DNA methylation status can be
60 associated with negative health outcomes such as embryonic lethality, tumor genesis, diabetes
61 and infertility.^{2,4-6} There is thus increasing evidence that adverse effects engendered by
62 chronic exposure to pollutants, even at very low concentrations, are linked to epigenetic
63 modifications in mammals.³ Despite such evidences, epigenetics research is surprisingly very
64 limited in ecotoxicology. As a major aim of ecotoxicology is to measure and predict the
65 effects of contaminants on natural populations, communities and ecosystems, the study of
66 epigenetics in an ecotoxicological context appears to be highly relevant.

67 Therefore, the aim of the present study was to investigate the potential impacts of
68 cadmium (Cd) exposure on the DNA methylation profile in an endangered fish species, the
69 European eel *Anguilla anguilla*. Cd is a widespread nonessential and highly toxic trace metal.
70 Cd is known to be a mutagenic metal and to induce apoptosis and oxidative stress.⁷
71 Anthropogenic activities such as mining or ore treatment have greatly increased the global

72 flux of Cd in the Earth's surficial environment leading to elevated concentrations in the
73 environment, notably in aquatic systems. Historically abundant and widespread in Europe,
74 European eel recruitment have suffered a sharp decline since early 1980s. European eel's
75 recruitment would represent now only one tenth of what it was in the early 1980s.⁸ In view of
76 these data, the European eel is currently considered as critically endangered and is listed on
77 the red list of threatened species. There is now some evidence that pollution, among other
78 factors, could contribute to the dramatic decline of the species. Their unusual and complex
79 life cycle makes them indeed particularly vulnerable to pollution.⁹⁻¹² Thus, the European eel
80 can be considered as a sentinel species in ecotoxicology and could provide a model to explore
81 recent hypotheses that human activities have led to a tremendous decrease in aquatic
82 biodiversity in the last century.¹³

83 To test whether chronic Cd exposure could induce changes in the DNA methylation
84 profile in a non-model but ecologically relevant species, European eels were exposed to
85 dissolved Cd at environmentally realistic concentrations ($[Cd]_{\text{water}} = 0.4$ and $4 \mu\text{g.L}^{-1}$) for 45
86 days.¹⁴ Thereafter, the global CpG methylation status of liver of both control and Cd-exposed
87 eels was determined by means of a home-made ELISA assay. In order to identify and
88 characterize genes and, by extension, biological processes, that are differentially methylated
89 between control and Cd-exposed eels, we used a methylation-sensitive arbitrarily primed PCR
90 (msAP-PCR) method, the McrBC-msAP-PCR method.¹⁵ Finally, the transcription level of
91 genes identified by this method was determined by quantitative RT-PCR.

92

93 **2. Materials and methods**

94 2.1. Experimental design

95 All procedures used in this experiment were approved by the Aquitaine fish-birds ethic
96 committee. Immature yellow eels (*Anguilla anguilla*), averaging 37 cm in length and 70 g in

97 weight (36.9 ± 2.5 cm and 70.4 ± 2.4 g, respectively, mean \pm SE, $n = 24$) were captured in
98 the Arcachon Bay (southwest of France) in May 2012. The animals were transferred to the
99 laboratory (Marine Biology Station of Arcachon) and kept in running aerated brackish water
100 (salinity 5 ‰, natural seawater dilution with aerated tap water) thermostated at 23°C over a 1-
101 month maintenance period, prior to experimentation. Over this period, fish were fed every day
102 with mussels and no lethality was observed.

103 The experiment was performed by means of a flow-through system consisting of three
104 separate 215 L experimental units (EU). Each tank was supplied with brackish water (salinity
105 = 5 ‰) by three water flowmeters and contained eight organisms. Fish in the first EU were in
106 uncontaminated water and constituted control animals. In the two other tanks, fish were
107 exposed to Cd at a dissolved metal concentration of 0.4 and 4 $\mu\text{g}\cdot\text{L}^{-1}$. Metal exposure was
108 initiated after one month of adaptation to the experimental conditions by adding Cd as CdCl_2
109 from a stock solution in water. To maintain constant Cd contamination over time,
110 contaminated tanks were fitted with a peristaltic pump which added Cd at the desired
111 concentration. During the experiment, the water column was permanently controlled for
112 temperature (23 ± 1 °C), pH (7.7 ± 0.1) and salinity (4.85 ± 0.7). Moreover, water samples
113 were collected three times a week. After acidification and dilution, they were checked in Cd
114 concentration and flows were adjusted if necessary. The average concentrations were $0.09 \pm$
115 0.01 $\mu\text{g}\cdot\text{L}^{-1}$, 0.43 ± 0.03 $\mu\text{g}\cdot\text{L}^{-1}$ and 4.00 ± 0.18 $\mu\text{g}\cdot\text{L}^{-1}$ (mean \pm SE, $n = 12$) in control and
116 contaminated tanks, respectively. During the 45 days of exposure, eels were fed every
117 evening in excess (7.5 % wet weight animal/day) with artificial food (fresh fish flesh mixed
118 with oil (1.3%) and agar-agar (0.8%)).

119 At the end of the exposure period, 5 eels per EU were removed and dissected. Two samples of
120 liver were immediately fixed in RNAlater solution and stored at -20°C until needed for
121 analyses.

122

123 2.2. DNA purification

124 Genomic DNA from liver tissue was isolated by overnight digestion with proteinase
125 K, followed by standard phenol/chloroform/isoamyl alcohol extraction and ethanol
126 precipitation. The pellet was resuspended in 100 μL of TE solution and treated with RNase A
127 ($2 \text{ mg}\cdot\text{mL}^{-1}$) at 37°C during 1 h. DNA was *de novo* treated with phenol/chloroform and
128 precipitated with absolute ethanol. The pellet was resuspended in 100 μL of TE.

129

130 2.3. Global CpG methylation assay

131 In order to facilitate maximum binding of DNA, 96-well polystyrene microplates
132 (Greiner-bio-one) were incubated overnight at 4°C with 200 μL per well of a 0.1% aqueous
133 solution of protamine sulfate (Sigma). The plates were then emptied by simple inversion and
134 washed five times with 200 μL of ultrapure milliQ water. These coated plates were then dried
135 at 37°C for 10 min and stored at 4°C in dark before use.

136 To establish a standard curve, DNA from several eels was treated with CpG
137 methyltransferase M.SssI (New England Biolabs) according to manufacturer instructions.
138 Briefly, 1 μL of DNA solution at $1 \mu\text{g}/\mu\text{L}$ was incubated at 37°C for 1h30 with 2 μL of SAM
139 at 32 mM, 2 μL of 10X NEBuffer, 1 μL of SssI methylase at 4 U/ μL and 14 μL of nuclease
140 free water. Methylated DNA was then isolated and purified by phenol/chloroform extraction
141 and ethanol precipitation. Methylated DNA was evaluated by spectrophotometry (Take3,
142 Epoch, Biotek). DNA standard was diluted with TE to obtain a standard solution at $10 \text{ ng}/\mu\text{L}$.
143 The solution was then aliquoted in 0.5 mL tubes and stored at -20°C until needed.

144 Two hundred microliters of standard solutions containing 8, 20, 40, 100 and 200 ng of
145 methylated-DNA as well as DNA samples containing 200 ng of DNA were heated at 94°C
146 for 2 min and immediately cooled in ice. Then, 50 μL of heat-treated DNA were added in the

147 desired number of wells and incubated 1h at 37°C. After DNA attachment, wells were washed
148 five times with 200 µL of PBS (NaCl 0.14M, Na₂HPO₄ 0.01M, pH 7.3). To diminish non-
149 specific antibody binding, each well was then filled with 200 µL of PBS-BSA solution (2%
150 BSA) and the plate was incubated 1h at 37°C. The plate was then washed five times with
151 PBS. Primary antibodies, i.e. anti-5-methylcytosine monoclonal antibody (Epigentek), were
152 diluted with PTB-BSA solution (PBS solution with BSA 2% and Tween-20 0.02%) at a final
153 concentration of 0.5 ng/µL. Wells were then filled with 50 µL of primary antibody solution
154 and the plate was incubated 1h at 37°C. After incubation, the plate was washed three-times
155 with PTB solution (PBS solution with Tween-20 at 0.02%) followed by two-times with PBS.
156 Secondary antibodies, i.e. Goat anti-Mouse IgG1 Antibody HRP Conjugated (Bethyl), were
157 diluted with PTB-BSA solution at a final concentration of 0.2 ng/µL. Wells were then filled
158 with 50 µL of secondary antibody solution and the plate was incubated 30min at 37°C. After
159 incubation, the plate was washed three-times with PTB solution followed by two-times with
160 PBS. Then, 150 µL of TMB solution (Pierce) was added to each well and the plate was
161 incubated 2min at room temperature. The reaction was stopped by the addition of 50 µl of
162 H₂SO₄ at 2N. The absorbance was then read at 450nm.

163

164 2.4. McrBC-msAP-PCR

165 The protocol described by Tryndyak et al. (2006) was used. Briefly, 20 µg of genomic DNA
166 was digested overnight at 37°C with 5 U.µg⁻¹ by *McrBC* endonuclease (New England
167 Biolabs). *McrBC*-digested DNA fragments were then separated on a 0.8% agarose gel
168 electrophoresis. Fragments larger than 1Kbp were purified by means of the QIAquick Gel
169 Extraction Kit (Qiagen), according to manufacturer's instructions. These fragments were then
170 digested overnight at 25°C with 20U.µg⁻¹ of methylation-sensitive restriction endonuclease
171 *SmaI* endonuclease (New England Biolabs). The reaction was stopped by heat treatment

172 (65°C, 25min). Then, fragments were digested overnight at 37°C with 40U.µg⁻¹ by *HpaII*
173 (New England Biolabs). For all enzymatic reactions, reactions were stopped by heat treatment
174 (65°C, 25min) and freezing (-20°C). Digested DNA fragments were PCR-amplified ((95°C
175 30s, 40°C 60s, 72°C 90s) x 5 cycles; followed by (95°C 15s, 55°C 15s, 72°C 60s) x 30
176 cycles) using 50 pmol of a single primer; MLG2 (5'-AACCCCTCACCCCTAACCCCGGG-3').
177 PCR products were resolved on 5% polyacrylamide gel. Bands presenting different intensities
178 among fish groups (Control and Cd-exposed eels) were excised. DNA was then extracted by
179 crush and soak method before to be PCR-amplified as described above. PCR products were
180 purified from a 0.8% agarose gel electrophoresis by means of the QIAquick Gel Extraction
181 Kit (Qiagen). Purified fragments were then cloned using pGEM-T easy vector system
182 (Promega) according to manufacturer's instructions. Selected clones were sequenced (GATC
183 Biotech). Sequence homologies were searched using the Blast+ program using *Anguilla*
184 *japonica* database.¹⁶

185

186 2.5. Quantitative RT-PCR

187 For each gene, specific primers were determined (Table 1) using the Primer3Plus
188 software and EeelBase.¹⁷ Total RNA was extracted from 20 mg of liver (20.41 ± 0.84 mg,
189 mean ± SE, n = 15) and treated with DNaseI using the Absolutely RNA Miniprep Kit
190 (Agilent), according to the manufacturer's instructions. For each sample, RNA quality was
191 evaluated by electrophoresis on a 1% agarose gel and RNA concentrations as well as purity
192 were determined by spectrophotometry (Take3, Epoch, Biotek). First-strand cDNA was then
193 synthesized from 1 µg of total RNA using the GoScript Reverse Transcription System
194 (Promega), according to the manufacturer's instructions. Following the reverse transcriptase
195 reaction, cDNA was diluted 10-fold. Real-time PCR reactions were then performed in an
196 MX3000P (Stratagene; 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C

197 for 30 s and 72°C for 30 s). Each 20 µL reaction contained 12.5 µL of GoTaq qPCR master
198 mix (Promega), 5 µL template and the specific primer pairs at a final concentration of 250 nM
199 each. Amplification efficiencies for all primer sets were calculated; all values proved to be
200 sufficient to allow direct comparison of amplification plots according to the $\Delta\Delta C_t$ method.¹⁸
201 Relative quantification of gene expression was achieved by concurrent amplification of the β -
202 *actin* endogenous control. Hence, during our experiment, total RNAs were quantified and 1
203 µg was used to be reverse-transcribed. During the subsequent qPCR amplifications, the output
204 cycle corresponding to the β -*actin* was examined. This output was always obtained around the
205 same output cycle; i.e. 21.87 ± 0.17 (mean \pm SE, n = 5) for control fish, 21.67 ± 0.36 (mean \pm
206 SE, n = 5) for Cd-exposed fish at $0.4 \mu\text{g.L}^{-1}$ and 21.27 ± 0.57 (mean \pm SE, n = 5) for Cd-
207 exposed fish at $4 \mu\text{g.L}^{-1}$, demonstrating the relevance of the β -*actin* as reference gene in our
208 conditions.

209

210 2.6. Statistical analyses

211 Comparisons among fish groups were performed by analysis of variance (ANOVA), after
212 checking assumptions of normality and homoscedasticity of the error terms. When the
213 assumptions were not met as deduced graphically and from ad-hoc tests, we used log and box-
214 cox data transformations. If significant effects were detected, the Least Square Deviation test
215 (LSD) was used to determine whether means between pairs of samples were significantly
216 different from one another. Computations were performed using STATISTICA version 6.1
217 software (StatSoft, USA). Numerical results are given as means \pm SE (n = 5 per condition).

218

219 3. Results

220 3.1. Effect of Cd exposure on global CpG methylation level and total RNA concentration

221 The calibration of our ELISA method is presented in Figure 1. A significant correlation (r^2
222 = 0.98, $P = 0.0009$) was observed between the quantity of methylated DNA and the
223 absorbance measured at 450 nm, allowing thus to investigate a possible effect of Cd exposure
224 on this parameter in eels. The effect of Cd exposure on the global CpG methylation status of
225 eel's liver is presented in Figure 2A. Increasing concentrations of Cd exposure were
226 associated with an increase in the global CpG methylation level in eels. However, this
227 increase was only significant in the case of eels exposed to the highest concentration (i.e.
228 $[Cd]_{\text{water}} = 4 \mu\text{g.L}^{-1}$). After 45 days of exposure, a significant 2-fold increase in global CpG
229 methylation level was detected in the liver of fish exposed to Cd at $4 \mu\text{g.L}^{-1}$ compared to non-
230 exposed eels. This increase in methylation level was associated with a significant decrease in
231 total RNA concentrations (Figure 2B). After 45 days of exposure, a significant 1.9-fold
232 decrease in total RNA concentration was detected in the liver of fish exposed to Cd at $4 \mu\text{g.L}^{-1}$
233 compared to controls. No significant effect of Cd was observed on this parameter at the
234 lowest Cd concentration tested, i.e. $0.4 \mu\text{g.L}^{-1}$.

235

236 3.2. Identification of differentially methylated sites

237 The McrBC-msAP-PCR method uses three methylation-specific restriction endonucleases
238 which cleave or, on the contrary, do not cleave DNA containing 5-methylcytosine. This
239 method was used on genomic DNA of eels in order to detect differentially methylated
240 fragments among control and Cd-exposed eels. Using this method, hypermethylation and
241 hypomethylation are visualized by the increase or the decrease in the band intensity of DNA
242 fingerprints. Results are presented in Figure 3. Genomic DNA from liver of Cd-contaminated
243 fish exhibited an emergence of novel sequences compared to control eels. A total of 24 bands
244 that showed differential intensities were excised from the gels, reamplified, cloned and
245 sequenced. The resulting sequences were compared to the genome of *Anguilla japonica* using

246 Blast+ program on Linux.¹⁹ For sequences that showed high homologies, a portion of the
247 *Anguilla japonica* genome containing the sequence plus 1000 bp upstream and 1000 bp
248 downstream was compared to Genbank database using Blast program. Only 3 fragments
249 showed high homologies with genes of known functions. These fragments were located near
250 or in the genes encoding for ADP-ribosylation factor 1 (ARF1), kinesin-like protein KIF21A
251 (KIF21A) and 1-acyl-sn-glycerol-3-phosphate acyltransferase delta (AGPAT4). All these
252 sequences presented on the gel an increase in band intensity in response to Cd exposure and
253 can be thus considered as hypermethylated in Cd contaminated fish. Whereas *kif21a* and
254 *agpat4* were found in both groups of Cd exposed eels, *arf1* was found only in eels exposed to
255 the lowest concentration tested; i.e. 0.4 $\mu\text{g}\cdot\text{L}^{-1}$. Using directly Genbank database and Blast
256 program, within sequences, some fragments were found to show highly significant
257 homologies with flanking regions (non-coding regions) of retrotransposon-like sequences.
258 Interestingly, such elements were even observed in the fragments containing the sequences of
259 *kif21a* and *agpat4* genes (Figure 4). All these sequences were found to be hypermethylated in
260 Cd contaminated fish.

261

262 3.3. Gene transcription level

263 To test whether changes in the DNA methylation status of fragments identified by
264 McrBC-msAP-PCR method could induce changes in the transcription level of the
265 corresponding genes, specific primer pairs were designed for *kif21a*, *agpat4*, and *arf1* genes.
266 In order to substantiate a potential effect of Cd on the methylation status and, concomitantly,
267 on the transcription level of TEs in eel, specific primer pairs were also designed for the
268 retrotransposon *line-1*. Among TEs, *line-1* is indeed the most abundant family of non-long
269 terminal repeat retrotransposons found throughout the genome. In consequence, its
270 methylation status is often used as a proxy to assess global DNA methylation.²¹ The

271 transcription level of these genes was then determined by quantitative RT-PCR. As a
272 significant decrease in total RNA concentration was observed in response to Cd exposure, we
273 also determined the transcription level of the gene encoding for 18s rRNA. Results are
274 presented in the Figure 5A. Cd exposure at 0.4 $\mu\text{g.L}^{-1}$ significantly triggered a down-
275 regulation of the gene encoding for ARF1. A significant decrease in the transcription level of
276 genes encoding for 18s and LINE-1 was also observed at the highest Cd concentration (4
277 $\mu\text{g.L}^{-1}$). Despite a trend to increase in response to Cd exposure, no significant effect of Cd
278 was observed on the transcription level of genes encoding for AGPAT4 and KIF21A.
279 Interestingly, the transcription levels of these two genes were found to be highly correlated
280 ($R^2 = 0.95$, $P < 0.0000001$, Figure 5B).

281

282 4. Discussion

283 Our results show that low-dose exposure to Cd induced changes in the CpG methylation
284 status of eel's hepatocytes. Cd exposure was associated with a significant increase in the
285 global CpG methylation status of eels. Such results are consistent with previous studies
286 carried out on human embryo lung fibroblast cells or in hens.^{5,22} For both studies, subchronic
287 Cd exposure triggered a concentration-related increase in the global DNA methylation level.
288 As epigenetic is still in its infancy, it is unclear why and how the genomic DNA methylation
289 of organisms can be modified by Cd exposure. Moreover, it is also unclear if changes in DNA
290 methylation in response to pollutant exposure represent an adaptive response to alleviate
291 pollutant toxicity or, on the contrary, a mechanism by which pollutants exert their toxicity.
292 For example, aberrant DNA methylation status was found to be an important factor in
293 tumorigenesis, including in the development of hepatocellular adenoma tumors in wild fish
294 from contaminated-environments.²³ However, it was shown in numerous models that global
295 genomic levels of DNA methylation are lower in cancer cells than in non-cancer tissues.²³ As

296 Cd is known to induce DNA damages²⁴, an increased in global DNA methylation could aim to
297 protect DNA and, by extension, organisms against Cd toxicity. Indeed, recent researches have
298 shown that chromatin compaction, a well-known effect of DNA methylation, protects DNA
299 from damage.²⁵⁻²⁶

300 Since DNA methylation is commonly associated with gene silencing¹, we then quantified
301 the amount of total RNA in eel liver. Our data indicate that Cd exposure induces a significant
302 decrease in total RNA concentration. Given that rRNAs represent at least 95% of the total
303 RNA in fish hepatocytes, such a decrease could be mainly explained by a decrease in
304 ribosome biosynthesis. In direct support of this hypothesis, a significant decrease in the
305 transcription level of the 18s rRNA gene was observed in Cd-exposed eels.

306 In order to obtain more information about genes and, by extension, biological processes
307 that are differentially methylated between control and Cd-exposed eels we used the McrBC-
308 msAP-PCR method. Our results indicate that Cd exposure can modify the methylation profile
309 of the eel genome. Three genes encoding for proteins involved in intracellular trafficking
310 (ARF1 and KIF21A) and phospholipid biosynthetic process (AGPAT4) were found to be
311 hypermethylated in response to Cd exposure.

312 For *arf1*, this gene was found to be hypermethylated only in eels exposed to the lowest Cd
313 concentration tested, i.e. 0.4 $\mu\text{g}\cdot\text{L}^{-1}$. In accordance to an increase in its methylation status, its
314 transcription level was found to be significantly lower in eels exposed to Cd at 0.4 $\mu\text{g}\cdot\text{L}^{-1}$.
315 Interestingly, ARF1 protein was recently found to be involved in intracellular Cd trafficking
316 and more precisely in the transport of Cd-metallothionein complex.²⁷ Metallothioneins (MT)
317 are cysteine-rich proteins involved in maintaining sufficient intracellular supplies of some
318 essential metals and in detoxifying excess intracellular metals (including non-essential metals
319 such as Cd). Using dominant-negative mutant for *arf1* (DN-*arf1*), authors have shown that the
320 transfer of Cd-MT complex to lysosome can lead to cytotoxicity. Cd toxicity was indeed

321 found to be significantly lower in DN-*arf1* than in wild type cells.²⁷ The hypermethylation of
322 *arf1* gene coupled with a down-regulation of its transcription level in response to metal
323 exposure could thus appear as a defense mechanism to alleviate Cd cytotoxicity.

324 Concerning *kif21a* and *agpat4* genes, despite these genes were found to be
325 hypermethylated in response to Cd exposure, no significant effect of Cd on their gene
326 transcription levels was observed. The gene *kif21a* encodes a microtubule-binding molecular
327 protein involved in intracellular trafficking. The gene *agpat4* encodes an enzyme involved in
328 lipid biosynthesis (i.e. triglycerides and phospholipids). It is interesting to note that *kif21a* and
329 *agpat4* genes, despite they seem to be involved in different biological processes, were found
330 to be highly co-expressed, suggesting a common regulatory mechanism. This could be
331 explained at least in part by the presence at the genomic level of a transposable element (TE)
332 at their proximity. TEs have long been considered as “selfish” or “parasitic” DNA elements
333 since active TEs are known to be highly mutagenic and to impair the fitness of their host.²⁸
334 However, the importance of TEs in genome evolution and transcription regulation has
335 received increasingly attention in recent years. Recent discoveries have demonstrated that TEs
336 are mostly inactive in host. Despite in most cases this is due to TE truncation, DNA
337 methylation was found to play a key role in keeping full-length TEs transcriptionally silent. It
338 is becoming increasingly clear that TEs play important roles in genome transcription
339 regulation since few authors have demonstrated that the activation of TEs by stresses can
340 change the transcriptional activity of neighboring genes.^{21,29-31} In our case, in accordance to
341 an increase in global CpG methylation and in the methylation level of TEs in Cd-exposed
342 eels, the transcription level of *line-1* gene was found to be down-regulated in response to Cd
343 exposure, suggesting an effect of chronic Cd exposure on the methylation level of TEs. It is
344 thus intriguing to speculate that dynamic DNA methylation changes within TEs in response to
345 Cd exposure have contributed among other factors to the (co-)regulation of *agpat4* and *kif21a*

346 transcription levels. Limited evidence exists in the literature about the relationships between
347 the methylation status of TEs and pollutant exposure or diseases (especially for Cd and fish).
348 Moreover, data are mainly restricted to epidemiologic studies and to the cancer field.³²⁻³³ In
349 most cases, TEs were found to be hypomethylated in response to pollutant (e.g. benzene, lead,
350 traffic particles) or to biotic²⁹ exposures. The response appears thus to be different for Cd in
351 our study. For a better understanding, it could be interesting to refer to the functions of
352 AGPAT4 and KIF21A. The product of AGPAT4, i.e. phosphatidic acid (PA), is both a
353 precursor of lipid biosynthesis and an emerging signaling lipid. PA is known to be involved in
354 the regulation of diverse cellular functions in plants and animals notably intracellular
355 trafficking but also microtubule organization, cytoskeleton reorganization and survival
356 signaling. In addition, it is interesting to note that proteomic investigations have shown that
357 kinesin-like proteins (KIF proteins) are PA-binding proteins.³⁴ It could thus explain why
358 *agpat4* and *kif21a* genes present common pattern of gene transcription. Intracellular levels of
359 PA are known to increase in response to abiotic stress. It has been proposed that PA is a
360 second messenger in a broad range of stress signaling pathways and that PA mediates
361 responses to various stresses.³⁵⁻³⁶ To our knowledge, there is no data on a potential role of PA
362 signaling pathway in the response to Cd exposure. As DNA methylation is commonly
363 associated to gene silencing (with few exceptions; see Vandegehuchte and Janssen, 2011¹),
364 our data could indicate that chronic Cd exposure *via* DNA methylation of TEs represses this
365 stress pathway, bringing the transcription level of *agpat4* and *kif21a* in Cd-exposed eels
366 closed to control eels. This could impair the capacity of eels to respond to Cd stress.
367 Alternatively, such a response could correspond to the setup of an adaptive response to
368 chronic Cd exposure. Indeed, under chronic exposure conditions, it could be difficult to
369 maintain over-time increased levels of PA, i.e. an energy-consuming stress response. In this
370 context, it is interesting to note that Takiguchi et al.³⁷ using rat liver cells showed that whereas

371 prolonged exposure to Cd (10 weeks) triggers DNA hypermethylation, a short term exposure
372 (1 week) to the same conditions induces, on the contrary, DNA hypomethylation. Further
373 kinetic studies are needed to test a potential effect of both acute and chronic Cd exposures on
374 PA biosynthesis.

375 Our study illustrates how epigenetics, and more precisely, DNA methylation can be
376 involved in the chronic stress response to Cd in a non-model fish species. Cd exposure was
377 found to increase the global CpG methylation status of eel liver. The methylation level of
378 three genes involved in intracellular trafficking and lipid biosynthesis was also increased in
379 Cd-exposed eels. Two of them were associated with a TE sequence at the genomic level. This
380 could reinforce the general hypothesis that DNA methylation of TEs could represent a
381 widespread response to abiotic stress in eukaryotic organisms by regulating the transcription
382 level of neighboring genes.

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396 **Figure captions**

397 **Figure 1.** Linear regression of log of ng 5-methylcytosine DNA versus absorption at 450 nm
398 (mean \pm SE, n = 3 technical replicates, the coefficient (R^2) and the level of significance (P) of
399 the correlation are reported on the graph).

400 **Figure 2.** Change in relative global CpG methylation level (A) and in total RNA
401 concentration (B) in liver of control and Cd exposed European eels ($[Cd]_w = 0.4$ and $4 \mu\text{g.L}^{-1}$)
402 ¹). Data are expressed as mean \pm SE (n = 5). Bars sharing same-case letters do not differ
403 significantly ($P > 0.05$).

404 **Figure 3.** Representative McrBC-msAP-PCR fingerprints of control (C) and Cd-exposed eels
405 ($[Cd]_w = 0.4$ and $4 \mu\text{g.L}^{-1}$, Cd0.4 and Cd4, respectively). Bands that appeared in Cd-exposed
406 eels and not in control eels and bands with increasing intensity of band in Cd-exposed eels
407 compared to control eels were considered as hypermethylation. M refers to molecular marker.
408 Open triangles refer to *kif21a*. Closed triangles refer to *agpat4*. Circle refers to *arf1*.

409 **Figure 4.** Schematic diagram detailing the fragments obtained by McrBC-msAP-PCR and
410 that contain both sequences with high homology for *agpat4* and *kif21a* genes from *Anguilla*
411 *japonica* and sequences with high homology for transposable elements (TE). Expect Evaluate
412 are indicated on the graph. These schematic diagrams were carried out using BLASTn
413 program and MEME software.²⁰

414 **Figure 5.** Effect of Cd exposure on gene transcription. (A) Change in gene transcription level
415 (mean \pm SE; n = 5) in liver of control and Cd exposed European eels ($[Cd]_w = 0.4$ and $4 \mu\text{g.L}^{-1}$)
416 ¹). Bars sharing same-case letters do not differ significantly ($P > 0.05$). (B) Relation between
417 the transcription level of *kif21a* and *agpat4* (n = 15, the coefficient (R^2) and the level of
418 significance (P) of the correlation are reported on the graph).

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421 **Figures**

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424 **Figure 1**

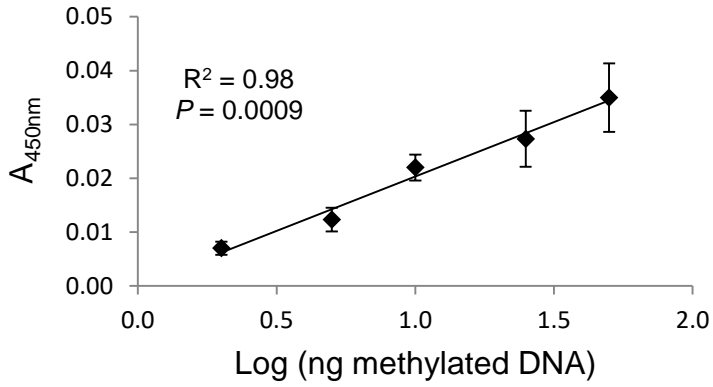
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433 **Figure 2**

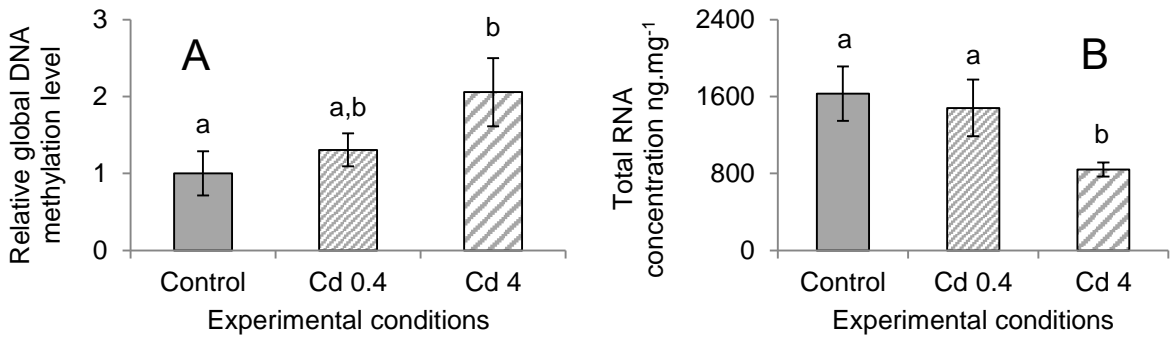
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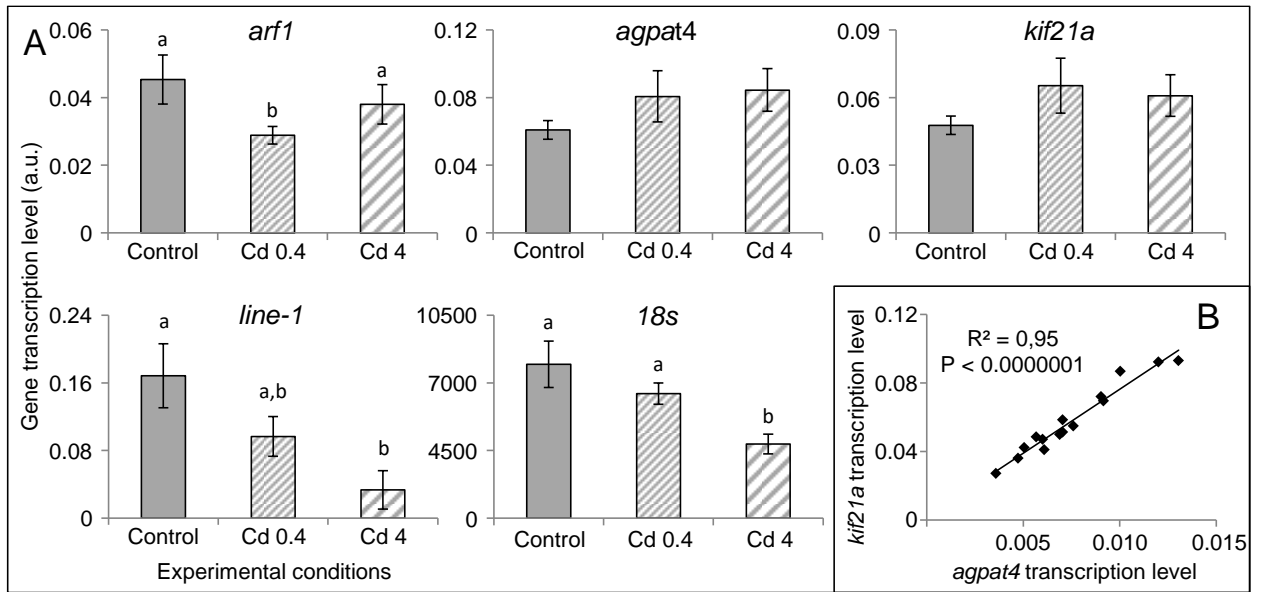
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462 **Figure 5**



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477 **Tables.**

478 **Table 1.**

479 Specific primer pairs used in quantitative real time PCR analysis

	Gene name	Primer sequences
481	<i>β-actin</i>	CAGCCTTCCTTCCTGGGT ^a AGTATTTGCGCTCGGGTG ^b
482	<i>arf1</i>	GCTGCTGGAAAAACAACCAT ^a CTAGTAGAACGGCGTCTCGG ^b
483	<i>kif21a</i>	CATCTGCTTCATCAGACGGA ^a GACTACGAGAAGCGGCTGAC ^b
484	<i>agpat4</i>	CATCTGCTTCATCAGACGGA ^a GACTACGAGAAGCGGCTGAC ^b
485	<i>line-1</i>	CGTCGCCTGTTCCATATTCT ^a AGCAGACAGTGGACAGCCTT ^b
486	18s	CATGGCCGTTCTTAGTTGGT ^a CTCTAAGAAGTTGGACGCCG ^b

487 ^a Forward primer, ^b Reverse primer

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491 5805.

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