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14 # Occurrence of DNA methylation in *Daphnia magna* and
15 # influence of multigeneration Cd exposure

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28

Abstract

Most of the research on the epigenetic phenomenon of DNA methylation has been performed with vertebrates and plants. Knowledge on DNA methylation in *Daphnia magna,* a key test organism in aquatic toxicology, is completely lacking. Through epigenetic inheritance, effects of transient chemical exposure could be transferred to non-exposed generations, which could have a major impact on ecological risk assessment procedures. In this study, we determined if CpG methylation occurs in *D. magna* and if this can be influenced by exposure to toxic substances. Homologs of human DNA methyltransferases DNMT1, DNMT2 and DNMT3A were found in the partially available *D. magna* genome. Using an optimized "Amplification of Intermethylated Sites (AIMS)" technique, two methylated fragments were discovered in *D. magna* DNA. No homology was found for these sequences. The methylation and the *D. magna* origin of the fragments were confirmed with Southern analysis. This optimized AIMS technique was then applied to DNA of *D. magna* which were exposed to 180 µg/L Cd for two generations. Exposure resulted in a significant decrease in reproduction. The same methylated fragments with the same band intensity were observed in DNA of both non-exposed and exposed daphnids. As such, it could not be demonstrated that Cd exposure altered DNA methylation. However, the presence of DNA methylation in *D. magna* shows that potentially epigenetic effects may occur in this species.

50

51 ## *Introduction*

52 Epigenetics is the study of changes in gene function that are mitotically and/or
53 meiotically heritable and that do not entail a change in DNA sequence (Wu and Morris
54 2001). One of the most studied epigenetic phenomena is DNA methylation, which is
55 the addition of a methyl group on the 5 position of cytosines in the DNA (Bird 2002;
56 Clark et al. 1994; Oakeley 1999; Watson and Goodman 2002). DNA methylation can
57 play an important role during development, cell differentiation, X-chromosome
58 inactivation and tumorigenesis (Santos et al. 2005). In mammals, methylation of DNA
59 in promoters is generally associated with silencing or reduced transcription of genes
60 (Bird 2002). It is hypothesized that CpG methylation represses transcriptional initiation,
61 not necessarily repressing transcription as such (Bird 1995). Recent research supports
62 this hypothesis for infrequently transcribed genes (Mandrioli 2007; Suzuki et al. 2007).
63 This explains the observation of methylation in transcribed genes. In *Arabidopsis*,
64 methylation has been found in a large number of moderately transcribed genes
65 (Zilberman et al. 2007). Methylated esterase genes in the aphid *Myzus persicae* were
66 shown to be actively transcribed and increasingly expressed (Field 2000), suggesting
67 that methylation plays a positive role in the expression of these genes. This could be
68 due to the above mentioned inhibition of spurious transcription.

69

70 Considerable research on DNA methylation and its function has been performed in
71 vertebrate organisms and plants (Chan et al. 2005; Reik and Dean 2001). Recently,
72 DNA methylation in invertebrates has received more attention. Some invertebrates
73 were long believed to have no DNA methylation at all, as suggested by (Urieli-Shoval

74 *et al.* 1982) for *Drosophila*. More recent research, however, has shown a low DNA
75 methylation status in *Drosophila* (Lyko et al. 2000). The methylation was higher in
76 early developmental stages compared to adults . It was not restricted to CpG
77 dinucleotides, as is the case in most of the mammalian DNA methylation, but it was
78 also observed in CpT and CpA dinucleotides. For other organisms, such as the
79 nematode *Caenorhabditis elegans*, no methylation has been found up to now (Lyko
80 2001). Most invertebrates show a mosaic DNA methylation pattern, with roughly
81 comparable amounts of methylated and unmethylated DNA (Simmen et al. 1999;
82 Tweedie et al. 1997) as opposed to vertebrates in which a predominantly highly
83 methylated genome is interrupted by unmethylated CpG islands.
84
85 Epigenetic changes can be triggered by environmental factors such as nutrition type or
86 exposure to xenobiotics due to the dynamic state of the epigenome (Szyf 2007). A large
87 proportion of offspring of mice that were fed with food high in folic acid, vitamin B12,
88 choline, and betaine content during pregnancy displayed a high frequency of CpG
89 methylation and heavily mottled and pseudo-agouti coat color. Conversely, a high
90 number of offspring of mice fed with a control diet had a lower frequency of CpG
91 methylation and a yellow or slightly mottled coat color (Waterland and Jirtle 2003).
92 Similarly, exposure to toxic substances can also result in epigenetic effects. Exposing
93 gestating female rats transiently to vinclozolin and methoxychlor caused reduced
94 reproduction effects in the progeny which correlated well with altered DNA
95 methylation patterns (Anway et al. 2005). This was observed up to four generations
96 after exposure, indicating the possibility of inheritance of epigenetic changes, even

97 after only a short transient exposure to environmental stress. Epigenetic inheritance has
98 also been demonstrated in *Drosophila* (Chong and Whitelaw 2004).
99 Through epigenetic inheritance, effects of transient chemical exposure could be
100 transferred to non-exposed generations. Populations can thus experience effects of their
101 ancestors' exposure. This could have a major impact on ecological risk assessment
102 procedures.
103 For *Daphnia magna*, a key test organism in aquatic toxicology and an important
104 species in many aquatic ecosystems, knowledge on DNA methylation is completely
105 lacking. This research was aimed at determining whether CpG methylation is an
106 epigenetic factor in *D. magna*, and if so, whether it can be influenced by exposure to
107 toxic substances.
108

109 *Materials and Methods*

110

111 **Sample collection and Daphnia cultures**

112 *D. magna* Straus (clone K6) used in all our experiments was originally collected from a
113 pond in Kiel (Antwerp, Belgium) and has been successfully cultured under controlled
114 laboratory conditions for more than 10 years. One batch of 250 organisms was cultured
115 as a control for four generations (F0 – F3) in M4 medium (Elendt and Bias 1990)
116 slightly modified to reach optimal conditions for daphnids (Muyssen and Janssen 2004).
117 A second batch of 250 organisms was cultured for two generations (F0 – F1) in the
118 same medium with 180 µg/L of added Cd. Organisms were daily fed with an algae mix
119 consisting of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3/1
120 cell number ratio.

121 Reproduction in *D. magna* in the control and Cd treatment was determined for two
122 weeks following the procedures of OECD 211 guideline (OECD 1998), using ten
123 replicates of individually held daphnids.
124 Algae-free juveniles of *D. magna* were obtained after 24 h from a batch of adults from
125 the lab culture that was fed suspended kaolin clay particles (Imerys, UK) in an algae-
126 free medium.
127
128 **DNA extraction**
129 DNA was extracted from 4 (juveniles), 21 or 27 (adults) day old daphnids with the
130 MasterPure™ kit (Epicentre, Madison, WI, USA), following the protocol provided by
131 the manufacturer. Fifty juveniles or 18-20 adult organisms were rinsed with deionized
132 water, blotted dry and shock frozen in liquid nitrogen. The frozen organisms were
133 subsequently transferred into a vial containing 300 µL Cell and Tissue Lysis buffer and
134 3 µL RNAse A solution (5 mg/mL) and then manually homogenized with a microfuge
135 tube pestle.
136 DNA was extracted from the algal mix in a similar manner. From the concentrated
137 algal solution used as daphnid food, 300 µL was centrifuged for 10 min at 10000 g in a
138 tabletop Hettich Mikro 200 R microcentrifuge (Andreas Hettich GmbH & Co KG,
139 Tuttlingen, Germany). The supernatant was discarded, the algal pellet was shock frozen
140 in liquid nitrogen and DNA was extracted using the MasterPure™ kit (Epicentre,
141 Madison, WI, USA) as described for *D. magna*.
142 DNA integrity was checked on a 1.5% 0.5x TAE agarose gel and the DNA
143 concentration was determined spectrophotometrically using a NanoDrop 1000
144 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

145

146 **AIMS**

147 CpG methylation was investigated by a restriction-enzyme based method derived from
148 the "Amplification of intermethylated sites (AIMS)" protocol described by (Frigola *et*
149 *al.* 2002). However, we used tetracutters *Hpa*II and *Msp*I instead of hexacutters *Sma*I
150 and *Psp*AI (Wang et al. 2006). One microgram DNA was digested with 10 units *Hpa*II
151 (New England Biolabs, Ipswich, MA, USA) for 16h at 37 °C. This enzyme is sensitive
152 to CpG methylation, hence it will not cut the restriction site if the inner C is methylated.
153 The sticky ends are then filled in with 2 units Klenow enzyme (New England Biolabs,
154 Ipswich, MA, USA) in the presence of a mix of 500 µM each dNTPs (Fermentas, St.
155 Leon-Rot, Germany), by incubating for 15 min at 37 °C. This reaction was stopped by
156 heating at 75 °C for 10 min. Subsequently, the DNA fragments were digested with 10
157 units *Msp*I (New England Biolabs, Ipswich, MA, USA) by incubating for 6 h at 37 °C.
158 This enzyme is insensitive to CpG methylation, hence it will cut regardless of the
159 methylation on the restriction site. Adaptors were prepared by adding 50 nmol oligo
160 "B" (5'-ATTCGCAAAGCTCTGA-3') to 50 nmol oligo "B-GC" (5'-
161 CGTCAGAGCTTTGCGAAT-3') (Eurogentec, Seraing, Belgium) in a total volume of
162 1 mL, incubating the mixture at 65 °C for 2 min and then cooling it to room
163 temperature for 30 to 60 min. Adaptors were ligated to the sticky ends created by the
164 *Msp*I digestion with T4 DNA ligase (New England Biolabs, Ipswich, MA, USA), by
165 incubating with 800 cohesive end units T4 DNA ligase for 16 h at 16 °C. The DNA
166 was then purified with the Illustra GFX PCR DNA and Gel Band Purification Kit (GE
167 Healthcare, Diegem, Belgium), following the manufacturer's protocol. Adaptor ligated
168 fragments were PCR-amplified using adaptor-specific primers, with the following PCR

169 conditions: 5 min at 95 °C, 35 cycles of (1 min at 95 °C, 45 s at 58 °C, 1 min 30 s at
170 72 °C), 6 min at 72 °C. The 15 µL reaction mixture contained 20 ng template (adaptor
171 ligated fragments), 0.33 µM primer, 1.67 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 50
172 mM KCl and 1 unit of Taq DNA polymerase (Invitrogen, Merelbeke, Belgium).
173 Selective nucleotides were added to the adaptor-specific primers (5'-
174 ATTCGCAAAGCTCTGACGG-3'). Primers without extra selective nucleotides and
175 primers with all possible sets of two extra nucleotides (i.e. 16 different primers) were
176 tested.
177 In a modification of the method, two DNA subsamples of 1 µg were used. One
178 subsample was digested with HpaII, while the other was digested with MspI. There was
179 no fill in of sticky ends with Klenow enzyme. All other steps were performed as
180 described above. A scheme of both the original and the modified AIMS methods is
181 shown in Fig. 1.
182 Primers were labeled with γ^{33}P-ATP (GE Healthcare, Diegem, Belgium) by
183 polynucleotide kinase (Fermentas, St.Leon-Rot, Germany). Reaction products were
184 analysed on large 5% denaturing polyacrylamide sequencing gels, cast and run in a
185 Sequi-Gen GT Sequencing Cell (Bio-Rad, Nazareth, Belgium). Gels were exposed for
186 two to four days to a sensitive film (Fujifilm, Düsseldorf, Germany) or for one or two
187 days to an imaging plate, after which they were analysed by a FLA-5100 imaging
188 system with MultiGauge Image Analysis software (Fujifilm, Düsseldorf, Germany).
189

190 **Cloning and sequencing of AIMS fragments**

191 Fragments of interest were excised from the polyacrylamidegel, reamplified by PCR,
192 ligated into the pGEM-T vector (Promega, Leiden, The Netherlands) and transformed

193 into heat shock competent *Escherichia coli* DH5α cells. Transformed cells were
194 selected on LB^+ medium containing 100 µg/mL carbenicillin and checked for inserts by
195 colony PCR using SP6 (5'-ATTTAGGTGACACTATAGAATACTCAAGC-3') and
196 T7 (5'-TAATACGACTCACTATAGGGCGAATTGG-3') primers. Plasmids of
197 positive colonies were isolated by miniprep (Birnboim and Doly 1979). Sequencing of
198 the insert was performed at the VIB Genetic Service Facility, Antwerp, Belgium.
199
200 **PCR on daphnid and algae DNA**
201 The total 30 µl PCR volume consisted of 4 µl genomic DNA (2 ng/µl), 2 µl Forward
202 primer (10µM), 2 µl Reverse primer (10 µM), 3 µl 10 x PCR buffer (Invitrogen,
203 Merelbeke, Belgium), 2 µl dNTPs (10 mM), 0.2 µl *Taq* polymerase (5 units/µl,
204 Invitrogen, Merelbeke, Belgium), 1.5 µl $MgCl_2$ (50 mM) and 15.3 µl water. Primers
205 were based on the obtained fragments after the AIMS procedure (Fig. 3): 5'-
206 GACTCGACCCATTCTGCAAT-3' and 5'-TTCGGAAGGGCTTTCCTTAT-3' as
207 forward and reverse primer for the first sequence DMMF1; 5'-
208 GGACGCGTGTACATTCACTG-3' and 5'-GGACCCATAAGACCCCTGTT-3' as
209 forward and reverse primer for the second sequence DMMF2. The PCR program
210 involved an initial denaturation for 4 min at 95°C, followed by 32 cycles of (1 min at
211 95 °C, 45 s at 54 °C and 1 min at 72 °C), and a terminal extension of 10 min at 72 °C.
212 PCR products were separated on 1.5% agarose 0.5x TAE gels. The MassRuler™ DNA
213 Ladder Mix (Fermentas, St. Leon-Rot, Germany) was loaded for reference. Ethidium
214 bromide stained agarose gels were analysed with UV-transillumination using a GelDoc
215 system (Bio-Rad, Nazareth, Belgium)
216

217 **Southern analysis for confirmation of methylation**

218 DNA fragments to be used as probe were amplified with PCR as described above and
219 radioactively labelled with [α-^{32}P]-dCTP (Perkin-Elmer, Zaventem, Belgium) using
220 Amersham Ready-To-Go DNA Labelling Beads (GE Healthcare, Diegem, Belgium).
221 Non-incorporated nucleotides were removed with Micro Bio-Spin 30 columns (Bio-
222 Rad, Nazareth, Belgium).

223 Digestion with MspI or HpaII was performed as described above, scaled up to 10 µg of
224 DNA. Digests were purified with the QIAquick Gel Extraction Kit (Qiagen, Venlo, The
225 Netherlands) and run overnight at 25 V on a 15 x 15 cm 0.8% agarose gel in 0.5x TBE
226 buffer. After electrophoresis, the gel was depurinated with 0.25 M HCl for 10 min,
227 denatured (1.5 M NaCl and 0.5 M NaOH) for 30 min and neutralized (1.5 M NaCl and
228 0.5 M Tris-HCl, pH7.4) for 45 min. This gel was blotted on an Amersham Hybond™-
229 N+ nylon membrane (GE Healthcare, Diegem, Belgium). After prehybridization in 5x
230 SSC, 5x Denhardt's solution (1 g/L Ficoll, 1 g/L polyvinylpyrrolidone, 1g/L BSA),
231 0.5 % SDS (all chemicals from Sigma, Bornem, Belgium) and 100 µg/mL denatured
232 salmon sperm DNA (Invitrogen, Merelbeke, Belgium), the blot was hybridized
233 overnight to 10 µL of denatured radioactive probe (approximately 150 – 200 ng) in a
234 solution with the same composition as the prehybridization buffer. The blot was rinsed
235 cold in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate) with 0.1% (w/v) sodium dodecyl
236 sulphate (SDS), then consecutively washed at 60°C in 2× SSC, 1× SSC and 0.5× SSC,
237 each time for 15 min. The membrane was exposed to an imaging plate for
238 approximately 1 h after which this was analyzed by a FLA-5100 imaging system with
239 MultiGauge Image Analysis software (Fujifilm, Düsseldorf, Germany).

240

241 **Statistical and bioinformatic analysis**

242 Significance of difference in reproduction between Cd exposed and non exposed
243 organisms was tested using a t-test in Statistica (Tulsa, USA) with $\alpha = 0.05$. Basic
244 Local Alignment Search Tool (BLAST, Altschul et al. 1997) analyses were performed
245 at the National Center for Biotechnology Information (NCBI) server
246 (http://www.ncbi.nlm.nih.gov) using BLASTn and both nucleotide collection and
247 expressed sequence tag (EST) databases and at the *Daphnia* Water Flea Genome
248 Database (http://wfleabase.org). For local BLAST searches ESTs of *D. magna* and *D.*
249 *pulex* were downloaded from NCBI (May 2008). The sequences were cleaned using
250 seqclean to remove contaminants, low quality and low-complexity sequences and
251 subsequently clustered using TIGR Gene Indices Clustering Tool TGICL (Pertea *et al.*
252 2003). A set of unigenes was obtained by combining the contigs and singletons. The
253 presence of *Daphnia* genes coding for DNA methyltransferase (DNMT) enzymes was
254 investigated by searching the partly assembled *D. magna* genome for homologs of
255 *Homo sapiens* DNMTs (DNMT1, A0AV63 / DNMT2, Q6ICS7 / DNMT3A,
256 Q9Y6K1.4 / DNMT3B, Q2PJS8) using tBLASTn at the *Daphnia* Water Flea Genome
257 Database.
258 Sequences were analysed using the programs getorf and showorf embedded in the
259 EMBOSS software package (Rice *et al.* 2000), http://emboss.sourceforge.net/).
260

261 ## *Results*

262 To detect whether *D. magna* is able to perform DNA methylation, we determined
263 whether the genes essential for this process are present in this species. A search with
264 tBLASTn in the partially assembled *D. magna* genome (version 2008_09) at the

265 *Daphnia* Water Flea Genome Database revealed unigenes with significant homology to
266 DNMT 1, DNMT 2 and DNMT 3A (Table 1). These results indicate that *D. magna*
267 possesses the molecular toolbox to perform DNA methylation.
268 After obtaining these indications for the occurrence of this type of modification, we
269 aimed to unequivocally demonstrate the presence of DNA methylation in *D. magna*. To
270 this end, *D. magna* DNA was analysed with the AIMS method. Amplification with
271 non-extended primers resulted in a non-resolved smear both on agarose and
272 polyacrylamide gels. Next, all 16 possible primers extended with two extra selective
273 nucleotides were used. This resulted in a total of 899 clear and reproducible bands, with
274 a nicely discernible pattern for most of the tested primers (Table 2). These fragments
275 were considered as flanked by at least one methylation site. As a confirmation of the
276 technique, the enzymes MspI and HpaII were used separately with two DNA
277 subsamples and not sequentially with one sample (Fig. 1). Surprisingly, 897 out of 899
278 bands that were found following sequential restriction with *Hpa*II and *Msp*I were also
279 detected, at a comparable intensity, in samples that were only restricted with the
280 methylation sensitive *Hpa*II. This probably indicates failed Klenow polymerization for
281 those specific fragments (M. Jorda, personal communication). When Klenow
282 polymerization fails filling up the sticky end of an unmethylated fragment after *Hpa*II
283 digestion, the adaptor is ligated to unmethylated fragments that will be amplified in the
284 PCR together with the methylated fragments. All except two bands observed after
285 restriction with MspI were also observed with comparable intensity after restriction
286 with HpaII, indicating the absence of methylation at the restriction sites of these
287 fragments.

288 Only samples amplified with a -GG extended primer resulted in two observed
289 methylation specific bands on the polyacrylamidegel (Fig. 2). These bands were
290 observed in samples that were treated with *Msp*I, but not in samples that were restricted
291 with *Hpa*II. This observation was confirmed in three independently isolated DNA
292 samples. The same procedure was executed with DNA taken from Cd-exposed juvenile
293 organisms from the 2nd generation to investigate the extent of the effect of Cd on DNA
294 methylation. Due to problems with the storage of the DNA samples, no analysis could
295 be performed on the F0 Cd exposed generation. The significant difference between the
296 total number of juveniles per surviving organism after two weeks of exposure of the
297 Cd-exposed versus the reproduction of non-exposed organisms in the F1 generation (8
298 versus 27 juveniles per replicate, p-value 0.000001) confirmed the adverse effect of Cd
299 at this concentration. Despite the clear effect at an organismal level, AIMS analyses
300 resulted in the same band pattern (with the same methylated bands) in both Cd-exposed
301 and non-exposed daphnids (Fig. 1).
302 The two "methylation specific" fragments DMMF1 and DMMF2 (*Daphnia magna*
303 *methylation specific fragment)* were excised from the polyacrylamide gel, reamplified
304 and sequenced (Fig. 3). The length of DMMF1 is 763 bp, whereas the length of
305 DMMF2 is 620 bp. No significant similarities were found when both sequences were
306 used in BLAST searches against the nucleotide collection database of NCBI (E-value
307 cutoff = 0.10). Analysis of the sequences against the partial *D. magna* genome (version
308 2008_09) and the *D. pulex* genome (version 2006_09) using BLASTn on the *Daphnia*
309 Water Flea Genome Database (http://wfleabase.org) also yielded no significant hits.
310 Despite this inability to identify similar sequences in the partial genome or in a related
311 genome, other elements indicate that the fragments might be coding for proteins. No

312 repeats were found in either sequence. DMMF1 exhibits a non-stop open reading frame.
313 The longest open reading frame that can be detected in DMMF2 is 495 nucleotides.
314 It was striking that no homology was found, despite the many *Daphnia* sequences
315 available in the public databases, including the roughly annotated *D. pulex* genome .
316 This observation might indicate that the detected specific fragments were amplified
317 from a contaminating source rather than from *Daphnia*. The best candidate for this
318 contaminant DNA is the algae mix used to feed *Daphnia* and which might still be
319 present in the gut when the daphnids are lysed for DNA extraction. Specific primers
320 were constructed on both fragments, and the primer pairs were used to amplify the
321 sequences from DNA extracted from *Daphnia* and algae. Whereas a band was obtained
322 with an expected size when DNA was used from *D. magna* or from juveniles that had
323 not been fed, the fragment could not be amplified from algal DNA (Fig. 4). This
324 finding thus demonstrated that the observed methylation specific fragments in the
325 *Daphnia* DNA did not originate from the algae ingested by the daphnids.
326 To confirm the methylation of the fragments, a Southern analysis was performed. DNA
327 was extracted from a batch of juveniles that had not been fed and had not had contact
328 with algae. One subsample was digested with HpaII, another subsample with MspI.
329 Hybridization of the amplified parts of the methylated fragments resulted for each
330 fragment in a band that was clearly visible in the lane with MspI digested DNA, but not
331 in the lane with HpaII digested DNA (Fig. 5). This confirms the methylation of these
332 specific fragments. The clear signal is a further confirmation that the fragments did
333 originate from *D. magna* DNA.

334 ## *Discussion*

335 *D. magna* homologs of enzymes involved in DNA methylation were found in the
336 partially sequenced *D. magna* genome. Three sequences showed a high degree of
337 similarity to the sequences coding for human DNA methyltransferases (DNMTs). In a
338 review of DNMTs in eukaryotes, DNMT2 is described as a strongly conserved and
339 widely distributed protein, which was observed in 20 out of the 23 investigated species
340 (Goll and Bestor 2005).
341 The AIMS approach confirmed the occurrence of methylation in *D. magna*. Two
342 differential bands were detected. The methylation was confirmed by a Southern blot
343 experiment in which the fragments were used as probe to hybridize against *D. magna*
344 DNA digested with methylation sensitive or insensitive restriction enzymes. Two
345 differential AIMS-bands correspond to a maximum of four methylated cytosines - one
346 at each side of the fragments. However, the actual number of methylation sites will be
347 higher since not all methylation sites can be detected with the applied AIMS approach.
348 Small fragments will not be discernible in the lower part of the gel whereas long
349 fragments will not be amplified under the PCR conditions used. Assuming that the
350 fraction of methylated sites is the same for both the visible and "invisible" fragments
351 on the gel it is calculated that $\frac{2}{899} = 0.22\%$ of CpG sites in *Daphnia magna* are
352 methylated, extrapolating methylation ratios for CCGG sites to methylation ratios for
353 all CpG sites. However, the two fragments can be flanked by four different methylated
354 restriction sites. Following the same reasoning, this thus results in 0.22% to 0.44%
355 methylated CpG sites. The cytosine-methylation level in *Drosophila melanogaster* is

356 0.1% to 0.4% (Lyko et al. 2000), which is the same range as what is predicted for *D.*
357 *magna* in this study.
358 Compared to cytosine methylation in other species this estimated degree of methylation
359 is low. The percentage of methylated cytosines in the DNA varies considerably: 5% in
360 mammals and birds, 10% in fish and amphibians, 0 to 10% in insects and more than
361 30% in some plants (Field et al. 2004). Little is known about DNA methylation in
362 crustaceans. In *Artemia*, no detectable methylation was found using HPLC-analysis
363 (Warner and Bagshaw 1984). In *Asellus aquaticus,* however, considerable DNA
364 methylation was observed with an immunolabeling technique (Barzotti 2006).
365 In vertebrates, DNA methylation occurs almost exclusively in the context of CpG
366 dinucleotides and also most invertebrate genomes exhibit methylation in a CpG context.
367 However, it should be noted that also non-CpG methylation, e.g. CpA or CpT
368 methylation has been described in plants, mammals and insects (Bernstein et al. 2007;
369 Lyko et al. 2000). Since this type of methylation can not be detected with the AIMS
370 method used in this study, it is possible that the total degree of methylation will be
371 higher than predicted.
372 No relevant matches were found when the methylated sequences were used as query in
373 BLAST searches against the partially sequenced *D. magna* genome, the fully
374 sequenced *D. pulex* genome or the nucleotide collection database at NCBI. This
375 suggests that these sequences are specific for *D. magna*. To obtain a general idea on the
376 number of species-specific genes in *D. magna*, a BLAST search was performed of all
377 unigenes from the *D. magna* EST dataset against the *D. pulex* EST dataset. With a
378 cutoff E-value of 1×10^{-3}, 1567 out of 5003 unigenes resulted in no hit. This means that
379 for approximately 31% of the currently known unigenes from the *D. magna* genome,

380 there is no similar gene in the *D. pulex* EST dataset. The latter dataset is considerably
381 larger than the *D. magna* dataset and consists of 32030 unigenes. This 31% is an
382 overestimation of the total fraction of *D. magna* specific genes. ESTs are always low
383 quality sequences that are only fragments of genes. Moreover, ESTs are only available
384 for genes that were expressed at the moment the EST database was created. It should
385 also be noted that although the *D. pulex* EST dataset is rather extensive, it does not
386 provide full coverage of all genes. This can be deduced from the relatively large
387 number of 12413 singletons compared to 19617 contigs. Nevertheless, the fact that
388 approximately 31% of the available *D. magna* unigenes had no similar counterpart in
389 the *D. pulex* EST dataset indicates that there is a large amount of species-specific DNA
390 in *D. magna*. This finding has also been reported for other congeneric species. For
391 *Caenorhabditis briggsae* for example, more than 4% of the genes have no detectable
392 matches in the related *Caenorhabditis elegans* (Stein et al. 2003).
393 Alternatively, the lack of sequence similarity could point to possible contamination by
394 non-*Daphnia* DNA. However, the fact that fragments could be amplified from DNA
395 obtained from juvenile *D. magna* that had not been exposed to algae, confirms that the
396 fragments indeed belong to the *D. magna* genome and not to the algae DNA (food).
397 This is further confirmed by the clear hybridization of the probes to the *D. magna* DNA
398 on the Southern blot.
399 The effect of Cd on the daphnids' reproduction corroborates the results from previous
400 studies with the same clone and same medium (Muyssen and Janssen 2004). For non-
401 acclimated organisms and based on R_0 values (net reproduction), a 21-day EC_{10} and
402 EC_{50} (95% confidence limits) of 167 (64-437) µg/L Cd and 254 (191-336) µg/L Cd,
403 respectively, was calculated. For organisms acclimated to 150 µg/L Cd for seven

404 generations, the 21-day EC_{10} and EC_{50} values (95% confidence limits) were 143 (66-
405 311) and 230 (154-344) µg/L Cd. In the present study, a 70% reduction of reproduction
406 was observed after 14 days at 180 µg/L Cd in organisms acclimated to 180 µg/L Cd for
407 two generations. Exposure to chemicals has been shown to alter DNA methylation in
408 several studies with mammalian cells. In vitro exposure of rat liver cells to 1.0 and 2.5
409 µM Cd in William's E medium caused hypomethylation after one week exposure and
410 hypermethylation after 10 weeks of exposure (Takiguchi et al. 2003).
411 Hypermethylation of DNA was also observed in human embryo lung fibroblast cells
412 exposed to to 1.2 and 1.5 µM Cd for 2 months (Jiang et al. 2008). Exposure of K562
413 cells to 2.0 µM of Cd resulted in hypomethylation of DNA after 24h and 48h (Huang et
414 al. 2008). Similar results have been obtained with plants. In white clover and hemp,
415 significant hypomethylation was observed after two weeks of growth in soils
416 contaminated with Cd, Ni and Cr (Aina et al. 2004). In our study, no changes in DNA
417 methylation pattern were observed in Cd exposed organisms. This suggests that Cd
418 exposure does not affect the DNA methylation status of *D. magna*. However, it should
419 be noted that not all CpG sites are visualized with the method used in this exploratory
420 study. Consequently, a potential effect of Cd exposure on CpG methylation cannot be
421 excluded. Other methods, in which a full genomic methylcytosine analysis is performed,
422 could elucidate this further.
423 Apart from DNA methylation, there are other mechanisms of epigenetic regulation.
424 Histone modifications such as acetylation, methylation and ubiquitination can regulate
425 DNA transcription (Grant 2001; Zhang 2003). RNA interference (RNAi) comprises
426 non-coding RNA sequences that can interfere with gene expression (Meister and
427 Tuschl 2004). These mechanisms can possibly also be influenced by environmental

428 factors. Exposure of male rats to cyclophosphamide and mating them with non-exposed
429 dams resulted in hyperacetylated histones in the pronuclei early after fertilization,
430 which may lead to erroneous gene expression (Feil 2006). These kinds of effects are
431 more difficult to investigate and literature concerning histone modification or RNAi
432 after environmental stress is scarce.
433
434 In conclusion, this research has discovered DNA methylation in the genome of *D.*
435 *magna* at CpG sites. The sequencing of the *D. magna* genome currently being
436 performed at the Centre for Genomics and Bioinformatics (Indiana University,
437 Bloomington, USA) will allow localization of the methylated sequences in the *D.*
438 *magna* genome.
439 The methylated sites were detected in both Cd-exposed and non-exposed daphnids. No
440 extra fragments were detected in DNA of Cd-exposed *D. magna*. As such, it could not
441 be demonstrated that exposure to Cd altered DNA methylation. However, the fact that
442 methylated DNA is observed, shows that potentially epigenetic effects may occur in *D.*
443 *magna*. The role and consequences of epigenetics in environmental toxicity testing and
444 ecological risk assessment certainly needs further investigation.
445

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565 Figure 1. Illustration of (A) the original AIMS procedure with one DNA sample and sequential digestions with both HpaII and MspI
566 restriction enzymes and (B) the modified procedure with two DNA subsamples and the single digestion with of either HpaII (B1) or MspI
567 (B2)

B2

568
569 Figure 2 – Detail of the fingerprint of two DNA-samples from *D. magna*: 4d old
570 control juveniles (first two columns) and 4d old juveniles which have been exposed to
571 180 μg/L Cd for 2 generations (last two columns). HpaII = only HpaII digestion
572 performed, MspI = only MspI digestion performed. Arrows indicate bands that appear
573 in the samples on which MspI digestion is performed, but not in the samples on which
574 HpaII digestion is performed. These indicate fragments which are methylated at the
575 CCGG site.
576

577
578 Figure 3 – Intermethylated sequences DMMF1 and DMMF2 in *D. magna*, reamplified
579 from the polyacrylamide gel. The sites of the internal primers for the PCR used to
580 confirm the origin of the sequences in the *D. magna* genome are underlined.
581
582

583

584 Figure 4 – Results from the PCR performed to amplify the methylated sequences

585 (DMMF1 and DMMF2) from DNA of: (J) juvenile daphnids that have never been in

586 contact with algae, (Dm+A) daphnids that have been fed algae and (A) only the algae

587 that were used as food source during the culturing of the daphnids. M stands for

588 MassRuler^{TM} DNA Ladder Mix (Fermentas, St. Leon-Rot, Germany).

589

590 Figure 5 – Image of Southern blot, in which *D. magna* DNA that was digested with
591 either the methylation insensitive restriction enzyme MspI or the methylation sensitive
592 restriction enzyme HpaII was hybridized with ^{32}P-labeled DMMF1 (*Daphnia magna*
593 methylated fragment 1) and DMMF2 (*Daphnia magna* methylated fragment 2) DNA
594 probes. The scale denotes fragment length (number of nucleotides) as read from a Mass
595 Ruler low range DNA ladder (Fermentas, St. Leon-Rot, Germany). Arrows indicate
596 hybridized fragments in MspI lanes at the expected length of DMMF1 and DMMF2,
597 absent in the HpaII lanes, confirming the methylation of the fragments.
598

599 Table 1 - *D. magna* DNA sequences showing homology with the sequences coding for

600 DNA methyltransferases in *Homo sapiens*

DNMT of *Homo sapiens* (accession number)	Homologous *D. magna* sequence	E value	Similarity at the protein level
DNMT 1 (A0AV63)	`contig_885354`	0.0	72%
DNMT 2 (Q6ICS7)	`contig_1141364`	3e-24	75%
DNMT 3A (Q9Y6K1.4)	`contig_1436800`	1e-13	52%

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606 Table 2 – Overview of the number of discernible fragments that could be detected on
607 the sequencing gel and the number of fragments that were detected as
608 methylated for each primer with specific extended nucleotides.

Selective nucleotides	Number of detected fragments	Number of methylated fragments
-AA	35	0
-AC	48	0
-AG	134	0
-AT	9	0
-CA	3	0
-CC	98	0
-CG	58	0
-CT	93	0
-GA	77	0
-GC	82	0
-GG	53	2
-GT	14	0
-TA	28	0
-TC	85	0
-TG	18	0
-TT	64	0

609