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Environmental Science & Technology
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Bisulfite Sequencing with *Daphnia* Highlights a Role for Epigenetics in Regulating Stress Response to *Microcystis* through Preferential Differential Methylation of Serine and Threonine Amino Acids

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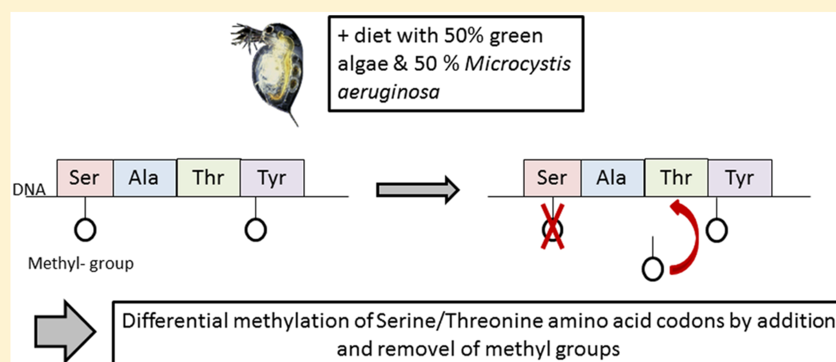
Supporting Information



ABSTRACT: Little is known about the influence that environmental stressors may have on genome-wide methylation patterns, and to what extent epigenetics may be involved in environmental stress response. Yet, studies of methylation patterns under stress could provide crucial insights on stress response and toxicity pathways. Here, we focus on genome-wide methylation patterns in the microcrustacean *Daphnia magna*, a model organism in ecotoxicology and risk assessment, exposed to the toxic cyanobacterium *Microcystis aeruginosa.* Bisulfite sequencing of exposed and control animals highlighted differential methylation patterns in *Daphnia* upon exposure to *Microcystis* primarily in exonic regions. These patterns are enriched for serine/threonine amino acid codons and genes related to protein synthesis, transport and degradation. Furthermore, we observed that genes with differential methylation corresponded well with genes susceptible to alternative splicing in response to *Microcystis* stress. Overall, our results suggest a complex mechanistic response in *Daphnia* characterized by interactions between DNA methylation and gene regulation mechanisms. These results underscore that DNA methylation is modulated by environmental stress and can also be an integral part of the toxicity response in our study species.

■ INTRODUCTION

The field of ecotoxicology and environmental risk assessment focus on understanding how organisms respond to environmental stressors, including anthropogenic and biotic stressors. Studying organismal response to environmental stressors requires a deep understanding of the molecular mechanisms involved. Of particular interest are epigenetic mechanisms, given that these induce additional phenotypic variability upon environmental stress exposure.[1,2] In the past five years, significant progress has been made in unraveling the role of methylation in invertebrates. Indeed, the increasing number of available invertebrate methylomes and meta-analyses of genome sequences have improved our understanding of DNA methylation.[3−8] Genome-wide differential methylation studies in invertebrates have primarily focused on differences among tissues, developmental stages, castes, or species.[3,5,9,10] DNA methylation in invertebrates seems to be primarily targeted to gene bodies in CpG context, while promoter or intergenic methylation and methylation in non CpG contexts are less frequently reported.[3,11,12] Although the role of DNA methylation in invertebrate responses to environmental

Received: August 3, 2016
Revised: November 21, 2016
Accepted: December 16, 2016
Published: December 16, 2016

ACS Publications © 2016 American Chemical Society

DOI: 10.1021/acs.est.6b03870
Environ. Sci. Technol. 2017, 51, 924−931

stressors is difficult to assess, preliminary studies suggest that alternative splicing, gene family size and gene functions play an integral part.[13,12,14] At present, existing studies lack sufficient resolution and depth to identify specific genes and mechanisms susceptible to changes in methylation upon environmental exposure.[15−20]

Here, we focus on genome-wide methylation patterns in the microcrustacean *Daphnia magna*, exposed to the toxic cyanobacterium *Microcystis aeruginosa* with the aim of identifying genes and molecular mechanisms responsible for toxicity response in *Daphnia*. *Daphnia* is a model organism in ecology, ecotoxicology and environmental risk assessment due to its ecological position, life cycle and physical properties.[21] Cyanobacteria in general, and *Microcystis* in particular, are known to have a poor nutritional quality compared to green algae, the common food source for *Daphnia*.[22−24] Feeding and life-history responses of *Daphnia* to *M. aeruginosa* are well documented and range from feeding inhibition to decreased reproduction and increased mortality.[25−27] We have previously observed significant changes in global methylation levels when *Daphnia* were fed with different diets including *Microcystis*.[19] In addition, gene expression studies have identified potential mechanisms involved in cyanobacterial toxicity.[28,29] Yet, it is unclear how *Microcystis* affects DNA methylation and to what extent changes in methylation may be involved in the mechanistic response to *Microcystis* stress. Here, we hypothesized that exposure to *Microcystis* would affect the methylation status of known genes involved in toxicity responses and that as such DNA methylation plays an active role in the stress response to *Microcystis* in *Daphnia*.

■ MATERIALS AND METHODS

Study Species. The strain Xinb3, used to generate the first genome assembly of *D. magna* (NCBI accession number: LRGB00000000[31]), was used to test methylation patterns associated with *Microcystis* exposure. The isolate was clonally propagated for at least 50 generations under standardized culture conditions in the lab prior to exposure to reduce the influence of maternal effects. Second broods were always used to establish the next generation. Animals were cultured in ADaM medium[32] under controlled conditions (20 ± 1 °C, 16 h:8 h light-dark cycle at a light intensity of 14 μmoles·m^{-2}·s^{-1}). They were fed daily with an algal mixture consisting of *Scenedesmus obliquus* and *Cryptomonas* sp. (SAG 26.80 http://sagdb.uni-goettingen.de) at a final concentration of 10^8 cells per liter in a 95:5 ratio on cell basis at a density of 20 animals per liter for the first 5 days and afterward 1.5×10^8 cells per liter in a 95:5 ratio on cell basis at a density of 10 animals per liter.

Experimental Setup. Neonates of the second brood (<24 h old) were isolated from the brood mother cultures of the Xinb3 strain and randomly placed in one of six 4L aquaria at a density of 20 animals per liter. All aquaria were kept under standardized laboratory conditions as described above. Half of these aquaria was fed a mixture of *S. obliquus* and *Cryptomonas* sp., whereas the other half a diet consisting of 50% of *M. aeruginosa*, with the same carbon content as 0.5×10^8 cells of the control diet, and 50% of the control diet, as described above, at a concentration of 0.5×10^8 cells per liter. All replicates were fed daily. Five days old animals were collected from the six aquaria, which will be hereafter referred to as biological replicates (i.e., three control aquaria or replicates and three treatment aquaria or replicates), for DNA extraction to capture early responses to *M. aeruginosa* treatment.

DNA Extraction, Library Construction, and Sequencing. DNA was extracted immediately from three biological replicates per treatment, each a pooled sample of all living individuals (at the same life stage), with the MasterPure kit (Epicenter, Madison, WI). Given the short exposure time and low exposure to *Microcystis*, mortality rate in all aquaria was lower than 10%. Library preparation and sequencing was executed by the BGI Sequencing Facility in Hong Kong. Next, the extracted DNA was fragmented by sonication to a mean size of approximately 300 bp. Library construction for pair-end sequencing of 90 bp reads consisted of blunt ending and 3′-end addition of dA, Illumina methylated adapters (Illumina, San Diego, CA) and was executed according to the manufacturer's instructions for the biological samples for bisulfite sequencing. The bisulfite conversion (C → U) was carried out after library preparation with the EZ DNA methylation Gold kit (Zymo Research, Irvine, CA) following manufacturer's instructions. All samples were sequenced using Illumina HiSeq-2000 (Illumina, San Diego, CA). Unmethylated lambda DNA (5 ng per ug of DNA sample) was added during the bisulfite conversion to assess the bisulfite conversion error rate. The conversion error rate, reported in Supporting Information (SI) Table S1, was defined as the percentage of reads yielding a methylation call after mapping to the unmethylated lambda phage control DNA. Sequencing data were processed with Illumina base-calling software (version 1.5), resulting in 90 bp reads. All data have been deposited to NCBI GEO under Series GSE83407.

Quality Assessment, Preprocessing, and Mapping. Read quality was assessed with the FastQC software (Babraham Institute, Cambridge, UK, version 0.11.5). Reads containing more than 5% N bases were omitted. Adapter sequences were trimmed from the reads using Trim Galore version 0.3.2 (Babraham Institute, Cambridge, UK). Next, reads were dynamically trimmed to the longest stretch of bases with a Phred score of at least 30 (i.e., at least 99.9% base-call accuracy). Trimmed reads were transformed in fully bisulfite converted forward and reverse reads. Reads were mapped against a fully bisulfite converted version of the genome (i.e., C → T and G → A converted) with Bowtie2 version 2.1.0[33] with the following scoring function: --score_min L,0,-0.6. PCR duplicates that might have occurred were removed with the Bismark function *deduplicate*.[34] The reference genome assembly v2.4, developed using the same *Daphnia* strain, was used for mapping (Bioproject PRJNA298946 in NCBI,[35]).

Methylation Levels. The methylation state per cytosine per read was inferred using Bismark version 0.9.0.[34] Only cytosines with at least 5× coverage in all three biological replicates were retained for further downstream analyses. Following general practices in literature,[3,5] a binomial testing procedure with multiple-testing correction was used to distinguish truly methylated cytosines from false positives using the calculated bisulfite conversion error rate for each replicate. Following,[3] all cytosines were assigned a methylation ratio defined by the number of methylated reads at the cytosine site divided by the total number of reads at the cytosine site.

Gene Body Methylation Levels. We used the gene models of *D. magna* described by,[35] developed for the same isolate as used here and available at http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/earlyaccess/. We only used genomic loci supported by the hybrid gene set generated using Evidential Gene,[35] denoted by dmag7finlm in the gff file and discarded all artificial or culled transcripts, as even though some of these might be valid loci,

DOI: 10.1021/acs.est.6b03870
Environ. Sci. Technol. 2017, 51, 924−931

insufficient evidence is available at this point. Cytosine-specific methylation levels for each gene were obtained by overlapping these gene models through BEDtools 2.17.0[36] with cytosine specific methylation levels as determined above. The methylation level of a gene body was calculated as the sum of all methylation ratios of the cytosines within the gene body divided by the number of cytosines in the gene body.[3] Methylation levels of exons and introns were determined similarly. To determine the methylation pattern across the entire gene, exons and introns were divided in 20 bins for which the methylation level was calculated. Again the methylation level per bin was defined as the sum of all methylation ratios of the cytosines within the bin divided by the number of cytosines in the bin. By using bins, all exons and introns are divided in the same number of bins despite the differences in length even though the size of the bin may differ (e.g., an exon of 500 bp has 20 bins of each 25 bp whereas an exon of 250 bp has 20 bins of each 10 bp.)

Promoter Methylation Levels. We studied the methylation in putative promoter regions, defined as 1500 basepairs upstream of the first exon or until the end of the previous gene model. Promoter methylation levels were determined similarly to the gene body methylation levels.

Differential Methylation Analysis. We determined differential methylation between treatments for each gene and promoter as well as for each individual cytosine by using a nonparametric t test at a significance level of $\alpha = 0.05$. We used a nonparametric test because the small sample size (i.e., two treatment groups with each three replicates) did not adhere to normality requirements (function *mt.teststat* in R, version 3.3.2). For each genomic feature (i.e., gene, promoter or cytosine), the null hypothesis was that there was no difference in methylation level between each two treatment groups. First, all features with zero methylation across the two treatments, that is, no methylated reads detected for any cytosine in the feature in any of the three biological replicates, were removed from the list. Second, we determined the differential methylation between the two treatments for each genomic feature with the nonparametric test. Then, p-values were adjusted using the Benjamini−Hochberg correction.[3] Next, for all genes and promoters with significant differential methylation between the two treatments, we tested for differential methylation for each cytosine within these genes or promoters.

Functional Analyses. Over- and underrepresentation analyses of annotation definitions within significantly differentially methylated genes and promoters consisted of Fishers-exact tests combined with Benjamini-Hochberg multiple testing correction. In addition, the respective amino acids codons were extracted for each differentially methylated cytosine position in a gene. The observed proportion of each amino acid within differentially methylated cytosines was compared with the proportion of amino acids in the genome by bootstrapping. Bootstrapping was used to assess the chance of observing the proportion of each amino acid by randomly selecting n amino acids (n equals the amount of significant codons) from all possible amino acid codons based on the proportion of amino acids in the genome and repeating the procedure 1000 times. This procedure generated a distribution for each amino acid, in which observed values lower than the 0.5% percentile were determined significantly lower than expected by chance and values higher than the 99.5% percentile were determined significantly higher than expected by chance. Seven amino acids were excluded from the analysis as they did not contain any CpG sites within their codons, that is, arginine, glutamine, glutamic acid, lysine, methionine, tryptophan, and the stop codon.

Analysis of Gene Expression Data for Alternative Splicing. Publically available RNA seq data of a similar experiment with the same *Daphnia* isolate and the same *Microcystis* strain conducted by Orsini et al.[35] was used. Specifically, the following four samples from the SRA archive SRX1057343, SRX1057342, SRX1056706, SRX1056709 were analyzed. The reads were mapped to the mRNA sequences of genes with significant differential methylation as defined above, known to have alternative splice variants. Genes with known alternative splice variants were extracted from the appropriate gff annotation file (dmagset7finalt9b.puban.mrna) at http://arthropods.eugenes.org/EvidentialGene/daphnia/daphnia_magna/Genes/earlyaccess/. Then, read counts per exon were analyzed in EdgeR according to[37] to identify alternative splicing events by testing for differential exon usage for each gene. The function diffSpliceDGE was used to test whether the log-fold changes differ between exons for the same gene. If such differences occur, it indicates alternative splicing events at a false discovery rate of 0.01.

RESULTS

Differential Methylation in Gene Bodies and Promoter Regions. We studied differential methylation in 21 870 gene models (75% of the total gene set reported by[35] as not all transcripts could be reliably mapped to genomic loci) all of which met all quality and coverage standards in both treatments as described in the Materials and Methods section. We observed 12 201 genes and 13 509 putative promoter regions

Table 1. List of All Genes with Significant Differential Methylation in the Promotor and the Gene[a]

GeneID	differential ML promotor	differential ML gene	annotation
Dapma7bEVm002017t1	0.0045	−0.0090	39S ribosomal protein L2, mitochondrial
Dapma7bEVm009522t1	0.0379	0.0017	ATP-dependent RNA helicase DDX4
Dapma7bEVm011744t1	−0.0069	0.0121	conserved protein
Dapma7bEVm008227t1	0.0698	−0.0074	HD domain-containing protein
Dapma7bEVm023350t1	0.0117	0.0184	Kinase C eta type
Dapma7bEVm007978t1	−0.0346	0.0041	microprocessor complex subunit DGCR8
Dapma7bEVm010354t1	−0.0122	−0.0040	mitogen-activated protein kinase kinase kinase
Dapma7bEVm005980t1	−0.0231	0.0133	myeloid differentiation primary response protein MyD88
Dapma7bEVm001156t1	−0.0466	−0.0058	myotubularin-related protein
Dapma7bEVm006630t1	−0.1165	−0.0047	transmembrane emp24 domain-containing protein
Dapma7bEVm007550t1	−0.1469	−0.0059	transmembrane protein 17A
Dapma7bEVm011289t1	0.1063	0.0256	uncharacterized protein
Dapma7bEVm008095t1	−0.0039	−0.0178	uncharacterized protein
Dapma7bEVm007389t1	−0.0572	0.0034	uncharacterized protein
Dapma7bEVm004499t1	0.1265	−0.0011	virilizer
Dapma7bEVm001891t1	−0.0107	−0.0248	zinc transporter ZIP14

[a]Differential methylation levels (ML) are expressed as ML_{Ctr}-ML_{MC}.

DOI: 10.1021/acs.est.6b03870
Environ. Sci. Technol. 2017, 51, 924−931

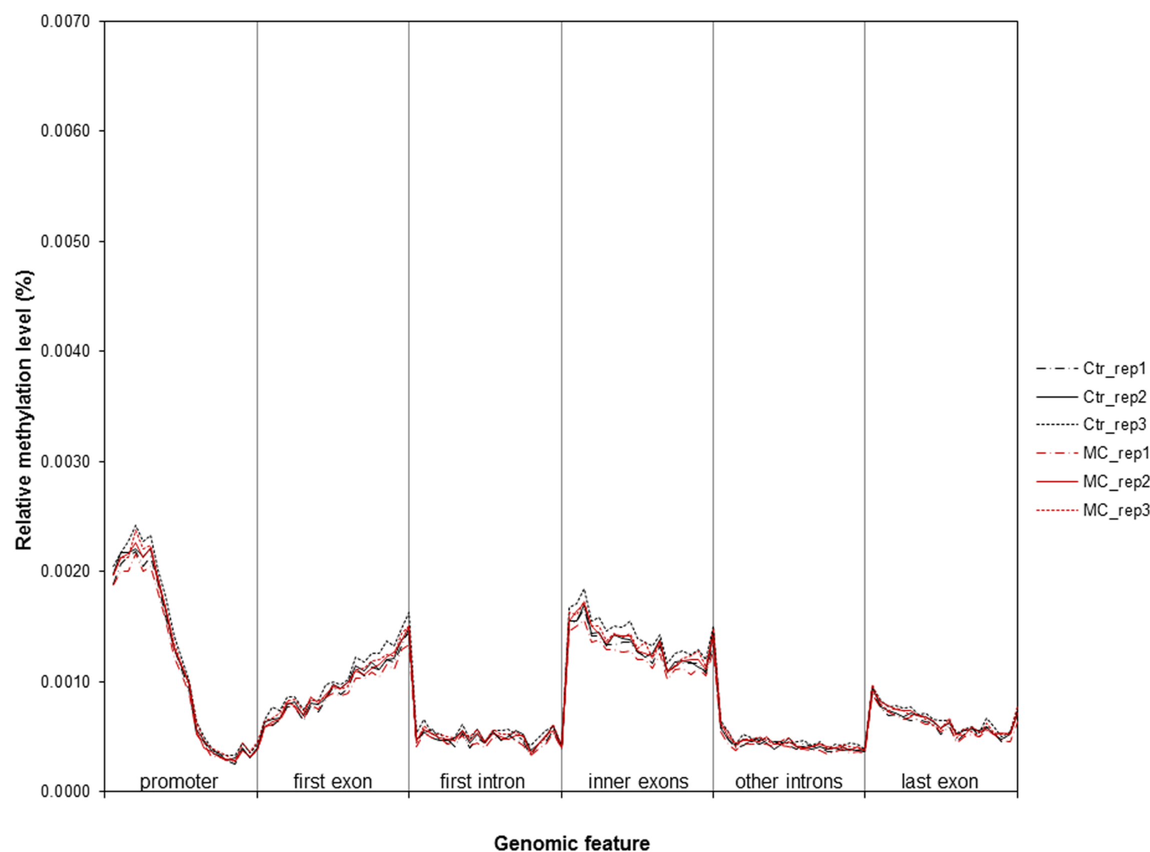


Figure 1. Relative methylation levels per treatment (Ctr = control in black and MC = *Microcystis* in red) for each replicate for all 21 870 available gene models across their genomic features. Promoters were defined as putative promoter regions 1500 basepairs upstream of the first exon. Different lines represent the different biological replicates.

with zero methylation, that is, not a single methylated read was detected for any cytosine within the gene or promoter in any of the three biological replicates, in both treatments. In contrast, 876 genes and 247 putative promoter regions were significantly differentially methylated (q-value <0.05) between the two treatments (SI Tables S2−S3). The distribution of differences in gene body methylation levels, i.e. the methylation level of the control treatment minus the methylation level of the *Microcystis* treatment, varied between −0.4 and 0.2; 490 genes had significantly higher methylation in the *Microcystis* treatment whereas 386 genes had significantly higher methylation levels in the control treatment. The distribution of differences in methylation levels in putative promoter regions varied between −0.25 and 0.16; 144 putative promoter regions had significantly higher methylation in the *Microcystis* treatment whereas 103 putative promoter regions had significantly methylation in the control treatment. When observing the relative change in methylation level (i.e., the difference in methylation level between the two treatments divided by the methylation level in the control treatment) about 50% of the genes showed a relative change of 10% or more with a small subset of genes (39) showing a 2-fold to 8-fold difference. A quarter of the genes with significant methylation showed a change of less than 5% in their methylation level. The relative changes in the putative promoter regions were larger with 79% of the promoters showing a relative change of 10% or more and 6 promoters having a 2-fold to 5-fold difference in methylation. Only 14 putative promoter regions showed a change of less than 5% in their differential methylation level. Sixteen genes showed significant differential methylation in both their gene body as well as their putative promoter region (Table 1).

Methylation Patterns Across Different Genomic Features. We compared methylation patterns across genomic features (i.e., promoters, exons, introns) for all genes between the two treatments (Figure 1) and for the significantly differentially methylated genes only (Figure 2). Overall, we observed the same pattern across all genes for the two treatments (Figure 1), in which promoter methylation decreases with decreasing distance to the first exon. Across the gene body, we observed an increase in methylation from the start of the first exon to a peak in the inner exons which decreased again in the last exon. Introns have lower methylation levels and seem to serve as breaking points. When taking into account only the 876 significant genes (Figure 2), the methylation levels are much higher and much more variable across the promoter and gene body. Even though the general pattern remains the same, large peaks of methylation can be observed in the promoter region, at the end of the first exon and at the end of the first intron. Overall, the intron−exon boundaries are less clear between the first intron and inner exons.

Functional Analysis of Genes and Promoters with Significant Differential Methylation. We also studied the functions of differentially methylated genes and the functions of genes corresponding to the differentially methylated putative promoter regions. We found six gene families out of 40 gene families with at least 1 differentially methylated gene, that were significantly enriched for differentially methylated genes, including ribosomal proteins, Rab family proteins and E3

DOI: 10.1021/acs.est.6b03870
Environ. Sci. Technol. 2017, 51, 924−931

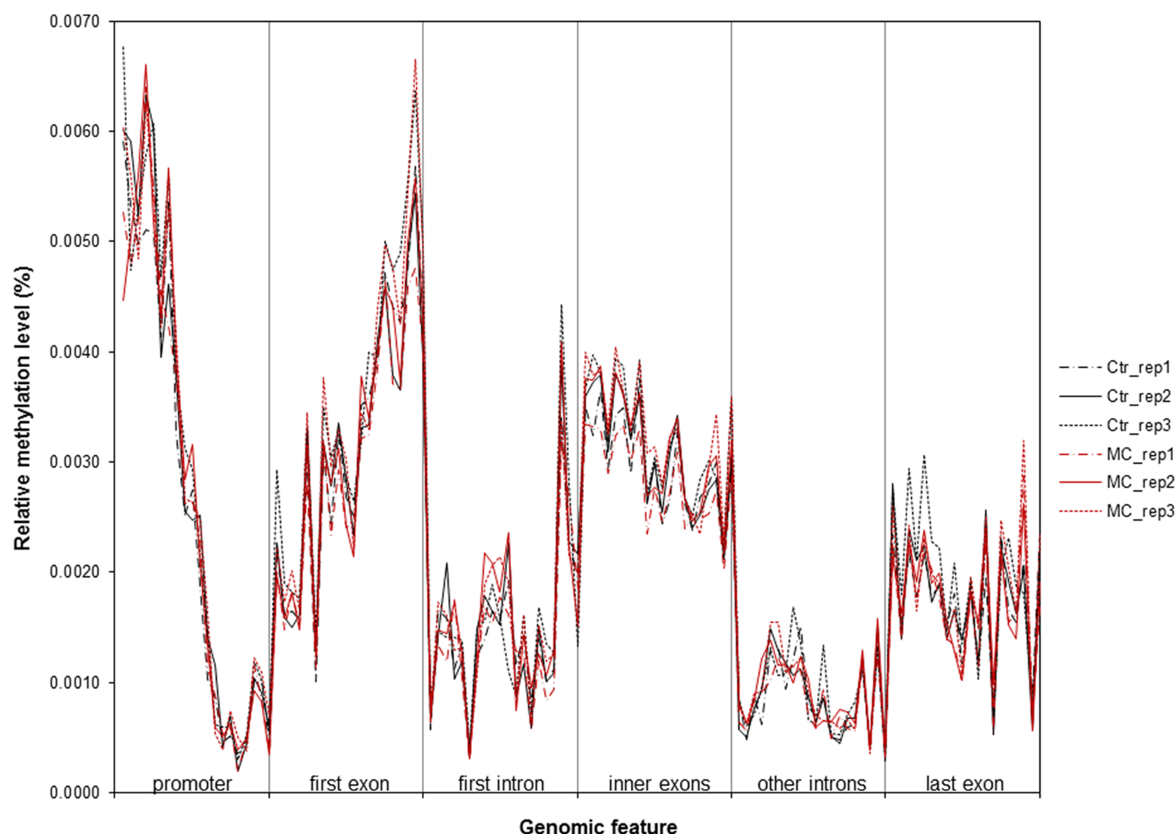


Figure 2. Relative methylation levels per treatment (Ctr = control in black and MC = *Microcystis* in red) for each replicate for all 876 genes which were significantly differentially methylation, across their genomic features (SI Table S2). Promoters were defined as putative promoter regions 1500 basepairs upstream of the first exon. Different lines represent the different biological replicates.

Table 2. Overrepresented Gene Families within the Genes with Significant Differential Methylation, Their Size within the Genome and the Corresponding Benjamini−Hochberg Correct *P*-Value

annotation definition	significant genes	family size in genome	*p*-value
transmembrane protein	8 (11%)	74	0.017
Ras-related protein Rab	5 (15%)	33	0.017
Ribosomal proteins	14 (8%)	174	0.019
integrator complex subunit	3 (25%)	12	0.020
nuclear pore complex protein	3 (18%)	17	0.044
E3 ubiquitin-protein ligase	8 (9%)	93	0.049
all genes	876 (4%)	21 870	

ubiquitin ligases (Table 2). We did not observe any enrichment of specific gene families for differentially methylated putative promoter regions.

Differential Methylation of Cytosines in Amino Acid Codons. Next, we studied whether differentially methylated cytosines in genes with significant differences in methylation were occurring more in specific amino acids than in others. We observed a significantly higher proportion of serine and threonine codons with differentially methylated cytosines than expected by chance (at the 1% confidence interval), while we observed less aspartic acid, glycine and leucine codons with differentially methylated cytosines than expected by chance (Table 3). Differentially methylated cytosines primarily occurred in exonic regions (78%) but we did not observe any significant differences between first, inner and last exons or the distance relative to the transcription start site (SI Table S4). Less than 10% of the differentially methylated cytosines were followed by a potential alternative stop codon.

Table 3. Observed Proportion (Relative to 1) of Each Amino Acid among the 303 Codons and the Corresponding Lower and Upper Limit at the 99% Confidence Interval (CI) after Random Bootstrapping[a]

amino acid	observed proportion	lower limit CI	upper limit CI
alanine	0.081	0.040	0.129
asparagine	0.063	0.026	0.092
aspartic acid	***0.025***	***0.033***	***0.112***
cysteine	0.020	0.007	0.056
glycine	***0.007***	***0.043***	***0.129***
histidine	0.023	0.016	0.072
isoleucine	0.038	0.033	0.106
leucine	***0.045***	***0.073***	***0.182***
phenylalanine	0.079	0.026	0.092
proline	0.097	0.036	0.106
serine	***0.256***	***0.066***	***0.168***
threonine	***0.188***	***0.046***	***0.122***
tyrosine	0.095	0.046	0.129
valine	0.033	0.013	0.076

[a]Amino acids in bold italic have a proportion significantly lower or higher than expected by chance.

Significant Differential Methylation and Alternative Splicing. Out of the 876 significantly differentially methylation genes, 371 genes were identified with known splice variants and could be studied for potential alternative splicing events. We

DOI: 10.1021/acs.est.6b03870
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observed 292 genes out the 371 genes with known splice variants, that showed differential exon usage between the two treatments, indicating alternative splicing events and expression differences (SI Table S5).

■ DISCUSSION

The increasing number of available invertebrate methylomes and meta-analyses of genome sequences have improved our understanding of the role of DNA methylation in this important taxonomic group.[3,4,6,8,7,13] Yet, little is known on how environmental stressors influence genome-wide methylation patterns as differential methylation studies in invertebrates have primarily focused on differences among tissues, developmental stages, castes, or species.[3,5,10]

Here, we report differential methylation in both promoter and gene bodies upon exposure to *Microcystis* with methylation primarily occurring in exonic regions as reported for other invertebrates.[3,9,11] We specifically observed enrichment of several gene families involved in protein regulation among differentially methylated genes. Furthermore, these gene families have been reported earlier as being differentially expressed in the literature in response to cyanobacterial exposure: ribosomal proteins, and Rab family proteins. Ribosomal proteins are differentially expressed upon exposure to *M. aeruginosa* related to the effects on protein synthesis in *Daphnia*.[28] In addition, E3 ubiquitin ligases, involved in protein degradation, and Ras related proteins of the Rab family, involved in protein transport, were also enriched in differentially methylated genes. Proteins of the Rab family were differentially expressed in zebrafish upon exposure to *M. aeruginosa*.[43]

These results suggest that *Daphnia* have to invest energy and resources to deal with protein regulation and potentially misfolded proteins under *Microcystis* stress, and that less energy is available for reproduction and growth. This shift in resource allocation may contribute to the reduced reproduction and growth observed under *Microcystis* stress.[25] In addition, the additional proteins may be produced to cope with the toxins of *Microcystis* or to cope with the different nutritional composition of *Microcystis*, which support previous findings in the literature.[23,28,30]

At the cytosine level, we observed enrichment of serine and threonine codons among differentially methylated cytosines. We therefore speculate that methylation of cytosines within serine and threonine codons may lead to alternative splicing mechanisms resulting in the expression of alternative or less sensitive proteins that are not affected by these phosphatase or protease inhibitors, i.e. the activity of these protease is less inhibited by the phosphatase or protease inhibitors produced by the cyanobacteria.[29] Indeed, our analysis of RNaseq data revealed that serine/threonine protein kinases were not only differentially methylated but also significantly spliced between treatments.

Methylation as a mechanism for regulating alternative splicing has been suggested in a number of invertebrate models.[11,13] Here, we put forward the hypothesis that the expression of new or alternate less sensitive proteins may be a consequence of alternative splicing and gene regulation mechanisms moderated by DNA methylation, in particular differential methylation of amino acid codons. Gene expression studies in different *Daphnia* species have observed the expression of less sensitive serine proteases and the alternate regulation of paralogous gene families in response to cyanobacterial stress.[29,38] Furthermore, tolerance to *Microcystis* in different *Daphnia* genotypes has been attributed to a differential sensitivity to protease inhibitors.[30] Thus, methylation may potentially play a role in adaptation to environmental stress by inducing polymorphisms and genetic variation, similar to what has been reported in host-parasite interactions.[39] In a similar manner, methylation may also play a role in microevolutionary effects as manifested changes in methylation upon exposure to environmental stress that can be passed on to subsequent generations. For example, cyanobacteria have been reported to potentially affect the genetic population structure of *Daphnia*, and in particular of protease genes, through microevolutionary effects, which could, based on our findings, potentially be regulated by DNA methylation.[40]

In addition, serine kinases and serine/threonine phosphatases, both known to be inhibited by microcystins, are known to be involved in the activities and localization of splicing factors within the cell, including serine/arginine rich splicing factors.[39−41] In addition, serine/threonine protein kinases are crucial in stress response as they help regulate of protein folding and help organisms cope with potentially misfolded proteins.[42]

Alternatively, preferential differential methylation of these codons could also be related to other gene regulation mechanisms through the adjustment of DNA accessibility as serine and threonine amino acids contribute to a large number of protein−DNA interactions while other amino acids including leucine and glycine are less likely to contribute to protein−DNA interactions.[5,44]

Overall, our results suggest a complex mechanistic response in *Daphnia* characterized by interactions between DNA methylation and gene regulation mechanisms. Even though the exposure was relatively short, only 4 days, the results correspond well with previous gene expression studies that detail longer exposures to *Microcystis*.[28−30] Furthermore, the overall gene expression patterns across genomic features are similar to reports in other invertebrates[4,11,45] and adult *Daphnia*.[14] At this point, it remains unclear to what extent these epigenetic changes could extend to future generations. The available literature does report on maternal effects of inducible tolerance against *Microcystis* in *Daphnia*,[46−48] which could be regulated through DNA methylation, but at present this connection remains unclear. So while the current study clearly demonstrates that DNA methylation modulated by environmental stress and can play an important role in the toxicity response by affecting gene regulation mechanisms, the potential effects on subsequent generations of unexposed offspring remain to be elucidated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b03870.

Table S1−S5 and is available free of charge on the ACS Publication Web site. Tables report the bisulfite conversion error for all samples (S1), differentially methylated genes (S2), differentially methylated promoters (S3), differentially methylated cytosines (S4) and genes with differential methylation and differential codon usage (S5). Raw sequencing data has been deposited at GEO: **Series GSE83407** (PDF)
(XLSX)

DOI: 10.1021/acs.est.6b03870
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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Jolien Depecker for performing the DNA extractions. Jana Asselman is a postdoctoral fellow of the FWO. Funding was received from the Research Foundation Flanders (FWO Project G.0614.11), from BELSPO (AquaStress project: BELSPO IAP Project P7/31), from KU Leuven research fund (xxxCenter of excellence SEEDSxxx). This research contributes to and benefits from the *Daphnia* Genomics Consortium.

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DOI: 10.1021/acs.est.6b03870
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