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1 **Mercury-methylating bacteria are associated with zooplankton: a proof-of-principle**
2 **survey in the Baltic Sea**

3

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7

8 **Abstract**

9 Methylmercury (MeHg) is a potent neurotoxin that biomagnifies in marine food-webs.
10 Inorganic mercury (Hg) methylation is generally considered to be conducted by bacteria
11 associated with sediment or detritus, but endogenous methylation by the gut microbiome of
12 animals in the lower food webs is another possible source. We examined the occurrence of the
13 bacterial gene (*hgcA*), required for Hg methylation, in the guts of dominant Baltic
14 zooplankters. A qPCR assay targeting the *hgcA* sequence in three main clades
15 (*Deltaproteobacteria*, *Firmicutes* and *Archaea*) was used in the field-collected specimens of
16 copepods (*Acartia bifilosa*, *Eurytemora affinis*, *Pseudocalanus acuspes* and *Limnocalanus*
17 *macrurus*) and cladocerans (*Bosmina coregoni maritima* and *Cercopagis pengoi*). All
18 copepods were found to carry *hgcA* genes in their gut microbiome, whereas no positive
19 amplification was recorded in the cladocerans. In the copepods, *hgcA* genes belonging to only
20 *Deltaproteobacteria* and *Firmicutes* were detected. These findings suggest that endogenous
21 Hg methylation can occur in zooplankton and may contribute to seasonal, spatial and vertical
22 MeHg variability in water column and food webs. Additional molecular and metagenomics
23 studies are needed to identify bacteria carrying *hgcA* genes and improve their quantification in
24 microbiota.

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25 **INTRODUCTION**

26 Mercury (Hg) is a global pollutant adversely affecting human and wildlife health due to its
27 toxicity and distribution in the environment.[1] Various processes, both natural and
28 anthropogenic, lead to the release of primarily inorganic Hg (IHg), which can undergo
29 methylation resulting in formation of neurotoxic monomethylmercury (MeHg). While both IHg
30 and MeHg can be taken up by biota, only MeHg bioaccumulates in aquatic food webs.[1,2]

31 The primary pathway for MeHg production is microbial Hg methylation,[3] and a bacterial gene
32 cluster associated with such methylation (*hgcAB*) has recently been discovered.[4,5] It was
33 previously thought that mainly sulfate-(SRB) and iron-(FeRB) reducing bacteria methylate Hg
34 in anoxic conditions.[6–8] However, the *hgcAB* gene cluster has been identified in a variety of
35 methanogens and syntrophic, acetogenic, and fermentative *Firmicutes* indicating a broader
36 phylogenetic representation of Hg methylators.[9] Recently, clade-specific quantitative PCR
37 (qPCR) assays were developed to quantify the abundance of *hgcA* gene of the main
38 methylators.[10] Hence, *hgcAB* and *hgcA* distribution can be used to predict occurrence of
39 potential Hg methylators in the environment[11]; moreover, the association between abundance
40 of *hgcA* and the rate of mercury methylation were found to be strong and present in different
41 environments.[10] Thus, understanding *hgcAB* and *hgcA* distribution is essential for estimating
42 MeHg production in the water column and biomagnification in food webs.[12]

43 Worldwide, great differences in MeHg accumulation have been reported for similarly structured
44 and geographically close food webs.[2,12] In aquatic environments, MeHg production takes place
45 in both sediment and water column;[12,13] however, in the oxygenated waters, Hg methylation
46 may occur in anoxic microenvironments on sinking organic matter.[8] In pelagia, MeHg,
47 bioconcentrated from the water column by phytoplankton, enters the food web via zooplankton
48 grazing, with subsequent transfer of zooplankton-associated MeHg to zooplanktivores.[12,14] An
49 additional source of MeHg and a possible contributor to the variability in food-web
50 bioaccumulation could be endogenous Hg methylation by gastrointestinal microbiota[4,15] with
51 subsequent MeHg uptake by the host. Therefore, endogenous Hg methylation in primary
52 consumers could constitute an unexplored MeHg source with consequences for higher trophic
53 levels. Exploring the Hg methylation capacity of gut microbiota has been attempted in various
54 animals using both analytical and molecular approaches.[15] While the gene cluster *hgcAB* has
55 been identified in the gut microbiome of some terrestrial arthropods,[4,15] its status in aquatic
56 invertebrates is so far unknown.

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57 In the Baltic Sea, Hg sources are historically high, due to both natural and anthropogenic
58 inputs,[16] which should promote Hg methylation ability in microorganisms[3] and facilitate
59 establishment of methylators in microbiota of filter-feeders, such as zooplankton. Here, we
60 report that the *hgcA* gene is present in the microbiome of Baltic copepods; this observation
61 represents the first record of potential methylators associated with zooplankton. Our findings
62 imply that endogenous Hg methylation can occur in primary consumers as a pathway by which
63 MeHg can enter the food webs.

64

65 **EXPERIMENTAL**

66 **Field zooplankton collections and sample preparation**

67 Zooplankton were collected at four coastal and open sea stations of the northern Baltic Proper
68 and the Bothnian Sea (Table 1, Figure S1). We focused on microcrustaceans, cladocerans and
69 copepods, which are the major groups of mesozooplankton in the Baltic Sea. These
70 microscopic animals are largely herbivorous, with parthenogenic cladocerans thriving in the
71 mixing layer and reproducing mostly during summer, whereas copepods usually reside at
72 deeper layers performing vertical migrations related to onthogeny, temperature and predation
73 risk.[17]

74 Animals retrieved from the cod-end were placed in 0.2-µm filtered aerated seawater and
75 supplied with an excess of the cryptophyte *Rhodomonas salina* (strain CCAP 978/24) to clear
76 the guts of any potential hgCA-containing microorganisms associated with their food items
77 and only retain those microbes closely associated with the gut mucosa. This procedure was
78 applied to all species except *Cercopagis pengoi*, a predatory onychopod, feeding by
79 puncturing exoskeleton of planktonic crustaceans and sucking soft body tissues.[18] Such
80 feeding mode leaves the chitinous gut of the prey intact in the discarded carcass, hence, the
81 contamination of the predator gut with prey microflora was considered unlikely, and *C.*
82 *pengoi* were not subjected to the gut clearance procedure. For the rest of the zooplankton,
83 randomly selected individuals with visibly reddish guts (indicating that the animals were
84 active and feeding during the incubation) were selected following two-hour incubation. All
85 specimens were preserved in groups using RNA*later* and stored at –20°C.[19]

86 From the RNA*later*-preserved samples, different species of copepods and cladocerans were
87 picked under a dissecting microscope with forceps, rinsed in artificial seawater, and

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88 transferred in groups (30-50 ind. sample^{-1}) into Eppendorf tubes. The following species and
89 developmental stages were selected for the analysis: (1) copepodites (CV–VI) of *Acartia*
90 *bifilosa* and *Eurytemora affinis*; these are small calanoids, dominant in the study area and
91 present all year round, mostly in the epipelagia; (2) copepodites (CIII-IV) of *Limnocalanus*
92 *macrurus* and *Pseudocalanus acuspes*; these are large calanooids, dominant zooplankton
93 below the halocline in the Northern Baltic, and important prey for zooplanktivores; (3)
94 cladoceran *Bosmina coregoni maritima* (females, >0.7 mm); a small zooplankter, often
95 reaching high abundance in the surface waters during summer and being occasionally
96 important prey for zooplanktivorous fish, and (4) cladoceran *Cercopagis pengoi* (Barb Stages
97 II and III); a large predatory zooplankter representing a secondary consumers a common prey
98 for fish during summer. Thus, except for *C. pengoi*, all analyzed species are primary
99 consumers and dominant species in the pelagic food web.

100 Reference samples used as a contamination control were hatched *Artemia* spp. nauplii (San
101 Francisco Bay Brand) grown on axenic culture of *R. salina* (5×10^4 cells mL^{-1}) in artificial
102 seawater (28 g L^{-1} of Instant Ocean synthetic sea salt; Aquarium Systems Inc., Sarrebourg,
103 France). The animals were sacrificed after reaching a body length of ~2 mm and treated in the
104 same way as the zooplankton samples. As no positive amplification was ever produced in the
105 reference samples with *Artemia* guts (3 replicates, 25 guts sample^{-1}), we consider bacterial
106 contamination during sample preparation to be either negligible or non-existent.

107

108 **DNA extraction**

109 From each specimen, the gut was excised with a sharp needle, a pair of ultrafine forceps and a
110 dissecting microscope; the instrumentation and glassware were sterile. In total, 36 samples,
111 25-50 guts sample^{-1}, were prepared (Table 1). The guts were transferred into 1.5 mL
112 centrifuge tubes for Chelex-based DNA extraction[20] following a protocol developed for
113 analysis of prokaryotes in zooplankton.[21] See Supporting Information for details and Table S1
114 for DNA yield in different species.

115

116 **qPCR assay**

117 Three main clades were considered as potential *hgcA*-targets, *Deltaproteobacteria*,
118 *Firmicutes*, and *Archaea*. For each clade, a separate qPCR assay was performed using a clade-
119 specific protocol of Christensen and co-workers.[10] As a standard, a synthetic DNA

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120 oligonucleotide[22] comprising the clade-specific target sequence was constructed using a
121 representative strain: *Dv. desulfuricans*, *Df. metallireducens*, and *Ml. hollandica*, for
122 *Deltaproteobacteria*, *Firmicutes*, and *Archaea*, respectively (Tables S2-S3). The standards
123 were cloned into plasmids and applied in five-step tenfold serial dilutions, 1.5×10^6 to 1.5×10^2
124 apparent copies of target DNA per reaction (Table S4, Figure S2). The qPCR primers and
125 amplification conditions[10] (Tables S3 and S4) were used for all test samples, reference
126 samples, NTC and standards. Under these conditions, qPCR yielded a single product in each
127 standard and in the test samples within an assay (Figure S3). No product was produced in the
128 reference samples and NTC (non-template control) within the assay range (30 cycles).

129

130 **Data analysis**

131 The number of *hgcA* copies detected by qPCR was used to calculate the number of *hgcA* copies
132 per individual and per μg of zooplankter wet weight (i.e., weight-specific number of Hg
133 methylators); individual zooplankter weights[23] were used for these calculations. Due to
134 substantial variations in the amplification efficiency and detection limits for these qPCR assays
135 among different bacterial strains (efficiency: 60 to 90%, detection limits: 10^2 to 10^6 *hgcA*
136 copies)[10], any statistical comparisons between species/sites were not meaningful[24]. Therefore,
137 we consider our results largely descriptive, indicative of the presence/absence of *hgcA* and, to
138 a lesser extent, of the interspecific or geographical variation.

139

140 **RESULTS AND DISCUSSION**

141 All four copepod species were tested positive for *hgcA* genes (Figure 1), whereas no positive
142 amplification was observed for the two cladocerans. Among the clades tested, the *hgcA* genes
143 of only *Deltaproteobacteria* and *Firmicutes*, but not *Archaea*, were found in the copepod guts.
144 Although there was a substantial imbalance in the sampling effort between copepods and
145 cladocerans (25 vs. 8 samples; Table 1), the occurrence of *hgcA*-positive samples for copepods
146 only is suggestive of a difference. However, the between-clade differences given the variability
147 in the limit of quantification between *Archaea* and the other two clades, and, to a lesser extent,
148 between *Deltaproteobacteria* and *Firmicutes* (Table S4) should be treated as indicative.
149 Moreover, although Archaea are commonly reported to occur in zooplankton guts,[25] the
150 contribution of this group can be low compared to bacteria.[26] This may have contributed to the
151 lack of *hgcA*-positive amplification. Also, considering the variability in the amplification

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152 efficiency among bacteria[10] and unknown composition of the *hgcA*-positive microbiota, only
153 rough interpopulation comparisons are possible. However, the overall findings suggest that
154 microbiota of zooplankters carries *hgcA* genes and thus may be capable of Hg methylation.

155 Whether bacteria-driven Hg methylation in zooplankton guts takes place depends not only on
156 the occurrence of *hgcA*-carrying bacteria but also on the functional performance of these
157 bacteria. To assess the *hgcA* expression, an analytical effort is required using available
158 molecular tools, such as RT-qPCR, RNA sequencing, and RNA-SIP. Furthermore, a better
159 understanding of community structure is needed. Although our results do not provide any
160 taxonomic identification of the bacteria involved, the observed prevalence of *Firmicutes* among
161 the *hgcA*-carriers (Figure 1) agrees well with a relatively high abundance of this bacterial group
162 in the microbiome of other copepods.[27–29] In future studies, a 16S rRNA gene diversity profiling
163 and *hgcAB* amplification with high-throughput sequencing should be combined with *hgcA*
164 quantification.[11] Broad-scale zooplankton sampling, including seasonal, spatial and vertical
165 coverage, should provide material for such an evaluation.

166 If gut Hg methylation occurs, zooplankton may serve as a primary MeHg entrance point of
167 global significance and affect variability in MeHg transfer to secondary consumers.[3] A mass-
168 balance budget for the herbivourous marine copepod *Calanus hyperboreus* suggested that
169 endogenous Hg methylation could account for up to 70% of the annual MeHg uptake in this
170 copepod.[30] If these estimates are correct, they might explain why reported drivers of MeHg
171 variability are often contradictory. Indeed, MeHg concentrations in herbivorous zooplankton
172 vary among taxa,[31,32] demographic population structure[33] and growth stoichiometry.[34] In wild
173 populations, however, these factors are difficult to disentangle,[35] partly due to their ultimate
174 dependence on body size. Todorova et al.[35] speculated that higher bioaccumulation of MeHg in
175 larger species resulted from higher filtration efficiency being a function of body size, whereas
176 Kainz et al.[32] attributed this size dependence to large zooplankton having larger anaerobic
177 intestinal niches, where Hg methylation can take place.[36] We found that larger copepods (but
178 not equally large *Cercopagis*) carried a greater number of *hgcA* copies, and not only in terms
179 of the individual- (thus supporting the view of Kainz et al.[32]) but also weight-specific values;
180 the latter implies phylogenetic differences. In the large-bodied *L. macrurus* and *P. acuspes*, our
181 estimate of *hgcA* genes yeilded up to 10-fold higher values compared to the small-bodied *A.*
182 *bifilosa* and *E. affinis,* with the difference being most pronounced for *Firmicutes* (Figure 1).
183 The group-specific variability may affect spatial and seasonal contribution of endogenous
184 MeHg to secondary consumers, because different zooplankton groups that vary in their ability

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185 to methylate Hg would have different capacity to contribute MeHg to bulk zooplankton. For
186 example, the relative importance of gut Hg methylation and MeHg uptake by zooplankton
187 would increase in winter due to the higher contribution of copepods to bulk zooplankton
188 biomass.[37]

189 The gut of copepods is likely to have anoxic conditions (at least in some species)[36] and thus a
190 suitable habitat for methylating microbes. Notably, the morphology of cladoceran gut
191 predisposes it to active oxygenation, and gut microbiota in these animals is dominated by clones
192 affiliated to aerobic or facultative anaerobic bacteria,[38] which may explain the lack of the
193 positive *hgcA* amplification in our cladoceran samples. Hg methylating genes have been
194 detected in invertebrate microbiota, including termites, beetles, and oligochaetes,[4,15] and in
195 some invertebrates the endogenous MeHg production has been documented.[39] As a life form,
196 intestinal microbiota exists in biofilms, and such communities are increasingly recognized as
197 important sites for environmental Hg methylation.[40,41] Commensal biofilms are present in both
198 planktonic and benthic animals that actively exchange gut and body-surface microbiota with
199 the ambient microbial communities and other animals.[42] We found no *hgcA* genes in the gut of
200 the predatory *Cercopagis pengoi*, which may indicate that the digestive system of predators
201 with this feeding mode (puncturing exoskeleton of planktonic crustaceans and sucking soft
202 body tissues) is less likely to become populated by methylating bacteria compared to filter-
203 feeders that have a more active exchange with diverse microbial communities of seston.

204 The presence of Hg methylating bacteria in copepod guts and, hence, in their carcasses and
205 fecal pellets, could be an important and yet unquantified source for MeHg production in the
206 water column.[43] Remineralization of organic matter is associated with elevated MeHg
207 production,[43,44] and Hg methylation potential is higher in fresh organic matter than in
208 decomposed material.[8,44] Zooplankton fecal pellets, a considerable fraction of marine organic
209 matter, are almost completely remineralized in the water column, while degraded
210 phytoplankton and terrestrial organic matter aggregates are more likely to reach the sea floor.[45]
211 The presence of active Hg methylating bacteria in fecal pellets could increase Hg methylation
212 efficiency compared to non-fecal organic matter, where a lag phase related to colonization time
213 is expected. In the latter case, the ecological niche for Hg methylating bacteria might not
214 become available until the most labile parts are already remineralized, resulting in lower MeHg
215 production. Ingestion of fecal pellets by mesopelagic zooplankters and benthic animals could
216 also facilitate spread of methylators among invertebrates and enrich these consumers with
217 microflora of epipelagic zooplankters. In addition, these pellets can become enriched in Hg

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218 methylators during the time spent in the water column. In line with this, we found higher *hgcA*
219 abundances in *P. acuspes* and *L. macrurus* residing in deeper water layers compared with *A.*
220 *bifilosa* and *E. affinis* inhabiting the epipelagic zone (Figure 1).

221 Endogenous Hg methylation in zooplankton could help explain spatial and temporal trends of
222 fish MeHg concentrations in the Baltic Sea. The strong decrease in Hg inputs to the Baltic Sea
223 during the last decades has not resulted in a consistent decrease in fish Hg levels across the
224 sea.[16,46] During this time, significant and basin-specific changes occurred in zooplankton
225 communities[47] in concert with alterations in climate, nutrient inputs and terrestrial runoff.[16,44]
226 It is plausible that synchronous shifts in the methylation capacity of zooplankton, at both the
227 individual microbiome and community levels, have taken place contributing to the MeHg
228 dynamics in the food web. Quantitative analysis of the interactions between biotic and abiotic
229 processes governing endogenous MeHg production is therefore essential if we are to understand
230 uptake and bioaccumulation of MeHg in water column and food webs.

231

232

233 **Conflicts of Interest**

234 There are no conflicts of interest to declare.

235

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Table 1. Summary of zooplankton samples used for qPCR analysis. Samples were taken by vertical tows with a WP2 net (mesh size 90 or 100 µm; diameter 57 cm) equipped with a cod end. At some stations, bottom to surface tows were taken, and at others, we used either stratified tows or sampled only an upper part of the water column. Species abbreviations for copepods: *Acartia bifilosa* (Ab, adults), *Eurytemora affinis* (Ea, adults), *Limnocalanus macrurus* (Lm, CIV), and *Pseudocalanus acuspes* (Pa, CIV), and cladocerans: *Bosmina coregoni maritima* (Bm, body length > 0.7 mm) and *Cercopagis pengoi* (Cp, > 2mm, excluding the tail spine). In total, 33 field-collected zooplankton samples and 3 reference samples (*Artemia* spp.) were analyzed.

Station	Location, area	Geographic coordinates and bottom depth	Month, Year	Sampling depth, m	Number of samples per species					
					Ab	Ea	Lm	Pa	Bm	Cp
H4	Himmerfjärden Bay, Northern Baltic Proper, Swedish coast	N 58°59', E 17°43'; 30 m	Jun 2007	28-0		3			3	2
BY31	Landsort Deep, Northern Baltic Proper, open sea	58°35' N, 18°14' E; 454 m	Jun 2009	100-60				3		
				30-0	3	3			3	
F64	Åland Sea, open sea	N 60°11', E 19°08'; 285 m	Sep 2009	100-0	3					
US5b	Bothnian Sea, open sea	N 62°35', E 19°58'; 214 m	Aug 2006	100-0	3		4	3		

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Fig. 1. Abundance of *hgcA* gene (mean ± SD; n = 3 in all cases, except *Limnocalanus macrurus*, where n = 4) in the Baltic copepods collected in different areas, ordered south to north; see Table 1 for the number of replicates and Figure S1 for sampling site map. No positive amplification was observed in any of the cladoceran samples. The individual-specific abundance (number of *hgcA* copies per individual) is shown in the upper panels and the weight-specific abundance (number of *hgcA* copies per µg wet weight of zooplankter) is shown in the lower panels (A and C: *Deltaproteobacteria* and B and D: *Firmicutes*; no positive amplification was observed for *Archaea*). Observe that selection of species is unique for every station; no value implies that no samples for the particular species was available for the analysis.