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1 **Tracking Functional Guilds: *Dehalococcoides* spp. in European River Basins**
2 **Contaminated with Hexachlorobenzene**

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18 **Abstract**

19 Hexachlorobenzene (HCB) has been widely used in chemical manufacturing
20 processes and as pesticide. Due to its resistance to biological degradation, HCB
21 mainly accumulated in fresh water bodies and agricultural soils. *Dehalococcoides*
22 spp., anaerobic dechlorinating bacteria that are capable of degrading HCB, were
23 previously isolated from river sediments. Yet there is limited knowledge about the
24 abundance, diversity and activity of this genus in the environment. This study focused
25 on the molecular analysis of the composition and abundance of active
26 *Dehalococcoides* spp. in HCB-contaminated European river basins. 16S ribosomal
27 RNA-based real-time quantitative PCR and denaturing gradient gel electrophoresis in
28 combination with multivariate statistics were applied. Moreover, a functional gene
29 array was used to determine reductive dehalogenase (*rdh*) gene diversity. Spatial and
30 temporal fluctuations were observed not only in the abundance of *Dehalococcoides*
31 spp. but also in the composition of the populations and *rdh* gene diversity.
32 Multivariate statistics revealed that *Dehalococcoides* spp. abundance is primarily
33 affected by spatial differences, whereas species composition is under the influence of
34 several environmental parameters, such as seasonal changes, total organic carbon
35 and/or nitrogen content and HCB contamination. This study provides new insight in
36 the natural occurrence and dynamics of active *Dehalococcoides* spp. in HCB
37 contaminated river basins.

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38 **Introduction**

39 Halogenated organic compounds are among the most widespread environmental
40 pollutants. Previously believed to be only anthropogenic, a large number of these
41 compounds, including aliphatic, aromatic and heterocyclic derivatives, are introduced
42 into the environment via biogenic and geogenic sources (9, 21). Hexachlorobenzene
43 (HCB) is believed to be persistent in the environment (22) due to its chemical stability
44 and its resistance to biodegradation. HCB is a hydrophobic and bio-accumulative
45 compound and is listed in the EC-Directive (14) as "priority hazardous substance". At
46 its peak production in the early 1980s, thousands of tons of HCB were produced to be
47 used as fungicide, wood-preservative, porosity-control agent, or in the manufacturing
48 of dyes. The usage of HCB is no longer allowed in most countries because of its
49 toxicity and carcinogenity towards fish and mammals. Nevertheless, it is still being
50 released to the environment as a by-product of various chemical processes, as a result
51 of incomplete combustion or from old landfills (4, 6, 7). HCB contamination has been
52 reported in different environments. Compared to rivers in sparsely populated regions,
53 lakes and sea (32, 42), significantly higher amounts of HCB could be found in river
54 water in agricultural areas, and in densely populated or highly industrialized areas.
55 HCB concentrations were shown to positively correlate with organic matter content of
56 sediments and soils, and European soils were observed to have the highest HCB
57 concentrations globally (38). Several authors reported on the fate and behavior of
58 HCB in the environment at regional or global scales. Nevertheless, our knowledge on
59 microbial degradation of this compound in natural environments remains limited. It
60 has been shown that HCB from air and water bodies can be removed via physical
61 processes like volatilization and photolysis (6, 43). Adsorption also plays an important
62 role in the removal of HCB from aquatic environments, but in turn results in

63 deposition in sediments. In these light scarce environments biodegradation offers a
64 great potential of transforming this persistent organic pollutant (7, 29). The only
65 known pathway for microbial dehalogenation of HCB is the reductive dechlorination
66 under anaerobic conditions, which results in formation of less chlorinated benzenes
67 (1).

68 The reductively dechlorinating bacteria isolated up to now belong to the
69 δ– and ε–*Proteobacteria* (*Geobacter*, *Sulfurospirillum*, *Desulfuromonas,*
70 *Desulfomonile*), the *Firmicutes* (*Desulfitobacterium* and *Dehalobacter*), or to the
71 *Chloroflexi* (*Dehalococcoides* and related groups) (51). So far, however,
72 *Dehalococcoides* is the only bacterial genus whose members are known to transform
73 HCB. Several *Dehalococcoides* strains were isolated that could grow with a broad
74 variety of chlorinated aliphatic and aromatic compounds, including chlorinated
75 benzenes and phenols, biphenyls, chloroethenes and dioxins. Nevertheless, until now
76 only two strains, *Dehalococcoides sp.* strain CBDB1 (3) and *Dehalococcoides*
77 *ethenogenes* 195 (17), which can transform HCB to tri- and dichlorobenzenes and use
78 the energy conserved in the process for growth, could be isolated. Besides HCB,
79 *Dehalococcoides sp.* strain CBDB1 can also reductively dechlorinate chlorinated
80 dioxins (11) and chlorophenols (2), whereas *Dehalococcoides ethenogenes* 195 can
81 dechlorinate various chlorinated ethenes, 1,2-dichloroethane (1,2-DCA) and vinyl
82 chloride (VC) (37).

83 Until now microbial community analyses of *Dehalococcoides* spp. largely
84 focused on chlorinated ethene-contaminated aquifers or soils. The presence of
85 *Dehalococcoides* spp. in uncontaminated and contaminated (PCE, TCE or VC) sites
86 from North America, Europe, and Japan was reported (24, 26, 30, 34, 60).
87 Furthermore, quantitative analyses targeting the *Dehalococcoides* spp. 16S ribosomal

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88 RNA (rRNA) gene in chlorinated ethene bioremediation sites showed that $8.6x10^3$-
89 $2.5x10^6$ copies/ g aquifer material (33) and $1.9x10^2$-$1.1x10^7$ copies/ g soil (50) could
90 be detected depending on the type of treatment applied. Although reductive
91 dechlorination by *Dehalococcoides* spp. is an energy yielding process, microcosm
92 studies conducted under controlled environmental conditions showed that growth of
93 the organisms is relatively slow (28). Moreover, the presence of other halorespiring
94 species may result in competition for chlorinated compounds or electron donors. This
95 may adversely affect the success of the reductive dechlorination of HCB in natural
96 environments. Hence, monitoring the indigenous dechlorinating species is needed to
97 understand their diversity and activity in contaminated sites.

98 The aim of this study was to assess the diversity of active *Dehalococcoides*
99 spp. in HCB polluted river basins and to reveal the links between species composition
100 and abundance with changing environmental parameters, using 16S rRNA- and
101 reductive dehalogenase-encoding gene-targeted molecular analyses, in combination
102 with multivariate statistics. River sediment, flood plain and agricultural soil samples
103 were collected from two European rivers, the Ebro (Spain) and the Elbe (Germany)
104 between 2004 and 2006. This study provides new insights on natural occurrence and
105 dynamics of reductively dechlorinating bacteria, generating important knowledge
106 towards understanding and predicting microbial HCB transformation.

107 **Materials and Methods**

108 *Study sites and sampling procedure*

109 Samples from two European rivers, the Ebro in Spain and the Elbe in Germany, were
110 collected at several locations (Fig. 1; for exact coordinates, see Table S1). The Ebro
111 River (928 km) is located in the northeast of Spain (Fig. 1A). The Ebro River delta

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112 (330 km^2) contains rice fields (210 km^2) and wetlands (80 km^2). Samples from the
113 Ebro River were taken in July 2004, February 2005 and February 2006. During the
114 last sampling campaign additional samples were taken from one of the upstream
115 locations (Flix, Tarragona), which has a chlor-alkali plant with more than one hundred
116 years of activity. At this location HCB concentrations in the river sediment are higher
117 than elsewhere in the Ebro River (19, 32). The Elbe River (1091 km) is one of the
118 longest rivers in Central Europe flowing from Czech Republic to its mouth at the
119 North Sea, Germany. Samples were taken from the Elbe River in October 2004, April
120 2005 and October 2005. The Elbe River was sampled at only one location,
121 Schönberg-Deich (Fig. 1B), and samples were taken from river sediment and flood
122 plain soil. The sampling site was located in the middle reach of the Elbe River,
123 downstream of the Bitterfeld-Wolfen industrial area. All samples were taken in
124 duplicate. River sediment samples were taken approximately 1.5 m away from the
125 river shore. At each location, sterilized PVC tubes (25 cm, internal diameter four cm)
126 were inserted vertically into sediment or soil, retracted, and immediately sealed from
127 the top and the bottom with rubber caps. Cores were frozen in liquid nitrogen,
128 transported on dry ice, and stored at -80°C. Under sterile laboratory conditions frozen
129 soil and sediment cores were cut into four slices (five cm thick, approximately 25
130 gram material), and homogenized by mixing with a spoon. This resulted in samples
131 representing 0-5cm, 5-10cm, 10-15cm and 15-20cm of depth in sediment or soil. The
132 slices were transferred into 50ml falcon tubes and stored at -80°C until use. Samples
133 were analyzed by AGROLAB (Al-West B.V., Deventer, The Netherlands) for
134 detection of geochemical parameters according to standardized methods. HCB
135 concentrations were measured according to ISO 10382 protocol.

136 *Nucleic Acid Extraction*

137 RNA was extracted using the FastRNA® Pro Soil-Direct Kit (Qbiogene, Carlsbad,
138 CA) according to the manufacturer's instructions with minor modifications. Briefly,
139 0.5 gram soil or sediment sample taken from the frozen stock was subjected to bead
140 beating with a Fastprep Cell Disruptor (Qbiogene, Carlsbad, CA), which was
141 followed by phenol/chloroform extraction and incubation at -20°C for one hour.
142 Total RNA was eluted with DEPC-treated distilled H_2O, which was supplied by the
143 manufacturer. RNA purity was checked by electrophoresis in 1.0% (wt/vol) low-melt
144 agarose gels. In case of DNA contamination RNA samples were treated with
145 amplification-grade DNase I (Promega, Madison, WI) as specified by the
146 manufacturer. DNA was isolated directly from soils (0.5 g) using the Fast
147 DNASPIN®Kit For Soil (Qbiogene, Carlsbad, CA) according to the manufacturers'
148 instructions. The RNA and DNA extraction yield was measured using a NanoDrop®
149 ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

150 *Reverse transcription (RT) and PCR amplification*

151 DGGE-PCR for *Dehalococcoides* spp. A nested RT-PCR approach was used to
152 specifically amplify *Dehalococcoides spp*. 16S rRNA fragments. Reverse
153 transcription of 16S rRNA and subsequent PCR amplifications were performed in the
154 same tube by using the Access RT-PCR System® (Promega, Madison, WI). The
155 reaction mix (50 µL total volume) consisted of 10-15 ng of total RNA, 0.8×AMV/Tfl
156 Reaction Buffer, 1 mM $MgSO_4$, 0.1 mM dNTP mix, 0.2 µM of each primer (DeF and
157 DeR, Table S2), 4U of AMV polymerase, 4U Tfl polymerase, 0.5 µL BSA
158 (20mg/mL, Roche). Reverse transcription and further PCR amplification was carried
159 out at 45°C for 45 min, 94°C at 2 min, 35 cycles of 94°C at 30 sec, 55°C at 30 sec,

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160 68°C at 1 min and final elongation at 68°C for 10 min. The products of RT-PCR were
161 then used as template for PCR for the generation of amplicons suitable for analysis by
162 denaturing gradient gel electrophoresis (DGGE) using previously described
163 conditions (53), with primers 968F -introducing a GC-clamp (40)- and DHC1350R
164 (Table S2).

165 Reverse transcription of RNA templates for quantitative PCR. Reverse transcription
166 (RT) of 16S rRNA was performed as described above, but with primers 27F and
167 1492R (Table S2) with second strand synthesis to produce double stranded cDNA
168 fragments.

169 *DGGE*

170 DGGE was performed according to the protocol of Muyzer et al.(41) using the Bio-
171 Rad gene detection system (BioRad, Hercules, CA) with denaturing gradients ranging
172 from 35% to 58%. The gels were stained with $AgNO_3$ (48) and analyzed with
173 BioNumerics 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The
174 Pearson product-moment correlation (23) was used to determine the similarity
175 between DGGE fingerprints by calculating the similarity indices of the densitometric
176 curves of the fingerprints. Bands were identified using the band search algorithm as
177 implemented in BioNumerics, and manually checked by comparison to the
178 corresponding densitometric curves. The Jaccard correlation coefficient was used to
179 compare fingerprints based on the presence or absence of individual bands in the
180 DGGE gels.

181 *Real-Time Quantitative PCR*

182 Real-time quantitative PCR (qPCR) was performed using an iQ5 iCycler (BioRad,
183 Veenendaal, Netherlands) with the thermocycling program as previously described

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184 (52) for 16S rRNA genes of dehalogenating bacteria (*Dehalococcoides*,
185 *Desulfitobacterium*, *Dehalobacter*) and total Bacteria using SYBR Green Dye. PCR
186 reactions were prepared in 25 µl total reaction volume containing 5 µl template cDNA
187 or DNA, 1× BioRad SYBR Green PCR master mix (BioRad, Veenendaal,
188 Netherlands), 0.2 µM of each primer (Table S2) and 6.5 µl sterilized milli Q. Samples
189 were analyzed in duplicate, and no-template controls were included. Standard curves
190 were generated from triplicate dilution series. qPCR standards were prepared by
191 cloning PCR-amplified 16S rRNA genes of targeted dehalogenating bacteria into the
192 pGEM-T Easy plasmid vector (Promega, Madison, WI). PCR-products amplified
193 from plasmid vectors using T7- and SP6-promotor targeted primers (Table S2) were
194 used as real-time PCR standards.

195 *Cloning and Sequencing*

196 For the construction of clone libraries, 16S rRNA fragments were amplified by nested
197 (RT-) PCR with primers DeF and DeR in the first PCR, and DeF and DHC1350R in
198 the second reaction. The clone library for the Ebro River was prepared from a sample
199 taken in winter 2005, from location Flix. For Elbe River the clone library was
200 constructed from a sediment sample obtained in spring 2005. Both libraries were
201 prepared from samples taken at a depth of 0-5 cm. The PCR products were cloned
202 using the pGEM-T Easy plasmid vector (Promega, Madison, WI), and *E. coli* XL1
203 blue cells (Stratagene, La Jolla, CA) according to the manufacturers' instructions. To
204 assess the diversity of cloned fragments, the 1.3-kb PCR products were digested with
205 the restriction enzyme MspI or/and AluI at 37°C for 3 hrs. Digestion mixtures (20 µl)
206 contained 5µl of the PCR product, 0.25 U of the respective restriction endonuclease
207 (Promega, Madison, WI), 0.1 mg of acetyl-bovine serum albumin (Promega,

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208 Madison, WI) and 1× restriction buffer (Promega, Madison, WI). The resulting
209 fragments were separated by electrophoresis for 1hr at 125V in 12% (wt/vol) pre-cast
210 Poly(NAT)® gels (Elchrom, Cham, Switzerland), using the Elchrom Submerged Gel
211 Electrophoresis System. Representative clones containing inserts with different
212 restriction patterns were selected and sequenced completely. The
213 CHECK_CHIMERA program of the Ribosomal Database Project (36), BLAST
214 searches and phylogenetic analyses of separate sequence domains identified one
215 potential chimeric artifact, which was excluded from further phylogenetic analyses.
216 Sequences belonging to two operational taxonomic units (OTUs) from the Ebro River
217 and eight OTUs from the Elbe River were deposited to NCBI database. Sequences
218 EU700499 and EU700500 originate from the Ebro River. Sequences EU700494-
219 EU700497 and EU700502- EU700505 are from the Elbe River. Sequences obtained
220 in this study were aligned with reference sequences using the online alignment tool
221 SINA available at http://www.arb-silva.de (44). The aligned sequences were imported
222 into the latest release of the ARB-Silva reference database (Silva96), and the
223 alignment was manually refined using tools available in the ARB software package
224 (35). A phylogenetic tree was constructed using the Neighbor Joining method as
225 implemented in ARB (35).

226 *Microarray Analysis*

227 The GeoChip (25) was used to detect functional genes in sediment- and agricultural
228 soil samples in the Ebro River. Since the current version of the GeoChip does not
229 include all the reductive dehalogenase (*rdh*) genes sequences currently deposited in
230 public databases, new probes were designed and added to the microarray to have
231 comprehensive coverage of these genes. Oligonucleotide probe design, synthesis and
232 fabrication was performed as described previously (25). A list of all *rdh* gene

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233 sequences for which additional probes were designed is given in a Supplementary
234 excel file. Samples from two locations, upstream (Flix) and downstream (Rice Fields),
235 and two depths (0-5 cm and 10-15 cm) were analyzed with the GeoChip. High
236 molecular weight DNA extraction was performed by lysis in a CTAB buffer at 60°C
237 using a phenol-chloroform purification protocol (61). Rolling circle amplification,
238 which has been shown to amplify total DNA from low biomass microbial
239 communities prior to microarray hybridization (58), was carried out using the
240 TempliPhi kit (Amersham, Piscataway, NJ) following manufacturer's instructions.
241 Spermidine (0.1 µg/µL) and single-strand binding protein (0.04 mM) were added to
242 the reaction to aid amplification. The reactions were incubated at 30°C for 3 hrs and
243 the enzyme was then inactivated by incubation at 60°C for 10 min. The amplification
244 products were labeled with Cystidine-5 (Cy-5) dye (Amersham, Piscataway, NJ).
245 Hybridizations were performed in a HS4800 Hybridization Station (TECAN US,
246 Durham, NC) as previously described (59) with following modifications. The first
247 wash was carried out at 50°C for 1 min with a pre-hybridization solution (5X SSC,
248 0.1% SDS and 0.1% BSA) followed by a 45 min pre-hybridization. The slides were
249 then washed four times with water at 23°C for 5 min with 30 sec soaking. Labeled
250 DNA dissolved in the hybridization solution was then injected at 60°C and
251 hybridization was carried out at 42°C for 10 hrs with high agitation. Slides were then
252 washed and dried under a flow of nitrogen gas. Arrays were scanned using a
253 ProScanArray microarray scanner (PerkinElmer, Boston, MA) at 633 nm using a laser
254 power of 95% and a PMT gain of 80%. Images were processed by ImaGene 6.0
255 (BioDiscovery, El Segundo, CA), where a grid of individual circles defining the
256 position of each DNA spot on the array was used to locate each fluorescent spot to be
257 quantified. Spot calling was based on the following parameters; (signal to noise ratio)

258 SNR<1.2 and (cumulative variance of background signal) CV<30% were chosen due
259 to highly variable total hybridization signal between the different samples. Spots with
260 two times higher signal intensity than the rest of the designated gene probes were
261 accepted as an outlier and removed from the analysis.

262 *Multivariate Analysis*

263 In order to relate the changes in the *Dehalococcoides* spp. community
264 composition and *rdh* gene variations to environmental variables, redundancy analysis
265 (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris,
266 Wageningen, The Netherlands). Presence/absence and relative abundance (peak areas)
267 of DGGE bands as well as normalized signal intensities of *rdh* genes were used as
268 predictors. The environmental variables tested were time of sampling, distance
269 between the sampling locations, sediment depth from which samples were taken,
270 *Dehalococcoides spp.* 16S rRNA copies/g sediment, hexachlorobenzene
271 concentration, water temperature and pH, and total organic carbon (TOC), total
272 Kjeldahl nitrogen (TKN) and total phosphorus (TP) measurements that were made on
273 soil and sediment samples. All of the environmental data except for pH data were also
274 transformed as log (1 + x). A Monte-Carlo permutation test based on 999 random
275 permutations was used to test the null hypothesis of "*Dehalococcoides* spp.
276 fingerprints are not related to environmental variables". The community structure was
277 visualized via ordination triplots with scaling focused on inter-sample differences.
278 Multivariate analysis of microarray data was performed using calculated relative
279 intensity (abundance) values for each hybridization signal, which were normalized
280 with Box-Cox transformation (10) prior to analysis. Redundancy analysis (CANOCO
281 4.5) was used to test the null hypothesis of "Variances in reductive dehalogenase
282 genes are not related to environmental variables". Gene distributions were plotted

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283 with scaling focused on inter-sample differences. For all statistical analyses,
284 correlations were considered highly significant at $p<0.05$ and significant at $p<0.10$
285 unless mentioned otherwise. All ANOVA and correlations analyses were performed
286 in R software.
287

288 **Results**

289 *Sediment and soil geochemistry*

290 The geochemistry of sediment and soils samples varied significantly between
291 different sampling locations in the Ebro River (Fig. 1A, Tables S1, S3 and S4).
292 Correlation between sediment (soil) TOC, TKN and TP content and sampling time
293 was not significant. Pesticide concentrations were highest in the upstream locations
294 (mainly in Flix), with dichlorodiphenyltrichloroethane (DDT) and HCB being the
295 main contaminants. Major differences were observed between sediment and soil
296 samples for TOC, TKN and TP in the Elbe River. In most of the cases, river
297 sediments had lower concentrations of these compounds than floodplain soils.
298 Moreover, pesticide contamination was significantly higher in floodplain soils than
299 river sediments. In Elbe, HCB was found to be the main contaminant, which was
300 followed by DDT and hexachlorocyclohexane (HCH).
301

302 *The impact of sediment (soil) geochemistry, spatial and temporal gradients on 16S*
303 *rRNA abundance of dechlorinating bacteria*

304 16S rRNA-targeted reverse transcription RT-qPCR assays were used for quantitative
305 detection of several dechlorinating genera in the river basins. *Dehalococcoides* spp.
306 and *Desulfitobacterium* spp. 16S rRNA could be detected in different quantities in the
307 sampling locations (Table S5), whereas *Dehalobacter* spp. rRNA could not be

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308 detected. Both absolute rRNA copy numbers and the relative abundances (i.e.
309 *Dehalococcoides* spp. 16S rRNA copies / total Bacteria 16S rRNA copies) were used
310 to calculate pairwise correlations (Spearman's correlation coefficient-r_s) of each genus
311 with environmental variables (Table S6). In the Ebro River, total bacterial rRNA copy
312 numbers were significantly higher in samples with high TOC, TKN and TP content.
313 Correlation between total bacterial rRNA copy numbers and spatial and temporal
314 gradients were not significant. In the Elbe, total bacterial rRNA copy numbers were
315 found to be decreasing (r_s=-0.81, p≤0.001) during the sampling period. No significant
316 correlations were found with geochemical parameters. There was no significant
317 correlation between HCB pollution levels and total bacterial rRNA copy numbers in
318 both rivers.

319 In the Ebro River *Dehalococcoides* spp. comprised on average 0.2% of the
320 bacterial 16S rRNA pool (up to 0.91%). *Dehalococcoides* spp. rRNA was consistently
321 and significantly more abundant in the upstream locations (Lleida and Flix) than in
322 downstream locations (Tortosa, Rice Fields and Estuary of the Ebro Delta) (r_s=-0.77,
323 p≤0.001) (Fig. 2A, Table S6). Other environmental variables, including HCB
324 pollution, did not significantly contribute to explain the changes in the relative
325 abundance of *Dehalococcoides* spp. 16S rRNA copies. To further investigate the
326 effect of the sampling location, additional samples were taken from upstream and
327 downstream of the regular sampling point in Flix in February 2006. Samples taken
328 from 2 km upstream and 100 m downstream of the regular sampling point were
329 analyzed to assess the spatial variation in the relative abundance of *Dehalococcoides*
330 spp. rRNA copies as well as the ratio of 16S rRNA copies to 16S rRNA gene copies
331 (rRNA/DNA) around the regular sampling point (Fig. S1). Results showed that
332 relative abundance and rRNA/DNA of *Dehalococcoides* spp. could vary remarkably.

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333 For example, in the upper 5cm, four to five-fold differences in the rRNA/DNA ratio
334 and two to 16-fold differences in the relative abundance between the locations could
335 be detected. There were no clear correlations between rRNA/DNA ratio and relative
336 abundance.

337 In the Elbe River, *Dehalococcoides* spp. had similar relative abundances as
338 observed for the Ebro, comprising on average 0.26% of the bacterial 16S rRNA pool.
339 However, for the flood plain soils rRNA abundances were considerably lower (0.05-
340 0.12%; Fig. 2B). During the sampling period species abundances tended to increase
341 (r_s=0.44, p≤0.10). *Dehalococcoides* spp. rRNA, when detected, had its highest
342 abundances - as high as 1% - in the upper layers of sediments (r_s=-0.40, p≤0.10).
343 Geochemical parameters, including HCB concentrations had no significant correlation
344 to *Dehalococcoides* spp. rRNA abundances.

345 The abundance of *Desulfitobacterium* spp. rRNA was similar in the Ebro
346 River in samples taken at different times and depths, but there were differences
347 between different sampling locations (r_s=-0.40, p≤0.10). Relative rRNA abundances
348 were in general 10-fold lower than for *Dehalococcoides* spp. (data not shown).
349 Throughout the sampling period, the relative abundance of *Desulfitobacterium* spp.
350 rRNA decreased in upstream locations and increased in the downstream locations in
351 the river delta. Furthermore, in the Elbe River significant increases in
352 *Desulfitobacterium* spp. rRNA relative abundances (r_s=0.88, p≤0.001) were detected
353 during the sampling period. In contrast to *Dehalococcoides* spp., relative abundances
354 of *Desulfitobacterium* spp. rRNA were higher in flood plain soils compared to river
355 sediment throughout the sampling period (r_s=0.37, p≤0.1). The highest relative
356 abundances were detected in deeper (5-15cm) layers of the soils (Fig. 2C).
357

358 *16S rRNA composition of Dehalococcoides spp. in the river basins*

359 Changes in the composition of *Dehalococcoides* spp. were followed by DGGE of
360 *Dehalococcoides*-specific 16S rRNA RT-PCR amplicons. The Pearson product-
361 moment correlation (23) was used to compare DGGE fingerprints. In the Ebro River,
362 Pearson correlation between all locations decreased from 68% in June 2004 to 13% in
363 February 2006 (Fig. S2). Pearson correlation within the sampling locations decreased
364 most drastically in Flix, namely from 93% to 19% during the sampling period.
365 Similarity indices among the Elbe River sediment and flood plain soils were as low as
366 9% (Fig. S3). Except for samples taken in October 2004, *Dehalococcoides* spp. 16S
367 rRNA fingerprints could only be generated for samples from the top 10cm of the river
368 sediment. *Dehalococcoides* spp. fingerprints showed 77% correlation in this fraction
369 of the sediment throughout the sampling period (data not shown). Variations observed
370 between DGGE fingerprints concerned differences in *Dehalococcoides* spp.
371 composition rather than variation in the intensity of the bands. This was supported by
372 the fact that pair-wise similarities and clustering based on either Jaccard or Pearson
373 correlation coefficients did not differ significantly (data not shown).

374 Clone libraries of the most diverse DGGE fingerprints were constructed from
375 16S rRNA fragments amplified by RT-PCR to confirm that all bands indeed
376 correspond to *Dehalococcoides* spp. related populations. Blast analysis (5) was
377 conducted for sequences from 10 different OTUs, as defined by RFLP analysis. All
378 the sequences from both rivers were affiliated with *Dehalococcoides* and close
379 phylogenetic relatives. In the Ebro River sequences had 91-98% identity to 16S rRNA
380 sequences of known *Dehalococcoides* spp. whereas the Elbe River sequences had 95-
381 99% identity (Fig. S4).

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383 *Redundancy Analysis (RDA) of Dehalococcoides spp. 16S rRNA abundance and*
384 *composition*
385 Multivariate statistics were used to determine to what extent environmental
386 parameters (i.e. spatial and temporal gradients, and sediment (or soil) geochemistry)
387 and 16S rRNA abundance contributed to the differences in the *Dehalococcoides*-
388 specific DGGE fingerprints. The analysis was conducted on band positions (i.e.
389 presence/absence). In the Ebro River the distribution of *Dehalococcoides* spp. in the
390 ordination space was most significantly correlated with the gradient "time" (sampling
391 period, $p \leq 0.001$) (Table 1). In addition, a Monte-Carlo significance test revealed that
392 also the geographical distances (sampling location) had a significant effect on
393 *Dehalococcoides* spp. composition. The model formed by the significant
394 environmental parameters could explain 37.7% of the variation in *Dehalococcoides*
395 spp. composition ($p=0.061$). When samples were grouped based on the sampling
396 period, samples from 2004 and 2006 did not intersect, indicating a significant change
397 in the community composition (Fig. 3A). A smaller number of species positively
398 correlated with increasing TOC content as compared to the effect of water
399 temperature and pH. Moreover, most of the populations negatively correlated with the
400 sampling period, indicating a decrease in richness in time. Correlations to depth
401 (sampling depth), 16S rRNA copy numbers, TKN, TP and HCB concentrations were
402 found to be insignificant.
403 The first two RDA axes could explain 48% of the total variation in the
404 *Dehalococcoides spp*. composition in the Elbe (Fig. 3B). The distribution of samples
405 in the ordination diagram was strongly influenced by the HCB contamination
406 ($p \leq 0.001$), which accounted for 16.7% of the variation in species composition (Table
407 1). Moreover, Monte-Carlo significance tests showed that variances can be

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408 significantly related to 16S rRNA copy numbers (p=0.006) and TKN (p=0.005).
409 Grouping the samples in river sediment and flood plain soils showed that these two
410 environments did not share the same species composition. Most of the species
411 negatively correlated with increasing HCB concentrations and increasing sampling
412 depth. In contrast to the results in the Ebro River, temporal gradients (sampling
413 period) and TOC did not significantly affect the species composition.
414

415 *GeoChip analysis of rdh gene diversity*

416 Functional gene array (GeoChip) analysis was used to assess the variation of the
417 reductive dehalogenase-encoding gene (*rdh* gene) diversity in upstream (Flix) and
418 downstream (Rice Fields) locations of the Ebro River basin. To assess the effect of
419 environmental parameters on *rdh* gene profiles, RDA was conducted using signal
420 intensities. Redundancy axes ($p \leq 0.01$) were found to explain 40.3% of the overall
421 variance within the *rdh* gene diversity. Monte-Carlo permutation tests showed that the
422 *rdh* gene diversity changed significantly between different sampling locations ($p \leq$
423 0.001). As a result, a clear separation could be observed between the upstream and
424 downstream samples (Fig. 4). Besides sampling location, TOC, TKN and TP were
425 found to strongly correlate with variation in *rdh* gene diversity. The upstream location
426 was mainly contained *rdh* genes of *Dehalococcoides* sp. CBDB1 (namely, cbdbA88,
427 cbdbA1535, cbdbA1578, cbdbA1582, cbdbA1595, cbdbA1624, cbdbA1638) and
428 *Dehalococcoides ethenogenes* 195 (namely, DET0088, DET0173, DET1522,
429 DET1545) (Table S7), which are the only cultivated anaerobic bacteria known to
430 degrade HCB (3, 17). These genes were amongst the most abundant *rdh* genes and
431 negatively correlated with increasing TOC and TKN concentrations (Fig. 4).
432 Downstream samples hybridized with probes specific for a variety of *rdh* genes from

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433 mainly *Desulfitobacterium* spp. and *Dehalococcoides* spp., including reductive
434 dehalogenases of strains FL2 (RdhA7), VS (*vcrA*), CBDB1 (cbdbA1582, cbdbA1535,
435 cbdbA1578, cbdbA1638) and *D. ethenogenes* 195 (DET0088, DET0173, DET1538,
436 DET1528). The top layers of the sediment and agricultural soil samples were
437 composed of genes originating from *Dehalococcoides* spp., whereas a mixture of
438 *Desulfitobacterium* spp., and *Dehalococcoides* spp. genes was detected in bottom
439 layers (Table S7). Abundance of *rdh* genes varied drastically during the sampling
440 period. Sampling depth, however, as well as sampling period and HCB concentrations
441 did not significantly affect the *rdh* gene diversity (Fig. 4).

442 **Discussion**

443 The aim of this study was to assess the composition and abundance of active
444 *Dehalococcoides* spp. in river basins polluted with HCB, using a set of
445 complementary cultivation-independent approaches. Previous biomolecular studies on
446 *Dehalococcoides* spp. have shown their presence in various environments and
447 geographical locations (24, 26, 30, 34, 60). However, to the best of our knowledge,
448 this study addressed for the first time the potential effects of temporal and spatial
449 gradients on species composition and relative abundance in river basins.

450 *Dehalococcoides* spp. 16S rRNA relative abundance changed significantly
451 between and within different sampling locations, depths and periods. In some
452 locations, relative abundance could reach up to 1% (Fig. 2A and 2B). The only
453 reported 16S rRNA relative abundance for *Dehalococcoides* spp. in the environment
454 is two to six percent in a PCE and TCE contaminated groundwater aquifer (16).
455 Additionally, HCB and PCE transforming batch scale enrichments from Ebro and
456 Elbe River sediment samples had a higher relative abundance of *Dehalococcoides*
457 spp. (two to six percent) than the corresponding environmental samples (54). In

458 enrichment cultures containing *D. ethenogenes* 195, 16S rRNA gene copy abundance
459 was calculated as 7-62% during PCE degradation (46). When taken together, our
460 results demonstrate that the relative abundance of *Dehalococcoides* spp. in the river
461 basins studied here is lower than in contaminated aquifers or enrichment cultures.

462 In geographically distant locations, as in the samples from the Ebro River,
463 variance between the different sampling locations and periods could be so influential
464 that the effects of other environmental parameters can be too small to explain the
465 variations in the species composition. Even though water temperature, pH and TOC
466 appeared to be relevant parameters in explaining the variation in the species
467 composition, they were not highly significant. It can not be excluded that other
468 factors, which could not be included in this study due to the lack of uninterrupted and
469 reproducible measurements, might be of importance. Sediment transport in the river
470 system and oxygen content in different depths of sediment (or soil), are two of these
471 factors. The flow of the Ebro River is highly (57%) regulated by reservoirs that are
472 used for irrigation and hydropower production. In the past years significant decreases
473 were reported in the flood discharges (8). Reservoirs were reported to trap most of the
474 sediment transported in the river stream, resulting in drop of the annual sediment
475 contribution of the Ebro to its delta by up to 99% in the past century (13, 47, 55).
476 During the sampling period dissolved oxygen content in Ebro River water varied
477 between 6.2-16.7 mg O_2/L (data not shown). Molecular oxygen is often being
478 depleted typically between 0.1-mm up to 1-cm depending on the carbon content of the
479 sediment (27), resulting in anoxic conditions in the deeper layers. Given the low flow
480 rates of the river and variable sediment deposition, however, it is not possible to
481 confidently estimate how much O_2 could be introduced to deeper layers of sediments
482 and soils. Especially in the Ebro Delta, due to agricultural practices, presumably more

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483 O_2 could be introduced to the soil. It can also not be excluded that anoxic micro-
484 environments can form even within otherwise oxic layers of the sediment, or the other
485 way around (20). In addition, varying salt concentrations (approx. 1-5 g/l from
486 seashore to inland at one meter depth) caused by seawater intrusion (49) could also
487 negatively influence the presence and activity of *Dehalococcoides* spp. in the river
488 delta. Previous surveys conducted in the sampling area between 1999-2003 and more
489 recent studies showed that besides HCB, DDT, PCE, and TCE, polycyclic aromatic
490 hydrocarbons (PAH's), polybrominated diphenyl ethers (PBDEs) and brominated
491 flame retardants could be detected in water, sediments and biota of this river (12, 15,
492 18). Therefore, the lack of significant correlation between HCB pollution and
493 *Dehalococcoides* spp. composition and relative abundances may suggest that
494 *Dehalococcoides* spp. in this river system does not depend only on HCB for their
495 growth and possibly use alternative electron acceptors.

496 In accordance with the above, the distribution and diversity of *rdh* genes in the
497 Ebro River could not be significantly related to the dominant contamination at the
498 sampling locations. However, selective pressure of the contaminants could be
499 demonstrated by differences in *rdh* gene distributions in the Ebro River up- and
500 downstream locations. Upper layers of the sediment sampled at the HCB
501 contamination hot-spot Flix were enriched with *rdh* genes from *Dehalococcoides sp.*
502 strain CBDB1 and *D. ethenogenes* 195, the only two cultured isolates currently
503 known for their HCB-dechlorinating activity (3, 17). In contrast, samples taken at
504 downstream locations within the Ebro Delta, which receives numerous halogenated
505 compounds, were found to contain a variety of *rdh* genes, including those from
506 various other species. From the detected *rdh* genes from *Dehalococcoides* spp. only
507 one was previously characterized; *vcrA* of *Dehalococcoides sp.* bacterium VS. The *vcrA*

508 gene product is involved in reductive dehalogenation of vinylchloride to ethene (39).
509 Even though the rest of the detected *rdh* genes are uncharacterized, DET0088,
510 DET0173 and DET1545 were reported to be up-regulated during PCE degradation in
511 ANAS enrichments and mixed cultures containing *D. ethenogenes* 195 (45, 57).
512 Furthermore, cbdbA1624 was found to be expressed during HCB degradation in batch
513 scale enrichments from Flix sediment (54).

514 Screening of the samples with 16S rRNA-targeted RT-qPCR and DGGE in the
515 Elbe River demonstrated that *Dehalococcoides* spp. are more active and have a higher
516 diversity in river sediments compared to flood plain soils (Fig. 3B). A major part of
517 their activity was located in the upper layers (0-10cm) of the river sediments that
518 could provide the desired conditions for the growth and activity of *Dehalococcoides*
519 spp. Unlike the situation in the Ebro River, HCB contamination was a significantly
520 explanatory variable. A smaller number of *Dehalococcoides* spp. rRNA copies was
521 found in flood plain soils, which had higher HCB contamination than river sediment.
522 However, the floodplain soils of the Elbe River were shown to be contaminated with
523 high concentrations of various heavy metals (31), which could be inhibitory for
524 *Dehalococcoides* spp., resulting in the observed low diversity and activity.

525 *Dehalococcoides* spp. emerged as the most abundant dechlorinating bacteria in
526 comparison to *Desulfitobacterium* spp. and *Dehalobacter* spp. in HCB contaminated
527 river basins. Active *Dehalobacter* spp. could not be detected in either river basin
528 during the two years of sampling. *Desulfitobacterium* spp., however, could be
529 detected in most locations, albeit usually in lower numbers than *Dehalococcoides* spp.
530 Unlike *Dehalococcoides* spp., relative abundances of *Desulfitobacterium* spp. were
531 higher in flood plain soils of the Elbe River. However, a similar trend was not
532 observed for the Ebro Delta. *Desulfitobacterium* spp. have not yet been reported to

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533 degrade chlorinated benzenes, and have only been associated with the degradation of
534 chlorinated ethenes and ethanes, and chlorophenols. The functional gene array
535 analysis of these samples confirmed the presence of potentially PCE and/or TCE
536 dechlorinating *Desulfitobacterium* spp. both in river sediment and agricultural soil.
537 Whereas at upstream locations *rdh* genes from *Desulfitobacterium hafniense* strains
538 DCB-2 and TCE1 (*pceA* and *pceB*) were detected, the Ebro Delta was also shown to
539 harbor a putative chloroethene reductive dehalogenase *rdhA* gene from
540 *Desulfitobacterium sp.* PCE1. Moreover, *Desulfitobacterium* spp. are more flexible in
541 their choice for electron acceptors than *Dehalococcoides* spp.. They can also use a
542 wide variety of non-chlorinated compounds, such as nitrate, sulfite, metals, and humic
543 acids (56). Therefore it cannot be excluded that numbers obtained via 16S rRNA
544 based detection of *Desulfitobacterium* spp. in the Elbe flood plains could also be
545 originating from non-dechlorinating members of the species.

546 ***Conclusions***

547 This study showed that high amounts of 16S rRNA *Dehalococcoides* spp. can be
548 detected in river sediments exposed to HCB for a long period of time. However,
549 spatial and temporal variations play a crucial role in affecting activity and diversity of
550 abundant populations. Our findings indicate that the *Dehalococcoides* spp. activity is
551 highly heterogeneous and varies significantly between different locations. In open
552 environments like river basins, it will remain challenging to unequivocally link
553 species composition and activity to changes in environmental conditions. From the
554 data presented here, it can be concluded that river sediment emerges as a preferred
555 environment for *Dehalococcoides* spp. as compared to agricultural or flood plain
556 soils. As could be expected from current knowledge on the ecophysiology of
557 halorespiring bacteria, *Dehalococcoides* spp. are more dominant in HCB polluted

558 locations within river basins than *Desulfitobacterium* spp. and *Dehalobacter* spp.
559 Hence, monitoring of *Dehalococcoides* spp. activity in HCB contaminated river
560 basins provides valuable information about changes in the environmental conditions
561 and contributes to our understanding of the life of these interesting bacteria in natural
562 environments.

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777 Table 1. Summary of the results obtained for the RDA test for the significance of
778 environmental variables in explaining the variance in *Dehalococcoides* spp. 16S
779 rRNA composition and reductive dehalogenase (*rdh*) gene diversity. Percentages
780 indicate the proportion of the variation in composition or diversity that could be
781 explained by the different parameters.

	River Elbe	River Ebro	
	Dehalococcoides spp 16S rRNA composition	*Dehalococcoides* spp 16S rRNA composition	*rdh* gene diversity
Sampling location	1.4% ns	6.2% *	12% ***
Sample depth	6.6% +	2.1% ns	6.8% ns
Sampling period	4.4% ns	16.9% ***	4.9% ns
Dehalococcoides spp. 16S rRNA copy / g sample	14.1% **	2.5% ns	na
Twater	na	5.0% +	na
pHwater	na	4.8% +	na
TOC	1.6% ns	4.8% +	9.0% *
TKN	12.8% **	3.4% ns	10.2% *
TP	3.4% ns	2.8% ns	9.1% *
HCB	16.7% ***	1.7% ns	4.4% ns
All	50.2% ***	37.7% **	40.3% **

782
783 na: not applicable; ns: not significant; +: P≤0.10; *P≤0.05; **: P≤0.01; ***: P≤0.001
784

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785 ***Figure Legends***
786
787 **Figure 1.** Sampling locations in the Ebro **(A)** and the Elbe **(B)** Rivers. Maps were re-
788 drawn from Google ® Maps. Black squares represent approximate locations of the
789 major cities closest to the sampling sites. Both maps are drawn according to scale as
790 mentioned. **(A)** The Ebro River is located in the north east of the Iberian peninsula.
791 Sampling was done in the downstream area of the River over a distance of
792 approximately 200 km. Black dots represent sampling locations. **(B)** The Elbe River
793 sampling points were river sediment (RS), flood plain soil 1 (FPS1) and flood plain
794 soil 2 (FPS2). The black arrow represents the location of the sampling site in
795 Germany. Light trimmed areas represent ponds created during flooding events. The
796 dotted white line with arrow heads represents the flow direction of the Elbe.
797
798 **Figure 2.** Changes in the relative abundance of 16S rRNA copies of dechlorinating
799 bacteria given as percentage of all bacterial 16S rRNA copies. The error bars
800 represent the standard deviation of duplicate measurements. (**A**) *Dehalococcoides spp.*
801 in Ebro River during sampling period in years 2004, 2005, 2006. The horizontal axis
802 shows relative abundance in percentages. The vertical axis represents sample depth.
803 **(B-C)** Changes in the relative abundance of *Dehalococcoides spp.* and
804 *Desulfitobacterium spp.* 16S rRNA copies in the Elbe River during sampling period of
805 1.5 years. The horizontal axis shows relative abundance in percentages. The vertical
806 axis represents sample depth. Locations of the floodplain soil 1 and 2 are indicated in
807 Figure 1.
808
809 **Figure 3.** Ordination triplots for RDA analysis. Species (each *Dehalococcoides spp.*
810 DGGE band) are displayed by triangles (▲). Samples are displayed by open circles,

811 squares and diamonds as indicated below. Arrows represent environmental parameters
812 ($p<0.1$). The length of each gradient (eigenvalue) is indicated on the corresponding
813 redundancy axis. The plot can be interpreted qualitatively by following the direction
814 of arrows for environmental parameters. The arrow length corresponds to variance
815 that can be explained by the environmental variable. The direction of an arrow
816 indicates an increasing magnitude of the environmental variable. The perpendicular
817 distance between species and environmental variable axes in the plot reflect their
818 correlations. The smaller the distance the stronger the correlation, whereas distances
819 among species symbols are not explanatory. **(A)** DGGE band analysis for samples
820 taken along the Ebro River. Samples are grouped according to sampling period (years
821 2004, □; 2005, ○; 2006, ◇). **(B)** DGGE band analysis for Elbe River samples.
822 Samples are grouped according to the sample type (soil, □; or sediment, ○).
823
824 **Figure 4.** Ordination triplot for RDA analysis of *rdh* gene diversity in the Ebro River
825 (based on GeoChip analysis). Each *rdh* gene is displayed by triangles (▲). Samples
826 are grouped according to sample location (upstream, □; or downstream, ○). This
827 grouping also represents sample type (agricultural soil or sediment). Arrows represent
828 environmental parameters ($p<0.1$). See the Fig. 3 legend for further explanation.
829

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Fig. 1

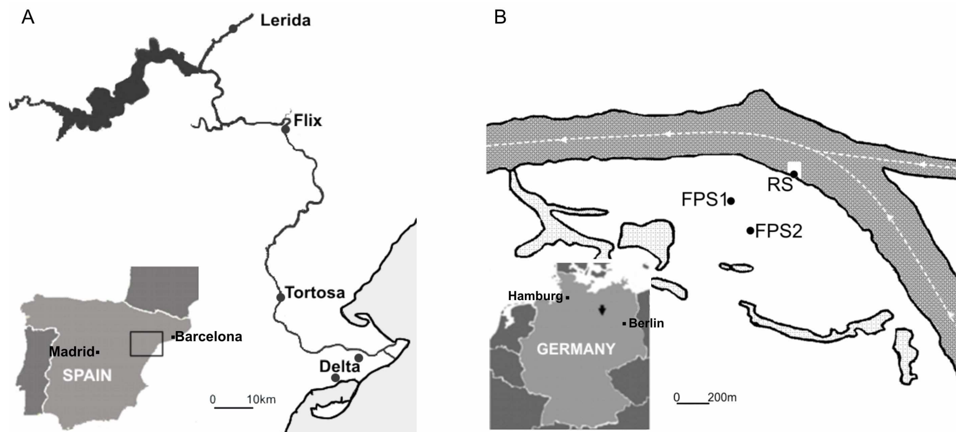

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Fig. 2

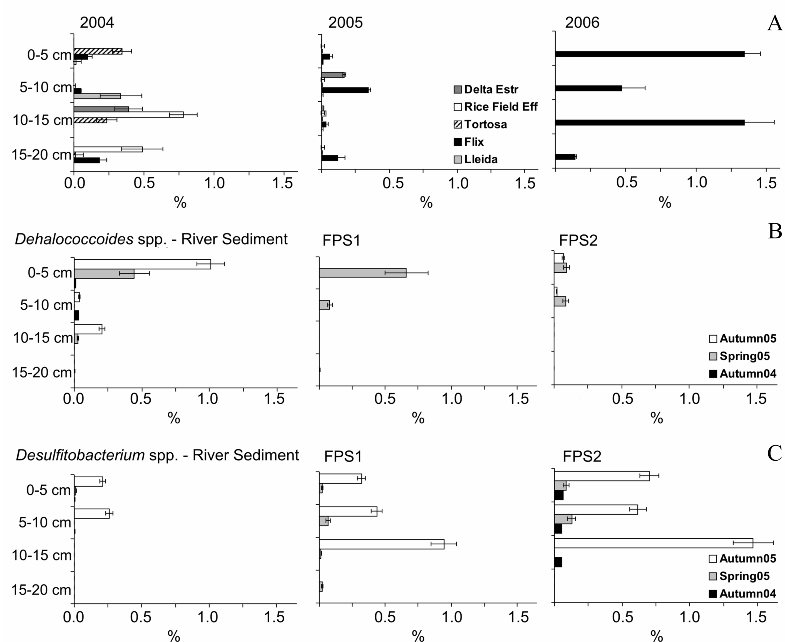

Fig. 3

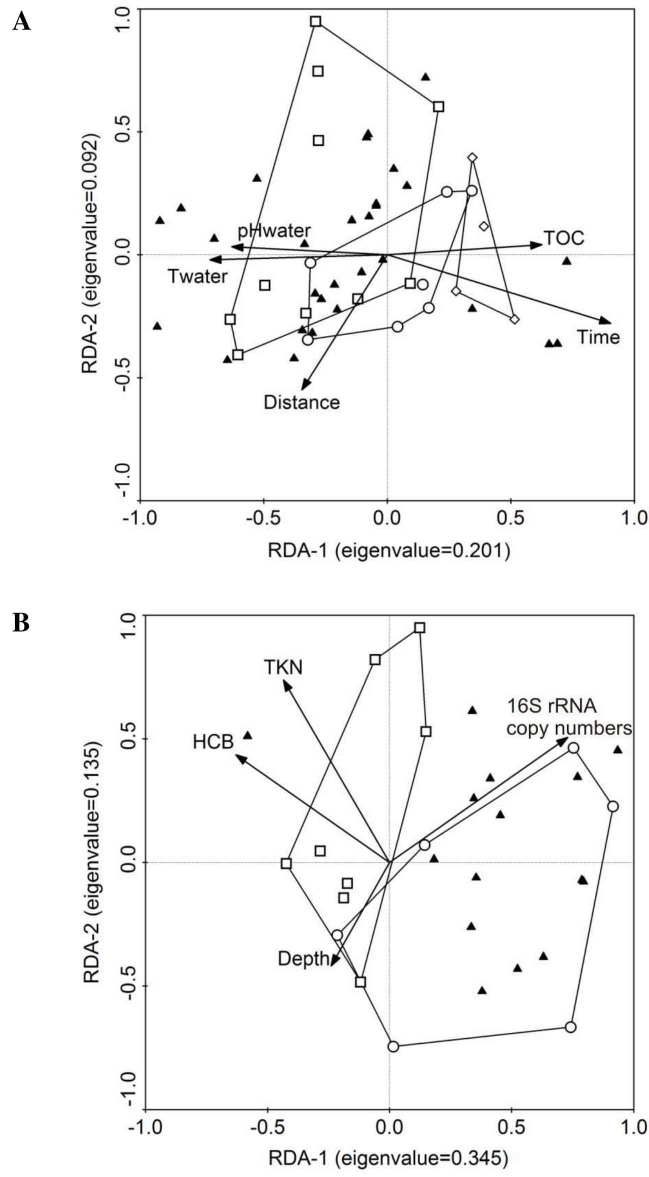

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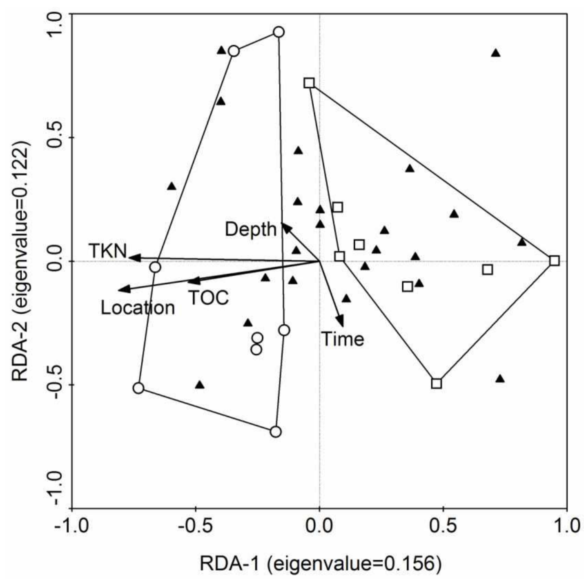
Fig. 4



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