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

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Abstract

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

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Introduction

Halogenated organic compounds are among the most widespread environmental pollutants. Previously believed to be only anthropogenic, a large number of these compounds, including aliphatic, aromatic and heterocyclic derivatives, are introduced into the environment via biogenic and geogenic sources (9, 21). Hexachlorobenzene (HCB) is believed to be persistent in the environment (22) due to its chemical stability and its resistance to biodegradation. HCB is a hydrophobic and bio-accumulative compound and is listed in the EC-Directive (14) as “priority hazardous substance”. At its peak production in the early 1980s, thousands of tons of HCB were produced to be used as fungicide, wood-preservative, porosity-control agent, or in the manufacturing of dyes. The usage of HCB is no longer allowed in most countries because of its toxicity and carcinogenity towards fish and mammals. Nevertheless, it is still being released to the environment as a by-product of various chemical processes, as a result of incomplete combustion or from old landfills (4, 6, 7). HCB contamination has been reported in different environments. Compared to rivers in sparsely populated regions, lakes and sea (32, 42), significantly higher amounts of HCB could be found in river water in agricultural areas, and in densely populated or highly industrialized areas. HCB concentrations were shown to positively correlate with organic matter content of sediments and soils, and European soils were observed to have the highest HCB concentrations globally (38). Several authors reported on the fate and behavior of HCB in the environment at regional or global scales. Nevertheless, our knowledge on microbial degradation of this compound in natural environments remains limited. It has been shown that HCB from air and water bodies can be removed via physical processes like volatilization and photolysis (6, 43). Adsorption also plays an important 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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RNA (rRNA) gene in chlorinated ethene bioremediation sites showed that $8.6x10^3$-$2.5x10^6$ copies/ g aquifer material (33) and $1.9x10^2$-$1.1x10^7$ copies/ g soil (50) could be detected depending on the type of treatment applied. Although reductive dechlorination by *Dehalococcoides* spp. is an energy yielding process, microcosm studies conducted under controlled environmental conditions showed that growth of the organisms is relatively slow (28). Moreover, the presence of other halorespiring species may result in competition for chlorinated compounds or electron donors. This may adversely affect the success of the reductive dechlorination of HCB in natural environments. Hence, monitoring the indigenous dechlorinating species is needed to understand their diversity and activity in contaminated sites.

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

Materials and Methods

Study sites and sampling procedure

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

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

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Nucleic Acid Extraction

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

Reverse transcription (RT) and PCR amplification

DGGE-PCR for *Dehalococcoides* spp. A nested RT-PCR approach was used to specifically amplify *Dehalococcoides spp*. 16S rRNA fragments. Reverse transcription of 16S rRNA and subsequent PCR amplifications were performed in the same tube by using the Access RT-PCR System® (Promega, Madison, WI). The reaction mix (50 µL total volume) consisted of 10-15 ng of total RNA, 0.8×AMV/Tfl Reaction Buffer, 1 mM $MgSO_4$, 0.1 mM dNTP mix, 0.2 µM of each primer (DeF and DeR, Table S2), 4U of AMV polymerase, 4U Tfl polymerase, 0.5 µL BSA (20mg/mL, Roche). Reverse transcription and further PCR amplification was carried 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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68°C at 1 min and final elongation at 68°C for 10 min. The products of RT-PCR were then used as template for PCR for the generation of amplicons suitable for analysis by denaturing gradient gel electrophoresis (DGGE) using previously described conditions (53), with primers 968F -introducing a GC-clamp (40)- and DHC1350R (Table S2).

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

DGGE

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

Real-Time Quantitative PCR

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

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

Cloning and Sequencing

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

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

Microarray Analysis

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

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

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

Multivariate Analysis

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

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

Results

Sediment and soil geochemistry

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

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

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

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

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

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

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

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

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

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

Clone libraries of the most diverse DGGE fingerprints were constructed from 16S rRNA fragments amplified by RT-PCR to confirm that all bands indeed correspond to *Dehalococcoides* spp. related populations. Blast analysis (5) was conducted for sequences from 10 different OTUs, as defined by RFLP analysis. All the sequences from both rivers were affiliated with *Dehalococcoides* and close phylogenetic relatives. In the Ebro River sequences had 91-98% identity to 16S rRNA sequences of known *Dehalococcoides* spp. whereas the Elbe River sequences had 95-99% 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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significantly related to 16S rRNA copy numbers (p=0.006) and TKN (p=0.005). Grouping the samples in river sediment and flood plain soils showed that these two environments did not share the same species composition. Most of the species negatively correlated with increasing HCB concentrations and increasing sampling depth. In contrast to the results in the Ebro River, temporal gradients (sampling period) and TOC did not significantly affect the species composition.

GeoChip analysis of rdh gene diversity

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

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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*

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

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

Dehalococcoides spp. emerged as the most abundant dechlorinating bacteria in comparison to *Desulfitobacterium* spp. and *Dehalobacter* spp. in HCB contaminated river basins. Active *Dehalobacter* spp. could not be detected in either river basin during the two years of sampling. *Desulfitobacterium* spp., however, could be detected in most locations, albeit usually in lower numbers than *Dehalococcoides* spp. Unlike *Dehalococcoides* spp., relative abundances of *Desulfitobacterium* spp. were higher in flood plain soils of the Elbe River. However, a similar trend was not 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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572 **References**

573 1. **Adrian, L., and H. Gorisch.** 2002. Microbial transformation of chlorinated
574 benzenes under anaerobic conditions. Res. Microbiol. **153:**131-137.
575 2. **Adrian, L., S. K. Hansen, J. M. Fung, H. Gorisch, and S. H. Zinder.** 2007.
576 Growth of *Dehalococcoides* strains with chlorophenols as electron acceptors.
577 Environ. Sci. Tech. **41:**2318-2323.
578 3. **Adrian, L., U. Szewzyk, J. Wecke, and H. Gorisch.** 2000. Bacterial
579 dehalorespiration with chlorinated benzenes. Nature **408:**580-583.

AEM Accepts published online ahead of print

4. **Agency for Toxic Substances and Disease Registry (ATSDR)** 2002, posting date. Toxicological profile for HCB. Agency for Toxic Substances and Disease Registry (ATSDR). [http://www.atsdr.cdc.gov/toxprofiles/tp90.html]

5. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.

6. **Bailey, R. E.** 2001. Global hexachlorobenzene emissions. Chemosphere **43:**167-182.

7. **Barber, J. L., A. J. Sweetman, D. van Wijk, and K. C. Jones.** 2005. Hexachlorobenzene in the global environment: Emissions, levels, distribution, trends and processes. Sci. Total Environ. **349:**1-44.

8. **Batalla, R. J., C. M. Gomez, and G. M. Kondolf.** 2004. Reservoir-induced hydrological changes in the Ebro river basin (NE Spain). J. Hydrol. **290:**117-136.

9. **Bossert, I. D., M. M. Haggblom, and L. Y. Young.** 2003. Microbial ecology of dehalogenation, p. 33-52, Dehalogenation: Microbial Processes and Environmental Applications.

10. **Box, G. E. P., and D. R. Cox.** 1964. An analysis of transformations. J. Roy. Stat. Soc. B **26:**211-252.

11. **Bunge, M., L. Adrian, A. Kraus, M. Opel, W. G. Lorenz, J. R. Andreesen, H. Gorisch, and U. Lechner.** 2003. Reductive dehalogenation of chlorinated dioxins by an anaerobic bacterium. Nature **421:**357.

12. **Confederación Hidrográfica del Ebro** 2008, posting date. *Control del Estado de las Masas de Agua Superficiales - Resultados analíticos*. [http://oph.chebro.es/DOCUMENTACION/Calidad/cemas/inicio.htm]

AEM Accepts published online ahead of print

13. **Day, J. W., Jr, E. Maltby, and C. Ibanez.** 2006. River basin management and delta sustainability: A commentary on the Ebro Delta and the Spanish National Hydrological Plan. Ecol. Eng. **26:**85-99.

14. **Decision No. 2455/2001/EC.** 2001. Decision No. 2455/2001/EC of the European Parliament and of the Council of 20 November 2001. The list of priority substances in the field of water policy and amending Directive 2000/60/EC. OJ. L. **331:**1-5.

15. **Eljarrat, E., and D. Barcelo.** 2007. Occurrence, fate, and behavior of brominated flame retardants in Ebro River Basin (Spain), p. 283-299, The Fate of Persistent Organic Pollutants in the Environment.

16. **Fazi, S., F. Aulenta, M. Majone, and S. Rossetti.** 2008. Improved quantification of *Dehalococcoides* species by fluorescence in situ hybridization and catalyzed reporter deposition. Syst. Appl. Microbiol. **31:**62-67.

17. **Fennell, D. E., I. Nijenhuis, S. F. Wilson, S. H. Zinder, and M. M. Haggblom.** 2004. *Dehalococcoides ethenogenes* Strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. Environ. Sci. Tech. **38:**2075-2081.

18. **Fernandez, M. A., C. Alonso, M. J. Gonzalez, and L. M. Hernandez.** 1999. Occurrence of organochlorine insecticides, PCBs and PCB congeners in waters and sediments of the Ebro River (Spain). Chemosphere **38:**33-43.

19. **Gomez-Gutierrez, A. I., E. Jover, L. Bodineau, J. Albaiges, and J. M. Bayona.** 2006. Organic contaminant loads into the Western Mediterranean Sea: Estimate of Ebro River inputs. Chemosphere **65:**224-236.

AEM Accepts published online ahead of print

20. **Gray, N. D., D. Comaskey, I. P. Miskin, R. W. Pickup, K. Suzuki, and I. M. Head.** 2004. Adaptation of sympatric *Achromatium* spp. to different redox conditions as a mechanism for coexistence of functionally similar sulphur bacteria. Environ. Microbiol. **6:**669-677.

21. **Gribble, G. W.** 2003. The diversity of naturally produced organohalogens. Chemosphere **52:**289-297.

22. **Hageman, K. J., S. L. Simonich, D. H. Campbell, G. R. Wilson, and D. H. Landers.** 2006. Atmospheric deposition of current-use and historic-use pesticides in snow at national parks in the western United States. Environ. Sci. Tech. **40:**3174-3180.

23. **Häne, B. G., K. Jäger, and H. G. Drexler.** 1993. The Pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. Electrophoresis **14:**967-972.

24. **He, J., K. M. Ritalahti, M. R. Aiello, and F. E. Loffler.** 2003. Complete detoxification of vinyl chloride by an anaerobic enrichment culture and identification of the reductively dechlorinating population as a *Dehalococcoides* species. Appl. Environ. Microbiol. **69:**996-1003.

25. **He, Z., T. J. Gentry, C. W. Schadt, L. Wu, J. Liebich, S. C. Chong, Z. Huang, W. Wu, B. Gu, P. Jardine, C. Criddle, and J. Zhou.** 2007. GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. ISME Journal **1:**67-77.

26. **Hendrickson, E. R., J. A. Payne, R. M. Young, M. G. Starr, M. P. Perry, S. Fahnestock, D. E. Ellis, and R. C. Ebersole.** 2002. Molecular analysis of

AEM Accepts published online ahead of print

Dehalococcoides 16S ribosomal DNA from chloroethene-contaminated sites throughout north America and Europe. Appl. Environ. Microbiol. **68:**485-95.

27. **Higashino, M., and H. G. Stefan.** 2005. Sedimentary microbial oxygen demand for laminar flow over a sediment bed of finite length. Water Res. **39:**3153-3166.

28. **Holliger, C., C. Regeard, and G. Diekert.** 2003. Dehalogenation by anaerobic bacteria, p. 115-157, Dehalogenation: Microbial Processes and Environmental Applications.

29. **Holliger, C., G. Schraa, A. J. Stams, and A. J. Zehnder.** 1992. Enrichment and properties of an anaerobic mixed culture reductively dechlorinating 1,2,3-trichlorobenzene to 1,3-dichlorobenzene. Appl. Environ. Microbiol. **58:**1636-44.

30. **Kittelmann, S., and M. W. Friedrich.** 2008. Identification of novel perchloroethene-respiring microorganisms in anoxic river sediment by RNA-based stable isotope probing. Environ. Microbiol. **10:**31-46.

31. **Krüger, F., R. Meissner, A. Gröngröft, and K. Grunewald.** 2005. Flood induced heavy metal and arsenic contamination of Elbe river floodplain soils. Acta. Hydroch. Hydrob. **33:**455-465.

32. **Lacorte, S., D. Raldua, E. Martinez, A. Navarro, S. Diez, J. M. Bayona, and D. Barcelo.** 2006. Pilot survey of a broad range of priority pollutants in sediment and fish from the Ebro river basin (NE Spain). Environ. Pollut. **140:**471-482.

33. **Lendvay, J. M., F. E. Loffler, M. Dollhopf, M. R. Aiello, G. Daniels, B. Z. Fathepure, M. Gebhard, R. Heine, R. Helton, J. Shi, R. Krajmalnik-Brown, C. L. Major, M. J. Barcelona, E. Petrovskis, R. Hickey, J. M.**

AEM Accepts published online ahead of print

Tiedje, and P. Adriaens. 2003. Bioreactive barriers: A comparison of bioaugmentation and biostimulation for chlorinated solvent remediation. Environ. Sci. Tech. **37:**1422-1431.

34. **Löffler, F. E., Q. Sun, J. Li, and J. M. Tiedje.** 2000. 16S rRNA gene-based detection of tetrachloroethene-dechlorinating *Desulfuromonas* and *Dehalococcoides* species. Appl. Environ. Microbiol. **66:**1369-74.

35. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Forster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. Konig, T. Liss, R. Lussmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucl. Acids Res. **32:**1363-1371.

36. **Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese.** 1996. The Ribosomal Database Project (RDP). Nucl. Acids Res. **24:**82-85.

37. **Maymo-Gatell, X., T. Anguish, and S. H. Zinder.** 1999. Reductive dechlorination of chlorinated ethenes and 1, 2-dichloroethane by "*Dehalococcoides ethenogenes*" 195. Appl. Environ. Microbiol. **65:**3108-13.

38. **Meijer, S. N., W. A. Ockenden, A. Sweetman, K. Breivik, J. O. Grimalt, and K. C. Jones.** 2003. Global distribution and budget of PCBs and HCB in background surface soils: Implications for sources and environmental processes. Environ. Sci. Tech. **37:**667-672.

39. **Muller, J. A., B. M. Rosner, G. von Abendroth, G. Meshulam-Simon, P. L. McCarty, and A. M. Spormann.** 2004. Molecular Identification of the

AEM Accepts published online ahead of print

catabolic vinyl chloride reductase from *Dehalococcoides* sp. strain VS and its environmental distribution. Appl. Environ. Microbiol. **70:**4880-4888.

40. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695-700.

41. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Leeuwenhoek **73:**127-41.

42. **Petty, J. D., J. N. Huckins, D. A. Alvarez, W. G. Brumbaugh, W. L. Cranor, R. W. Gale, A. C. Rastall, T. L. Jones-Lepp, T. J. Leiker, C. E. Rostad, and E. T. Furlong.** 2004. A holistic passive integrative sampling approach for assessing the presence and potential impacts of waterborne environmental contaminants. Chemosphere **54:**695-705.

43. **Plimmer, J. R., and U. I. Klingebiel.** 1976. Photolysis of hexachlorobenzene. J Agr Food Chem **24:**721-723.

44. **Pruesse, E., C. Quast, K. Knittel, B. M. Fuchs, W. Ludwig, J. Peplies, and F. O. Glockner.** 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucl. Acids Res. **35**:7188-7196.

45. **Rahm, B. G., R. M. Morris, and R. E. Richardson.** 2006. Temporal expression of respiratory genes in an enrichment culture containing *Dehalococcoides ethenogenes*. Appl. Environ. Microbiol. **72:**5486-91.

AEM Accepts published online ahead of print

725 46. **Rahm, B. G., and R. E. Richardson.** 2008. Correlation of respiratory gene
726 expression levels and pseudo-steady-state PCE respiration rates in
727 *Dehalococcoides ethenogenes*. Environ. Sci. Tech. **42:**416-421.
728 47. **Rovira, A., and C. Ibàñez.** 2007. Sediment management options for the lower
729 Ebro river and its delta. JSS **7:**285-295.
730 48. **Sanguinetti, C. J., E. Dias Neto, and A. J. G. Simpson.** 1994. Rapid silver
731 staining and recovery of PCR products separated on polyacrylamide gels.
732 Biotechniques **17:**915–919.
733 49. **Sierra, J. P., A. Sánchez-Arcilla, P. A. Figueras, J. G. Del Río, E. K.**
734 **Rassmussen, and C. Mösso.** 2004. Effects of discharge reductions on salt
735 wedge dynamics of the Ebro River. River Res. Appl. **20:**61-77.
736 50. **Sleep, B. E., D. J. Seepersad, K. Mo, C. M. Heidorn, L. Hrapovic, P. L.**
737 **Morrill, M. L. McMaster, E. D. Hood, C. LeBron, B. SherwoodLollar, D.**
738 **W. Major, and E. A. Edwards.** 2006. Biological enhancement of
739 tetrachloroethene dissolution and associated microbial community changes.
740 Environ. Sci. Tech. **40:**3623-3633.
741 51. **Smidt, H., and W. M. de Vos.** 2004. Anaerobic microbial dehalogenation.
742 Annu. Rev. Microbiol. **58:**43-73.
743 52. **Smits, T. H. M., C. Devenoges, K. Szynalski, J. Maillard, and C. Holliger.**
744 2004. Development of a real-time PCR method for quantification of the three
745 genera *Dehalobacter*, *Dehalococcoides*, and *Desulfitobacterium* in microbial
746 communities. J. Microbiol. Meth. **57:**369-378.
747 53. **Sousa, D. Z., M. A. Pereira, H. Smidt, A. J. M. Stams, and M. M. Alves.**
748 2007. Molecular assessment of complex microbial communities degrading

AEM Accepts published online ahead of print

long chain fatty acids in methanogenic bioreactors. FEMS Microbiol Ecol **60:**252-265.

54. **Taş, N.** 2009. *Dehalococcoides* spp. in River Sediments: Insights in Functional Diversity and Dechlorination Activity. PhD dissertation, Wageningen University, Wageningen.

55. **Vericat, D., and R. J. Batalla.** 2005. Sediment transport in a highly regulated fluvial system during two consecutive floods (lower Ebro River, NE Iberian Peninsula). Earth Surf Process Landforms **30:**385-402.

56. **Villemur, R., M. Lanthier, R. Beaudet, and F. Lepine.** 2006. The *Desulfitobacterium* genus. FEMS Microbiol. Rev. **30:**706-733.

57. **West, K. A., D. R. Johnson, P. Hu, T. Z. DeSantis, E. L. Brodie, P. K. H. Lee, H. Feil, G. L. Andersen, S. H. Zinder, and L. Alvarez-Cohen.** 2008. Comparative Genomics of "*Dehalococcoides ethenogenes*" 195 and an Enrichment Culture Containing Unsequenced "*Dehalococcoides*" Strains. Appl. Environ. Microbiol. **74:**3533-3540.

58. **Wu, L., X. Liu, C. W. Schadt, and J. Zhou.** 2006. Microarray-based analysis of subnanogram quantities of microbial community DNAs by using whole-community genome amplification. Appl. Environ. Microbiol. **72:**4931-4941.

59. **Yergeau, E., S. Kang, Z. He, J. Zhou, and G. A. Kowalchuk.** 2007. Functional microarray analysis of nitrogen and carbon cycling genes across an Antarctic latitudinal transect. ISME Journal **1:**163-179.

60. **Yoshida, N., K. Asahi, Y. Sakakibara, K. Miyake, and A. Katayama.** 2007. Isolation and quantitative detection of tetrachloroethene (PCE)-dechlorinating bacteria in unsaturated subsurface soils contaminated with chloroethenes. J. Biosci. Bioeng. **104:**91-97.

AEM Accepts published online ahead of print

774 61. **Zhou, J., M. A. Bruns, and J. M. Tiedje.** 1996. DNA recovery from soils of
775 diverse composition. Appl. Microbiol. Biotechnol. **62:**316-22.
776

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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,

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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

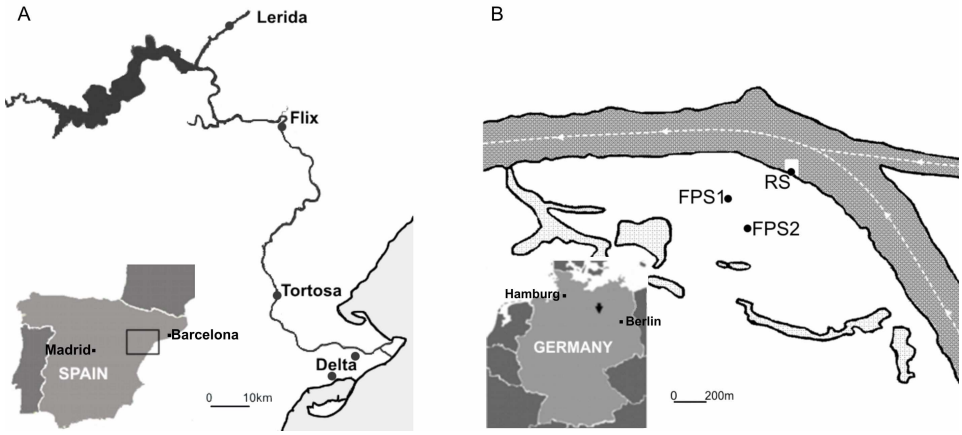

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Fig. 2

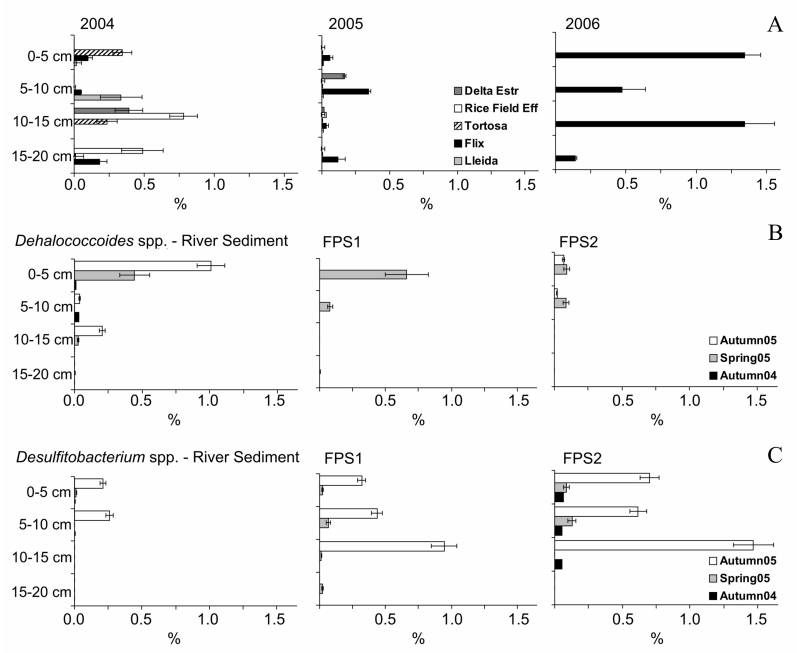

Fig. 3

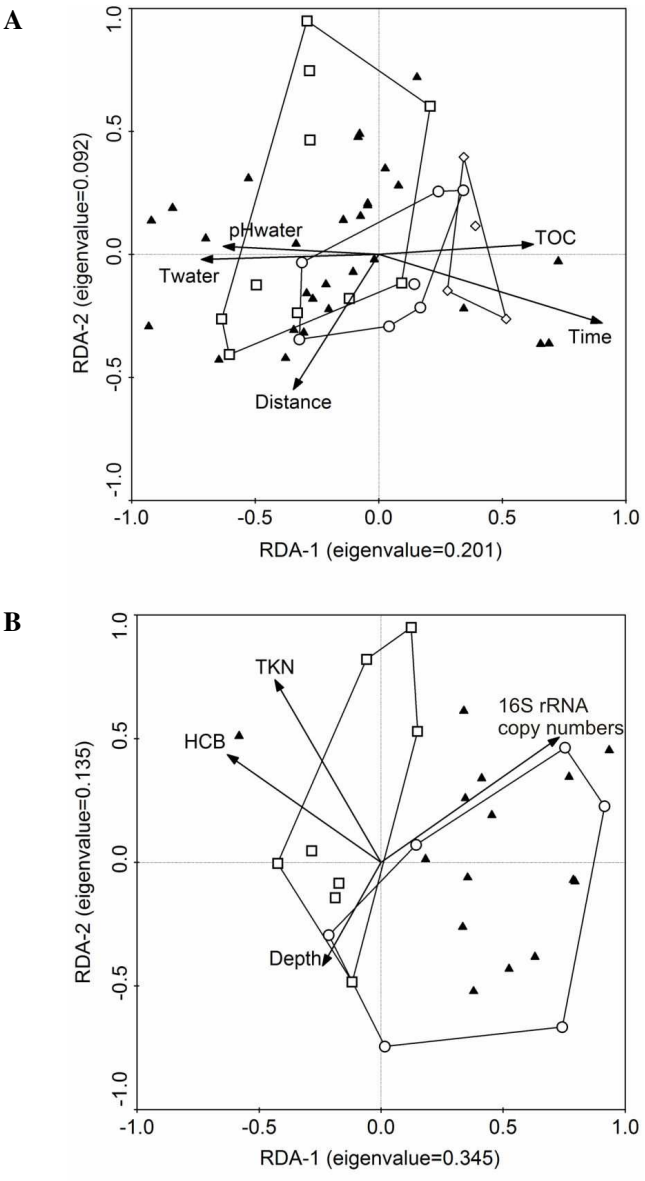

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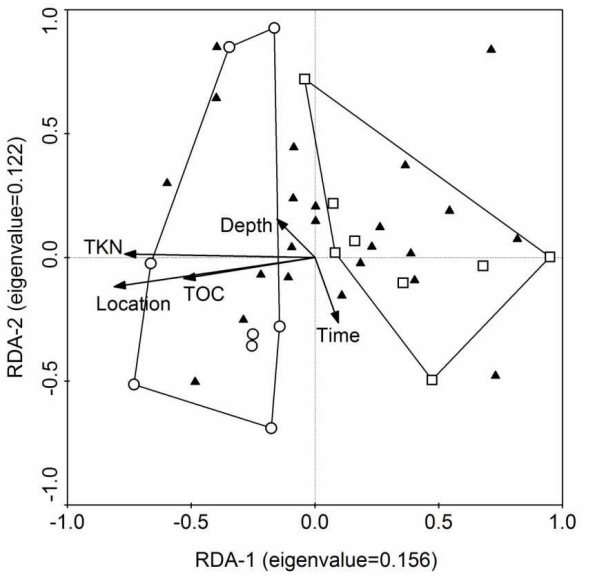
Fig. 4



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