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

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Hexachlorobenzene (HCB) has been widely used in chemical manufacturing processes and as a pesticide. Due to its resistance to biological degradation, HCB has mainly accumulated in freshwater 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 rRNA-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* sp. 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 into the natural occurrence and dynamics of active *Dehalococcoides* spp. in HCB-contaminated river basins.

Halogenated organic compounds are among the most widespread environmental pollutants. Although these compounds were previously believed to be only anthropogenic, a large number of them, 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 bioaccumulative compound and is listed in the EC Directive (15) as a "priority hazardous substance." At the peak production of HCB in the early 1980s, thousands of tons were produced to be used as fungicides, wood preservatives, and porosity control agents or in the manufacturing of dyes. The use of HCB is no longer allowed in most countries because of its toxicity and carcinogenicity toward fish and mammals. Nevertheless, it is still being released into 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 the sea (32, 42), significantly larger 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 on regional or global scales. Nevertheless, our knowledge of 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 deposition in sediments. In these light-limited environments, biodegradation offers great potential for transforming this persistent organic pollutant (7, 29). The only known pathway for microbial dehalogenation of HCB is reductive dechlorination under anaerobic conditions, which results in formation of less chlorinated benzenes (1).

The reductively dechlorinating bacteria isolated up to now belong to the *Deltaproteobacteria* and *Epsilonproteobacteria* (*Geobacter*, *Sulfurospirillum*, *Desulfuromonas*, and *Desulfomonile*), the *Firmicutes* (*Desulfitobacterium* and *Dehalobacter*), or the *Chloroflexi* ("*Dehalococcoides*" and related groups) (51). So far, however, *Dehalococcoides* is the only bacterial genus whose members are known to transform HCB. Several *Dehalococcoides* strains that could grow with a broad variety of chlorinated aliphatic and aromatic compounds, including chlorinated benzenes and phenols, biphenyls, chloroethenes, and dioxins, were isolated. Nevertheless, until now only two strains,

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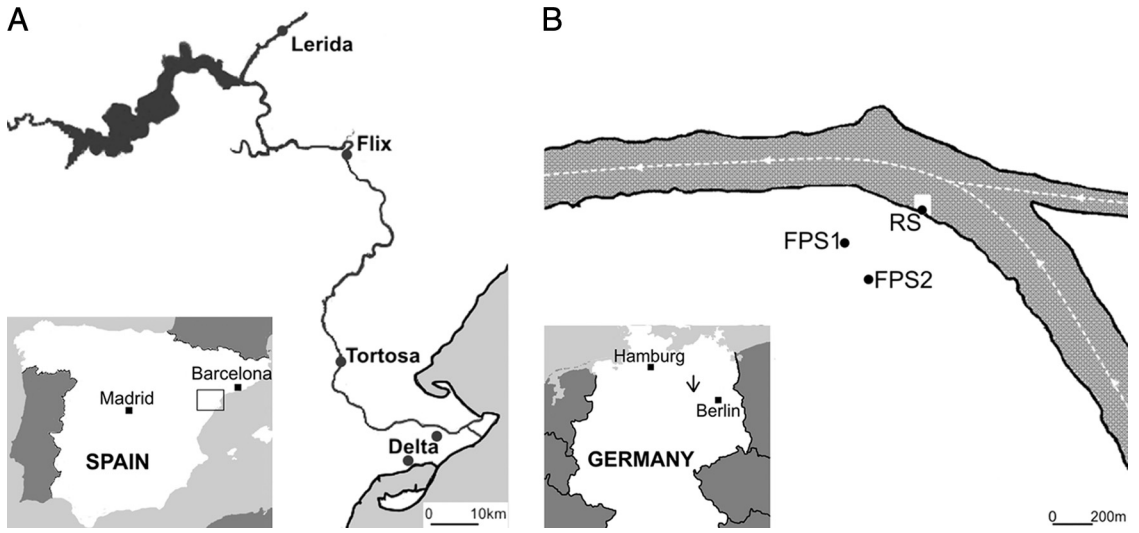

FIG. 1. Sampling locations in the Ebro (A) and the Elbe (B) rivers. Maps were redrawn from OpenStreetMap (http://www.openstreetmap.org). Black squares represent approximate locations of the major cities closest to the sampling sites. Both maps are drawn according to scale as mentioned. (A) The Ebro River is located in the northeast of the Iberian peninsula. Sampling was done in the downstream area of the river over a distance of approximately 200 km. Black dots represent sampling locations. (B) The Elbe River sampling points were river sediment (RS), floodplain soil 1 (FPS1), and floodplain soil 2 (FPS2). The black arrow represents the location of the sampling site in Germany. The dotted white line with arrowheads represents the flow direction of the Elbe.

Dehalococcoides sp. strain CBDB1 (3) and "*Dehalococcoides ethenogenes*" 195 (17), which can transform HCB to tri- and dichlorobenzenes and use the energy conserved in the process for growth, could be isolated. Besides HCB, *Dehalococcoides* sp. strain CBDB1 can also reductively dechlorinate chlorinated dioxins (11) and chlorophenols (2), whereas *Dehalococcoides ethenogenes* 195 can dechlorinate various chlorinated ethenes, 1,2-dichloroethane, and vinyl chloride (37).

Until now, microbial community analyses of *Dehalococcoides* spp. largely focused on chlorinated ethene-contaminated aquifers or soils. The presence of *Dehalococcoides* spp. in uncontaminated and contaminated (with tetrachloroethene [PCE], trichloroethene [TCE], or vinyl chloride) sites from North America, Europe, and Japan was reported elsewhere (24, 26, 30, 34, 60). Furthermore, quantitative analyses targeting the *Dehalococcoides* 16S rRNA gene in chlorinated ethene bioremediation sites showed that 8.6×10^3 to 2.5×10^6 copies/g aquifer material (33) and 1.9×10^2 to 1.1×10^7 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 of 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, floodplain, 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 the natural occurrence and dynamics of reductively dechlorinating bacteria, generating important knowledge toward 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 in the supplemental material). The Ebro River (928 km) is located in the northeast of Spain (Fig. 1A). The Ebro River delta (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 100 years of activity. At this location, HCB concentrations in the river sediment are higher than those elsewhere in the Ebro River (19, 32). The Elbe River (1,091 km) is one of the longest rivers in central Europe, flowing from the 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 floodplain 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 polyvinyl chloride tubes (25 cm; internal diameter, 4 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 (5 cm thick; approximately 25 g material) and homogenized by being mixed with a spoon. This resulted in samples representing 0 to 5 cm, 5 to 10 cm, 10 to 15 cm, and 15 to 20 cm of depth in sediment or soil. The slices were transferred into 50-ml 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 ac-

cording to standardized methods. HCB concentrations were measured according to the ISO 10382 protocol.

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 g 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 1 hour. Total RNA was eluted with diethyl pyrocarbonate-treated distilled H_2O, which was supplied by the manufacturer. RNA purity was checked by electrophoresis in 1.0% (wt/vol) low-melting-point 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 DNA spin 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).

RT and PCR amplification. (i) DGGE-PCR for *Dehalococcoides* spp. A nested reverse transcription-PCR (RT-PCR) approach was used to specifically amplify *Dehalococcoides* 16S rRNA fragments. RT 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 to 15 ng of total RNA, 0.8× avian myeloblastosis virus/Tfl reaction buffer, 1 mM $MgSO_4$, 0.1 mM deoxynucleoside triphosphate mix, 0.2 μM of each primer (DeF and DeR [see Table S2 in the supplemental material]), 4 U of avian myeloblastosis virus polymerase, 4 U Tfl polymerase, and 0.5 μl bovine serum albumin (20 mg/ml; Roche). RT and further PCR amplification were carried out at 45°C for 45 min, 94°C for 2 min, and 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, followed by 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) under previously described conditions (53), with primers 968F—introducing a GC clamp (40)—and DHC1350R (see Table S2 in the supplemental material).

(ii) RT of RNA templates for quantitative PCR. RT of 16S rRNA was performed as described above but with primers 27F and 1492R (see Table S2 in the supplemental material) with second-strand synthesis to produce double-stranded cDNA fragments.

DGGE. DGGE was performed according to the protocol of Muyzer and Smalla (41) using the Bio-Rad gene detection system (Bio-Rad, 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.

qPCR. Real-time quantitative PCR (qPCR) was performed using an iQ5 iCycler (Bio-Rad, Veenendaal, The Netherlands) with the thermocycling program as previously described (52) for 16S rRNA genes of dehalogenating bacteria (*Dehalococcoides*, *Desulfitobacterium*, and *Dehalobacter*) and total bacteria using SYBR green dye. PCR mixtures were prepared in a 25-μl total reaction volume containing 5 μl template cDNA or DNA, 1× Bio-Rad SYBR green PCR master mix (Bio-Rad, Veenendaal, The Netherlands), 0.2 μM of each primer (see Table S2 in the supplemental material), 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 by using T7 and SP6 promoter-targeted primers (see Table S2 in the supplemental material) 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 the Flix location. For the 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 to 5 cm. The PCR products were cloned using the pGEM-T Easy plasmid vector (Promega, Madison, WI) and *Escherichia 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 and/or AluI at 37°C for 3 h. Digestion mixtures (20 μl) contained 5 μl of the PCR product, 0.25 U of the respective restriction endonuclease (Promega, Madison, WI), 0.1 mg of acetyl bovine serum albumin (Promega, Madison, WI), and 1× restriction buffer (Promega, Madison, WI). The resulting fragments were separated by electrophoresis for 1 h at 125 V in 12% (wt/vol) precast 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 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*) gene 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 were performed as described previously (25). A list of all *rdh* gene sequences for which additional probes were designed is given in an Excel file in the supplemental material. Samples from two locations, upstream (Flix) and downstream (rice fields), and two depths (0 to 5 cm and 10 to 15 cm) were analyzed with the GeoChip. High-molecular-weight DNA extraction was performed by lysis in a cetyltrimethylammonium bromide 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 the manufacturer's instructions. Spermidine (0.1 μg/μl) and single-strand binding protein (0.04 mM) were added to the reaction mixture to aid amplification. The reaction mixtures were incubated at 30°C for 3 h, and the enzyme was then inactivated by incubation at 60°C for 10 min. The amplification products were labeled with Cy5 dye (Amersham, Piscataway, NJ). Hybridizations were performed in an HS4800 hybridization station (Tecan US, Durham, NC) as previously described (59) with the following modifications. The first wash was carried out at 50°C for 1 min with a prehybridization solution (5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate, and 0.1% bovine serum albumin) followed by a 45-min prehybridization. The slides were then washed four times with water at 23°C for 5 min with 30 s of soaking. Labeled DNA dissolved in the hybridization solution was then injected at 60°C, and hybridization was carried out at 42°C for 10 h with high agitation. Slides were then washed and dried under a flow of nitrogen gas. Arrays were scanned using a ProScanArray microarray scanner (Perkin-Elmer, Boston, MA) at 633 nm using a laser power of 95% and a photomultiplier tube 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: a signal-to-noise ratio of <1.2 and a cumulative variance of background signal of <30% were chosen due to highly variable total hybridization signal between the different samples. Spots with signal intensities twice as high as those of the rest of the designated gene probes were accepted as outliers and removed from the analysis.

Multivariate analysis. In order to relate the changes in the *Dehalococcoides* 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, number of *Dehalococcoides* 16S rRNA copies/g sediment, HCB 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 that *Dehalococcoides* fingerprints are not related to environmental variables. The community structure was visualized via ordination triplots with scaling focused on intersample differences. Multivariate analysis of microarray data was performed using calculated relative intensity (abundance) values for each hybridization signal, which were normalized with Box-Cox trans-

formation (10) prior to analysis. RDA (CANOCO 4.5) was used to test the null hypothesis that variances in reductive dehalogenase genes are not related to environmental variables. Gene distributions were plotted with scaling focused on intersample differences. For all statistical analyses, correlations were considered highly significant at $P < 0.05$ and significant at $P < 0.10$ unless mentioned otherwise. All analyses of variance and correlations analyses were performed in R software.

Nucleotide sequence accession numbers. Sequences belonging to two operational taxonomic units (OTUs) from the Ebro River and eight OTUs from the Elbe River were deposited in the NCBI database. Sequences EU700499 and EU700500 originate from the Ebro River. Sequences EU700494 to EU700497 and EU700502 to EU700505 are from the Elbe River.

RESULTS

Sediment and soil geochemistry. The geochemistry of sediment and soil samples varied significantly between different sampling locations in the Ebro River (Fig. 1A; see also Tables S1, S3, and S4 in the supplemental material). 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 did floodplain soils. Moreover, pesticide contamination was significantly higher in floodplain soils than in river sediments. In the Elbe, HCB was found to be the main contaminant, which was followed by DDT and hexachlorocyclohexane.

Impact of sediment (soil) geochemistry and spatial and temporal gradients on 16S rRNA abundance of dechlorinating bacteria. 16S rRNA-targeted RT-qPCR assays were used for quantitative detection of several dechlorinating genera in the river basins. *Dehalococcoides* and *Desulfitobacterium* 16S rRNA could be detected in different quantities in the sampling locations (see Table S5 in the supplemental material), whereas *Dehalobacter* rRNA could not be detected. Both absolute rRNA copy numbers and the relative abundances (i.e., *Dehalococcoides* 16S rRNA copies/total bacterial 16S rRNA copies) were used to calculate pairwise correlations (Spearman's correlation coefficient, r_s) of each genus with environmental variables (see Table S6 in the supplemental material). In the Ebro River, total bacterial rRNA copy numbers were significantly higher in samples with high TOC, TKN, and TP contents. Correlations 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* rRNA was consistently and significantly more abundant in the upstream locations (Lleida and Flix) than in the downstream locations (Tortosa, rice fields, and estuary of the Ebro Delta) ($r_s = -0.77, P \leq 0.001$) (Fig. 2A; see also Table S6 in the supplemental material). Other environmental variables, including HCB pollution, did not significantly contribute to explaining the changes in the relative abundance of *Dehalococcoides* 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* rRNA copies as well as the ratio of 16S rRNA copies to 16S rRNA gene copies (rRNA/DNA) around the regular sampling point (see Fig. S1 in the supplemental material). Results showed that relative abundance and rRNA/DNA of *Dehalococcoides* spp. could vary remarkably. For example, in the upper 5 cm, fourfold to fivefold differences in the rRNA/DNA ratio and 2- 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 relative abundances similar to those observed for the Ebro, comprising on average 0.26% of the bacterial 16S rRNA pool. However, for the floodplain soils rRNA abundances were considerably lower (0.05 to 0.12% [Fig. 2B]). During the sampling period, species abundances tended to increase ($r_s = 0.44, P \leq 0.10$). *Dehalococcoides* rRNA, when detected, was most abundant in the upper layers of sediments ($r_s = -0.40, P \leq 0.10$), with proportions as high as 1%. Geochemical parameters, including HCB concentrations, had no significant correlation with *Dehalococcoides* rRNA abundances.

The abundances of *Desulfitobacterium* rRNA were 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 those for *Dehalococcoides* spp. (data not shown). Throughout the sampling period, the relative abundance of *Desulfitobacterium* rRNA decreased in upstream locations and increased in the downstream locations in the river delta. Furthermore, in the Elbe River significant increases in *Desulfitobacterium* 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* rRNA were higher in floodplain soils than in river sediment throughout the sampling period ($r_s = 0.37, P \leq 0.1$). The highest relative abundances were detected in deeper (5- to 15-cm) layers of the soils (Fig. 2C).

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 (see Fig. S2 in the supplemental material). 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 floodplain soils were as low as 9% (see Fig. S3 in the supplemental materials). Except for samples taken in October 2004, *Dehalococcoides* 16S rRNA fingerprints could be generated only for samples from the top 10 cm of the river sediment. *Dehalococcoides* fingerprints showed 77% correlation in this fraction of

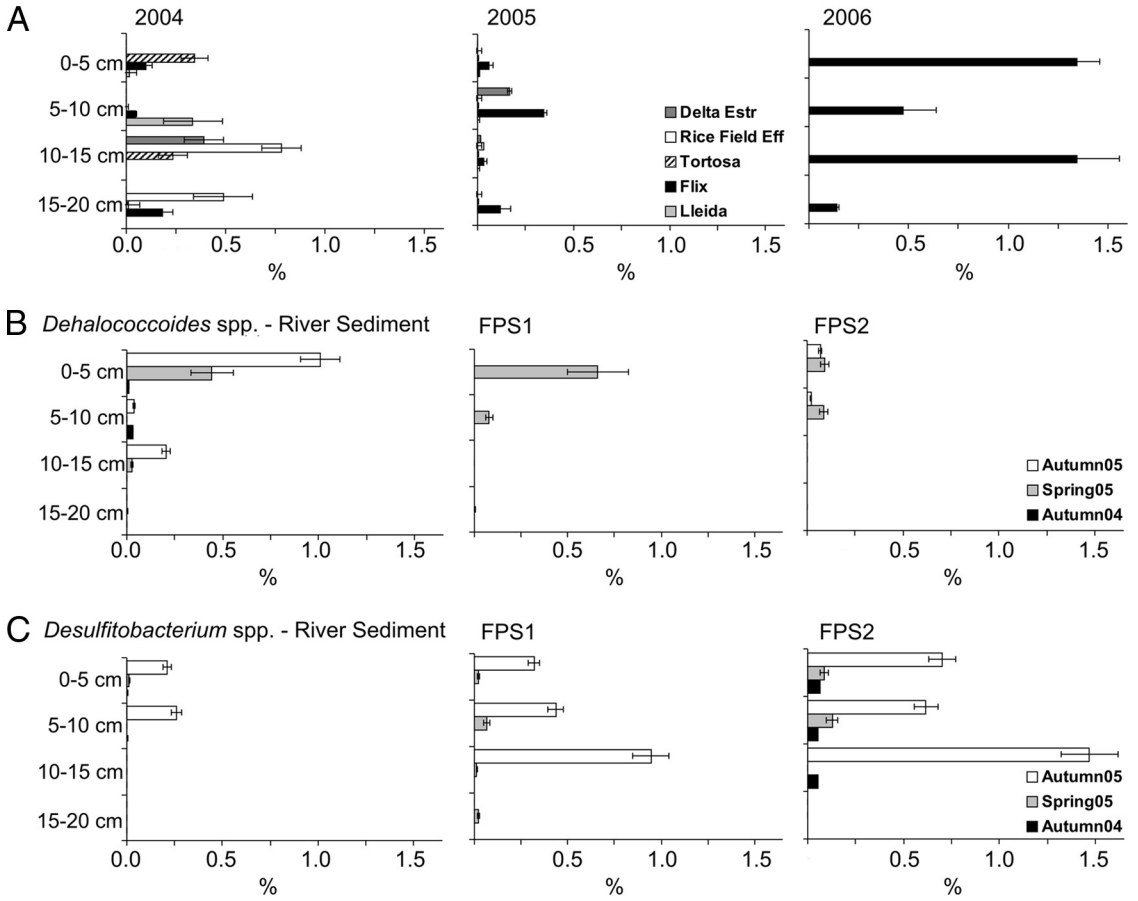

FIG. 2. Changes in the relative abundance of 16S rRNA copies of dechlorinating bacteria given as percentages of all bacterial 16S rRNA copies. The error bars represent the standard deviations of duplicate measurements. (A) *Dehalococcoides* spp. in the Ebro River during the sampling period in 2004, 2005, and 2006. The horizontal axis shows relative abundances as percentages. The vertical axis represents sample depth. (B and C) Changes in the relative abundances of *Dehalococcoides* and *Desulfitobacterium* 16S rRNA copies in the Elbe River during a sampling period of 1.5 years. The horizontal axis shows relative abundances as percentages. The vertical axis represents sample depth. Locations of the floodplain soils 1 and 2 are indicated in Fig. 1.

the sediment throughout the sampling period (data not shown). Variations observed between DGGE fingerprints concerned differences in *Dehalococcoides* composition rather than variation in the intensity of the bands. This was supported by the fact that pairwise 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*-related populations. BLAST analysis (5) was conducted for sequences from 10 different OTUs, as defined by restriction fragment length polymorphism analysis. All the sequences from both rivers were affiliated with *Dehalococcoides* and close phylogenetic relatives. In the Ebro River, sequences had 91 to 98% identity to 16S rRNA sequences of known *Dehalococcoides* spp., whereas the Elbe River sequences had 95 to 99% identity (see Fig. S4 in the supplemental material).

RDA of *Dehalococcoides* 16S rRNA abundance and composition. Multivariate statistics were used to determine to what extent environmental parameters (i.e., spatial and temporal gradients and sediment [or soil] geochemistry) and 16S rRNA abundance contributed to the differences in the *Dehalococcoides*-specific DGGE fingerprints. The analysis was conducted on band positions (i.e., presence/absence). In the Ebro River, the distribution of *Dehalococcoides* spp. in the ordination space was most significantly correlated with the gradient "time" (sampling period, $P \leq 0.001$) (Table 1). In addition, a Monte Carlo significance test revealed that also the geographical distances (sampling location) had a significant effect on *Dehalococcoides* composition. The model formed by the significant environmental parameters could explain 37.7% of the variation in *Dehalococcoides* composition ($P = 0.061$). When samples were grouped based on the sampling period, samples from 2004 and 2006 did not intersect, indicating a significant change in the community composition (Fig. 3A). A smaller number of species positively correlated with increasing TOC content than with the effect of water temperature and pH. Moreover, most of the populations negatively correlated with the sampling period, indicating a decrease in richness over time. Correlations with depth (sampling depth), 16S rRNA copy numbers, and TKN, TP, and HCB concentrations were found to be insignificant.

The first two RDA axes could explain 48% of the total

TABLE 1. Summary of the results obtained for the RDA test for the significance of environmental variables in explaining the variance in *Dehalococcoides* 16S rRNA composition and reductive dehalogenase (*rdh*) gene diversity

Parameter	% of variation in composition or diversity explicable by parameter (significance[a])		
	River Elbe	River Ebro	
	Dehalococcoides 16S rRNA composition	*Dehalococcoides* 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)
No. of *Dehalococcoides* 16S rRNA copies/g sample	14.1 (**)	2.5 (NS)	NA[b]
Temp of water	NA	5.0 (+)	NA
pH of water	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 (**)

[a] NS, not significant; +, $P \leq 0.10$; *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.
[b] NA, not applicable.

variation in the *Dehalococcoides* composition in the Elbe (Fig. 3B). The distribution of samples in the ordination diagram was strongly influenced by the HCB contamination ($P \leq 0.001$), which accounted for 16.7% of the variation in species composition (Table 1). Moreover, Monte Carlo significance tests showed that variances can be significantly related to 16S rRNA copy numbers ($P = 0.006$) and TKN ($P = 0.005$). Grouping the samples in river sediment and floodplain 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 mainly contained *rdh* genes of *Dehalococcoides* sp. strain CBDB1 (namely, cbdbA88, cbdbA1535, cbdbA1578, cbdbA1582, cbdbA1595, cbdbA1624, and cbdbA1638) and *Dehalococcoides ethenogenes* 195 (namely, DET0088, DET0173, DET1522, and DET1545) (see Table S7 in the supplemental material), which are the only cultivated anaerobic bacteria known to degrade HCB (3, 17). These genes were among 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 mainly *Desulfitobacterium* spp. and *Dehalococcoides* spp., including reductive dehalogenases of strains FL2 (RdhA7), VS (*vcrA*), CBDB1 (cbdbA1582, cbdbA1535, cbdbA1578, and cbdbA1638), and *D. ethenogenes* 195 (DET0088, DET0173, DET1538, and DET1528). The top layers of the sediment and agricultural soil samples were com-

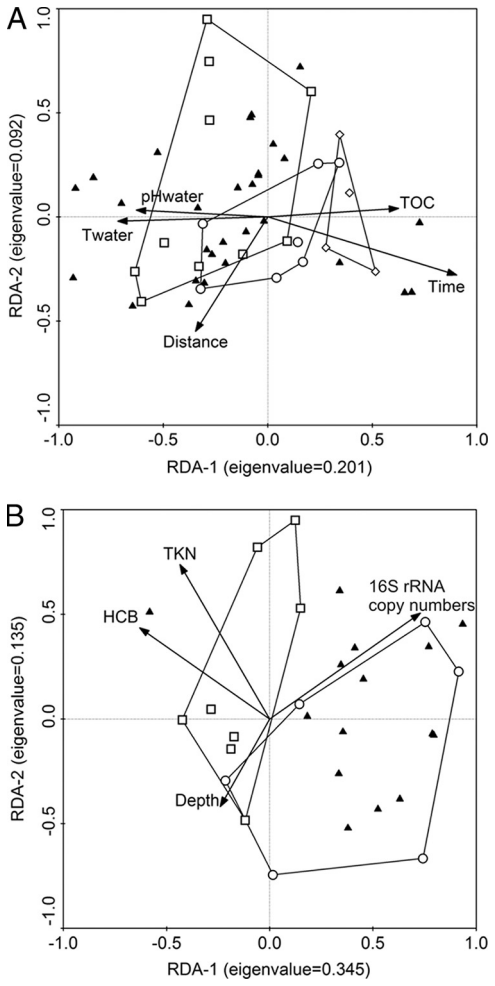

FIG. 3. Ordination triplots for RDA. Species (each *Dehalococcoides* DGGE band) are indicated by triangles (▲). Samples are indicated by open circles, squares, and diamonds as indicated below. Arrows represent environmental parameters ($P < 0.1$). The length of each gradient (eigenvalue) is indicated on the corresponding redundancy axis. The plot can be interpreted qualitatively by following the direction of arrows for environmental parameters. The arrow length corresponds to variance that can be explained by the environmental variable. The direction of an arrow indicates an increasing magnitude of the environmental variable. The perpendicular distance between species and environmental variable axes in the plot reflects their correlations. The smaller the distance, the stronger the correlation, whereas distances among species symbols are not explanatory. (A) DGGE band analysis for samples taken along the Ebro River. Samples are grouped according to sampling period (2004 [□], 2005 [○], and 2006 [◇]). (B) DGGE band analysis for Elbe River samples. Samples are grouped according to the sample type (soil [□] or sediment [○]).

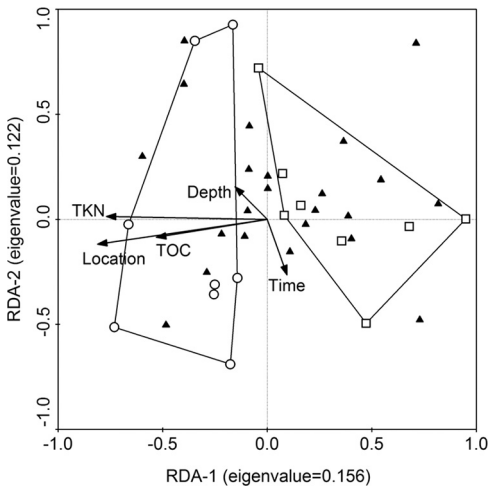

FIG. 4. Ordination triplot for RDA of *rdh* gene diversity in the Ebro River (based on GeoChip analysis). Each *rdh* gene is indicated by a triangle (▲). Samples are grouped according to sample location (upstream [□] or downstream [○]). This grouping also represents sample type (sediment or agricultural soil). Arrows represent environmental parameters ($P < 0.1$). Further explanations may be found in the Fig. 3 legend.

posed of genes originating from *Dehalococcoides* spp., whereas a mixture of *Desulfitobacterium* and *Dehalococcoides* genes was detected in bottom layers (see Table S7 in the supplemental material). The abundance of *rdh* genes varied drastically during the sampling period. Sampling depth, however, as well as sampling period and HCB concentrations did not significantly affect the *rdh* gene diversity (Fig. 4).

DISCUSSION

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

Dehalococcoides 16S rRNA relative abundance changed significantly between and within different sampling locations, depths, and periods. In some locations, relative abundance could reach up to 1% (Fig. 2A and B). The only reported 16S rRNA relative abundance for *Dehalococcoides* spp. in the environment is 2 to 6% in a PCE- and TCE-contaminated groundwater aquifer (16). Additionally, HCB and PCE transforming batch scale enrichments from Ebro and Elbe River sediment samples had a higher relative abundance of *Dehalococcoides* spp. (2 to 6%) than did the corresponding environmental samples (54). In enrichment cultures containing *D. ethenogenes* 195, 16S rRNA gene copy abundance was calculated as 7 to 62% during PCE degradation (46). When taken together, our results demonstrate that the relative abundance of *Dehalococcoides* spp. in the river basins studied here is lower than that in contaminated aquifers or enrichment cultures.

In geographically distant locations, as in the samples from the Ebro River, variance between the different sampling locations and periods could be so influential that the effects of other environmental parameters could be too small to explain the variations in the species composition. Even though water temperature, pH, and TOC appeared to be relevant parameters in explaining the variation in the species composition, they were not highly significant. It cannot be excluded that other factors, which could not be included in this study due to the lack of uninterrupted and reproducible measurements, might be of importance. Sediment transport in the river system and oxygen content in different depths of sediment (or soil) are two of these factors. The flow of the Ebro River is highly (57%) regulated by reservoirs that are used for irrigation and hydropower production. In past years, significant decreases were reported in the flood discharges (8). Reservoirs were reported to trap most of the sediment transported in the river stream, resulting in a drop of the annual sediment contribution of the Ebro to its delta by up to 99% in the past century (13, 47, 55). During the sampling period, dissolved oxygen content in Ebro River water varied between 6.2 and 16.7 mg O_2/liter (data not shown). Molecular oxygen is often being depleted typically between 0.1 mm and up to 1 cm depending on the carbon content of the sediment (27), resulting in anoxic conditions in the deeper layers. Given the low flow rates of the river and variable sediment deposition, however, it is not possible to confidently estimate how much O_2 could be introduced to deeper layers of sediments and soils. Especially in the Ebro Delta, due to agricultural practices, presumably more O_2 could be introduced to the soil. It can also not be excluded that anoxic microenvironments can form even within otherwise oxic layers of the sediment, or the other way around (20). In addition, varying salt concentrations (approximately 1 to 5 g/liter from seashore to inland at 1-meter depth) caused by seawater intrusion (49) could also negatively influence the presence and activity of *Dehalococcoides* spp. in the river delta. Previous surveys conducted in the sampling area between 1999 and 2003 and more recent studies showed that besides HCB, DDT, PCE, and TCE, polycyclic aromatic hydrocarbons, polybrominated diphenyl ethers, and brominated flame retardants could be detected in water, sediments, and biota of this river (12, 14, 18). Therefore, the lack of significant correlation between HCB pollution and *Dehalococcoides* composition and relative abundances may suggest that *Dehalococcoides* spp. in this river system do not depend only on HCB for their growth and possibly use alternative electron acceptors.

In accordance with the above data, the distribution and diversity of *rdh* genes in the Ebro River could not be significantly related to the dominant contamination at the sampling locations. However, selective pressure of the contaminants could be demonstrated by differences in *rdh* gene distributions in the Ebro River upstream and downstream locations. Upper layers of the sediment sampled at the HCB contamination hot spot Flix were enriched with *rdh* genes from *Dehalococcoides* sp. strain CBDB1 and *D. ethenogenes* 195, the only two cultured isolates currently known for their HCB-dechlorinating activity (3, 17). In contrast, samples taken at downstream locations within the Ebro Delta, which receives numerous halo-

genated compounds, were found to contain a variety of *rdh* genes, including those from various other species. From the *rdh* genes detected from *Dehalococcoides* spp., only one was previously characterized, *vcrA* of *Dehalococcoides* sp. strain VS. The *vcrA* gene product is involved in reductive dehalogenation of vinyl chloride to ethene (39). Even though the rest of the detected *rdh* genes are uncharacterized, DET0088, DET0173, and DET1545 were reported to be upregulated during PCE degradation in Alameda Naval Air Station 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 than in floodplain soils (Fig. 3B). A major part of their activity was located in the upper layers (0 to 10 cm) 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* rRNA copies were found in floodplain soils, which had higher HCB contamination than did 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 2 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 floodplain soils of the Elbe River. However, a similar trend was not observed for the Ebro Delta. *Desulfitobacterium* spp. have not yet been reported to degrade chlorinated benzenes and have been associated only with the degradation of chlorinated ethenes and ethanes and chlorophenols. The functional gene array analysis of these samples confirmed the presence of potentially PCE- and/or TCE-dechlorinating *Desulfitobacterium* spp. both in river sediment and in agricultural soil. Whereas in upstream locations *rdh* genes from *Desulfitobacterium hafniense* strains DCB-2 and TCE1 (*pceA* and *pceB*) were detected, the Ebro Delta was also shown to harbor a putative chloroethene reductive dehalogenase *rdhA* gene from *Desulfitobacterium* sp. strain PCE1. Moreover, *Desulfitobacterium* spp. are more flexible in their choice for electron acceptors than are *Dehalococcoides* spp. They can also use a wide variety of nonchlorinated compounds, such as nitrate, sulfite, metals, and humic acids (56). Therefore, it cannot be excluded that numbers obtained via 16S rRNA-based detection of *Desulfitobacterium* spp. in the Elbe floodplains could also be originating from nondechlorinating members of the species.

Conclusions. This study showed that large amounts of 16S rRNA from *Dehalococcoides* spp. can be detected in river sediments exposed to HCB for a long period of time. However, spatial and temporal variations play a crucial role in affecting activity and diversity of abundant populations. Our findings indicate that the *Dehalococcoides* activity is highly heterogeneous and varies significantly between different locations. In open environments like river basins, it will remain challenging to unequivocally link species composition and activity to changes in environmental conditions. From the data presented here, it can be concluded that river sediment emerges as a preferred environment for *Dehalococcoides* spp. compared to agricultural or floodplain soils. As could be expected from current knowledge on the ecophysiology of halorespiring bacteria, *Dehalococcoides* spp. are more dominant in HCB-polluted locations within river basins than are *Desulfitobacterium* spp. and *Dehalobacter* spp. Hence, monitoring of *Dehalococcoides* activity in HCB-contaminated river basins provides valuable information about changes in the environmental conditions and contributes to our understanding of the life of these interesting bacteria in natural environments.

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