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Reduction of Nitrated Diphenylamine Derivatives under Anaerobic Conditions

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2-Nitrodiphenylamine, 4-nitrodiphenylamine, and 2,4-dinitrodiphenylamine were anaerobically metabolized in sediment-water batch enrichments inoculated with mud from the German North Sea coast. The first intermediate in 2,4-dinitrodiphenylamine degradation was 2-amino-4-nitrodiphenylamine, which appeared in large (nearly stoichiometric) amounts before being completely reduced to 2,4-diaminodiphenylamine. Of the second theoretically expected metabolite, 4-amino-2-nitrodiphenylamine, only traces were detected by gas chromatographic-mass spectrometric analysis in highly concentrated extracts. In addition, low levels of 4-nitrodiphenylamine, which may be the product of *ortho* deamination of intermediately produced 2-amino-4-nitrodiphenylamine, were observed. 2-Nitrodiphenylamine and 4-nitrodiphenylamine were primarily reduced to 2-aminodiphenylamine and 4-aminodiphenylamine, respectively. Diphenylamine was never detected in any experiment as a theoretically possible intermediate. Results from studies with dense cell suspensions of anaerobic, aromatic-compound-mineralizing bacteria confirmed the transformation reactions, which were carried out by microorganisms indigenous to the anaerobic coastal water sediment.

Some single-base propellants and some explosives spontaneously decompose under normal storage conditions. The main products of decomposition are nitrogen oxides (e.g., NO and NO_2) which catalyze and hence accelerate the decomposition process, which may lead to self-heating and autoignition (27). Therefore, a stabilizer is added to prevent autocatalytic reactions. Its primary function is to react with the oxides of nitrogen formed, thereby being converted into the corresponding nitroso and nitro derivatives.

Diphenylamine (DPA) is the most commonly used stabilizer for nitrocellulose-containing explosives and propellants (19, 20). As a result of nitration of DPA, mainly *N*-nitroso-DPA, mononitro-DPAs, dinitro-DPAs, and to a minor extent trinitro-DPAs and some nitro-*N*-nitroso-DPAs, will occur (2, 8). In some cases also 2-nitro-DPA was used for stabilizing propellants and explosives (17). Nevertheless, 19 nitration products of DPA (including also tetranitro-, pentanitro-, and hexanitro-DPA) have been detected in aged nitrocellulose-base propellants by colorimetric, ultraviolet, infrared, and chromatographic techniques (12, 16).

While many investigators have reported on the analysis and identification of DPA and its reaction products, there is only one report on the biodegradability of this class of compounds. Gardner et al. (9) reported the aerobic microbial degradation of radiolabeled stabilizer diphenylamine in a laboratory model sewage sludge system. In addition to the lack of biodegradation studies of DPA derivatives, there is only poor information about the biotoxicity of these compounds, mainly concerning DPA and 4-amino-DPA. Recently published studies reported the first ecotoxicological data for nitrated and aminated derivatives of DPA and other nitroaromatic compounds (4, 6). These data clearly demonstrate that nitro- and amino-DPAs endanger the aquatic environment and indicate that they may cause pollution of drinking water. To our knowledge this is the first report which presents results of the bacterial metabolism of nitro-DPAs (2-nitro-DPA, 4-nitro-DPA, and 2,4-dinitro-DPA) under anaerobic conditions obtained by using sediment-water batch enrichments and dense cell suspensions of anaerobic, aromatic-compound-mineralizing pure cultures.

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MATERIALS AND METHODS

Chemicals. 2-Nitro-DPA and 4-nitro-DPA were purchased from Aldrich Chemical Company (Steinheim, Germany). 2,4-Dinitro-DPA and 4-amino-DPA were products of Arcos Chimica (Geel, Belgium), and 2-amino-DPA was obtained from Fluka AG (Buchs, Switzerland). All chemicals were of the highest purity available. 2-Amino-4-nitro-DPA and 2,4-diamino-DPA were synthesized according to the procedures of Delétra and Ullmann (3) and Nietzki and Almenräder (21). After these chemicals were recrystallized three times, their identities were confirmed by melting point, infrared spectral, and gas chromatographic (GC)-mass spectrometric (MS) analyses.

Source of sediment. The batch enrichments were started with anaerobic, black, marine sediment, which was collected from the tidal shallows near Wilhelmshaven (Lower Saxony, Germany) at the German North Sea coast. The sediment samples were obtained at a depth of approximately 25 cm, where high contents of ferrous sulfide as a result of sulfate reduction by sulfate-reducing bacteria clearly demonstrate strictly anaerobic conditions. The samples were stored at 4°C in 1-liter plastic containers flushed with N_2 and sealed with air-tight snap lids until usage.

Anaerobic bacteria. Pure cultures of *Desulfococcus multivorans* (DSM 2059), *Desulfobacterium anilini* (DSM 4660), *Desulfotomaculum* sp. strain Groll (15), *Desulfoarculus* sp. strain SAX (7), a sulfate-reducing diculture (5), and *Pseudomonas* sp. strain K172 (28), all able to mineralize some aromatic compounds as sole carbon and energy sources, were used for dense cell suspension experiments.

Media and growth conditions. Three different anaerobic media were used for sediment-water batch enrichments. The medium for sulfate-reducing bacteria was previously described (7). For denitrifying bacteria the medium was prepared as described by Tschech and Fuchs (28) without addition of sources of nitrate and ammonium, and the medium described by Kaiser and Hanselmann (14) was used for enrichments of fermentative-methanogenic communities (without addition of NH_4Cl). Enrichments were carried out in 500-ml serum bottles, which were inoculated with 10% (wt/vol) anaerobic, black, marine sediment and filled up to the top to minimize any headspace. The bottles were closed with butyl rubber stoppers. All enrichments were incubated at 36°C in the dark, whereas cultures of dense cell suspensions were incubated at different temperatures as listed in Table 1. The media for dense cell suspension experiments with sulfate-reducing bacteria differed only in NaCl concentration (see Table 1). Resazurin (0.4 mg · liter^{-1}) was added as a redox indicator to all culture media. Different electron donors were added from sterile stock solutions prior to inoculation. The enrichments were allowed to outgas over 4 to 6 weeks; were exposed to 100 μM

TABLE 1. Product formation from metabolism of 2-nitro-DPA, 4-nitro-DPA, and 2,4-dinitro-DPA found in dense cell suspension experiments with different anaerobic bacteria after an incubation time of 3 months

Organism (growth conditions [OD_{578}])[a]	Products (% or amt) found for transformation of[b]:		
	2N-DPA	4N-DPA	2,4DN-DPA
Desulfococcus multivorans (36°C, 0.5% NaCl [9.8])[c]	2N-DPA (74), 2A-DPA (18)	4N-DPA (97), 4A-DPA (0.5)	2,4DN-DPA (51), 2A4N-DPA (32), 2,4DA-DPA (0)
Desulfobacterium anilini (36°C, 1.5% NaCl [0.8])[d]	2N-DPA (54), 2A-DPA (33)	4N-DPA (98), 4A-DPA (traces)	2,4DN-DPA (82), 2A4N-DPA (11), 2,4DA-DPA (2), 4N-DPA (traces)
Desulfotomaculum sp. strain Groll (36°C, 0.1% NaCl [7.2])[c]	2N-DPA (94), 2A-DPA (2)	4N-DPA (98), 4A-DPA (traces)	2,4DN-DPA (97), 2A4N-DPA (traces), 2,4DA-DPA (0)
Desulfoarculus sp. strain SAX (30°C, 2% NaCl [2.4])[e]	2N-DPA (97), 2A-DPA (traces)	4N-DPA (96), 4A-DPA (traces)	2,4DN-DPA (97), 2A4N-DPA (traces), 2,4DA-DPA (0), 4N-DPA (traces)
Sulfate-reducing diculture (36°C, 0.1% NaCl [3.2])[e]	2N-DPA (78), 2A-DPA (13)	4N-DPA (62), 4A-DPA (19), 4-OH-DPA (ND)[f]	2,4DN-DPA (74), 2A4N-DPA (20), 2,4DA-DPA (2)
Pseudomonas sp. strain K172[g] (30°C [2.1])[e]	2N-DPA (92), 2A-DPA (5)	4N-DPA (97), 4A-DPA (traces)	2,4DN-DPA (91), 2A4N-DPA (4), 2,4DA-DPA (0.5), 4N-DPA (traces)

[a] OD_{578}, optical density at 578 nm.
[b] Amounts of products mediated by chemical reduction in the culture media are subtracted (approximately 6% for 100 μM nitro-DPA); a total of 100% was never reached because of the unidentified metabolites (see the text). Traces means ≤5 μM. The data are the means of the values from triplicate measurements of duplicate incubations. N, nitro; DN, dinitro; A, amino; DA, diamino.
[c] Addition of 500 μM nitro-DPAs.
[d] Addition of 150 μM nitro-DPAs.
[e] Addition of 250 μM nitro-DPAs.
[f] ND, not determined (4-hydroxy-DPA standard not available).
[g] Now classified as *Thauera aromatica.*

2-nitro-DPA, 4-nitro-DPA, or 2,4-dinitro-DPA (nitro compounds dissolved in acetone); and were repeatedly exposed to the nitroaromatics after their disappearance. All enrichments were carried out in duplicate. Uninoculated controls (chemical metabolism) and killed controls (autoclaved with inoculum) containing the test chemicals were without bacterial activity.

Experiments with dense cell suspensions. Benzoate-pregrown cells were harvested from 1-liter media in the late exponential growth phase in an anoxic chamber under an atmosphere of N_2-H_2 (90:10). Cell pellets were obtained by centrifugation in gastight polycarbonate bottles at 25,000 × *g* for 15 min at 4°C and washed once with culture medium. After another centrifugation step, different cell densities were adjusted (depending on the different growth yields of the test strains with benzoate as the carbon and energy source [Table 1]). All experiments were performed in duplicate with 20-ml aliquots in 50-ml serum bottles under an atmosphere of N_2-CO_2 (80:20). For toxicological reasons, the concentrations of nitroaromatics used in dense cell suspensions depended on the different adjusted optical densities of the test strains; the concentrations are given in Table 1. Samples for extractions (1 ml) were taken with sterile syringes and were immediately analyzed or frozen at −20°C until analyzed by high-pressure liquid chromatography (HPLC) and/or GC.

Extraction procedures. Two different extraction procedures were performed. To also obtain low-level and trace compounds, 100-ml aliquots of aqueous phases of each enrichment were filtered, adjusted to pH 3 with H_3PO_4, and then extracted with 100 ml of ethyl acetate (nanograde quality) for 24 h. The organic phases were evaporated to dryness at 55°C in a vacuum rotary evaporator. The residue was dissolved in 1 ml of ethyl acetate for HPLC, GC-electron capture detection, and GC-MS analyses. These extractions were carried out in an anoxic chamber to avoid chemical condensation reactions of intermediates mediated by oxygen in air as observed in preliminary studies. The second extraction procedure was carried out for the determination of degradation kinetics in enrichments and dense cell suspension studies. For these experiments 1-ml aliquots of the aqueous phases were centrifuged, adjusted to pH 3 with H_3PO_4, and extracted with 1 ml of ethyl acetate by ultrasonication in closed centrifuge tubes for 30 min. After ultrasonic treatment, organic and inorganic phases were clearly separated by a centrifugation step at −4°C and 2,000 × *g* for 20 min. Of the organic layer, 350 μl was used for HPLC and 350 μl was used for GC analyses. Levels of products formed during nitro-DPA biodegradation were determined in relation to calibration values for authentic and self-synthesized standards.

Analytical methods. Levels of nitroaromatic, aminonitroaromatic, and aminoaromatic compounds were analyzed by reversed-phase HPLC with a Pharmacia liquid chromatograph equipped with a model 2150 solvent pump, a model 2151 variable-wavelength monitor, a model 2157 autosampler, and a model 2152LC controller. The eluent was methanol-water (45:55 [vol/vol]). For determination of levels of nitro and aminonitro compounds 20-μl aliquots were injected into a temperature-controlled (34 to 35°C) Nucleosil-100-C8 (25 cm by 4 mm; 5 μM) column. The solvent flow rate was 0.7 ml/min, and the UV detector was set at 254 nm. The same column was used to detect amino compounds. But in that case, the flow rate of the same solvent was 0.6 ml/min, 10-μl aliquots were injected, and the detector was set at 281 nm. Products were verified and quantified by comparison with authentic standards (if available). Low levels of nitro and aminonitrodiphenylamines were measured by GC with electron capture detection. GC analysis was carried out by using a Varian GC model Star 3400CX (Varian Instruments, Sunnyvale, Calif.) equipped with a 60-m DB-5 fused-silica column (J&W Scientific, Folsom, Calif.; inside diameter, 0.25 mm). The injector temperature was 280°C (split 1:20), and the detector was held at 300°C. The carrier gas was H_2 (3 ml/min), and the flow rate of the makeup gas N_2 was 25 ml/min. The temperature program for the column was as follows: 200°C for 5 min, 10°C/min for 10 min, and 300°C for 10 min. The injection volume was 1 μl, and a Varian Autosampler 8200 CX was used.

Peaks of interest on liquid and/or gas chromatograms were analyzed by GC-MS. The GC-MS analyses were performed in electron ionization mode (70 eV) on a Hewlett-Packard 5890 II system equipped with a Hewlett-Packard 5971 A detector. The samples were chromatographed on a DB-5 capillary column with a gradient temperature program. The initial temperature, 90°C, was held for 5 min; then the temperature was increased to 130°C within 1 min, held for 2 min, increased to 280°C by 10°C/min, and finally held for 2.5 min. The temperature of the injector and the detector was kept at 300°C. The helium flow was 20 cm/s, and the injection volume was 1 μl (splitless).

RESULTS

Screening for microbial enrichments with capacity to biodegrade nitro-DPA. Batch enrichment experiments were carried out to screen for bacteria with the capability to metabolize or mineralize nitro-DPA under anaerobic conditions. The time course of adaptation and biodegradation after more than 1 year of incubation of enrichments with 2,4-dinitro-DPA under denitrifying and fermentative-methanogenic conditions is shown in Fig. 1. The data under both denitrifying and fermentative-methanogenic conditions were similar, and therefore they are grouped and presented together in Fig. 1 (and also in Fig. 2). The rate of microbially mediated disappearance of 2,4-dinitro-DPA reached a maximum after a lag and adaptation phase, which lasted between 4 and 6 months. Subsequently added concentrations of 100 μM 2,4-dinitro-DPA disappeared within 3 to 4 weeks; this was indicated visually by color shifts from orange to colorless in the culture media. The adaptation and degradation time courses for 2-nitro-DPA and 4-nitro-DPA were in close agreement with those for 2,4-dinitro-DPA (color shifts from yellow to colorless) and therefore are omitted. Degradation of nitro-DPAs in enrichments under sulfate-

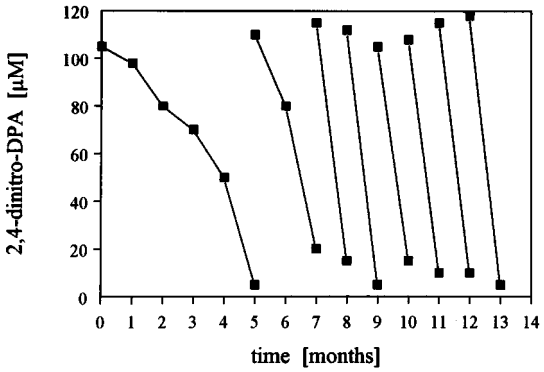

FIG. 1. Time course of adaptation and microbially mediated disappearance of 2,4-dinitro-DPA in sediment-water batch enrichments under anaerobic conditions. The data are the means of the values from triplicate measurements of duplicate incubations.

reducing conditions lasted three times longer than degradation in those under denitrifying and fermentative-methanogenic conditions (approximately 10 to 12 weeks). Therefore, results presented in this study are from experiments performed under denitrifying and fermentative-methanogenic conditions. In these enrichments metabolism of nitro compounds and degradation of the solvent acetone resulted in the production of CO_2 and CH_4. The redox potentials (E_h) of all enrichments were below −300 mV, clearly demonstrating strict anaerobic conditions.

2-Nitro-DPA degradation and accumulation of intermediates in sediment-water batch enrichments. In acclimated batch enrichments 100 μM 2-nitro-DPA was anaerobically transformed into the corresponding amine (2-amino-DPA) in nearly stoichiometric quantities within 3 to 4 weeks (Fig. 2A). Subsequent additions of 100 μM portions of 2-nitro-DPA led to an accumulation of 2-amino-DPA in the culture media. Only a slight decrease of accumulated amine could be observed during the incubation time. The fate of disappearing 2-amino-DPA has remained unknown so far, but analyses to determine possible further metabolites are in progress.

4-Nitro-DPA degradation and accumulation of intermediates in sediment-water batch enrichments. In a manner similar to that of 2-nitro-DPA in the above-mentioned degradation studies, 100 μM 4-nitro-DPA was anaerobically transformed into 4-amino-DPA in large quantities in all batch enrichments within 3 to 4 weeks (Fig. 2B). Repeated additions of 100 μM portions of 4-nitro-DPA primarily led to an accumulation of 4-amino-DPA in the culture media, but a subsequent decrease of 4-amino-DPA levels was measured. In HPLC and GC-MS analyses a new peak was observed in the chromatograms. During the decrease of 4-amino-DPA (retention times in HPLC and GC-MS of 18.7 to 18.9 min and 17.4 min, respectively), the newly produced compound (with retention times of 14.8 to 15.1 min in HPLC and 17.1 min in GC-MS), which corresponded to 4-hydroxy-DPA in mass spectrum ($M^+ = 185$ [Fig. 3]), as proposed by comparison of the fragment spectra of this compound and of 4-amino-DPA, appeared. Both fragment spectra were very similar, but the loss of the fragment at $m/z = 107$ (NH_2-C_6 H_4-NH-) and its replacement by the fragment at $m/z = 108$ (OH-C_6 H_4-NH-) support the suggestion that 4-hydroxy-DPA was a product of anaerobic 4-amino-DPA metabolism. 4-Hydroxy-DPA was not available commercially, and so we were unable to use it as a standard. Identification of further metabolites is in progress.

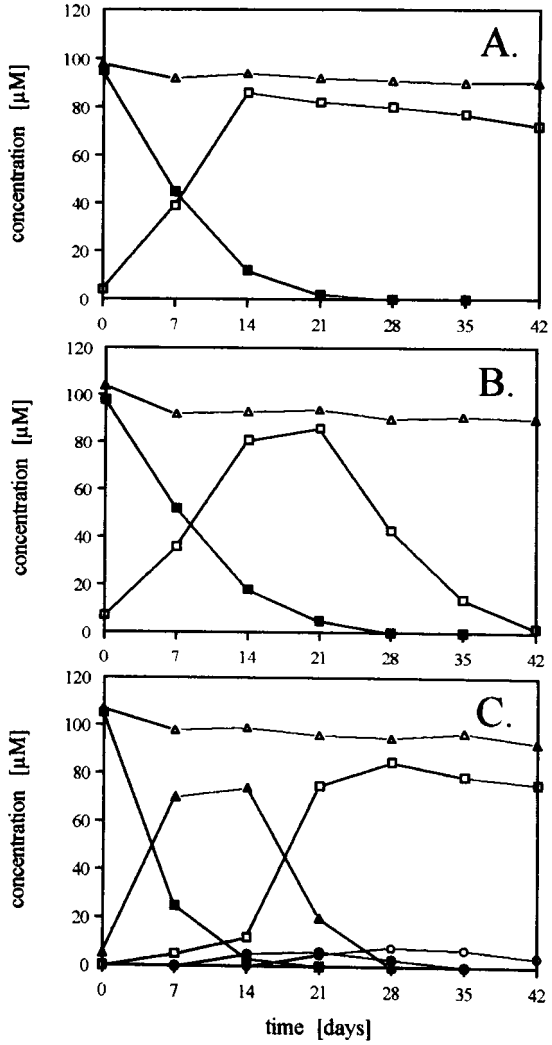

FIG. 2. Microbially mediated disappearance of nitro-DPA from sediment-water microcosms under anaerobic conditions. (A) Concentrations of 2-nitro-DPA (■) and accumulation and disappearance of 2-amino-DPA (□) are shown. (B) Concentrations of 4-nitro-DPA (■) and accumulation and disappearance of 4-amino-DPA (□) are shown. (C) Concentrations of 2,4-dinitro-DPA (■) and accumulation and disappearance of 2-amino-4-nitro-DPA (▲), 2,4-diamino-DPA (□), 4-nitro-DPA (●), and 4-amino-DPA (○) in the culture media are shown. For all experiments, values are also shown for controls consisting of nitro-DPA (△) without bacterial activity (see Materials and Methods). The data are the means of the values from triplicate measurements of duplicate incubations.

2,4-Dinitro-DPA degradation and accumulation of intermediates in sediment-water batch enrichments. The degradation of 2,4-dinitro-DPA in acclimated enrichments was observed for several weeks, and the results are presented in Fig. 2C. 2,4-Dinitro-DPA was 100% removed by bacterial activity within 2 to 3 weeks. It was primarily converted to 2-amino-4-nitro-DPA (the mass spectrum in Fig. 4 was supported by a synthetic standard), which appeared during the first 2 weeks before being completely reduced to the diamino derivative (2,4-diamino-DPA). The conversion of 2,4-dinitro-DPA via 2-amino-4-nitro-DPA to 2,4-diamino-DPA was achieved within 3 to 4 weeks of incubation and was indicated by color shifts from orange (dinitro-DPA) to yellow (aminonitro-DPA) and finally to colorless (diamino-DPA) in the culture medium. The second theoretically produced intermediate, 4-amino-2-nitro-DPA, was detected only in traces by GC-MS analysis in

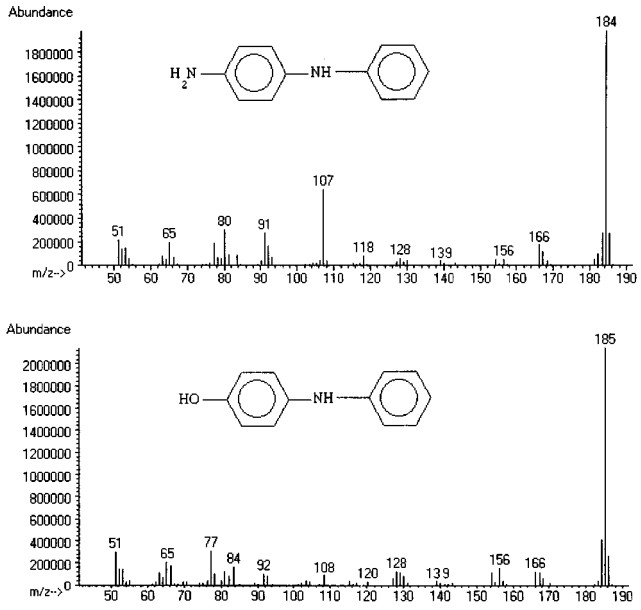

FIG. 3. Mass spectra of the two metabolites found during metabolism of 4-nitro-DPA in sediment-water batch enrichments, 4-amino-DPA (top; M^+ = 184) and its deaminated and hydroxylated product 4-hydroxy-DPA (bottom; M^+ = 185).

highly concentrated extracts. The concentration of 2,4-diamino-DPA decreased very slightly after 4 weeks, but the fate of the disappearing diamino compound is yet unknown. Analyses to determine further metabolites are in progress. After 2 weeks, low levels of 4-nitro-DPA, which were subsequently reduced to the corresponding amine, were also detected. 4-Amino-DPA concentration decreased after 4 weeks (Fig. 2C). Further identifiable metabolites of 4-amino-DPA degradation (e.g., 4-hydroxy-DPA) were not observed.

In controls that lacked sediment as an inoculum (chemical metabolism of the media) and in autoclaved controls (with sediment) the concentrations of nitroaromatic compounds (100 μM 2-nitro-, 4-nitro-, or 2,4-dinitro-DPA) decreased by approximately 6% because of the reductive potential of the media maintained by dithionite and sulfide addition during preparation of the media. Therefore, only low levels of amino-DPAs and aminonitro-DPA from controls inoculated with nitro-DPAs were measured (Fig. 2; for better survey low levels of amino derivatives produced in controls are omitted). 2,4-Diamino-DPA was never detected in controls exposed to 2,4-dinitro-DPA.

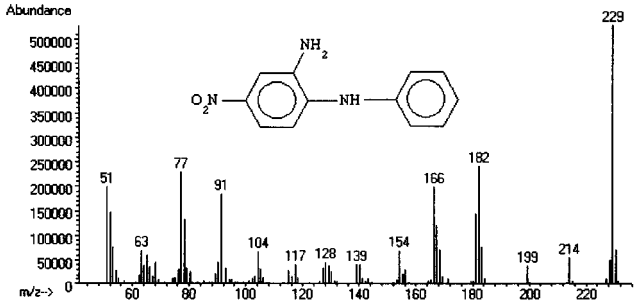

FIG. 4. The mass spectrum of the first metabolite in 2,4-dinitro-DPA-degrading sediment-water batch enrichments as detected by GC-MS in ethyl acetate extracts (2-amino-4-nitro-DPA; M^+ = 229).

Degradation of nitrated DPAs in dense cell suspensions. The products of anaerobic, microbially mediated nitro-DPA metabolism performed in sediment-water batch enrichments were also found in experiments with dense cell suspensions of pure cultures of anaerobic, aromatic-compound-mineralizing bacteria. These strains were chosen to investigate the question of whether they are able to break down the nitro-DPA structure to produce mineralizable compounds (to be used as carbon and energy sources). The strains were not representative of the sediment used in our enrichment studies, but they were useful to determine a possible potential for the biodegradability of nitro-DPAs. The anaerobic bacteria of the marine sediment were not characterized. Table 1 summarizes the amounts of metabolites produced during degradation of 2-nitro-, 4-nitro-, and 2,4-dinitro-DPA after an incubation time of 3 months. 2-Nitro-DPA was effectively reduced to the corresponding amine by pure cultures of *D. multivorans*, *D. anilini*, and a sulfate-reducing diculture, whereas *Pseudomonas* sp. strain K172 showed only low nitroreduction activity. 4-Nitro-DPA was reduced to 4-amino-DPA only in dense cell suspensions containing the sulfate-reducing diculture. Additionally, traces of 4-hydroxy-DPA were detected in those cell suspensions. All other bacterial strains tested in this study were incapable of reducing this *para*-substituted nitro compound. Analogous to the results obtained with 2-nitro-DPA, 2,4-dinitro-DPA was also effectively reduced by *D. multivorans*, *D. anilini*, and the sulfate-reducing diculture and less effectively reduced by *Pseudomonas* sp. strain K172. These strains preferably reduce the nitro group in the *ortho* position of 2,4-dinitro-DPA, and so 2-amino-4-nitro-DPA was the major metabolite. A reduction of the second nitro group (in the *para* position) to yield 2,4-diamino-DPA was detected only at very low levels (Table 1). 4-Nitro-DPA as a deamination product of 2-amino-4-nitro-DPA was detected only in traces. *Desulfotomaculum* sp. strain Groll and *Desulfoarculus* sp. strain SAX were not able to reduce any of these nitro compounds.

DISCUSSION

The results of the present study indicate that the microbial communities indigenous to North Sea coastal water sediment are capable of transforming 2-nitro-, 4-nitro-, and 2,4-dinitro-DPA. In all cases, complete disappearance of the source compound from the dissolved phase was achieved within 3 to 4 weeks by reduction of all nitro compounds to the corresponding amines. The reduction of nitroaromatic compounds is rather common among anaerobic bacteria and is well established (10, 11, 13, 25, 26). The bacteria in the batch enrichments as well as some sulfate-reducing bacteria (Table 1) produced aromatic amino compounds from nitro compounds, indicating that they may have nitroreductase activity. Rafii et al. (26) demonstrated nitroreductase activity for some anaerobic *Clostridium* species isolated from human feces in one of several studies which show that the potential for enzymatic nitroreduction may be widespread in anaerobic or anoxic areas. In our enrichment experiments nitroreduction was microbially mediated during catabolism of the solvent acetone, in which the nitro-DPAs were dissolved. The anaerobic mineralization of acetone is well documented in the literature (22–24). In our enrichments gas production stopped if no further exposures of the substrate (nitro-DPA plus acetone) were carried out, but additions of acetone as the sole source of carbon and energy reactivated the production of gas (CO_2 and CH_4), clearly showing the mineralization of the solvent. Attempts to

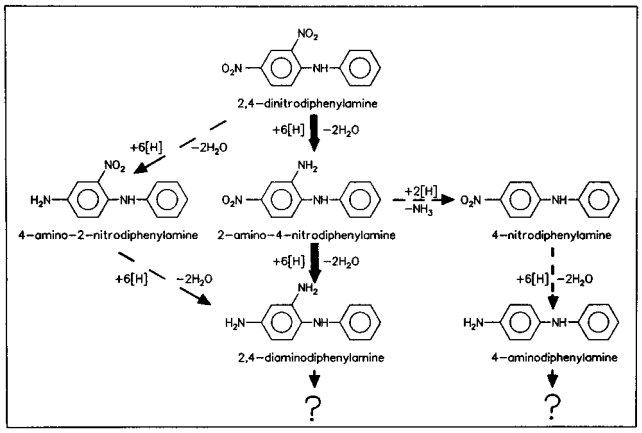

FIG. 5. Proposed pathway for the first steps of anaerobic 2,4-dinitro-DPA metabolism. The pathway of major intermediates is indicated by thick arrows, whereas the pathways of minor intermediates are indicated by thin, interrupted arrows.

obtain stable mixed or pure cultures of anaerobic bacteria from these enrichments with the capability of mineralizing nitro- or amino-DPAs have failed until now. After the first or second transfer into fresh medium containing acetone and nitro- or amino-DPA, the bacteria were not able to grow. Only with acetone as the sole source of carbon little growth was observed. Addition of yeast extract enhanced bacterial growth in these transfers, and nitroreduction activity was observed visually by decolorization of the yellow to orange culture media. With yeast extract we were able to maintain bacterial communities with nitroreduction capacities, but mineralization (the object of our efforts) was not achieved.

Besides 4-amino-DPA, 4-hydroxy-DPA seems to be another transformation product in anaerobic 4-nitro-DPA metabolism. 4-Hydroxy-DPA may be produced by removal of the amino group of 4-amino-DPA with subsequent addition of a hydroxyl substituent. Replacement of the amino group by a hydroxyl group may be achieved by the activity of hydrolytic enzymes to provide ammonia as a nitrogen source for the bacteria in the nitrogen-limited enrichments. The explanation that a reductive enzyme (reductive deamination) accompanied by a hydroxylating enzyme was responsible for the production of 4-hydroxy-DPA is unlikely, because DPA was never observed intermediately. Enzymological determinations were not the subject of this study, and therefore only suppositions can be made. Experiments with added nitrogen sources were not carried out under denitrifying and fermentative-methanogenic conditions. Under sulfate-reducing conditions the media were prepared with 0.3 g of NH_4Cl per liter (7) and the cultures led to the same transformation products, but more time was needed to produce these metabolites. Besides aniline and indole, Gardner et al. (9) found 4-hydroxy-DPA and an unidentified isomeric compound as products of a progressive oxidative metabolism of DPA in an aerobically performed study using a laboratory model sewage sludge system.

In 2-nitro-DPA metabolism only 2-amino-DPA was observed as the major metabolite under anaerobic conditions.

A proposed pathway for the first steps of anaerobic metabolism of 2,4-dinitro-DPA is illustrated in Fig. 5. The first step in this metabolism is the reduction of the *ortho*-substituted nitro group, yielding 2-amino-4-nitro-DPA as the first intermediate. In this case the bacteria follow chemical principles, because in chemical studies partial reduction of 2,4-dinitro-DPA always begins at the *ortho* position of the molecule, resulting in the formation of 2-amino-4-nitro-DPA. This finding is supported by the enrichments, dense cell suspension experiments, and controls without sediment (chemical reduction). 4-Amino-2-nitro-DPA was observed only in traces, and so the reduction step of the *para*-substituted nitro group seems to be more difficult; therefore, this step represents a minor pathway. The fact that the *ortho*-nitro group was reduced most readily is consistent with the findings of previous investigations of fungal 2,4-dinitrotoluene transformation in which this nitro compound was reduced to 2-amino-4-nitrotoluene (29); however, in another study, the major intermediate products of microbial 2,4-dinitrotoluene transformation were 4-amino-2-nitrotoluene and, to a lesser extent, 2-amino-4-nitrotoluene (1), indicating that the nitroreductase activities were less specific. In a second minor pathway, small amounts of the intermediately formed 2-amino-4-nitro-DPA were deaminated to 4-nitro-DPA, which subsequently is reduced to 4-amino-DPA. The fact that 4-nitro-DPA first appeared after 2 weeks and not at the start of incubation leads us to suppose that it was a transformation product of the intermediately produced 2-amino-4-nitro-DPA, rather than a product of 2,4-dinitro-DPA after removal of the *ortho*-substituted nitro group as free nitrite. Free ammonia or nitrite was not found in any of the nitrogen-limited test systems; this may be due to immediate consumption of these limited nitrogen sources. Finally, both aminonitro-DPAs are completely reduced to 2,4-diamino-DPA, which represents the major pathway. The fate of disappearing amino compounds (2-amino-, 4-amino-, and 2,4-diamino-DPA) is yet unclear, but investigations to determine it are in progress. DPA was never found in any experiment as a theoretically possible transformation product in nitro- or amino-DPA metabolism. During HPLC analyses the occurrence of peaks with short retention times was observed in all studies. These peaks have not yet been identified. These more polar compounds (also with aromatic properties as determined at A_{254}) are probably further metabolites in nitro-DPA degradation.

The microbial and chemical reduction of nitro compounds to their corresponding amines increases the water solubility of these compounds because of their higher polarities (11). From a toxicological point of view, only a complete reduction of 2,4-dinitro-DPA to 2,4-diamino-DPA reflects a real detoxification reaction, whereas 4-amino-DPA shows nearly the same toxicological potential as 4-nitro-DPA and 2-amino-DPA was more toxic than 2-nitro-DPA according to results from luminescence inhibition studies with *Vibrio fischeri* NRRL-B-11177 (6). Therefore, it is important to study the fate of the amino-DPAs which disappear during anaerobic incubation. The results of dense cell suspension studies indicate that some strains showed no nitroreduction activity, whereas others reduced the *ortho*-substituted nitro group but were not able to reduce the *para*-substituted nitro group of 2-amino-4-nitro-DPA to produce 2,4-diamino-DPA. In toxicological experiments with the luminescent bacterium *V. fischeri* it was demonstrated that 2-amino-4-nitro-DPA was more toxic than 2,4-dinitro-DPA (6). Thus, it is possible that the bacteria poisoned themselves when producing large amounts of 2-amino-4-nitro-DPA. This theory is supported by microscopically observed morphological changes of the pure cultures, e.g., changes involving loss of motility, sporulation, cell deformation, and cell lysis.

The microbial metabolism of explosives (10) and propellants and their additives, e.g., stabilizers, waxes, plastics, and liquid polymers (18), requires further investigations to determine the complete dimensions of the environmental hazard and to introduce possible (microbial) cleanup techniques for sites contaminated with these compounds.

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