Microbial population changes during bioremediation of nitroaromatic- and nitramine-contaminated lagoon ☆

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Nitration reactions of aromatic compounds are commonly involved in military industrial processes. Military industries treated their process effluents using lagoon systems for many years. In this study, the sediment of a lagoon was investigated from a bioremediation objective. The physico-chemical characterization of the sediments showed the organic nature of the sediment (25.4% carbon with a C:N = 3) highly concentrated in RDX (hexahydro-1,3,5-trinitro-1,3,5-triazine), HMX (octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine) as well as two herbicides Dinoterb (2-*tert*-butyl-4,6-dinitrophenol) and Dinoseb (2-*sec*-butyl-4,6-dinitrophenol). Analysis of the 16S rRNA gene clone library revealed the presence of three dominant families, *Geobacteriacea*, *Clostridiaceae* and *Pseudomonaceae*. A bioremediation assay was carried out in anaerobic conditions in order to degrade organic compounds. In these conditions, 100% of Dinoterb and Dinoseb were degraded after 75 days of culture, while RDX and HMX were not consumed. The 16S rRNA gene clone library analysis of this incubation showed a drastic reduction of the final biodiversity composed by clones related to *Enterobacteriaceae* (especially *Leclercia adecarboxylata*) and *Pseudomonaceae* family. It was then suggested that *Enterobacteriaceae* and *Pseudomonaceae* were potentially involved in biodegradation of these two herbicides. To confirm this hypothesis, cultures were carried out with isolated species of *Pseudomonas putida*, *Pseudomonas citronellolis* and *L. adecarboxylata* in the presence of Dinoterb. The data confirmed that in the presence of glucose, these microorganisms are able to consume Dinoterb.

Keywords: Nitrated compounds; Dinoterb; Biodegradation; Phylogenetic analysis; Pseudomonas

1. Introduction

A wide range of nitroaromatic and nitramine molecules have been produced for pyrotechnic applications (e.g. RDX, HMX, TNT (2,4,6-trinitrotoluene), and picric acid) as well as for herbicide synthesis (e.g. Dinoterb, Dinoseb). In the past, military and chemical industries treated their process effluents using lagoon systems (Funk et al., 1993; Orupold et al., 2000). Sediments from these lagoons contained complex mixtures of different pyrotechnic compounds. Considering the persistence of nitroaromatic and nitramine compounds in the environment, these sediments are today sources of contamination for soil, groundwater and surface water (Beller and Tiemer, 2002; Myler and Sisk, 1991; Talmage et al., 1999). In addition, most of them are toxic. RDX and its metabolites are currently reported to damage soil and water organisms (Mukhi et al., 2005; Robidoux et al., 2001; Zhang et al., 2006). Robidoux et al. (2004) have described significant effects of HMX on growth and reproduction of the earthworm (*Eisenia andrei*). The Dinoseb 96 h LC50 value for Chinook salmon alevins has been determined to be 70.6 ppb and a 87% mortality for Chinook Salmon alevins has been observed after a 96 h-exposure to 100 ppb (Viant

☆ This paper describes the chemical profile and the bacterial ecology of a sludge before and after successful bioremediation for two compounds: Dinoseb and Dinoterb.

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et al., 2006). Dinoterb is reported to be hepatotoxic between 10 and 100 μmol l^{-1} after 2 h of incubation and becomes much more toxic when undergoing a 24 h exposure to rat hepatocytes. Dinoterb was withdrawn from sale in April 1998 by the European Authorities (98/269/CE decision) and nitrophenols are rated as priority pollutants (HR-3 grade) by the US Environment Agency.

Several bacteria and fungi are reported to degrade nitroaromatic compounds such as TNT (Schakmann and Müller, 1991) or nitramines. *Rhodococcus* sp. Strain DN22 (Coleman et al., 1998), *Stenotrophomonas maltophilia* (Binks et al., 1995) and the fungus *Phanerochaete chrysosporium* (Fernando et al., 1990) aerobically degrade RDX. *Klebsiella pneumoniae* (Zhao et al., 2002) and other members of *Enterobacteriaceae* family (Kitts et al., 1994) metabolize it in facultative anaerobic conditions. In addition, some strict anaerobes of the *Clostridiaceae* family are able to transform RDX (Regan and Crawford, 1994; Zhao et al., 2003). Zhao et al. have also isolated some psychrophilic RDX- and HMX-degrading bacteria from a contaminated marine sediment (Bhatt et al., 2006; Zhao et al., 2004; Zhao et al., 2005). Novel remediation pathways have been proposed in extreme environments due to nitroaromatic pollutants (Symons and Bruce, 2006). Moreover, several mechanisms can be involved in cyclic nitramine biodegradation processes: formation of a nitramine free radical and loss of nitrofunctional groups, reduction of nitrofunctional groups, direct enzymatic cleavage, hydroxylation, and hybrid ion transfer. Degradation pathways involved in nitramine biodegradation have also been studied (Crocker et al., 2006).

Like explosive compounds, dinitrophenol compounds are made of nitro groups, Dinoseb can be completely degraded undergoing anaerobic microbial consortium action (Kaake et al., 1992). *Clostridium bifermentas* KMR-1 has been shown to have Dinoseb degradation properties with the help of fermentable carbohydrates (Hammill and Crawford, 1996). Very little is known about Dinoterb biodegradation.

Our contaminated material, sludge from an old lagoon which has received military industry effluent from nitration processes for several decades, contains at least four nitrified cyclic compounds: RDX, HMX, Dinoseb and Dinoterb (Fig. 1). This paper describes the chemical profile and the bacterial ecology of this sludge before and after successful bioremediation for two compounds: Dinoseb and Dinoterb.

2. Materials and methods

2.1. Chemicals

The explosives RDX and HMX standards were obtained from Restek (Paris, France). The herbicides Dinoseb and Dinoterb standards were purchased from Cluzeau (Paris, France). Methanol, acetonitrile and HPLC (high performance liquid chromatography) grade water were obtained from Fischer (Illkirch, France).

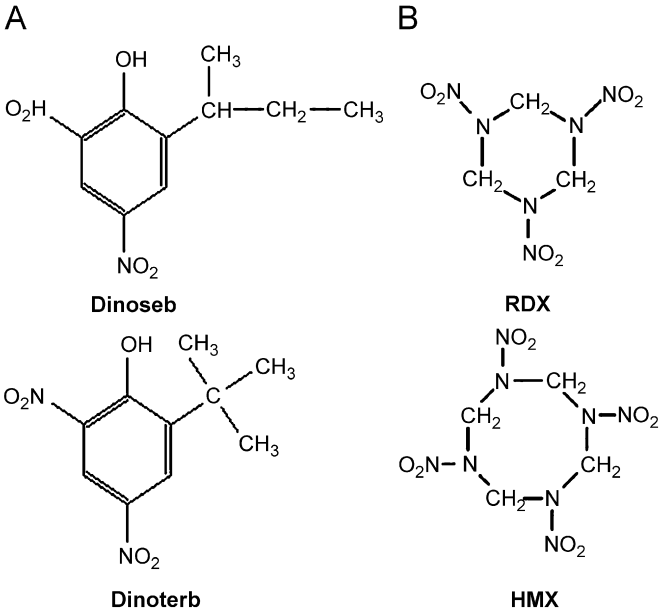

Fig. 1. Molecular structure of the herbicides: (A) and explosives (B) studied.

2.2. Sediment sample and physico-chemical analysis

Sediment was collected from a site in the South of France that produced Dinoseb, Dinoterb, RDX and HMX for several decades. Production wastewater was stored in a lagoon where the sediment samples were taken. Sample (Z4) used in this experiment was in the most contaminated location (15 m from the wastewater evacuation). All the samples were stored at 4 °C. Ten grams of sludge were resuspended in hot neutralized H_2O_2 in order to extract the mineral phase. An opalescent liquid with particle weight less than 10 mg was observed, consequently, the grain size and mineralogy were not determined. The wet sludge had a reddish brown color (Munsell: 2.5 YR 4/4). Organic elemental analysis (C, H, O, N, S) was carried out by dry combustion in a CHN Micro analyzer, Fisons Instruments, UK. In order to determine the physico-chemical properties of the material, 15 g of sludge were suspended in 1 l of distilled water. The pH of the sludge was measured with a Denver Ultrabasic pH meter Station. Major cations (Na^+, K^+, Ca^{2+}, Mg^{2+}) and anions (Cl^-, NO_2^-, NO_3^-, HPO_4^{2-}, SO_4^{2-}) in the sludge were measured using a Dionex DX-100 Ion Chromatograph.

2.3. Bioremediation trails

Bioremediation trails were run in 1.5 l reactors sealed in anaerobic conditions under nitrogen flow at 35 °C under stirring. Fifteen grams of sediment were suspended in 1 l of Winogradsky culture medium containing K_2HPO_4 5 g l^{-1}, $MgSO_4$ 2.5 g l^{-1}, NaCl 2.5 g l^{-1}, $FeSO_4$ 0.05 g l^{-1}, $MnSO_4$ 0.05 g l^{-1}, with glucose 5 g l^{-1} (AA4) or without glucose (Control). Each trail was run in triplicate. Dinoseb, Dinoterb, HMX and RDX present in the sediment served as the sole N source. The initial concentrations of Dinoseb, Dinoterb, HMX and RDX in the reactors were: 1.34, 2.87, 1.4 and 25 mg l^{-1}, respectively. For each run, samples were taken every 15 days.

2.4. Extraction and analysis of explosives and herbicides

The pH of each sample (15 ml) was measured and adjusted to 7.5, in order not to disturb organic compound solubility and spectrophotometric properties. Enrichments were then filtered through 3μ filters from Fischer (France). Explosive and herbicide compounds were extracted with RDX

Porapak cartridges from Waters (Massachussets, USA). Due to the high concentrations of organic compounds, only 5 ml of sample were added to the cartridge. Explosives and herbicides were eluted with 5 ml of acetonitrile.

The concentrations of Dinoseb and Dinoterb were determined using an HPLC–mass spectrometry (HPLC–MS) system (Surveyor Pump, Thermo; Surveyor Autosampler, Thermo, USA). Ten microliters of extract was injected into the HPLC system equipped with a reversed-phase column (Hypersil ODS C18, 3μ, 4.6 mm i.d. × 150 mm, Thermo). The solvent systems used were solvent A (Methanol) and solvent B (HPLC grade water). Compounds were eluted at a flow rate of 0.8 ml min^{-1} by using a gradient starting with 26% of solvent A, followed by a gradual increase to 74% solvent A in 18 min.

Mass spectrometry was performed with an ion trap spectrometer (LCQ Advantage Max, Thermo). The following settings were used: capillary temperature: 280 °C, sheath gas: 40, auxiliary gas: 10, source voltage: 4.5 kV. The concentrations of RDX and HMX were determined using a high performance liquid chromatography-UV detector (HPLC-UV) using a PDA detector from Thermo. Absorbance of explosive compounds was read at 235 nm.

2.5. DNA extraction and 16S rDNA gene amplification

Bacterial DNA was extracted from Z4 and AA4 samples (1 g) using the ULTRACLEAN™ Soil DNA extraction Kit (Mo Bio Laboratories Inc. (Lane, 1991), Carlsbad, CA). The16S rDNA genes were then amplified by PCR from extracted DNAs. PCR mixture consisting of 5 μl of PCR buffer, 2.5 μl of $MgCl_2$ (50 mM), 0.5 μl of dNTP (20 mM), 0.5 μl of each primer (8f 20 mM) (Weisburg et al., 1991) and 1489R 20 mM, 5 μl of DNA extraction product and 0.5 μl of Taq polymerase (Eurobio). Milli-Q water was added until its final volume reached 50 μl. Reactions were cycled in a PTC200 thermocycler (MJ Research, USA). The conditions were an initial denaturation step at 94 °C for 5 min, followed by 35 cycles at 94 °C for 45 s, 52 °C for 45 s and 72 °C for 1 min. A final step at 72 °C for 10 min corresponded to the final elongation step. PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Uppsala, Sweden).

2.6. 16S rRNA gene clone library

The purified PCR amplicons were ligated in the Topo vector supplied with the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. The ligation mixture was transformed into TOPO 10F' competent cells supplied in the same TOPO TA cloning kit. Recombinant vectors were selected on LB agar plates (NaCl 10 g l^{-1}, tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, pH 7.5) containing X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside) and ampicillin (50 μg ml^{-1}) for overnight incubation at 37 °C. Ninety-four recombinant clones were reamplified by PCR. The PCR mixture consisted of 2.5 μl of PCR buffer, 0.75 μl of $MgCl_2$ (50 mM), 0.5 μl of dNTP (20 mM), 0.5 μl of each primers (M13 F, 20 mM and M13 R, 20 mM) and 0.5 μl of Taq polymerase (Eurobio). Milli-Q water was added until to reach a final volume of 25 μl. The PCR temperature cycle was performed using a PTC 200 apparatus (MJ Research, USA). The conditions were an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 52 °C for 45 s and 72 °C for 1 min. A final step of 72 °C of 10 min corresponded to the final elongation step. PCR products were randomly verified by electrophoresis on a 1% agarose-TAE gel. PCR products were purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Uppsala, Sweden).

2.7. RFLP (restriction fragment length polymorphism) analysis and sequencing of 16S rRNA gene clones

Eighty purified PCR products of each sample were selected and digested with *Hae*III and *Hinf* I (New England Biolabs). The digestion mixture consisted of 1 μl of buffer no. 2, 5 U of enzyme, 2 μl of a purified clone PCR product. Milli-Q water was added until a final volume of 10 μl. Electrophoresis was performed on the digests (4 h, 25 V) on an 88-well 3% agarose TBE gel stained with ethidium bromide. In samples Z4 and AA4, restriction profiles with at least two representatives were selected for sequencing. The selected clones were named using the sample name followed by the clone ID number. Selected clones underwent a sequencing reaction using Big Dye Terminator v 3.1 cycle sequencing kit (Applied Biosystem). Sequencing was performed at the *Plateforme Genomique Fonctionnelle* at the University of Bordeaux I. The length of the sequences ranged from 615 to 833 bp.

2.8. Phylogenetic analysis of 16S rRNA gene sequences

Sequences were compared with existing GenBank 16S rRNA gene sequences, using BLASTN: http://www.ncbi.nlm.nih.gov/BLAST/. The closest uncultured 16S rRNA gene sequences and the closest organism 16S rRNA gene were aligned with CLUSTALX 1.83 (Thompson et al., 1997). Sequences were treated with Proseq 2.91 (Filatov, 2002) to homogenize sequence lengths. The phylogenetic tree was constructed with 582 bp length sequences using MEGA3.1 (Kumar et al., 2004).

2.9. Nucleotide sequence accession numbers

The sequences for clones Z4 88, Z4 40, Z4 19, Z4 65, Z4 20, Z4 58, Z4 59, Z4 74, AA4 74, AA4 52, AA4 57, AA4 44, AA4 23, AA4 8, AA4 32, AA4 35 determined in this study have been submitted to the EMBL database and assigned accession nos. EF379136, EF379137, EF379138, EF379139, EF379140, EF379141, EF379142, EF379143, EF379144, EF379145, EF379146, EF379147, EF379148, EF379149, EF379150, and EF379151, respectively.

2.10. Dinoterb degradation tests

Degradation tests were carried out in anaerobic conditions under nitrogen flow at 35 °C in sealed 30 ml bottles. *Pseudomonas citronellolis*, *Pseudomonas putida* and *Leclercia adecarboxylata* were obtained from "*Institut Pasteur*", France. Initial concentrations of Dinoterb in bottles were of 1.5 μg ml^{-1}. Experiments were carried out in the presence or absence of glucose (5 g l^{-1}). The cell growth was evaluated by measuring the $OD_{680\,nm}$ with a spectrophotometer Biomate 3 (Thermo Spectronic) between 0 and 5 days of culture. Each test was carried out in triplicate. Samples were taken after 5 days of culture and extracted with RDX Porapak cartridges and analyzed by HPLC as described above.

3. Results

3.1. Sediment analysis

Physico-chemical analysis of the sediment was performed for the most highly contaminated location in the lagoon (Z4). The pH of the sediment in this area was 6.55. C, H, O, N, S findings revealed high levels of carbon (25.4%), oxygen (18.7%) and nitrogen (8.4%) and a smaller but significant level of sulfur (0.6%). The C:N ratio was therefore 3. The sediment solution (1 l) was found to contain only three cations Ca^{2+} (3.5 mM), Na^{+} (1.1 mM), Mg^{2+} (0.42 mM) and one anion Cl^{-} (1.7 mM) in significant concentrations. NO_2^-, NO_3^- and SO_4^{2-} were also present but in low concentrations: 0.2, 0.1 and 0.1 mM, respectively.

3.2. Bioremediation trails

Time courses of nitramine and nitroaromatic compound biodegradation were investigated in anaerobic conditions in the presence and absence of glucose. In the absence of glucose, only HMX was degraded (Fig. 2). The HMX transformation began slowly after 15 days of culture and reached a maximum value (20%) after 45 days (Fig. 2B). During HMX degradation, no variation of pH was observed (data not shown).

In the presence of glucose, RDX and HMX concentrations remained unchanged (Fig. 2A and B), whereas Dinoterb and Dinoseb were totally degraded (Fig. 3A and B). Dinoseb and Dinoterb degradation started rapidly. After 30 days of culture, 79% and 80% of Dinoterb and Dinoseb were degraded, respectively. At 75 days, pesticides were no longer detected. During the time course of pesticide degradation, a decrease of pH from 6.8 to 5.8 was observed. The pH slowly decreased during the first 15 days and remained constant until 75 days.

3.3. Phylogenetic analysis

Sixty-four clones were analyzed from sample library Z4 by RFLP showing 47 different profiles. Clones which have only one profile are named singletons. Singletons represented 65% of RFLP analyzed clones (Fig. 4). In addition, eight

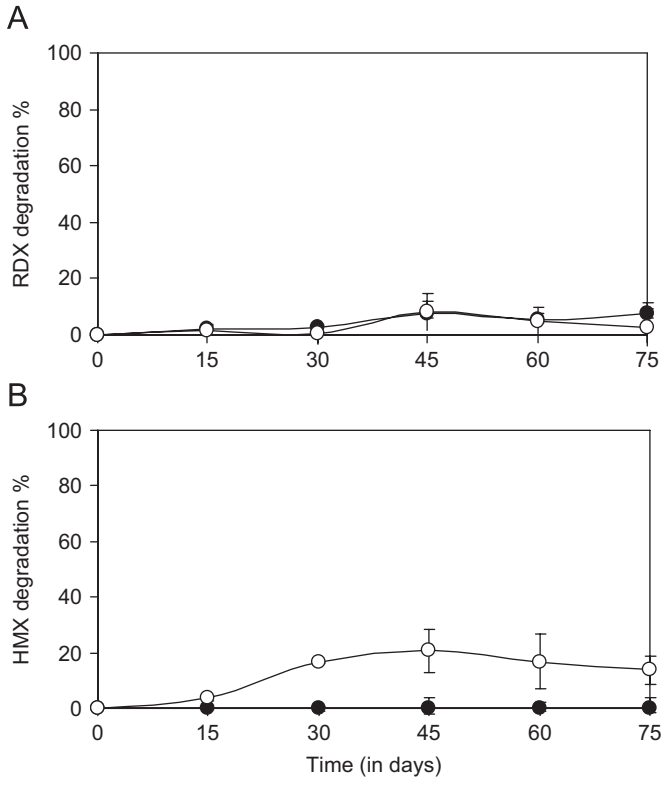

Fig. 2. Time course HPLC analyses of sample filtrate fractions in remediation culture assay: (○) medium without glucose, (●) medium supplemented with glucose; (A) RDX degradation and (B) HMX degradation. Error bars represent standard deviations of three separate experiments.

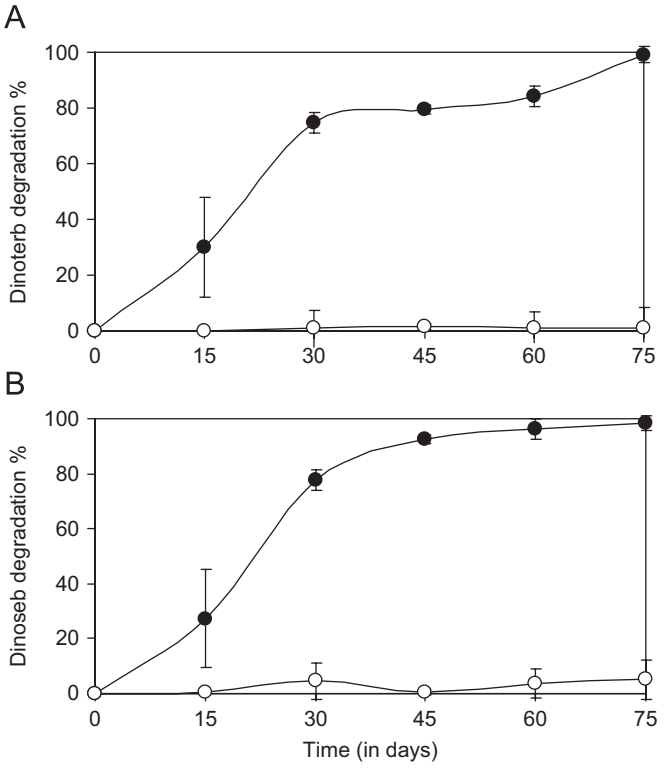

Fig. 3. Time course HPLC analyses of sample filtrate fractions in remediation culture assay: (○) medium without glucose, (●) medium supplemented with glucose; (A) Dinoterb degradation and (B) Dinoseb degradation. Error bars represent standard deviations of three separate experiments.

restrictions profiles presented two or more clones. These eight restriction profiles were selected for sequencing. Clones Z4-20, Z4-65, Z4-40 and Z4-88 were related to δ *Proteobacteriaceae* and represented 21% of total analyzed clones. Clones Z4-20 and Z4-65 were closely related to the *Geobacter* species (99% and 100% homology, respectively) (Fig. 5). However, we were unable to find a clear affiliation for Z4-40 and Z4-88. Clones Z4-19, Z4-74 and Z4-58 were related to the Pseudomonads group. Finally, clone Z4-59 was related to Firmicutes. The closest cultured bacterium was *Catabacter hongkongensis*, but only presented 93% homology.

Seventy-two clones from the AA4 sample library were analyzed by RFLP. We discriminated only eight different restriction profiles although singletons represented 5% of the RFLP clones analyzed (Fig. 4). The library was dominated by clones related to *L. adecarboxylata* (AA4-52, AA4-44, and AA4-35). Clones AA4-23, AA4-67 and AA4-8 gave a 63% match with *Enterobacter ludwigii* and *Pantoea agglomerans*. The *Pseudomonaceae* family was present in the AA4 sample with *P. citronellolis* which showed 99% homology with clones AA4-74 and AA4-32.

3.4. Dinoterb degradation test by Pseudomonas and Leclercia species

The study of Dinoterb degradation by different microorganisms in the presence or absence of glucose showed

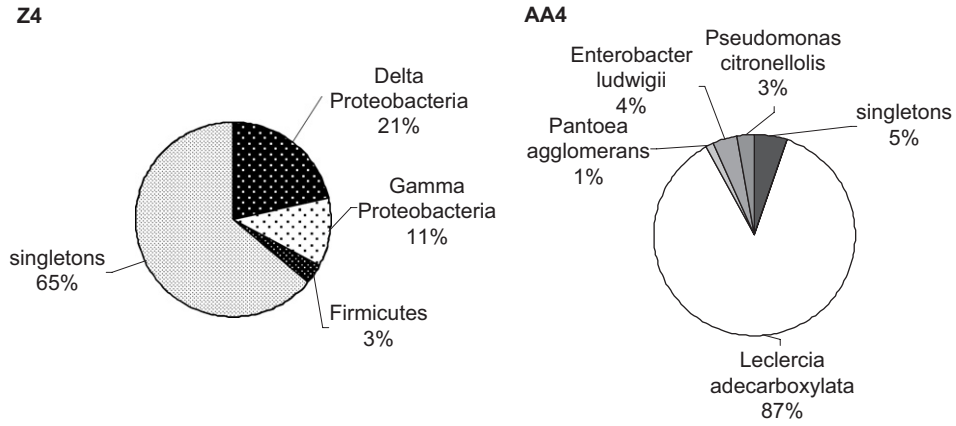

Fig. 4. Identification of representative groups of clones detected in, respectively, Z4 and AA4. Z4 represents families of microorganisms found in the sediment. AA4 represents species of microorganisms (all γ proteobacteria) found in the enrichment.

that in the absence of glucose, the growth of microorganisms was slight (5–10%) and insignificant, whatever the microorganism. In the presence of glucose, a significant increase in the growth of the three microorganisms was observed. However, the variation of the OD_{680} between 0 and 5 days differed significantly according to the bacteria. The development of *L. adecarboxylata* was 10 times higher in the presence of glucose than in its absence after 5 days of culture (data not shown). For *Pseudomonas* species, the growth was six times higher for *P. citronellolis* and only three times higher for *P. putida*. Dinoterb biodegradation test was carried out in the presence or absence of glucose (Fig. 6). It showed that in the absence of glucose, the Dinoterb was not metabolized by *L. adecarboxylata*. On the other hand, for the *Pseudomonas* species, Dinoterb was metabolized up to 26.3% and 47.7%, respectively, by *P. putida* and *P. citronellolis*. In the presence of glucose, all bacteria were able to metabolize Dinoterb. In these experimental conditions, a 60.7% decrease in Dinoterb concentration was observed for *L. adecarboxylata*, a 61% decrease for *P. putida*, and 81.8% for *P. citronellolis*.

4. Discussion

Physico-chemical analysis of sediment from an old industrial lagoon revealed that the sediments can be assimilated to an industrial sludge. The high organic carbon content and the low C:N ratio (equal to 3), confirmed that the sludge resulted purely from the sedimentation of wastewater over more than 50 years. Few studies have been carried out on such material, containing both herbicides and explosives. In addition, the concentrations of nitramines and nitroaromatic compounds are higher than at other military sites previously described (Kaake et al., 1992; Simini et al., 2003). The nature and the diversity of these compounds illustrate the complexity and the originality of the material studied.

The bacterial populations identified in sample Z4 belonged to Proteobacteria and to Firmicutes mainly distributed within three families: *Clostridiaceae*, *Pseudomonadaceae* and *Geobacteriacea*. It has been shown that *C. bifermentas* KMR-1 can degrade dinitrophenols, like Dinoseb (Hammill and Crawford, 1996). *Clostridiaceae* are also involved in degradation of explosives, *Clostridium* sp. EDB2 is able to produce NO_2^- from RDX, HMX and CL20. The NO_2^- produced can play an important role in the recruitment of other bacteria able to increase the degradation process (Bhushan et al., 2004). *Pseudomonadaceae* is one of the predominant groups of soil microorganisms (Schakmann and Müller, 1991). It has been largely claimed that it degrades nitroaromatic compounds, particularly *p*-nitrophenol and TNT (Ajithkumar et al., 2003; Caballero et al., 2005; Esteve-Nùñez et al., 2000; Kulkarni and Chaudhari, 2006; Nam et al., 2003). *Geobacteriacea* are also anaerobes commonly found in the environment, however, studies of biodiversity of bacterial communities associated with such pollutants are scarce.

In the lagoon, the high concentrations in nitrophenols and nitramines measured 20 years after the end of production, associated with a low global mineral content suggest a weak biological activity. Moreover, in multi-solute systems, herbicide combination implies competition for sorption sites and could thus influence biodegradation processes by modifying bioavailability to microorganisms (Martins and Mermoud, 1998). In view of the high concentration of organic compounds, for bioremediation, a significant dilution of the sludge and the addition of mineral nutrients are necessary. In order to improve the biodegradation of these nitramines and nitroaromatic compounds, a source of carbon that could constitute both a co-substrate and an energy source was tested. In this aim, a Winogradsky culture medium supplied with or without glucose was selected for bioremediation trials. In the absence of glucose, 20% biotransformation of HMX was observed after 45 days of culture. Under nitrate reducing conditions, Boopathy (2001) showed that 77% HMX was removed with production of two metabolites, chloroform and methanol. In this study, only one metabolite identified as mononitroso derivative of HMX appeared in the culture

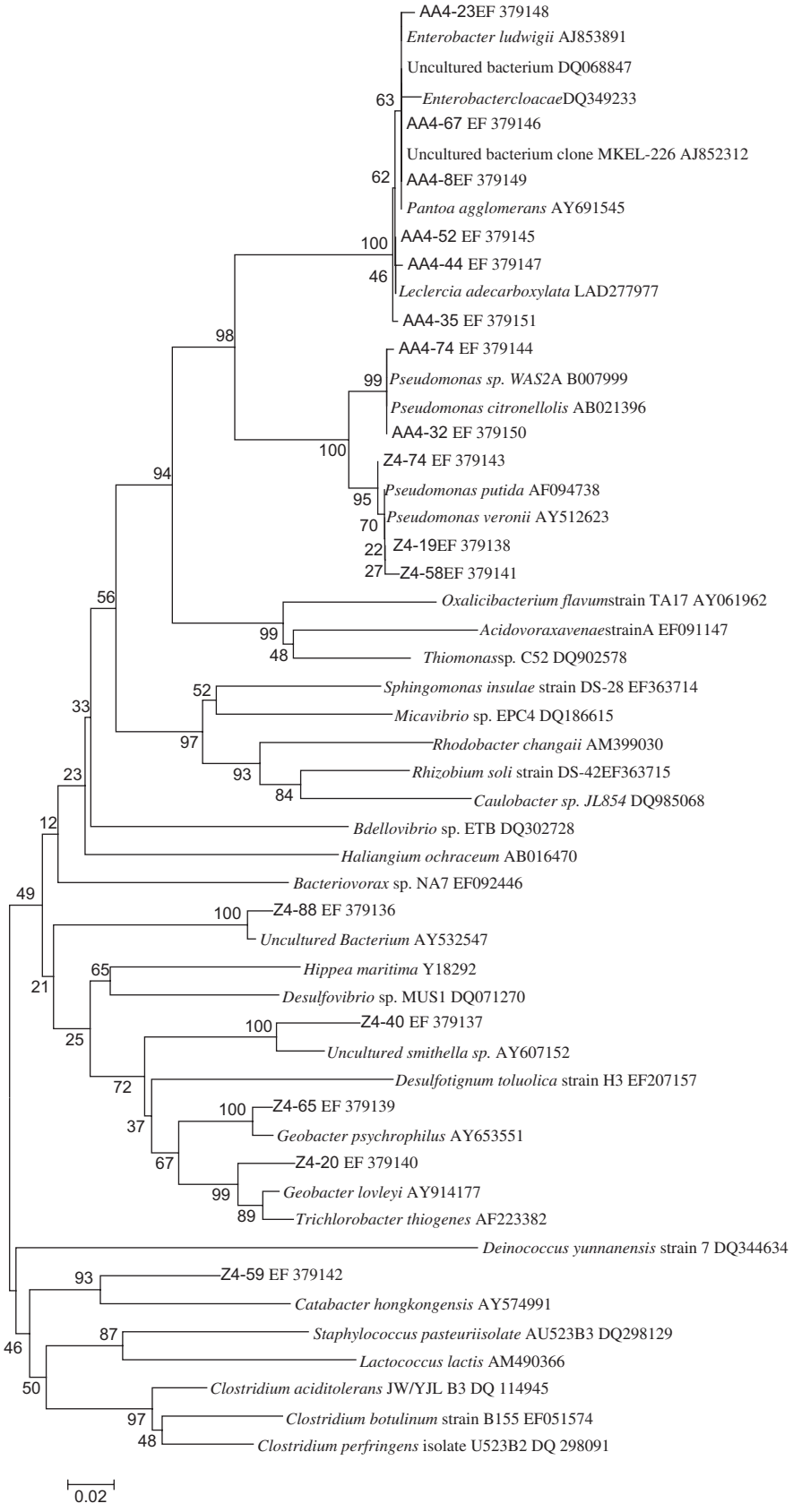

Fig. 5. Phylogenetic tree of clones isolated in Z4 and AA4 libraries and related bacteria based on 16 s RNA sequences. Bar = 1 inferred base change per 100 nucleotides. Bootstrap values greater than 75% are shown at the branch points.

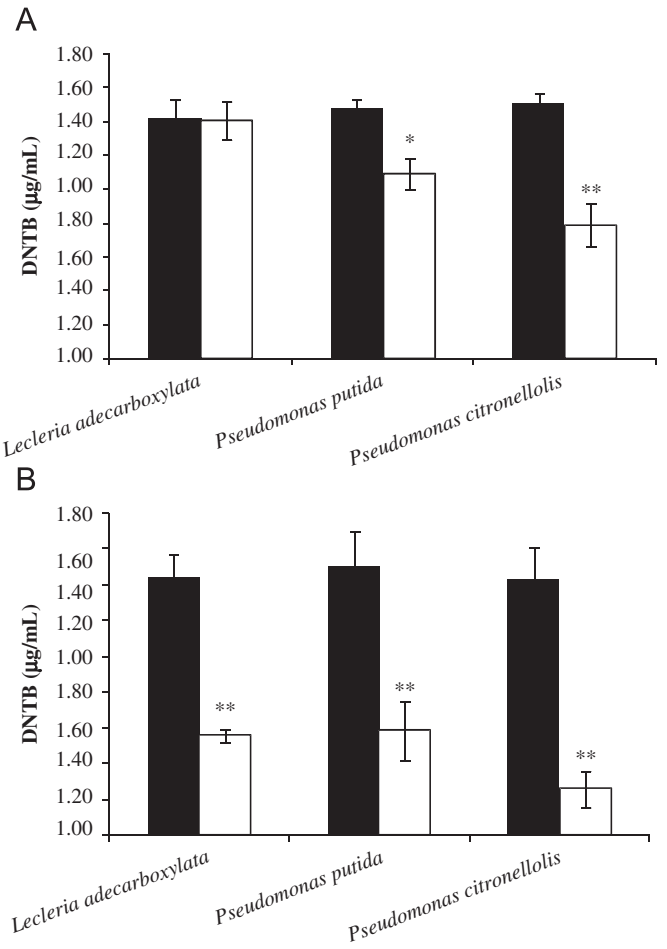

Fig. 6. Dinoterb biodegradation tests in the presence or absence of glucose. The study of Dinoterb degradation in the absence (A) or presence (B) of glucose between 0 (■) and 5 (□) days of culture. The mean represents the average value of three experiments ±S.D. * and ** represent a significant value with, respectively, $p<0.01$ and $p<0.05$ (Tukey's test).

medium after 15 days of culture until 75 days at very low level (data not shown). RDX and HMX have similar structures, and their degradation depends on the presence of electron acceptors or electron donors (Shen et al., 2000). HMX degradation in the absence of glucose suggests that HMX transformation could occur without electron donors. Previous studies showed that RDX was generally more easily degraded than HMX (Shen et al., 2000). In these conditions, the absence of RDX decrease suggests an unknown mechanism for HMX transformation.

Treatment AA4 (Winogradsky culture medium + glucose at 37 °C in anaerobic conditions) led a close-to-100% biotransformation of nitrophenols Dinoseb and Dinoterb (Fig. 3). In these culture conditions, no metabolites were detected in extraction medium (data not shown). It has been reported that Dinoseb can be partially degraded in 20 days with an unidentified intermediate that remains intact for up to 200 days (Roberts et al., 1993). Dinoseb transformation did not occur without glucose. Glucose was probably used as a co-substrate in nitrophenol transformation. Indeed, most herbicides degradation studies are carried out in the presence of co-substrates. Hammill and Crawford (1996) showed that *Clostridium* was able to degrade Dinoseb in anaerobic conditions in the presence of a supplementary carbon source.

The sequencing comparison of the 16S rRNA clone library, revealed a predictable reduction in microbial diversity paralleling the enrichment process. In the presence of glucose, the dominance of clones related to the *Enterobacteriaceae* and *Pseudomonaceae* family was observed. After 75 days incubation, the microflora in the AA4 treatment was strongly dominated by bacteria associated to *L. adecarboxylata*. The presence of *Pseudomonas* in anaerobic conditions suggests the presence of nitrate or nitrite in AA4 enrichment. As shown previously, zone Z4 was very poor in inorganic nitrogen and Winogradsky medium has no nitrogen source. In the reactor, the nitrate and nitrite concentrations were about 15 M. This concentration of mineral nitrogen appeared to be too low to provide sufficient levels of electron acceptor for respiration. In these conditions, the only source of reducible nitrogen is the nitrited functions of organic contaminants. *P. putida* is known to degrade *p*-nitrophenol irrespective of the nitrogen sources in the culture medium (Kulkarni and Chaudhari, 2006). In the reactor, the two nitrophenolic herbicides, which present the same structural nitro groups as TNT, would serve as electron acceptors in anaerobic conditions. This could constitute the first step in Dinoseb and Dinoterb degradation.

The potential role of *Pseudomonas* and *Leclercia* species on Dinoterb transformation has been studied with pure cultures of *P. putida*, *P. citronellolis* and *L. adecarboxylata*, in the presence or absence of glucose. The results clearly confirm that in the presence of glucose all these microorganisms were able to transform Dinoterb, ranked by efficacy as *P. citronellolis* > *P. putida* = *L. adecarboxylata*. In addition, in the absence of glucose only the *Pseudomonaceae* were able to transform Dinoterb with a significant difference between *P. putida* (26.3%) and *P. citronellolis* (47.6%). These data revealed the potential of *Pseudomonas* species and particularly *P. citronellolis* for Dinoterb transformation. In this study, no Dinoterb metabolites were found in the culture medium using mass spectrometry, whatever the conditions tested. Although it is possible for nitro groups to be reduced to hydroxylamino or amino derivatives which subsequently bind to organic compounds in the sediment, this did not occur in Winogradsky mineral medium. Likewise, non-specific adsorption of Dinoterb metabolites on cell surfaces remains possible, but this hypothesis is not supported by the difference noted in the decrease in the levels of Dinoterb in the medium in the absence or presence of glucose. Although *P. citronellolis* was able to consume 47.6% of the Dinoterb with only 10% of growth in the absence of glucose, in the presence of glucose it consumed 81.7% of the Dinoterb with a 10-fold increase in growth. Studies with Dinoterb are very scarce, but tests with TNT on *Pseudomonas* sp. revealed that 85%

of the total nitrogen in the nitroaromatic compounds was incorporated as cell biomass (Esteve-Nùñez et al., 2000). In trials using [^{14}C] TNT, only 1% of the radioactivity was detected as $^{14}CO_2$ but 45% was associated to trichloroacetic acid-precipitable cell material. Overall, the data therefore argue for incorporation and transformation of Dinoterb in the cells.

In conclusion, studies of distribution of microbial communities in industrial lagoons are useful for understanding their phylogenetic positions, the potential roles they carry out in environmental processes and their mechanisms of adaptation to such extreme habitats. The analysis of microflora evolution during the AA4 bioremediation process, in anaerobic conditions, reveals a strong decrease of the biodiversity and the selection of clones. However, the AA4 assay is only a static picture after 75 days of culture, and it is difficult to conclude as to the chronology and the precise roles of bacteria on the two nitrophenolic compounds. Data obtained with pure cultures indicated a key role for the *Pseudomonas* identified and particularly for *L. adecarboxylata* in herbicide degradation.

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