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ORIGINAL PAPER

Biodegradation of *N*,*N* diethylaniline in a contaminated aquifer: laboratory- and field-scale evidences

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Abstract The effectiveness of biosparging to mitigate *N*,*N* diethylaniline in aquifer was evaluated by measuring the time course of decrease in concentration of the aforementioned compound in aerobic microcosm experiments. The first-order kinetic constant for *N*,*N* diethylaniline aerobic biodegradation was estimated from microcosm data (0.037 ± 0.004 d^{-1}), and the value was consistent with the best-fitting value in the transport and reaction model of the aquifer (0.020 d^{-1}). Furthermore, the biodegradability of the compound was evaluated under anaerobic condition in microcosm experiments, which was supported by field modelling. There was no significant degradation in the anaerobic microcosm experiments, confirming the recalcitrance of *N*,*N* diethyl aniline under the aforementioned aquifer condition.

Keywords *N*,*N* diethylaniline · Anilines · Aquifer · Groundwater · Bioremediation · Biodegradability

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Introduction

Anilines are widely distributed environmental pollutants resulting from the manufacturing of dye materials (Meyer 1981) and agricultural chemicals such as herbicides (Kearney and Kaufmann 1975). Many of them are known to be toxic, mutagenic and carcinogenic (Crabtree et al. 1991; Chung et al. 1997; Bhunia et al. 2003). Thus, the fate of anilines in the environment is of great concern. The chemical and physical properties as well as biodegradability of these compounds have been extensively studied (Bollag et al. 1978; Lyons et al. 1984, 1985; Gheewala and Annachhatre 1997).

Among the family of anilines, many studies have been carried out to assess the biodegradability in aerobic condition of aniline and substituted anilines harbouring chloride or methyl groups on the aromatic ring (Bollag et al. 1978; Qureshi et al. 2007). Recently, aniline biodegradation under denitrifying conditions has been reported in standardised biodegradability tests (Vazquez-Rodriguez et al. 2008) and in microcosm experiments simulating river sediment conditions (Wu et al. 2008). With respect to the biochemistry of the aforementioned chemicals, only the aerobic biodegradation pathway of aniline has been elucidated (Urata et al. 2004). Anilines are activated upon their oxidation to catechol by dioxygenase which is then transformed into central intermediates by *ortho*- or *meta*- pathways. Aerobic biodegradation of mono- and di-chloroanilines has

been observed in soil microcosms (Tongarun et al. 2008) and in a pure culture of *Pseudomonas fluorescens* 26-K (Travkin et al. 2003). Recently, *Stenotrophomonas* HPC 135 strain has been isolated from hydrocarbon-contaminated soil which was able to degrade 4-nitroaniline in pure culture (Qureshi et al. 2007).

Little is known about the biodegradability of anilines which carry alkyl residues on the amino group (Resnick et al. 1996; Taupp et al. 2006). Available information in literature is not sufficient to assess the biodegradability of *N*,*N* diethylaniline in environment, and no studies have been carried out to evaluate the enhanced biodegradation and natural attenuation of *N*,*N* diethylaniline in aquifers in which anaerobic conditions are likely to occur. In this paper, we present the results of a laboratory- and field-scale project aimed at assessing the applicability of biosparging technology in bioremediation of *N*,*N* diethylaniline. An estimation of natural attenuation in the anaerobic part of the aquifer was also undertaken. Both aerobic and anaerobic microcosm experiments were supported with actual field data collection.

Materials and methods

Description and history of the site

During the late 1990s, a pharmaceutical factory located in north Italy was responsible for the spilling of a massive amount of aromatic amines, causing the presence of the contaminants in the unsaturated layer of soil. During the following years, the contamination leached through the soil, reaching the aquifer which stands at about 5.50 m under the ground.

Investigation on the site started in 2000 and revealed the presence of aniline and anilines substituted both on the aromatic ring and on the amino group (*N*-ethylaniline, *N*,*N* diethylaniline, *N* methylaniline, *N*,*N* dimethylaniline, *o*,*p* toluidine, 2-chloro 4-nitro aniline).

As a consequence of the characterisation, a hydraulic barrier and a biosparging system were installed to capture and treat contaminated waters and to enhance aerobic bacterial degradation, respectively. Biosparging technology was proven to be ineffective, due to oxygen transfer limitation in subsurface environment. However, in the course of the remediation activities, most of the contaminants were removed from the site and only *N*,*N* diethylaniline remained at high concentrations in the aquifer as unique contaminant after 5 years of operation. Details about the site and the remediation activities have been previously reported (Porto et al. 2008).

The evidences of the biodegradability of *N*,*N* diethylaniline were investigated at both laboratory- and field-scale by the means of (1) the assessment of Terminal Electron Accepting Processes (TEAPs) active in the site, (2) microcosm experiments and (3) groundwater modelling.

Assessment of TEAPs active in the site

On two different groundwater samples, outside and within the contaminated plume (Pz9 and Pz5) of the site, chemical and microbiological analyses were carried out.

Microcosm experiments

Microcosms were set up both in aerobic and anaerobic conditions with aquifer material and groundwater samples from the site. For each condition, five separate microcosm experiments were constructed. Each microcosm experiment consisted of 40 g of aquifer material sampled from the site (sieved: 2 mm) and groundwater artificially contaminated with *N*,*N* diethylaniline (Sigma–Aldrich, St. Louis, MO, USA) which were placed into 117-ml serum bottles. The microcosms for anaerobic conditions were prepared under inflatable glove chamber (I2R, Cheltenham, Pennsylvania) equilibrated with N_2, while stable aerobic condition was maintained in the aerobic microcosms leaving 25 ml of headspace and by weekly shaking of the bottles. The oxygen present in the headspace was fivefold the amount necessary for the complete mineralisation of the added *N*,*N* diethylaniline. Serum bottles were closed using butyl rubber PTFE-faced stoppers and crimp-sealed. Bottles were kept at room temperature in the dark. Control-replicated microcosms, poisoned with $HgCl_2$ (200 mM), were also prepared to account for abiotic losses. Groundwater was sampled from at least two replicate bottles through sterile syringes at different times and microbiological and chemical analyses were carried out. Anilines (aniline, *N*,*N* dimethylaniline, *N*,*N* diethylaniline), total heterotrophic aerobic bacteria and total

heterotrophic anaerobic bacteria were determined on both aerobic and anaerobic microcosms while Fe(II), Mn(II), sulphates, nitrates, anaerobic denitrifying bacteria and anaerobic sulphate-reducing bacteria were determined only on anaerobic microcosms. At different times, duplicate bottles were "destroyed" for soil analysis of residual anilines (aniline, *N*,*N* dimethylaniline, *N*,*N* diethylaniline).

Chemical analyses

Concentration of chemicals in soil and groundwater were determined according to standard protocols: EPA 3510 C and EPA 8270 D for analyses of anilines in soil samples; EPA 3535 A and EPA 8270 D for anilines in groundwater samples; UNI EN ISO 10304-1 for nitrates and sulphates; EPA 200.8 for Fe(II) and Mn(II).

Microbiological analyses

Viable bacteria analyses

Total heterotrophic bacteria were enumerated by dilution plating on LD medium (per litre): yeast extract 5 g, tryptone 10 g and NaCl 5 g (Maniatis et al. 1982). Plates were then incubated at 30°C; for anaerobic bacteria the plates were incubated in anaerobic condition in GENbox systems (BioMérieux). Sulphate-reducing bacteria (SRB) and denitrifying (DNB) bacteria were enumerated by three-tube Most Probable Number (MPN) method. Serial dilution of the samples were inoculated in Nitrate Broth (DIFCO) for DNB and Skerrman medium (Skerrman 1967) for SRB. Tubes were incubated at 30°C for 15 days in anaerobic condition in GENbox systems (BioMérieux). The presence of a bubble and the consumption of nitrate, verified by colorimetric assay, were used as indicators of dissimilatory nitrate consumption (Tiedje et al. 1982). The presence of black precipitated was an index of dissimilatory sulphate consumption.

DNA extraction and polymerase chain reaction conditions

Aquifer samples from microcosm experiments were stored at −20°C until the DNA extractions were carried out. DNA was extracted from the samples with the FastDNA Kit (BIO 101 Inc., Vista, CA, USA) following manufacturer's instructions. 16S rRNA gene was amplified in a 50 µl volume with 20 ng of template DNA and 2 U of Taq DNA Polymerase (Promega Corporation, Madison, WI, USA) using Com1f primer (Schwieger and Tebbe 1998) labelled with 6-carboxyfluorescein and 1495r primer (Mengoni et al. 2001).

Terminal restriction fragment length polymorphism of 16S rRNA gene

The amplified products were purified with the Wizard SV PCR purification kit (Promega Corporation, Madison, WI, USA). Subsequently, 200 ng of amplified DNA were separately digested with 20 U of *Msp* I, *Hha* I and *Hae* III (Promega Corporation) for 3 h at 37°C. An aliquot of the digested products was resolved by capillary electrophoresis on an ABI310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using ROX 500 (Applied Biosystems) as size standard for Peak Scanner (Applied Biosystems) analysis. Fragment sizes from 35 to 500 bp were considered for profile analysis. Only fragments with fluorescence intensity >50 arbitrary units of fluorescence were considered (Mengoni et al. 2001).

Analysis of the T-RFLP profiles

The T-RFLP profiles were visualised by Peak Scanner Software (Applied Biosystems), and the lengths of the terminal fragments were retrieved from the profiles obtained for each enzyme. Reference terminal fragments for each restriction enzyme were obtained by "in silico" restriction analysis using CLC DNA Workbench software (CLC bio A/S). The sequences of the 16S rRNA gene of all the type strains present in the Ribosomal Database Project (RDP) (Wang et al. 2007) were used as reference sequences. All the possible triplets composed of one fragment for each enzyme (*Hha* I, *Hae* III, *Msp* I), obtainable from the three T-RFLP profiles of the same sample, were computed. One or more bacteria of the reference sequences were associated to each fragment triplet when the lengths of all the three fragments of the reference bacterium coincided with the respective fragment lengths of the triplet in a ±5-bp range. These associated bacteria were considered

the phylogenetic relatives of the bacteria potentially present in the microbial community. The database interrogations were carried out using specific software developed in Access 2007 (Microsoft) environment. The software and the Fasta file of the reference sequences are available upon request to the authors.

Groundwater flow and biodegradation modelling

In order to re-project the hydraulic barrier and to confirm the laboratory results a numerical model was developed. The model area has an extension of 1.6 km^2 and for its discretisation we used an irregular grid with closely spaced nodes near the factory where the barrier had to be modelled. This allowed us to better analyse the water balance and the leakage terms. The 3D mathematical model was implemented with the numerical model Modflow for flux and MT3D for transport in addition with Groundwater Vistas interface.

The model is composed of four layers. The first and the second layers represent the shallow aquifer while the fourth one represents the confined aquifer; the third one is used to represent the aquitard that separates the two aquifers. The hydrogeological sequence was reconstructed on the basis of the well logs relative to the model area and nearby. After the setting of geometric parameters of the model, the next step was the definition of the recharge/discharge relationships; a small river and numerous irrigation channels were considered as a term of recharge, in addition to precipitation.

Results and discussion

The investigated site has been contaminated by a mixture of different anilines, substituted both on the aromatic ring and on the amino group. The *N*,*N* diethylaniline was still present in the aquifer as residual contaminant despite the previous treatments.

Assessment TEAPs active in the site

Table 1 shows the chemical and microbiological characteristics of pristine and contaminated subsurface environment. The contaminated sample (Pz9) was characterised by stronger anaerobic conditions as indicated by higher concentration of Fe(II), Mn(II) and lower concentration of nitrates than the uncontaminated sample (Pz5). Furthermore, the number of anaerobic bacteria (both DNB and SRB) was greater in the contaminated sample. These results suggested that the mixture of contaminants underwent to an extensive biodegradation that caused the consumption of the most thermodynamic favourable electron acceptors (nitrates, Fe(III) and Mn(IV)). However, these data did not allow concluding that *N*,*N* diethylaniline was degraded in the site in aerobic or in anaerobic conditions since it could be the residual and most recalcitrant contaminant in the aquifer after the biodegradation of the other compounds.

Table 1 Chemical and microbiological analyses on contaminated (Pz9) and uncontaminated (Pz5) portions of the aquifer

	Pz5	Pz9
N,*N* diethylaniline (μg l^{-1})	0.1	350
Nitrates (mg l^{-1})	29.3	<2.9
Sulphates (mg l^{-1})	87.5	173
Fe(II) (μg l^{-1})	5.1	9,700
Mn(II) (μg l^{-1})	<2.0	3,670
Total aerobic bacteria (CFU ml^{-1})	2.0×10^3	1.7×10^2
Denitrifying bacteria (MPN ml^{-1})	9.0×10^2	4.0×10^3
Sulphate-reducing bacteria (MPN ml^{-1})	<30	4.5×10^2

Microcosm experiments

Microcosm experiments were set up in order to simulate the site conditions during biosparging operation (aerobic condition) and in a posttreatment natural attenuation (anaerobic conditions). Table 2

Table 2 Chemical and microbiological characterisation of the sample used for microcosm experiments

N,*N* diethylaniline (μg kg^{-1})—soil	5,340 ± 1,560
N,*N* diethylaniline (μg l^{-1})—groundwater	1,611 ± 904
Nitrates (mg l^{-1})	0.89 ± 0.52
Sulphates (mg l^{-1})	180 ± 1.5
Fe(II) (μg l^{-1})	59 ± 11
Mn(II) (μg l^{-1})	3,930 ± 549
Total aerobic bacteria (CFU ml^{-1})	$(9.1 \pm 5.3) \times 10^3$
Total anaerobic bacteria (CFU ml^{-1})	$(4.5 \pm 3.2) \times 10^3$
Denitrifying bacteria (MPN ml^{-1})	$(1.3 \pm \text{nd}) \times 10^3$
Sulphate-reducing bacteria (MPN ml^{-1})	<30

Errors are computed as standard deviation of genuine replicates

presents the results of chemical and microbiological analyses on the groundwater used for the microcosms low concentrations of nitrates, and relative high concentrations of Mn(II) indicate that anaerobic conditions (i.e. use of terminal electron acceptors alternative to oxygen) were present in this portion of the aquifer. The high concentration of sulphate and the low presence of SRB suggested that the red/ox conditions were less reducing than sulphate reduction (Chappelle 2003).

Microcosm experiments (Table 3) revealed that an extensive biodegradation of *N*,*N* diethylaniline occurred in aerobic conditions while the concentration of the contaminant in anaerobic conditions remained substantially constant during the 4 month duration of the experiments. As reported in Fig. 1, in the aerobic microcosms a slight increase of total aerobic bacteria and a one order of magnitude decrease of total anaerobic bacteria were observed. This is probably due to the imposed oxic conditions which promoted the growth of aerobic bacteria over anaerobic ones. The decrease of denitrifying bacteria to undetectable values at the end of the experiment and the values of SRB always below the detection limit confirmed this hypothesis (data not shown).

Figure 2 illustrates the trends of total aerobic, anaerobic and denitrifying bacteria in anaerobic microcosms. The general decrease over time of the all considered microbial populations suggested that the biological activity also decreased in anaerobic microcosms. This low activity was confirmed by the negligible consumption of electron acceptors (nitrate and sulphate) and by the negligible production of reduced compounds of anaerobic respiration (Fe(II) and Mn(II)) (data not shown).

Biodegradation is usually computed in groundwater models using a first-order kinetic constant. In order to verify the kinetic behaviour and to estimate a laboratory-scale kinetic constant, the total amount

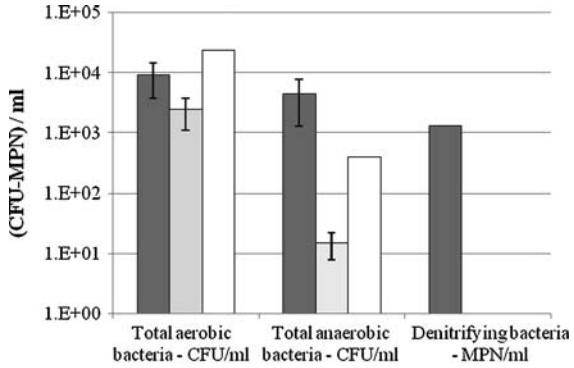

Fig. 1 Total aerobic, anaerobic and denitrifying bacteria in aerobic microcosms. 0 days (*dark grey*), 30 days (*light grey*), 120 days (*white*)

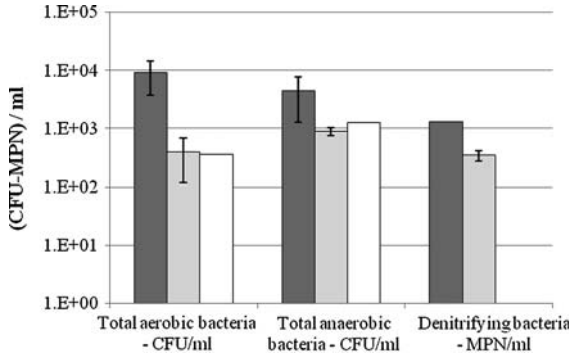

Fig. 2 Total aerobic, anaerobic and denitrifying bacteria in anaerobic microcosms. 0 days (*dark grey*), 30 days (*light grey*), 120 days (*white*)

(ng) of *N*,*N* diethylaniline in each microcosm at each time was considered. Data were log-transformed and interpolated by linear equation showing a good fitting ($R^2 = 0.990$). In Fig. 3, the data analysis of *N*,*N* diethylaniline degradation in aerobic and anaerobic microcosms is reported showing the best-fitting linear equation. The estimated kinetic constant ($k - \mathrm{d}^{-1}$) was 0.037 ± 0.004 which corresponds to a half-life decay time of 18.7 ± 1.9 d. No accumulation of

Table 3 Residual concentration of *N*,*N* diethylaniline at different times in microcosm experiments

Time (days)	Aerobic microcosms		Anaerobic microcosms	
	Soil (μg kg^{-1})	Groundwater (μg l^{-1})	Soil (μg kg^{-1})	Groundwater (μg l^{-1})
0	5,340 ± 1,560	1,611 ± 904	8,110 ± 640	8,650 ± 1,626
30	2,200 ± 60	175 ± 49	9,780 ± 600	6,900 ± 636
120	160 ± 150	<dl	9,130 ± 750	16,731 ± 4,764

Errors are computed as standard deviation of genuine replicates

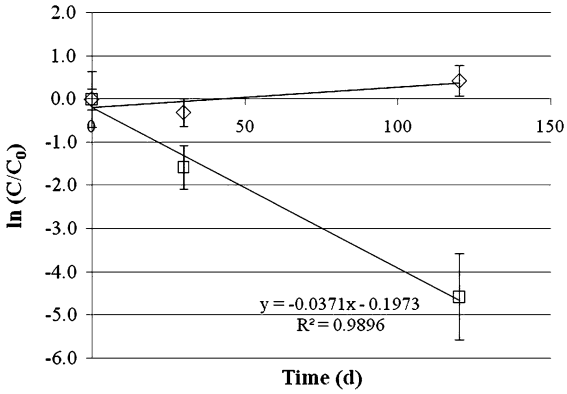

Fig. 3 Degradation of *N*,*N* diethylaniline in aerobic (*square*) and anaerobic (*diamond*) microcosms. *C*: total mass (ng) at time *t*; C_0: total mass (ng) at time 0

aniline and *N*,*N* dimethylaniline was observed and no others peaks appeared in the chromatograms during the experiment leading to suppose that *N*,*N* diethylaniline was degraded without accumulation of intermediates even if specific studies are necessary to definitely assess this point.

Little information is present in literature regarding enzymatic activities on anilines substituted on the amino group. The methyl substituted amino group seems to be more reactive than the ethyl substituted one. Among various reactions catalysed by naphthalene dioxygenase in *Pseudomonas* NCIB 9816, the conversion of *N* methylaniline and *N*,*N* dimethylaniline into aniline could potentially drive the degradative pathway of these compounds into the previously described aniline degradation pathway (Resnick et al. 1996). To the best of our knowledge, only Taupp et al. (2006) reported bacterial enzymatic activities on *N*,*N* diethylaniline describing *ortho*- and *para*-hydroxylation of aromatic ring by *Bacillus megaterium*. Despite this activity, microorganisms able to mineralise *N*,*N* diethylaniline have not been described so far. Furthermore, results from the few studies on the environmental biodegradability of *N*,*N* diethylaniline are very contrasting. In 1987, Niemi et al. classified *N*,*N* diethylaniline as "persistent chemical" since it did not show detectable BOD_5 values and resulted more recalcitrant than other anilines with alkyl substitutes on the amino group. In a more recent study, both *N*,*N* dimethylaniline and *N*,*N* diethylaniline showed half-lives of 1.3 and 1.4 h, respectively, in a simulated river bed sediment environment (Bornick et al. 2001).

Analysis of T-RFLP profiles

Figure 4 shows the three T-RFLP profiles for each time. Both at the beginning of the experiment (T0) and after 120 days (T120), the microbial communities were dominated by few *taxa* as suggested by the low number of fragments in the profiles. However, some significant differences between T0 and T120 profiles revealed that the composition of the microbial community changed during the growth on *N*,*N* diethylaniline. The 183-bp fragment is present only at T120 in *Hha* I profiles, while the peak of the 225-bp fragment is significantly higher at T120 than at T0 in *Hae* III profiles. The developed software allowed us to associate with these two fragments 177 strains retrieved from the RDP reference sequences. All these strains share the same terminal fragment lengths for *Hha* I, *Hae* III and *Msp* I (182, 225 and 37 bp, respectively). Moreover, the "in silico" 37-bp fragment can be associated to the 34- and 37-bp fragments present in *Msp* I profiles both at T0 and at T120. Almost all these strains (175 of 177) belong to the order *Actinomycetales*. Among them, the genera *Pseudonocardia* (45 strains), *Nocardiopsis* (22 strains) and *Streptomyces* (16 strains) are already described as hydrocarbon degraders and herbicide degraders (Omer et al. 1990; Kampfer and Kroppenstedt 2004; Taupp et al. 2006; Vainberg et al. 2006).

Groundwater flow and biodegradation modelling

The numerical model needed a calibration before its utilisation; it was calibrated both in steady and transient state. In steady state conditions, calibration of water recharge and conductance was performed by trial and error on field piezometric data. In transient state conditions, the flux was estimated on the basis of a pumping test conducted on well no. 4 with monitoring of the drawdown in observation well Pz8 (Porto et al. 2008). After this first step of calibration of the model, the hydraulic barrier was re-projected in order to optimise the amount of water extracted from the aquifer, on the basis of the estimated flux value. The final calibration step introduced also transport modelling, and it was performed by matching the field analyses and the laboratory results. In fact, the

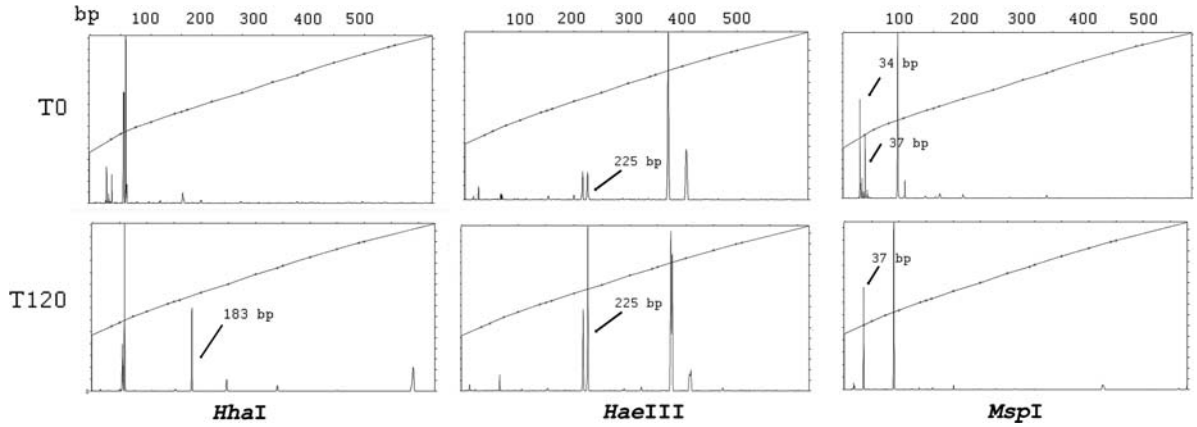

Fig. 4 T-RFLP profiles obtained from aerobic microcosms at the beginning (T0) and at the end (T120) of the experiment after restriction with *Hha* I, *Hae* III and *Msp* I

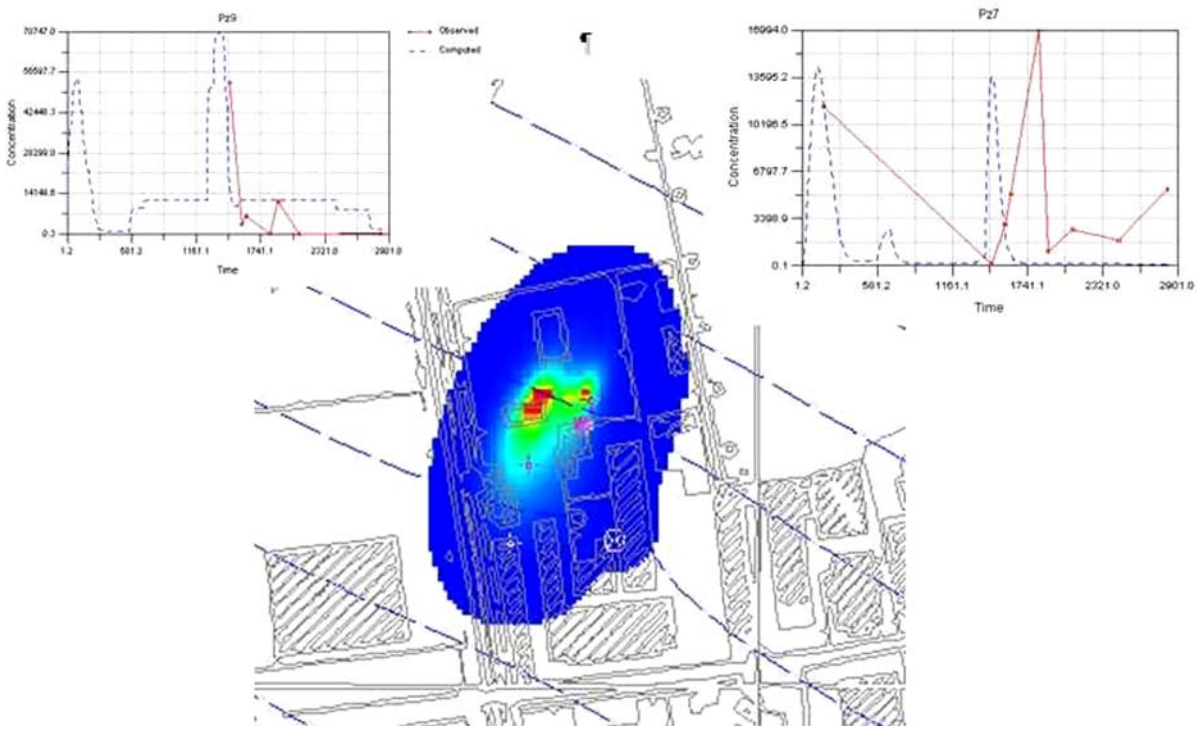

Fig. 5 Comparison between field and computed data in two different wells (Pz9 and Pz7) using the half-life decay parameter obtained in microcosm experiments as reactive term in transport model

main parameter to insert in MT3D is the half-life decay time, which was one of the results obtained by the laboratory tests. The carried out simulation consisted in a sensitivity analysis in order to verify whether the laboratory value could provide a good match with the field observed data. The result is showed in Fig. 5. The final value (31.0 d) is close to the one computed from laboratory data, meaning that the latter is a good estimation of actual field values.

Conclusions

To the best of our knowledge, our work provides the first evidences of biodegradation of *N*,*N* diethylaniline in aquifer environment under aerobic condition. These evidences come from both laboratory and field data. The high biodegradation rate and the undetectable concentration of the contaminant reached after 120 d allowed supposing that biostimulation by air/oxygen release (biosparging) is a suitable technology for remediation of *N*,*N* diethylaniline-contaminated aquifers. Despite we indicated that members of *Actinomycetales* order are potentially present in the enriched community after the aerobic growth on *N*,*N* diethylaniline, further experimental effort is needed to elucidate the microorganisms and the biodegradation pathway involved. The negligible degradation observed in anaerobic microcosms is consistent with the persistence of *N*,*N* diethylaniline in the aquifer as residual contaminants of a mixture of different anilines. However, it does not absolutely reveal that *N*,*N* diethylaniline is recalcitrant to biodegradation under anaerobic conditions. In many situations, the degradation of aromatic compounds in the environment started after several months of lag-time in which no degradation occurred (Chappelle 2003). Furthermore, some aromatic compounds such as benzene are known to be more recalcitrant under denitrifying conditions than under sulphate-reducing conditions (Foght 2008) even if the thermodynamic conditions are more favourable. Therefore, it is not excluded that *N*,*N* diethylaniline biodegradation occurs in aquifer environment under different TEAPs and due to the activity of different and more acclimated microbial communities.

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