Atrazine mineralization by indigenous and introduced
Pseudomonas sp. strain ADP in sand irrigated with municipal wastwater and amended with composted sludge

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Abstract

Indigenous soil bacteria significantly mineralized atrazine irrespective of sand depth or treatment type. After 32 d, the mineralization ranged from 0.3 to 75%, with a variable lag period before the initiation of mineralization, indicating the presence of genes for atrazine mineralization. Soil DNA extraction followed by magnetic capture hybridization-PCR revealed the presence of the genes atzA, atzB and atzC, indicating potential mineralization via the same pathway as in Pseudomonas sp. strain ADP (P.ADP). When P.ADP was inoculated into the sands, its atzA copy number declined after 1 d from the initial inoculation size (7.5 × 10^6 copies g^-1 sand) by at least two orders of magnitude (<3.9 × 10^4 copies g^-1 sand) with no significant recovery after 18 d. In spite of atzA low copy number in the sand, 40 and 75% atrazine mineralization occurred after 1 week when the sand was irrigated with tap water or wastewater, respectively. Amendment with composted sludge, resulted in a similar mineralization rate to that in the sands irrigated with wastewater alone, when the K_d value for atrazine was less than 1.17 l kg^-1, regardless of the irrigation water quality. In two replicates of the 10–20-cm layer, with K_d values of 1.57 and 2.79 l kg^-1, only 23 and 5%, respectively, of the applied atrazine was mineralized. These observations suggest that, even though sludge amendment or wastewater irrigation increased the competition between indigenous populations and introduced bacteria, P.ADP was able to continue mineralizing atrazine. The atzA copy numbers remain in the treated sand in low but stable (and active) concentrations. The high organic matter content of the sludge was the main factor affecting atrazine mineralization, because of its atrazine sorption ability. © 2000 Published by Elsevier Science Ltd. All rights reserved.

Keywords: Atrazine; Mineralization; Sludge; Wastewater treatment; Survival; MCH-PCR; Pseudomonas

1. Introduction

Reuse of wastewater for agricultural irrigation, together with sludge amendment to soil has become a common practice in recent years (Pescod, 1992). This practice is further encouraged by the increasing attention of the regulatory authorities to protection of water quality by restricting waste disposal into rivers, lakes and the marine environment. In Europe commu-
tural and environmental factor that should be taken into account is the effect of such soil amendments on the microbial degradation of herbicides (Masaphy and Mandelbaum, 1997). Irrigation with effluents or sludge amendment can modify the degradation of pesticides in soil by indigenous or introduced microorganisms, through a number of factors: (i) direct bio-toxicity of residual organic compounds or heavy metals in the wastes (Brooks, 1995; McGrath et al., 1995); (ii) indirect effects such as shifts in the soil microbial biostasis (Roane and Kellogg, 1996); (iii) sorption of the target pesticides by soluble or suspended organic matter originating in the sludge, which may change their bioavailability and distribution in space and time (Scow, 1996); (iv) bioavailability of nitrogen components from the amendment can reduce the utilization of nitrogen-containing herbicides as a nitrogen source for bacteria (Cook, 1987).

Bacteria which are introduced into soil can serve as probes to detect a stressed soil environment since their survival is affected by similar factors, e.g. competition with indigenous or sludge-borne microorganisms (van Elsas and Heijnen, 1990; Morita, 1991); toxic substances and antibiotics from the sludge (Vasseur et al., 1996). Bogosian et al. (1996) reported that introduced *E. coli* showed greater persistence in sterile than in untreated soil, and suggested that the bacteria were unable to compete with and, perhaps, were being consumed by the indigenous inhabitants of these environmental samples. Chaudri et al. (1992) found that long-term exposure to high concentrations of heavy metals, such as those released from continuous soil application of industrial sludge, impaired the survival and activity of the nitrogen fixing bacteria, rhizobia; they also determined the effects of 12 organic compounds found in sewage sludge, on the soil indigenous population of rhizobia (Chaudri et al., 1996) and found pentachlorophenol to be the only toxic compound in a concentration above 75 mg kg$^{-1}$ soil. Introduced bacteria, which are capable of degrading specific soil pollutants, can usually survive and proliferate better when such compounds are present in the soil. Jacobsen and Rasmussen (1992) found that a 2,4-d-degrading strain was able to grow to the same count size in soil amended with 2,4-d, irrespective of the amount of soil inoculation, whereas in non-amended soil its count maintained at the inoculation level. Flemming et al. (1994) reported that *Pseudomonas aeruginosa* UG2L, which is capable of degrading oil, survived better in oil-contaminated soils. In contrast to these observations van Dyke et al. (1996) noted that the survival of a PCB-degrading *Alcaligenes eutrophus* H850 strain in sandy loam soil was not affected by the absence or presence of PCBs over 56 d.

The study of the fate of bacteria necessitates accurate counting methods, and the ability to grow microorganisms in cultures is a prerequisite for such approaches as viable plating and most-probable-number (MPN) analysis. However, bacteria can become “viable but nonculutable” in the environment (Roszak and Colwell, 1987; Pedersen and Jacobsen, 1993; van Elsas et al., 1997), therefore, direct detection methods are advantageous for monitoring the fate of introduced bacteria (Roszak and Colwell, 1987). Molecular techniques such as DNA extraction, PCR and DNA/DNA hybridization complements the traditional methods (Leung et al., 1995). In our study we implemented a novel method to overcome limitations associated with the purity and yields of target DNAs obtained in the soil extracts; it was based on a unique technique developed by Jacobsen (1995): magnetic capture-hybridization PCR (MCH-PCR). This method combines an initial DNA extraction purification step, including solution hybridization with a single-stranded DNA probe on magnetic beads, and a subsequent PCR amplification of the extracted target gene.

Our objectives were to evaluate the effects of the application to sand of treated municipal wastewater and composted sludge, on atrazine mineralization by indigenous and introduced *P. ADP* bacteria and on the dynamic of its *atzA* gene.

### 2. Materials and methods

#### 2.1. Chemicals

Atrazine of 98% chemical purity and [U-ring-$^{14}$C]a-trazine (chemical purity 97.3%, specific activity 14.6 mCi mg$^{-1}$, radiochemical purity 98.6%) were a gift from the Novartis Corporation, Greensboro, NC. All basic chemicals were of analytical grade and purchased from Merck, Darmstadt, Germany or from Sigma Chemical, St. Louis, USA.

#### 2.2. Lysimeters

Eight aerial lysimeters (200-l barrel, each) were lined internally with an 0.1-mm thick polyethylene sleeve (Fine et al., 1997). A 0.1-m layer of limestone pebbles was placed on the bottom, overlain by an 1 mm mesh nylon net on top of which was a 70-cm dune-sand (quarts, <250 μm) column. Anaerobically digested, dried, activated sludge from Haifa, Israel that had been composted for 3 months in windrows without a bulking agent, was applied to half of the lysimeters. After composting the sludge characteristic was: total organic carbon (TOC) — 25.85%, cation exchange capacity (CEC) — 57 meq 100 g$^{-1}$, total N — 4.35%, pH$_w$ — 6.3 and EC$_w$ — 6.3 dS m$^{-1}$. The composted sludge was mixed with sand at 250 g kg$^{-1}$ and placed on top of the sand column to form an upper 0.2-m
layer. The amount of composted sludge applied was 17.5 kg lysimeter\(^{-1}\), equivalent to 625 Mg ha\(^{-1}\). Before the experiments began the lysimeters had been irrigated for 3 yr (except during winters) with tap water (pH — 7.8 and TOC — 7.8 mg l\(^{-1}\)) or with secondary effluent (pH — 8.0 and EC — 1.96 dS m\(^{-1}\)) pumped directly from an adjacent facultative oxidation pond. The quality of the secondary effluent was relatively poor, as indicated by a high TOC concentration (190 ± 11 mg l\(^{-1}\)), the low DOC-to-TOC ratio (0.21 ± 0.03) and the high BOD (≈ 134 mg l\(^{-1}\)). Irrigation was computer controlled and monitored to attain a leaching fraction (LF) ≈ 1 (the irrigated volume was nearly equal to the volume leached from the lysimeter). Water was applied to the sand surface, three times daily. Each lysimeter received its water from one or two 8-l regulated drippers that were subdivided into eight semi-regulated distribution tubes.

2.3. Sand collection

After 3 yr of irrigation, each lysimeter was sampled by coring the sand from the surface to the bottom and removing subsamples from three depths along the lysimeter profile (10–20, 20–30 and 30–50 cm), for further testing. From each sample a subsample was kept at 4°C (for the determination of biological components), and the rest was air dried and sieved (0.2 mm), for chemical analysis.

2.4. Sand analysis

The sand analysis was carried out in two replicates per layer in each lysimeter (total of four replicates per treatment). Total organic carbon was determined by high-temperature potassium dichromate oxidation and back titration with ferrous ammonium sulfate (Jackson, 1958). Total reduced N (NH\(_4^+\)+organic N) was determined as follows: 0.5–1.0 g sand plus 4–8 ml concentrated H\(_2\)SO\(_4\) were placed in a 100-ml glass tube and combusted on an aluminum heating block. Small portions (0.2 ml) of H\(_2\)O\(_2\) were added intermittently to the cooled digest, which was subsequently reheated. The clear digest was diluted prior to the colorimetric determination of NH\(_4^+\) by the Berthelot reaction (Bremner, 1965). The concentration of heavy metals was determined after hot 4 N HNO\(_3\) extraction, according to del Castilho et al. (1993) and inductively coupled plasma (ICP) analysis was performed (ICP-AES, Spectro, Germany). The distribution coefficient, \(K_d\) (l kg\(^{-1}\)) was calculated from the Freundlich equation, \(x/m(K_fC)^{1/n}\). The \(n\) values of the treated sands found to be nearly one (data not shown), therefore we used the \(K_d\) coefficient instead of \(K_f\). The experimental procedure was carried out according to Shapir and Mandelbaum (1997). Sand characteristics are presented in Table 1.

Sand toxicity was monitored by means of the Microtox test (M600 Analyzer, Microbics Corporation, CA) according to the manufacturers toxicity-testing handbook (6-1-1994).

2.5. Indigenous soil microorganisms activity measurements

The measurements of indigenous soil microorganisms activity carried out in two replicates per layer in each lysimeter (total of four replicates per treatment).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Depth (cm)</th>
<th>Total N (mg kg(^{-1}))</th>
<th>TOC(^{b}) (%)</th>
<th>Cr (mg kg(^{-1}))</th>
<th>Cd (mg kg(^{-1}))</th>
<th>Zn (mg kg(^{-1}))</th>
<th>Pb (mg kg(^{-1}))</th>
<th>Bacteria CFU(^{c}) (cells g(^{-1}) sand)</th>
<th>FDA(^{d}) (mg kg(^{-1}) 30 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waste water</td>
<td>10–20</td>
<td>264 ± 27</td>
<td>0.19 ± 0.02</td>
<td>5.0 ± 0.7</td>
<td>0.04 ± 0.06</td>
<td>12.6 ± 2.0</td>
<td>ND</td>
<td>7.9 ± 0.3 × 10(^7)</td>
<td>4.02 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>20–30</td>
<td>166 ± 9</td>
<td>0.13 ± 0.01</td>
<td>4.3 ± 0.4</td>
<td>0.05 ± 0.05</td>
<td>10.5 ± 2.7</td>
<td>ND</td>
<td>2.1 ± 0.5 × 10(^7)</td>
<td>2.26 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>30–50</td>
<td>50 ± 14</td>
<td>0.07 ± 0.01</td>
<td>3.7 ± 0.3</td>
<td>0.02 ± 0.05</td>
<td>7.5 ± 2.3</td>
<td>ND</td>
<td>7.9 ± 2.0 × 10(^7)</td>
<td>2.03 ± 0.57</td>
</tr>
<tr>
<td>Tap water</td>
<td>10–20</td>
<td>15 ± 7</td>
<td>0.04 ± 0.00</td>
<td>3.7 ± 0.1</td>
<td>ND(^{e})</td>
<td>17.8 ± 12.0</td>
<td>ND</td>
<td>3.2 ± 2.0 × 10(^5)</td>
<td>1.69 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>20–30</td>
<td>27 ± 2</td>
<td>0.03 ± 0.00</td>
<td>3.6 ± 0.0</td>
<td>ND</td>
<td>15.7 ± 10.0</td>
<td>ND</td>
<td>6.3 ± 0.6 × 10(^5)</td>
<td>2.07 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>30–50</td>
<td>19 ± 3</td>
<td>0.03 ± 0.00</td>
<td>3.5 ± 0.1</td>
<td>ND</td>
<td>11.4 ± 2.6</td>
<td>ND</td>
<td>5.0 ± 3.6 × 10(^5)</td>
<td>1.31 ± 0.76</td>
</tr>
<tr>
<td>Waste water sludge</td>
<td>10–20</td>
<td>1137 ± 227</td>
<td>1.20 ± 0.30</td>
<td>10.0 ± 2.2</td>
<td>0.8 ± 0.2</td>
<td>123.4 ± 1.3</td>
<td>11.4 ± 2.6</td>
<td>7.9 ± 7.0 × 10(^5)</td>
<td>7.89 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>20–30</td>
<td>61 ± 18</td>
<td>0.08 ± 0.02</td>
<td>1.8 ± 0.0</td>
<td>0.1 ± 0.0</td>
<td>10.9 ± 0.1</td>
<td>1.0 ± 0.0</td>
<td>2.5 ± 0.9 × 10(^5)</td>
<td>1.80 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>30–50</td>
<td>78 ± 26</td>
<td>0.07 ± 0.01</td>
<td>1.8 ± 0.1</td>
<td>ND</td>
<td>7.6 ± 2.7</td>
<td>1.1 ± 0.1</td>
<td>3.2 ± 0.3 × 10(^5)</td>
<td>0.73 ± 0.21</td>
</tr>
<tr>
<td>Tap water sludge</td>
<td>10–20</td>
<td>1569 ± 933</td>
<td>1.53 ± 1.05</td>
<td>14.0 ± 7.7</td>
<td>0.9 ± 0.1</td>
<td>125.0 ± 4.6</td>
<td>13.7 ± 5.6</td>
<td>4.0 ± 3.0 × 10(^5)</td>
<td>6.13 ± 0.82</td>
</tr>
<tr>
<td></td>
<td>20–30</td>
<td>345 ± 389</td>
<td>0.35 ± 0.43</td>
<td>4.9 ± 4.5</td>
<td>0.3 ± 0.0</td>
<td>78.6 ± 76.3</td>
<td>3.8 ± 3.7</td>
<td>2.5 ± 0.7 × 10(^5)</td>
<td>2.36 ± 2.69</td>
</tr>
<tr>
<td></td>
<td>30–50</td>
<td>50 ± 33</td>
<td>0.07 ± 0.01</td>
<td>2.2 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>31.1 ± 9.9</td>
<td>1.3 ± 0.0</td>
<td>4.0 ± 3.0 × 10(^5)</td>
<td>0.56 ± 0.65</td>
</tr>
</tbody>
</table>

\(^{a}\) Results are presented as means ± standard deviation.

\(^{b}\) TOC is total organic carbon.

\(^{c}\) CFU is colony forming units.

\(^{d}\) FDA is fluorescein diacetate.

\(^{e}\) Not detected.
The fluorescent diacetate (FDA) hydrolysis method was used to determine soil enzymatic activity, according to Schnurer and Rosswall (1982). The bacterial count was monitored by drop plating (Miles and Misra, 1938) on 4% tryptic soy agar medium (Difco, Detroit, USA) containing the fungicide nystatin in final concentration of 50 µg ml⁻¹.

2.6. Microbial culture

An atrazine-degrading bacterium, Pseudomonas strain ADP was used; it has been described by Mandelbaum et al. (1995). The culture was grown in 250 ml of liquid atrazine medium at 35°C on an orbital shaker (125 rev min⁻¹), according to Mandelbaum et al. (1993). After 24 h of incubation, the cells were harvested by centrifugation (6000 g), washed twice with 0.1 M phosphate buffer (pH 7.4) and resuspended in the same solution to a final concentration of 2.5 × 10⁸ cells ml⁻¹.

2.7. P.ADP atrazine mineralization activity and its atzA gene dynamic

Atrazine mineralization activity by P.ADP was measured in 50-ml capped Erlenmeyer flasks containing sand samples (5 g dw) taken from each treatment and each depth. In each Erlenmeyer, an uncapped Eppendorf tube containing 750 µl of liquid atrazine medium at 35°C was used; it has been described by Mandelbaum et al. (1993). The culture was grown in 250 ml of liquid atrazine medium at 35°C on an orbital shaker (125 rev min⁻¹), according to Mandelbaum et al. (1995). The culture was grown in 250 ml of liquid atrazine medium at 35°C on an orbital shaker (125 rev min⁻¹), according to Mandelbaum et al. (1993). After 24 h of incubation, the cells were harvested by centrifugation (6000 g), washed twice with 0.1 M phosphate buffer (pH 7.4) and resuspended in the same solution to a final concentration of 2.5 × 10⁸ cells ml⁻¹.

2.8. Soil DNA extraction

Soil DNA was extracted by a modification of the method published by Yeates and Gillings (1998). Sand samples (0.5 g dw) were placed in 2-ml Eppendorf tubes containing 320 mg of 106-µm glass beads, eight 1-mm glass beads, 900 µl extraction buffer (aqueous solution of 100 mM Tris-base, 100 mM EDTA, 100 mM Na-phosphate and 1% CTAB [pH 8]) and 100 µl 20% SDS. The mixture was ground in a FP120 Fastprep machine (Bio101, Savant Instruments, Holbrook NY) for 30 s at 5.5 m s⁻¹, centrifuged for 5 min at 14000 × g, and the supernatant was transferred to a new tube. A 200-µl aliquot of 2.5 M KCl was added to the supernatant, the solution was mixed by gentle hand shaking and centrifuged for 12 min at 14000 × g, and the supernatant was collected as crude extract of soil DNA.

Table 2
Sequences and positions of oligonucleotides in the MCH-PCR detection of Pseudomonas sp. strain ADP atzA, atzB and atzC genes

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Gene</th>
<th>Position in the gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner forward</td>
<td>atzA</td>
<td>472–489</td>
<td>5′GCA–CGG–ACG–TCA–ATT–CTA</td>
</tr>
</tbody>
</table>
2.9. MCH-PCR: preparation of internal probe, and its attachment to magnetic beads

The internal MCH-probe (Table 2) spanning 96 bp of the atzA gene was prepared by Gibco-BRL (Life Technologies, MD) with a biotin molecule on a five-carbon atom spacer arm incorporated on the 5' end of the oligonucleotide. The oligonucleotide had been purified by polyacrylamide gel electrophoresis (PAGE) by the supplier, to ensure that all synthesized DNA actually carried the biotin molecule. For a total of 10 reactions, 100 ng of the internal probe was attached to 200 μl of a 10-mg ml⁻¹ suspension of magnetic M-280 streptavidin beads (Dynal, Skøyen, Norway). Conjugation steps between the beads and the probe were well characterized by Jacobsen (1995).

2.10. MCH-PCR: hybridization and capture of target DNA

Samples (50 μl) of crude extract of soil DNA (including positive control of boiled P,ADP cell) or DNA-free samples (negative control for MCH-PCR), 20 μl of the magnetic probe and 330 μl of hybridization solution were hybridized overnight in a rotating hybridization oven at 62°C. The hybridization solution contained 5 × SSC, blocking reagent (1% (w/v)) (Boehringer, Mannheim, Germany), N-laurylsarcosine (0.1% (w/v)) and SDS (0.02% (w/v)).

After hybridization, the beads were concentrated with an Eppendorf tube rack with a built-in magnet (Dynal, Skøyen, Norway). The hybridization solution, including soil particles and noncomplementary DNA, was carefully withdrawn with a pipette. The beads containing probe and complementary DNA were resuspended in Milli-Q-purified water at room temperature and were again concentrated on the magnetic rack. After removal of water with a pipette, the beads were resuspended in 50 μl of water, decimal diluted for quantitative estimation, and used for PCR analysis. The concentration of the gene calculated according to the most diluted subsample that amplified a PCR product.

2.11. PCR amplification of captured target DNA

Three sets of primers were selected to amplify products of 444-, 407- and 424-bp oligonucleotides from the atzA, atzB and atzC genes, respectively (Table 2). The PCR mixture contained 15 μl of captured target DNAs from sand samples, 3 μl of PCR buffer without MgCl₂ (Advanced Biotechnology, Epsom, UK), 2 mM MgCl₂, 2 μl of each primer (16.7 μM) (Gibco-BRL, Life Technologies, MD), 0.2 mM of each deoxynucleoside triphosphate (Boehringer, Mannheim, Germany) and 1.5 U of Red Hot Taq polymerase enzyme (Advanced Biotechnology, Epsom, UK). The reaction mixture was adjusted to a total volume of 30 μl with sterile Milli-Q-purified water. Samples were amplified in a Deltacycler I System (Ericomp, San Diego, USA) with one cycle of 5 min of denaturation at 98°C followed by 35 cycles of 1 min of denaturation at 95°C, 1.5 min of annealing at 55°C and 1.5 min of extension at 72°C. The final extension at 72°C was performed for 5 min. A 10-μl volume of the reaction product was separated by electrophoresis in a 1.2% agarose gel containing 0.4 μg of ethidium bromide in TAE buffer (Maniatis et al., 1989).

2.12. Statistical analysis

Data collected from the atzA gene dynamic experiment were statistically analyzed by multifactor ANOVA, with the JMP software (SAS Inst.). When the interaction between the main factors was significant (P < 0.05), a contrast (multirange test) test was performed between treatments at each incubation period.

3. Results

3.1. Effect of organic amendment and irrigation with wastewater on sand characteristics

The influence of composted sludge addition and of the irrigated water quality (wastewater or tap water) on the chemical properties of the sand were measured (Table 1). Control sands (sands not amended with sludge and irrigated with tap water) showed very low concentrations of total organic carbon (TOC) (<0.04%), total nitrogen (<27 mg kg⁻¹) and heavy metals, regardless of the depth in the lysimeters. Wastewater irrigation had a notable influence on the concentration of all the measured components (except heavy metals) in all layers, but the effect decreased with increasing depth. The TOC increased by a factor of 4 in the 10–20-cm (upper) and 20–30-cm (middle) layers, and by a factor of 2 in the 30–50 cm (bottom) layer. The total nitrogen concentration increased from less than 27 mg kg⁻¹ to 50–264 mg kg⁻¹. Wastewater irrigation had only minor effects on the heavy metal concentrations: Zn and Pb concentration did not change significantly, and only a limited increase was monitored in the concentrations of Cr and Cd. The greatest effects of the composted sludge were found in the application layer. Regardless of the irrigation water quality, TOC concentrations in the upper layer reached >1.2% and the total nitrogen increased to more than 1000 mg kg⁻¹. Similar effects of sludge amendment were monitored in the heavy metal con-
centrations, which were increased significantly, especially in the amended layer. The $K_d$ values for atrazine were positively correlated ($R^2=0.99$) with the TOC (data not shown). The upper layers in the sludge-treated sands, where the TOC was high, showed the highest $K_d$ values (more than 1.09 l kg$^{-1}$).

3.2. Microbial counts and activity of indigenous microorganisms

Bacterial counts on tryptic soy agar and hydrolytic enzymatic activity, were influenced by wastewater irrigation and amendment with composted sludge (Table 1). Sludge amendment or wastewater irrigation had similar effects on the bacterial counts, which increased by more than one order of magnitude at all depths (compared with tap water irrigation treatment), with highest counts in the upper sand layer. In contrast to the bacterial count results, the highest change in hydrolytic activity occurred in the upper sand layer whereas lower activity was recorded in the middle and bottom layers, similar to the activity in control treatment (irrigated with tap water only). Sludge addition increased the hydrolytic activity in the upper layer threefold over that activity in the deeper layers, while wastewater irrigation only doubled the activity.

In the deeper layers of the sludge-amended, tap water irrigated lysimeters, the two replicates varied widely in their activity, as indicated by the large standard deviation. The Microtox test did not indicate any toxic effect on luminescent bacteria in any of the replicates that showed lower hydrolytic activity.

3.3. Atrazine mineralization by indigenous soil bacteria

In most of the uninoculated treatments, the indigenous microflora could not mineralize atrazine (data not shown) or mineralize it in very reduced rate. However, in some of the replicates significant mineralization occurred irrespectively of layer depth or sand treatment (Fig. 1). The mineralization after 32 d ranged from 0.3 to 75%, with variable lag periods before initiation of mineralization. Most of the uninoculated treatments, which showed atrazine mineralization activity, mineralized less than 4% of the initial atrazine concentration after 32 d. The highest activity (75%) was measured in one of the replicates of the deeper layers from lysimeters that did not receive sludge, regardless of the irrigated water quality.

Using the sensitive method, MCH-PCR, it was found that all sand samples that mineralized atrazine (even if less than 1% from the initial concentration was mineralized) contained the $atzA$ gene for atrazine dechlorination (Fig. 2). In all samples, including pure $P$.ADP DNA (positive control), a 444-bp PCR fragment was detected, indicating the presence of the $atzA$ gene. Two samples with the highest band intensities were in agreement with the highest atrazine mineralization activity; $atzA$ was not detected in sands that did not exhibit atrazine mineralization.

The treatment not receiving sludge and irrigated with tap water was also tested for the presence of $atzB$ and $C$ genes. The presence of these genes (Fig. 3) indicated that the same genes from the atrazine mineralization pathway as in $P$.ADP were present and active.

3.4. Atrazine mineralization by introduced $P$.ADP bacteria

The influence of water quality (tap water or wastewater) and sand depth on the rate of atrazine mineralization by $P$.ADP was measured. Since similar mineralization patterns were obtained from all sand layers in each irrigated water quality (data not shown), the data were pooled (Fig. 4). It is evident that irrigation with wastewater significantly increased the atrazine mineralization rate by the introduced $P$.ADP bacteria (SigmaStat, Jandel Corporation). The highest difference occurred during the first 6 d in which 77% of the atrazine was mineralized in the wastewater treatment and only 40% in the tap water treatment. After 18 d, 80 and 58% mineralization were recorded in the wastewater and tap water-irrigated sands, respectively.

When sands irrigated with wastewater were amended with sludge (Fig. 5A), the mineralization rates at all depths reached 80% after 18 d, except for one replicate from the upper sand layer, which reached only 23% mineralization. Nutrients supplied by the sludge improved atrazine mineralization, which was 20%
higher than those in the sands irrigated with tap water. The wide variability between replicates, in atrazine mineralization in the upper layer can be partly explained by the large difference in the sand $K_d$ values, 1.57 and 1.09 $l\ kg^{-1}$ in replicates one and two, respectively. A positive correlation ($R^2=0.99$) was found between the TOC and the $K_d$ values (data not shown). This atrazine sorption phenomenon was most clearly exhibited in the two replicates of the upper sand layers in the treatment amended with sludge and irrigated with tap water (Fig. 5B). One replicate showed 80% atrazine mineralization after 18 d, and the other only 5%. Their respective $K_d$ values were 1.17 and 2.79 $l\ kg^{-1}$. In one of the replicates of the tap water irrigation sludge amendment treatments, atrazine mineralization in the middle and bottom layers did not exceed 30%. This low mineralization rate could not be explained by atrazine bioavailability limitation, because of the lower $K_d$ values measured ($K_d=0.1, 0.08\ l\ kg^{-1}$). The Microtox test did not indicate any toxic effect upon luminescent bacteria.

3.5. AtzA copy number in the treated sands

The effects of water quality, sludge amendment, atrazine presence (10 $mg\ kg^{-1}$ sand) and sand layer

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**Fig. 2.** Gel electrophoresis in 1.2% agarose of MCH-PCR amplified $atzA$ gene from various depths of uninoculated sands that showed atrazine mineralization activity. Lanes: (M) size marker (Boehringer Mannheim VI); (1) ww20–30-cm layer; (2) ww30–50-cm layer; (3) tw30–50-cm layer; (4) tw20–30-cm layer; (5) sww10–20-cm layer; (6) sww20–30-cm layer; (7) sww20–50-cm layer; (8) stw10–20-cm layer; (9) stw20–30-cm layer; (10) control sample with no atrazine mineralization activity; and (11) positive control using $P$.ADP cell extract as template. (s=composted sludge, ww=wastewater and tw=tap water).

**Fig. 3.** Gel electrophoresis in 1.2% agarose of three MCH-PCR amplified $atz$ genes in the 30–50-cm layer of sand treatment irrigated with tap water. Lanes: (M) size marker (Boehringer Mannheim VI); (1) fragment amplified from $atzA$ gene; (2) fragment amplified from $atzB$ gene; (3) fragment amplified from $atzC$ gene; and (4) control sample showing no atrazine mineralization activity (the amplified gene was $atzA$).

**Fig. 4.** Atrazine mineralization in sands inoculated with $P$.ADP and irrigated with wastewater (■) or with tap water (●). Differences between the pooled data from all depth taken from wastewater (WW) or tap water (TW) irrigated sands were significantly different ($P<0.05$) after 2 d from the initiation of the experiment (all samples to the right of the broken line).
depth, on the *atzA* gene dynamic were monitored 1 and 18 d after inoculation (Fig. 6). Sand depth and atrazine concentration did not significantly influence the *atzA* copy number (*P* > 0.05), therefore, these treatments were used as replicates for the analysis of the effects of sludge and water quality. The effects of sand treatment, at each incubation period, were subjected to an ANOVA contrast test, because of the interaction between these factors. In all treatments, *atzA* counts declined by at least two orders of magnitude from the inoculation size (7.5 × 10<sup>6</sup> cell g<sup>−1</sup> sand). However, in the treatments irrigated with tap water or wastewater but not amended with sludge, some recovery was detected by 18 d after inoculation. By 24 h after inoculation the copy number of *atzA* was significantly higher in sand irrigated with tap water and amended with sludge, but its counts declined by one order of magnitude after 18 d.

4. Discussion

The use of treated wastewater for irrigation and the application of sludge have been demonstrated to affect soil microbial activity (Metge et al., 1993) and microbial degradation of pesticides (Masaphy and Mandelbaum, 1997). Since the safe use of pesticides is essential for environmentally compliant agriculture, it was important to evaluate the effects of sludge amendment and of irrigation with effluents on pesticide degradation. Currently the rates and frequencies of application of sludge and treated wastewater are calculated according to the risk of distribution of pathogens and the contents of heavy metals and nitrates, to avoid soil and groundwater contamination (Masaphy and Mandelbaum, 1997). We used atrazine as a model compound, because of its widespread use in many parts of the world and its relative recalcitrance to biodegradation (Cook, 1987).

4.1. Indigenous atrazine mineralization activity

In recent years, few bacterial isolates that mineralize atrazine were isolated in different places in the world (deSouza et al., 1998). Surprisingly, atrazine mineralization by indigenous soil bacteria occurred in some of the test sands that most likely had not previously been exposed to atrazine. Moreover, there was no correlation between sand treatment and the atrazine mineralization capacity in the sand (Fig. 1). The detection method (MCH-PCR) for the presence of the atrazine-dechlorinating gene (*atzA*) enabled us to identify very low concentrations of the target genes which are involved in the mineralization, even in sands where less than 1% of the amended atrazine was mineralized after 32 d. Using this sensitive technique allowed us to conclude that atrazine mineralization processes are probably fully biological (not chemical) even when mineralization is very low. The presence of *atzB* and...
atZC genes, which were detected by the same MCH-PCR method, indicated that the indigenous population had the potential to mineralize atrazine via an identical pathway to P.AD (hydroxyatrazine pathway). Therefore, for further experiments we used P.AD (originally isolated from soils contaminated with atrazine) amended sands, to shorten experimental time and to enhance phenomena that might have been more difficult to study when the genes were present in the sand in low copy numbers.

4.2. Effect of sludge and irrigation with wastewater on the atrazine mineralization activity of P.AD

Nutrients such as those added when the sand is amended with wastewater and sludge may influence the indigenous microbial activity in the soil. Therefore, in our study, it was assumed that wastewater irrigation and sludge amendment could temporarily upset the biostasis and competition between indigenous bacteria and known atrazine degraders. Irrigation with wastewater mainly affected the upper layers, and had less effect in deeper sand strata. Sludge amendment, however, caused significant changes in the soil nutritional value, because of its high content of organic matter, in consequence of which, bacterial numbers, both in the top layer and in lower depths, increased. Interestingly, this increase in bacterial counts was not necessarily correlated with similar changes in bacterial activity. Changes in enzymatic activity of the sands were significant only in the upper sand depths, irrespectively of the treatment. The increase in the activity was limited to the 10–20-cm depth, where wastewater made little change, but sludge amendment increased the activity.

Many studies have showed decreased survival of target bacteria because of competition from the soil indigenous community (van Elsas and Heijnen, 1990; Morita, 1991). Interestingly, irrigation with wastewater enhanced both the concentration of soil bacteria as compared with that present under irrigation with tap water (Table 1), and the atrazine mineralization activity of the introduced P.AD bacteria (Fig. 4). Moreover, there was no difference among the layers, in atrazine mineralization rate, in spite of their differing soil microbial communities. The same phenomenon was observed in the sludge amended treatments, irrespective of the water quality (Fig. 5). In most sludge amended treatments the atrazine mineralization rate was nearly the same as in the wastewater treatment without sludge (Figs. 4 and 5). There was no apparent influence of the sludge amendment or irrigation water quality on the copy number of the atZA gene (Fig. 6). In all treatments, atZA copy number dropped by two to three orders of magnitude 1 d after inoculation, and recovery had not occurred even after 18 d. The presence of atrazine did not seem to enhance the gene copy number. These results are in contrast to those of other studies which found enhanced survival of introduced bacteria when they were co-inoculated with a pollutant degraded only by them, because of their consequent nutrient supply advantage (Jacobsen and Rasmussen, 1992; Flemming et al., 1994). The atrazine mineralization activity of P.AD bacteria was not negatively influenced by the addition of wastewater and sludge, which increased the soil bacterial population and decreased the copy number of the atZA gene because of competition. In spite of the decrease in atZA counts the gene remains at a low but stable (and active) concentration.

Additionally, atrazine mineralization proceeded in spite of the high content of nitrogen-containing compounds in the sand. The effect of nitrogen-rich conditions on atrazine mineralization remains unclear, because high concentrations of nitrogen have been reported to inhibit (Stucki et al., 1995) or to have no effect on s-triazine degradation (Shapir et al., 1998a,b).

The organic matter from sludge which had been added to the upper 20-cm sand layer was weathered over 3 yr, and leached into lower sand layers in the lysimeters (Table 1). The transport was demonstrated by the spread throughout the soil profile of the organic matter and the heavy metals associated with the sludge. The Kd values for atrazine, which mainly indicate its sorption to the organic matter, were positively correlated \((R^2 = 0.99)\) with the TOC percentage (data not shown). High values of \(K_d\) \( (> 1.17 \text{ kg}^{-1})\), which indicate low availability of free atrazine, delayed the mineralization of the herbicide by the P.AD bacteria in some of the replicates from the upper sand layers (Fig. 5A and B). These observations show a negative correlation between atrazine mineralization and TOC percentage, in agreement with the results of Radosevich et al. (1996). It is, therefore, considered that the organic matter concentration influences P.AD activity by at least two distinct mechanisms. When organic matter is present in low concentrations, it supports the activity by supplying nutrients; but high concentrations prevent herbicide mineralization because atrazine sorption reduces its availability for biodegradation.

4.3. The influence of toxic materials on P.AD activity

Sludge or wastewater amendment of the sand can enrich the environment with heavy metals and organic chemicals that may be toxic to microorganisms. According to Chaudri et al. (1992) long-term exposure of soil bacteria to high concentrations of heavy metals, such as those arising from continuous soil application of industrial sludge may reduce their survival and activity. In our study, in spite of the large amount of added sludge and continuous irrigation with waste-
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