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ORIGINAL PAPER

Atrazine degradation by aerobic microorganisms isolated from the rhizosphere of sweet flag (*Acorus calamus* L.)

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Abstract In presented study the capability of microorganisms isolated from the rhizosphere of sweet flag (*Acorus calamus*) to the atrazine degradation was assessed. Following isolation of the microorganisms counts of psychrophilic bacteria, mesophilic bacteria and fungi were determined. Isolated microorganisms were screened in terms of their ability to decompose a triazine herbicide, atrazine. Our results demonstrate that within the rhizosphere of sweet flag there were 3.8×10^7 cfu of psychrophilic bacteria, 1.8×10^7 cfu of mesophilic bacteria, and 6×10^5 cfu of fungi per 1 g of dry root mass. These microorganisms were represented by more than 20 different strains, and at the first step these strains were grown for 5 days in the presence of atrazine at a concentration of 5 mg/l. In terms of the effect of this trial culture, the bacteria reduced the level of atrazine by an average of about 2–20%, but the average level of reduction by fungi was in the range 18–60%. The most active strains involved in atrazine reduction were then selected and identified. These strains were classified as *Stenotrophomonas maltophilia*, *Bacillus licheniformis*, *Bacillus megaterium*, *Rahnella aquatilis* (three strains), *Umbelopsis isabellina, Volutella ciliata* and *Botrytis cinerea*. Culturing of the microorganisms for a longer time resulted in high atrazine degradation level. The highest degradation level was observed at atrazine concentrations of 5 mg/l for *S. maltophilia* (83.5% after 15 days of culture) and for *Botrytis* sp. (82% after 21 days of culture). Our results indicate that microorganisms of the sweet flag rhizosphere can play an important role in the bioremediation of atrazine-contaminated sites.

Keywords Atrazine · Bioremediation · Mesophilic bacteria · Fungi · Psychrophilic bacteria · Rhizosphere microorganisms

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Introduction

Herbicides are widely used in modern agricultural practices in many countries around the world. There are currently billions of tons of chemicals that are passing through the soil, from where they can pollute the environment (soil, water, air).

Atrazine (2-chloro-4-ethylamino-1,3,5 triazine) is a herbicide widely used in weed control, especially in corn cultivation. The long life of this herbicide and good water solubility (33 ppm) makes atrazine a relatively mobile compound. It can migrate into deeper layers of the soil profile and further into groundwater. Atrazine is the most common herbicide that persists in groundwater and surface water (Pang and Close 2001; Tasli et al. 1996; Liu et al. 1999).

Atrazine shows high toxicity in populations of fish, amphibians, and phytoplankton. There are also increasing numbers of reports indicating a relationship between environmental atrazine contamination and increased incidence of many human diseases (Fan 1999; Whalen et al. 2003). Atrazine is the most commonly detected pesticide in food and drinking water. According to WHO regulations, the upper limit for atrazine concentration in water is 2.00 μg/l (EU Directive 80/778/EEC 1980). Thus, it is very important to find efficient methods of detoxication of this substance. Atrazine can be removed or degraded in soil, sediments and water environment by either biotic or abiotic processes, but bioremediation is more effective and remains a very promising approach (Hequet et al. 2001). A major role in the process of biological degradation of this compound is played by plants and microorganisms. The capability of plants to degrade xenobiotics has been employed to develop new technologies of environmental decontamination known as phytoremediation. In the phytoremediation processes, plants are often supported by rhizosphere microorganisms.

It is well known that organic contaminants commonly disappear more quickly from planted soils (Anhalt et al. 2000). Plants have a significant effect on an increase in the number and activity of the microbial population. The substances produced by plants and released to the rhizosphere increase the biological activity of microorganisms, thus increasing the degradation rate of xenobiotics. Plant metabolites released to the environment also increase microbial resistance to stress factors (Burken and Schnoor 1996; Glick 2003). It has also been demonstrated that species with well-developed fibrous rooting systems are most effective in terms of enhancing degradation rates, effectively increasing the counts of microorganisms within their rhizosphere (Fang et al. 2001).

Sweet flag (*Acorus calamus* L.) is an example of wetland species. These species are very effective in the degradation of xenobiotics and have a great phytoremediation potential because of their high growth rate, high level of biomass production, and well-developed rooting systems, where organic compounds can be cached (Wilson et al. 2000; Knuteson et al. 2000). Because of high atrazine toxicity to aquatic organisms the ability of sweet flag and its rhizosphere microorganisms to degrade this herbicide could be a promising method of restoring lake and river quality.

The objectives of this study were to determine the efficiency of atrazine degradation by microorganisms isolated from sweet flag rhizosphere.

Materials and methods

Chemicals and media

Atrazine (Fluka) used in these investigations was 98.0% pure. The pesticide was prepared as ethanol stocks at the concentration of 10.0 mg/ml, sterilized by filtering through a Millipore 0.22 μm filter unit, and added to the growth media. For bacteria isolation and growth, we used a nutrient broth containing 10.0 g/l glucose. Malt broth (6°Blg) was used for the isolation and growth of fungi.

Microorganism isolation, identification, and culture

Roots of sweet flag were placed into 100 ml of 0.85% NaCl and shaken on a rotary shaker (100 rpm) for 30 min. The obtained microorganism suspension was diluted and inoculated into Petri dishes. Incubation was performed for 5 days at different temperatures: 15°C for psychrophilic bacteria, 20°C for fungi, and 2 days at 37°C for mesophilic bacteria. The colonies were then counted and different strains were evaluated on the basis of their morphological properties.

Isolated strains of different microorganisms were first tested for their ability to reduce atrazine contents. They were grown in the presence of 5.00 mg/l of atrazine for 5 days. Cultures were shaken on a rotary shaker (100 rpm) at different temperatures for each group of microorganisms. On the last day of culturing, the samples were taken off the shaker and the level of atrazine was determined.

For the most effective strains, the growth rate and atrazine reduction rate were evaluated. Cultures were grown for 15 days (bacteria) and for 21 days (fungi) at concentrations of 5.00 and 20.0 mg/l atrazine, respectively. During this time, samples were collected and atrazine levels and microbial counts were determined. All the experiments were repeated twice in triplicate.

The most effective microorganisms were identified from microscopic observations (cell morphology,

gram staining) and 16S RNA for bacteria or 18S rRNA for fungi analysis. Total DNA from bacteria was extracted using a Genomic Mini AX Bacteria Kit (A&A Biotechnology, Gdańsk, Poland) and from fungi using a Genomic Mini AX Yeast Kit (A&A Biotechnology, Gdańsk, Poland) after 1 h initial incubation at 37°C in 40 mg/ml lysozyme (Sigma) or chitinase solution (supplied by the manufacturer of the kits), respectively. Sequences encoding small subunits of rRNA were amplified in PCR reactions using primers SDBact0008aS20 and SUniv1492bA21 for 16S rDNA (Suau et al. 1999) and NS1 and ITS4 for 18S-5.8S rDNA with an ITS spacer as described by de Souza et al. 2004. PCR products were purified using a Clean-Up Kit (A&A Biotechnology, Gdańsk, Poland) and sequenced at the Molecular Biology Laboratory at the Faculty of Biology, Adam Mickiewicz University, Poznań, Poland with primers used for PCR and additionally for inner sequences with GTGCCAGCMGCCGCGGTAA (for 16S rDNA) and CTTTTACTTCCTCTAAATGACC (for 18S rDNA). Obtained sequences were arranged into contigs and identified in the BLAST service of the GenBank database (Altschul et al. 1990).

HPLC analysis

Determination of atrazine was carried out using a MERCK-HITACHI system consisting of an autosampler (model L-7250), a pump (model L-7100) and a DAD L-7455 set at 220 nm. Analyses were performed isocratically at a flow rate of 0.60 ml/min, at 30°C on a Lichrospher® RP-18 250 × 4.60 mm column (MERCK). Acetonitryle and 1 mM sodium acetate (35:65) were used as a mobile phase. Samples were filtered (0.22 µm, Millex-GS, Millipore) and injected at 30.0 µl. A standard was used to identify peaks in chromatograms, and a peak area was used to determine sample concentrations. The identity of each peak was confirmed by comparing the spectrum of the standard with that of the positive peak in the sample after normalization. This was accomplished via computer integration (Chromatography Data Station Software, MERCK-HITACHI) operated in the mode of the external standard. A standard calibration solution was prepared within the range of 0.10–5.00 mg/l and 0.10–20.0 mg/l and depended on the variant of the experiment.

Statistical analysis

Data from each experiment were analyzed via analysis of variance (ANOVA) to detect significant differences between treated groups and the control. Standard deviations and standard errors were also calculated. The probability of α (type I error) was 5.00% ($P < 0.05$).

The rate of atrazine degradation was described by the one-phase exponential decay equation:

$$y = a \cdot \exp(-k \cdot t) + b$$

where y is the concentration of atrazine (mg/l), t is time (days), a is the coefficient which represents the distance from the starting point to the bottom plateau, b is the bottom plateau value, and k is the rate constant (days^{-1}). For each experiment, the half-life ($t_{1/2}$) of atrazine was also calculated. The half-life is the time required for half of the atrazine concentration to decay. The half-life and decay rate constant are related by the equation:

$$t_{1/2} = \frac{\mathrm{In}(2)}{k} = \frac{0.693}{k}$$

Statistical analysis was carried out using STATISTICA (data analysis software system), version 6.0, by Statsoft Inc. (2004).

Results

Microorganism isolation and identification

Microorganisms were isolated from the sweet flag rhizosphere to evaluate their role in the degradation of atrazine. We recorded 3.8×10^7 cfu of psychrophilic bacteria, 1.8×10^7 cfu of mesophilic bacteria, and 6.0×10^5 cfu of fungi per 1 g of dry root mass. Following macroscopic observations, seven strains of psychrophilic bacteria (BP), nine strains of mesophilic bacteria (BM), and six strains of fungi (GR) were selected for further analysis from the grown microorganisms. All these strains were tested for their atrazine degradation ability (Figs. 1–3).

The highest atrazine reduction level was observed for fungi (Fig. 3), with levels varying from 18% to almost 60% of initial atrazine concentration.

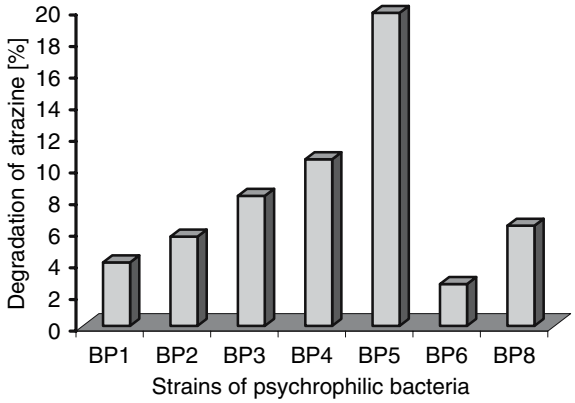

Fig. 1 Atrazine degradation level after 5 days of psychrophilic bacterial growth in medium supplemented with 5 mg/l of herbicide

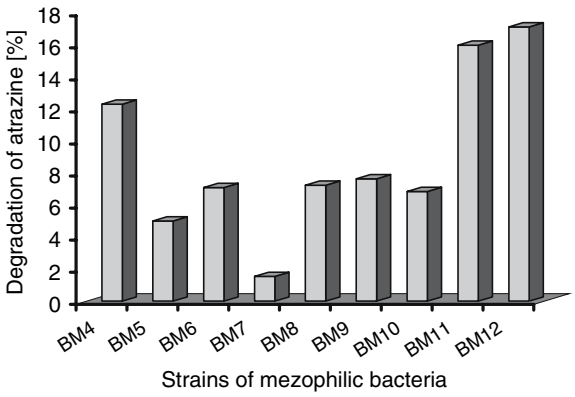

Fig. 2 Atrazine degradation level after 5 days of mesophilic bacterial growth in medium supplemented with 5 mg/l of herbicide

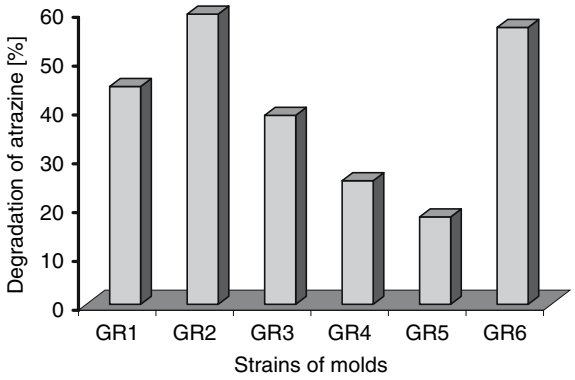

Fig. 3 Atrazine degradation level after 5 days of mold's growth in medium supplemented with 5 mg/l of herbicide

Psychrophilic (Fig. 1) and mesophilic bacteria (Fig. 2) reduced much less atrazine than fungi, with reduction levels below 20%. Among all the three groups, the most active microorganisms within each group were selected by statistical analysis for further examination on the basis of their greatest impact on atrazine removal. The chosen strains were as follows: psychrophilic bacteria BP3, BP4, and BP5; mesophilic bacteria BM4, BM11, and BM12; and fungi GR1, GR2, and GR6. The strains coded as BP3, BP4, BP5, and BM4 were evaluated as Gram-negative, non-lactose fermentating bacteria, while BM11 and BM12 were Gram-positive bacillus strains. In addition, appropriate molecular analyses were performed and respective species were identified (Table 1).

Atrazine degradation rate in batch culture of selected microorganisms

Selected strains (Table 1) were tested for their ability to decompose atrazine at different concentrations (5.00 and 20.0 mg/l) during 15 or 21 days of culture (molds were grown for 21 days because of their slower growth rates). The rate of atrazine reduction was evaluated for each strain, and the results are shown in Figs. 4–6.

The highest reduction level was observed for fungi and mesophillic bacteria (Figs. 5–6). During 21 days of fungi culture, on average 73.4 ± 13.2% of atrazine was removed from the medium at the concentration of 5.00 mg/l, and 47.7 ± 6.16% removed at the concentration of 20.0 mg/l. The most effective strain was *Botrytis cicerea*. Mesophillic bacteria (Fig. 5)

Table 1 Identification results of the most effective atrazine-degrading bacteria strains

Strain names abbreviations	Names of identified microorganisms strains
BM4	*Stenotrophomonas maltophilia*
BM11	*Bacillus licheniformis*
BM12	*Bacillus megaterium*
BP3	*Rahnella aquatilis* strain 1
BP4	*Rahnella aquatilis* strain 2
BP5	*Rahnella aquatilis* strain 3
GR1	*Umbelopsis isabellina*
GR2	*Volutella ciliata*
GR3	*Botritis cinerea*

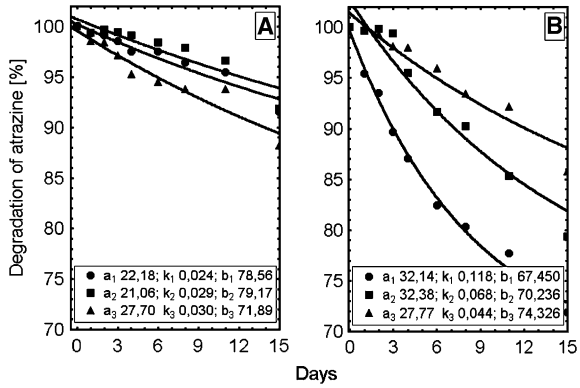

Fig. 4 Atrazine degradation model in selected psychrophilic bacteria culture. (**A**) Concentration of atrazine 5 mg/l, (**B**) Concentration of atrazine 20 mg/l. Symbols: ⬣ *Rahnella aquatilis strain 1*, ■ *Rahnella aquatilis strain 2*, ▲ *Rahnella aquatilis strain 3*

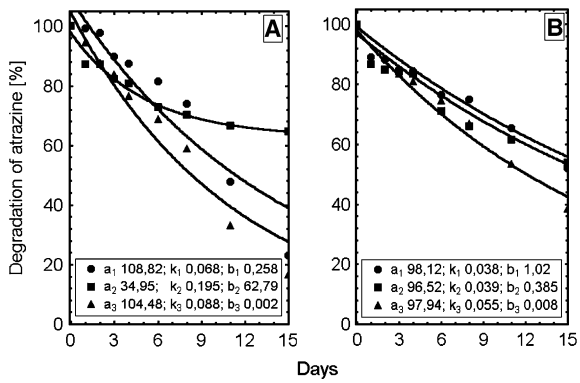

Fig. 5 Atrazine degradation model in selected mesophilic bacteria culture. (**A**) Concentration of atrazine 5 mg/l, (**B**) Concentration of atrazine 20 mg/l. Symbols: ⬣ *Bacillus licheniformis*, ■ *Bacillus megaterium*, ▲ *Stenotrophomonas maltophilia*

were also effective in this process, with the highest reduction level being 83.5 ± 3.60% for *Stenotrophomonas maltophilia* grown at 5.00 mg/l atrazine concentration. Psychrophilic bacteria (Fig. 4) were less active, with an average of 10.0 ± 2.35% degradation level at atrazine concentration of 5.00 mg/l.

A statistical model, represented by the equation $y = a \cdot \exp(-kt) + b$, was used to compare the rate, constant *k*, and half-life of atrazine degradation recorded for each of the tested strains. The shortest atrazine degradation time was observed for *Bacillus megaterium*, grown in the presence of 5.00 mg/l atrazine. Half of the total degraded xenobiotic was

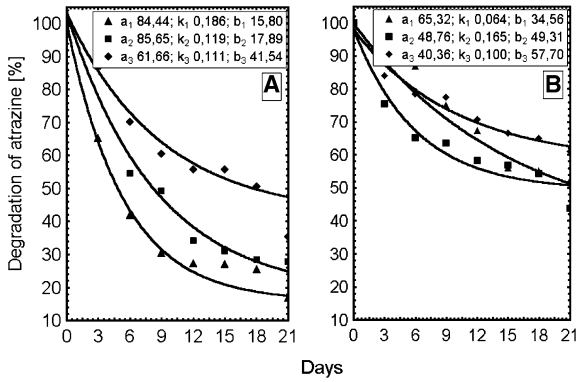

Fig. 6 Atrazine degradation model in selected molds culture. (**A**) Concentration of atrazine 5 mg/l, (**B**) Concentration of atrazine 20 mg/l. Symbols: ▲ *Botritis* cinerea ■ *Volutella ciliata* ◆ *Umbelopsis isabellina*

removed after 3.5 days (Fig. 5). Similar results were observed for *Botrytis cinerea*, where half of the total reduced atrazine was removed from the medium after 3.7 days (Fig. 6). Generally, for mesophilic bacteria and molds the higher atrazine concentration (20.0 mg/l) resulted in a slower degradation process (Figs. 5–6). Only for *Volutella ciliata* grown on a medium containing atrazine at a concentration of 20.0 mg/l was a shorter half-life of herbicide degradation recorded (4.2 days instead of 5.8 days), although in this case the final level of degradation was low (47.7 ± 6.16%; Fig. 6).

Comparing all the tested strains, only the psychrophilic bacteria were less active in the process of atrazine degradation, with low *k* values and low degradation levels recorded for all tested strains regardless of atrazine concentration (Fig. 4).

Anyhow, no full mineralization of atrazine was found in any of the treatments, although it was converted into metabolites (data unpublished). However, the lowest levels of deetylatrazine, deisopropylatrazine and hydroxyatrazine were observed in the *Stenotrophomomas maltophilia* culture, which may suggest that this strain is able to perform the mineralization of atrazine.

Growth rate in batch cultures of selected microorganisms

We also evaluated the impact of atrazine presence and concentration on the growth rate of selected strains. Every 3 days of culture samples were

extracted and the number of cells was calculated using the pouring plate method; results are shown in Figs. 7–9.

There were no statistical differences between growth rates of different strains of fungi and bacteria in the presence of 5 mg of herbicide. In most cases the number of growing cells was identical as in the control culture. Only for the mesophilic bacteria strain (*Bacillus megaterium*) lower counts of cells were observed (Fig. 8A).

Increased atrazine concentration (20.0 mg/l) resulted in a significantly reduced growth rate of fungi: a 50% reduction in cell numbers was observed for all three strains of molds. However, some bacterial strains, e.g. *Stenotrophomonas maltophilia, Bacillus licheniformis*, and *Rahnella aquatilis* (strain 2) were positively affected by the higher atrazine concentration. In these cases, the number of cells was greater than 4×10^{10}, 1×10^{11}, and 6×10^{11} cfu/ml, respectively. In any case, the larger number of cells did not significantly influence the atrazine reduction level (Figs. 7–9).

Discussion

Microorganisms are of great importance in environmental cleaning and herbicide degradation. In microbially active soil, the mean half-life of atrazine is 2.4 times lower than in sterile soil (Accinelli et al. 2001). In case of atrazine, several strains of bacteria and fungi have been identified as atrazine degrading

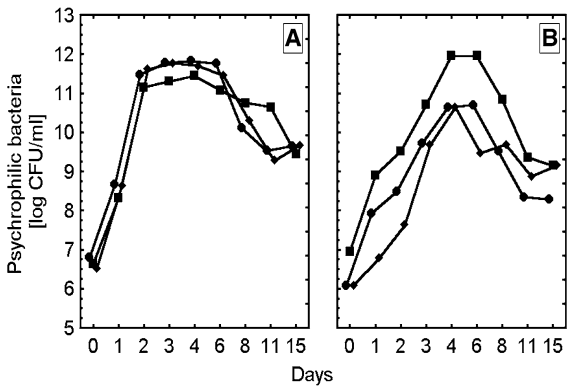

Fig. 7 Growth kinetics of selected psychrophilic bacteria strains culture in the presence of atrazine. (**A**) Concentration of atrazine 5 mg/l, (**B**) Concentration of atrazine 20 mg/l. Symbols: ⬣ *Rahnella aquatilis strain 1*, ■ *Rahnella aquatilis strain 2*, ◆ *Rahnella aquatilis strain 3*

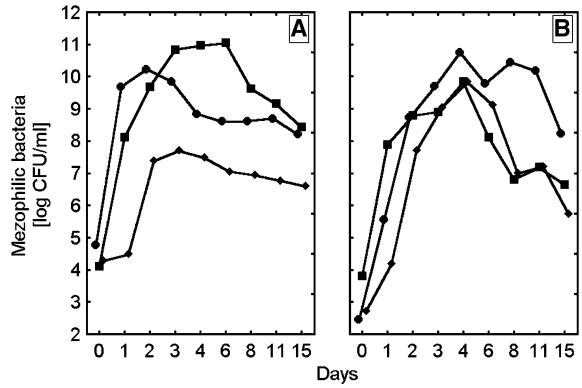

Fig. 8 Growth kinetics of selected mesophilic bacteria strains culture in the presence of atrazine. (**A**) Concentration of atrazine 5 mg/l, (**B**) Concentration of atrazine 20 mg/l. Symbols: ■ *Bacillus licheniformis*, ◆ *Bacillus megaterium*, ⬣ *Stenotrophomonas maltophilia*

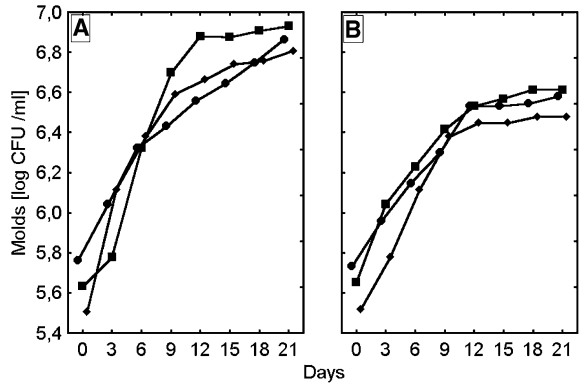

Fig. 9 Growth kinetics of selected molds strains culture in the presence of atrazine. (**A**) Concentration of atrazine 5 mg/l, (**B**) Concentration of atrazine 20 mg/l. Symbols: ⬣ *Botritis cinerea* ■ *Volutella ciliata* ◆ *Umbelopsis isabellina*

microorganisms. Many of these microorganisms may perform atrazine dealkylation, deamination or dechlorination, but not complete mineralization. In this way atrazine is converted to hydroxyl atrazine (HA), deetyl atrazine (DEA), deisopropyl atrazine (DIA), deethyl deisopropyl atrazine (DEIA) or dethyl deisopropyl hydroxyl atrazine (DEIHA). These derivatives of atrazine conversion are still hazardous for the environment, and harmful for human health (Gosh et al. 2004; Seffernick et al. 2000).

Pseudomonace species have been described as bacteria capable of utilizing hundreds of different compounds as their source of carbon and energy. *Pseudomonas* sp. ADP strain is one of the best-

known bacteria which show the ability to metabolize atrazine. *Pseudomonas* sp. ADP strain can use atrazine as a sole nitrogen source, but for growth require the addition of a carbon source. This strain of bacteria contains the plasmid-borne genes *atzA, atz B*, and *atzC*, which encode the aminohydrolase enzymes useful for the complete biodegradation of atrazine to ammonia, chloride and carbon dioxide. This *Pseudomonas* strain mineralized 63% of atrazine after 72 h of culture (Clausen et al. 2002). This kind of *Pseudomonas* activity could be helpful in explaining the results obtained in our study, as *Stenotrophomonas maltophilia*, which is very effective in the atrazine degradation process (83.5 ± 3.60% of atrazine reduction level), was previously taxonomically classified as *Pseudomonas maltophilia* (Schreckenberger 1995). Genes which are homologues to *atzA, atz B*, *atzC* present in *Pseudomonas* sp. ADP strain have also been isolated from *Ralstonia picketti* D, *Rhizobium* strain PATR, *Pseudoaminobacter* strains C147, C195 and *Alkaligenes* strain SG1, *Agrobacterium radiobacter*, which may suggest that these genes are widespread in the world of microorganisms (Struthers et al. 1998; Seffernick et al. 2000; Rousseaux et al. 2001; Clausen et al. 2002).

The atrazine degradation level depends on the kind of used microorganisms and the other abiotic factors or supplements. Struthers et al. (1998) tested *Agrobacterium radiobacter* in the presence of atrazine as the only source of nitrogen. A 94% reduction in atrazine was observed after 72 h of culture. Up to 48% of atrazine degradation level was recorded by Ghosh et al. (2004). This degradation level was reached in wastewater containing 5 mg/l of atrazine and 300 mg/l of dextrose under anaerobic culture of mixed microbial cosortium. However, *Pseudomonas* sp. ADP strain could also degrade atrazine under anaerobic conditions, but the degradation level was lower (55%) than under aerobic conditions (63%) (Clausen et al. 2002). Acinelli at al. (2001) observed that under anaerobic conditions, atrazine degradation was markedly slower than under aerobic conditions, with half-life of 124 and 407 days in surface soil and subsoil, respectively.

Strains isolated and identified in our experiments are not commonly described in the papers as atrazine degrading microorganisms; however, but some of them could degrade other xenobiotics. *Stenotrophomonas maltophilia* is mentioned in terms of the degradation of xenobiotics such as 2,4-dichlorophenoxyacetic acid (2,4-D), hexachlorocyclohexane (HCH), polycyclic aromatic hydrocarbons (PAHs), cyclotrimethylenetrinitroamine derivatives (RDX), and oil product derivatives (Binks et al. 1995; Rousseaux et al. 2001; Samanta et al. 2002; Tesar et al. 2002; Osterreicher-Cunha et al. 2003). Other bacterial strains evaluated in our study exhibited lower atrazine degradation capabilities, although Texier et al. (1999) found that *Bacillus megaterium* could support the photodegradation process of atrazine. These strains are also known as bioremediation tools for other xenobiotics. *Bacillus megaterium* efficiently degrades trifluralin (α,α,α-trifluoro-2,6-dinitro-N,N-dipropyl-*p*-toluidine): after 30 days of incubation, a 21% reduction in the herbicide level was observed (Bellinaso et al. 2003). Pentachlorophenol is also degraded by this species, with a 99% reduction level observed after 6 weeks of incubation (McGrath and Singleton 2000).

The mechanism of atrazine degradation by fungi is not so well known. The ability of fungi to degrade atrazine and other xenobiotics such as dioxins, polychlorinated biphenyls (PCBs) could be due to the action of enzymes involved in the lignine degrading system (LDS). Extracellular peroxidases—lignin peroxdases (LiPs) and manganese dependent peroxidases (MNPs) and laccases are key components of the LDS (Reddy 1995). White-rot fungi are the most active degraders of a wide variety of toxic xenobiotics. *Phanerochaete chrysosporium* is capable of degrading 48% atrazine after four days of incubation (Reddy 1995). A similar effect was observed for white-rot fungi *Pleurotus pulmonarius.* In this case atrazine transformation was enhanced by the addition of manganese [Mn(II)]. Up to 98% decrease in atrazine concentration was recorded in a culture of soil mycelial fungus INBI 2–26 (-). The presence of atrazine in the medium of this culture at a concentration of 20 and 50 mg/l increased fungal growth and the synthesis of the degrading enzyme (cellobiose dehydrogenase) (Khromonygina et al. 2004).

Among the fungi isolated from the sweet flag rhizosphere in our experiment, *Botrytis cinerea* was identified as most active in the atrazine degradation process. Even if according to the published data fungi are considered to be less important in the degradation of xenobiotics, atrazine reduction recorded in our

experiments was 82% for *Botrytis cinerea*. Previously Bordjiba et al. described the positive role of *Botrytis cinerea* in the degradation of triazine herbicides. A 50% reduction in metribuzine at a concentration of 100 mg/l was found after 5 days culture of this fungi (Bordjiba et al. 2001).

Other fungi (*Umbelopsis isabellina* and *Vollutella ciliata*) isolated in our study are seldom mentioned as xenobiotic degraders, although *Umbelopsis isabellina* is able to degrade syntetic dyes and exhibits enzymatic activity of manganese-dependent peroxidase (Yang et al. 2003). These strains of fungi could also enhance phytoremediation processes by the degradation of phytotoxins in the rhizosphere (Voughan et al. 1993).

Conclusion

The positive effect of microorganisms in the process of xenobiotic degradation can be explained in terms of a variety of different species and a broad spectrum of produced enzymes. Microorganisms can therefore use different sources of carbon and nitrogen to adapt to different environments. The process of microbial atrazine degradation is conducted via the detachment of chlorine, N-dealkilation of the lateral chain, dissociation of the triazine ring, and then mineralization to NH_4, CO_2, and Cl. These processes can be carried out by one microorganism or by a mixture of different species of microorganisms adapted to an environment contaminated by triazines and able to perform herbicide degradation (Struthers et al. 1998; Seffernick et al. 2000). Introduction of selected microbial communities to atrazine contaminated sites (bioaugumentation) could be a helpful method of enhancing environment remediation.

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