Szent István University

Genetic and ecophysiological survey of atrazine-degrading soil and sediment bacteria

Ph. D. thesis

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1. Introduction

Xenobiotic pollution of agricultural soils, surface and subsurface waters is one of the most urgent issues of current environmental protection. Pollution by various pesticides involves especially extensive areas, due to their widespread – and often irresponsible – use.

Atrazine is an *s*-triazine herbicide (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) used since the 1950s. It is applied in various fields of agriculture, mainly corn production and forestry for the selective elimination of broad-leaf weeds. Its usage was restricted in the European Union, however, its consumption exceeds 1000 tons in Hungary and 40 000 tons worldwide. Due to its intense use, atrazine is frequently detected in agricultural soils, groundwater and surface waters (Radosevich et al., 1996; Boyd et al., 2000). Though the acute human toxicity of atrazine is low, long term repeated interaction is proved to cause kidney, lung and endocrine damage, and it is a presumptive carcinogen.

Half-life of atrazine is generally 50-70 days in soil. Its concentration is influenced by both biotic and abiotic processes. Atrazine can be transported to streamwaters by runoff precipitation or leached to subsurface soil and groundwater. In these environments, its half-life is considerably longer (up to several years). Atrazine is readily adsorbed on soil or sediment particles, the extent of sorption depends mostly on the clay- and organic matter content of the environment (Németh-Konda et al., 2002). This process enables the accumulation of the compound and decreases its bioavailability. Early studies on the degradation of atrazine attributed it mainly to abiotic processes such as photolysis, or chemical hydrolysis. Subsequent studies however confirmed the principal importance of microbial degradation (Assaf and Turco, 1994). Since the 80-ies, various microorganisms were isolated, capable of the dechlorination or dealkylation of atrazine. In the middle of the previous decade, almost simultaneously, several research groups observed fast and complete degradation in soil and isolated atrazine mineralizing bacteria (Mandelbaum et al, 1995; Radosevich et al, 1996). Currently known atrazine-degrading isolates are taxonomically diverse, including various *Pseudomonas* strains, and representatives of the *Stenotrophomonas*, *Alcaligenes*, *Clavibacter*, *Rhizobium*, *Pseudaminobacter*, *Nocardioides*, *Rhodococcus* and *Arthrobacter* genera (de Souza et al., 1998; Topp et al., 2001; Rousseaux et al, 2001; Struthers et al., 1998). The possible end-products of atrazine degradation are hydroxiatrazine, monoalkyl-ammeline or –ammelide derivatives, cyanuric acid, or inorganics (carbon-dioxide and ammonia). Metabolic pathway of *Pseudomonas* ADP was investigeted in detail (atrazine → hydroxiatrazine → ethylammelide → cyanuric acid → biuret →urea), and to our present knowledge is the most widespread mechanism (Martinez et al., 2000). Enzymes and genes participating in atrazine were also identified, and subsequently detected in taxonomically and geographically diverse bacteria (Rousseaux et al., 2001).

Theoretically, carbons and nitrogens of the aminoalkyl groups and nitrogens from the triazine ring are utilized for bacterial growth during biodegradation (Bichat et al., 1999). Most previously described atrazine-degrading microorganisms use atrazine as a nitrogen-source, though some of them can grow on atrazine as a sole source of carbon as well. Recent studies state that the latter quality enhances the survival and *in vivo* biodegradative activity of atrazine-degrading inocula in soils, and thus is essential for bioremediation (Topp et al., 2001).

Like every microbial activity, biodegradation of atrazine is influenced by various environmental factors. However, previous data on the optimal parameters of degradation is scarce. Highest catabolic activity was observed between 20 and 30 ºC in bacterial culture, at lower temperature efficiency of biodegradation decreased, and under 15 ºC (or according to other studies, 7 ºC) it ceased completely. Optimal pH range was 5.5 to 8.5, lower pH inhibited degradation in liquid culture (Mandelbaum et al., 1995). Unfavorable effect of acidic pH was even more pronounced in soils, under pH=6.5 inhibition was complete (Houot et al., 2000). Beside the inhibition microbial activity, at low pH values soil adsorption of atrazine and hydroxiatrazine is increased. Formation of irreversibly bound residues is also a limiting factor of biodegradation. Effect of various carbon and nitrogen amendments was investigated more thoroughly, though different studies gave contradicting results (Abdelhafid et al., 1998). For instance, presence of inorganic or easily degraded nitrogen sources inhibited the biodegradation of atrazine in liquid culture of some isolates, while it had no significant effect on other organisms. Similar differences were observed in soil. Complex carbon sources generally increased the efficiency of biodegradation. Easily utilized carbon sources may have limiting effect, while in other studies some of them (e. g. citrate, mannite, glucose) proved to enhance atrazine catabolic activity.

Several physico-chemical techniques were developed for the clean-up of atrazine pollution (e. g. thermal desorption, or various combinations of UV, peroxides, and metal-oxides). These treatments however are generally expensive, may involve the formation of toxic by-products, and in some cases the end-product requires further treatment. Bioremediation – biological (generally microbial) degradation of the pollutant – is a cost-effective and environmentally friendly alternative.

The term bioremediation includes a variety of treatments, depending on the quantity and quality of the pollutant, parameters of the polluted area, presence and amount of biodegrading bacteria (Hurst et al, 1997). If the pollutant is degraded easily, stimulation of the autochthon microbial communities is usually sufficient (e. g. amendment of carbon, nitrogen, phosphorus or trace elements, watering, airing etc.). A more resistant pollutant or high amount of it might require bioaugmentation (inoculation of the polluted area by xenobiotic degrading bacterial culture from strain banks or isolated from the site). This technique however involves several further difficulties:

inocula are often subdued by the endogenous microbial communities, some inoculating strains are potential pathogens, recombinant inocula mean environmental hazard. Cell-free bioremediation methods (e. g. application of crude cell extract or purified catabolic enzymes) eliminate these problems. A recently discovered possibility is the utilization of horizontal gene transfer for bioremediation purposes. Horizontal gene transfer is the principal source of genetic variability in the domains Bacteria and Archea. Thus it is involved in the adaptation to pollutants, and has an important role in the unassisted biodegradation (bioattenuation) of xenobiotic pollutions. Three basic mechanisms of horizontal gene transfer are known. During transformation, bacterial cells take up naked double-stranded DNA fragments. Conjugation means the transfer of mobile genetic elements from a donor to a recipient cell, it requires contact of the cells. Phages perform the transfer in transduction. Transfer of catabolic genes for various xenobiotics (e. g. naphtalene, 2,4-D) was observed in bacterial culture and soil (Herrick et al., 1997).

Since atrazine degrading plasmids were found to be located on plasmids in at least some of the described atrazine degrading bacteria, it is possible, that this gene region has evolved in one or few locations as a result of long-term atrazine pollution, and was then dispersed through horizontal gene transfer. This hypothesis provides a possible explanation for the nearly simultaneous appearance of atrazine mineralizing isolates.

During the present work, biodegradation of atrazine in sediment and soil was studied. My aim was to survey the possibility and conditions of bioremediation using multiple approach. The study of atrazine degradation in river sediment was performed in an artificially polluted laboratory microcosm. Soil samples were collected in a site of ling atrazine history. Specific aims of the study were the following:

- Survey on the unassisted biodegradation in soil. Detection and identification of produced and accumulated metabolites, comparison to those previously described.
- Investigation of the microbiological effects of atrazine. Alterations in the total and atrazine-degrading viable counts, and microbial diversity as an effect atrazine pollution in soil.
- Trace acute and chronic atrazine pollution in a laboratory microcosm containing native Danube gravel sediment. (The microcosm is a model of bank-wall filtered wells.) Assessment of the relative significance of adsorption and biodegradation. Identification of produced/accumulated metabolites in the model system, Danube water and the water of bank-wall filtered wells, comparison of the results.
- Assessment of the effect of pollution on the sediment microbial community (total and atrazine-degrading viable count and diversity).
- Isolation of atrazine-degrading bacteria from sediment and soil. Characterization and identification of the isolates.

- Survey on the nutrition preference and biomass production on atrazine of the isolated strains.
- Characterization of atrazin degradation mechanism (metabolites, pathway, speed-limiting steps) of the isolates.
- Based on the results of the above investigations, selection of efficient atrazine degrading strains as potential inocula for bioremediation of atrazine pollution.
- Determination of the optimal conditions for the growth and biodegradative activity of the isolates. Investigation of the effect of nutrient amendments (carbon and nitrogen sources, vitamins).
- Investigation of the atrazine catabolic genes, detection of previously described atrazine-degrading genes in the isolated strains.
- Investigation of horizontal gene transfer in the sediment. Transfer of plasmid-encoded antibiotic resistance genes from a donor organism to the autochton microbes. Detection, isolation and characterization of the recipients. Survey on the conditions of horizontal gene transfer, extension of the investigation to catabolic genes.

2. Materials and methods

2.1. Soil sampling and characterization

Soil samples were collected in Ják village (Vas County), in the surroundings of a mansion used formerly as herbicid storage-distribution site. Samples were taken at 7 randomly chosen points, from the upper 10-15 cm layer of the soil. Samples were homogenized and sieved on a 2 mm sieve. Particle size distribution was determined by further sieving and sedimentation (Stefanovits et al., 1999). Total organic carbon and total Khejhldal nitrogen was determined spectrophotometrically following a digestion with concentrated sulphuric acid and hydrogen peroxide (for nitrogen evaluation) or potassium-dichromate (for TOC). For the determination of the pollutant content, soil samples were extracted by solvent (aceton-hexane) extraction, and extracts analyzed by reverse phase HPLC-MS. Assessment of viable count was performed by the Most Probable Number method in nutrient broth for the total, and atrazine-mannose broth for atrazine-degrading viable count (Hurst et al., 1997).

2.2. Construction of the model system

Six sediment core samples (length 70-80 cm, ø 12 cm) were taken at Szentendrei Island, near the Kisoroszi I. bank-wall filtered well from the under-water gravelbed. The samples (transferred to PVC columns) were used for the construction of a laboratory model system. The model was fed with native Danube water, water exchange was maintained by a peristaltic pump.

2.3. Collection and characterization of water samples

Water samples were obtained from the outlet water and side sampler openings of the model system. Feed water (i. e. native Danube water) was also analyzed. Concentration of ions relevant for drinking water quality (nitrate, nitrite, ammonia and phosphate) were determined spectrophotometrically. Conductivity and pH were measured by specific analytical electrodes. Determination of the total viable count was preformed by the Most Probable Number method in nutrient broth.

2.4. Degradation of atrazine in soil

The entire process of atrazine degradation was not followed in the soil samples, metabolism was presumed based on the detected metabolites. Residual atrazine and its degradation products were determined by solvent (aceton-hexane) extraction and RP-HPLC/MS analysis.

2.5.Degradation of atrazine in sediment

Background values for atrazine and atrazine metabolites were determined in Danube and the bank-wall filtered wells (at the site where the sediment samples were collected) by solid phase extraction and RP-HPLC/MS. Acute atrazine pollution was simulated in the laboratory model system in 2 subsequent studies by loading first 450, then 80 μg atrazine on the S5 column dissolved in 10 mL (2 mL) water. Outlet water was collected in 300 (100) mL fractions, and analyzed by RP-HPLC/MS for atrazine and atrazine metabolites. For the investigation of long-term atrazine pollution, S5 column was fed for 5 months on water amended with 100 μg L^{-1} atrazine. Appearing metabolites were determined by RP-HPLC/MS. Alteration of total viable count as an effect of atrazine pollution was measured by MPN in nutrient broth. Number of atrazine degraders was assessed on atrazine-ammonia plates.

2.6. Isolation of atrazine-degrading bacteria

Atrazine degraders were isolated from the soil samples after enrichment in atrazine containing media on solid media containing atrazine as a sole carbon or nitrogen source. Water samples from the model system were directly streaked on agar containing plates. Colonies showing considerable growth after one week were isolated.

2.7. Characterization of atrazine degrading strains

Colony morphology was characterized by or stereomicroscopy, bacterial cell morphology was studied by phase contrast microscopy. Cell wall structure was analyzed by Gram stain and Japanese Gram test. Schaeffer-Fulton spore stain was performed for the determination of endospore formation. Basic biochemical tests (catalase reaction, Kovacs oxidase test, Hugh-Leifson sugar

utilization test) were also perfomed. For the sediment bacteria, carbon utilization spectra was determined using BIOLOG fast identification system (Gerhard et al., 1994).

2.8. Identification of bacterial isolates by 16S rDNA sequence comparison

Bacterial cells were broken by enzymatic reaction, using lysosim, proteinase K and SDS. DNA was extracted by phenol-chloroform extraction, and purified by a silicamartix-based kit (Prep-A-Gene). Species-specific 16S rDNA fragment was amplified by polimerase chain reaction (PCR), using universal Eubacteria primers. DNA and PCR-products were detected by ethydium-bromide stain following gel electrophoresis in 1 % agarose gel. Identical isolates were screened by ARDRA (amplified ribosomal DNA restriction analysis) method. PCR products were digested by Taq I. and Hin 6 I. restriction enzymes, then fragments were separated in 1.5 % agarose gel by gel electrophoresis. Strains having identical restriction pattern were grouped in ARDRA groups (Stackebrandt and Goodfellow, 1991). PCR product of group representatives was sequenced by labelled dye terminated cyclic sequencing reaction. ABI Prism 300 sequence analysis machine was used for the determination of nucleotide sequence. For the identification, sequences were compared to those described previously in Internet databases by BLAST sequence fitting computer program.

2.9. Atrazine utilization and biomass production of the isolates under C or N limited conditions

Strains were streaked on plates containing atrazine as a sole source of nitrogen or carbon for the determination of their preference for atrazine as a nutrient. Biomass production was assessed in atrazine containing liquid growth culture by the measurement of net wet cell mass.

2.10. Degradation of atrazine in liquid culture

Atrazine containing media was inoculated by the isolates, and grown shaking for 2 weeks. Cells were partially broken by sonication and removed by centrifugation. 1 mL of the supernatant was extracted by solid phase extraction and analyzed by normal-phase HPLC/MS for atrazine and its metabolites.

2.11. Investigation of atrazine degradation on microplate-scale.

A method was developed for the investigation of atrazine degradation on microplates. It is more simple and cost-effective than the traditional methods. 24-well microplates were used. Liquid media containing atrazine as a sole carbon or nitrogen source was pipetted to the wells, then inoculated with the isolates. Samples were analyzed as described above.

2.12. Determination of the atrazine metabolic pathway

The method described above is suitable for the determination of the exact metabolic pathway and dynamics of the isolates. In this case, all wells on the microplate were inoculated with the same

organism. Each week, content of one row was harvested and analyzed. Thus the process was followed for 6 weeks.

2.13. Optimal ecological parameters of atrazine degradation

Optimal conditions of growth and atrazine catabolic activity of the isolated strains was performed on microplates. Atrazine containing liquid media was filled to the wells of a 96-well plate, and inoculated with the strains. Bacterial growth was indicated by the color shift of resasurin redox indicator, and detected spectrophotometrically on 405, 450 and 600 nm. Composition of the media varied according to the investigated parameters. All of the contained the same salt stock, phosphate-buffer, resasurin indicator and atrazine. Mannit, glucose, sodium-nitrate, ammonium-sulphate or vitamin stock solution was added for the survey on the effects of various nutrient amendments. Effect of temperature and pH were also studied at 4, 12, 20, 28, 37, 42, 50 ˚C and 3, 4, 5, 6, 7, 8, 9 and 10 pH values.

2.14. Investigation of atrazine catabolic genes

Of the previously described atrazine catabolic genes *atzA* and *atzB* of *Pseudomonas* ADP strain were chosen for investigation. Presence of the genes was investigated by PCR, using specific primers designed based on literature data. Touch-down thermocycle profile was used (were anellation temperature changed in each cycle). PCR products were detected in 1 % agrose gel by ethydium-bromide stain.

2.15. Microbial diversity of the soil samples

Molecular diversity was investigated by two methods of different resolution. Total DNA of the soil samples was extracted using glass-bead method or the Fast-DNS kit. Extracts were purified on silicamatrix (Prep-A-Gene). Species specific 16S rDNA fragment was then amplified by PCR. Universal Eubacteria primers were used for amplification in the community ARDRA study, then PCR product was digested by Taq I. and Hin 6 I. restriction enzymes. Digestion fragments were separated in 2 % agarose gel, the pattern was visualized by ethydium-bromide stain under UV (Stackebrandt and Goodfellow, 1991). For the denaturing gradient gel electrophoresis (DGGE) specific GC primers (i. e. primers with long, high GC clamp) were used for amplification (Hurst et al., 1997). Electrophoresis was performed in 8 % poliakrylamide gel, denaturing gradient was set from 40 to 70 %. Denaturing agents were urea and formamide. After electrophoresis, gel was stained with ethydium-bromide. DNA fragments were detected under UV by LCD camera.

2.16. Horizontal gene transfer in the sediment

Viable count of coliform and antibiotic resistent organisms in the sediment column were assessed on ENDO and antibiotic (Amp, Km, Tet) amended ENDO agar plates. Escherichia coli XL1 Blue strain (having plasmid-encoded Tet resistance) was used as a donor organism. Liquid culture of the donor strain was ussed for the inoculation of the sediment column S4, then outlet water and water from side samplers were analyzed. Recipient cells (i. e. organisms having acquired Tet resistance) were isolated on Tet- amended ENDO and LB plates. LB+Tet broth was also inoculated by the water samples. Presence and alterations of Tet resistant microbial community was detected by community ARDRA (method described in 2.15.) (Clerc and Simonet, 1998).

2.17. Analytical methods

Sample preparation of the water samples was performed using octadecyl-silica solid phase extraction (SPE) columns. Pollutants were extracted from soil samples by acetone-hexane solvent extraction. For the analysis of bacterial growth cultures, cells were disrupted by sonication, cell debris was removed by centrifugation and the supernatant extracted by SPE. Environmental samples were analyzed by a purpose-developed reverse-phase HPLC method, which has very low detection limit for atrazine and its primary metabolites. Microbial degradation was investigated by normal-phase HPLC. The latter technique is less sensitive, however it is suitable for the detection of all atrazine metabolites in a single run.

3. Results and Discussion

3.1. Characterization of the soil samples

General soil type of the area is brown forest soil (Luvisol). Particle size distribution and organic matter content of the samples were diverse. C:N ratio was slightly lower than 10 for most samples, pH was slightly acidic. Atrazine concentration ranged from 0.7 to 86 $\mu g\ kg^{-1}$, some samples also contained gasoline and other pollutants. Total viable count was 105-107 CFU mL-1, count of atrazine degraders ranged from 103 to106. The former showed correlated to organic nitrogen content of the samples, while the latter to the atrazine concentration.

3.2. Characterization of water samples

Chemical parameters of water samples are good indicators of the filtration efficiency. Concentration of nitrite and ammonia decreased significantly during filtration in the sediment column, while the amount of nitrate has changed only moderately. This phenomenon suggests mainly aerobic conditions in the sediment sample. Phosphate concentration and Conductivity was stable, pH was

less alkaline within the column than that of the Danube water. Overall data is in accordance with the results from a previous in situ investigation (Zalmum, 1997).

3.3. Atrazine degradation in soil

10 years after the last contamination, samples still contained atrazine up to 30 times the environmental safety limit. High residual concentration implies large initial amounts of pollutant, as well as low efficiency of degradation. Since atrazine degraders were present at the site in ample quantity, limiting condition was presumably the acidic pH of the soil. Accumulated metabolites were hydroxi-atrazine and ethyl-ammelide.

3.4. Atrazine degradation in the sediment

Atrazine and hydroxi-atrazine (a total of 0.75 μg L^{-1}) were detected in Danube water, while their monodesalkyl metabolites were dominant in the bank-wall filtered wells. Subsequent to a single load of atrazine on the sediment column, predominantly adsorption was observed, 20-25 μg was retained permanently. This suggests considerably lower sorption capacity than that of the soil. Residence time was several days, equal to several weeks in the natural sediment. Following 5 months of continuous load, 90 % of atrazine was degraded. Stable metabolite was hydroxi-atrazine, similarly to results of previous investigations in aqueous environments. Pollution did not affect the total microbial count, though quantity and diversity of atrazine degraders increased.

3.5. Isolation and characterization of atrazine degrading bacteria

25 maintainable strains were isolated from the soil and 17 from the sediment samples. Former group consisted exclusively of Gram negative, motile, non-sporeforming, oxidase and catalase positive, rod-shaped organisms. Sediment bacteria were more diverse, Gram positive, pleomorphic organisms were dominant. Carbon utilization spectra was also diverse, simple sugars were utilized by most isolates, while none of them grew on methylated sugars and some other compounds.

3.6. Identification of atrazine degrading strains

Soil isolates were grouped in 16 ARDRA groups. All strains were Proteobacteria, with the exception of one non-identified organism, which belonged to the Cytophaga-Flexibacter group. 16 of the isolates were Peudomonas, 4 Stenotrophomonas, an Achromobacter, Sphingomonas, Rhizobium and Vaiovorax species were identified (Balows et al. 1992). For the sediment bacteria, ARDRA grouping was not necessary, due to their lower number and higher phenotypical variability. 4 strains were not identified. Actinomycetales ordo was represented in the greatest number, with Rhodococcus as the most numerous genera (4 strains). A Deinococcus and a Bacillus strain were also identified. Gram negative isolates were Proteobacteria (Pseudomonas Xanthomonas

and Delftia strains). Most of the above genera contains previously described xenobiotic degrading bacteria, though only few of them was associated with atrazine degradation.

3.7. Atrazine utilization and biomass production under carbon and nitrogen limited conditions

Most of the isolates preferred nitrogen-limited conditions regardless of the method of isolation. The only exceptions were two Pseudomonas and an Acinetobacter strains. Other Pseudomonas isolates were inhibited by the presence of inorganic nitrogen. Biomass production in liquid culture did not correlated with the growth observed on solid media or microbial activity (indicated by the change in redoxpotential). Highest biomass producers are Pseudomonas, Stenotrophomonas and Agrobacterium strains.

3.8. Biodegradative pathway of atrazine

Biodegradation of 11 strains were resolved. Hydroxi-atrazine was the only metabolite detected in the culture of Bacillus strain. Most Actinomycetales isolates and two unidentified strains produced hydroxi-atrazine as the initial metabolite, which was further dealkylated to ammeline, ammelide or cyanuric acid. Two of the strains were capable of the direct dealkylation of atrazine as well. Deinococcus sp. also hydrolyzed both the amino-alkyl chains and chloride, but its end-product was monoalkyl-ammeline. Biodegradation pathway of Pseudomonas ADP, which is – to our current knowledge – the most widespread mechanism, was nost detected in any of our isolates. Metabolites suggesting cleavage of the triazine ring were only detected in the culture of Delftia acidovorans D24 strain, thus this was selected for further investigation.

3.9. Metabolism of atrazine in D. acidovorans D24

Biodegradation of atrazine was followed for 6 weeks in liquid culture of Delftia acidovorans D24. Hydroxi-atrazine was the first detected metabolite, followed by aminoalkyl-hydrolysis. Cleavage of the triazine ring was verifyed by the detection of biuret and urea. Accumuleted metabolites were hydroxi-atrazine, ethylammelide and cyanuric acid, suggesting that the degradation of these compounds are the time-limiting steps of the pathway.

3.10. Effect of various growth factors on atrazine degradation

All of the investiated strains utilized atrazine more readily as a nitrogen source. Glucose was the preferred additional carbon source. Optimal temperature range was between 20 and 40 °C, thugh some of the strains were active even above 50 °C. Acidic pH inhibited growth on atrazine, optimal pH was slightly alkaline (8-9). Addition of vitamins accelerated growth and shifted the optimal temperature range to higher values.

3.11. Atrazine catabolic genes

AtzA enzyme catalyses dehalogation, which was observed in all of out investigated strains, and its product, hydroxi-atrazine was detected in both water and soil. However, atzA gene was not detected in any of the isolates. AtzB gene, though it encodes a more specific enzyme was detected in three strains (two Pseudomonas and a Stenotrophomonas isolate). Metabolic pathway of the above strains was not revealed so far, but it is expected to be similar to that of Pseudomonas ADP. Attempt to detect atzA or atzB in the soil DNA was unsuccessful, though according to the detected metabolites both enzymes might be present.

3.12. Assessment of soil biodiversity

Community ARDRA (having lower resolution of the two methods) revealed no significant differences in the pattern, only the intensity of bands varied. This suggests a dominantly similar microbial community throughout the area, despite the diverse physico-chemical properties of the samples. Denaturing gradient gel electrophoresis gave a more detailed picture. Dominant bands lined up with those of the atrazine degrading isolates, indicating that these organisms may become dominant as a result of long-term atrazine pollution. Dominant bands were so intense that secondary (and more variable) pattern was difficult to distinguish.

3.13. Horizontal gene transfer in the sediment

Endogenous microbiota of the sediment column did not include a detectable number of tetracyclin resistent organisms. Single inoculation with the donor cell did not yield new resistant organisms, and the original strain was washed out of the system after a few days. Maintaing a stable, relatively high donor cell concentration, horizontal transfer of the antibiotic resistance genes was verified by both culturing and molecular methods. Thus the transfer of atrazine catablic genes might also be possible in the sediment.

4. Thesis

- Effects of atrazine pollution in water and sediment were compared. Pollution did not affect total bacterial count, however, number and morphological diversity of atrazine degraders increased. In case of soil samples, dominance of atrazine degraders in the community was confirmed by DGGE.
- Ethyl-ammelide was detected during biodegradation in soil. This metabolite was described previously in bacterial culture only.
- Atrazine degradation was investigated for the first time in Danube sediment. Previous research concerning degradation in river sediment is scarce, and significant biodegradation was generally

not observed. Hydroxi-atrazine was confirmed to be the primary metabolite in aquifer environment.

- Irreversible adsorption of atrazine (described previously in soil) was observed in the sediment. Absorbed quantity however was one order of magnitude lower, due to the lower organic matter ad clay content of the sediment.
- Atrazine degrading strains (a total of 17) were isolated for he first time from river sediment. Five of the isolates (four Rhodococcus and a Pseudomonas) belong to genera previously associated with atrazine degradation, 6 of them however are novel biodegradative organisms (*Aeromicrobium, Micrococcus, Microbacterium, Deinococcus, Delftia, Xanthomonas* strains).
- Previously only a mixed atrazine degrading community was described from Hungarian soil. 25 isolates were obtained from soil, capable of atrazine degradation in pure culture. 21 of these belong to previously reported atrazine degrading genera (16 *Pseudomonas*, 5 *Stenotrophomonas* és 1 *Rhizobium* strain). Three organisms are novel atrazine degraders (*Achromobacter*, *Sphingomonas* és *Variovorax* species). Since the soil environment was examined thoroughly, smaller ratio of novel degrarders is understandable, and considering the low number of known atrazine-degrading organisms, still significant.
- Hypothetical biodegradation pathway was revealed for 11 strains. One of these removes only the chloride, most of them degrade to cyanuric acid or its mono- and diamino derivatives. One strain (*Delftia acidovorans* D24) is capable of ring cleavage, followed by complete mineralization. Four different pathways were revealed, none of them is identical with those previously described.
- Presence of the previously identified *atz*B gene (coding an amidohydrolase in Pseudomonas ADP) was confirmed in 3 strains (two *Pseudomonas* sp. and a *Stenotrophomonas maltophilia* isolate).
- Optimal conditions of growth and atrazine degradation was revealed for the first time for sediment bacteria (previous such investigations dealt with soil isolates). Isolates preferred atrazine as a nitrogen source. Highest activity was observed between 20 and 40 °C, at slightly alkaline pH. Results correlated with those observed for soil bacteria. Inhibition of atrazine degradation by acidic pH was confirmed.
- Horizontal transfer of antibiotic resistance gene was confirmed in sediment for the first time. The process requires high concentration of the donor cell.

5. Conclusions and suggestions

Summarizing the above results, degradation of atrazine by endogenous microorganisms is possible in soil and sediment after adaption (i.e. long-term atrazine pollution). Ratio and

morphological diversity of degrading organisms increased in the meanwhile. Accumulating metabolite under the investigated circumstances was the non-toxic hydroxi-atrazine, and its concentration was significantly lower than the initial amount of atrazine.

A temperature range of 20 to 40 ºC and pH 8 were found optimal for atrazine degradation. In case of *in situ* bioremediation, pH optimalization (by adding Ca(OH)2) is necessary for acidic soils. Elevation of temperature by constructing soil prisms will also enhance degradation.

In accordance with previous observations, our investigations have shown inhibiting effect of inorganic nitrogen sources (e.g fertilizer residues). Thus addition of carbon sources promotes atrazine degradation by both the general activation of metabolism and the shift of C/N ratio to a more favorable value. Of the tested simple carbon sources, glucose was most efficient in activation. Further goal is to test these assumptions in a soil microcosm experiment.

Delftia acidovorans D24 was the sole strain of those tested so far that could mineralize atrazine, thus it seems to be appropriate for bioremediation purposes. Further potential candidates are the efficient *Stenotrophomonas* sp. AAT9 and *Pseudomonas* sp. AMT6 strains. The isolates producing higher biomass (*Rhizobium radiobacter* AMD1 and *Pseudomonas syringae* AMD7) are potential plant pathogens, and thus are not eligible for this purpose. Generation of a stable mix culture of strains having a diverse atrazine metabolism (including both Gram negative and Gram positive strains) is possibly the most promising solution. Generation and testing of such mixed culture is also one of the future aims of study.

Horizontal transfer of antibiotic resistance genes was confirmed in Danube sediment. Though it is not revealed so far, if the genes for atrazine degradation are located in the genome or on a plasmid in our isolates, literature data suggests the possibility of the latter. This is especially true for those organisms, where previously described catabolic genes were found. Thus investigation of horizontal transfer of atrazine catabolic genes is also an area of future interest.

6. List of related publications

Publication in interntional journal

Z. Takáts, Vargha M., K. Vékey (2001) Investigation of Atrazine Metabolism in Riverine Sediment by HPLC-MS. Rapid Communications in Mass Spectrometry 15: 1735-1742

Publication in national journal

Vargha M., Márialigeti K. Szabó G. (2000) A dunai kavicsüledék mikrobiális víztisztító kapacitása I. *Hidrológiai Közlöny*, 2000/4

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In Hungarian, complete

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M. Vargha[1, 2], K. Márialigeti[1] Effect of ecological parameters on the growth and degrading capacity of atrazine utilizing sediment bacteria *Training Course organized by the Hungarian Society for Microbiology and the UNESCO-Hebrew University of Jerusalem International School for Molecular* Biology, Microbiology and Science for Peace, Keszthely, August 23-27, 2000

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