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Abstract The *s*-triazine herbicide terbuthylazine (TERB) has been used as the main substitute of atrazine in many EU countries for more than 10 years. However, the ecological consequences of this substitution are still not fully understood. Since the fate of triazine herbicides is primarily dependent on microbial degradation, in this paper, we investigated the ability of a mixed bacterial culture, M3-T, originating from *s*-triazine-contaminated soil, to degrade TERB in liquid culture and soil microcosms. The M3-T culture grown in mineral medium with TERB as the N source and citrate as the C source degraded 50 mg L^{-1} of TERB within 3 days of incubation. The culture was capable of degrading TERB as the sole C and N source, though at slower degradation kinetics. A thorough LC-MS analysis of the biodegradation media showed the formation of hydroxyterbuthylazine (TERB-OH) and *N*-*t*-butylammelide (TBA) as major metabolites, and desethylterbuthylazine (DET), hydroxydesethylterbuthylazine (DET-OH) and cyanuric acid (CA) as minor metabolites in the TERB degradation pathway. TBA was identified as a bottleneck in the catabolic pathway leading to its transient accumulation in culture media. The supplementation of glucose as the exogenous C source had no effect on TBA degradation, whereas citrate inhibited its disappearance. The addition of M3-T to sterile soil artificially contaminated with TERB at 3 mg kg^{-1} of soil resulted in an accelerated TERB degradation with $t_{1/2}$ value being about 40 times shorter than that achieved by the native microbial community. Catabolic versatility of M3-T culture makes it a promising seed culture for accelerating biotransformation processes in *s*-triazine-contaminated environment.

Keywords Terbuthylazine · Biodegradation · Bacterial culture · *s*-Triazines

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Introduction

The *s*-triazine herbicides, mainly atrazine, are among the most widely used herbicides in the world for selective weed control. However, the detection of atrazine and its dealkylated metabolites in aquatic ecosystems beyond the maximum allowable concentration limits along with its potential adverse impacts on different organisms and human health has led to the gradual restriction and finally ban of its production and uses all over the European countries (Sass and Colangelo 2006), including Croatia. As a consequence, terbuthylazine (2-chloro-4-ethylamino-6-*t*-butylamino-1,3,5-triazine) has been introduced as a substitute of atrazine due to its favourable physico-chemical properties as compared to atrazine. Because of its lower water solubility and higher adsorption coefficient, terbuthylazine (TERB) is expected to be retained in the soil significantly longer, and therefore, it poses a lower risk for aquifer contamination (EFSA 2011). However, frequent detection of this herbicide in surface and ground waters at levels exceeding the regulatory limits renders it a significant environmental contaminant (Bottoni et al. 2013; Guzzella et al. 2006; Hildebrandt et al. 2008). This fact along with its

approved use in the EU market until 2021 and potential toxicological and ecotoxicological properties (EFSA 2011) have raised concerns regarding the risk that TERB might pose for both the environment and human health.

The fate of *s*-triazines in the environment is mainly dependent on biotic degradation by microorganisms, while abiotic degradation can be considered a minor dissipation route (Mandelbaum et al. 2008). The current understanding of the biodegradation mechanisms of *s*-triazines mostly results from the studies involving atrazine. Atrazine biodegradation has been reported to occur via two main pathways: oxidative *N*-dealkylation and hydrolytic mineralization (reviewed in Udikovic-Kolic et al. 2012). While the first pathway results in the formation of dealkylated chlorometabolites, the second one involves a series of hydrolytic cleavage reactions of chloro, amino and alkylamino substituents from the *s*-triazine ring converging to cyanuric acid. Cyanuric acid is further subjected to hydrolytic ring cleavage to produce CO_2 and NH_4^+. The enzymes TrzN/AtzA, AtzB and AtzC hydrolyze atrazine to cyanuric acid, while the enzymes TrzD/AtzD, AtzE and AtzF degrade cyanuric acid to inorganic compounds.

Contrary to atrazine, knowledge of the fate of TERB in the environment is still relatively limited, especially regarding the assessment of its transformation products. Experiments with ^{14}C ring-labelled terbuthylazine showed very limited mineralization by native soil microorganisms (Dousset et al. 1997; Kristensen et al. 2001; Langenbach et al. 2001). The main biotransformation pathway of TERB in soils and water is *N*-dealkylation, leading to the formation of desethylterbuthylazine (DET) or detertbutylterbuthylazine (DTT), and dechlorination, which results in the formation of hydroxyterbuthylazine (TERB-OH; Caracciolo et al. 2010, 2005; Di Corcia et al. 1999; Guzzella et al. 2003; Karanasios et al. 2013). Hydroxydealkylated metabolites hydroxydesethylterbuthylazine (DET-OH) and hydroxydetertbutylterbuthylazine (DTT-OH) may be further formed by dechlorination of DET or DTT or by dealkylation of TERB-OH (Caracciolo et al. 2005; Di Corcia et al. 1999). Dealkylated metabolites are more water soluble than TERB and therefore have a greater potential to reach aquatic environment (Guzzella et al. 1996, 2003). In contrast, TERB itself and its hydroxylated metabolites have lower water solubility and are considered to be retained in soil (EFSA 2011).

Elucidation of the transformation pathways of herbicides used in agriculture by native microorganisms is an important part of the comprehensive risk assessment. However, an additional important issue is the enrichment of bacterial cultures possessing an enhanced potential for the degradation of triazine herbicides, which could be used as seed cultures for improved removal of these compounds from contaminated soils. Up to date, only scarce data are available on degradation of TERB by means of bacterial isolates/consortia (Caracciolo et al. 2010; Grenni et al. 2009). In our previous investigations, we enriched a mixed bacterial culture M3-T from *s*-triazine-contaminated soil with atrazine as the sole C and N source (Udikovic et al. 2003). Using that culture, we thoroughly characterized the underlying atrazine-mineralization mechanism at both genetic and biochemical levels (Kolic et al. 2007).

The aims of the current study were (1) to assess the M3-T efficacy in TERB degradation in liquid culture and soil microcosms and (2) to explore the biochemical mechanism of TERB degradation by M3-T culture with an emphasis on further degradation of dealkylated chlorotriazine metabolites. Moreover, this study offers a basis for the development of an improved bioremediation method for the reclamation of sites polluted with triazine herbicides.

Materials and methods

Chemicals and solutions

Technical TERB (97 %) was provided by Herbos (Sisak, Croatia). Analytical standards of TERB, TERB-OH (2-*t*-butylamino-4-etylamino-6-hydroxy-1,3,5-triazine) and cyanuric acid (CA; 2,4,6-trihydroxy-1,3,5-triazine) were purchased from Fluka (Buchs, Switzerland). DTT (2-amino-4-etylamino-6-chloro-1,3,5-triazine), desethylatrazine (DEA; 2-isopropyl-4-amino-6-chloro-1,3,5-triazine), desethyldetertbutylterbuthylazine (DEDTT; 2,4-diamino-6-chloro-1,3,5-triazine), DET (2-*t*-butylamino-4-amino-6-chloro-1,3,5-triazine), DET-OH (2-*t*-butylamino-4-amino-6-hydroxy-1,3,5-triazine) and DTT-OH (2-amino-4-etylamino-6-hydroxy-1,3,5-triazine) were purchased as analytical standards from Riedel-de Haën (Seelze, Germany). *N*-Ethylammelide (EA) and *N*-*t*-butylammelide (TBA) were not commercially available and were synthesized at the Rudjer Bošković Institute as previously described (Diels 1899; Thurston et al. 1951) and as described in Electronic Supplementary Material, respectively.

All chemicals used for the growth media were of analytical grade purity and supplied by Kemika (Zagreb, Croatia). Nutrient agar was purchased from Biolife (Milan, Italy). Liquid chromatography-mass spectrometry (LC-MS) grade solvents (acetonitrile and methanol) were products of J.T. Baker (Deventer, the Netherlands). The individual stock solutions (1 mg mL^{-1}) of triazine compounds and their hydroxy analogues were prepared in methanol and methanol containing 1 % of formic acid, respectively. The mixed standard solutions (0.01 and 0.001 mg mL^{-1}) of all target compounds were prepared in methanol. The standard solutions used for the external calibration during LC-MS quantification were prepared from the mixed standard solutions either in the mineral salts medium (MSM), which was used for biodegradation experiments in liquid cultures, or in acetonitrile/water (2/8; *v*/*v*) for the extracts obtained from the soil experiments.

Bacterial culture

The bacterial culture M3-T used in this study and available on request to the scientific community was enriched previously with atrazine, as the sole C and N source, from the soil exposed to long-term contamination with atrazine and other *s*-triazine compounds (Udikovic et al. 2003). The adaptation of this culture to TERB was performed by repeated batch cultivation in MSM (Mandelbaum et al. 1993) supplemented with sodium citrate (0.1 %) as the C source (MSM-citrate) and increasing concentrations of TERB as the N source (10–80 mg L^{-1}). The repeated batch cultivation started from TERB concentration of 10 mg L^{-1} and then gradually increased to 20, 30, 40, 50, 60, 70 and 80 mg L^{-1}. The stock solution of TERB (20 mg mL^{-1}) prepared in methanol was filtered through a 0.2 μm and added to an empty Erlenmeyer flask. After evaporation of methanol, 100 mL of MSM-citrate was added to the flasks which were then sonicated in an ultrasonic bath for 10 min to ensure complete dissolution of TERB. The flasks were then inoculated with 1 mL of frozen enrichment culture and incubated on a rotary shaker (180 rpm) at 28 °C in the dark. Every 7–10 days, 2 % of the culture was transferred to a fresh medium with increased TERB concentration and incubated under the same conditions. After seven subculturings, biomass was centrifuged (12,000 rpm, 10 min), resuspended in phosphate buffer (pH 7.5) and stored at −20 °C under glycerol (20 % *v*/*v* as the final concentration).

Biodegradation experiments

Inoculum preparation

Inocula for all degradation experiments were prepared by growing bacteria in shake flasks at 28 °C using MSM-citrate (100 mL) supplemented with 80 mg L^{-1} of TERB for 2 weeks. Cultures were pelleted by centrifugation (12,000 rpm, 8 min), cells were rinsed with MSM with or without citrate supplement (0.1 %) and resuspended in the same medium. Cell numbers were quantified by plating on nutrient agar plates.

Biodegradation of terbuthylazine in liquid culture

Duplicate flasks (300 mL) containing MSM (100 mL) with or without citrate supplement (0.1 %) and TERB (50 mg L^{-1}) were inoculated with M3-T cells to give an initial density of 1.33×10^6 colony forming units (CFU) mL^{-1}. The cultures were incubated at 28 °C on a rotary shaker at 180 rpm. Control experiment without the addition of inoculum was performed as well. Aliquots (1.5 mL) were withdrawn periodically, centrifuged immediately (10,000 rpm, 10 min), and the concentrations of TERB and intermediates in supernatants were determined by LC-MS. Culture growth was monitored by plating appropriate dilutions on nutrient agar plates.

Biodegradation of dealkylated chlorotriazine compounds

The activity of M3-T culture in the degradation of dealkylated *s*-triazine metabolites was verified using the same procedure as previously reported (Udiković Kolić et al. 2008). Briefly, MSM-citrate containing four *s*-triazine compounds (the initial concentration of individual compounds was 5 mg L^{-1}) was inoculated with M3-T culture at initial cell density of 1×10^8 CFU mL^{-1}, and cultures were subsequently shaken at 28 °C for 4 days. The HPLC analysis was used for quantitative determination of the initial and residual concentrations of *s*-triazines (Udikovic et al. 2003). All experiments were performed in duplicate, and non-inoculated flasks were used as abiotic controls.

Biodegradation of N-t-butylammelide in liquid culture

This study was performed in 300-mL flasks containing MSM (100 mL) with *N*-*t*-butylammelide as the sole C and N source (30 mg L^{-1}) and with the addition of other C sources (citrate, 0.1 %; glucose, 1 %). Duplicate flasks were inoculated with 1.34×10^6 CFU mL^{-1} and incubated on a rotary shaker (180 rpm, 28 °C) for 45 days. Aliquots (1.5 mL) were withdrawn at selected time intervals, centrifuged (10,000 rpm, 10 min), and supernatants were analyzed by LC-MS. Bacterial growth was monitored by plating on nutrient agar plates.

Biodegradation of terbuthylazine in soil microcosms

The samples of agricultural surface soil (0–30 cm) were collected at the experimental station Šašinovečki Lug, located 20 km northeast of Zagreb (Croatia). The soil was sampled either from the plot contaminated with TERB at a mass fraction of 550 μg kg^{-1} of dry soil or from the control plot with no *s*-triazine application history. The soil was classified as silty-loam (sand 5.8 %, silt 77.8 % and clay 16.4 %) with pH 7.27, organic carbon content 1.56 % and organic nitrogen content 0.35 %.

Soils were passed through a 2-mm sieve, and 200 g (dry weight basis) portions of uncontaminated and contaminated soils were distributed into 500-mL glass bottles. The bottles with uncontaminated soil were sterilized by the three repeated autoclaving steps (30 min at 121 °C) with 2-day breaks between each step. The sterile soil microcosms were then artificially contaminated with methanolic solution of TERB at a mass fraction of 3 mg kg^{-1}. After evaporation of methanol, the fortified soils were mixed thoroughly with a sterilized spatula and aged for 2 months prior to the inoculation with M3-T culture. Duplicate soil microcosms were treated as follows: (1) sterile soil was inoculated with 50 mL of M3-T suspension in MSM; (2) sterile soil was amended only with 50 mL of MSM (abiotic control); (3) non-sterile soil was amended with 50 mL of MSM; (4) sterile soil was inoculated

with 50 mL of M3-T suspension in MSM-citrate; and (5) sterile soil was amended with 50 mL of MSM-citrate (control). The inoculated microcosms received initial cell density of 1.79×10^7 CFU g^{-1} of soil. Initial cell density in the microcosm containing native soil was 1.43×10^7 CFU g^{-1} of soil. The final moisture content of 22 % was maintained at a constant level throughout the experiment by adding sterile purified water as necessary. The microcosms were incubated in the dark at 28 °C for 35 days. Immediately after treatment and at regular time intervals thereafter, subsamples (≈10 g) from each bottle were removed for the quantitative analysis of TERB residues and its intermediates by LC-MS, determination of soil dry matter by the gravimetric method (ISO 11465 (1993)) and bacterial count by plating the soil suspension (1 g of soil suspended in 9 mL of sterile 0.9 % NaCl) on nutrient agar plates.

Analytical procedures

Ultrasonic extraction

Extraction of triazine compounds from wet soil was carried out similar to the procedure of Stipicevic et al. (2003). Soil samples (5 g) were extracted with 20 mL of methanol in an ultrasonic bath for 5 min. After centrifugation (3,000 rpm, 10 min), the supernatant was decanted, and the extraction of soil repeated with a new 10-mL portion of methanol. The combined extract was evaporated to dryness under a gentle stream of nitrogen, and the dry residue was redissolved into 1 mL of acetonitrile/water (8/2; *v*/*v*) and filtered through a 0.45-μm Teflon filter before the LC-MS analysis.

To evaluate the extraction procedure, freshly spiked soil samples were prepared by adding 100 μL of methanolic mixture containing 150–169 μg mL^{-1} of each triazine compound (Table S1; Electronic Supplementary Material) to 5 g of untreated wet soil. The samples were manually shaked, left overnight at room temperature until the solvent evaporated and then were extracted as mentioned above.

LC-MS analysis

The analysis of TERB and its potential transformation products was performed using ultrahigh-performance liquid chromatography (UHPLC) coupled to quadrupole-time-of-flight mass spectrometry (QTOF-MS). UHPLC separation was performed using a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA), equipped with a binary solvent delivery system and autosampler. The chromatographic separations employed a column (50 mm×2.1 mm) filled with a 1.7 μm BEH C_{18} stationary phase (Waters Corp., Milford, MA, USA). A binary gradient at a flow rate of 0.4 mL min^{-1} was applied for the elution. The eluents A and B were water and acetonitrile, both containing 0.1 % (*v*/*v*) of formic acid. The elution gradient started at 0 % of eluent B, which was kept for 1 min, then increased to 50 % in 4 min and to 60 % in 1 min. The initial conditions were then achieved in 20 s. The total run-time, including column conditioning to reach initial conditions, was 7.2 min. The injection volume was 5 μL.

Mass spectrometry was performed on a QTOF Premier instrument (Waters Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray interface as described in Terzic et al. (2011). The analysis included a detailed screening of the samples with the purpose of identifying possible transformation products as well as quantitatively determining TERB and its major metabolites. Details of the applied conditions for the LC-MS analyses are given in Electronic Supplementary Material. It should be noted that all triazine compounds except CA were analyzed in the positive ionization mode, while CA was determined in the negative ionization mode. The quantitation was achieved using external calibration curves of the corresponding reference standard solutions.

Method validation

The analytical procedure used in our experiments has been fully validated (described in Electronic Supplementary Material), and the results of the validation experiments are presented in Table S1 (Electronic Supplementary Material). Briefly, the precision of the method was very good (<5.5 %) for all investigated compounds, while relatively high extraction efficiency (65–94 %) was achieved for all compounds except CA (29 %). The LC-MS method detection limits were in the range 0.002–0.008 mg kg^{-1} and 0.003–0.059 mg L^{-1} for soil samples and liquid culture samples, respectively.

Data analysis

The half-life values ($t_{1/2}$) were estimated based on the first-order dissipation kinetics using the equation $C_t=C_0e^{-kt}$, where C_t is the TERB mass fraction in the soil after time t, C_0 is the apparent initial mass fraction, and k and t are the rate constant and degradation period, respectively. Mean, standard deviation, correlation analyses and t test were performed using the Prism computer software programme version 6.00 (GraphPad Software, San Diego, CA, USA).

Results

Biodegradation of terbuthylazine and identification of catabolic intermediates

The results of TERB degradation experiments by M3-T culture in liquid medium under two different conditions are presented in Fig. 1 and Fig. S1 (Electronic Supplementary Material). When TERB was the sole C and N source, degradation was slow, eliminating about 90 % of the initially

applied TERB within 2 weeks of incubation (Fig. S1). The addition of citrate as an additional C source increased the degradation rate, resulting in complete disappearance of 50 mg L^{-1} TERB within 3 days of incubation (Fig. 1). In both cases, degradation was accompanied by the production of intermediates and culture growth, confirming the capability of M3-T culture to use TERB as the N source or both C and N source (Figs. 1 and S1). In order to illustrate the mass balance of TERB and its main transformation products, all concentrations were presented in molar units. It should be noted that the added concentration of TERB (50 mg L^{-1} or 218 μM) was above its water solubility (8.5 mg L^{-1} or 37 μM), and the measured concentrations at the beginning of the experiment were lower than the nominal concentration. However, during the experiment, TERB was converted to more soluble triazine species, and they emerged in the dissolved fraction at much higher concentrations.

In order to identify any stable intermediates formed during biotransformation of TERB, culture supernatants were thoroughly analyzed by the LC-MS technique using accurate mass feature (Terzic et al. 2011). The samples were screened for all possible TERB transformation products based on the knowledge of triazine degradation products from the literature (Mandelbaum et al. 2008; see Table S2; Electronic Supplementary Material). The LC-MS analysis of culture supernatants revealed the presence of five intermediates in the culture medium, two of which can be considered major intermediates, which were prominent in the total ion current chromatograms (Fig. 2). Based on their accurate mass spectra and comparison with analytical grade reference materials, these two transformation products were identified as TERB-OH (protonated molecule at *m/z* 212.1511) and TBA (protonated molecule at *m/z* 185.1039; Fig. S2; Electronic Supplementary Material). TERB-OH transiently accumulated in the medium reaching maximum after 47 h and then it was gradually removed within 4 days. In contrast, TBA showed an accumulative behaviour with a gradual increase over the entire 11 days of incubation (Fig. 1). In fact, the final mass balance analysis (Table S3; Electronic Supplementary Material) confirmed the stoichiometrical transformation of TERB to TBA. Apart from the two prominent transformation products, we identified three additional minor metabolites, which did not have a significant influence on the mass balance of triazine compounds but were important as diagnostic intermediates for the interpretation of transformation pathways. These included DET (protonated molecule at *m/z* 202.0859), DET-OH (protonated molecule at *m/z* 184.1198) and CA (deprotonated molecule at *m/z* 128.0097). DTT and DEDTT, as well as their hydroxy analogues as possible intermediates (see Table S2), were not detected in culture supernatants throughout the experiment. There was no transformation of TERB in non-inoculated controls.

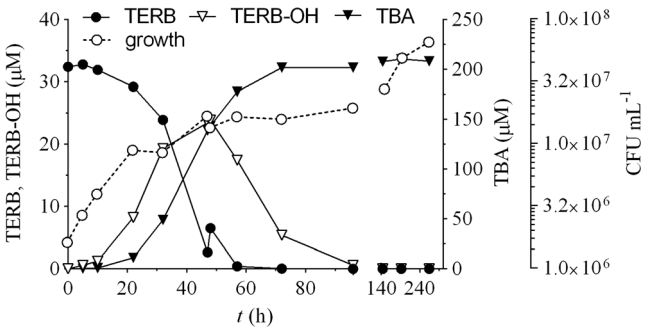

Fig. 1 Growth and terbuthylazine (*TERB*) degradation by M3-T culture. The culture was cultivated in MSM containing TERB (50 mg L^{-1}) as N source and citrate as C source (0.1 %). *TERB-OH* hydroxyterbuthylazine, *TBA N-t*-butylammelide

Capability of M3-T culture to degrade dealkylated chlorotriazine metabolites

In order to assess the role of alkyl substituents of *s*-triazine ring in the degradation efficiency, the range of *N*-dealkylated triazines was catabolized by M3-T in MSM-citrate containing mixture of four *s*-triazine compounds supplied as the N sources. The degradation patterns of *s*-triazine metabolites, i.e. DTT, DEA, DET and DEDTT, are presented in Fig. 3. As can be seen, a complete transformation of DTT and DEA was achieved after 2 and 4 days of incubation, respectively. In contrast, after 4 days, only 50 % of DET and 20 % of DEDTT was degraded under the same conditions. The DEDTT concentration was halved after an extended incubation of 34 days.

Capability of M3-T culture to use and degrade *N-t*-butylammelide

To evaluate further transformation of TBA as the major intermediate during TERB degradation in liquid culture, TBA was added to MSM with or without the C source (glucose or citrate) and inoculated with the M3-T culture. The results of these studies presented in Fig. 4a, b revealed that M3-T was capable of degrading and using TBA under all tested conditions; however, the kinetics was much slower than that for TERB. When M3-T was grown in MSM containing TBA as the sole C and N source, a degradation of approximately 70 % was obtained after 45 days with transient formation of CA, which reached its maximum of about 1.3 % of the initial TBA concentration on the 11th and 18th day (Fig. 4a). The presence of glucose as the exogenous C source had no effect on the TBA disappearance resulting in the degradation pattern similar to that seen with TBA as the sole C and N source (Fig. 4a). In contrast, the addition of citrate as the C source repressed TBA dissipation as only 20 % degradation compared to control was achieved until the end of the experiment (45 days).

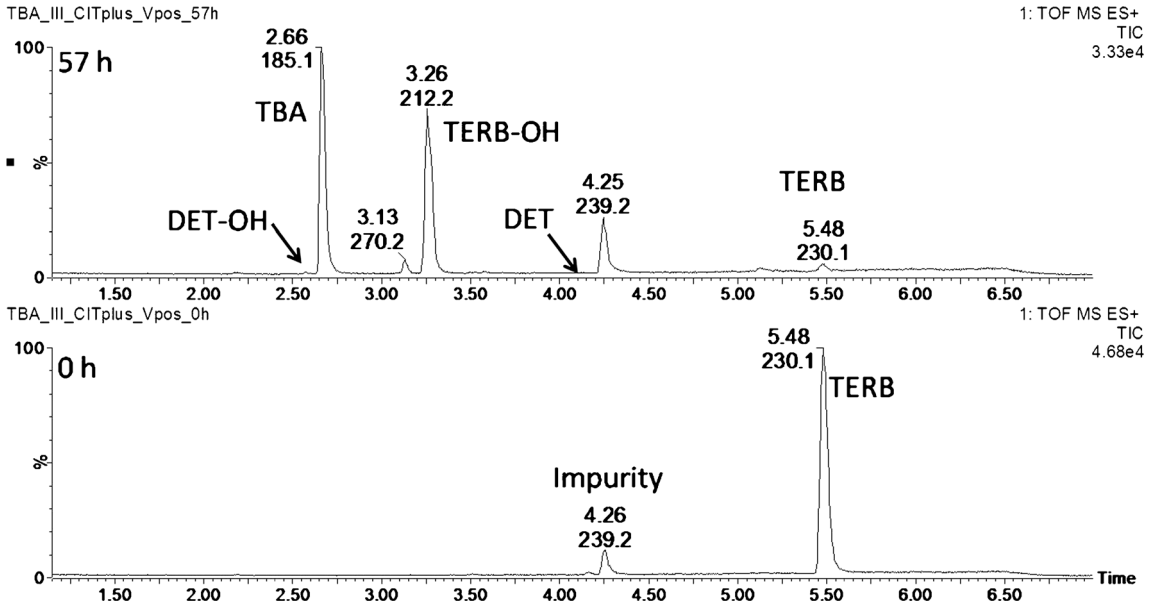

Fig. 2 Total ion current chromatograms (*m/z* 50–1,000 Da) of two selected samples (*t*=0 and 57 h) obtained during cultivation of M3-T culture in MSM containing terbuthylazine (50 mg L^{-1}) as N source and citrate as C source (0.1 %). *TERB* terbuthylazine, *DET* desethylterbuthylazine, *DET-OH* hydroxydesethylterbuthylazine, *TERB-OH* hydroxyterbuthylazine, *TBA N-t*-butylammelide

Degradation of terbuthylazine by M3-T culture in soil microcosm

To test survival and the degrading potential of M3-T culture in soil under controlled laboratory conditions, we added M3-T into a sterile soil contaminated with TERB at 3 mg kg^{-1}. The bacterial growth and degradation curves for TERB and its major degradation products in soil are presented in Fig. 5. The observed degradation of TERB in inoculated soil was fast with $t_{1/2}$ value of 35±2.4 h. In contrast, slow TERB dissipation was achieved in native soil without M3-T addition ($t_{1/2}$=61.3± 5.5 days), indicating a limited capability of native soil microbes to significantly degrade TERB under laboratory conditions. No significant TERB degradation (less than 10 % mass fraction decrease) occurred in sterile control soil, which remained sterile for the entire length of the experiment, as determined by the plating assays. This indicates that microorganisms are responsible for TERB degradation in native soil.

The presence of small quantities of the two metabolites, TBA and TERB-OH, in soil at the beginning of incubation is a consequence of their introduction into the soil with M3-T inoculum, because these metabolites were not detected in uninoculated soils. Although caution was taken during

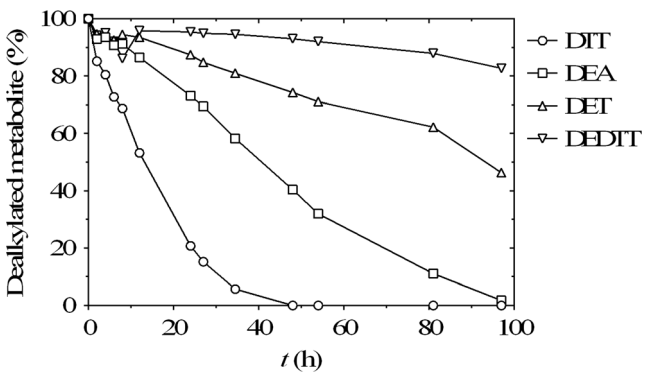

Fig. 3 Degradation curves of mixture of dealkylated chlorotriazine compounds during cultivation of M3-T culture in MSM with citrate addition (0.1 %). Initial concentration of individual *s*-triazine compounds was 5 mg L^{-1}. *DTT* detertbutylterbuthylazine, *DEA* desethylatrazine, *DET* desethylterbuthylazine; *DEDTT* desethyldetertbutylterbuthylazine

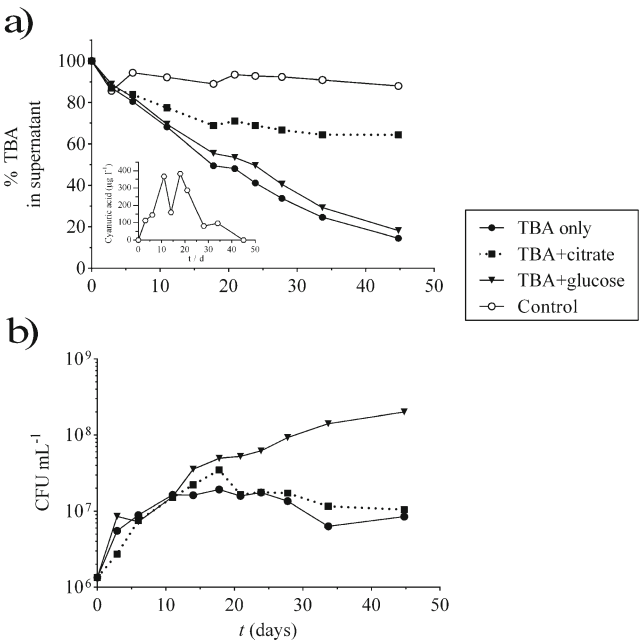

Fig. 4 Comparison of *N-t*-butylammelide degradation (*TBA*) by M3-T culture (**a**) and growth of M3-T culture (**b**) during cultivation in MSM with TBA (30 mg L^{-1}) as the only C and N source and in the same medium supplemented with 1 % glucose or 0.1 % citrate as C source

inoculum preparation (formed intermediates were washed away), traces of these intermediates probably remained adsorbed on the cells and thus were introduced into the soil. Similar to liquid culture, TBA was detected as a major transient intermediate. Its formation increased gradually by the 34th hour reaching a maximum of 59.1 % of the initial TERB dose and then decreased slowly to 9.5 % of the initial dose at the end of the study. All other transformation products occurred at much lower concentrations. The formation of TERB-OH was also only transient and preceded the formation of TBA. Residues of DET were detected in soil at low concentrations (up to 0.016 nmol g^{-1}) and were fully degraded within 9 days.

In addition, bacterial growth assays, done using plating counts, showed that M3-T population density increased from 1.79×10^{7} CFU g^{-1} at inoculation to 2.32×10^{10} CFU g^{-1} after 5 days of incubation (Fig. 5). This increase in bacterial abundance was correlated ($p<0.01$) with TERB degradation (80 % of the applied dose degraded). Morphological evaluation of the colonies grown on nutrient agar plates indicated the predominance of yellow colony types previously identified as belonging to the genus *Arthrobacter* with confirmed capability to transform atrazine to cyanuric acid (Kolic et al. 2007). From the 5th to 22nd day of incubation, population density of M3-T remained stable and declined to 2.19×10^{9} CFU g^{-1} at the end of the experiment (35th day). Adding citrate to M3-T-inoculated soil did not induce significant effects ($p>0.05$) either on TERB dissipation, TBA degradation or culture growth (Fig. S3; Electronic Supplementary Material).

Disscussion

Ubiquitous distribution and potential deleterious effects of triazine herbicides require a careful assessment of their environmental fate. Most of the studies that have been performed so far focused on atrazine, which resulted in the recommendation for its phasing out in many countries (Sass and Colangelo 2006). In contrast, a comprehensive environmental assessment, including biodegradation, of its recommended substitute TERB is still missing. Our previous studies (Kolic et al. 2007; Udikovic et al. 2003) have shown that the four-member bacterial culture enriched from *s*-triazine-contaminated soil was capable of mineralizing atrazine. The degradation mechanism involved the formation of hydroxyatrazine, *N*-isopropylammelide and CA as intermediates by means of *trzN*, *atzB*, *atzC* and *trzD* gene products (Fig. 6). In the present study, we used this culture to evaluate its potential to degrade the structurally related and agriculturally relevant compound TERB. As expected, M3-T culture exhibited growth and substantial activity in the degradation of TERB (50 mg L^{-1} degraded within 3 days) during incubation in MSM supplemented with citrate as the C source. The culture was also capable of growing and degrading TERB as the sole C and N source, although with a slower degradation kinetics. This observation corroborates previous studies which showed the stimulatory effects of additional C sources on *s*-triazine degradation (Mandelbaum et al. 1995; Sajjaphan et al. 2010).

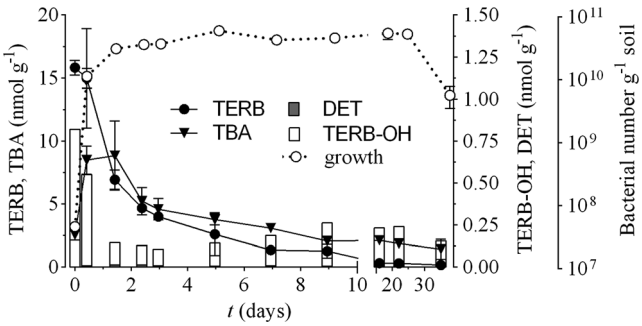

Fig. 5 Growth and terbuthylazine (*TERB*) degradation by M3-T culture in sterilized soil contaminated with 3 mg TERB g^{-1} soil without citrate amendment. *DET* desethylterbuthylazine, *TERB-OH* hydroxyterbuthylazine, *TBA N-t*-butylammelide. DET is detected at very low mass fractions (0.002 to 0.016 nmol g^{-1}) compared to other metabolites

Based on the intermediates identified during the TERB degradation, we proposed the mechanism of TERB degradation by our M3-T culture (Fig. 6). The main degradation intermediates are analogous to those observed during atrazine degradation (Kolic et al. 2007), but the advanced analytical technique applied in this study allowed a more detailed and comprehensive insight into the composition of the triazine intermediates formed. This culture initiated TERB catabolism via hydrolytic dechlorination to yield TERB-OH as the dominant initial transformation product. In parallel, TERB was transformed to DET via oxidative *N*-deethylation which was a minor metabolic route, presumably catalyzed by the non-specific P450 monooxygenase as suggested previously (Nagy et al. 1995). Both intermediates (i.e. TERB-OH and DET) were subsequently transformed into TBA. TERB-OH was converted to TBA by the hydrolytic removal of the ethyl side chain, whereas the conversion of DET to TBA proceeded through DET-OH as an intermediate. The dechlorination steps were expected to be catalyzed by the TrzN enzyme, whereas subsequent transformation was carried out presumably by the AtzB enzyme, resulting in the release of ethylamine from TERB-OH, which allowed culture to use it as a growth substrate (Kolic et al. 2007). TBA was further transformed to CA by AtzC much less efficiently than the analogous intermediate *N*-isopropylammelide in atrazine catabolic pathway (Fig. 6). This may be due to a reduced ability of AtzC to accommodate the bulky *t*-butyl substituent into its active site, indicating that this reaction could be rate-limiting for TERB degradation. In support of this hypothesis, we also observed that, when exposed to a mixture of dealkylated chlorotriazine compounds, the M3-T culture preferentially degraded those having smaller alkyl substituents (DTT>DEA>DET). This finding along with earlier observations (Shapir et al. 2002; Topp et al. 2000) supports the

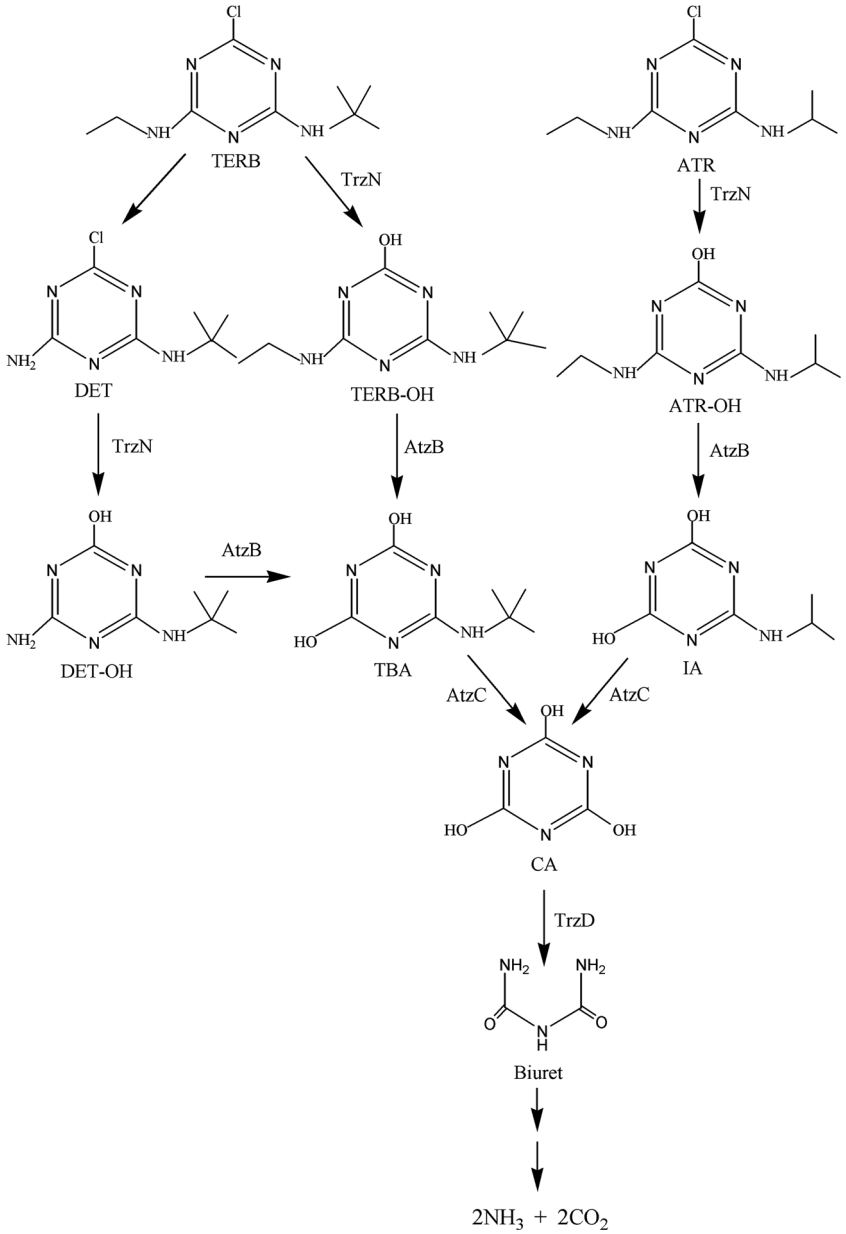

Fig. 6 Comparison of the proposed pathways for degradation of terbuthylazine (*TERB*) and atrazine (*ATR*) by M3-T culture. Atrazine-mineralization pathway has been elucidated previously (Kolic et al. 2007). The enzymes catalyzing each step are indicated. *DET* desethylterbuthylazine, *DET-OH* desethylhydroxyterbuthylazine, *TERB-OH* hydroxyterbuthylazine, *TBA N-t*-butylammelide, *ATR-OH* hydroxyatrazine, *IA N*-isopropylammelide, *CA* cyanuric acid

idea that AtzC determines the degradation rate of *s*-triazines with a variable *N*-alkyl chains. We propose that transformation of TERB to CA is catalyzed by *Arthrobacter* populations harbouring *trzN*, *atzB* and *atzC* catabolic genes (Kolic et al. 2007). Further, CA which was produced from TBA in a rate-limiting step catalyzed by AtzC was detected only at very low levels, suggesting that it was degraded as soon as it was formed. This was most probably facilitated by the action of the TrzD enzyme from two other culture members, i.e. *Ochrobactrum* sp. and *Pseudomonas* sp. (Kolic et al. 2007; Udikovic-Kolic et al. 2011). Taken together, contrary to previous studies which suggested that native soil bacteria metabolized TERB mainly or solely via oxidative *N*-dealkylation (Dousset et al. 1997; Grenni et al. 2012; Guzzella et al. 2003), we showed that our culture degraded TERB mainly through the hydrolytic pathway with some minor simultaneous formation of DET (<30 μg L^{-1}). TBA was identified to be the bottleneck in this hydrolytic mineralization pathway due to the faster rate of hydrolytic deethylation in comparison with detertbutylation of the *s*-triazine ring.

The addition of M3-T culture to sterile soil contaminated with TERB at a dose of 3 mg kg^{-1}, which is relevant for sites having moderate TERB pollution, resulted in accelerated TERB degradation with $t_{1/2}$ being about 40 times shorter than that achieved by the native microbial community. We showed that our culture degraded over 80 % of the added TERB within 5 days after inoculation, which was accompanied by a significant increase in cell number from approximately 10^7 to 10^{10} CFU g^{-1}, with dominance of *Arthrobacter* populations.

This cell number was maintained roughly constant until day 22 after inoculation, which indicated that the introduced M3-T culture successfully proliferated and persisted in the soil, providing a basis for the degradation of TERB and its degradation intermediates. The major intermediate TBA showed only a short-lived accumulation in soil microcosms followed by an efficient depletion. In contrast to the degradation experiment in the liquid medium, the stoichiometric transformation of TERB into TBA was not observed. It is therefore likely that TBA was formed and degraded simultaneously which seemed to be related to high density of bacteria in our soil microcosms, particularly *Arthrobacter* populations harbouring catabolic genes for the transformation of TERB into CA. However, the TBA and TERB-OH residues remaining in soil (total 10 % of the initial mass fraction) indicate that biodegradation after 35 days was still incomplete.

In conclusion, the results presented in this study reveal that the degradation of TERB can result in a number of detectable metabolites. These findings have significant implications for the assessment of environmental fate of TERB. Moreover, the observed catabolic activity of M3-T culture toward a range of environmentally important chlorodealkylated *s*-triazine intermediates has an additional ecotoxicological relevance, since these compounds can be phytotoxic and are more mobile than the parent compounds and therefore more easily leached into groundwater from contaminated soils (Caracciolo et al. 2010; Guzzella et al. 2003; Krutz et al. 2010). The aforementioned catabolic versatility of our culture in *s*-triazine transformation is important for developing a bioremediation strategy for accelerating biotransformation processes in *s*-triazine-contaminated soils. A follow-up study will focus on evaluating the full degrading potential of our culture in non-sterile soil to test how the culture competes with degradation capabilities of native soil microorganisms and with changing environmental conditions.

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