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Profiling 5-tolyltriazole biodegrading sludge communities using next-generation sequencing and denaturing gradient gel electrophoresis

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ABSTRACT

Efficient biodegradation of 5-tolyltriazole (5-TTri) in wastewater treatment would minimize its potential detrimental effects on aquatic systems. Therefore, in order to profile 5-TTri biodegrading activated sludge communities (ASC) by DGGE and NGS, acclimation experiments with (i) easily degradable substrates, and (ii) various complex substrates mimicking wastewater conditions were performed. DGGE revealed four genera: *Aminobacter* (family *Phyllobacteriaceae*), *Flavobacterium* (family *Flavobacteriaceae*), *Pseudomonas* (family *Pseudomonaceae*), and *Hydrogenophaga* (family *Comamonadaceae*). Metagenomics (DNA) revealed the dominant families *Alcaligenaceae*, *Pseudomonadaceae* and *Comamonadaceae* that also represented the most active families at the RNA level (metatranscriptomics), which might indicate their importance for 5-TTri biodegradation. ASC acclimation and the composition of the substrate significantly affected 5-TTri biodegradation and the development of biodegrading communities. Using acetate only, a moderate 5-TTri degrading community was detected with a very low biodiversity and *Pseudomonas* spp. as dominant organisms. In contrast, setups fed 'sludge supernatant' (a complex substrate) efficiently biodegraded 5-TTri and formed a more diverse microbial community but with *Hydrogenophaga* spp. as the dominant group. Finally, a hypothetical 5-TTri biodegradation pathway was constructed based exclusively on the detected, biodegradation-related, *Hydrogenophaga* spp. genes.

Introduction

The xenobiotic 5-tolyltriazole (5-TTri) is a commonly used corrosion inhibitor for protecting various surfaces. The mixture tolyltriazole, consisting of 5-TTri and 4-TTri in varying proportions, is used in metal finishing, cooling systems [44,43], as an anticorrosive additive in aircraft deicing or braking fluids [5], in household dishwashing detergents [14,45], and as a UV filter and sun screen agent [23,46]. Unlike 4-TTri, which is not biodegradable and thus might accumulate in the environment [8], 5-TTri is well biodegraded in most cases and thus was used here to study its impact on microbial community structure. Due to its high production volume and widespread usage, 5-TTri is almost omnipresent in aquatic compartments [19,16,29,22]. Wastewater treatment plants are incapable of completely removing 5-TTri during treatment [23,35], although removal efficiencies (using transformation or mineralization) of up to 90% have been reported [1]. Besides small-scale diffuse entry pathways from road runoffs [34,21], sewage treatment comprises the major source of 5-TTri discharge found in river systems [20,9], as well as even groundwater used to generate drinking water [21].

However, even more concerning is that 5-TTri has shown acute toxicity at concentrations above 6.0 mg L^{-1} in Microtox® tests (*Vibrio fischeri* light emission) [31,18], while chronic effects have already been observed at 0.40 mg L^{-1} when 5-TTri showed adverse effects on the aquatic organism *Daphnia galeata* [37]. 5-TTri is regarded as potentially hazardous for aquatic systems [3], implying the need to enhance the removal efficiency during sewage treatment. Laboratory biodegradation experiments conducted to examine the behavior of 5-TTri, showed that it can be eliminated biologically in activated sludge systems [22,10] and within aquifer material [25]. However, information on the composition of 5-

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Table 1
Composition, DOC to N ratio, and applications of the media used in the biodegradation experiments.

Medium	Components [g L^{-1}]	DOC:N ratio of the substrate (DOC:N [mg L^{-1}])	Application
MSM (mineral salt medium)	KH_2PO_4 (0.08), K_2HPO_4 (0.2), Na_2HPO_4 (0.3), $MgSO_4$*7 H_2O (0.02), $CaCl_2$*2 H_2O (0.04), $FeCl_3$*6 H_2O	–	Growth with 5-TTri as sole C and N sources
MSM-CN (carbon and nitrogen)	MSM medium supplied with sodium acetate (0.3) and NH_4NO_3 (0.0075)	33:1 (90:2.7)	Growth with 5-TTri and easily degradable substrate
MSM-SS (sludge supernatant)	MSM medium supplied with 10% heat inactivated sludge supernatant	2.7:1 (162:62)	Growth with 5-TTri and complex substrates mimicking natural wastewater conditions

5-TTri (10 mg L^{-1}) and Hoagland trace elements (0.1 mL L^{-1}) were added to all media.
pH was adjusted to 7.4 in all media.

TTri degrading activated sludge communities (ASC) and a 5-TTri biodegradation pathway is still missing but could help to understand why 5-TTri, although structurally closely related to 4-TTri, is more easily biodegraded.

This study evaluated the community structure of 5-TTri biodegrading and non-biodegrading ASC. The effect of different substrate compositions and microbial acclimation on 5-TTri were analyzed. Results from denaturing gradient gel electrophoresis (DGGE) were compared to next-generation sequencing (NGS) data that included metagenomic and metatranscriptomic analyses. This approach revealed information concerning ASC composition and specific gene expression levels, thus allowing a detailed insight into the abundance of ASC organisms, their gene-related activity during 5-TTri biodegradation, and the creation of a possible 5-TTri biodegradation pathway.

Materials and methods

Chemicals and reagents

5-tolyltriazole (5-TTri; CAS 136-85-6) was purchased from Sigma-Aldrich (Steinheim, Germany), whereas all other media components were obtained from Merck KGaA (Darmstadt, Germany). DNA and RNA cleanup kits were acquired from Qiagen GmbH (Hilden, Germany).

Experimental setups

Activated sludge sampling and acclimation procedure

Activated sludge was obtained from a wastewater treatment plant (CAS-M) in the Munich area, Germany. Acclimation was performed over eight generations (1–8), as follows: A first generation setup was inoculated with 1 mL (dry solids 3 g L^{-1}) PBS-washed activated sludge and incubated until biodegradation occurred between 24 and 48 h (detected by UV-absorbance measurements, UV-AM [11]). Then, 1 mL of this reactor was used to inoculate a subsequent setup (400 mL medium) corresponding to generation 2. Subsequent continuous repetition led to generations 3–8.

Reactor setup and nutrient supply

Biodegradation was monitored in three different setups (three replicates each) containing mineral salt medium (MSM). Abiotic controls without biomass were operated to detect potential losses due to sorption. The MSM setup was not supplemented with carbon and nitrogen, but the MSM-CN and MSM-SS setups were fed with different substrates (Table 1). MSM-CN (carbon and nitrogen) contained only acetate as a carbon source and ammonium nitrate as a nitrogen source. MSM-SS (sludge supernatant) contained complex nutrients mimicking natural wastewater. All experiments were batch setups spiked with 20 mg L^{-1} 5-TTri, kept in the dark, and agitated at 150 rpm (dissolved oxygen >2 mg L^{-1}). The temperature was kept constant at 20 °C (±2 °C) and the pH was maintained at 7.4. All reactor setups (400 mL medium in 1 L flasks with air-permeable caps) were incubated for 22 days and inoculated with either 1 mL of original or subsequent ASC generations. DGGE (DNA) and NGS (DNA and RNA) samples were taken after nine days by withdrawing 100 mL of the reactor suspended biomass.

Biodegradation monitoring of 5-TTi

Removal of 5-TTri was monitored by microplate UV-absorbance measurements (UV-AM) at 262 nm (decreasing values indicated biodegradation) [11] on a regular basis and, for specific 5-TTri analysis, by GC-MS/MS at days 1 and 9 according to Ref. [12].

DNA and RNA extraction

Pellets from 50 mL samples (30 min, 4 °C, 10,000 g) were resuspended in either 500 μL PBS-Buffer (NaCl 8.0 g L^{-1}, KCl 0.2 g L^{-1}, Na_2HPO_4 2.7 g L^{-1}, KH_2PO_4 0.2 g L^{-1}) for DNA extraction or 1000 μL RNAlater RNA Stabilization Reagent (Qiagen) for RNA extraction, and kept at −80 °C. Extractions were performed with the DNeasy Blood and Tissue Kit (DNA) and RNeasy Mini Kit (RNA) both from Qiagen, according to the manufacturer's protocol. After concentration measurements (Nanodrop™, Thermo Fisher Scientific, Schwerte, Germany), DNA and RNA (DNAse treated and PCR confirmed) were stored in elution buffer at −80 °C.

Denaturing gradient gel electrophoresis (DGGE)

The V1–V3 region of the bacterial 16S rDNA gene was amplified by PCR from the genomic DNA using primers 27f-GC (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCGCCCCCGCCCCGG-AGAGTTTGATCMTGGCTCAG), and 517r (5′-GTA TTA CCG CGG CTG CTG GC) [26]. Additionally, the V3–V5 regions were amplified using primers 341f-GC (5′-CCTACGGGAGGCAGCAG, same GC clamp) and 907r (5′-CCGTCAATTCMTTTGAGTTT). PCR was performed using GoTaq® Hot start master mix (Promega, Mannheim, Germany), with TAs of 55 °C (27f/517r) and 52 °C (341f/907r), and 30 cycles. DGGE was carried out on a DCode™ universal mutation detection system (Bio-Rad, Munich, Germany). Ten μL of PCR product were loaded on a 6% (w/v) polyacrylamide gel that was run in TAE buffer at 60 V for 16 h at 55 °C (denaturing gradient 50%–80% with a 100% denaturing solution defined as 7 M urea and 40% (v/v) formamide). The gel was stained for 10 min in ethidium bromide/TAE buffer (0.5 μg mL^{-1}). Prominent bands (boxed, Fig. 2) were excised, incubated in 100 μL sterile TE buffer (overnight, 20 °C) and re-amplified with their corresponding primers 27f (without GC-clamp) — 517r or 341f (without GC-clamp) — 907r.

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