Aerobic biodegradation of tramadol by pre-adapted activated sludge culture: cometabolic transformations and bacterial community changes during enrichment

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Abstract

The biodegradation of biorecalcitrant opioid drug tramadol (TRAM) was studied in a model biodegradation experiment performed with an enriched activated sludge culture pre-adapted to high concentration of TRAM (20 mg/L). TRAM and its transformation products (TPs) were determined by applying ultrahigh-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS), the sludge culture was characterized using a 16S rRNA gene amplicon sequencing, whereas ecotoxicological evaluation was performed based on determination of toxicity to freshwater algae. Tramadol removal was much faster ($t_{1/2}$ = 1.3 days) and more efficient in glucose-containing mineral medium (cometabolic conditions) than in a medium without glucose. The elimination of the parent compound resulted in the formation of five TPs, two of which (TP 249 and TP 235) were identified as N-desmethyltramadol (N-DM TRAM) and N,N-didesmethyltramadol (N,N-diDM TRAM). The remaining 3 TPs (TP 277a-c) were isomeric compounds with an elemental composition of protonated molecules $C_{16}H_{24}NO_3$ and a putative structure which involved oxidative modification of the dimethylamino group. Pronounced changes in the taxonomic composition of the activated sludge were observed during the enrichment, especially regarding an enhanced percentage of 8 genera (*Bacillus*, *Mycobacterium*, *Enterobacter*, *Methylobacillus*, *Pedobacter*, *Xanthobacter*, *Leadbetterella* and *Kaistia*), which might be related to the observed transformations. The removal of TRAM resulted in proportional reduction of algal toxicity, implying a positive result of the accomplished transformation processes.

Key words: tramadol; biotransformation; taxonomic characterization; sludge culture; transformation products; ecotoxicological assessment.

1. Introduction

Tramadol (TRAM) is a synthetic opioid drug frequently used in a mild to severe pain treatment. Up to 30% of the consumed TRAM dose is excreted unchanged, whereas the key TRAM metabolites include different proportions of O-desmethyltramadol (O-DM TRAM), N-desmethyltramadol (N-DM TRAM), N,O-didesmethyltramadol (N,O-diDM TRAM), N,N-didesmethyltramadol (N,N-diDM TRAM) and N,N,O-tridesmethyl tramadol (N,N,O-triDM TRAM), either in free and conjugated forms (Baselt, 2008; Lintz et al., 1981). The literature shows that TRAM should be considered a highly recalcitrant water contaminant whose removal efficiency in classical mechanical-biological wastewater treatment plants (WWTPs) is low (e.g., Falås et al., 2016; Kasprzyk-Hordern et al., 2009; Rúa-Gómez and Püttmann 2012a). Consequently, TRAM is widely present in treated and untreated wastewaters (few hundreds ng/L to few μg/L) (e.g., Baker and Kasprzyk-Hordern, 2013; Hummel et al., 2006; Mackuľak et al., 2016; Krizman-Matasic et al., 2018), as well as in surface waters (few ng/L – few tens ng/L) (e.g. Campos-Mañas et al., 2018; Kasprzyk-Hordern et al., 2009; Krizman-Matasic et al., 2018; Park et al., 2018; Rúa-Gómez and Püttmann, 2012b). Moreover, TRAM has recently been pointed out as one of the three substances contributing predominantly to the acute mixture toxic pressure in Swedish waterbodies (Lindim et al., 2019). Furthermore, according to the recent literature (Buřič et al., 2018), environmentally relevant TRAM concentrations (μg/L) may affect behavior of marbled crayfish *Procambarus virginalis*.

In order to reduce the potential environmental risks associated with the exposure to this widely present water contaminant, new treatment approaches suitable for the reduction of its environmental concentrations are needed. The removal of pharmaceutical compounds, either by abiotic or biotic processes, results very frequently in the formation of persistent transformation products (TPs), which may possess negative ecotoxicological properties. Consequently, a comprehensive assessment of the treatment outcome should take into account

the contribution of the formed TPs (Escher and Fenner, 2011). For example, it was shown that the removal of TRAM using advanced oxidation (e.g., ozon, ferrate, chlorine) and phototransformation processes was associated with the formation of different TPs (Cheng et al., 2015; Zimmerman et al., 2012; Bergheim et al., 2012), some of which being more toxic than the parent compound itself (Bergheim et al., 2012; Bergheim et al., 2014). In contrast, biodegradation of TRAM was not achieved by applying any of the three most widely used OECD biodegradability tests, including the closed bottle test, the manometric respirometry test and the Zahn-Wellens test (Bergheim et al., 2012). A more recent study by Falås et al. (2016) reported an improved TRAM bioremoval under anaerobic conditions. To the best of our knowledge, an oxidative biodegradation of TRAM was not achieved in any of the published studies and, consequently, the information on possible oxidative microbial transformation pathways is still missing.

The aims of this study were to generate a pre-adapted microbial culture derived from activated sludge from a municipal WWTP capable of degrading TRAM and to use this enriched culture to study TRAM biodegradation under aerobic conditions. Biodegradation study combined determination of TRAM removal kinetics and identification of formed TPs with the taxonomic characterization of the applied mixed microbial culture. In addition, the ecotoxicological outcome of the achieved biotransformation was assessed using toxicity to freshwater algae.

2. Materials and methods

2.1. Chemicals and reagents

TRAM was purchased from Sigma-Aldrich (Steinheim, Germany). N-DM TRAM and deuterated tramadol (TRAM-d_3) were purchased from Lipomed (Arlesheim, Switzerland).

The purity of the reference materials used for confirmatory purposes was ≥ 98%. The individual stock solutions (10 mg/mL and 1 mg/mL) of TRAM and N-DM TRAM used for quantitative purposes were prepared in methanol (LC-MS grade). Chemicals used for growth media were of analytical grade purity whereas those for molecular analyses were of molecular grade and were supplied by Sigma-Aldrich (USA) and Kemika (Zagreb, Croatia). Formic acid (LC-MS grade) and ammonium formate (purity ≥ 99%) were purchased from Sigma-Aldrich. LC-MS grade solvents (acetonitrile and methanol) were products of J.T. Baker (Deventer, the Netherlands). Phenol (purity ≥ 99.5%) and glucose (purity ≥ 99.5 %) were purchased from Sigma-Aldrich. Sulfuric acid (95–97%) was purchased from Merck. Ultrapure water was produced using an Elix-Milli-Q system (Millipore, Bedford, MA, USA).

Solid-phase extraction (SPE) cartridges Oasis HLB (60 mg/3 mL) were supplied by Waters (Milford, MA, USA).

2.2. Enrichment of tramadol-degrading microbial culture

The original activated sludge, used as an inoculum for the microbial culture enrichment, was collected from the aeration tank of the central WWTP of the city of Zagreb, which is equipped with a conventional activated sludge treatment having the following characteristics: hydraulic retention time 12 h; MLSS concentration 3.5 g/L; solids retention time 3 days. Before being used in biodegradation experiments, the collected sludge was subjected to a prolonged enrichment procedure using TRAM as a selection agent. The enrichment was performed in 50-mL Erlenmeyer flasks containing 30 mL of modified phosphate-buffer minimal salts medium (MSM; Bedard et al., 1987; for the exact composition see Supplementary material), which was supplemented with 20 mg/L of TRAM. TRAM was added to the empty flask in the form of methanol solution (60 µL; 10 mg/ml). Before adding MSM, methanol was allowed to evaporate, in order to avoid its possible influence on the biomass growth. The enrichment

media were supplemented with different amounts of glucose and ammonium chloride (NH_4Cl) as additional C and N sources. The C/N ratios examined in the preliminary phase of the enrichment are listed in Table S1 (Supplementary material) along with other modifications tested. Based on these experiments, the medium containing 1 g/L of glucose and 170 mg/L of NH_4Cl (C/N ratio of 10) was found optimal and it was selected for all further experiments. The enrichment started with inoculation of the modified MSM with 3 mL of the fresh activated sludge. The Erlenmeyer flasks containing inoculated media were incubated in the dark at 30^{o}C on a rotary shaker operated at 180 rpm (9279 g). Every 7-10 days, over the entire enrichment period, 10% (3 mL) of the microbial culture was transferred to a fresh medium (30 mL) and the incubation was continued under the same conditions. The ability of the enrichment culture to degrade TRAM was periodically examined (Table S2; Supplementary material) by determining the concentration of TRAM and screening samples to detect possible TPs.

2.3. Tramadol biodegradation experiments

The biodegradation experiments were performed with the culture which was obtained after one year of enrichment (approximately 40 transfers in the optimized medium; C/N ratio of 10). The inoculum for the biodegradation experiments was prepared by growing the enriched sludge culture in the optimized medium supplemented with TRAM, for 3 days. The inoculum culture was pelleted by centrifugation (8944 g; 5 min) and the cells were resuspended (10 g/L; wet weight) in fresh MSM (30 mL), supplemented with NH_4Cl and TRAM (20 mg/L). Half of the experimental flasks were additionally supplemented with glucose (w/ glucose), whereas the remaining flasks did not contain glucose (w/o glucose). The abiotic control experiments were also performed using non-inoculated MSM. All biodegradation experiments were performed in duplicate. The incubation conditions during biodegradation experiments were identical to those applied during the enrichment. The duration of biodegradation experiments

was 14 days. At selected time intervals aliquots of the degradation media were collected for chemical (1 mL) and ecotoxicological analyses (5 mL). The supernatant for the analysis of the dissolved residual TRAM was obtained after centrifugation at 8944 g. The changes in the biomass concentration were determined by measuring changes in optical density (OD) at 600 nm. Pellets obtained by centrifugation were stored at -20^{o}C and subsequently used for DNA extractions.

2.4. Taxonomic characterization

Taxonomic characterization of the activated sludge culture included extraction of the total genomic DNA by using Nucleospin Microbial DNA extraction kit (Macherey-Nagel, Germany) and subsequent sequencing of the obtained 16S rRNA amplicon libraries. Total genomic DNA was extracted from pelleted cells (8944 g; 10 min), following manufacturer instructions, obtained from both the original sludge culture (initially collected from the WWTP Zagreb) and from samples of the enriched sludge culture collected during biodegradation experiment at days 0 and 4. Details on the extraction are provided in Supplementary material. Quality and quantity of the extracted DNA was determined on Biospec Nano (Shimadzu).

V4 variable region of the 16S rRNA gene marker was PCR-amplified at MR DNA Laboratory (www.mrdnalab.com, Shallowater, TX, USA) in a single-step 30-cycle PCR using the HotStarTaq Plus Master Mix Kit, standard primers 515/806 with barcode on the forward primer (Qiagen, USA) and extracted DNA as a template. Prepared Illumina 16S rRNA libraries were sequenced on a MiSeq platform following manufacturer's guidelines (MR DNA; www.mrdnalab.com, Shallowater, TX, USA). Obtained data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA) with OTUs taxonomically classified using BLASTn against a database derived from RDPII (http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov). More details can be found in Kostanjevecki et al. (2019).

178 Similarities between microbial communities were tested using non-metric multidimensional
179 scaling (nMDS), the PRIMER 6 v.6.1.11. and PERMANOVA +v.1.0.1. statistical software
180 packages (Clarke and Gorley, 2006). Similarities were calculated for each abundance
181 resemblance matrix based on Bray–Curtis (di)similarity index (Clarke and Warwick, 1994).
182
183 ***2.5. Kinetics analysis***
184 The degradation kinetics was modelled for the first-order kinetics as follows:
185 $c_t = c_0 * e^{(-kt)}$,
186 where c_0 is the initial concentration of TRAM, c_t is the concentration of the TRAM at time t, k
187 is the degradation rate constant (day^{-1}) and t is the degradation period in days.
188 Degradation rate constants were calculated by performing a nonlinear least squares regression
189 analysis. The biodegradation half-life ($t_{1/2}$) was calculated using the equation:
190 $t_{1/2} = \ln 2/k$
191
192 ***2.6. Analyses of tramadol and its transformation products***
193 Before being analyzed by ultrahigh-performance liquid chromatography (UHPLC) coupled to
194 quadrupole-time-of-flight mass spectrometry (Q-TOF-MS), the collected samples were
195 diluted (10 times) in 50 mM aqueous solution of ammonium formate. The UHPLC/Q-TOF-
196 MS analyses were performed using an instrument consisting of a Waters Acquity UPLC
197 solvent delivery system (Waters Corp., Milford, MA, USA) coupled to Q-TOF Premier
198 instrument (Waters Micromass, Manchester, UK) equipped with an orthogonal Z-spray-
199 electrospray interface. Argon and nitrogen were used as the collision gas and ion
200 desolvation/nebulizing gas, respectively. The desolvation gas flow and temperature were set
201 at 700 L/h and 300°C. The cone nitrogen flow and the source temperature were set to 25 L/h
202 and 120°C, respectively. The capillary voltages in the positive and negative ionization

polarity modes were 3500 V and 3000 V, respectively, whereas the cone voltage in both modes was set to 30 V. The MS data were acquired between m/z 50 and m/z 1000, applying collision energy of 4 eV. All spectra were recorded using the extended dynamic range (DRE) option. Leucine enkephaline was used as an independent reference mass to provide accurate mass measurements. The data were collected in the centroid mode. The scan and interscan time were 0.08 s and 0.02 s, respectively.

In addition, structural identification of the TRAM TPs was performed based on a number of targeted MS2 and in-source fragmentation experiments, which were performed by applying different combinations of preselected fixed collision energies (CE: 15, 20, 25, 30, 40 eV) and sample cone voltages (CV: 30, 40, 50, 55, 60, 65, 70 V).

In both polarity modes, chromatographic separation was performed on an Acquity BEH C_{18} column (50 x 2.1 mm; 1.7 μm) using gradient elution at a flow rate of 0.4 mL/min. In the positive ionization mode, the eluents A and B were water and acetonitrile, respectively, each supplemented with formic acid (0.1%). The same solvents, but without formic acid supplementation, were applied in the negative ionization mode acquisition. The applied elution gradient started at 5% B. The percentage of solvent B was then linearly increased to 50% in 8 minutes, after 1 min of isocratic hold. The system was then returned to the initial conditions in 0.5 min. The initial conditions were kept for additional 2 min before the next sample was injected. The recorded data were processed using the MassLynx software incorporated in the instrument. The quantification of TRAM and N-DM TRAM was performed by using external calibration. The reference standards (0.02 - 2.5 μg/mL) used for the qualitative and quantitative LC-MS analyses were prepared in 50 mM aqueous solution of ammonium formate. The analytical quality assurance data are given in Supplementary material (Table S3). The instrument performance was checked by running quality check (QC) analyses (TRAM standard solutions 2 ng/μL) after every 10-12 injections. When reference

standards were not available (TRAM TP 277a-c), semiquantitative estimates were performed by assuming the equal molar responses of the TPs and the parent compound.

2.7. Glucose concentration measurements

Glucose concentrations in biodegradation media were determined spectrophotometrically, using the phenol-sulfuric acid method (Dubois et al., 1956). Briefly, after sample centrifugation to remove biomass (8944 g; 5 min), supernatant aliquots (10 μL) were diluted with ultrapure water (990 μL) and thoroughly mixed with phenol solution (1 mL; 0.53 mol/L) and concentrated sulfuric acid (5 mL). The prepared samples were shaken and then incubated for 1 h in the dark. The resulting absorbance of the samples was measured in a quartz cuvette (1 cm pathlength) at 490 nm, using a Varian Cary 50 probe UV-Visible Spectrophotometer. The quantitation was performed using a 7-point calibration curve, constructed in the concentration range from 0.1 mg/L to 15 mg/L. The method repeatability and quantitation limit were 2% and 0.1 mg/L, respectively.

2.8. Chronic toxicity evaluation

Chronic toxicity of the biodegradation samples was assessed using the freshwater green algae *Desmodesmus subspicatus* (86.81 SAG) grown in ISO/FDIS 8692 culture medium, as described in detail in ISO (2004). In order to pre-concentrate the samples and to remove the salts present in the biodegradation medium, the original protocol was modified to include a SPE step. The samples (5 mL) for the evaluation of algal toxicity were percolated through preconditioned (3 mL of methanol, ultrapure water and spring water) Oasis HLB cartridges. After the sample enrichment, the residual salts were washed out from the cartridge with ultrapure water (3 mL) and the adsorbed analytes were eluted with methanol (2 mL). The methanol extract was evaporated to dryness and re-dissolved in 0.5 mL of the ISO/FDIS 8692

culture medium. The recovery and precision of the applied SPE sample preparation procedure for TRAM were 79% and 1.6%, respectively. The recovery and precision for TRAM TPs were not experimentally determined and they were assumed to be equal to that of TRAM. The toxicity test was conducted in 96 microwell plates as described previously (Blaise et al., 1986; Smital et al., 2011) with slight modifications (for details see Kostanjevecki et al., 2019). The average specific growth rate was calculated and subsequently fitted to a three-parameter sigmoid dose–response equation to calculate the inhibition. The dose–response curve of potassium dichromate ($K_2Cr_2O_7$) was included as a reference standard in all experiments.

3. Results and discussion

3.1. Biodegradation of tramadol

The original activated sludge culture, collected at WWTP Zagreb, was unable to remove TRAM from the biodegradation media (Fig. S1). The first significant degradation (30%) was observed after 7 months (total of about 20 culture transfers). Progression of the culture's capability to degrade TRAM over the enrichment period is presented in Table S2 (Supplementary material). The biodegradation experiments, performed by using the finally optimized enriched culture obtained after 1-year enrichment period, are presented in Fig. 1. In order to investigate possible role of an additional source of carbon for the microbial removal of TRAM, the experiments were performed in two different media, one containing 1 g/L of glucose (w/ glucose) and the second one without the addition of glucose (w/o glucose). Both media contained 170 mg/L of NH_4Cl (C/N ratio of 10). A pronounced biomass growth was determined at both biodegradation conditions during the first experimental day (Fig. 2). However, immediately after that, the stagnation and gradual decrease of biomass concentration was recorded in the glucose-lacking media. By contrast, in the glucose-containing media, the highest biomass concentration was achieved between the 3rd and 4th day

of the experiment with a gradual decrease in the subsequent 10-days period. Under the selected experimental conditions, removal of TRAM was more efficient (82%) at cometabolic conditions (w/ glucose) than in the media w/o glucose (33%), which is in line with the results recently obtained for another amine-containing opioid drug, methadone (Kostanjevecki et al., 2019). The real removal efficiency might have been even slightly higher, since the result presented in Fig. 1 was not corrected for the media evaporation losses (5-10%) which occurred during the experiment. Furthermore, the contribution of adsorption to the overall TRAM removal, was estimated to be rather low (2-6%), based on K_d value of 64 L/kg (unpublished data for the activated sludge in WWTP of the city of Zagreb) and the highest achieved biomass concentration, whereas a photo-transformation was excluded by performing the experiments in the dark. Consequently, the observed removal of TRAM was primarily assigned to biotransformation processes, which was additionally supported by the appearance of TPs in total ion chromatograms (Fig. S2 and Fig. S3). At cometabolic conditions, the elimination of TRAM (Fig. 1) was well correlated ($y = 0.7617\ x + 0.0011$; $r^2 = 0.984$) with the removal of glucose (Fig. 2), indicating a possible importance of cometabolic processes in TRAM biodegradation. Interestingly, almost no degradation of glucose and TRAM was obtained after the 4^{th} day of the experiment, which coincided with a decreasing trend in biomass concentration. The observed stagnation of TRAM and glucose removal might have been caused by unfavourable C/N ratios in that period, however the validity of this assumption requires further investigation. Since the degradation in both experiments virtually stopped after the 4^{th} experimental day, the degradation kinetics was determined by fitting the removal data obtained during the initial four days to first-order kinetic model (Table 1). The degradation rate constants obtained in the media with (w/) and without (w/o) glucose were 0.525 $days^{-1}$ and 0.136 $days^{-1}$. The degradation half-life in the experiment with glucose was 1.3 days. In contrast, the removal of TRAM from the medium w/o glucose stopped at

303 approximately 30% and never reached 50%. This indicated a much faster and more efficient
304 TRAM degradation at cometabolic conditions.
305
306 ***3.2. Biotransformation products of tramadol***
307 Microbial removal of TRAM was accompanied with the formation of several TPs, all of
308 which were detected by applying a positive polarity ionization mode (Fig. S2). By contrast,
309 the aquisitions performed in negative polarity ionization mode did not indicate a formation of
310 any additional TPs (Fig. S3). All identified TPs along with their corresponding retention time,
311 elemental composition, theoretical *m*/*z* values of protonated molecules, mass errors and
312 proposed chemical structures are presented in Table 2, whereas their extracted ion
313 chromatograms and mass spectra are given in the Supplementary material (Figs. S4-S9). The
314 identification of the detected TPs was based on their chromatographic and accurate mass
315 spectrometric data and, whenever possible, included confirmation by comparison with the
316 authentic reference standards. The identification confidence reported in Table 2 was
317 performed using a five-level system proposed by Schymanski et al. (2014). The 1st level of
318 confidence was assigned to compounds confirmed by reference standards, whereas the
319 confidence levels of 2b and 3 were assigned as probable structures and tentative candidates,
320 respectively, based on the mass-spectrometric data. The TP 249 and TP 235 were identified as
321 N-DM TRAM and N,N-diDM TRAM, implying N-demethylation as one of the key
322 mechanisms of TRAM microbial transformation. The literature shows that N-demethylated
323 derivatives of TRAM were also formed by metabolic transformation in freshwater crustaceans
324 (Jeon et al., 2013). Furthermore, the same biotransformation pathway was recently reported
325 for structurally related pharmaceutical, venlafaxine (Llorca et al., 2019). N-demethylation was
326 also one of the main mechanisms of microbial transformation of another amine-containing
327 opioid, methadone (Kostanjevecki et al., 2019). The results obtained in this study are in line

with the hypothesis made by Gulde et al. (2016) that mammalian and microbial transformations of a number of different amine-containing contaminants are fairly similar, which can be of use in suspect screening of possible TPs in environmental samples. In addition to biotic transformations, N-demethylation was also reported as an important abiotic transformation mechanism involved in the photocatalytic degradation of TRAM (Antonopoulou and Konstantinou, 2016).

By contrast, the remaining TRAM TPs (TRAM TP 277a-c) observed in our study, have not yet been reported in the literature. These TPs included three chromatographically separated isomeric compounds, having a *m*/*z* value 278.1756, corresponding to the elemental composition of protonated molecule $C_{16}H_{24}NO_3$. Furthermore, all three isomeric TPs were chromatographically eluted after the parent compound itself (Table 2, Figs. S2 and S4) suggesting a relative increase in lipophilicity. The presence of the product ion *m*/*z* 201.1279 in the mass spectra of TRAM (Fig. S5) and all 3 isomeric TPs (Fig. S8), corresponding to elemental composition $C_{14}H_{17}O$, clearly indicated that the transformations leading to the formation of these 3 isomeric TPs must have occurred on dimethylamino moiety, whereas the remaining parts of the molecule remained intact. This conclusion was additionally supported by the presence of the product ions *m*/*z* 159 ($C_{11}H_{11}O$) and *m*/*z* 121 (C_8H_9O), which were produced by further fragmentation of the ion *m*/*z* 201, in the MS spectra of both TRAM and the TPs 277a-c (Figs. S5 and S8). Based on the available mass-spectrometric evidence and existing knowledge on biotransformation mechanisms of amine-containing micropollutants (Gulde et al., 2016; Kostanjevecki et al., 2019), two tentative structures were proposed for these TPs (Table 2) whereas the structure of the third isomeric TP remained unknown. The first proposed structure (Table 2, structure I) could have been formed either by N-formylation of N-DM TRAM or by α-C-oxidation of TRAM to formamide, whereas the second proposed structure (Table 2, structure II) might have been produced by N-acetylation of N,N-diDM

TRAM. All isomeric TRAM TPs exhibited a pronounced predisposition to form sodium adduct ions (Fig. S9). This is in agreement with the proposed structures containing amide moiety (Kruve et al., 2013). The obtained mass spectra did not include any diagnostic ions which would enable an unequivocal assignment of the proposed isomeric structures to individual chromatographic peaks in the absence of the reference standards.

Nevertheless, some additional indication can be obtained from the formation dynamics of individual TPs during the biodegradation experiments (Fig. 3). The presence of all identified TPs was confirmed at both investigated conditions except for N,N-diDM TRAM, which was detected only in glucose-supplemented media. The predominant N-demethylated TP was N-DM TRAM which showed rather similar formation dynamics under both experimental conditions. It is interesting to note that the formation of TP 277a in glucose-containing media started with a delay of 2 days, only after an initial accumulation of N,N-diDM TRAM, and coincided with the decrease of N,N-diDM TRAM. Consequently, it seems plausible to conclude that TRAM TP 277a was actually a N-acetyl derivative of N,N-diDM TRAM (Table Table 2, structure II). This result indicated a possible enhancement of some specific metabolic pathways, including N-acetylation, in the presence of glucose, however, further investigation is needed to confirm a validity of this assumption. The isomers TP 277b and 277c can very probably be assigned as formylated derivatives (see Table 2 for the proposed structure) of the major TP N-DM TRAM which represented 80 ± 5% of the overall concentration of N-demethylated TPs. There is a theoretical alternative that one of these two isomers could have been N-oxoethyl, N-desmethyl TRAM which has the same elemental composition as the acetylated and formylated derivatives but we are not aware of any biochemical mechanism which would lead to this structure. Based on the structural assignments discussed above we proposed a tentative biotransformation pathway during aerobic degradation of TRAM carried out in this study (Figure S10; Supplementary material).

An attempt to provide a provisional mass balance based on semi-quantitative estimates of molar concentrations of TRAM and all detected TPs, is given in Fig. S11 (Supplementary material). In spite of significant uncertainties related to TP quantitation in the absence of reference standards, the summed-up concentrations of TRAM and its TPs were rather stable over the course of the entire experiment which underlines the persistent character of the TPs formed during our aerobic degradation experiments (Fig. S11).

3.3. Taxonomic characterization of the microbial culture

Next generation sequencing of the 16S rRNA libraries allowed us to determine the diversity and community composition of both, the original culture collected from the WWTP aeration tank and the culture established after one year of the selective enrichment. With bacteria representing > 99% of the identified operational taxonomic units (OTUs) both cultures included members of the phyla *Bacteroidetes, Actinobacteria, Firmicutes*, *Proteobacteria, Chloroflexi* and *Planctomycete*s, with the last two representing only a minor fraction within the community (1-5%). Comparative 16S rRNA sequence analyses further revealed a clear switch in the composition of the two investigated activated sludge cultures (Fig. 4, Fig. S12). While in the original sludge culture *Proteobacteria* (with *Alphaproteobacteria* representing 50% of all proteobacterial sequences) was a dominant phylum, accounting for 60% of all sequences, in the enriched culture *Firmicutes* (represented solely by *Bacilli*) reached high abundances and represented 30% of the community. Even though microbial structure of the WWTP biomass is highly dependent on the type of technological system, wastewater characteristics and geographic location, our results are in line with other studies showing that in these systems phylum *Proteobacteria* usually predominates (30–60%), with subdominant phyla including *Firmicutes*, *Bacteroidetes* and *Actinobacteria* (average 8%) (Cydzik-

Kwiatkowska and Zielińska, 2016; Zhang et al., 2012). Interestingly, *Alphaproteobacteria* are usually not predominant *Proteobacteria* within microbial biomass.

Chao, Shannon and Simpson indices in the original sludge culture reached values of 2937, 7.7 and 0.98 (Table S4), representing average values usually observed in WWTP (Zhang et al., 2012). This highly diverse community identified in the original sludge was however comprised of species with low abundances, mostly not exceeding 1% of the total community. Within this community only 3 genera (*Tabrizicola*, *Arthrobacter* and *Amaricoccus),* not usually found as core members of sludge cultures, contributed > 5% to the total community (Fig. S13). However, a study conducted in 15 WWTPs (Zhang et al., 2012) indicated clear differences between these activated sludge communities at the genera level and contribution of individual community members rarely exceed 2-3% of the total community.

As expected, enrichment resulted in lowering the diversity (Table S4: 924, Chao; 4.1, Shannon; 0.78, Simpson) within the original sludge community with a sharp decrease in the number of individual genera and species observed (Fig. S13). Under selected conditions, enrichment led to selection of 8 genera, including different species of *Bacillus* (30% community), *Mycobacterium* (10% community), *Enterobacter* (8% community), *Methylobacillus* (6% community), *Pedobacter* (6% community), *Xanthobacter* (6% community), *Leadbetterella* (5% community) and *Kaistia* (5% community), comprising altogether > 70% of the total community. Species within the genera *Bacillus*, *Enterobacter*, *Methylobacillus, Pedobacter* and *Xanthobacter* are among strains being isolated using culture-based methodologies from different sludges, with many of them showing biodegradation potential towards different environmental pollutants (Emanuelsson et al., 2009; Hu et al., 2009; Lolas et al., 2012).

In order to identify possible community members responsible for the observed biotransformation activity, we followed changes in the community structure from day 0 to day

4 of the biodegradation experiments (w/ and w/o glucose). Even though chemical analysis showed a clear difference in TRAM removal efficiencies as well as in the dynamics of biotransformation products between the enriched activated sludge culture grown w/ and w/o glucose, taxonomic analysis did not strongly concur with these results (Figs. S12 and S13). The cultures growing under different C regimes could be discriminated only based on 10% differences in their community structures. In the condition w/o glucose, genera found to proliferate included *Sphingobacterium*, *Xantobacter*and and *Methylobacillus*, representing 21% of the community, whereas *Bacillus* accounted for 18%. By contrast, in the experiment with glucose supplementation, the strongest enrichment was found for *Enterobacter* (13% of the community) and *Bacillus* (30% of the community). Even though enrichment of these different populations could be linked to the difference in C regime, role of these microorganisms in the elimination of TRAM should not be disregarded. Based on the observed differences we hypothesize that consortium comprised of several bacterial populations (*Bacillus* / *Methylobacillus* / *Enterobacter* / *Xantobacter* / *Sphingobactreium)* might have been involved in the removal of TRAM. These populations in the conditions w/ and w/o glucose reached different biomasses that might consequently influence TRAM degradation rate as well as the rate and extent of biotransformation products formation. Unfortunately, currently no bacterial strains can be linked to tramadol biotransformation and therefore more detailed studies are needed to identify a bacterial strain or bacterial consortium responsible for the observed TRAM biotransformation activity. Slower degradation rates obtained in the conditions w/o glucose indicated importance of optimizing growth factors during biodegradation experiments concerning C source, i.e. C/N ratio that is known to affect metabolic activities of microorganisms in degradation of environmental contaminants (Falås et al., 2016; Teng et al., 2010).

3.4. *Toxicity evaluation*

Tramadol is rather non-toxic to algae (EC_{50} = 47.5 mg/L), however, some of its TPs could potentially be more toxic. The removal of TRAM in all performed experiments was incomplete (33-81%) and, consequently, the media at the end of the performed biodegradation experiments contained mixtures of parent compound and its TPs in different proportions. At both investigated experimental conditions, the extent of the final reduction of parent compound concentration and algal growth inhibition was rather similar (Fig. 5), implying a rather positive ecotoxicological result of the achieved transformation processes. Since the recoveries of TPs were not experimentally verified, there is a possibility that a loss of TPs during sample preparation also contributed to reduced toxicity. Moreover, the study included only one endpoint and it is possible that different outcomes might have resulted if some other assays were performed. For instance, Bergheim et al. (2014) showed that irradiated TRAM samples exhibited significantly higher toxicity to *Pseudomonas putida* and *Vibrio fischeri* as compared to non-irradiated samples. Consequently, additional ecotoxicological tests are needed to confirm the general validity of the conclusion drawn from the algal bioassays.

2. Conclusions

Tramadol is an extremely recalcitrant water contaminant whose biodegradation can be achieved only by carefully enriched microbial cultures. Our experiments suggest that several genera of the phylum *Firmicutes* may have played a role in the biotransformation. Moreover, the results of this study suggest that the transformation occurs primarily at cometabolic conditions in the presence of easily degradable organic matter supporting a high concentration of microbial biomass and therefore it is not likely to expect its extensive oxidative microbial transformation in real aquatic environments such as surface waters. Furthermore, the biotransformation does not lead to a complete mineralization to CO_2 and water but the

transformation leads to the formation of TPs which are rather stable at aerobic conditions. Despite the fact that an algal bioassay indicated a significant reduction of toxic effects in biotransformed media, the formation of rather persistant TPs requires an additional ecotoxicological evaluation.

Declaration of interest

The authors declare no conflict of interest.

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602

603 **Table 1.** Tramadol (TRAM) biodegradation kinetic parameters achieved by using enriched sludge
604 culture in a medium with (w/) and without (w/o) glucose.

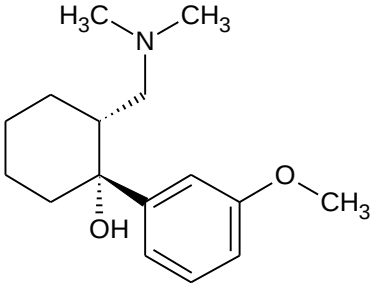
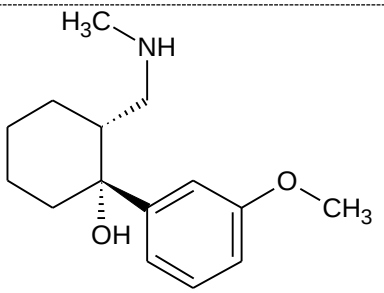
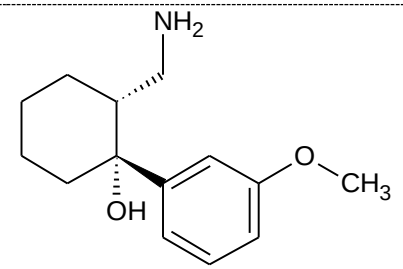
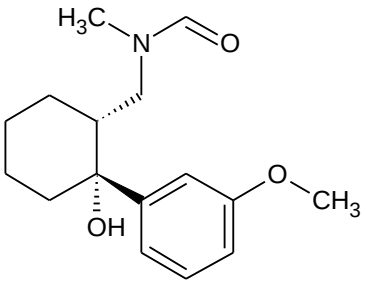
	k (day^{-1})	$t_{1/2}$ (days)	r^2	Kinetic model
w/o glucose	0.136	N/A	0.9592	1st order kinetics
w/ glucose	0.525	1.3	0.9592	1st order kinetics

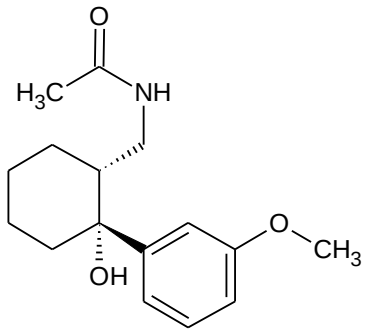
605 N/A – not applicable

606

607

608 **Table 2.** Chromatographic and mass spectrometric characteristics of tramadol (TRAM) and proposed
609 structures of its biotransformation products (TPs).

Compound	RT (min)	Elemental composition $[M+H]^+$	*m/z* theoretical	Error (mDa)	Structure	Level of confidence *
TRAM	3.71	$C16H26NO2$	264.1964	-1.2		NA
TRAM TP 249 = N-DM TRAM	3.78	$C_{15}H_{24}NO_2$	250.1806	-0.3		1 (confirmed structure)
TRAM TP 235 = N,N-diDM TRAM	3.68	$C_{14}H_{22}NO_2$	236.1651	+2.4		2b (probable structure by diagnostic evidence)
TRAM TP 277b, c	5.26; 5.69; 5.98	$C_{16}H_{24}NO_3$	278.1756	+0.1; +1.4; -2.0	**I)** 	3 (tentative candidate)
TRAM TP 277a					**II)**	3 (tentative candidate)



610 *according to Schymanski et al., 2014; TRAM = tramadol; N-DM-TRAM = N-desmethyltramadol; N,N-
611 di-DM-TRAM = N,N-didesmethyltramadol; RT – retention time; NA – not applicable

612

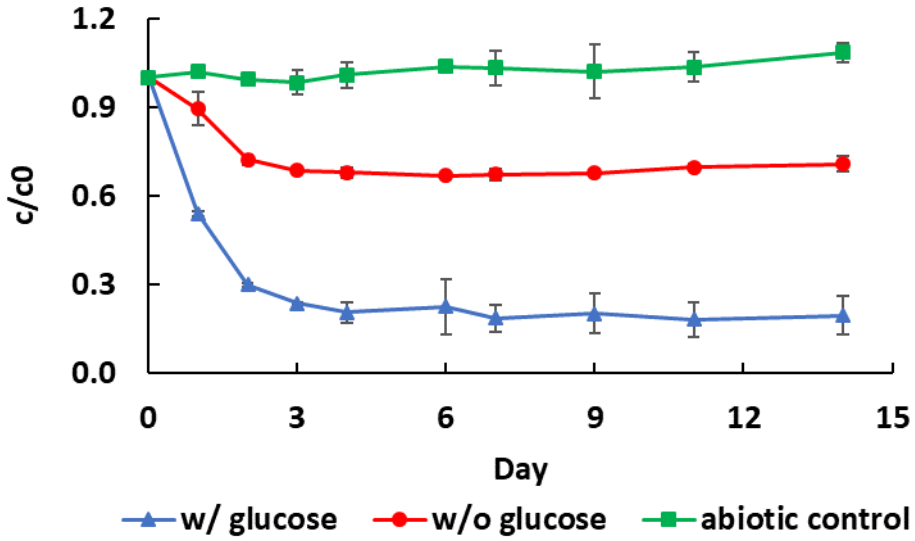


Fig. 1. Removal of tramadol in a biodegradation experiment performed in the medium with (w/) and without (w/o) glucose. Error bars represent standard deviations

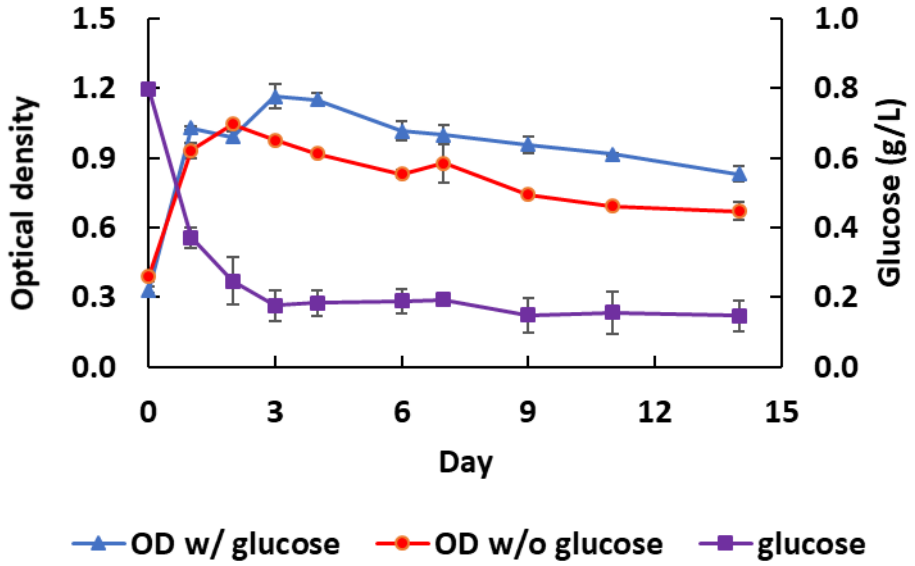
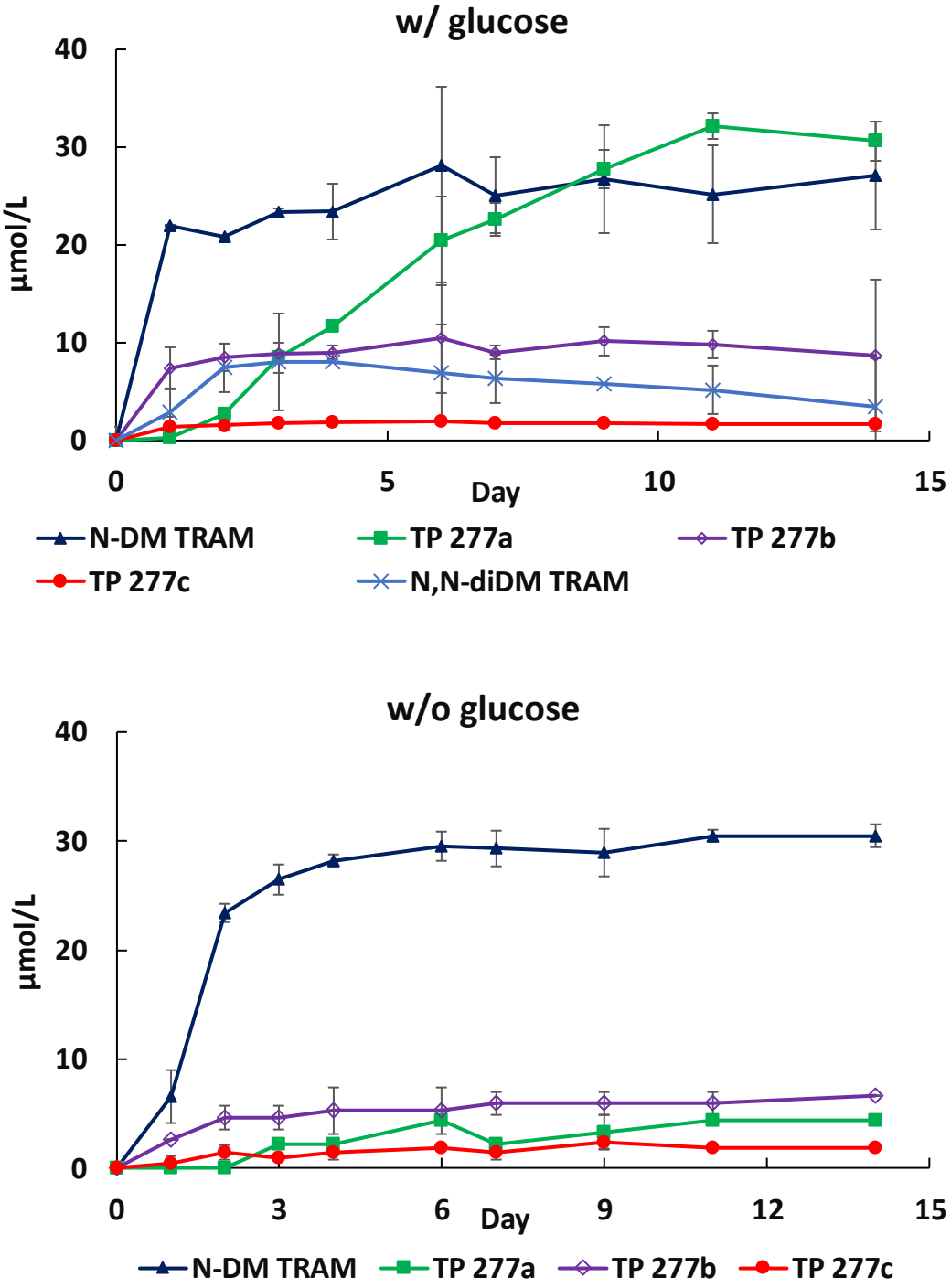


Fig. 2. Biomass growth, measured as optical density (OD), in biodegradation media with (w/) and without (w/o) glucose and glucose consumtion in the glucose-containing media. Error bars represent standard deviations.



624

625 **Fig. 3.** Dynamics of tramadol transformation products (TRAM TPs) in the biodegradation
626 experiments performed in a media with (w/) and without (w/o) glucose. Error bars represent
627 standard deviations.

628

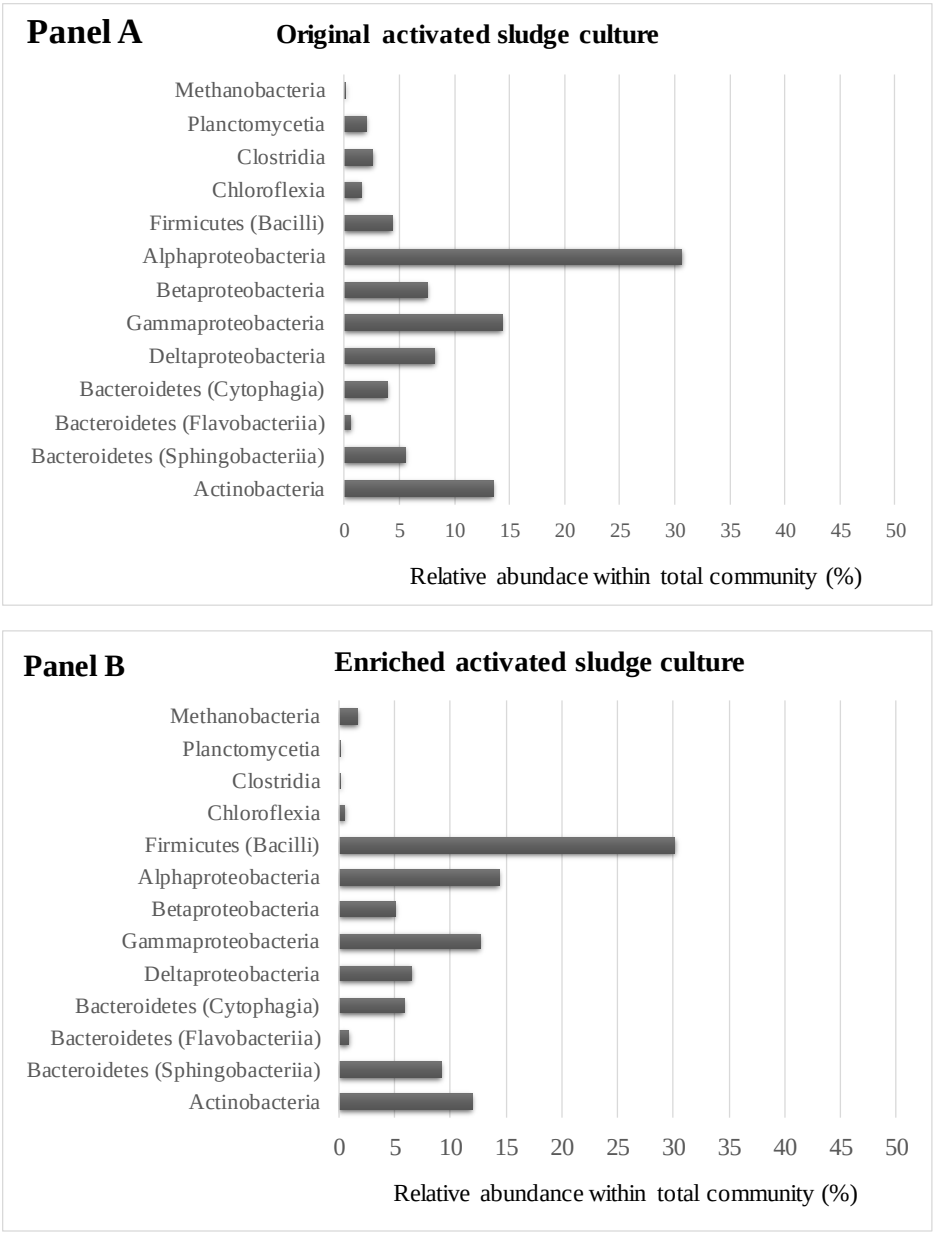
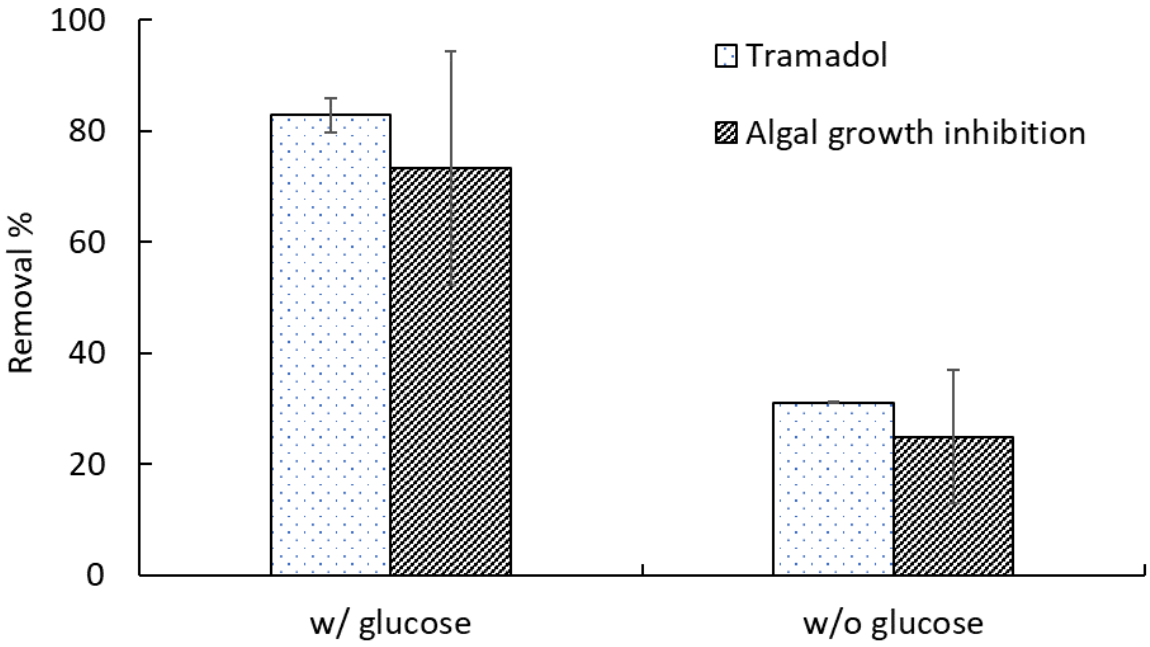


Fig. 4. Differences in the microbial community composition between original activated sludge culture collected at the WWTP (Zagreb) site (**Panel A**) and enriched activated sludge culture established after 12 months of the selective enrichment (**Panel B**), presented on the class level, determined based on sequencing of the V4 variable region of the 16S rRNA gene marker.



637

638 **Fig. 5.** Removal of tramadol and algal growth inhibition in the media with (w/) and without
639 (w/o) glucose. Error bars represent standard deviations.

640