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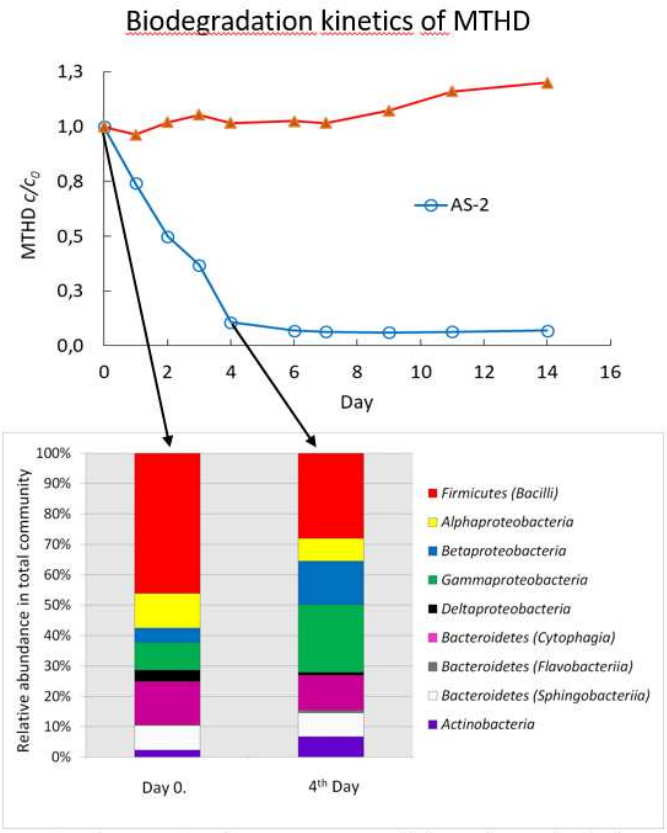
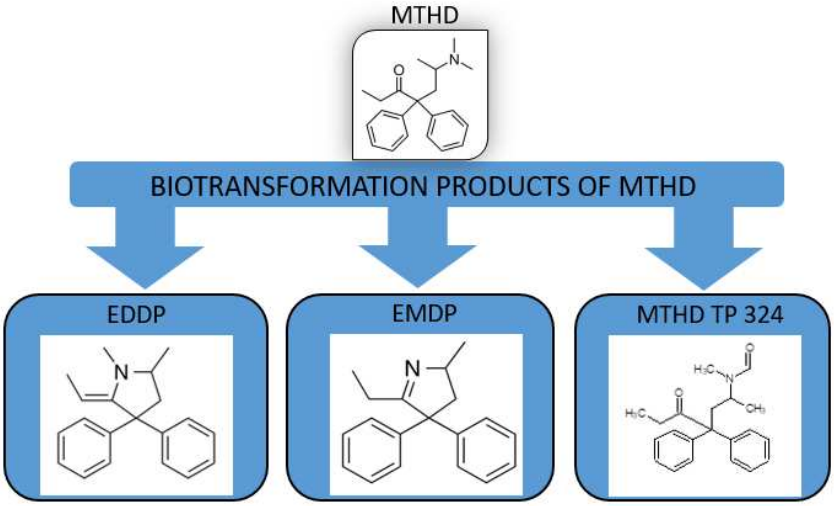


Taxonomic changes in the structure of the adapted sludge culture



Biodegradation study of methadone by adapted activated sludge: elimination kinetics, transformation products and ecotoxicological evaluation

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Abstract

The biotransformation study of difficult-to-degrade opioid analgesic methadone (MTHD) was performed by activated sludge culture adapted to high concentration of methadone (10 mg/L). The study included determination of elimination kinetics of the parent compound, taxonomic characterization of microbial culture, identification of biotransformation products (TPs) and assessment of ecotoxicological effects of biotransformation processes. The chemical analyses were performed by ultra-performance liquid chromatography/quadrupole-time-of-flight mass spectrometry, whereas the ecotoxicological assessment was made based on determinations of toxicity to freshwater algae. Changes of the adapted sludge culture during the experiment were followed using the 16S rRNA gene amplicon sequencing. Depending on the experimental conditions, the elimination efficiency of methadone (10 mg/L) varied from 9% to 93% with the corresponding half-lives from 11.4 days and 1.5 days. A significantly faster elimination ($t_{1/2}$ from 1.5 days to 5.8 days) was achieved at cometabolic conditions, using glucose-containing media, as compared to the experiments with MTHD as a single organic carbon source ($t_{1/2}$ = 11.4 days). Moreover, increased biotransformation rate following the additional supplementation of ammonia, revealed a possible importance of nitrogen availability for the transformation at cometabolic conditions. The elimination of parent compound was associated with the formation of 3 different TPs, two of which were identical to main human metabolites of MTHD, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3- diphenyl-1-pyrroline (EMDP). EDDP represented over 90% of the total TP concentration at the end of experiment. The biodegradation of MTHD was associated with a pronounced drop in algal toxicity, confirming a rather positive ecotoxicological outcome of the achieved biotransformation processes.

Key words: methadone, biodegradation, biotransformation, transformation products, EDDP, ecotoxicological evaluation.

1. Introduction

Methadone is a synthetic opioid with analgesic activity. It is commonly used to treat addictions to opiates, especially to heroin, as well as in treatments of moderate to severe pain. After consumption, MTHD is excreted either in its original or metabolized form. The major excretion products include the parent compound itself, 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP; major human metabolite) and 2-ethyl-5-methyl-3,3- diphenyl-1-pyrroline (EMDP; minor human metabolite), with large individual variations in excretion percentages (e.g. Preston et al., 2003; Kreek et al., 1983; Baselt, 2008). Based on the available published data, the average EDDP/MTHD ratios in urine and untreated wastewater were estimated to be 2.06 and 1.97, respectively (Thai et al., 2016). Like many other pharmaceuticals, MTHD is a widely present water contaminant, whose removal in wastewater treatment plants (WWTPs) has been reported to be rather low (e.g. Boleda et al., 2009; Terzic et al., 2010). Consequently, it is rather ubiquitous contaminant which is frequently detected in wastewater treatment plant effluents (from several ng/L to several hundred ng/L) (e.g. Berset et al., 2010, Bijlsma et al., 2012., Boleda et al., 2009; Castiglioni and Zuccato, 2010; Terzic et al., 2010; Krizman et al., 2016; Cosenza et al., 2018), surface waters (from several ng/L to several tens ng/L) (e.g. Baker and Kasprzyk-Hordern, 2011; Berset et al., 2010; Castiglioni and Zuccato, 2010; Mastroianni et al., 2016; Mendoza et al., 2014) as well as in tap water (from < 1 ng/L to several ng/L) (Boleda et al., 2009; Mendoza et al., 2016). Moreover, it was shown that WWTPs, receiving substantial inflows from pharmaceutical formulation facilities, can become significant hot-spots with dramatically enhanced opioid concentrations (Phillips et al., 2010). Consequently, there is a need to improve the knowledge on the approaches suitable for the reduction of MTHD environmental concentrations to mitigate the potential environmental risks associated with the exposure to MTHD. However, it should not be neglected that both abiotic and biotic removal of parent compounds may potentially be

78 associated with the formation of different transformation products (TPs), some of which
79 might be characterized by rather high persistence and/or unfavorable ecotoxicological
80 properties (Escher and Fenner, 2011). Abiotic transformations of MTHD have been studied
81 by several research groups (e.g. Gonzalez-Marino et al., 2015; Hsieh et al., 2018; Postigo et
82 al., 2011), who reported a prominent EDDP formation either in water chlorination as well as
83 in photodegradation experiments. By contrast, the knowledge on MTHD biodegradability as
84 well as its biotransformation products is still rather limited. Some of the model in-sewer
85 stability experiments indicated rather high in-sewer stability of MTHD (van Nuijs et al., 2012;
86 Senta et al., 2014), whereas some other studies (Ramin et al., 2016; Gao et al., 2017) indicated
87 rather efficient elimination of MTHD in the rising main sewer and gravity sewer. The latter
88 studies, however, were focused exclusively on the parent compounds, while the mechanisms
89 included in MTHD removal remained unknown.

90 The aim of the present study was, therefore, to study the ability of the activated sludge culture
91 adapted to high concentration of MTHD (10 mg/L) to degrade MTHD under aerobic
92 conditions. The study included growth of a mixed microbial culture in the laboratory
93 conditions in the presence of MTHD, its taxonomic characterization, determination of the
94 removal kinetics of the parent compound at elevated concentration typical of pharmaceutical
95 formulation facilities, identification of biotransformation products (TPs) and ecotoxicological
96 evaluation of the biotransformation.

97

98 **2. Materials and methods**

99 ***2.1. Chemicals and reagents***

100 Methadone (MTHD) and 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) as well
101 as their deuterated analogues (MTHD-d3 and EDDP-d3) were purchased from Lipomed

(Arlesheim, Switzerland), whereas 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) was obtained from Sigma-Aldrich (Steinheim, Germany). The purity of the reference materials used for confirmatory purposes was ≥ 98%. Chemicals used for growth media were of analytical grade purity while those for molecular analyses were of molecular grade and were supplied by Sigma-Aldrich (USA) and Kemika (Zagreb, Croatia). Difco LB agar was obtained from BD (USA). Ammonium chloride (purity > 99.5%) was purchased from Gram-mol (Zagreb, Croatia). Formic acid (LC-MS grade) and ammonium formate (purity ≥ 99%) were purchased from Sigma-Aldrich. All other chemicals used for biodegradation media were of analytical grade purity and supplied by Kemika (Zagreb, Croatia). LC-MS grade solvents (acetonitrile and methanol) were products of J.T. Baker (Deventer, the Netherlands). 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). The individual stock solutions (10 mg/mL and 1 mg/mL) of MTHD were prepared in LC-MS grade methanol.

2.2. Selection and preparation of microbial cultures for preliminary biodegradation experiments

Four activated sludge cultures (AS-1, AS-2, AS-3 and AS-4) were selected for preliminary experiments of MTHD degradation (Table S3). The cultures AS-1, AS-2 and AS-3 originated from the aeration tank of the Central wastewater treatment plant of the city of Zagreb and were previously enriched on three different amine-moiety containing compounds: azithromycin (10 mg/L), tramadol (20 mg/L) and MTHD (10 mg/L), respectively. The cultures AS-1 and AS-2 were previously proven to be able to degrade macrolide antibiotics (Terzic et al., 2018) and tramadol (unpublished data), respectively. Before being used in

preliminary MTHD biodegradation experiments, the culture AS-1 was adapted to high concentrations of MTHD (10 mg/L) for 9 months, whereas tramadol-degrading culture AS-2 had no prior MTHD adaptation period. The microbial culture AS-4 was collected from the aeration tank of a small membrane bioreactor used for treatment of hospital wastewaters and enriched on MTHD (10 mg/L) for 10 months.

For both adaptations and enrichments, all activated sludge cultures were grown in phosphate-buffer minimal salts medium pH 7.3 (MSM) (Petric et al., 2007) with details on its composition provided in the Supplementary material. Different combinations and ratios of organic carbon (glucose) and nitrogen (NH_4Cl) source were used during growth experiments with the aim to test ability of sludge cultures to grow on MTHD used as a sole organic carbon or nitrogen source or to grow cometabolically and included: (i) addition of glucose (500 mg/L), (ii) addition of NH_4Cl (100 mg/L), (iii) addition of glucose and NH_4Cl (C/N=10:1) and (iv) addition of glucose and NH_4Cl (C/N=18:1) in the MSM. During both adaptation and enrichment growth activated sludge cultures were every 7-10 days transferred (10% of the culture) into fresh MSM supplemented with MTHD (10 mg/L) and flasks were shaken on a rotary shaker (180 rpm, 30^{o}C). MTHD, dissolved in methanol, was added into empty flasks, evaporated under sterile hood after which MSM and supplements were added to the flasks. Only those cultures showing turbidity, i.e. changes in their optical density (OD) during growth, were selected for further preliminary MTHD degradation experiments.

2.3. Methadone biodegradation experiments and culture growth

2.3.1. Inoculum and media preparation for biodegradation experiments

Inoculum for all biodegradation experiments was prepared by growing activated sludge cultures in MSM supplemented with MTHD (10 mg/L), NH_4Cl (100 mg/L) and glucose (500 mg/L) for 3 days on a rotary shaker (180 rpm, 30^{o}C). Cultures were pelleted by centrifugation

(8944 g) for 5 min and cells were resuspended in fresh MSM. In the preliminary MTHD biodegradation experiments, the inoculum was prepared at the final concentration of 5 g/L (wet weight) and for the main MTHD biodegradation experiments, the final concentration of inoculum was increased to 10 g/L (wet weight).

2.3.1.1. Preliminary MTHD biodegradation experiments

Inoculum prepared as indicated above was added into Erlenmeyer flasks containing 30 mL of MSM, supplemented with MTHD (10 mg/L), glucose (added as C source) and NH_4Cl (added as N source). Preliminary experiments included four activated sludge cultures (Table S3). Abiotic control experiments (without the addition of bacterial cells) were performed as well. Cultures prepared in this way were incubated for 13 days on a rotary shaker (180 rpm, 30°C). Culture samples and controls (1 ml) were collected at day 0 and day 13, centrifuged (8944 g, 5 min), and recovered supernatant was used for preliminary analyses in order to gather information necessary for the optimization of the main experiments.

2.3.1.2. MTHD biodegradation experiments with selected AS-2 culture

Based on the results of the preliminary experiments, the most active culture AS-2 was selected for all further MTHD biodegradation experiments, which were conducted in 45 ml of the MSM, supplemented with MTHD (10 mg/L) and NH_4Cl (100 mg/L).

In order to determine the relative importance of cometabolic conditions, the experiments were performed in two ways: i) with (w/) and ii) without (w/o) addition of glucose (500 mg/L). These biodegradation experiments were performed in duplicate.

In order to test the importance of nitrogen availability, an additional separate biodegradation experiment was carried out at cometabolic conditions (glucose-containing media), in two

inoculated Erlenmeyer flasks. On the 2nd day of this experiment, one of the flasks was subjected to an additional N-supplementation (100 mg/L of NH_4Cl).

All biodegradation experiments included abiotic controls performed in uninoculated flasks.

During 14-day biodegradation experiments conducted on a rotary shaker (180 rpm, 30°C), samples were collected with the following dynamics: (i) for chemical analyses and OD measurements (1 mL) at days 0; 1; 2; 3; 4; 6; 7; 9; 11 and 14; (ii) for molecular-based analyses (1 mL) at days 0; 3; 4 and 7 (iii) for ecotoxicological analyses (5 mL) at days 0 and 14. For chemical and ecotoxicological analyses supernatant was collected after centrifugation (8944 g), whereas the pellet obtained after centrifugation was stored at -20°C and subsequently used for DNA extractions.

Changes in biomass concentration during MTHD biodegradation experiments were followed by measuring changes in OD, which was determined spectrophotometrically at 600 nm, in 1 mL aliquots .

2.4. DNA extraction, 16S rRNA amplicon sequencing and analysis

Total genomic DNA was extracted from the bacterial cultures collected at days 0 and 4 after beginning of the biodegradation experiment by using Nucleospin Microbial DNA extraction kit (Macherey-Nagel, Germany) following manufacturer instructions. Details on the extraction are given as a Supplementary material.

Extracted DNA samples were sent for 16S rRNA library preparation and Illumina MiSeq sequencing to MR DNA (www.mrdnalab.com, Shallowater, TX, USA). 16S rRNA gene V4 variable region was amplified in the samples by using primers 515/806 with barcode on the forward primer in a single-step 30 cycle PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) with PCR program included in the Supplementary material. Multiple samples

were pooled together (e.g., 100 samples), purified using calibrated Ampure XP beads and used to prepare illumina DNA library.

Sequencing was performed on a MiSeq platform following the manufacturer's guidelines (MR DNA; www.mrdnalab.com, Shallowater, TX, USA) and the obtained data were processed using a proprietary analysis pipeline (MR DNA, Shallowater, TX, USA). OTUs were taxonomically classified using BLASTn against a database derived from RDPII; http://rdp.cme.msu.edu) and NCBI (www.ncbi.nlm.nih.gov). All sequence data are currently being deposited in the National Centre for Biotechnology Information Sequence Read Archives (SRA).

2.5. Kinetics analysis

The degradation kinetics was modelled for the first-order kinetics as follows:

$$c\,(t) = c_0 * e^{(-kt)}$$

where c_0 is the initial concentration of MTHD at time zero, $c(t)$ is the concentration of the MTHD at time t, k is the degradation rate constant (d^{-1}) and t is the degradation period in days. Degradation rate constants were estimated by performing a nonlinear least squares regression analysis. Goodness of fit was assessed using the fitting value r^2.

The biodegradation half-life ($t_{1/2}$) was calculated using a following equation:

$$t_{1/2} = \ln 2/k$$

2.6 ***Analyses of methadone and its transformation products***

Before LC-MS analyses the collected samples (1mL) were centrifuged (8944 g) and diluted 5 times in 50 mM ammonium formate. The analysis was performed using ultrahigh-performance liquid chromatography (UPLC) coupled to quadrupole-time-of-flight mass spectrometry (QTOFMS). UPLC 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 x 2.1 mm) filled with a 1.7 µm BEH C_{18} stationary phase (Waters Corp., Milford, MA, USA). Binary gradients at a flow rate of 0.4 mL/min were applied for the elution. In the positive ionization (PI) mode the eluents A and B were 0.1% HCOOH in water and 0.1% HCOOH in acetonitrile, respectively. The eluents used in the negative ionization (NI) mode consisted of (A) water and (B) acetonitrile. The analyses in both polarity modes were performed by applying the following gradient: the elution started at 5% B and after a 1 min of isocratic hold, the percentage of B was linearly increased to 50% in 8 min.

The mass spectrometry was performed on a QTOF Premier instrument (Waters Micromass, Manchester, UK) using an orthogonal Z-spray-electrospray interface. Nitrogen was used as a drying gas and nebulizing gas, whereas argon was used as a collision gas in MS-MS experiments. The desolvation gas flow was set to 700 L/h at a temperature of 300°C. The cone gas flow was adjusted to 25 L/h, and the source temperature to 120°C. The capillary voltages in the PI and NI mode were 3500 V and 3000 V, respectively, whereas the cone voltage in both modes was set to 30 V. The MS data were collected between *m*/*z* 50 and *m*/*z* 1000, applying a collision energy of 4 eV.

All spectra were recorded using the extended dynamic range (DRE) option to correct for possible peak saturations, and the data were collected in the centroid mode with a scan time of 0.08 s and an interscan time of 0.02 s. To ensure maximum accuracy and reproducibility of the system, all acquisitions were performed using an independent reference spray via the lock spray interface. Leucine enkephaline was applied as a reference mass both in PI and NI mode. Targeted MS2 experiments were performed in order to identify MTHD TPs.

The data were processed using the MassLynx software incorporated in the instrument. The quantification of the parent compounds was performed by using the external calibration curves. The reference standards used for the qualitative and quantitative LC-MS analyses

were prepared in 50 mM ammonium formate in the concentration range of 0.02 - 2.5 µg/mL. The analytical quality assurance data are given in Supplementary Material (Table S1 and S2). The instrument performance was checked by running quality check (QC) analyses after every 10-12 injections. When reference standards were not available (MTHD TP 324), a semiquantitative estimates were performed by assuming the same molar response of the TP and the parent compound.

2.7. *Chronic toxicity*

2.7.1. Sample preparation

To eliminate the salts contained in the medium used for biodegradation studies, the samples for the evaluation of algal toxicity were previously percolated through Oasis HLB columns. The sample (5 mL) was percolated through the extraction cartridges previously preconditioned with 3 mL of methanol, ultrapure water and spring water. After the sample enrichment, the residual salts were washed out from the cartridge with 3 mL of ultrapure water and discarded, while the adsorbed analytes were eluted with 2 mL of methanol by applying a gravity flow. The methanol was evaporated, and the dry residue was re-dissolved in 0.5 mL of the ISO/FDIS 8692 culture medium. The recovery and precision of the applied sample preparation procedure were 69% and 6.9%, respectively.

2.7.2. Chronic toxicity evaluation

Chronic toxicity of samples was evaluated using the freshwater green algae *Desmodesmus subspicatus* (86.81 SAG) grown in ISO/FDIS 8692 culture medium, as described in detail in ISO (2004). The test was conducted in 96 microwell plates as described previously (Blaise et al., 1986; Smital et al., 2011) with slight modifications (for details see Supplementary Material). The average specific growth rate was calculated and subsequently used to calculate

the inhibition, and then fitted to a three-parameter sigmoid dose–response equation. The dose–response curve of $K_2Cr_2O_7$ was included as a reference standard in all experiments.

3. Results and discussion

3.1. Selection of microbial culture for methadone biodegradation experiments

Four activated sludge cultures (Table S3) were preliminarily tested for their capability to degrade elevated concentrations of MTHD (10 mg/L) at aerobic conditions. The experiments were performed by applying 2 different carbon to nitrogen ratios (C:N = 10 and C:N = 18). Only 1 out of 4 tested sludge cultures exhibited capability to partially degrade MTHD (Fig. S1). The main criterium for the assessment of degradation capability of the tested sludge cultures was loss of MTHD with concurrent formation of transformation products. The active microbial culture AS-2 removed 33% of MTHD in 13 days, whereas the concentrations of MTHD remained virtually unchanged in the experiments performed with the remaining 3 microbial cultures (AS-1, AS-3, AS-4). Slightly negative removal figures obtained for some of these cultures, probably can be attributed to evaporation losses (about 6%) through the cotton plug. Regarding different C:N ratios, the highest MTHD removal (33%) was obtained at C:N ratio of 10. Consequently, all further biodegradation experiments were performed with the culture AS-2 in the medium having C:N ratio of 10. It should be stressed that the enrichment procedure was essential for the successful degradation of MTHD, since the original activated sludge from WWTP of the city of Zagreb was found to be virtually inactive. The experiments indicated that the enrichment of an active microbial culture was rather time consuming and it is possible that a prolonged (>10 months) enrichment of microbial cultures AS-3 or AS-4 would also eventually result in their ability to degrade/transform methadone.

3.2. Biodegradation of methadone by selected sludge culture

3.2.1. Biodegradation of methadone in the media with and without additional organic carbon source

The sludge culture AS-2 (see Table S3) selected in the initial biodegradation experiment was adapted to MTHD (10 mg/L) over a period of approximately 4 months before being used in study of biodegradation efficiency and removal kinetics of MTHD. The experiment was performed in MSM containing NH_4Cl (100 mg/L) in the presence (500 mg/L; cometabolic conditions) and in the absence of glucose. The MTHD removal curves as well as the abiotic control curves are presented in Fig. 1. At cometabolic conditions (glucose-containing biodegradation medium), the enriched microbial culture AS-2 exhibited improved MTHD removal efficiency of 70% in 14 days, whereas its concentration in the abiotic control was rather stabile. Based on the K_d value of 230 L/kg, determined for the activated sludge in WWTP of the city of Zagreb, and the residual concentration of MTHD in the final medium (2.4 mg/L), the removal of MTHD by adsorption was estimated at 11 %. Moreover, the possible impact of photo-transformation (Postigo et al., 2011) was excluded by performing the experiments in the dark. Consequently, the MTHD removal determined under cometabolic conditions was primarily attributed to biotransformation processes (82% of the total). This conclusion was additionally supported by the concomitant formation of TPs. The removal efficiency achieved in the medium containing MTHD (c_0 = 10 mg/L) as a sole external organic C source, was much lower (9% in 14 days).

The removal curves of MTHD (Fig. 1) were fitted with the first-order kinetic model (Table 1). The removal kinetics of MTHD was much faster at cometabolic conditions ($t_{1/2}$ = 4.1 days) then when MTHD was used as a sole organic carbon source ($t_{1/2}$ = 11.4 days). This probably can be related to a higher initial biomass growth achieved in the presence of glucose as an additional labile carbon source.

3.2.2. Impact of additional nitrogen supplementation on biomass growth and methadone biodegradation

A possible role of nitrogen (N) defficiency in the decrease of MTHD degradation rate, which was observed after the second day of the biodegradation experiment performed at cometabolic conditions (see Fig. 1A), was investigated in an additional separate experiment, by comparing the degradation kinetics and biomass growth achieved with and without additional nitrogen supplementation. MTHD removal curves as well as biomass growth (presented as changes of optical density) determined in this experiment are presented in Fig. 2. As before, no pronounced changes were observed in the abiotic control throughout the experiment, whereas the microbial culture AS-2 confirmed the ability to lower MTHD concentration, without and with additional N-supplementation. However, much faster MTHD removal kinetics (Table 1) and higher removal efficiency (Fig. 2) was obtained in the flask receiving the additional N-supplementation ($t_{1/2}$ = 1.5 days; 93% removal efficiency) than in the flask without the additional N-supplementation ($t_{1/2}$ = 5.8 days; 63% removal efficiency). The additional N-supplementation on the 2nd day of the experiment was associated with an additional biomass growth on following day (Fig. 2B), whereas the biomass concentration in the flask without additional N-supplementation remained rather stable after the 2nd day of the experiment. This indicated that a fast N-depletion during the first two days of the experiments was probably the reason of the incomplete MTHD elimination in the experiments without additional N-supplementation.

3.2.3. ***Taxonomic characterization of the microbial culture AS-2 capable of MTHD biodegradation***

Phylogenetic structure of the adapted sludge culture AS-2 and changes in its community composition occurring during MTHD biodegradation experiment were determined based on the amplicon sequencing of the V4 variable region of the 16SrRNA gene marker. Sequencing depth was shown to be around 30 000 reads per sample, being sufficient for adequate description of AS-2 culture community. Rarefaction curve shown to reach asymptote, confirming that the read depth was sufficient, given these taxonomic classifiers.

Analyzed samples included culture collected at Day 0, representing inoculum AS-2 culture, and culture collected at Day 4, representing time point with highest observed MTHD transformation activity (experiment performed in cometabolic conditions with the additional N supplementation). Sequencing results, presented in the Fig. 3, provided high-resolution analysis of AS-2 microbial community composition, up to the taxonomic level of bacterial species. Both species richness estimates (Chao1), diversity indices (Shannon and Simpson) (Table S4) and taxonomic identification indicated changes in both diversity and structure of the adapted sludge culture AS-2 during the biodegradation experiment. Culture AS-2 analyzed at Day 0 had lower Chao1, Shannon and Simpson values when compared to the one taken at Day 4 (694.125 vs. 1027.018; 3.59 vs. 4.28; 0.738 vs. 0.848), indicating an increase in the diversity within the community 4 days after the beginning of the biodegradation experiment. At this time point purple nonsulfur bacteria *Rhodoplanes* and two *Bacteroidetes/Chlorobi* group members, *Flavobacterium* and *Pedobacter*, being characterized as proficient biopolymer-degrading chemoorganotrophs, were shown to appear as new community members, however, were represented in low numbers (1% of the total community). All three bacterial genera are commonly isolated from the activated sludge (Liang et al., 2010; Liu et al., 2018, Zhang et al., 2017). At highest taxonomic level of phyla (Fig. 3A) inoculum culture at Day 0 was characterized by the dominance of *Firmicutes* (representing cca. 50% of the total community), comprised solely by the members of class

374 *Bacilli*, and followed by different members of *Proteobacteria* (30%) and *Bacteroidetes* (20%)
375 phylum. At the Day 4, switch in the community was observed with the enrichment of
376 *Gammaproteobacteria, Betaproteobacteria* and *Actinobacteria* representing more than 40%
377 of the total bacterial community.
378 Taxonomic affiliations obtained at the level of genera (Fig. 3B, Table S5) gave more clear
379 indications on the changes observed at the higher taxonomy level. Inoculum culture AS-2 was
380 dominated by several members of the *Bacillus* genera with majority of sequences identified as
381 *B. megaterium* and *B. flexus*. Their presence in the adapted sludge culture can be explained by
382 their stability (spore formation), easily preparation and antagonistic effects on pathogens
383 (Hong et al., 2005) which lead to the wide use of different *Bacillus* species in wastewater
384 facility inoculums. At day 4, when high MTHD transformation activity was achieved,
385 *Bacillus* members were outcompeted by *Mycobacterium (Actinobacteria), Methylobacillus*
386 *(Betaproteobacteria), Enterobacter* and *Pseudomonas (Gammaproteobacteria)* species.
387 *Mycobacterium pulveris, Methylobacillus flagellatus* and *Pseudomonas (P. plecoglossicida,*
388 *P. putida, P. sp.),* lowly represented within the inoculum culture (avg. 2%), at Day 4
389 triplicated in their abundance. Even though there has been no research into methadone effect
390 on microorganisms, enrichment with the observed populations combined with
391 ecotoxicological assessment data, could indicate tolerance of these species to MTHD
392 metabolite EDDP found in high concentration at this time point. This, in combination with the
393 low abundance of these populations in the experiment, showing low MTHD biodegradation
394 activity (data not shown), further implied their possible role in the biotransformation of
395 MTHD. With no information available on biodegradation of MTHD, and with results only
396 based on the DNA analysis, it is difficult to define AS-2 community member responsible for
397 the observed MTHD transformation. However, bacterial strains identified as *Pseudomonas*
398 and *Methylobacillus* are known to degrade an array of different environmental pollutants (e.g.

Lister at al., 1999; Mardal et al., 2017). *Pseudomonas* with vast metabolic versatility are those species most often studied for their bioremediation capabilities (Kahlon et al., 2016). Different *Methylobacillus* strains were identified as degraders of different contaminants, including the amine-moiety containing pesticide carbofuran (Hanson and Hanson., 1996, Topp et al., 1993). However, to our knowledge, *Methylobacillus* or *Pseudomonas* strains have not previously been linked to MTHD biodegradation. Since this experiment included likewise additional N supply, growth of specific bacterial groups could be likewise linked to nitrification. No presence of autotrophic nitrifiers (*Nitrosomonas, Nitrospira, Nitrobacter*) indicated that oxidation of ammonium during the experiment was probably conducted by heterotrophs, such as *Pseudomonas* sp., previously found to conduct heterotrophic nitrification in laboratory-scale aerobic experiments (Cydzik-Kwiatkowska et al., 2015). Role in the N cycling could be likewise correlated to the *Bacillus* strains, known to possess good nitrogen removal properties (Rout et al., 2017). Results implied the importance of further studies on the possible inhibitory effects of MTHD and its metabolites on microbially-mediated biogeochemical processes. At the end, in addition to their possible ecological roles, presence of *Enterobacter hormaechei* and *Mycobacterium pulveris*, indicated that clinically and environmentally relevant bacterial strains are surviving in the treated municipal wastewater (Amha et al., 2017).

3.2.4. Biotransformation products of methadone

Microbial elimination of MTHD was associated with the formation of 3 different transformation products (TPs). Figures S2 and S3 in the Supplementary Material show the corresponding total ion chromatograms of the biodegradation media at the beginning and at the end of MTHD biodegradation acquired in the PI and NI mode. All 3 identified MTHD TPs were detected in PI mode (Table 2, Fig. S4 and S5) whereas no additional TPs were

424 detected in the NI mode. The full list of identified TPs as well as their retention time,
425 elemental composition, theoretical *m*/*z* values, mass errors and chemical structures are
426 presented in Table 2. The structural identification of the detected TPs was performed based on
427 the elucidation of the chromatographic and accurate mass-spectrometric data as well as MS2
428 experiments, and when possible, by confirmation with reference standards. The identification
429 confidence reported in Table 2 followed the five-level system proposed by Schymanski et al.
430 (2014). The identification of 2 out of 3 identified MTHD TPs (EDDP and EMDP) was
431 achieved at the confidence level 1 (confirmation with reference materials) whereas confidence
432 level 2b (probable structure by diagnostic evidence: MS, MS2, experimental data) was
433 assigned to the proposed structure of MTHD TP 324. The comparison of the MS2 spectra of
434 MTHD TPs, which were identified as EDDP and EMDP, with the corresponding MS2 spectra
435 of reference materials is given in Supplementary Material (Fig. S6 and S7). EDDP is a MTHD
436 TP formed by N-demethylation and cyclization, whereas EMDP formation includes additional
437 N-demethylation of EDDP. To the best of our knowledge, this is the first evidence that EDDP
438 and EMDP, which are well-known human metabolites of MTHD, are also products of
439 microbial transformations. This finding is, however, in a good agreement with a report by
440 Gulde et al. (2016), who pointed out that microbial and mammalian transformation reactions
441 of a number of different amine-containing contaminants were fairly similar, suggesting that
442 predicted as well as experimental transformation data from the mammalian metabolism of
443 pharmaceuticals can be used for suspect screening approaches targeted at TPs in the
444 environment. These findings are in contrast with the studies of in-sewer stability of MTHD
445 (Ramin et al., 2016, Gao et al., 2017), which indicated the lack of the EDDP net formation
446 associated with the observed MTHD loss. In addition to EDDP and EMDP, a minor novel
447 product (MTHD TP 324) was also detected in the biotransformation experiments. The
448 production of the MTHD TP 324 (see Table 2), included a transformation which must have

449 occurred at a dimethylamino group, as indicated by MS2 spectra presented in Fig. S8. This TP
450 is most probably formed by α-C-oxidation to formamide (Gulde et al., 2016). The alternative
451 mechanism, which would include N-demethylation and subsequent N-formylation, seems to
452 be less likely due to the tendency of N-demethylated MTHD to be stabilized by cyclization to
453 EDDP. For the same reason, the alternative isobaric TP, formed by N-acetylation of doubly
454 N-demethylated MTHD, does not seem very likely. The proposed TP structure containing an
455 amide-group is additionally supported with the presence of the sodium adducts in the MS
456 spectra of MTHD TP 324 (Fig. 5; *m*/*z* 346.1783). Finally, the proposed structure is in a good
457 agreement with the transformation mechanisms involved in the biotransformation of amine-
458 containing micropollutants in activated sludge (Gulde et al., 2016).
459 The most prominent MTHD TP throughout the biodegradation experiment was EDDP, whose
460 formation was tightly related to MTHD removal (Fig. 4). In fact, EDDP represented 82-92%
461 of the overall MTHD TP concentration throughout the experiment, whereas the prevalence of
462 the remaining two MTHD TPs was much lower. Furthermore, rather constant summed-up
463 molar concentration of MTHD and its TPs throughout the performed biodegradation
464 experiment indicated rather high stability of the formed TPs, primarily EDDP.
465
466 **3.2.5.** ***Toxicity evaluation***
467 All performed biodegradation experiments were associated with the formation of stable TPs
468 and none of them achieved complete (> 99%) elimination of MTHD. Therefore, the
469 biodegradation media at the end of the individual biodegradation experiments, performed at
470 different experimental conditions, contained variable proportions of the parent compound
471 (MTHD) and individual TPs, which was reflected on the extent of algal growth inhibition in
472 these experiments. The removal of the parent compound was associated with a decrease in
473 algal toxicity. Moreover, rather good correlation (r^2 = 0.8895) was obtained between the

residual MTHD concentrations and the rate of algal growth (Fig. 5), indicating lower toxicity of MTHD biotransformation products as compared to MTHD itself. This result is fully supported by EC_{50} values determined in this study, which confirmed higher toxicity of MTHD (EC_{50} = 5.42 mg/L) as compared to EDDP (EC_{50} = 10.9 mg/L) and EMDP (EC_{50} = 15.5 mg/L). Consequently, the results obtained in this study confirmed a rather positive ecotoxicological outcome of the biotransformation processes. Similar outcome was reported by Postigo et al. (2011), who assessed photochemical degradation of MTHD using Vibrio fischery bioassay. Furthermore, several previous studies, carried out with several other amine-moiety containing xenobiotics, indicated that transformation on that site might result in a reduction of toxicity (Lange et al., 2006; Rusch et al., 2015; Terzic et al., 2018). Nevertheless, it should be noted that the ecotoxicological evaluation in our study was performed based on one selected endpoint, so we can not exclude the possibility that a rather different outcome might have been obtained using some other endpoints. For example, the preliminary ecotoxicological assessment performed with US EPA TEST software predicted 16 times higher EDDP than MTHD toxicity for Daphnia magna (Gonzalez-Marino et al., 2015). Although these findings were not experimentally confirmed, they, together with a rather high persistence of the formed TPs (see Fig. 4), warrant further ecotoxicological assessment.

4. Conclusions

One of the most important strategies for the reduction of the exposure to emerging contaminants via the aquatic route is their biodegradation. However, for contaminants like MTHD, which are not easy-to-degrade, this goal can be reached only by using enriched microbial cultures at appropriate experimental conditions. MTHD biotransformation is markedly enhanced in the presence of additional, more labile organic carbon source

(cometabolic conditions), whereas the degradation rate at cometabolic conditions strongly depends on nitrogen availability. Microbial degradation of MTHD is associated with a formation of rather persistent TPs, two of which are identical to its human metabolites, EDDP and EMDP. This confirms that microbial and mammalian transformations of amine-containing pharmaceuticals might be similar, which can be of use in suspect screening approaches of environmental samples (Gulde et al., 2016). The effect-driven evaluation of the biotransformation process, based on toxicity to algae, indicated a significant reduction of toxic effects, however the formation of stable TPs warrants further ecotoxicological assessment.

Conflict of interest

The authors of this study declare no conflict of interest.

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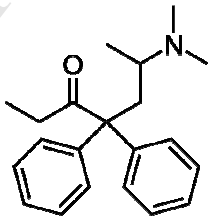
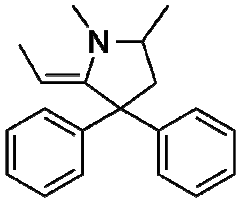
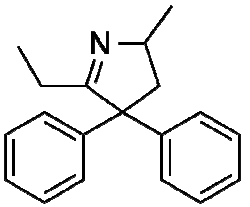
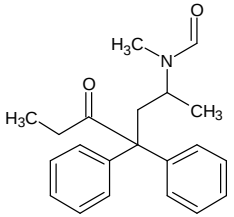
Table 1. Methadone (MTHD) biodegradation kinetic parameters achieved by using adapted sludge culture AS-2 by applying different experimental conditions. (w/o glucose - media containing MTHD as a single organic carbon source; w/ glucose – media containing glucose; w/ additional N supplementation - additional N supplementation on the 2nd day of the experiment.

	k (day^{-1})	$t_{1/2}$ (days)	r^2	Kinetic model
w/o glucose	0.061	11.4	0.8621	1st order kinetics
w/ glucose	0.169	4.1	0.9414	1st order kinetics
w/o additional N supplementation	0.119	5.8	0.974	1st order kinetics
w/ additional N supplementation	0.450	1.5	0.9338	1st order kinetics

Table 2. Chromatographic and mass spectrometric characteristics of methadone (MTHD) and its transformation products identified in biodegradation experiments performed with adapted sludge culture AS-2.

Compound	RT (min)	Elemental composition $[M+H]^+$	*m/z* theoretical	Error (mDa)	Structure	Level of confidence *
MTHD	6.33	$C_{21}H_{28}NO$	310.2171	- 0.3		NA
MTHD TP 278 EDDP	5.67	$C_{20}H_{24}N$	278.1909	+ 0.1		1
MTHD TP 264 EMDP	5.79	$C_{19}H_{22}N$	264.1752	+1.5		1
MTHD TP 324	8.19	$C_{21}H_{26}NO_2$	324.1964	-1.3		2b

*according to Schymanski et al., 2014; EDDP = 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine ; EMDP = and 2-ethyl-5-methyl-3,3- diphenyl-1-pyrroline; RT – retention time; NA – not applicable

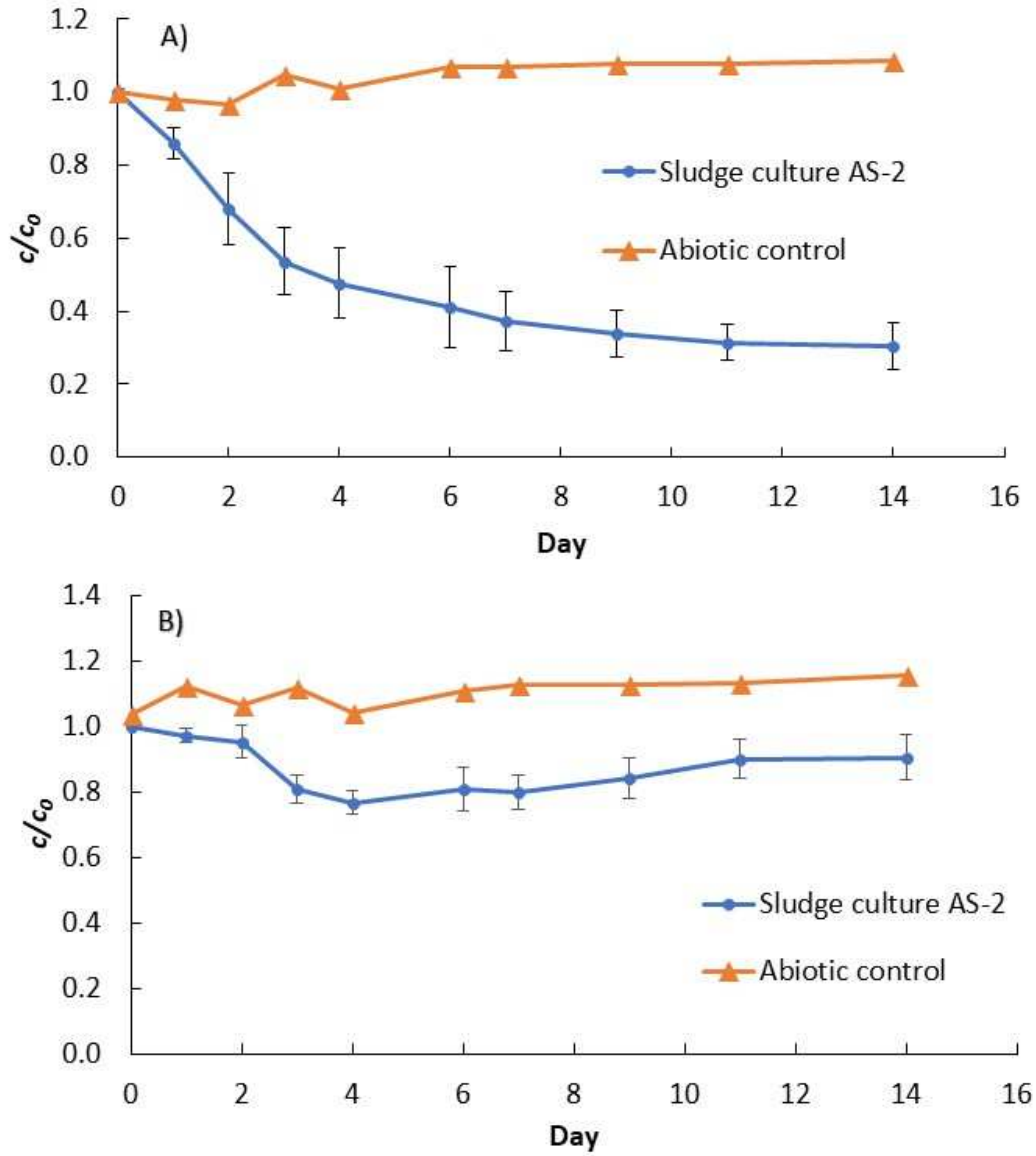


Fig. 1. Biodegradation kinetics of methadone (MTHD; c_0 = 10 mg/L) in model experiments performed by adapted sludge culture AS-2 in glucose-containing media (cometabolic conditions) (A) and in media containing MTHD as a sole organic carbon source (B).

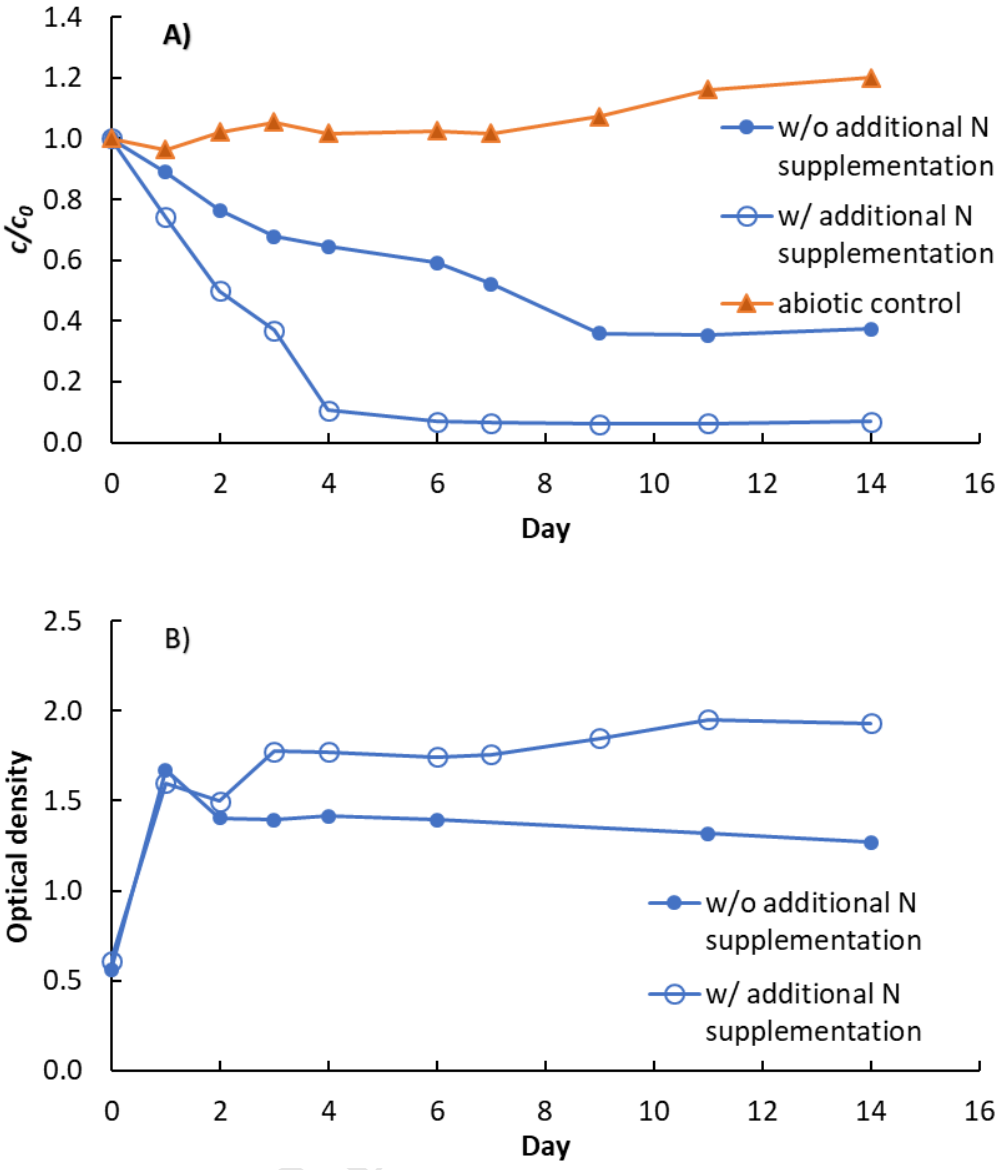


Fig. 2. Impact of additional nitrogen supplementation on biodegradation kinetics of methadone (MTHD; c_0 = 10 mg/L) by adapted sludge culture AS-2 in glucose-containing media (cometabolic conditions) (A) and on biomass concentration measured as optical density (B).
w/o – without; w/ - with

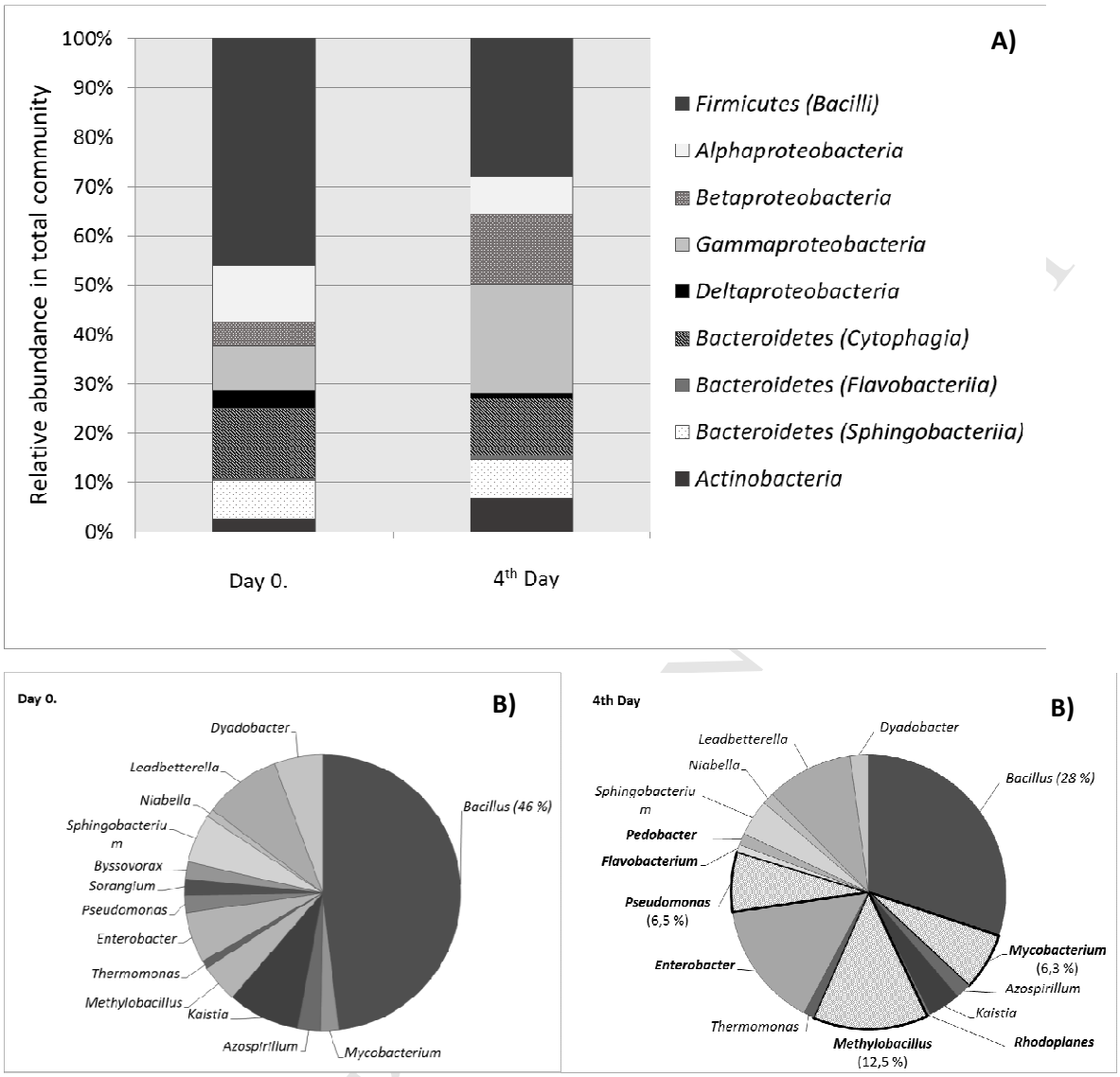


Fig. 3. Changes in the structure within the adapted sludge culture AS-2 during MTHD biodegradation experiment (at Day 0. and 4th Day) grown in glucose-containing media (cometabolically) as revealed by high throughput amplicon sequencing of the 16SrRNA gene marker represented at the higher level of bacterial phyla/classes (**A**) and at the level of major genera (with > 1% relative sequence contribution) (**B**)

* Genera with bolded names are enriched within the culture

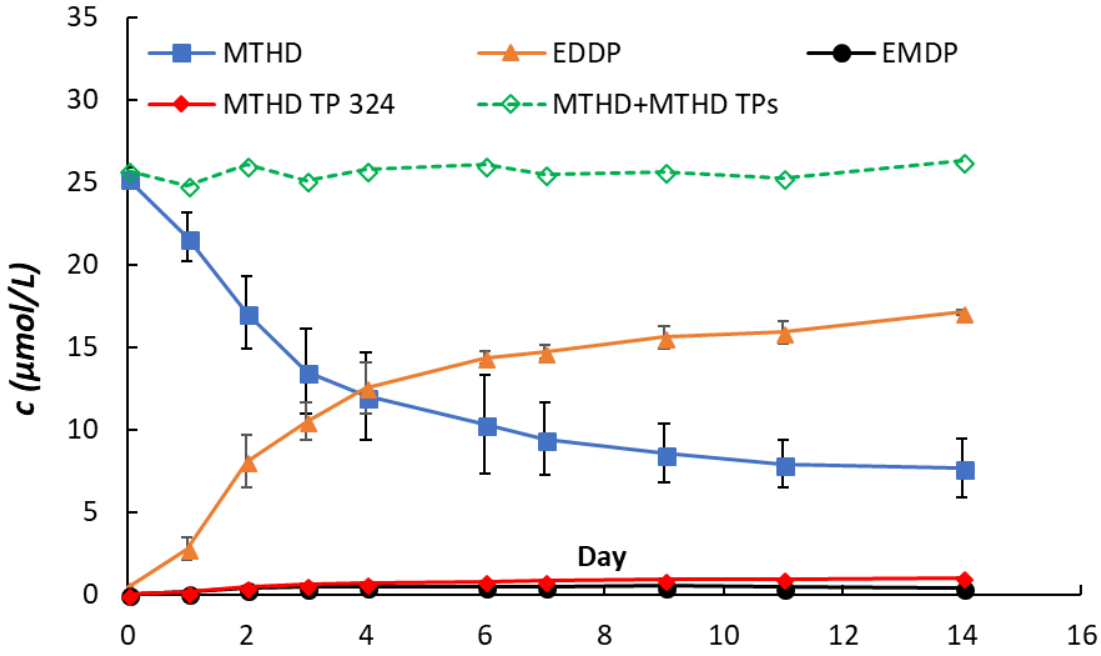


Fig. 4. Removal of methadone (MTHD), formation of transformation products (EDDP, EMDP, MTHD TP 324) and summed-up concentration of MTHD and its TPs (MTHD + MTHD TPs) in biodegradation experiment performed by adapted sludge culture AS-2 in glucose containing media (cometabolic conditions). EDDP = 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine; EMDP = 2-ethyl-5-methyl-3,3- diphenyl-1-pyrroline.

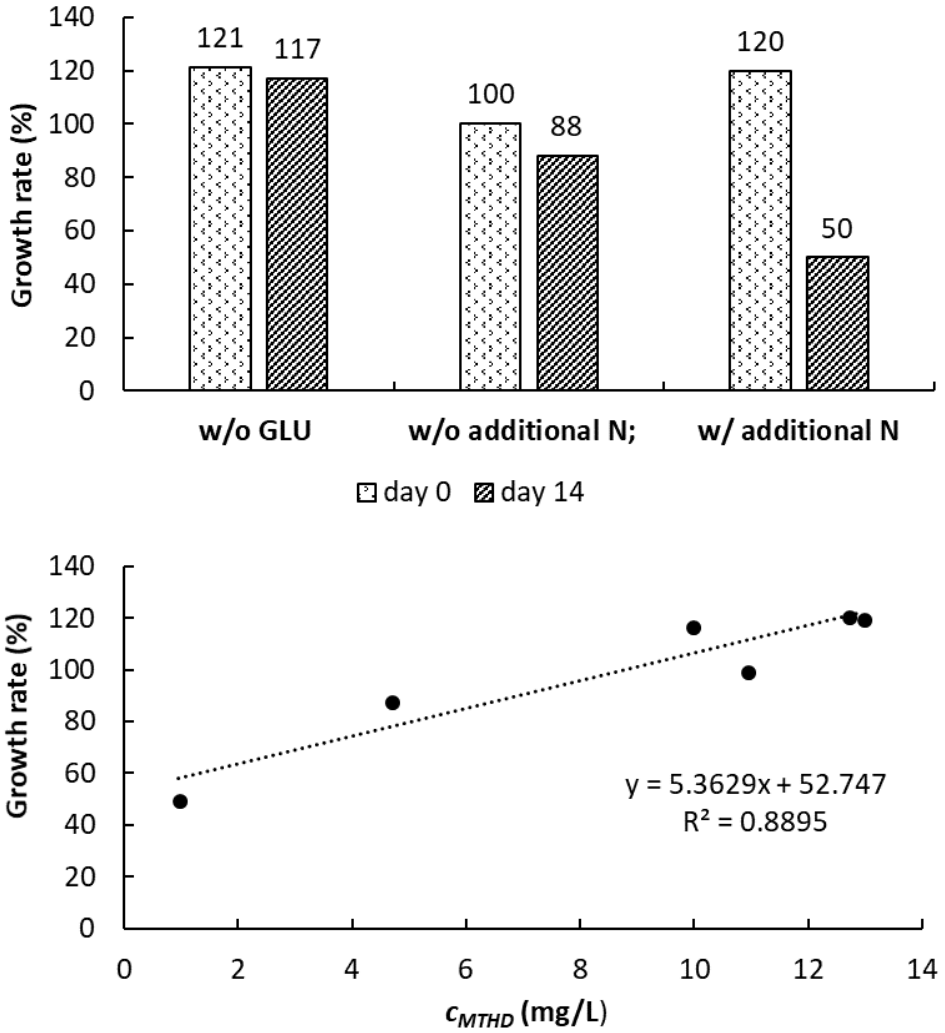


Fig. 5. Ecotoxicological evaluation of biotransformation of methadone (MTHD) as reflected by corresponding changes in algal toxicity. Test organism: *Desmodesmus subspicatus* (86.81 SAG).

Highlights

- Methadone degrading microbial culture was enriched from municipal activated sludge
- Three major transformation products were identified and quantified by UPLC/QTOF-MS
- Two transformation products were identical to human metabolites, EDDP and EMDP
- The changes in the enriched culture were followed using 16S rRNA gene sequencing
- Biotransformation of methadone reduced toxicity to algae