SUPPLEMENTARY MATERIAL

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

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Methodology details

Preparation of the phosphate-buffer minimal salts medium

Growth media composed of K_2HPO_4 (4.4 g/L), KH_2PO_4 (1.7 g/L) and yeast extract (50 mg/L) was sterilized by autoclaving (121^oC, 20 min.) after which it was supplemented with 10% salts medium. Filter sterilized (0.20 μm) 100x salts stock contained $MgSO_4x7H_2O$ (19.5 g/L), $MnSO_4x4H_20$ (6.6 g/L), $FeSO_4x7H_20$ (1 g/L), $CaCl_2x6H_20$ (0.447 g/L) and several drops of concentrated H_2SO_4 to prevent precipitation of basic salts. Depending of the experiment organic carbon source in the form of glucose and nitrogen source, in the form of NH_4Cl, were filter sterilized and added to the growth medium.

DNA extraction procedure, PCR amplifications and analysis details

Cultures were centrifuged (10 000 g, 10 min) and pellets were resuspended in the elution buffer. DNA was extracted from the harvested cells by combining chemical (addition of Lysis buffer and Proteinase K) and mechanical lysis steps (agitation for 12 min at max. speed, Vortex-Genie (MoBio). After centrifugation (11 000 g, 30 s) supernatant containing DNA was collected and transferred onto the Nucleospin Microbial DNA Column. DNA was purified by washing silica membrane with 2 different buffers following centrifugation steps (11 000 g, 30 s). DNA was eluted from the column after the addition of the elution buffer, incubation at room temperature (1 min) and subsequent centrifugation step (11 000 g, 30 s). Quality and quantity of the extracted DNA was assessed by NanoDrop spectrophotometer (BioSpec-nano, Shimadzu). PCR program used for amplification of the V4 16S rRNA region with primers 515/806 included: 94°C (for 3 min), 30 cycles (5 cycle used on PCR products) of 94°C (for 30 s), 53°C (for 40 s) and 72°C (for 1 min), after which a final elongation step at 72°C (5 min) was performed. After amplification,

PCR products are checked on 2% agarose gel of bands. After MiSeq platform analysis sequences were depleted of barcodes and primers, then sequences < 150 bp removed, sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were also removed. Sequences were denoised, OTUs generated and chimeras removed.

Table S1. The combinations of growth conditions tested in the enrichment period.

No	**Growth conditions**	**Yeast extract (mg/L)**	**Tramadol (mg/L)**	**Glucose (mg/L)**	**Glycerole (mg/L)**	**NH_4Cl (mg/L)**	**NH_4NO_3 (mg/L)**	**C:N***
1	Tramadol as C and N source	50	20	-	-	-	-	NA
2	Glucose as C source, Tramadol as N source	50	20	1000	-	-	-	NA
3	NH_4Cl as N source, Tramadol as C source	50	20	-	-	170	-	NA
4	Glucose as C source; NH_4Cl as N source	50	20	1150	-	1000	-	2:1
5	Glucose as C source; NH_4Cl as N source	50	20	500	-	200	-	4:1
6	Glucose as C source; NH_4Cl as N source	50	20	1000	-	100	-	18:1
7	Glucose as C source; NH_4Cl as N source	50	20	1000	-	170	-	10:1
8	Glycerol as C source; NH_4Cl as N source	50	20	-	600	100	-	10:1
9	Glucose as C source; NH_4NO_3 as N source	50	20	1000	-	-	130	10:1

*the initial C:N ratio established by supplementation of a minimal salt medium (MSM) with the selected carbon (glucose, glycerol) and nitrogen sources (NH_4Cl, NH_4NO_3).

Table S2. Data on tramadol (TRAM) removal and presence of transformation products (TPs) during the enrichment performed in the minimal mineral mediu (MSM) containing TRAM and different combinations of selected carbon (C) and nitrogen (N) sources.

	TRAM removal (%)					TP presence				
MINIMAL SALT MEDIUM (MSM) ammended with:	2 months	4 months	7 months	8 months	12 months	2 months	4 months	7 months	8 months	12 months
TRAM + NH_4Cl	<1	N/A	N/A	N/A	N/A	ND	N/A	N/A	N/A	N/A
TRAM + C (glu)	<1	N/A	N/A	N/A	N/A	ND	N/A	N/A	N/A	N/A
TRAM + [C (glu) + N (NH_4Cl)]; C: N = 2:1	<1	N/A	N/A	N/A	N/A	ND	N/A	N/A	N/A	N/A
TRAM + [C (glu) + N (NH_4Cl)]; C: N = 4:1	<1	<1	N/A	N/A	N/A	ND	ND	N/A	N/A	N/A
TRAM + [C (glu) + N (NH_4Cl)]; C: N = 10:1	<1	<1	33	60	83	ND	Trace*	D**	D**	D**
TRAM + [C (glu) + N (NH_4Cl)]; C: N = 18:1	<1	<1	N/A	N/A	N/A	ND	ND	N/A	N/A	N/A
TRAM + [C (gly) + N (NH_4Cl)]; C: N = 10:1	<1	<1	N/A	N/A	N/A	ND	ND	N/A	N/A	N/A
TRAM + [C (glu) + N (NH_4NO_3)]; C: N = 10:1	<1	N/A	N/A	N/A	N/A	ND	N/A	N/A	N/A	N/A

Glu – glucose; gly – glycerol, N/A – not applicable; ND – non-detectable; D - detectable; *- trace amount of N-desmethyl tramadol; ** detectable presence of all five TRAM TPs

Validation of the analytical protocol

The validation of the applied analytical protocol comprised determination of linearity range, method quantitation limit (MQL), instrumental precision, method precision, quality check (QC) variability and measurement accuracy.

Linearity of the response was determined from 8-point external standard calibration curves, constructed by injecting standard solutions containing analytes in the concentration range from 0.02 ng/µl to 2.5 ng/µl.

MQL was defined as the lowest point of the calibration curve.

Instrumental precision was assessed as a relative standard deviation of 4 replicate analyses of a standard solution.

Method precision was assessed as a relative standard deviation of 4 replicates prepared by aplying all steps included in the analytical protocol, including a sample centrifugation and dilution before the analysis.

QC variability was assessed as a relative standard deviation of QC sample (2 ng/µL) which was analysed after every 10-12 sample analyses.

Matrix effect (signal suppression or enhacement) was determined by comparing the average response (n = 4) of deuterated TRAM and N-DM-TRAM spiked (0.2 ng/mL) into the diluted sample (A_{SAMPLE}) collected at the beginning (t_0) and at the end (t_{end}) of the biodegradation experiment with their average response (A_{STD}; n = 4) in a matrix-free standard solution (0.2 ng/mL), as follows.

$$ME\ (\%) = (1 - A_{SAMPLE}/A_{STD}) * 100$$

The measurement accuracy was determined by comparing the average concentration determined in QC samples with a nominal concentration of 2 ng/µL.

The determined validation parameters are presented in the Table S3.

Table S3. Method validation parameters

	TRAM	**N-DM-TRAM**
Linearity (r^2)	0.9961	0.9991
MQL (mg L^{-1})	0.2	0.2
Instrumental precision (%)	1.3	1.7
Method precision (%)	0.9	1.8
QC variability (%)	1.5	1.9
Matrix effect t_0	3.3	ND
Matrix effect t_{end}	9.9	ND
Determination accuracy (%)	96.7	100.7

ND – not determined

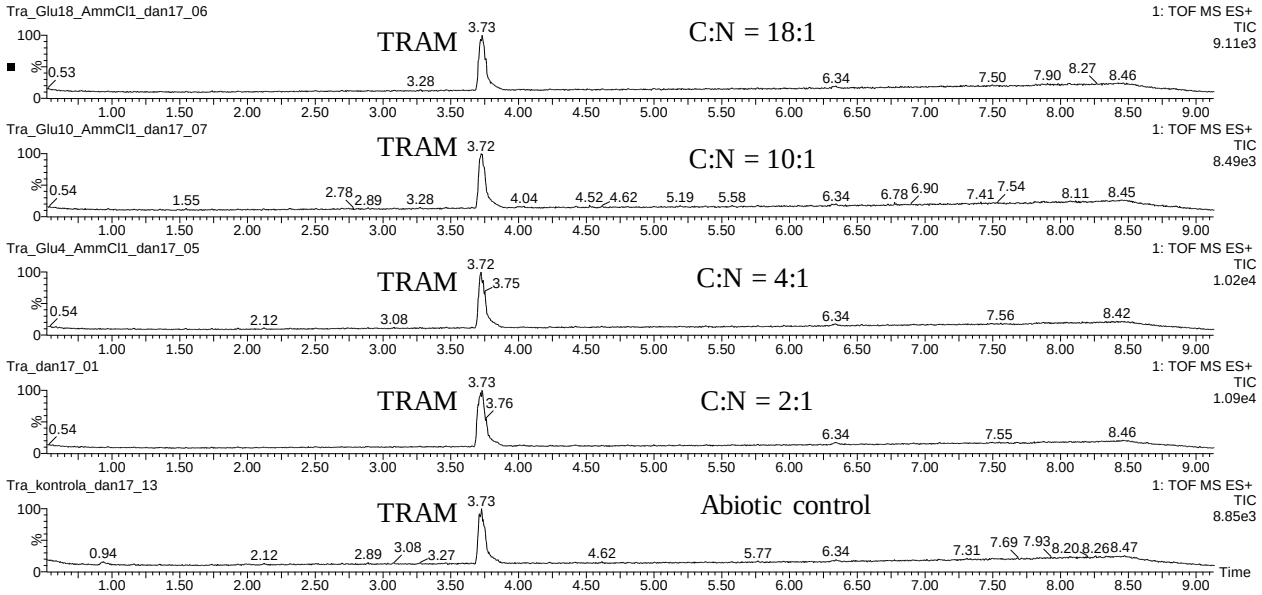

Fig. S1. Total ion chromatograms of the samples collected at the end of the preliminary biodegradation experiment (2 weeks) performed with the original sludge culture at four selected carbon (C) to nitrogen (N) ratios; C source - glucose; N source - ammonium chloride

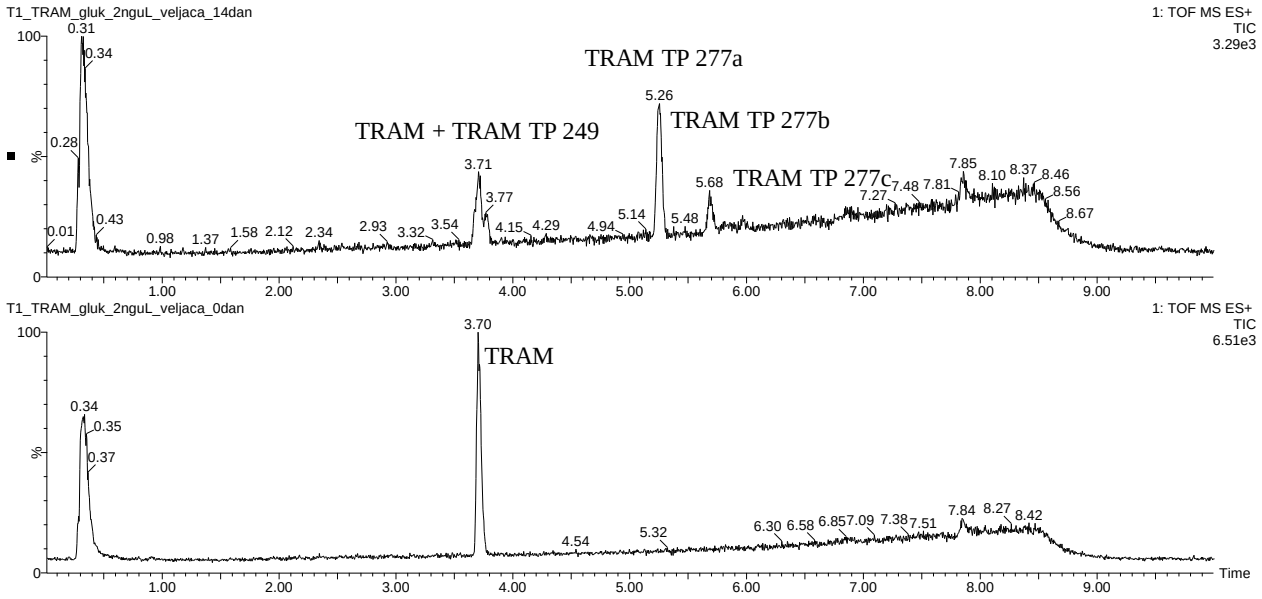

Fig. S2. Total ion chromatograms (TIC) of the samples collected at the beginning (bottom) and at the end (top) of the biodegradation experiment performed with an enriched sludge culture, acquired in a positive ionization (PI) mode. TRAM – tramadol; TP – transformation product

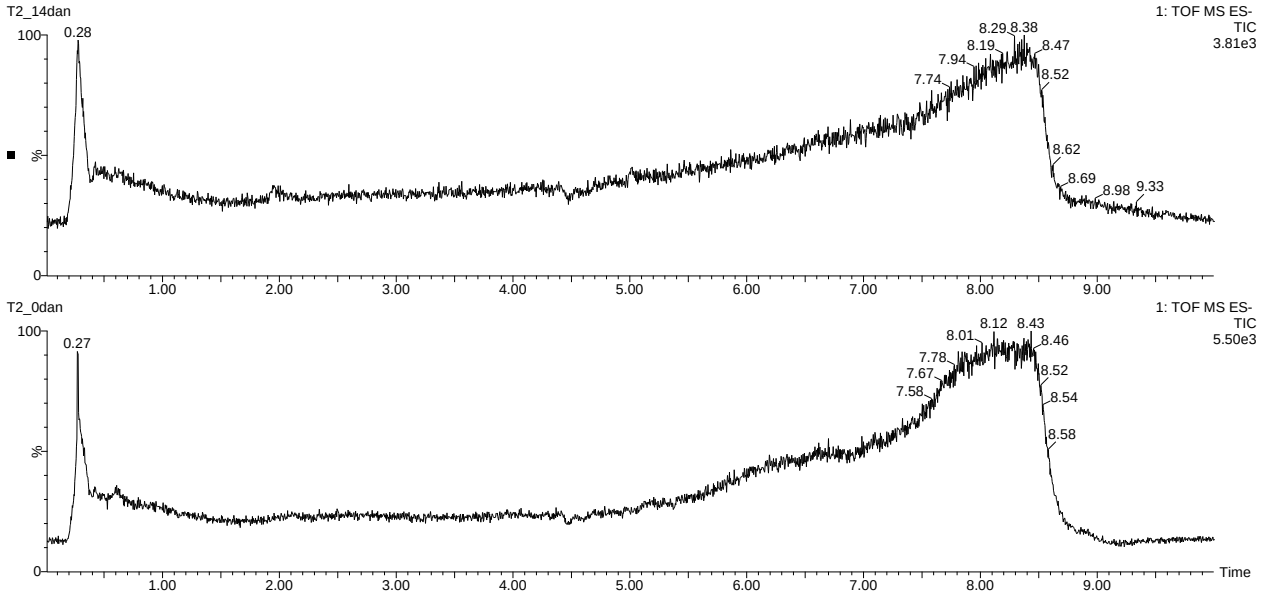

Fig. S3. Total ion chromatograms (TIC) of the samples collected at the beginning (bottom) and at the end (top) of the biodegradation experiment performed with an enriched sludge culture, acquired in a negative ionization (NI) mode.

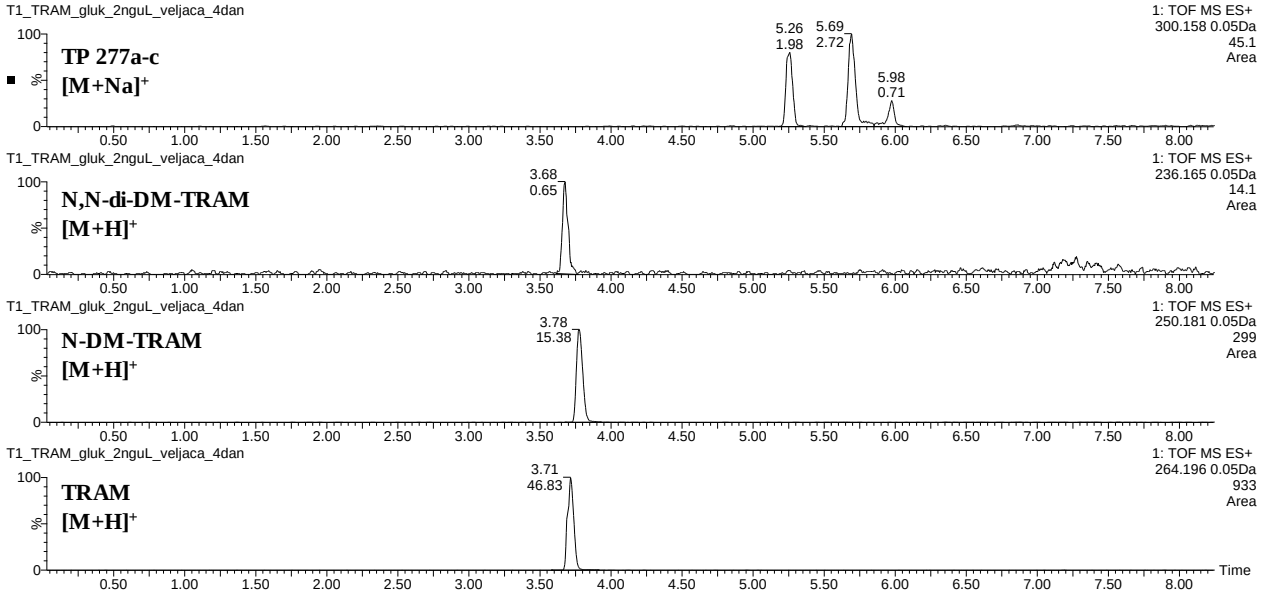

Fig. S4. Extracted ion chromatograms (XIC) of TRAM and its biotransformation products (TPs) of the sample from TRAM biodegradation experiment with glucose supplementation (day 4).

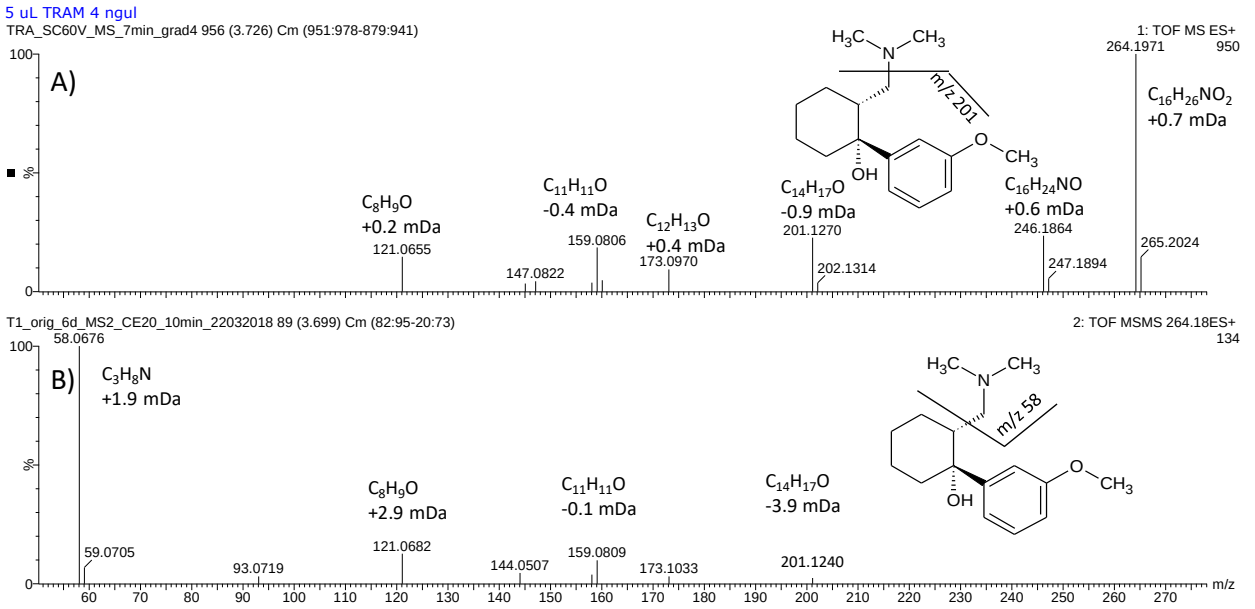

Fig. S5. Accurate mass spectra of tramadol (TRAM) obtained by applying two different mass-spectrometric conditions: A) Sample cone voltage = 60 V (in-source fragmentation); B) Sample cone voltage = 30 V and Collision energy (CE) = 20 eV

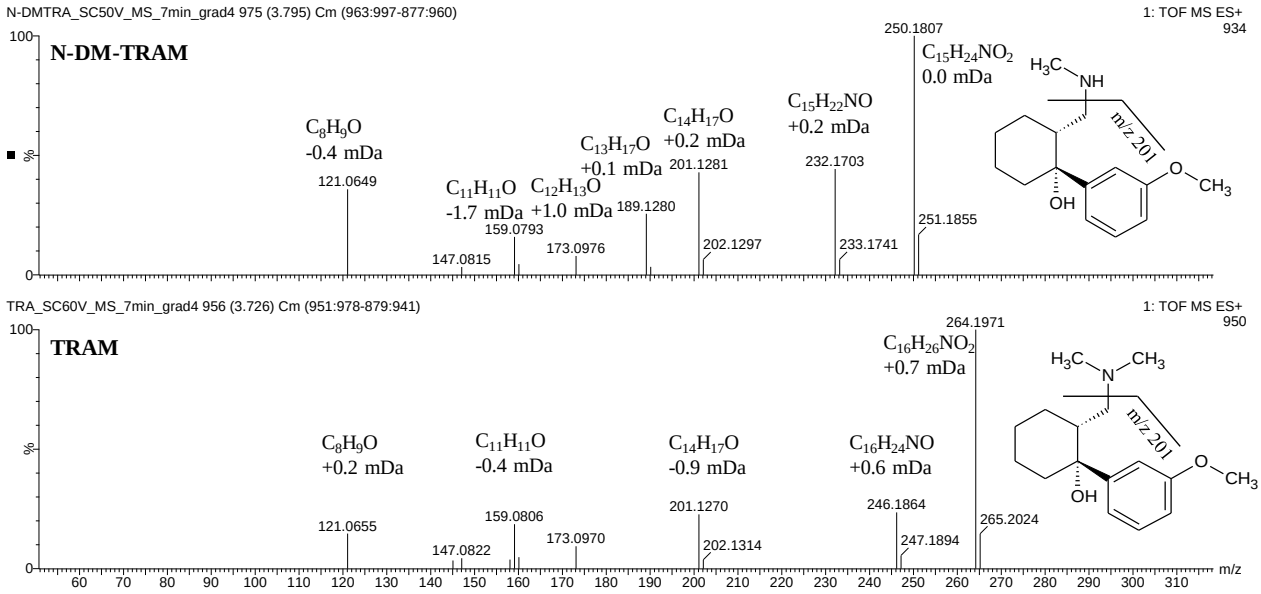

Fig. S6. Accurate mass spectra of tramadol (TRAM) and N-desmethyltramadol (N-DM-TRAM) acquired by sample cone voltages of 60 V and 50 V (in-source fragmentation), respectively. Sample from TRAM biodegradation experiment with glucose supplementation (day 6).

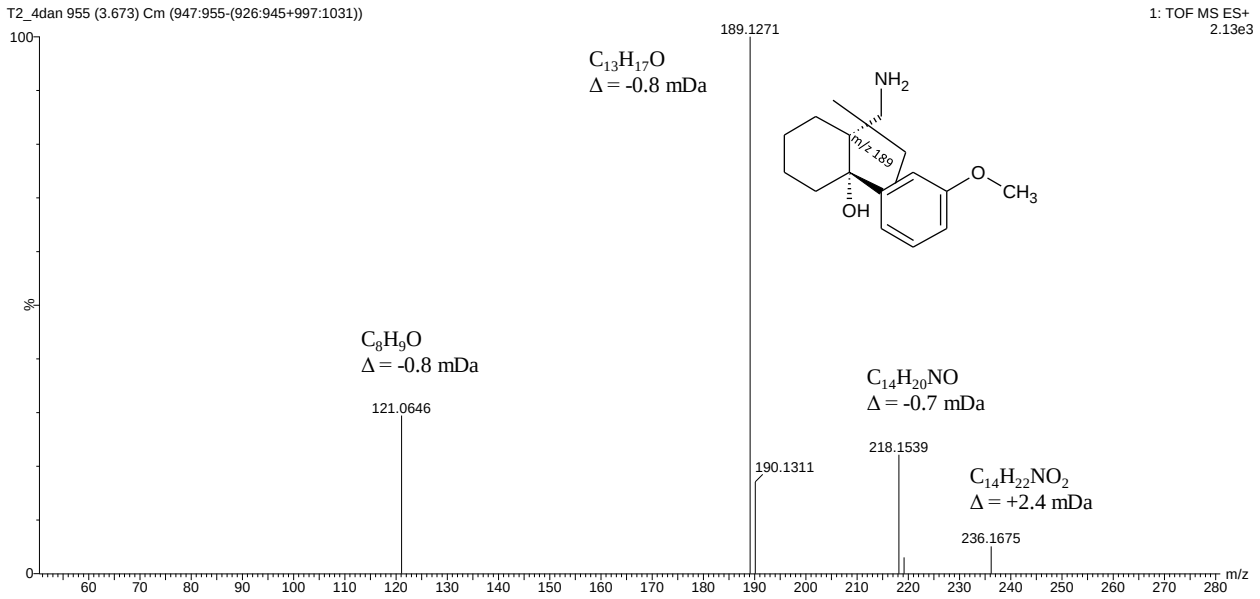

Fig. S7. Accurate mass spectra of N,N-didesmethyltramadol (N,N-di-DM-TRAM). Sample from TRAM biodegradation experiment with glucose supplementation (day 4).

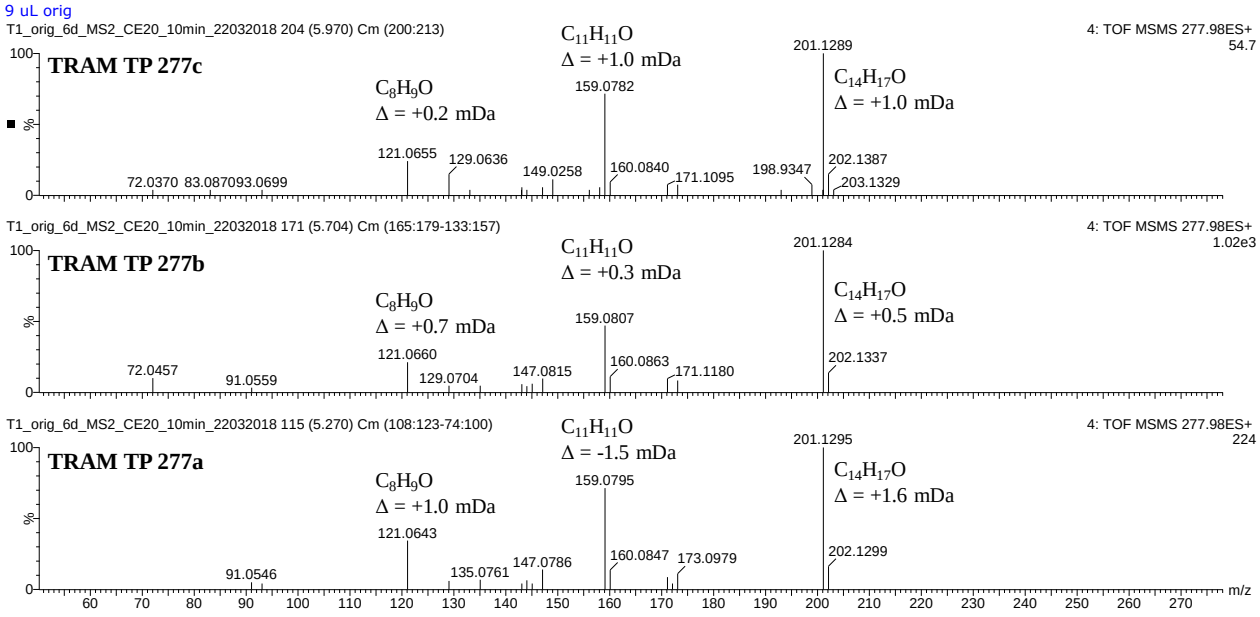

Fig. S8. Accurate mass spectra of tramadol transformation products 277a,b,c acquired by applying a collision energy, CE = 20 eV and sample cone voltage, CV = 30 eV. Sample from TRAM biodegradation experiment with glucose supplementation (day 6).

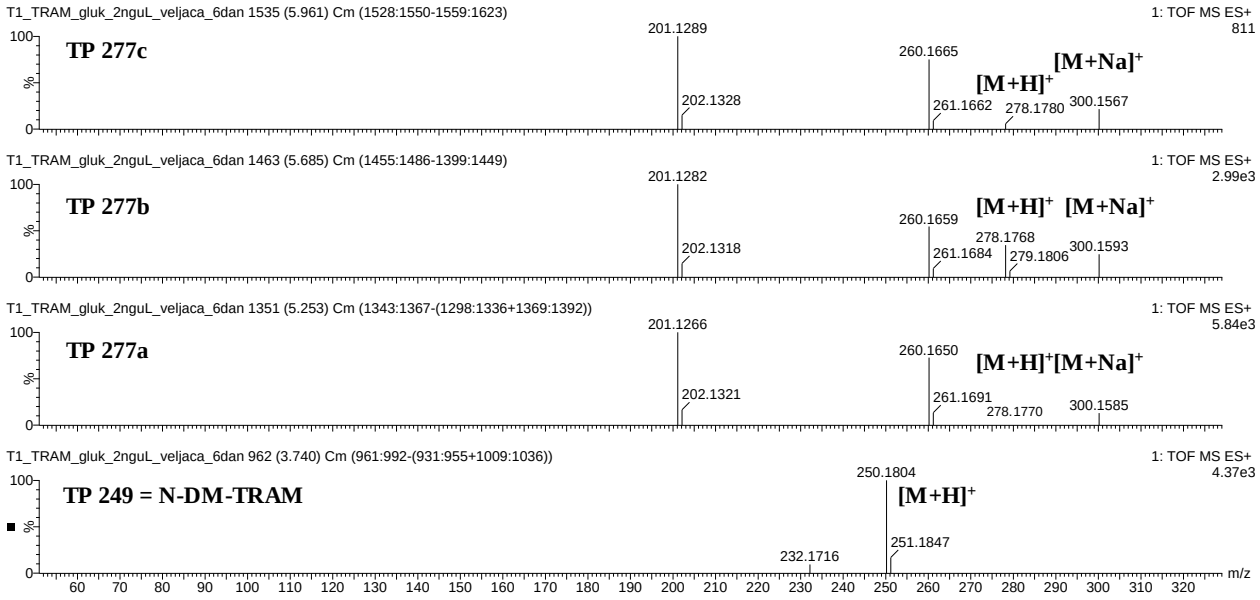

Fig. S9. Accurate mass spectra of N-desmethy tramadol (N-DM TRAM) and TRAM TPs 277a-c. Sample from TRAM biodegradation experiment with glucose supplementation (day 6).

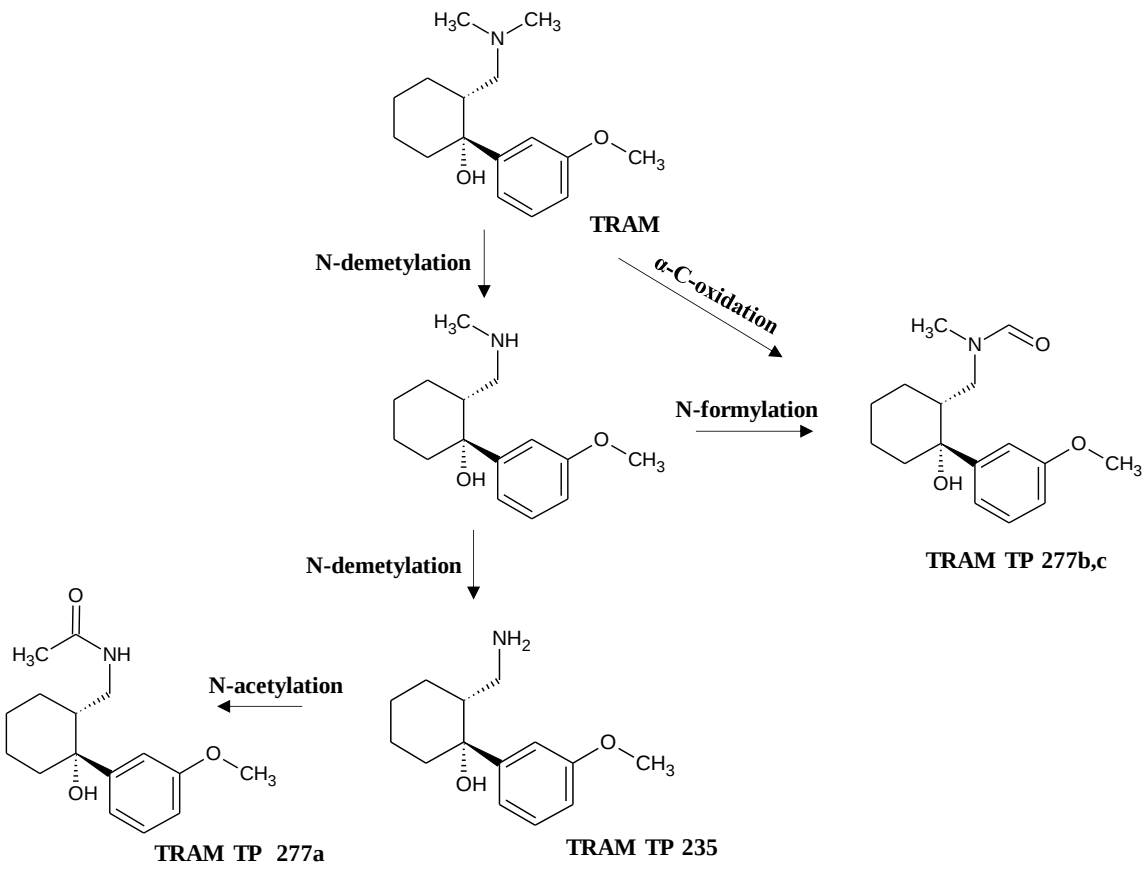

Fig. S10. Tentative biotransformation pathway of tramadol (TRAM) observed under aerobic conditions. TP = transformation product.

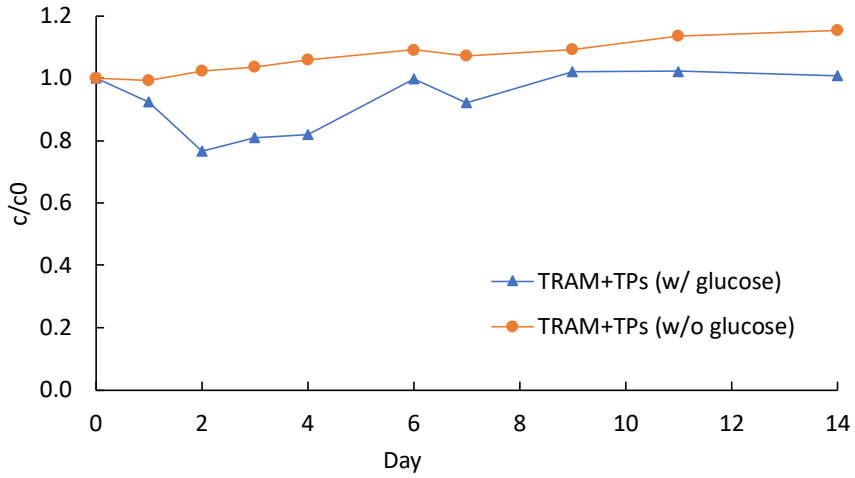

Fig. S11. Ratios of summed-up molar concentration of TRAM and its TPs determined during the aerobic biodegradation experiment performed with (w/) and without (w/o) glucose supplementation. (c –concentration at selected sampling-pointt; c0 – initial concentration

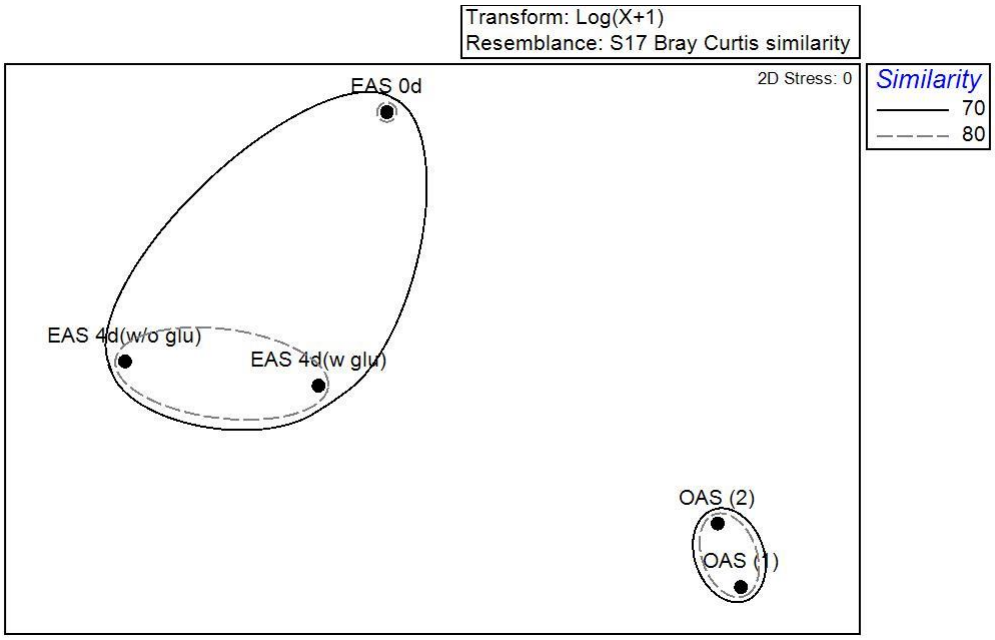

Fig. S12. Non-metric multi-dimensional scaling (nMDS) plot showing similarities in community structure (genus level) between original activated sludge culture collected at the WWTP (Zagreb) site (OAS) and the enriched activated sludge culture sampled at day 0. (EAS 0d) and 4th Day (EAS 4d) of the biodegradation experiment. Samples are clustered in a single group based on the DNA sequences. Results of the cluster analysis are based at the Bray-Curtis distance (70%; 80%) and superimposed on the MDS ordination.

EAS 0d = enriched activated sludge culture sampled at Day 0
EAS 4d (w glu) = enriched activated sludge culture growing with glucose; sampled at Day 4
EAS 4d (w/o glu) = enriched activated sludge culture growing without glucose; sampled at Day 4

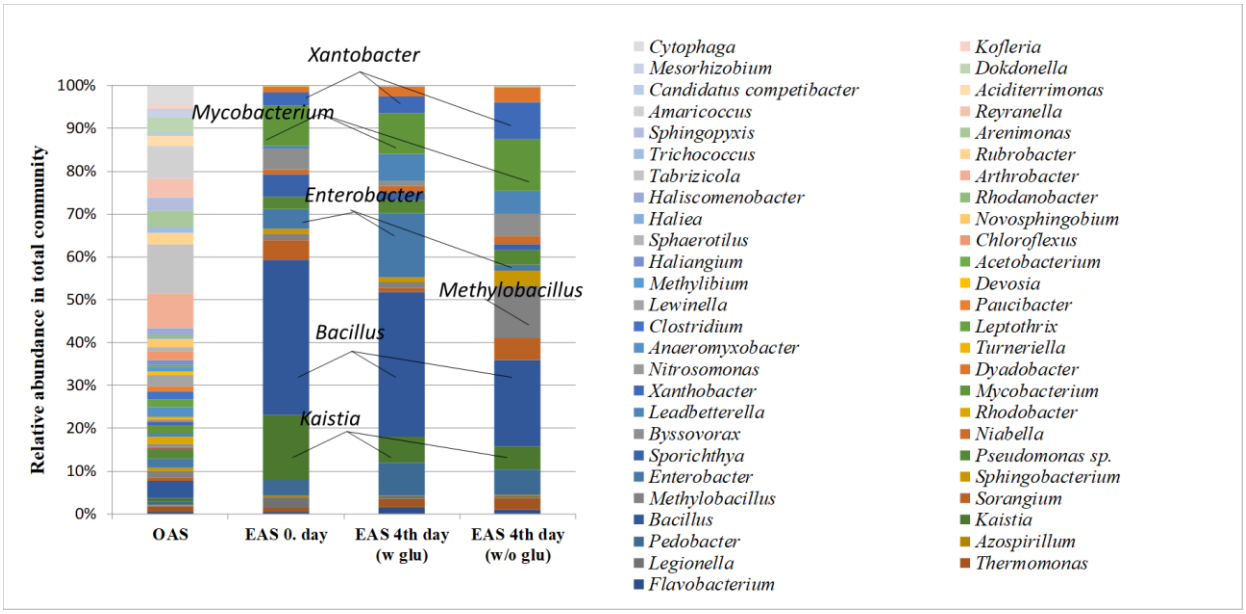

Fig. S13. Differences in the microbial community composition between original activated sludge culture **(OAS**) collected at the WWTP (Zagreb) site enriched activated sludge culture during TRAM biodegradation experiment, represented on the level of different bacterial genera, determined based on sequencing of the V4 variable region of the 16S rRNA gene marker.

EAS 0. day = enriched activated sludge culture sampled at Day 0
EAS 4d (w glu) = enriched activated sludge culture growing with glucose; sampled at Day 4
EAS 4d (w/o glu) = enriched activated sludge culture growing without glucose; sampled at Day 4

Table S4. Number of the observed species (OTUs, 97% cutoff value) within original activated sludge culture and enriched activated sludge culture collected and analyzed during TRAM biodegradation experiment and their correspondent diversity indices.

Culture	**Observed species (OTUs)**	**Chao**	**Shannon**	**Simpson**
Original Sludge culture	1526	2937	7.7	0.98
Enriched activated sludge culture 0. day	494	924	4.1	0.78
Enriched activated sludge culture (w glu) 4^{th} day	469	815	4.0	0.79
Enriched activated sludge culture (w/o glu) 4^{th} day	512	887	4.8	0.90