Reactor staging influences microbial community composition and diversity of denitrifying MBBRs- Implications on pharmaceutical removal

Torresi, Elena; Gülay, Arda; Polesel, Fabio; Jensen, Marlene Mark; Christensson, Magnus; Smets, Barth F.; Plósz, Benedek G.

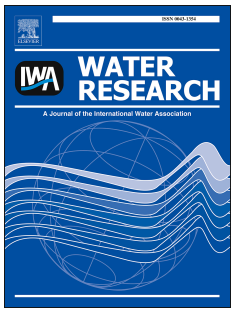
Published in:
Water Research

Link to article, DOI:
10.1016/j.watres.2018.03.014

Publication date:
2018

Document Version
Peer reviewed version

Accepted Manuscript



Reactor staging influences microbial community composition and diversity of denitrifying MBBRs- Implications on pharmaceutical removal

Elena Torresi, Arda Gülay, Fabio Polesel, Marlene M. Jensen, Magnus Christensson, Barth F. Smets, Benedek Gy. Plósz

PII: S0043-1354(18)30194-5

DOI: 10.1016/j.watres.2018.03.014

Reference: WR 13633

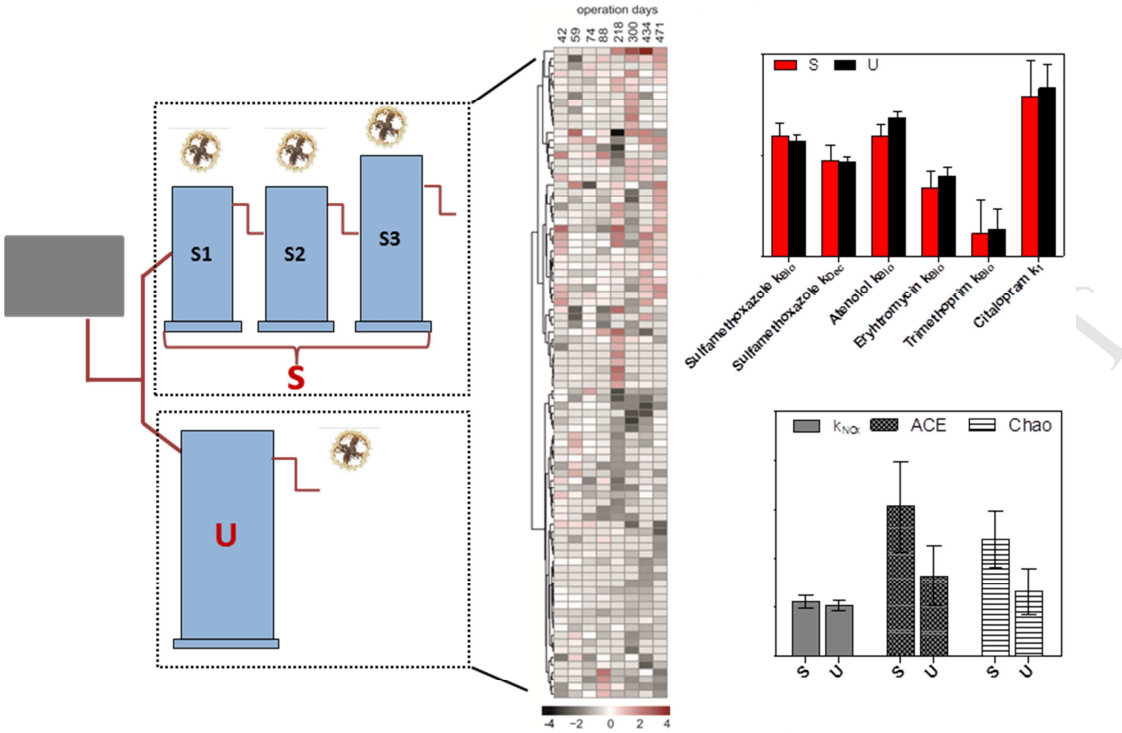
To appear in: *Water Research*

Received Date: 9 November 2017

Revised Date: 16 February 2018

Accepted Date: 6 March 2018

Please cite this article as: Torresi, E., Gülay, A., Polesel, F., Jensen, M.M., Christensson, M., Smets, B.F., Plósz, B.G., Reactor staging influences microbial community composition and diversity of denitrifying MBBRs- Implications on pharmaceutical removal, *Water Research* (2018), doi: 10.1016/j.watres.2018.03.014.



Reactor staging influences microbial community composition and diversity of denitrifying MBBRs-Implications on pharmaceutical removal

Elena Torresi[1,2,**], Arda Gülay[1], Fabio Polesel[1], Marlene M. Jensen[1], Magnus Christensson[2], Barth F. Smets[1*], Benedek Gy. Plósz[1,3]

[1]DTU Environment, Technical University of Denmark, Bygningstorvet B115, 2800 Kongens Lyngby, Denmark

[2]Veolia Water Technologies AB, AnoxKaldnes, Klosterängsvägen 11A, SE-226 47 Lund, Sweden

[3]Department of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY, UK

[*]Corresponding authors: elto@env.dtu.dk, bfsm@env.dtu.dk

Abstract

The subdivision of biofilm reactor in two or more stages (i.e., reactor staging) represents an option for process optimisation of biological treatment. In our previous work, we showed that the gradient of influent organic substrate availability (induced by the staging) can influence the microbial activity (i.e., denitrification and pharmaceutical biotransformation kinetics) of a denitrifying three-stage Moving Bed Biofilm Reactor (MBBR) system. However, it is unclear whether staging and thus the long-term exposure to varying organic carbon type and loading influences the microbial community structure and diversity. In this study, we investigated biofilm structure and diversity in the three-stage MBBR system (S) compared to a single-stage configuration (U) and their relationship with microbial functions. Results from 16S rRNA amplicon libraries revealed a significantly higher microbial richness in the staged MBBR (at 99% sequence similarity) compared to single-stage MBBR. A more even and diverse microbial community was selected in the last stage of S (S3), likely due to exposure to carbon limitation during continuous-flow operation. A core of OTUs was shared in both systems, consisting of *Burkholderiales*, *Xanthomonadales*, *Flavobacteriales* and *Sphingobacteriales*, while MBBR staging selected for specific taxa (i.e., *Candidate division WS6* and *Deinococcales*). Results from quantitative PCR (qPCR) showed that S3 exhibited the lowest abundance of 16S rRNA but the highest abundance of *atypical nosZ*, suggesting a selection of microbes with more diverse N-metabolism (i.e., not-complete denitrifiers) in the stage exposed to the lowest carbon availability. A positive correlation ($p<0.05$) between removal rate constants of several pharmaceuticals with abundance of relevant denitrifying genes was observed, but not with biodiversity. Despite the previously suggested positive relationship between microbial diversity and functionality in macrobial and microbial ecosystems, this

34 was not observed in the current study, suggesting a need to further investigate structure-function
35 relationships for denitrifying systems.

36

37 **Keywords**: Moving Bed Biofilm Reactors, micropollutant removal, organic carbon, structure-function
38 relationships, heterotrophic denitrification

39

1. Introduction

The presence of micropollutants (e.g., pharmaceutical and personal care products) in municipal wastewater effluent is well documented (Barbosa et al., 2016) and has been associated to several environmental risks (Painter et al., 2009).

Existing processes in conventional wastewater treatment plants (WWTPs) do not represent an efficient barrier against the release of micropollutants with treated effluent streams (Carballa et al., 2004). Hence, a number of engineering solutions are being explored to optimize the removal of micropollutants via biological wastewater treatment (Falås et al., 2016; Torresi et al., 2017, 2016).

The subdivision of biological reactors in two or more stages (i.e. reactor staging) has recently been proposed to enhance the removal of conventional pollutants (i.e., organic carbon, nitrogen) and pharmaceuticals in biofilm systems such as Moving Bed Biofilm Reactors (MBBRs) (Escolà Casas et al., 2015; Polesel et al., 2017). In MBBRs, biofilms grow on specifically designed plastic carriers, which are suspended and retained in the system (Ødegaard, 1999).

Due to presence of different fractions of organic carbon (e.g., from readily and slowly biodegradable to recalcitrant) in wastewater (Roeleveld and Van Loosdrecht, 2002), biofilm retained in different MBBR stages can be likely exposed to different substrate type and availability conditions. Based on long and short-term laboratory experiments, our previous work (Polesel et al., 2017) showed that the first stage of a pre-denitrifying three-stage MBBR system (S1) was effectively exposed to higher loadings of easily degradable organic carbon compared to the last stage (S3). Consequently, the first and last stage of the staged MBBR system exhibited, respectively, the highest and lowest denitrification and micropollutant biotransformation rate constant during targeted batch experiments (Polesel et al., 2017). However, it remains unclear: (i) if these differences in denitrification and biotransformation kinetics

are related to the microbial community structure and diversity, induced by organic carbon availability; and (ii) if this results in major differences in the overall performance of three-stage MBBR system compared to a single-stage configuration.

Wastewater organic carbon availability has previously been shown to differently shape the structure and diversity of microbial communities of denitrifying (Xia et al., 2010), aerobic MBBR (Fu et al., 2010), and in aquifer sediment system (Li et al., 2012, 2013), an effect that was found to correlate with micropollutants removal efficiency (Alidina et al., 2014). Hence, elucidating the microbial structure and diversity in biofilm systems and its influence on the overall microbial activity is fundamental for providing a basis to improve design and operation of MBBR systems towards pharmaceuticals removal. Additionally, although denitrification is a widespread process in biological wastewater treatment, substantial knowledge gaps remain concerning microbial communities under denitrifying condition (Lu et al., 2014).

Investigating microbial composition and diversity (i.e., α-diversity) in biological systems appears especially important when assessing rare microbial functions, such as biotransformation of micropollutants (Helbling et al., 2015; Johnson et al., 2015a). The existence of a relationship between microbial diversity and activity has been debated but a positive relationship between biodiversity and ecosystem functionality is commonly accepted (Briones and Raskin, 2003). This relationship has been observed with respect to the biotransformation of several micropollutants in both full-scale (Johnson et al., 2015a) and laboratory- scale bioreactors (Torresi et al., 2016; Stadler at al., 2016), suggesting that communities with higher diversity are likely to have more functional traits (Johnson et al., 2015b). Accordingly, biofilms, potentially exhibiting more microbial niches and thus higher biodiversity than conventional activated sludge (Stewart and Franklin, 2008), can represent a valid option to enhance micropollutant removal. Furthermore, the exposure of biofilm to varying carbon types and conditions

85 through bioreactor staging could additionally positively impact biofilm microbial diversity– the core
86 hypothesis tested herein.
87 In this study we evaluated the long-term effects of three-stage (S=S1+S2+S3) and single-stage
88 configurations (U) of pre-denitrifying MBBR on the biofilm microbial community composition and
89 diversity. High-throughput sequencing of 16S rRNA gene amplicon and quantitative PCR (qPCR) were
90 used to assess microbial diversity at local (S1, S2, S3) and system (S, U) level and the abundance of
91 relevant denitrifying genes, respectively. Thus, the main objectives of the study were:

92 1) to investigate the effect of organic carbon availability tiered by staging MBBRs on microbial
93 composition and diversity at local and system level, benchmarked against a single-stage
94 configuration;
95 2) to assess the dynamics in microbial community composition and denitrifying genes abundance
96 in the two MBBR systems during long term operation;
97 3) to assess associations between micropollutant biotransformation, local/system diversity and
98 denitrifying functionalities.

2. Methods

2. 1. Continuous-flow operation of the MBBRs and batch experiments.

A detailed description of the three- and single stage MBBR systems is given in Polesel et al. 2017. Briefly, two laboratory scale pre-denitrifying MBBR system with K1 carriers (AnoxKaldnes, Lund, Sweden) were operated in parallel under continuous-flow conditions for 1.5 years.

The single-stage system included a single bioreactor (U) with an operating volume of 6 L. The three-stage configuration included three reactors in series (S1, S2, S3) with a total operating volume of 6 L (1.5 L for S1 and S2 and 3 L for S3). The two systems were operated under identical conditions, i.e. influent flow rate, hydraulic residence time (HRT= 8.9 h), filling ratio (33%), ambient temperature, medium characteristics (pre-clarified wastewater from Mølleåværket WWTP, Lundtofte, Denmark), influent nitrate concentration (~103 mgN L^{-1}), sparging of N_2 gas for mixing and to ensure anoxic conditions (see Table S1 in Supplementary Information). The systems were started with MBBR carriers collected from the post-denitrification zone of Sjölunda WWTP (Malmö, Sweden), operated with external methanol dosing, as carbon source.

Two batch experiments were performed with the aim to assess denitrification rates and biotransformation rate constants of micropollutants at day 100 (Batch 1, after reaching a stable COD and nitrogen removal of ca. 70% and 44%, respectively) and day 471 (Batch 2, at the end of the experiment) (Polesel et al., 2017). For the batch experiments, the flow to and between reactors was stopped and the reactors were drained. Subsequently, the reactors were filled with pre-clarified wastewater (grab sampled with an initial NO_3-N concentration of 100-104 mgNL^{-1}) and carriers from U, S1, S2 and S3 (20% and 10% of filling ratio for Batch 1 and 2, respectively). The experiment lasted for 24 and 49 h for Batch 1 and 2, respectively, and 10 samples of aqueous solution were withdrawn

for micropollutants analysis at regular intervals (0, 0.3, 0.7, 1.2, 1.7, 2.2, 4.3, 7.5, and 10.3, 20 h for Batch 1 and additionally at 49 h for Batch 2). A fixed number of carriers was removed to maintain a constant filling ratio during batch experiments. The experiments were performed at ambient temperature (i.e., 20.3 ± 0.05 ºC in Batch 1 and 16.9 ± 0.4 ºC in Batch 2) and pH was adjusted to 8 ± 0.5 with 1M HCl spikes. Further details on the batch experiment can be found in Polesel et al. (2017).

2.2 DNA extraction and quantitative PCR.

To characterize microbial composition and its variation over long-term operation of the two MBBR systems, biofilm carriers for each MBBR were collected at day 0 (inoculum sample), 42, 59, 74, 88, 218, 300, 434 and 471 of operation. Samples were collected with the highest frequency during the first 100 days of operation to assess biomass adaption to the new operational conditions (i.e., staged pre-denitrification without methanol addition). A prior analysis of biological replicates (2 different carriers) at day 0 showed that the biomass from one carrier provideed a sufficient descriptor of the entire community (results are discussed in section S1 of supplementary information and in Fig. S1). Each time, biomass was detached from one carrier using a sterile brush (Gynobrush, Dutscher Scientific) and sterile-filtered tap water, centrifuged (10000 rpm for 5 minutes), and the supernatant was removed. The sample was stored at -20 ^{o}C until further analysis. DNA was extracted from attached biomass of one carrier using a Fast DNA spin kit for soils (MP Biomedicals, USA) following manufacturer's instructions. The quantity and quality of DNA were measured and checked by its 260/280 ratio by NanoDrop (Thermo Scientific™). Quantitative PCR (qPCR) was performed to estimate the abundance of total bacteria (EUB) with non-specific 16S rRNA gene targeted primers, and the abundance of a suite of genes encoding relevant functions: nitrate reductase (*narG*), cytochrome cd1 and copper nitrite reductases (*nirK* and *nirS*, respectively) nitrous oxide reductase of the *Proteobacteria nosZ* variant

144 (*nosZ* typical) and of the *nosZ* variant (*nosZ* atypical). Reported total microbial abundances are
145 expressed as number of gene copies per gram of biomass. Primers and conditions for quantification of
146 each gene are listed in Table S2.
147
148 **2.3. 16S rRNA gene amplification, sequencing and bioinformatic analysis.**
149 DNA extracted as described in 2.2 was also used for 16S rRNA analysis by Illumina MiSeq. PCR
150 amplification and sequencing were performed at the DTU Multi Assay Core Center (Kgs Lyngby, DK).
151 Briefly, extracted DNA was PCR amplified using 16S rRNA bacterial gene primers PRK341F (5'-
152 CCTAYGGGRBGCASCAG-3') and PRK806R (5'-GGACTACNNGGGTATCTAAT-3')(Yu et al.,
153 2005) targeting the V3 and V4 region. PCR products were purified using AMPure XP beads
154 (Beckman-Coulter) prior to index PCR (Nextera XT, Illumina) and sequencing by Illumina MiSeq.
155 Paired-end reads were assembled and screenings were implemented using mothur (Schloss, 2009).
156 High quality sequences were then transferred to the QIIME environment and OTUs were picked at 93,
157 95, 97 and 99% sequence similarity using the UCLUST algorithm with default settings, and
158 representative sequences from each were aligned against the Silva123_SSURef reference alignment
159 using SINA algorithm. Aligned sequences were then used to build phylogenetic trees using the Fast
160 Tree method (Price et al., 2009).
161 Taxonomy assignment of each representative sequence at all similarity levels was implemented using
162 the BLAST algorithm against the Silva128_SSURef database. Sequences with reference sequence hit
163 below 90% were called unclassified.
164 Meta communities were created by combining OTU libraries of S1, S2 and S3 reactors and adding into
165 the OTU tables of original and subsampled further to the lowest sample size. α-diversity of OTU
166 libraries was measured using the Chao1, Shannon, and ACE metrics as implemented in R using

Phyloseq package. Microbial evenness was estimated as H_1/H_0 as described in Johnson et al. (2015a). Distance matrices were constructed using the Bray-Curtis algorithms in R. Moving windows analysis was implemented using the microbial community of the inoculum as the reference point as described in Marzorati et al. (2008). Most abundant taxa and enriched taxa in S and U reactors were visualized using the Pheatmap package in R. The 100 most abundant taxa within the samples taken at 218, 300, 434, 471 days of operation were selected and compared.

2.4 Denitrification in continuous-flow and batch experiments

During continuous-flow operation, overall denitrification performance in the two MBBR systems was assessed by calculating (i) COD removal rate r_{COD} (gCOD m^{-2} d^{-1}) normalized per available surface area (Table S1) in each reactor, based on measured influent and effluent COD concentration in each stage; (ii) the denitrification rate normalized to surface area of reactor $r_{NOx\text{-}N}$ (gN m^{-2} d^{-1}), based on the measured influent and effluent concentration of NO_X-N (accounting for both NO_3-N and NO_2-N; Sözen et al., 1998) in each stage. Measurements were taken semi-weekly during the first 100 days of operation to ensure stable start-up of the systems and semi-monthly subsequently.

During batch experiments, biomass-specific denitrification rates k_{NOx} (mgN g^{-1} d^{-1}) were derived through linear regression of NO_X-N utilization curves for each sub-stage MBBR (Polesel et al., 2017). Specific denitrification rate at system level in S was calculated according to Eq. 1:

$$k_{NOX,S} = \frac{k_{NOX,S1}*X_{S1}*V_{S1}+k_{NOX,S2}*X_{S2}*V_{S2}+k_{NOX,S3}*X_{S3}*V_{S3}}{\sum X_{SMBBR}*V_S} \quad \text{(Eq.1)}$$

Where $k_{NOx,S1}$, $k_{NOx,S2}$, $k_{NOx,S3}$, (mgN g^{-1} d^{-1}) indicate specific denitrification rates in S1, S2 S3 respectively. X (g L^{-1}) and V (L) at the nominator in Eq. 1 indicate the biomass concentration and the

188 volume of each MBBR stage, respectively, while X_{SMBBR} (g L^{-1}) and V_S (L) at the denominator are the
189 biomass and volume of the staged system as a total MBBR (S).
190
191 **2.5 Micropollutants in continuous-flow and batch experiments**
192 Only indigenous pharmaceuticals occurring in municipal wastewater were quantified, as no reference
193 pharmaceuticals were spiked during continuous-flow and batch experiments. Twenty-three
194 pharmaceuticals were targeted and can be grouped in five categories: beta-blockers, sulfonamide
195 antibiotics, anti-inflammatory drugs, psycho-active drugs, X-ray contrast media. The complete list of
196 targeted pharmaceuticals quantified using HPLC-MS/MS analysis is reported in section S2 of the
197 Supplementary Information.
198 Continuous-flow samples were taken in two separate monitoring campaigns before the execution of
199 Batch 1 (100 days) and 2 (471 days) experiments. Removal efficiencies were calculated by measuring
200 influent and effluent concentration in the two systems (Polesel et al., 2017).
201 During batch experiments three main micropollutant removal mechanisms were observed or
202 hypothesized: (1) biotransformation, (2) retransformation to parent compounds (e.g.., deconjugation),
203 and (3) enantioselective biotransformation (Ribeiro et al., 2013). Pseudo first-order transformation
204 kinetics k_{Bio} (1, L g^{-1} d^{-1}), retransformation rates k_{Dec} (2, L g^{-1} d^{-1}), biotransformation rate constant of
205 enantiomer 1 and 2, $k_{bio,1}$ and $k_{bio,2}$, (3, L g^{-1} d^{-1}) were estimated as described in Polesel et al. (2017)
206 using the Activated Sludge Model framework for Xenobiotics (ASM-X), accounting for sorption
207 processes (Plósz et al., 2012).
208 Subsequently, the estimated k_{Bio} and k_{Dec} in each sub-reactor of the staged MBBR configuration (Table
209 S3) were used to calculate the following kinetic parameters:

(i) system-level biotransformation/retransformation rate for S for each micropollutant. As described for specific denitrification rate (Eq. 1), system-level $k_{Bio,S}$ (and similarly $k_{Dec,S}$) were calculated according to Eq. 2:

$$k_{\mathrm{Bio,S}} = \frac{k_{\mathrm{Bio,S1}} * X_{S1} * V_{S1} + k_{\mathrm{Bio,S2}} * X_{S2} * V_{S2} + k_{\mathrm{Bio,S3}} * X_{S3} * V_{S3}}{\sum X_{SMBBR} * V_S} \quad \text{(Eq.2)}$$

(ii) *collective* rate constants of multiple pharmaceuticals (*collective* k_{BioS1}-k_{DecS1}, k_{BioS2}-k_{DecS2}, k_{BioS3}-k_{DecS3}, k_{BioU}-k_{DecU}) to compare the performance of each sub-stage of S MBBR with U MBBR in terms of micropollutant biotransformation. The rate *collective* rate constants of multiple pharmaceuticals were calculated using the multifunctionality measure described by Zavaleta et al. (2010) and Johnson et al. (2015a). Briefly, the rate constants of each pharmaceuticals were scaled (to a mean of 0, standard deviation of 1) and the *collective* rate constants were obtained by averaging of the scaled rates.

2.6 Analytical methods

In all batch and continuous-flow samples, concentrations of conventional pollutants, i.e. NO_3-N, NO_2-N, NH_4-N, total and soluble COD (sCOD) were measured with Hach-Lange/Merck colorimetric kits followed by spectrophotometry, as previously described in Polesel et al. (2017). Wastewater fractionation and quantification of the organic carbon availability (mg L^{-1} of biodegradable COD with its two sub-fractions, i.e., readily (Ss) and slowly (Xs) biodegradable COD) was performed according to Roeleveld and Van Loosdrecht (2002) by combination with BOD-analysis and measure of total and soluble COD. Samples for micropollutants were analyzed using HPLC-MS/MS as described in Escolà Casas et al. (2015) and in Polesel et al., (2017). Briefly, 4 mL of aqueous samples were sampled and

stored in glass vials with addition of 1.4 mL pure methanol (99.9%, Merck Millipore). Samples were frozen at -20 ^{o}C prior analysis. For the analysis 1.5 mL of each sample were transferred to an HPLC vial and centrifuged (6000 rpm, 10 min). Subsequently, 100 µL of internal standard solution was added to 900 mL of the centrifuged samples solution. Samples were analyzed with an injected volume of 100 µL. Information regarding targeted micropollutants, HPLC-MS/MS and mass spectrometry conditions, limit of quantification and detection are shown in Escolà Casas et al. (2015).

2.7 Statistical analysis.

Correlation between k_{Bio}, k_{Dec}, *collective* rate constants k_{Bio}-k_{Dec} and denitrification rates k_{NOx}, biodiversity indices (Shannon, ACE, Chao and evenness indices), denitrifying gene abundance were estimated using Graph Prism 5.0. The statistical methods comprise (i) one way analysis of variance (ANOVA) with Bonferroni post-hoc test (significance level at $p<0.05$); (ii) Pearson correlation analysis (r values reported) and adjusted p-values (two-tailed), and (iii) paired Wilcoxon test. Although the Wilk-Shapiro test of normality may suggest a normal distribution ($p<0.05$) as the underlying distribution for the obtained biotransformation rate constants, bias could occur due to the small sample size (equal to 4). Pearson coefficients were reported as an indication of the strength of the association between the targeted parameters and micropollutant biotransformation rate constants.

3. Results and discussion

3.1 Comparison of continuous-flow operation performance in S and U MBBR systems

3.1.1. Denitrification

The loading of readily biodegradable (S_S) and slowly biodegradable (X_S) COD fractions in influent wastewater varied significantly through the experimental time (Fig. S2), with X_S typically contributing to more than 50% of biodegradable COD (bCOD). Most of influent S_S was utilized in the first stage S1 (on average 70%, Fig. S3a), leading to lower carbon loadings in the following stages (1.6 ± 0.4, 0.78 ± 0.2, 0.6 ± 0.2 gCOD d^{-1} in S1, S2, S3 respectively, Polesel et al., 2017). A decrease in the surface-normalized COD removal rates (r_{COD}, gCOD d^{-1} m^{-2}) could be observed after approximately 70 days of operation for the three-stage and the single-stage MBBR, due to differences in carbon loading (Fig. S2). No major differences in COD removal were observed between the two systems (Fig. 1a, (iii) p=0.46 with Wilcoxon test), with the exception of 3 sampling days when r_{COD} was up to 2-fold higher in the three-stage MBBR. Higher variability of performance was observed in terms of NO_X-N removal in the two systems (Fig. 1b). The three-stage MBBR generally outperformed the single-stage system (up to 30% higher, for ~60% measurements) in terms of r_{NOx-N} after 50 days of operation, however the difference was found statistically insignificant (p=0.07, Wilcoxon test). Fluctuations in r_{NOx-N} were also caused by the variance in the influent bCOD. Biomass concentration rapidly increased in the first 100 days of operation (Fig. S4), reaching values (average ± standard deviation) of 4.9 ± 0.9, 5.2 ± 1.9, 4.7 ± 1.2, 4.47 ± 1.3 g L^{-1}, for S1, S2, S3 and U, respectively.

Overall, data during continuous-flow operation suggest an enhancement in denitrification performance in the three-stage MBBR, possibly explained based on reaction kinetics principles (Plósz, 2007), e.g.,

maximization of the uptake rate of S_S and less degradable organic carbon in the stages of S configuration. Additionally, differences in nitrogen oxide reduction with a similar COD utilization were also observed during batch experiments, resulting in different calculated observable yield $Y_{H,obs}$ (mgCOD mgCOD^{-1}) in the four MBBRs (Polesel et al., 2017).

<Fig.1 >

3.1.2 Micropollutant removal

During continuous-flow operation, 11 of the 23 targeted compounds were detected in the pre-clarified wastewater, including compounds such as atenolol, citalopram, diclofenac, sulfamethoxazole, erythromycin and iohexol (Fig. 1 c, d). During the two sampling campaigns (at ~100 and 470 days of operation), atenolol and citalopram exhibited the highest removal efficiency (72% and 56–67%, respectively, calculated according to Eq. S1), while the overall balance of sulfamethoxazole removal showed a net formation of parent chemicals (> -150%) as a results of de-conjugation of human metabolites (Polesel et al., 2017). Overall, the removal efficiency of the measured pharmaceuticals was not significantly different between U and S MBBR system in the two sampling campaigns ($p>0.05$, Wilcoxon test in Fig. 1c and d), while diclofenac (DCF) presented higher removal in U (~10%) compared to S (~-20%).

3.2 Microbial community composition and diversity in S and U MBBR systems

3.2.1 Microbial diversity at local and system level

Microbial diversity in the two MBBR systems was assessed at 93%, 95%, 97% and 99% sequencing similarities cut-offs to maximize the resolution of the α-diversity analysis between the four reactors.

After the implementation of quality control measures, a total of 3178345 high quality sequences were obtained for each clustering, subsequently rarefied to 15800 sequences per sample.

α-diversity (expressed as Shannon diversity, ACE and Chao richness indices) increased overall with increasing sequence similarity cut-offs (Fig. 2), as expected (Birtel et al., 2015). As the two MBBR systems followed similar patterns over the time in terms of community diversity (Fig. 2), the α-diversity was likely influenced by variations in influent wastewater composition in terms of COD and microbial community in the influent wastewater, as result of frequent change of the influent medium. Accordingly, linear regression analysis (Fig. S5) suggested a significant ($p<0.05$, ANOVA) positive linear relationship between influent soluble COD with microbial richness (ACE and Chao) in U (R^2 of 0.88, n=6) and S1 (R^2 of 0.80, n=6) at 99% similarity, but not for S, S2 and S3.

<Fig.2>

No major differences were observed in terms of Shannon diversity and evenness indices over time between S and U (Fig. 2), while ACE and Chao richness presented overall higher values in S compared to U (with increasing differences at increasing sequences similarity cut-offs, from 23% to 30% for 93% and 99, respectively).

Furthermore, we assessed how the difference in the microbial community diversity in S and U (β-diversity) changed over the duration of the experiment and estimated the time needed for the MBBR microbial communities to reach a steady composition that was dissimilar from the inoculum (Fig. 3). Moving window analysis (MWA) was implemented using the reciprocal of Bray-Curtis indices measured at different sequence similarities (Fig. 3). Microbial community similarity significantly decreased from the same inoculum sample during the first 200 days of operation, subsequently, reaching a relatively stable composition for the rest of the experiment. Bray-Curtis indices profiles (Fig. 3) for S and U decreased to the highest extent at 99% sequence similarity cut-off.

317 As the MBBR microbial communities and the degree of dissimilarity from the inoculum community
318 appeared stable after approximately 200 days of operation based on MWA, the Shannon, richness and
319 evenness indices were averaged after 200 days of operation (n=4) to assess statistical difference
320 between the two systems and for each sub-stage of S (Fig. 4). For all four tested sequence similarity
321 levels, no significant difference was observed for the Shannon diversity and evenness for the microbial
322 communities prevailing in S and U (reported at 97 and 99%, Fig. 4a–b). On the other hand, microbial
323 richness (ACE and Chao) was higher in S than to U at both sequence similarities (at system level), with
324 significant difference at 99% sequence similarity cut-off (ANOVA, $p<0.05$, Fig. 4d).

325 <Fig.3>

326 Hence, our findings (Fig. 2 and Fig. 4) suggest that the exposure of microbial communities to a
327 gradient of organic carbon availability, achieved through reactor staging, results in significantly higher
328 microbial richness compared to a single-stage configurations. Additionally, average Shannon diversity,
329 evenness and richness were higher (although not significantly different, ANOVA, $p>0.05$) in S3
330 compared to S1 and S2 at 99% sequence similarity (Fig. 4). It is likely that the more refractory and
331 slowly biodegradable carbon, to which S3 was exposed during continuous-flow operation (Fig. S3), led
332 to the co-existence of a more diverse microbial community due to substrate competition (Huston,
333 1994). On the contrary, the easily degradable carbon mostly utilized in S1 may have favoured
334 microbial groups that dominate the microbial community.

335 Similar observations were previously reported in managed aquifer recharge systems (MAR), where
336 higher community diversity was observed at more oligotrophic depths compared to the depths where
337 more easily degradable carbon was available (Li et al., 2013, 2012). Increased taxonomic richness was
338 also associated with lower influent ambient nitrogen and carbon availability in full-scale wastewater
339 treatment plant microbial communities (Johnson et al., 2015b). Conversely, higher microbial diversity

(expressed as Shannon index) was found in the first stage of an aerobic two-stage nitrifying MBBR treating landfill leachate (Ciesielski et al., 2010).

<Fig.4>

3.2.2 Temporal variability in the selection of taxa by substrate availability in S and U MBRR systems

We further investigated the development of microbial structure in S and U over 471 days of operation to elucidate whether staging the MBBR system resulted in a selection of specific taxa. Hence, we computed heatmaps of the 100 most abundant OTUs at order level sorted by most abundant OTUs after 200 days of operation (218, 300, 434, 471 days) (Fig. 5 (a) and (b)). In both systems, the methanol-utilizing bacteria *Methylophilales* that were enriched in the inoculum (methanol dosing was applied to full-scale WWTP) decreased over time, eventually disappearing after approximately 200 days. A core of OTUs was shared in both systems, consisting of *Burkholderiales*, *Xanthomonadales*, *Flavobacteriales* and *Sphingobacteriales*. *Burkholderiales* (isolated from different environmental sources) has been shown to be able to biotransform a vast array of aromatic compounds (Pérez-Pantoja et al., 2012), as well as to be enriched by using methanol as carbon source (Kalyuzhnaya et al., 2008). Bacterial strains affiliated to the order *Burkholderiale*, *Xanthomonadales* and *Sphingobacteriales* were previously identified in a four-stage MBBR with a pre-denitrification configuration (Villemur et al., 2015) and they were suggested to play an important role in the biofilm development, due to excretion of exopolymeric substances (EPS) for biofilm formation (Pal et al., 2012). *Flavobacteriales* and *Burkholderiales* were associated to the biotransformation of venlafaxine and ranitidine, respectively, in WWTP community (Helbling et al., 2015). Notably, taxa such as *AKYG1722*, *Caldilineales*, *JG30-KF-CM4*5 and *Candidate division WS6* were enriched in both MBBR systems during 300 days of operation. To effectively identify the microbial organisms that were differently selected in the two

362 configurations, we considered the most abundant OTUs of S and U MBBRs and reported the log of the
363 ratio of the sequence abundance in S and U (log(S/U)) (Fig. 5 (c)). A similar approach was used for the
364 taxa in S3 and S1 (log(S3/S1), Fig. 5 (d)). Notably, the three-stage MBBR (S) selected for the OTU
365 *Bifidobacteriales* and *Candidate division WS6* after day 218. *Candidate division WS6* have been
366 previously identified as abundant community members in anoxic/anaerobic environments of
367 hydrocarbon-and chlorinated-solvent-contaminated aquifer (Dojka et al., 1998, 2000). *Candidate*
368 *division WS6* and *Deinococcales* were enriched in S3 over S1 (Fig. 5 (4)), suggesting a correlation of
369 these OTUs with low readily biodegradable carbon availability in S3 during continuous-flow operation.
370 This is in disagreement with Dojka et al. (2000) that observed *Candidate division WS6* mainly in
371 organic-rich, anaerobic redox environment. The family *Deinococcaceae* is widely studied, since
372 organisms from this groups have been observed to exhibit remarkable resistance to radiation
373 (Chaturvedi and Archana, 2012). Conversely, *Dictyoglomales*, *Microgenomates*_4 and subgroup 4 of
374 *Acidobacteria* were mostly enriched in U over S after 218 days (Fig. 5 (3)). Compared to other
375 subgroups, the abundance of *Acidobacteria subgroup 4* has been negatively associated with organic
376 carbon availability and C-to-N ratio in grassland soils (Naether et al., 2012).
377 Overall, we observed generally dynamic microbial communities, with only few taxa consistently
378 enriched after 218 days in the three-stage configuration compared to single-stage one. Considering the
379 long-term operation of the two systems with actual pre-clarified wastewater influent, it is likely that
380 (besides the organic substrate availability) continuous and random immigration by the microbial
381 community present in the influent wastewater played an important role in shaping the microbial
382 communities. The importance of microbial immigration was shown by calibrating a neutral model
383 community assembly with dynamic observations of wastewater treatment communities (Ofiteru et al.,
384 2010), in full-scale WWTP (Wells et al., 2014), as well as in a pilot-scale membrane bioreactor system

385 (Arriaga et al., 2016). Additionally, cross-inoculation between staged reactors may have been occurred,
386 as previously observed in staged bioprocesses in full-scale WWTP (Wells et al., 2014).

387 <Fig. 5>

3.2.3 Microbial and denitrifying gene abundance in S and U MBBR systems

Quantification of 16S rRNA (total bacteria) and denitrifying genes was performed to investigate differences in denitrifying microbial communities in the four MBBR reactors (Fig. S6). For the U and S1 MBBR, microbial abundance of total bacteria during 471 days of operation could be associated with the influent substrate concentration – expressed as influent soluble COD (Fig. S7, R^2 of 0.8 and 0.5, respectively). Conversely, no association was found for S2 and S3 with respect to influent sCOD.

As informed by moving window analysis, qPCR data for all reactors were averaged from the point when the microbial community was stable (i.e., after 200 days of operation) (Table 1). The lowest ($p<0.05$) abundance of 16S rRNA gene (copies $g_{biomass}^{-1}$) was measured in S3, mostly adapted to carbon limitation during continuous-flow operation, as previously observed in simulated managed aquifer recharge (Li et al., 2013). Overall, the measured *nirS* gene fraction was up to 10 times higher than *nirK* (in agreement with other studies in aquatic ecosystems, e.g., Braker et al., 2000;), while no differences were observed between the four reactors in terms of *nirS* gene fraction (Table 1). Previous studies have suggested lower densities of *nirK*-containing denitrifiers in aquatic ecosystems (Braker et al., 2000). The decrease of *nirS* from the inoculum sample (day 0), adapted to methanol (Fig. S6) is consistent with the previous observation that utilization of methanol as a readily biodegradable substrate can select for *nirS*-expressing denitrifiers (Hallin et al., 2006). Furthermore, a change to different carbon sources can result in a loss of *nirS* density (Hallin et al., 2006). The S3 reactor was continuously exposed to the lowest C-to-N influent ratio (average values of C, expressed as sCOD, -to-N ratio of 1, 0.8 and 0.7 for S1 and U, S2, S3 respectively), and previous research reported increased N_2O production at lower C-to-N ratios (Zhang et al., 2016). However, S3 exposed to lower C-to-N ratios during continuous-flow operation exhibited the highest ($p<0.05$) abundance of atypical *nosZ* gene and a

lower nitrite reductase and nitrous reductase ration (Table 1), which could indicate a more effective N_2O removal with respect to other reactor stages. Furthermore, S3 and S1 had the highest and lowest ratio of atypical to typical *nosZ*, respectively (Table 1). Typical *nosZ* genes have been associated with bacteria capable of complete denitrification (thus encoding all the enzymes for converting nitrate to nitrogen) (Sanford et al., 2012). In contrast, atypical *nosZ* genes were also found in non-denitrifying bacteria with more-diverse N-metabolism (e.g., missing *nirK* and *nirS*) (Orellana et al., 2014; Sanford et al., 2012), and are commonly present at concentrations higher than typical *nosZ* in soil (Orellana et al., 2014). Hence, our results suggest a microbial selection driven by the substrate gradient through the MBBR stages, where most of the complete denitrifiers (carrying typical *nosZ*) are selected in S1 (with the highest readily biodegradable substrate availability). On the other hand, microbes with more diverse N-metabolism (carrying atypical *nosZ*) are selected in S3. Although, based on prior reports, the highest N_2O production was expected in MBBR stages with low influent C-to-N ratio, the selection of non-denitrifying bacteria containing atypical *nosZ* genes (which code for high affinity N_2O reductase) may well imply a reduced accumulation of nitrous oxide. This factor suggests biofilm reactor staging as a process optimisation means to reduce N_2O emissions through wastewater treatment, which requires further research.

<Table 1>

3.3. Linking activity, community composition and diversity with micropollutant biotransformation in batch experiments

3.3.2 System level (S and U)

Based on the results of the batch experiments, biotransformation rate constants of the pharmaceuticals were calculated at system level for the three-stage MBBR system (Eq. 2) and compared with rate constants for the single-stage system for Batch 1 (Fig. S11) and Batch 2 (Fig. 6 a). We did not observe a significant difference in specific denitrification rate at system level (calculated as in Eq. 1) but higher ($p<0.05$) microbial richness was measured in S compared to U in Batch 2 (Fig. 6b). Nonetheless, no significant difference was observed in the biotransformation of the targeted micropollutants between the two MBBR systems (Fig S11 and Fig. 6a).

<Fig. 6>

3.3.1. Local level (S1, S2, S3 and U)

As reported in Polesel et al., 2017, the decreasing loading and availability of carbon during continuous-flow operation led to a decreasing trend of pharmaceuticals biotransformation from S1 to S3 (Table S3).

Pearson's coefficients r were used to evaluate associations between biotransformation rate constant k_{Bio} /k_{Dec} and (i) biodiversity indices (at 99% sequence similarity, Fig. S9); (ii) denitrifying gene abundance (Fig. S6); (iii) specific denitrification rates $\bar{k}_{NOX}$ (mgN g^{-1} d^{-1}) (Table S3). Only relevant k_{Bio} and k_{Dec} values (>0.1 L g^{-1} d^{-1} and corresponding to a removal >20% according to the classification presented in Joss et al., 2006) were included in the analysis. Notably, correlations were performed by using only taxonomic diversity (based on 16S rRNA amplicon sequencing), rather than data combining functional

diversity (based on the phenotypes inferred from taxonomic descriptors and on mRNA sequencing), which was beyond the scope of this study. Although it has been observed that taxonomic and functional diversity associate with each other in wastewater treatment systems (Johnson et al., 2015b), additional information could be derived by the combination of both analyses.

In Batch 1, only few positive correlations ($p<0.05$) were observed between diversity indices and biotransformation rate constants of pharmaceuticals. i.e., sulfamethoxazole, trimethoprim and metoprolol (Fig. S10). In Batch 2, k_{Bio} and k_{Dec} of most detected pharmaceuticals were negative or not significantly correlated with microbial richness (Fig. 7), but positively correlated ($p<0.05$) with specific denitrification rates k_{NOx}. The obtained correlations are reported in Fig. S8. Positive correlations ($p<0.05$) were found with k_{Bio} of erythromycin, trimethoprim and *collective* pharmaceuticals as well as with the abundance of denitrifying genes *narG*, *nirS*, and *nosZ* typical, but not atypical *nosZ.*

The difference in the results between Batch 1 and 2 may stem from the variation in the denitrification rates k_{NOX} obtained during batch experiment, that affected the cometabolic biotransformation of the pharmaceuticals (Polesel et al., 2017). Due to lower biotransformation rate constants obtained in Batch 2 compared to Batch 1, adaption of the biomass to targeted pharmaceuticals is unlikely. Previous studies on soil and wastewater activated sludge concluded that pre-exposure of the biomass to trace organic chemicals may not affect the removal efficiency of micropollutants (Falås et al., 2016) neither the microbial community structure nor its function (analyzed by metagenomes) (Alidina et al., 2014).

As discussed previously, a stable microbial community was observed only after 200 days of operation and results from Batch 2 (at 471 days) may be considered representative of the long-time operation of the two MBBR systems.

Biotransformation of several micropollutants has been related to the lack of specificity of enzymes such as ammonia monooxygenase (Khunjar et al., 2011; Sathyamoorthy et al., 2013). To our knowledge,

478 cometabolism of micropollutants by respiratory denitrifying enzymes (e.g., *narG*, *nirS*, *nor*, *nosZ*) has
479 not been documented. Thus, the unexpected association between denitrifying genes and
480 biotransformation of micropollutants may be the result of a genuine but nevertheless non-causal
481 relationship (Johnson et al., 2015c). Further research is required to examine the cause of this
482 correlation.

483 As mentioned previously, positive relationships between microbial diversity (and in particular α-
484 diversity) and biotransformation rate constants of micropollutants have been observed in activated
485 sludge (Johnson et al., 2015a), in sequencing batch lab-reactors (Stadler and Love, 2016) and in
486 nitrifying MBBRs (Torresi et al., 2016). Yet, similar negative correlation has been observed between
487 biodiversity and removal of natural and synthetic estrogens in suspended biomass (Pholchan et al.,
488 2013; Tan et al., 2013) and for sulfonamide antibiotics (sulfadiazine, sulfamethoxazole, sulfamethizole)
489 in nitrifying MBBRs (Torresi et al., 2016). Among others, two phenomena may explain the lack of an
490 observable (positive) relationship:

491 (i) A positive relationship between biodiversity (or richness) would emerge (a) if the microbial
492 community consisted of a number of microorganisms with unique niche partioning or (b) if
493 facilitative interactions (i.e., complementarity effects) occurred (Cardinale, 2011) .
494 However, functional redundancy (i.e., different taxa coexist to perform the same
495 functionality) could be sufficient to mask this positive interaction (Johnson et al., 2015b).
496 Accordingly, if the biotransformation of a specific compound is performed by a large
497 number of taxa, the increase of biodiversity may not necessary positively impact the
498 biotransformation as it is not limited by the number of taxa that can perform it (Stadler and
499 Love, 2016). Taken together, the negative correlation observed in this study between
500 biotransformation rate constants and biodiversity, combined with the positive correlation

501 with kinetics of denitrification, could suggest a redundancy of the denitrifying microbial
502 community towards the biotransformation of these targeted pharmaceuticals.
503 This observation might further suggest that denitrifying systems exhibit higher
504 biotransformation rates of pharmaceuticals compared to aerobic systems, due to the higher
505 number of taxa performing this function. Hence, we compared the average
506 biotransformation rate constants obtained in this study (under pre-denitrification conditions)
507 and in post-denitrification MBBRs (Torresi et al., 2017) with kinetics obtained for aerobic
508 nitrifying MBBRs (Torresi et al., 2016) (Fig. S12). While we observed comparable
509 biotransformation kinetics for aerobic and pre-denitrifying MBBRs (this study) (Fig. S12a),
510 post-denitrifying MBBRs indeed exhibited higher biotransformation rate constants for more
511 than 60% of the examined pharmaceuticals (Fig. S12b) compared to aerobic MBBRs. In the
512 post-denitrifying MBBRs (Torresi et al., 2017), additional carbon sources (i.e., methanol or
513 ethanol) were dosed in the systems, which are known to be readily biodegradable substrates.
514 Thus, in the absence of catabolic limitation (i.e., in the presence of excess easily degradable
515 organic carbon), biotransformation of several targeted pharmaceuticals may be more
516 expedient under anoxic versus aerobic conditions.
517 (ii) An increase in biodiversity might not translate into differences in microbial functionality if
518 the microbial community presents sufficient biodiversity to begin with, that can saturate the
519 possible effects (Johnson et al 2015a). While this effect was not observed for suspended
520 biomass in full-scale WWTP (i.e., microbial communities were not sufficiently diverse to
521 maximize the collective rate of multiple micropollutant biotransformation, Johnson et al.,
522 (2015a)), this may be different for biofilm systems that can already potentially harbor higher
523 microbial diversity compared to suspended biomass (Stewart and Franklin, 2008).

Overall, our results (at both global and system level) suggest that despite the general positive association between microbial diversity and ecological activity (Cardinale, 2011; Emmett Duffy, 2009), this association is not fully understood for microbial communities in biological wastewater treatment regarding micropollutant biotransformation. On the other hand, additionally information could be obtained by targeting a broader number of micropollutants.

Nonetheless, the relationships between microbial diversity and system stability and resilience in wastewater treatment plant has been discussed (Lu et al., 2014), as a result of functional redundancy (Briones and Raskin, 2003). Accordingly, it has been recommended that if two denitrifying configurations perform equally efficiently, the configuration with higher functional diversity should be preferentially selected to ensure higher system stability (Lu et al., 2014).

4. Conclusions

The microbial communities of two pre-denitrifying MBBR systems operated in parallel in single- (U) and three-stage (S) configurations using pre-clarified wastewater as influent and native concentration of micropollutants were investigated during long-term operation.

- The decreasing gradient of organic carbon loading and availability (created through MBBR staging) led to an increase of α-diversity of the microbial community (although not significant) within the three-stage system and an overall increased richness (ANOVA, $p<0.05$) of the microbial community in the S configuration compared to U.
- The microbial community became stable only after 200 days of operation, when the two configurations shared a core of OTUs such as *Burkholderiales*, *Xanthomonadales*, *Flavobacteriales* and *Sphingobacteriales*. The staged configuration (and in particular in the last stage MBBR, S3) selected for OTUs such as *Candidate division WS6* and *Deinococcales*.
- S3 exposed to lower C-to-N ratios during continuous-flow operation exhibited the highest ($p<0.05$) abundance of atypical *nosZ* gene and a lower nitrite reductase and nitrous reductase ration, suggesting a more effective N_2O removal with respect to other reactor stages.
- Specific and *collective* bio- and re-transformation rate constants of the targeted pharmaceuticals positively correlated with specific denitrification rates and abundance of denitrifying genes (*narG*, *nirS* and *nosZ* typical), rather than biodiversity.

Overall, staging of MBBR systems under denitrifying conditions resulted in enhanced denitrification rate and increased microbial diversity compared to a single-stage configuration, although no major improvement was observed in the removal of the selected trace organic pharmaceuticals.

Acknowledgements

This research was supported by MERMAID: an Initial Training Network funded by the People Programme (Marie-Curie Actions) of the European Union's Seventh Framework Programme FP7/2007-2013/under REA grant agreement n. 607492. The authors thank Luca Loreggian and Saverio Conti for technical support.

References

Alidina, M., Li, D., Ouf, M., Drewes, J.E., 2014. Role of primary substrate composition and concentration on attenuation of trace organic chemicals in managed aquifer recharge systems. J. Environ. Manage. 144, 58–66.

Arriaga, S., De Jonge, N., Lund Nielsen, M., Andersen, H.R., Borregaard, V., Jewel, K., Ternes, T.A., Lund Nielsen, J., 2016. Evaluation of a membrane bioreactor system as post-treatment in waste water treatment for better removal of micropollutants.

Barbosa, M.O., Moreira, N.F.F., Ribeiro, A.R., Pereira, M.F.R., Silva, A.M.T., 2016. Occurrence and removal of organic micropollutants: An overview of the watch list of EU Decision 2015/495. Water Res. 94, 257–279.

Birtel, J., Walser, J.-C., Pichon, S., Bürgmann, H., Matthews, B., 2015. Estimating Bacterial Diversity for Ecological Studies: Methods, Metrics, and Assumptions. PLoS One 10.

Braker, G., Zhou, J., Wu, L., Devol, A.H., Tiedje, J.M., 2000. Nitrite reductase genes (nirK and nirS) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. Appl. Environ. Microbiol. 66, 2096–104. Briones, A., Raskin, L., 2003. Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. Curr. Opin. Biotechnol. 14, 270–276.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., García-Jares, C., Rodriguez, I., Gomez, M., Ternes, T., 2004. Behavior of pharmaceuticals, cosmetics and hor- mones in a sewage treatment plant. Water Res. 38 (12), 2918e2926.

Cardinale, B.J., 2011. Biodiversity improves water quality through niche partitioning. Nature 472, 86–9.

589 Chaturvedi, R., Archana, G., 2012. Novel 16S rRNA based PCR method targeting Deinococcus spp.
590 and its application to assess the diversity of deinococcal populations in environmental samples. J.
591 Microbiol. Methods 90, 197–205.
592 Ciesielski, S., Kulikowska, D., Kaczowka, E., Kowal, P., 2010. Characterization of bacterial structures
593 in a two-stage moving-bed biofilm reactor (MBBR) during nitrification of the landfill leachate. J.
594 Microbiol. Biotechnol. 20, 1140–1151.
595 Dojka, M.A., Harris, J.K., Pace, N.R., 2000. Expanding the known diversity and environmental
596 distribution of an uncultured phylogenetic division of bacteria. Appl. Environ. Microbiol. 66,
597 1617–21.
598 Dojka, M., Hugenholtz, P., Haack, S., Pace, N., 1998. Microbial diversity in a hydrocarbon-and
599 chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ.
600 Microbiol. 64, 3869–3877.
601 Emmett Duffy, J., 2009. Why biodiversity is important to the functioning of real-world ecosystems.
602 Front. Ecol. Environ. 7, 437–444.
603 Escolà Casas, M.E., Chhetri, R.K., Ooi, G., Hansen, K.M.S., Litty, K., Christensson, M., Kragelund,
604 C., Andersen, H.R., Bester, K., 2015. Biodegradation of pharmaceuticals in hospital wastewater
605 by staged Moving Bed Biofilm Reactors (MBBR). Water Res. 83, 293–302.
606 Falås, P., Wick, A., Castronovo, S., Habermacher, J., Ternes, T.A., Joss, A., 2016. Tracing the limits of
607 organic micropollutant removal in biological wastewater treatment. Water Res. 95, 240–249.
608 Fu, B., Liao, X., Ding, L., Ren, H., 2010. Characterization of microbial community in an aerobic
609 moving bed biofilm reactor applied for simultaneous nitrification and denitrification. World J.
610 Microbiol. Biotechnol. 26, 1981–1990.
611 Hallin, S., Throbäck, I.N., Dicksved, J., Pell, M., 2006. Metabolic profiles and genetic diversity of

612 denitrifying communities in activated sludge after addition of methanol or ethanol. Appl. Environ.
613 Microbiol. 72, 5445–5452.
614 Helbling, D.E., Johnson, D.R., Lee, T.K., Scheidegger, A., Fenner, K., 2015. A framework for
615 establishing predictive relationships between specific bacterial 16S rRNA sequence abundances
616 and biotransformation rates. Water Res. 70, 471–484.
617 Huston, M.A., 1994. Biological Diversity: the Coexistence of Species. Cambridge University Press.
618 Johnson, D.R., Helbling, D.E., Lee, T.K., Park, J., Fenner, K., Kohler, H.P.E., Ackermann, M., 2015a.
619 Association of biodiversity with the rates of micropollutant biotransformations among full-scale
620 wastewater treatment plant communities. Appl. Environ. Microbiol. 81, 666–675.
621 Johnson, D.R., Lee, T.K., Park, J., Fenner, K., Helbling, D.E., 2015b. The functional and taxonomic
622 richness of wastewater treatment plant microbial communities are associated with each other and
623 with ambient nitrogen and carbon availability. Environ. Microbiol. 17, 4851–4860.
624 Johnson, D.R., Helbling, D.E., Men, Y., Fenner, K., 2015c. Can 613 meta-omics help to establish
625 causality between contaminant biotransformations and genes or gene products? Environ. Sci. Water
626 Res. Technol. 1, 272–278.
627 Joss, A., Zabczynski, S., Göbel, A., Hoffmann, B., Löffler, D., McArdell, C.S., Ternes, T. A.,
628 Thomsen, A., Siegrist, H., 2006. Biological degradation of pharmaceuticals in municipal
629 wastewater treatment: Proposing a classification scheme. Water Res. 40, 1686–1696.
630 Kalyuzhnaya, M.G., Hristova, K.R., Lidstrom, M.E., Chistoserdova, L., 2008. Characterization of a
631 novel methanol dehydrogenase in representatives of Burkholderiales: implications for
632 environmental detection of methylotrophy and evidence for convergent evolution. J. Bacteriol.
633 190, 3817–23.
634 Khunjar, W. O.; Mackintosh, S. A.; Skotnicka-Pitak, J.; Baik, S.; Aga, D. S.; Love, N. G. Elucidating

the relative roles of ammonia oxidizing and heterotrophic bacteria during the biotransformation of 17alpha-Ethinylestradiol and trimethoprim. Environ. Sci. Technol. 2011, 45 (8), 3605−3612. (14)

Li, D., Sharp, J.O., Saikaly, P.E., Ali, S., Alidina, M., Alarawi, M.S., Keller, S., Hoppe-Jones, C., Drewes, J.E., 2012. Dissolved organic carbon influences microbial community composition and diversity in managed aquifer recharge systems. Appl. Environ. Microbiol. 78, 6819–6828.

Li, D., Alidina, M., Ouf, M., Sharp, J.O., Saikaly, P., Drewes, J.E., 2013. Microbial community evolution during simulated managed aquifer recharge in response to different biodegradable dissolved organic carbon (BDOC) concentrations. Water Res. 47, 2421–30.

Lu, H., Chandran, K., Stensel, D., 2014. Microbial ecology of denitrification in biological wastewater treatment. Water Res. 64, 237–254.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D., Verstraete, W., 2008. How to get more out of molecular fingerprints: practical tools for microbial ecology. Environ. Microbiol. 10, 1571–81.

Naether, A., Foesel, B.U., Naegele, V., Wüst, P.K., Weinert, J., Bonkowski, M., Alt, F., Oelmann, Y., Polle, A., Lohaus, G., Gockel, S., Hemp, A., Kalko, E.K. V, Linsenmair, K.E., Pfeiffer, S., Renner, S., Schöning, I., Weisser, W.W., Wells, K., Fischer, M., Overmann, J., Friedrich, M.W., 2012. Environmental factors affect Acidobacterial communities below the subgroup level in grassland and forest soils. Appl. Environ. Microbiol. 78, 7398–406. Ofiteru, I.D., Lunn, M., Curtis, T.P., Wells, G.F., Criddle, C.S., Francis, C.A., Sloan, W.T., 2010. Combined niche and neutral effects in a microbial wastewater treatment community. Proc. Natl. Acad. Sci. 107, 15345–15350.

Orellana, L.H., Rodriguez-R, L.M., Higgins, S., Chee-Sanford, J.C., Sanford, R.A., Ritalahti, K.M., Löffler, F.E., Konstantinidis, K.T., 2014. Detecting nitrous oxide reductase (NosZ) genes in soil metagenomes: method development and implications for the nitrogen cycle. MBio 5, e01193-14.

Painter, M.M., Buerkley, M.A., Julius, M.L., Vajda, A.M., Norris, D.O., Barber, L.B., Furlong, E.T., Schultz, M.M., Schoenfuss, H.L., 2009. Antidepressants at environmentally relevant concentrations affect predator avoidance behavior of larval fathead minnows (Pimephales Promelas). Environ. Toxicol. Chem. 28, 2677.

Pal, L., Kraigher, B., Brajer-Humar, B., Levstek, M., Mandic-Mulec, I., 2012. Total bacterial and ammonia-oxidizer community structure in moving bed biofilm reactors treating municipal wastewater and inorganic synthetic wastewater. Bioresour. Technol. 110, 135e143

Pérez-Pantoja, D., Donoso, R., Agulló, L., Córdova, M., Seeger, M., Pieper, D.H., González, B., 2012. Genomic analysis of the potential for aromatic compounds biodegradation in Burkholderiales. Environ. Microbiol. 14, 1091–1117.

Pholchan, M.K., de Baptista, J.C., Davenport, R.J., Sloan, W.T., Curtis, T.P., 2013. Microbial community assembly, theory and rare functions. Front. Microbiol. 4, 1–9.

Plósz, B.G., 2007. Optimization of the activated sludge anoxic reactor configuration as a means to control nutrient removal kinetically. Water Res. 41, 1763–1773.

Plósz, B.G., Langford, K.H., Thomas, K. V, 2012. An activated sludge modeling framework for xenobiotic trace chemicals (ASM-X): assessment of diclofenac and carbamazepine. Biotechnol. Bioeng. 109, 2757–69.

Polesel, F., Torresi, E., Loreggian, L., Casas, M.E., Christensson, M., Bester, K., Plósz, B.G., 2017. Removal of pharmaceuticals in pre-denitrifying MBBR – Influence of organic substrate availability in single- and three-stage configurations. Water Res. 123, 408–419.

Price, M.N., Dehal, P.S., Arkin, A.P., 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol. Biol. Evol. 26, 1641–50.

Ribeiro, A.R., Afonso, C.M., Castro, P.M.L., Tiritan, M.E., 2013. Enantioselective HPLC analysis and

biodegradation of atenolol, metoprolol and fluoxetine. Environ. Chem. Lett. 11, 83e90.

Roeleveld, P.J., Van Loosdrecht, M.C.M., 2002. Experience with guidelines for wastewater characterisation in The Netherlands. Water Sci. Technol. 45, 77–87.

Sanford, R.A., Wagner, D.D., Wu, Q., Chee-Sanford, J.C., Thomas, S.H., Cruz-García, C., Rodríguez, G., Massol-Deyá, A., Krishnani, K.K., Ritalahti, K.M., Nissen, S., Konstantinidis, K.T., Löffler, F.E., 2012. Unexpected nondenitrifier nitrous oxide reductase gene diversity and abundance in soils. Proc. Natl. Acad. Sci. U. S. A. 109, 19709–14.

Sathyamoorthy S, Chandran K, Ramsburg CA (2013) Biodegradation and cometabolic modeling of selected beta blockers during ammo- nia oxidation. Environ Sci Technol 47:12835–12843

Schloss, P.D., 2009. Introducing mothur: A Computational Toolbox for Describing and Comparing Microbial Communities. Abstr. Gen. Meet. Am. Soc. Microbiol. 109.

Stadler, L.B., Love, N.G., 2016. Impact of microbial physiology and microbial community structure on pharmaceutical fate driven by dissolved oxygen concentration in nitrifying bioreactors. Water Res. 104, 189–199.

Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. Nat Rev Micro 6, 199–210.

Sözen, S., Çokgör, E.U., Orhon, D., Henze, M., 1998. Respirometric analysis of activated sludge behaviour—II. Heterotrophic growth under aerobic and anoxic conditions. Water Res. 32, 476–488.

Tan, D.T., Arnold, W.A., Novak, P.J., 2013. Impact of organic carbon on the biodegradation of estrone in mixed culture systems. Environ. Sci. Technol. 47, 12359–12365.

Torresi, E., Fowler, J.S., Polesel, F., Bester, K., Andersen, H.R., Smets, B.F., Plosz, B.G., Christensson, M., 2016. Biofilm thickness influences biodiversity in nitrifying MBBRs –

Implications on micropollutant removal. Environ. Sci. Technol. 50, 9279–9288.

Torresi, E., Casas, M.E.C., Polesel, F., Pl, B.G., Bester, K., 2017. Impact of external carbon dose on the removal of micropollutants using methanol and ethanol in post-denitrifying moving bed biofilm reactors 108, 95–105.

Villemur, R., Juteau, P., Bougie, V., Ménard, J., Déziel, E., 2015. Development of four-stage moving bed biofilm reactor train with a pre-denitrification configuration for the removal of thiocyanate and cyanate. Bioresour. Technol. 181, 254–262.

Wells, G.F., Wu, C.H., Piceno, Y.M., Eggleston, B., Brodie, E.L., DeSantis, T.Z., Andersen, G.L., Hazen, T.C., Francis, C.A., Criddle, C.S., 2014. Microbial biogeography across a full-scale wastewater treatment plant transect: Evidence for immigration between coupled processes. Appl. Microbiol. Biotechnol. 98, 4723–4736.

Xia, S., Li, J., Wang, R., Li, J., Zhang, Z., 2010. Tracking composition and dynamics of nitrification and denitrification microbial community in a biofilm reactor by PCR-DGGE and combining FISH with flow cytometry. Biochem. Eng. J. 49, 370–378.

Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89, 670–9.

Zavaleta, E.S., Pasari, J.R., Hulvey, K.B., David Tilman, G., 2010. Sustaining multiple ecosystem functions in grassland communities requires higher biodiversity. Proc. Natl. Acad. Sci. 104, 1443–1446.

Zhang, Y., Ji, G., Wang, R., 2016. Drivers of nitrous oxide accumulation in denitrification biofilters with low carbon:nitrogen ratios. Water Res. 106, 79–85.

Ødegaard, H., 1999. The Moving Bed Biofilm Reactor. Water Environ. Eng. Reuse Water 250–305.

Tables

Table 1. Results from qPCR targeting 16S rRNA and functional genes in the four MBBRs (S1, S2, S3, U). Values result from the average of the last four sampling days (218, 300, 434 and 471) after microbial community stabilization according to MWA. Values are reported with the corresponding standard deviation (n=8). Statistical differences ($p<0.05$) were estimated according to one way analysis (ANOVA).

	S1	**S2**	**S3**	**U**
16S rRNA (copies/$g_{biomass}$)	2.13×10^{11} ± 44%	1.57×10^{11} ± 16%	7.24×10^{10} ± 7%(1)	2.2×10^{11} ± 19%
narG (%)*	48 ±20	22 ± 7(2)	53 ±22	27 ± 9
nirS (%)*	58 ± 10	54 ± 9	63 ± 6	64 ± 9
nirK (%)*	8 ± 4	12 ± 7	11 ± 5	11 ± 7
nirS/nirK	6.9 ± 3	8.3 ± 3	6.3 ± 3	10.9 ± 11
nosZ_typ (%)*	9 ± 3	9 ± 1	9 ± 1	9 ± 2
nosZ_atyp (%)*	6 ± 1	8 ± 5	15± 8(3)	11 ± 5
nosZ_atyp/nosZ_typ	0.72 ± 0.23	0.93 ± 0.53	1.60 ± 0.71(3)	1.36 ± 0.62
(nirK+nirS)/ (nosZ_atyp+nosZ_typ)	4.68+1.14(4)	3.86+0.59	3.51+1.11	3.96+0.70

* % of 16S rRNA gene abundance

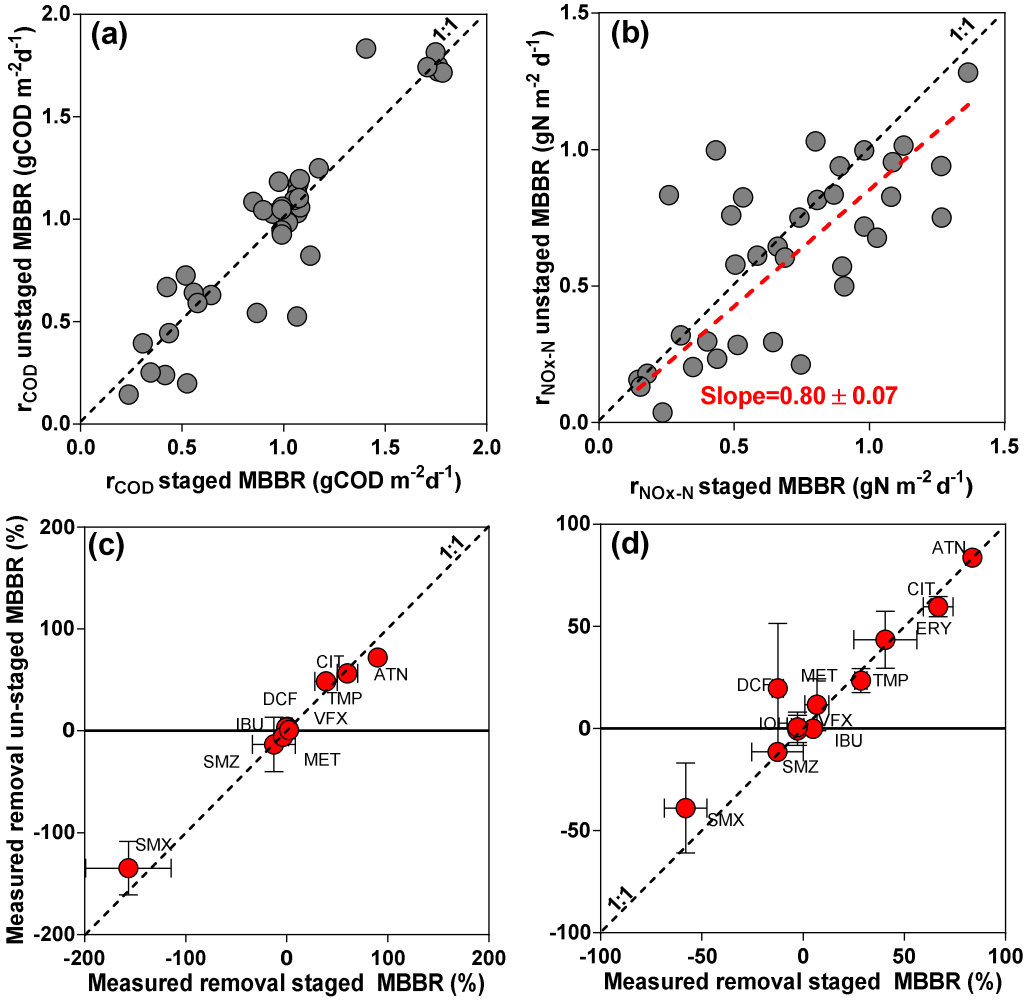
(1) significantly lower than S1 and U (95% confidence interval)

(2) significantly lower than S1 and S3 (95% confidence interval)

(3) significantly higher than S1 and S2 (95% confidence interval)

737 [4] significantly higher than S3 (95% confidence interval)

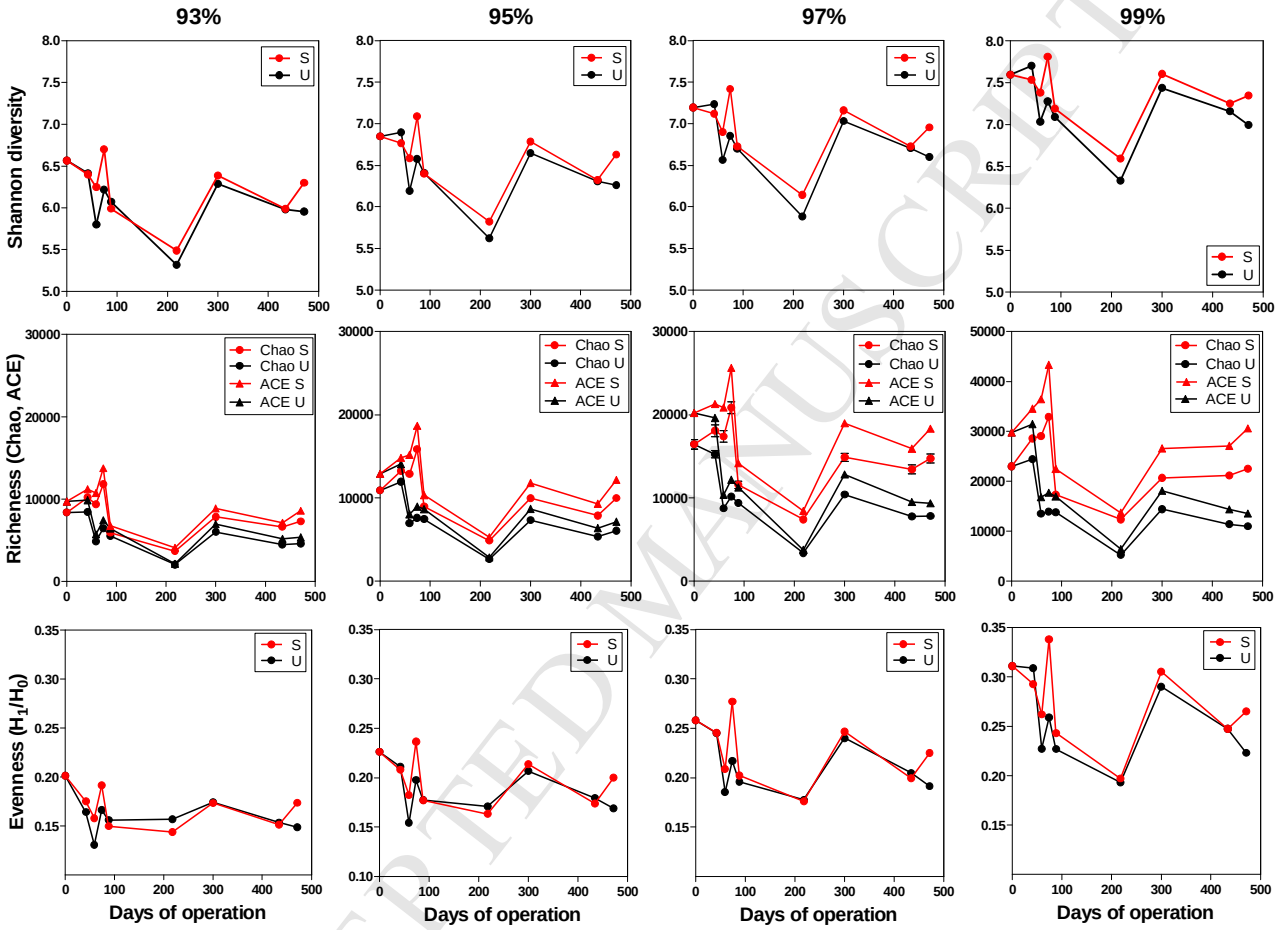
738 **Figures**



739

740 **Figure 1.** Measured data from continuous-flow operation. Comparison between COD removal rates (r_{COD},
741 gCOD d^{-1} m^{-2}) (a) and denitrification rate (r_{NOx-N}, gN m^{-2} d^{-1}) (b) in the three-(S) and single-stage (U) MBBR
742 configuration (n=40); comparison between micropollutant removal (%) in S and U in the first (c, ~100 days of
743 operation) and second (d, ~470 days of operation) campaigns (Polesel et al., 2017). Dashed red line in (b) shows
744 linear regression (slope 0.80 ± 0.07, p<0.001). Abbreviations: ATN = atenolol; CIT = citalopram; TMP =

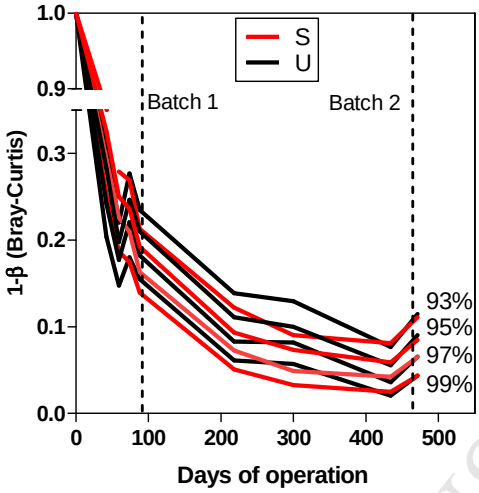
745 trimethoprim; DCF = diclofenac; IBU= ibuprofen; MET = metoprolol; SMX = sulfamethoxazole; SMZ =
746 sulfamethizole; VFX = venlafaxine; ERY = erythromycin; IOH = iohexol.



747
748

749 **Figure 2.** Shannon, richness (Chao and ACE) and evenness (H_1/H_0) indices measured at different time points for
750 the three-(S) and single-stage (U) MBBR configuration at 93, 95, 97 99% of sequencing similarity**.** Error bars
751 define standard errors.

752



753

754 **Figure 3.** Moving window analysis (MWA) using reciprocal of Bray-Curtis indices (β) from the initial biofilm

755 inoculum measured at different sequence similarity (93-99%) for S and U over 471 days of operation.

756

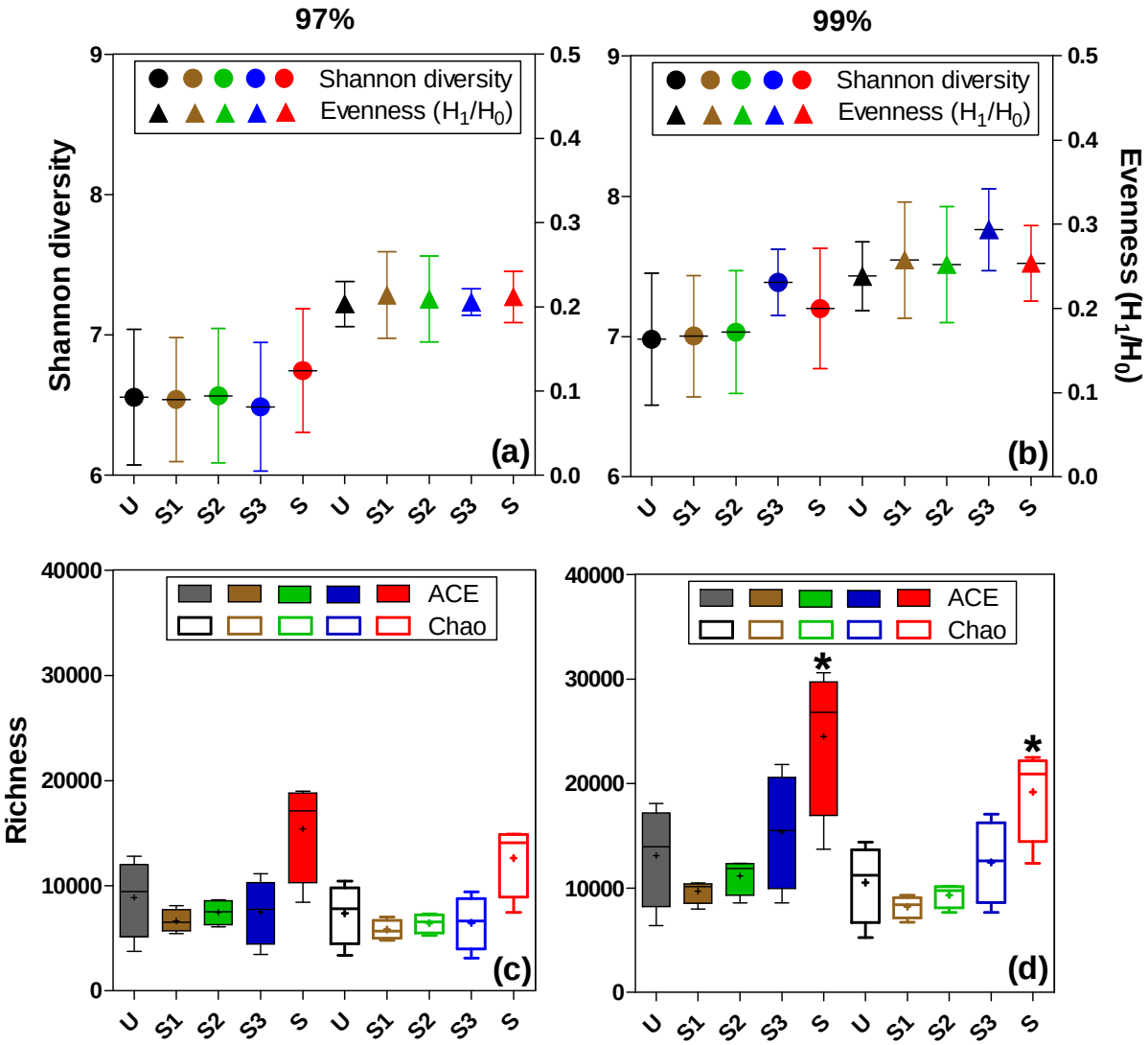


Figure 4. Averaged values of Shannon diversity, evenness, ACE and Chao indices after 200 days of operation (n=4) at 97% (a, c) and 99% (b, d) sequence similarity cut-offs for the three stages MBBR at local (S1, S2, S3) and system (S) level and the single stage system (U). Asterisks indicate significance difference (ANOVA, $p<0.05$). Mean is shown as +.

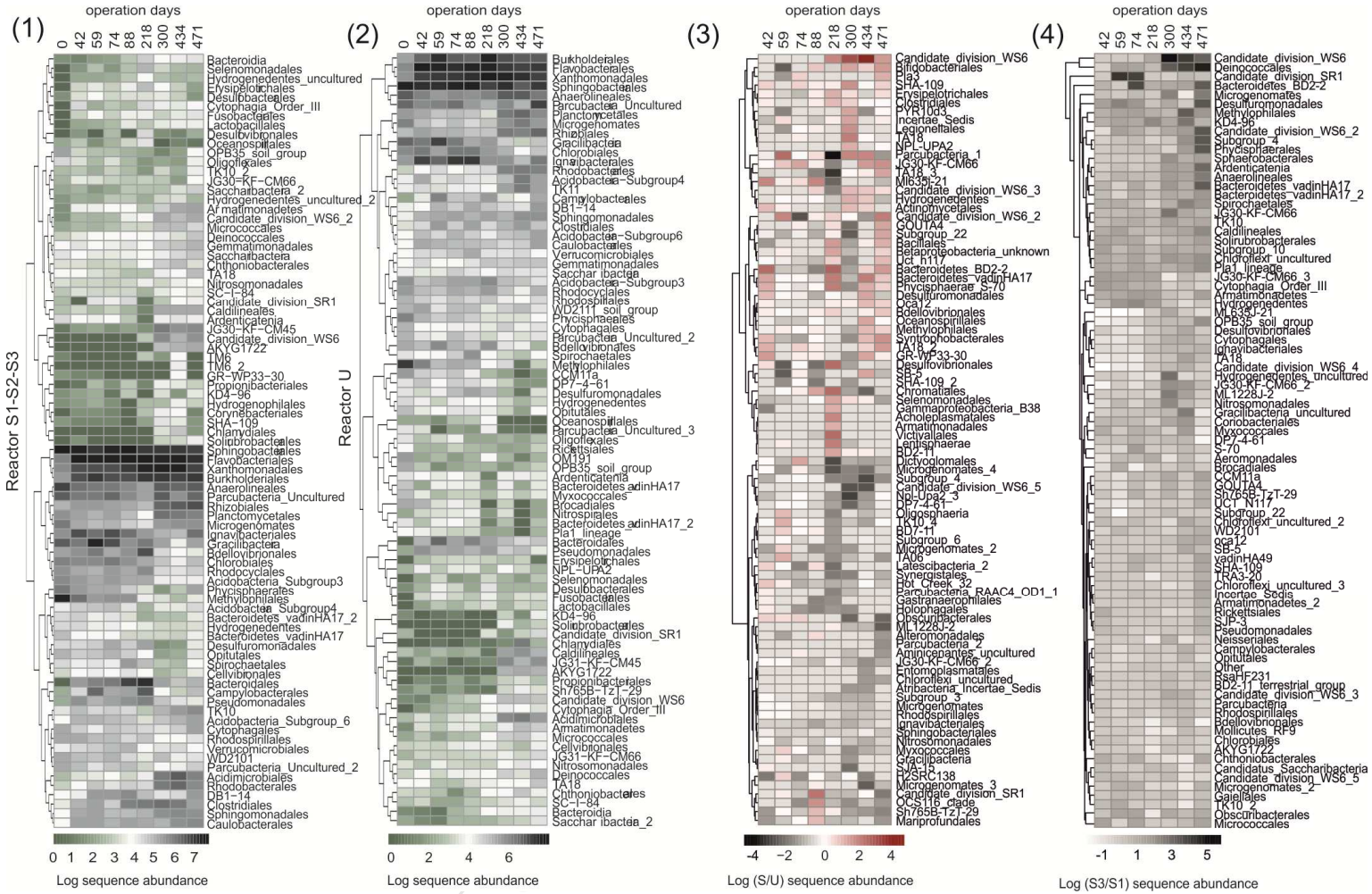
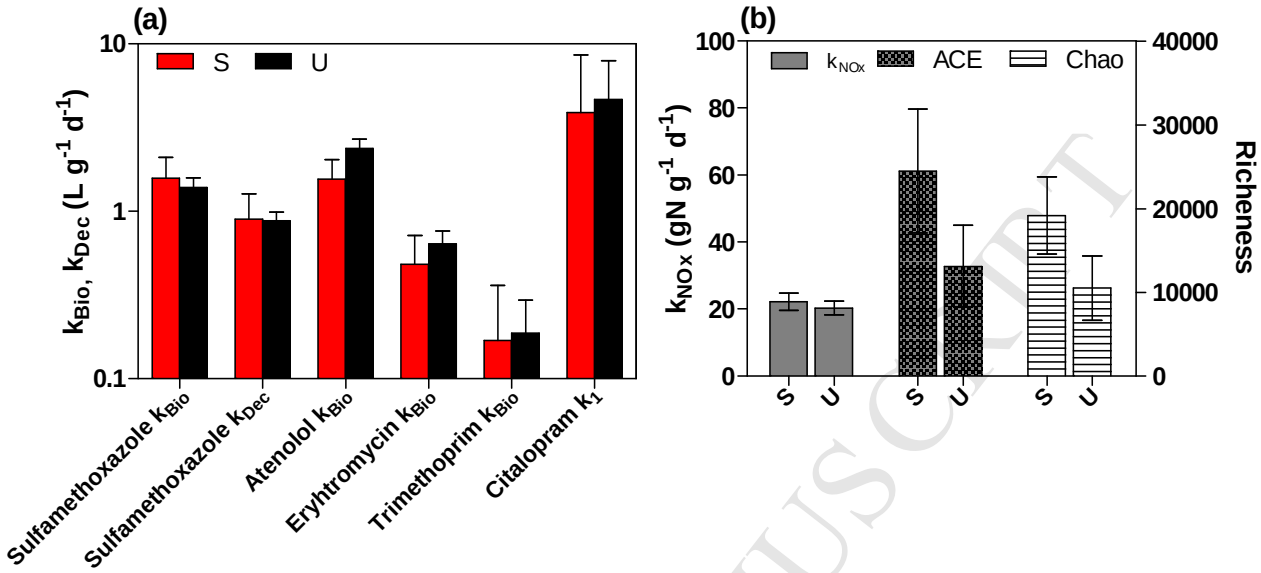


Figure 5. Heatmaps of the 100 most abundant order level taxa in the staged MBBR, S (a) and un-staged MBBR, U (b). The most shifted abundant taxa (expressed as log sequence abundance) of S and S3 were selected to perform the ratio of S/U (c) and S1/S3 (d) to effectively identify the selected taxa in S compared to U, and S3 compared to S1

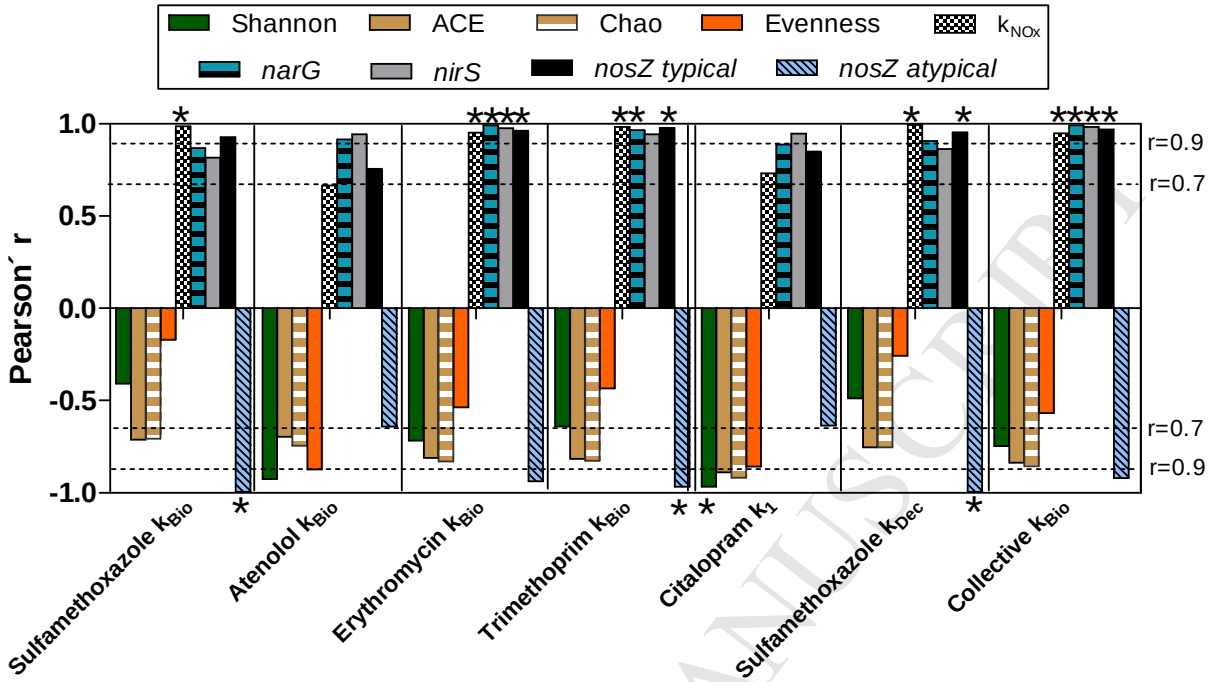


767
768

769 **Figure 6.** Biotransformation and retransformation rate constants k_{Bio} and k_{Dec} (L g^{-1} d^{-1}) for each micropollutant
770 (a) and specific denitrification rate (k_{NOx}), ACE and Chao indices (b) calculated at system level for S for U
771 (Polesel et al., 2017) MBBR in Batch 2. Abbreviations: k_1, biotransformation rate constant of enantiomer 1.

772

773



774
775 **Figure 7.** Pearson´s coefficient (r) of the correlation between biotransformation (k_{Bio}), retransformation (k_{Dec}) of
776 micropollutant, collective k_{Bio} with Shannon biodiversity, richness (ACE and Chao), evenness indices (at 99%
777 sequences similarity) and specific denitrification rate k_{NOx} (mgN g^{-1} d^{-1}) for Batch 2. Asterisks indicate
778 significance (ANOVA, $p<0.05$).

779

780

Highlights

- Microbial composition and diversity in single- and three stage MBBR investigated
- Reactor staging increased overall microbial richness and evenness in staged MBBR
- Microbial diversity increased with decreasing carbon loading in staged MBBR
- Biotransformation of pharmaceuticals positively correlated with denitrifying genes abundance
- Biotransformation of pharmaceuticals did not correlate with microbial diversity