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Torresi, Elena; Fowler, Jane; Polesel, Fabio; Bester, Kai; Andersen, Henrik Rasmus; Smets, Barth F.; Plósz, Benedek G.; Christensson, Magnus

Published in:
Environmental Science and Technology

Link to article, DOI:
[10.1021/acs.est.6b02007](https://doi.org/10.1021/acs.est.6b02007)

Publication date:
2016

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Torresi, E., Fowler, J., Polesel, F., Bester, K., Andersen, H. R., Smets, B. F., Plósz, B. G., & Christensson, M. (2016). Biofilm Thickness Influences Biodiversity in Nitrifying MBBRs-Implications on Micropollutant Removal. *Environmental Science and Technology*, 50(17), 9279-9288. <https://doi.org/10.1021/acs.est.6b02007>

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1 **Biofilm thickness influences biodiversity in nitrifying MBBRs**
2 **– Implications on micropollutant removal**

3 Torresi, E.^{1,2,*}, Fowler, S.J.¹, Polesel, F.¹, Bester, K.³, Andersen, H.R.¹, Smets, B.F.¹, Plósz, B.G.^{1,*},
4 Christensson, M.².

5 ¹ Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet B115, 2800 Kgs.
6 Lyngby, Denmark

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

8 ³ Department of Environmental Science, Aarhus University, Frederiksborgvej 399, 4000 Roskilde, Denmark

9
10 * *Corresponding authors:*

11 Email: elto@env.dtu.dk; Phone: +45 45251474

12 beep@env.dtu.dk; Phone: +45 45251694

14 **Abstract**

15 In biofilm systems for wastewater treatment (e.g., moving bed biofilms reactors—MBBRs) biofilm
16 thickness is typically not under direct control. Nevertheless, biofilm thickness is likely to have a
17 profound effect on the microbial diversity and activity, as a result of diffusion limitation and thus
18 substrate penetration in the biofilm.

19 In this study, we investigated the impact of biofilm thickness on nitrification and on the removal of
20 more than 20 organic micropollutants in laboratory-scale nitrifying MBBRs. We used novel carriers
21 (Z-carriers - AnoxKaldnesTM) that allowed controlling biofilm thickness at 50, 200, 300, 400, and
22 500 μm . The impact of biofilm thickness on microbial community was assessed via 16S rRNA gene
23 amplicon sequencing and ammonia monooxygenase (*amoA*) abundance quantification through
24 quantitative PCR (qPCR). Results from batch experiments and microbial analysis showed that: (i)
25 the thickest biofilm (500 μm) presented the highest specific biotransformation rate constants (k_{bio} , L
26 $\text{g}^{-1} \text{d}^{-1}$) for 14 out of 22 micropollutants; (ii) biofilm thickness positively associated with
27 biodiversity, which was suggested as the main factor for the observed enhancement of k_{bio} ; (iii) the
28 thinnest biofilm (50 μm) exhibited the highest nitrification rate ($\text{gN d}^{-1} \text{g}^{-1}$), *amoA* gene abundance
29 and k_{bio} values for some of the most recalcitrant micropollutants (i.e., diclofenac and targeted
30 sulfonamides). Although thin biofilms favored nitrification activity and the removal of some
31 micropollutants, treatment systems based on thicker biofilms should be considered to enhance the
32 elimination of a broad spectrum of micropollutants.

33

34

35 **Introduction**

36 The presence of micropollutants in the effluents of municipal wastewater treatment plants
37 (WWTPs) is well documented^{1,2} and has received increased attention due to the potential threat that
38 they pose to environmental recipients³. Optimization of biological wastewater treatment
39 technologies has been explored to improve removal of micropollutants in WWTPs⁴ and to minimize
40 the use of advanced tertiary treatment processes. Laboratory and full-scale studies have previously
41 demonstrated enhanced micropollutant removal under nitrifying conditions⁵⁻⁹, which was associated
42 with non-specific cometabolic activity of the ammonia monooxygenase gene (*amoA*) by ammonia
43 oxidizing bacteria (AOB)^{7,8,10}. On the other hand, the enrichment of nitrifying bacteria is generally
44 linked to a longer solid retention time (SRT) compared to heterotrophic bacteria and a positive
45 association between SRT and micropollutant removal was observed in a number of studies¹¹⁻¹⁷.
46 Increased biotransformation potential at longer SRT was hypothesized to be induced by an
47 enrichment of slow growing bacteria and by the increased diversity of “microbial specialists” able
48 to biotransform the recalcitrant chemical structure possessed by many micropollutants¹⁷.

49 Over the past two decades, research has shown the importance of biodiversity in biological
50 systems¹⁹⁻²², and microbial communities with higher richness (the number of species in a
51 community) were found to have higher functionality and stability than microbial communities with
52 lower richness¹⁹⁻²². However, biodiversity is a complex concept which includes species richness
53 and evenness (the relative abundance of the species)²³. While most studies focus on microbial
54 richness, Wittebolle et al.²³ demonstrated that highly uneven communities (dominated by one or
55 few species) can be less resistant to environmental stress than more even communities. Johnson et
56 al.¹⁸ further showed that both richness and evenness are positively associated with the removal of
57 some micropollutants in full scale wastewater treatment.

58 Based on these observations, biofilm systems exhibiting longer SRT (due to enhanced physical
59 retention) and potentially higher biodiversity than conventional activated sludge (CAS) can
60 represent an option to enhance micropollutant removal. Among biofilm systems, moving bed
61 biofilm reactors (MBBRs) seem to be a promising alternative compared to CAS for the elimination
62 of recalcitrant micropollutants, e.g. diclofenac and X-ray contrast media²⁴⁻²⁶.

63 MBBRs, in which biofilm is grown on specifically designed plastic carriers²⁷, are usually operated
64 without direct control of biofilm thickness. However, biofilm thickness can potentially impact
65 biofilm structure and activity. The diffusive transport of substrates, in particular oxygen, from the
66 bulk liquid into the biofilm is the major rate-limiting process in MBBR^{28,29}, thereby creating

67 substrate gradients through the biofilm³⁰. Increasing biofilm thickness thus results in greater
68 concentration gradients and stratification of metabolic processes throughout the biofilm, likely
69 leading to a more heterogeneous and biodiverse biofilm. However, it is presently unclear how
70 biofilm thickness influences biodiversity and functionality (e.g., micropollutant removal) in biofilm
71 systems, partly due to a lack of technology enabling controlled biofilm thickness.
72 Therefore, the objectives of the present study were: (i) to investigate the impact of biofilm thickness
73 on nitrification and on the removal of 22 micropollutants in laboratory-scale nitrifying MBBRs by
74 using novel designed carriers (Z-carriers - AnoxKaldnesTM), which allowed the development of
75 biofilms of five different thicknesses (50, 200, 300, 400 and 500 μm); (ii) to assess how biofilm
76 thickness influence the diversity of microbial communities in terms of richness and evenness; (iii)
77 to evaluate relationships between biofilm activity (i.e., nitrification), biodiversity and
78 micropollutant biotransformation. Overall, this study aims at optimizing the efficiency of biofilm
79 systems towards micropollutant removal by discriminating between the effects of using thin vs
80 thicker biofilms during operation of biofilm based technologies.

81

82 **Material and methods**

83 **Description of the Z-carriers and controlled biofilm thickness.**

84 To obtain biofilms of different thicknesses, newly designed carriers from AnoxKaldnesTM (Z-
85 carriers) were used (Figure S1 in Supporting Information, SI). The Z-carriers are made of
86 polyethylene and, unlike the conventional MBBR carriers, have a saddle shaped grid covered
87 surface, which allows the biofilm to grow on the outside of the carrier rather than in an inside void,
88 as in e.g., the K1-type carrier²⁹. As the carriers continuously scrape against each other during
89 reactor operation, the height of the grid wall corresponds to the maximum biofilm thickness.
90 Five different Z-carriers (named Z50, Z200, Z300, Z400 and Z500) were used in the experiment,
91 with the numbers indicating the grid wall height (equal to the controlled biofilm thickness) in μm .
92 Except for the grid wall height, the Z-carriers Z200, Z300, Z400 and Z500 are identical in design,
93 and thus the exposed biofilm area is expected to be the same. Notably, the Z50 type carriers differ
94 slightly from the other Z-carriers by having a flat shape and 10% lower surface area (Table S1).
95 Although determining exact biofilm thickness requires detailed measurements, the design of the Z-
96 carriers enables a fairly precise control of the biofilm thickness solely by its design²⁹. Further details
97 on the Z-carriers used in this study can be found in Table S1.

98 **MBBRs configuration.**

99 **Continuous-flow operation.**

100 The laboratory-scale experiment was conducted in two parallel aerobic MBBRs continuously
101 operated using Z-carriers. Reactor 1 (R1) had a working volume of 3 L, containing 200 carriers of
102 each type (Z200, Z300, Z400, Z500) with a total exposed surface area of 1.02 m² (Table S1).
103 Reactor 2 (R2) had a volume of 1.5 L, containing 293 Z50 carriers with a total exposed surface area
104 of 0.33 m². To enable differentiation between the 5 types of Z-carriers, they were produced in
105 different colors. The enrichment of nitrifying biofilm was performed by feeding the reactors (in
106 continuous-flow mode) with effluent wastewater from a local municipal treatment plant (Källby,
107 Lund, Sweden), spiked with ammonium (50 mg L⁻¹ of NH₄-N as NH₄Cl) and phosphorus (0.5 mg L⁻¹
108 of PO₄-P as KH₂PO₄). For details about the start-up of the MBBR systems, readers should refer to
109 S1 in SI. Hydraulic residence time (HRT) after the start-up procedure was kept equal to 2 hours for
110 both reactors. Temperature was set at 20 °C using a thermostat bath and pH was kept at 7 ± 0.5 by
111 using 400 mg L⁻¹ as CaCO₃ of alkalinity (in the form of NaHCO₃) and sodium hydroxide (20 mg L⁻¹
112 ¹). Aeration intensity was set so that an average dissolved oxygen concentration (DO) of 4.5 ± 0.5
113 mg L⁻¹ could be maintained in both biofilm reactors. Thus, R1 and R2 reactors were fed using the
114 same influent quality and with identical operational conditions (i.e., HRT, DO, temperature).
115 MBBR R2 was initiated 45 days after the start-up of MBBR R1 as Z50 carriers were produced at a
116 later time. In order to maintain the same HRT and filling ratio as R1, the volume of R2 is different.
117 Samples were analyzed for bulk chemicals (NH₄-N, NO₃-N, NO₂-N, COD, alkalinity, PO₄³⁻)
118 semiweekly. R1 and R2 were operated for approximately 300 days under continuous-flow operation
119 (Figure S2).

120 **Batch operation.**

121 Two different batch experiments to assess micropollutant removal were performed (Figure S2).
122 Batch experiment 1 was performed with five different types of Z-carriers after reaching stable
123 ammonia removal (at day 168 for R1 and at day 123 for R2) and using the exact same feed as in
124 continuous operation (batch-feed 1) without micropollutant spiking. Batch experiment 2 was done
125 using Z50, Z200 and Z500 at day 275 and 230 of operation for R1 and R2 respectively. Batch
126 experiments were done using batch-feed 1 with additional spiking of 23 micropollutants with an
127 initial concentration of 1 µg L⁻¹ for most of the compounds and of 15 µg L⁻¹ for the X-ray contrast
128 media as they are usually found at higher concentrations in the effluent wastewater³¹.
129 Micropollutants were added from a stock solution (40 mg L⁻¹) containing the chemical compounds

130 dissolved in methanol. To minimize the increase of organic substrates in the nitrifying system,
131 micropollutant stock solutions were first spiked into an empty glass beaker and methanol was
132 allowed to evaporate in the fumehood for approximately 1 hour. Prior batch experiments, the
133 continuous-flow systems R1 and R2 were disconnected, the five types of Z-carriers (200 each) were
134 manually separated through color recognition and placed in separated batch glass reactors
135 (operating volume of 1 L). Samples (n= 12) for micropollutant analysis and nitrogen species were
136 taken at regular intervals (Table S2) from the reactors over 24 h. To maintain the same biomass
137 concentration over the duration of the experiment, three carriers were withdrawn from the reactors
138 each time a sample (14 mL) was taken for analysis. pH and DO were continuously monitored and
139 manually adjusted to 7.5 (using pH buffer) and 4.5 mg L⁻¹, respectively, during the experiment. An
140 additional reactor was used as a control experiment to assess abiotic degradation of micropollutants.
141 The experiment was divided into two parts as previously proposed²⁵, (i) without plastic carriers and
142 using only filtered (with 0.2 µm pore size Munktell MG/A glass fiber filter) effluent wastewater to
143 assess abiotic degradation and sorption onto glass walls; and (ii) with new carriers added to filtered
144 effluent wastewater to investigate sorption onto plastic carriers (Table S2). Batch sorption
145 experiments were also performed with biomass inhibition by using allythiourea (10 mg L⁻¹) and
146 sodium azide (0.5 mg L⁻¹) to estimate the sorption coefficient K_d. A description of the experimental
147 method and K_d values are presented in S2.

148

149 **Chemicals.**

150 Twenty-three environmentally relevant micropollutants were selected for this study, which included
151 some of the most frequently detected pharmaceuticals in wastewater effluents³¹. Furthermore, to
152 investigate possible trends among groups of pharmaceuticals, the targeted pharmaceuticals were
153 grouped in six categories according to their use. The micropollutants included: (i) four beta-blocker
154 pharmaceuticals atenolol (ATN), metoprolol (MET), propranolol (PRO) and sotalol (SOT); (ii) five
155 X-ray contrast media diatrizoic acid (DIA), iohexol (IOH), iopamidol (IOP) iopromide (IOPR),
156 iomeprol (IOM); (iii) sulfonamide antibiotics sulfadiazine (SDZ), sulfamethizole (SMZ) and
157 sulfamethoxazole (SMX), one combination product, trimethoprim (TMP), and one metabolite
158 acetyl-sulfadiazine (AcSDZ); (iv) three anti-inflammatory pharmaceuticals phenazone (PHE),
159 diclofenac (DCF), ibuprofen (IBU); (v) three anti-epileptic/anti-depressants carbamazepine (CBZ),
160 venlafaxine (VFX) and citalopram (CIT); (vi) three macrolide antibiotics, erythromycin (ERY),

161 clarithromycin (CLA) and roxithromycin (ROX). For information regarding CAS numbers and
162 chemical suppliers, the reader should refer to Escolà Casas et al.²⁴.

163

164 **Analytical methods.**

165 Samples taken for analysis of conventional pollutants (NH₄-N, NO₃-N, NO₂-N, COD, alkalinity,
166 PO₄³⁻) were filtered through 0.45 µm glass fiber filters (Sartorius, Göttingen, Germany). Hach
167 Lange kits (LCK 303, LCK 339, LCK 341 and LCK 342) were used and analyzed using a
168 spectrophotometer (Hach Lange DR2800).

169 The attached biomass concentrations were calculated from the difference in weight of 3 dried
170 carriers (105 °C for >24 h) before and after biofilm removal (using 2M H₂SO₄ with subsequent
171 brushing), as previously considered^{24,32,33}. These results were used to normalize the nitrification and
172 biotransformation rate constants. The volatile suspended solids (VSS) measurement, needed to
173 normalize the results from microbial characterization, was conducted by scraping and dissolving the
174 attached biofilm in tap water and measured according to APHA standard methods³⁴. Micropollutant
175 concentrations in the liquid phase were analyzed by sampling 4 ml of water sample from each
176 reactor with a glass pipette. Micropollutants were determined successively by direct injection to
177 HPLC-MS/MS as described by Escolà Casas et al.²⁴ and as reported in supplementary information
178 (S3). Information regarding HPLC-MS/MS and mass spectrometry data, limit of detection (LOD)
179 and quantification (LOQ) of compounds is shown in Escolà Casas et al.²⁴.

180

181 **Nitrification.**

182 Nitrification rates were calculated as (i) ammonia uptake rate per gram of attached biomass $r_{\text{NH}_4\text{-B}}$
183 (gN-NH₄⁺ d⁻¹ g⁻¹) and (ii) ammonia uptake rate per carrier surface area $r_{\text{NH}_4\text{-S}}$ (gN-NH₄⁺ d⁻¹ m⁻²).
184 Nitrification rates were derived through linear regression of NH₄⁺-N concentration during batch
185 experiments under non-limiting ammonia conditions (NH₄⁺ > 20 mg L⁻¹). To estimate kinetic
186 parameters a 1-D two-step nitrification biofilm model, including growth and decay of ammonia
187 (AOB) and nitrite oxidizing bacteria (NOB), was implemented in Aquasim 2.1d³⁵. Maximum
188 specific growth rates for AOB and NOB ($\mu_{\text{max,AOB}}$ and $\mu_{\text{max,NOB}}$), and affinity constants for
189 ammonium and nitrite ($K_{\text{NH}_4\text{-AOB}}$ and $K_{\text{NO}_2\text{-NOB}}$) were estimated by considering values of yield
190 coefficient (Y_{AOB} , Y_{NOB}) derived from literature³⁶. The parameters were estimated by calibrating the
191 model to measured concentrations of NH₄⁺-N, NO₂⁻-N and NO₃⁻-N during batch experiments,

192 attached biomass concentration and estimated AOB and NOB fractions from microbial analysis
193 (qPCR) to define initial conditions. Details of the model are presented in S4.

194

195 **Micropollutant biotransformation.**

196 Model structure to describe micropollutant removal in batch 1 and 2, were identified using the
197 Activated Sludge Model framework for Xenobiotics (ASM-X)^{16,37,38}. Accordingly, pseudo first-
198 order biotransformation rate constants k_{bio} ($L\ gTSS^{-1}\ d^{-1}$), biomass normalized, were calculated
199 according to Eq.1:

200

$$201 \frac{dC_{LI}}{dt} = - \frac{k_{bio}}{(1+K_d X)} C_{LI} X \quad (1)$$

202 where C_{LI} denotes the aqueous micropollutant concentration ($ng\ L^{-1}$) measured in the reactor and X
203 the attached biomass concentration on Z-carriers ($g\ L^{-1}$). Sorption onto biofilm can influence the
204 availability of the aqueous micropollutant for biodegradation³⁴ and sorption coefficient K_d ($L\ g^{-1}$)
205 was included in Eq. 1, by assuming instantaneous sorption equilibrium^{39,40}. Biotransformation rate
206 constants k_{bio} were estimated from the measured data using least-square optimization without
207 weighting with GraphPad Prism 5.0.

208 A retransformation-biotransformation model was developed and retransformation rates k_{Dec} ($L\ g^{-1}\ d^{-1}$)
209 were estimated using the secant method embedded in Aquasim 2.1d³⁵ according to Eq. 2:

$$210 \frac{dC_{LI}}{dt} = - \frac{k_{bio}}{(1+K_d X)} C_{LI} X + k_{Dec} C_{CJ} X \quad (2)$$

211 where C_{CJ} accounts for the fraction of micropollutant present as e.g., conjugate undergoing
212 retransformation to the parent compound. Further details of biokinetics estimation are presented in
213 S5. As the estimation of micropollutants biokinetics considers the total amount of attached biomass,
214 we note that the estimated k_{bio} lumps biotransformation by nitrifying and heterotrophic bacteria,
215 which were subsequently estimated using qPCR. The effect of diffusion into biofilm on the removal
216 of micropollutants from bulk aqueous phase was lumped in the biotransformation rate constants, as
217 previously considered^{24-26,40}.

218 Biotransformation rate constants normalized to the surface area of the MBBR, k_S ($m^{-2}\ d^{-1}$) were
219 calculated to compare the performance of the three MBBR batch systems, regardless of biomass
220 concentration.

221

222 **DNA extraction and qPCR.**

223 Duplicates of biomass samples for each Z-carrier were collected before batch 2 and stored in
224 sterilized Eppendorf tubes at -20 °C. Biomass was detached using a sterile brush (Gynobrush,
225 Dutscher Scientific, United Kingdom) and tap water, the sample was centrifuged (10000 rpm for 5
226 minutes), and the supernatant was removed. The collected biomass was subject to DNA extraction
227 using the MP FastDNA™ SPIN Kit (MP Biomedicals LLC., Solon, USA) following manufacturer's
228 instructions. The concentration and purity of extracted DNA were measured by spectrophotometry
229 (NanoDrop Technologies, Wilmington, DE, USA). Quantitative PCR (qPCR) targeting 16S rRNA
230 and functional genes was carried out according to Pellicer-Nàcher et al.⁴¹ to estimate the abundance
231 of total bacteria (EUB), ammonia oxidizing bacteria (AOB, based on 16S rRNA-gene and *amoA* -
232 gene), ammonia oxidizing archaea (*amoA*- gene), and nitrite oxidizing bacteria (NOB, *Nitrospira*
233 *spp.* and *Nitrobacter spp.*, based on 16S rRNA- gene). Primers are reported in Table S4.

235 **16S rRNA gene amplification, sequencing and bioinformatic analysis.**

236 PCR amplification and sequencing were performed at the DTU Multi Assay Core Center (Kgs
237 Lyngby, DK). Briefly, DNA was PCR amplified using 16S rRNA bacterial gene primers PRK341F
238 (5'- CCTAYGGGRBGCASCAG-3') and PRK806R (5'-GGACTACNNGGGTATCTAAT-3')⁴²
239 targeting the V3 and V4 region. The thermocycling protocol is reported in S6. PCR products were
240 purified using AMPure XP beads (Beckman-Coulter) prior to index PCR (Nextera XT, Illumina)
241 and sequencing by Illumina MiSeq. Paired-end reads were assembled and low quality sequencing
242 reads were removed using mothur⁴³. Taxonomic assignment and calculation of alpha diversity
243 metrics (Shannon, ACE and Chao extrapolated richness) was performed in mothur using the RDP
244 reference taxonomy. To identify the relative fraction of aerobic and anaerobic bacteria, sequences
245 were clustered at the family level and their electron acceptor preference based on literature^{44,45}.
246 Additional diversity indices were calculated according to Hill⁴⁶. Microbial evenness was estimated
247 as H_1/H_0 as described in Johnson et al¹⁸.

248

249

250

251

252

253 **Statistical analysis.**

254 The statistical methods used comprise: (i) Pearson correlation analysis (parametric test) to assess
255 possible association between k_{bio} of individual micropollutant and biofilm thickness; (iii) one-way
256 analysis of variance (ANOVA) with non-parametric test (Kruskal-Wallis test) to evaluated
257 significance difference between parameters of alpha-diversity measured for the different biofilm
258 thicknesses. The statistical analysis was computed in Prism 5.0. In addition, non-parametric rank
259 correlation and permutation tests were performed (see section S10 for details).

260

261

262 **Results and discussion**

263 **Continuous-flow operation of the MBBR systems.**

264 During the start-up phase, which lasted approximately 80 d for R1, and 50 d for R2, the gradual
265 increase of the ammonium loading resulted in a rapid increase of nitrification rate for the two
266 MBBRs (Figure S4). After 88 and 50 d of operation, R1 and R2 reached an average ammonium
267 removal rate of $1.88 \pm 0.22 \text{ gN d}^{-1} \text{ m}^{-2}$ and $2.38 \pm 0.47 \text{ gN d}^{-1} \text{ m}^{-2}$ respectively, which stabilized to
268 an average value of $2.21 \pm 0.23 \text{ gN d}^{-1} \text{ m}^{-2}$ for the duration of the experiment. Removal of the
269 fraction of biodegradable COD from the effluent wastewater ($\sim 10 \text{ mg L}^{-1}$ averaged concentration)
270 was less than 11% in R1 and 8% in R2, suggesting the presence of active heterotrophic biomass
271 which was subsequently estimated by qPCR (Table S5). Nitrite production was negligible ($< 0.1 \text{ gN}$
272 $\text{d}^{-1} \text{ m}^{-2}$) in both reactors with the exception of two sampling points where failure in the system
273 resulted in temporary nitrite accumulation with subsequent performance recovery within 5 d.
274 Measurements performed on the Z-carriers at different days of operation revealed increased
275 attached biomass concentration with increased biofilm thickness, with the biomass concentration in
276 Z500 being approximately 4.5- fold higher than in Z50 (Figure S5).

277

278 **Influence of biofilm thickness on nitrification.**

279 Two sets of batch experiments (batch 1 and batch 2) were used to assess biokinetics of nitrification
280 and micropollutant removal (Figure S2). Here we present results only from batch 2, whereas results
281 from batch 1 (where no micropollutant spiking was performed) are reported in Figure S7 and S12.

282 The nitrification rates $r_{\text{NH}_4\text{-B}}$ ($\text{gN-NH}_4^+ \text{d}^{-1} \text{g}^{-1}$) obtained from linear regression of ammonium
283 concentration profiles (Figure S6) and normalized by biomass concentration were 3- and 4-fold
284 higher in Z50 compared to Z200 and Z500 respectively (Figure 1 A). Likewise, nitrification rates
285 normalized by surface area $r_{\text{NH}_4\text{-S}}$ ($\text{gN-NH}_4^+ \text{d}^{-1} \text{m}^{-2}$) were found higher for Z50 compared to the
286 other biofilm thicknesses although with marginal difference. We also observed that an increase of
287 biofilm thickness beyond 200 μm did not result in any significant increase in nitrification rates.
288 Similar results were previously found through 1-D biofilm modeling of nitrifying MBBR
289 demonstrating that biofilm thickness over approximately 200 μm did not influence effluent
290 ammonium concentrations⁴⁷. Higher nitrification rates for thinner biofilms (20-30 μm) has
291 previously been hypothesized to be a result of a more active aerobic upper biofilm layer compared
292 to thicker biofilms where accumulation of less-active biomass occurs in deeper layers⁴⁸. Less
293 diffusion limitation in the thin biofilms could also lead to nitrifying communities with higher
294 functional attributes compared to those in thicker biofilms. To test this hypothesis, we estimated the
295 maximum specific growth rate (μ_{max}) of AOB by defining the initial biomass concentration of the
296 autotrophic bacteria based on the fraction of AOB and NOB estimated by qPCR analysis as
297 described in section S4 and S7. We observed that the estimated specific growth rates μ_{maxAOB}
298 followed a trend similar to $r_{\text{NH}_4\text{-B}}$ (Fig 1A), i.e., significantly decreased $r_{\text{NH}_4\text{-B}}$ with increasing
299 biofilm thickness. We also observed higher values of substrate (ammonium) affinity constant
300 $K_{\text{NH}_4\text{-AOB}}$ within Z50 with a decreasing trend over biofilm thickness (Table S6). Values of μ_{maxAOB}
301 obtained for the thinnest biofilm (Figure 1A) are slightly higher compared to that estimated for
302 activated sludge⁴⁹ (Table S6). Nevertheless, the estimated specific growth kinetics supports the
303 hypothesis of functional differentiation, in terms of nitrification, with varying biofilm thickness.
304 Previous studies identified a large diversity of nitrifiers^{50,51}, and modelling of microbial competition
305 in nitrifying biofilms showed how their spatial distribution can follow *r*- and *K*- selection
306 theory^{49,52,53}. Accordingly, a vertical distribution of different autotrophic microorganisms was
307 observed where *K*-strategist (with lower substrate affinity constant and maximum growth rate
308 compared to *r*-strategists) populated all layers of the biofilm equally while *r*-strategists were only
309 present in the active surface of the biofilm. Accordingly, in our study fast-growing organisms that
310 adapted to high substrates availability (i.e., ammonia), characterized by high μ_{maxAOB} and high
311 values of substrate affinity constant $K_{\text{NH}_4\text{-AOB}}$ (*r*-strategists) have mainly populated the surface
312 layers of thin biofilms (Z50-Z200) unlike thicker biofilms due to reduced diffusion limitation.

313

314 **Micropollutant removal kinetics.**

315 Most of the investigated chemicals were removed according to first-order kinetics (goodness of fit
316 is presented in Table S7), allowing for the estimation of removal rate k_s (Figure S19) and
317 biotransformation rate constant k_{bio} (Figure 2). However, two compounds (i.e., DCF and SMX)
318 were removed according to different kinetics. DCF exhibited an initial increase of concentration
319 given by the retransformation of its human metabolites such as sulfate and glucuronide conjugates⁵⁴
320 (possibly present in the effluent wastewater used in the batch experiment) to parent compound
321 (Figure 2). Hence, kinetics of retransformation k_{Dec} were estimated along with k_{bio} (Table S8),
322 according to Plósz et al.¹⁶. SMX data showed different process kinetics in batch 1 and 2 (Figure 2;
323 Figure S8). SMX was removed according to first-order kinetics in batch 2 (Figure S10). On the
324 other hand, in batch 1, SMX concentration profiles obtained with the Z500 and Z200 carriers
325 suggest significant cometabolic effects (enhancement by ammonia availability), unlike that obtained
326 with Z50 and all profiles obtained in batch 2. Biotransformation of SMX was previously predicted
327 using a cometabolic kinetic model^{7,16}, whereby the primary substrate affects (enhances or
328 competitively inhibits) micropollutants biotransformation.

329 Ibuprofen was completely removed in the first 15 minutes of the experiment, preventing the
330 estimation of k_{bio} . Finally, micropollutants removal measured during the control experiment was
331 less than 10% (Figure S11), indicating that no abiotic removal was observed during batch
332 experiment.

333

334 **Impact of biofilm thickness on removal of individual micropollutants.**

335 Biotransformation rates k_{bio} were estimated for 22 spiked chemical compounds (Figure 3). We
336 tested the correlation between k_{bio} and biofilm thickness by estimating Pearson's coefficients, r .
337 Results were classified as (i) positive correlations when $r > 0$ and (ii) negative correlation when $r <$
338 0 . We observed three important outcomes: (i) for 14 over 22 spiked chemical compounds, k_{bio}
339 positively correlated with biofilm thickness ($r > 0.8$), (ii) k_{bio} showed low correlation with four
340 compounds ($-0.2 \leq r < 0.2$), and (iii) for the three sulfonamide antibiotics (SMX, SDZ and SMZ)
341 and DCF, the estimated k_{bio} showed negative correlation ($r = -0.9$) with biofilm thickness. Pearson's
342 coefficients (r) are reported in Table S9. The results from the rank correlation and permutation tests
343 (Figure S17 and S18) showed that the positive correlation between k_{bio} and biofilm thickness found
344 for 14 of 22 spiked micropollutants is significant different ($p < 0.05$), suggesting a dependence of
345 biotransformation rate constants on biofilm thickness at 95% confidence level (see section S10).

346 **Compounds with positive correlation.**

347 *Beta-blockers.* Previous studies have shown, with the exception of ATN^{5,6}, moderate
348 biodegradability of beta-blockers in activated sludge (Table S8) and no direct link to ammonia
349 oxidation^{55,56}. In our study, removal of ATN, MET, and PRO was higher in the Z500 biofilm, with
350 ATN presenting the highest k_{bio} among the beta blockers in agreement with previous studies^{24,57}.
351 Significantly higher k_{bio} with Z500 were found for ATN and PRO compared to previous
352 observations in activated sludge⁵⁵ and MBBR²⁴. On the other hand, ATN and PRO presented high
353 sorption affinity to biofilm (highest for Z500, Figure S3) possibly indicating underestimation of
354 biotransformation rate constants in previous studies that neglected sorption²⁴. SOT was removed to
355 a very low extent and the k_{bio} obtained was significantly lower than in previous studies using not
356 enriched nitrifying communities^{24,55}. This suggests that the removal of SOT might not be linked to
357 autotrophic activity. Although biotransformation of beta-blockers (with exception of SOT) was
358 enhanced in the nitrifying MBBR in the present study in comparison to activated sludge, their
359 removal seems to be related to the biofilm microbial community and not necessarily to nitrification
360 activity.

361

362 *Iodinated X-ray contrast media (ICM).* While in batch 1 we observed extremely low removal of the
363 iodinated contrast media (IOH, IOM, IOP, IOPR) across all biofilm thicknesses (Figure S7), after
364 approximately 230 days of operation the thick biofilm Z500 had developed the capability to degrade
365 these compounds. On the other hand, less than 2% of the ICM were removed during either batch
366 experiments by Z50 biofilm. Overall, ICM showed lower removal rate constants compared to other
367 targeted compounds in this study, with k_{bio} values comparable to those reported in previous
368 investigations^{24,26}. No sorption of ICM was observed in this study (Figure S3). ICM have high
369 polarity and are designed to be resistant to human metabolism⁵⁸. It has been suggested that the slow
370 biotransformation of ICM is due to steric hindrance caused by the iodine atoms which prevent
371 enzyme to access the aromatic rings⁵⁸. The slowest removal of IOP (Table S8), having the greatest
372 steric hindrance, supports this hypothesis. Our results further suggest that increased biofilm
373 thickness is beneficial for the removal of ICM. Biotransformation of ICM occurs mainly via
374 deiodination, a process which includes reductive dehalogenation at low redox potential^{26,59}. Higher
375 diffusion limitation of oxygen in the thicker biofilm may have led to a lower redox potential that
376 facilitated dehalogenation of ICM compounds.

377

378 *Anti-depressants/ Anti-epileptics*. The comparably low k_{bio} estimated for the antidepressant VFX is
379 in line with previous studies^{24,40}. VFX removal was previously associated with ammonia oxidation
380 activity⁵ but in our study no removal of venlafaxine was observed in Z50 carriers (Figure S10),
381 exhibiting the highest nitrification activity. CIT, which was found to be moderately removed in
382 activated sludge¹², exhibited k_{bio} values for Z500 similar to what reported from another study on
383 aerobic MBBRs²⁴.

384 *Antibiotics*. The biotransformation rate constants of two macrocyclic (CLA and ERY) antibiotics
385 and TMP showed positive correlation with biofilm thickness. Controversial results have been found
386 for TMP, the removal of which was positively associated with nitrification⁶⁰, as well as with
387 heterotrophic activity^{24,61}. Although it is difficult to identify the process involved in TMP removal,
388 our study suggests that microbial species other than nitrifiers could play a role in its
389 biotransformation. Estimated k_{bio} for ERY and CLA agrees well with previous studies using
390 activated sludge and MBBR^{7,24}. As observed for beta-blockers, significantly higher sorption of
391 ERY and CLA was observed in Z500 (Figure S3) compared to Z50 and Z200.

392

393 **Compounds with negative correlation.**

394 Biotransformation kinetics of DCF, SDZ, SMZ, and SMX were found to be negatively correlated
395 with biofilm thickness, which, in turn, suggests an association of biotransformation processes with
396 nitrification activity (see e.g., $\mu_{\text{max,AOB}}$ in Figure 1A). Studies on the biotransformation of DCF in
397 activated sludge suggested cometabolic enhancement by growth substrate, with k_{bio} obtained in the
398 absence and presence of growth substrate around 0.1 and 1.2 L gSS⁻¹ d⁻¹, respectively¹⁶. The latter
399 value agrees well with k_{bio} values for thickest biofilm estimated in this study¹⁶. On the other hand,
400 k_{bio} values obtained for the thinnest biofilm were about four times higher than those in the thickest
401 biofilm, supporting the hypothesis that DCF removal is positively associated with nitrification.
402 Likewise, the removal of SDZ, SMZ and SMX supports the same hypothesis (Figure 2). This is in
403 agreement with a recent study in which SMX removal positively associated with nitrification in
404 synthetic wastewater⁵⁸, while no previous studies have investigated this link for SDZ and SMZ. The
405 main human metabolites of SDZ, AcSDZ⁶³, did not to follow the same trend as the parent
406 compound as its biotransformation (via de-acetylation) was significantly enhanced with increasing
407 biofilm thickness.

408

409

410 **Compounds with low correlation.**

411 The removal kinetics of the antibiotic ROX, the analgesic PHE, the antidepressant CBZ and the X-
412 ray contrast media DIA were found to be weakly correlated with biofilm thickness ($-0.2 \leq r < 0.2$),
413 partly because of the negligible removal measured with Z50 biofilm (Figure S8 and S9). k_{bio} values
414 obtained for CBZ agrees well with that obtained with activated sludge¹⁶. For ROX, k_{bio} value
415 obtained for Z200 ($0.7 \text{ L g}^{-1} \text{ d}^{-1}$) was significantly lower than that obtained in activated sludge
416 nitrifying reactors^{15,60}. As for DIA ($k_{\text{bio}} < 0.1 \text{ L g}^{-1} \text{ d}^{-1}$) and PHE ($k_{\text{bio}} \sim 0.6 \text{ L g}^{-1} \text{ d}^{-1}$) our results were
417 in line with previous evidence on activated sludge^{16,39} and MBBR²⁴ (Table S8).

418

419 **Impact of biofilm thickness on community structure.**

420 To investigate the impact of increasing biofilm thickness on the community structure, we quantified
421 the relative abundance of targeted AOB and NOB using 16S as well as ammonia monooxygenase
422 (*amoA*) functional gene by qPCR (further details are reported in S8). In addition, we calculated total
423 community biodiversity using 16S rRNA amplicon sequencing. Overall, the thinner biofilm (Z50)
424 exhibited significantly higher ($p < 0.05$) AOB (based on 16S and *amoA*) and NOB relative
425 abundance per gram of biomass (quantified as volatile suspended solids, VSS) compared with the
426 other biofilm thicknesses (Figure S13 and Fig 1B), in accordance with the higher nitrifying activity
427 found in the thin biofilm. We estimated by qPCR the fraction of heterotrophic bacteria to be
428 between 10 and 53%, being the lowest in Z50 biofilms (Table S5). In all the biofilms, Archaea
429 *amoA* were below the detection limit. For all of the carriers, 374 970 high quality sequences were
430 recovered by 16S rRNA amplicon sequencing. Samples were normalized to 19313 sequences per
431 sample and clustered into an average of 856 observed OTUs at 97% sequence similarity. Shannon
432 taxonomic diversity and evenness index were significantly lower in Z50 compared to thicker
433 biofilms. (Figure 1C and in Table S10). This suggests that increasing biofilm thickness over 200 μm
434 does not substantially increase functionality (as observed for nitrification activity) or biofilm
435 biodiversity. Although ACE and Chao (extrapolated taxonomic) richness metrics were observed to
436 increase somewhat with biofilm thickness, this change was not significant (Figure 1C, Table S10).
437 As biodiversity lumps together both microbial richness and evenness, the significant increase of
438 Shannon index and evenness index (H_1/H_0) with biofilm thickness, coupled with an insignificant
439 change in species richness, points towards an increase in evenness with thickness (Figure 1D).
440 Finally, we investigated the relative fraction of aerobic and anaerobic bacteria in the different
441 biofilms, observing that more than 65% of the community of the Z50 biofilm was aerobic and

442 aerobic/facultative bacteria, with decreasing fraction over thickness (Figure S15). Overall, this
443 suggested a shift from a more aerobic but less biodiverse microbial community in the Z50 to a less
444 aerobic but more biodiverse and most importantly, more evenly distributed community with
445 increasing biofilm thickness.

446

447 **Correlations of activity and community structure with micropollutant** 448 **biotransformation.**

449 We assessed the correlation of the micropollutant biotransformation rates of each compound – at
450 different biofilm thickness - with nitrification rates ($r_{\text{NH}_4\text{-B}}$) and microbial community structure
451 (Figure 4). For all the investigated chemicals, the correlation between k_{bio} , Shannon and Evenness
452 indices followed a linear model (Figure S16). Conversely, correlations between k_{bio} , nitrification
453 and *amoA* abundance were predicted more accurately with a logarithmic model (i.e, with a
454 decelerating shape, Figure S16). A decelerating shape was previously observed to better describe
455 the correlation between biodiversity and micropollutant multifunctionality in a full-scale study on
456 activated sludge¹⁸.

457 Pearson correlation analysis (values reported in Table S11-14) indicated that most of the
458 micropollutant biotransformation rate constants that were positively correlated with biofilm
459 thickness (L_F in Figure 4) were also positively associated with Shannon taxonomic diversity ($r > 0.8$)
460 (Figure 4). Hence, the removal of this group of chemicals (mainly beta-blockers, ICM, the anti-
461 depressants CIT and VFX and the antibiotics TMP and ERY) could be enhanced by a more even
462 microbial community. In agreement with the findings by Wittebolle et al.²³, we observed that a
463 microbial community with a more even distribution can maximize its functionality even in non-
464 stressed conditions. Furthermore, for the micropollutants with the lowest k_{bio} ($< 0.4 \text{ L g}^{-1}$; SOT,
465 AcSDZ and the ICM IOM), stronger correlations ($r > 0.98$) with both Shannon and evenness indices
466 (Table S13 and S14) were shown, suggesting the importance of maximizing biodiversity in biofilms
467 to enhance removal of the most recalcitrant compounds. This finding is supported by previous
468 studies¹⁸, where strong associations with biodiversity were observed for rare micropollutant
469 biotransformations (e.g. VFX). Likewise, compounds such as SOT, IOM, AcSDZ, VFX and TMP
470 (with biotransformation rate constants positively correlated with biofilm thickness) exhibited
471 negative correlation between k_{bio} and *amoA* abundance or nitrification rate (Figure 4), indicating
472 that their removal is possibly related to the biodiversity of the heterotrophic and not autotrophic
473 community.

474 We further observed a positive association between biotransformation kinetics, nitrification rate
475 $r_{\text{NH}_4\text{-B}}$ ($r > 0.9$) and *amoA* abundance ($r > 0.7$) for compounds with k_{bio} negatively correlated with
476 biofilm thickness (with the exception of SDZ). The relationship between micropollutants removal
477 and *amoA* abundance has been previously observed^{61,64}, and *Nitrosomonas europaea* is known to
478 catalyze hydroxylation reactions with aromatic compounds and estrogens⁶⁵. SMX and DCF mainly
479 undergo biotransformation via hydroxylation to hydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-
480 sulfonamide and 4'-hydroxydiclofenac respectively^{66,67}, supporting the hypothesis that their
481 biotransformation is linked to the abundance of *amoA* gene and nitrification. Furthermore, a recent
482 study observed suppressed SMX removal in a nitrifying SBR when *amoA* was inhibited by
483 allylthiourea⁵⁹. SDZ and SMZ have very similar chemical structures and biotransformation
484 pathways similar to SMX were predicted by the EAWAG-BBD pathway prediction systems⁶⁸.
485 Although, association with *amoA* abundance and nitrification were not previously investigated for
486 SDZ and SMZ, it is likely that hydroxylation is also the primary pathway involved in the removal
487 of these compounds and that higher abundance of *amoA* could potentially enhanced their removal.
488 Nonetheless, all the targeted sulfonamides and DCF exhibited stronger correlation to nitrification
489 rate ($r > 0.9$) compared to *amoA* abundance ($r > 0.7$) (Figure 4). Helbling et al.⁵ observed a strong
490 correlation between k_{bio} , nitrification rate and archaeal *amoA* for a number of micropollutant (i.e.,
491 isoproturon, ranitidine and VFX) in activated sludge but inhibition of ammonia monooxygenase
492 activity had little effect on their biotransformation (undergoing mainly via oxidative reactions).
493 Thus, they suggested that other enzymes involved in nitrification besides *amoA* (e.g.,
494 hydroxylamine oxidoreductase) could be responsible for the removal of these compounds.
495 Overall, the examination of micropollutant biotransformation, nitrification and microbial
496 community structure contributed to understanding the effect of using thin or thick biofilms in
497 biofilm-based technologies. We have shown that by using a thicker biofilm (500 μm), which
498 resulted in increased microbial biodiversity, the biotransformation kinetics of more than 60% of the
499 targeted compounds were maximized. This is also supported by the estimated transformation rates
500 k_{S} normalized by biofilm surface area (Figure S19), which in Z500 were higher than or comparable
501 to other biofilm thicknesses for most compounds. In full-scale operation, thicker biofilm (~500 μm)
502 could potentially optimize the removal of most of the micropollutants targeted in this study.
503 On the other hand, we demonstrated that a thin biofilm (50 μm) could increase the removal of four
504 of the targeted compounds (SDZ, SMZ, SMX and DCF), which have previously been considered

505 recalcitrant³⁹. It is likely that the removal of these compounds is enhanced by the significantly
506 higher nitrification rate and *amoA* abundance of a less diffusion limited thin biofilm (50 µm).
507 Finally, our results suggest that although thin biofilm (~50 µm) can achieve complete nitrification
508 and increase the removal of some key compounds, biofilm technologies based on thicker biofilms
509 could enhance the removal of a major number of micropollutants.

510

511 **Acknowledgements**

512 This research was supported by MERMAID: an Initial Training Network funded by the People
513 Programme (Marie-Curie Actions) of the European Union's Seventh Framework Programme
514 FP7/2007-2013/ under REA grant agreement n. 607492. The authors thank the technical assistance
515 provided by VA SYD at Källby wastewater treatment plant and Associate Professor Olivier Thas
516 (Ghent University) for the support on the statistical analyses.

517

518 **Associated content**

519 Supporting Information Available. This information is available free of charge via the Internet at
520 <http://pubs.acs.org>. The Supporting Information is divided in ten sections containing relevant tables
521 (Table S1-S17) and figures (Figure S1-S19).

522

523

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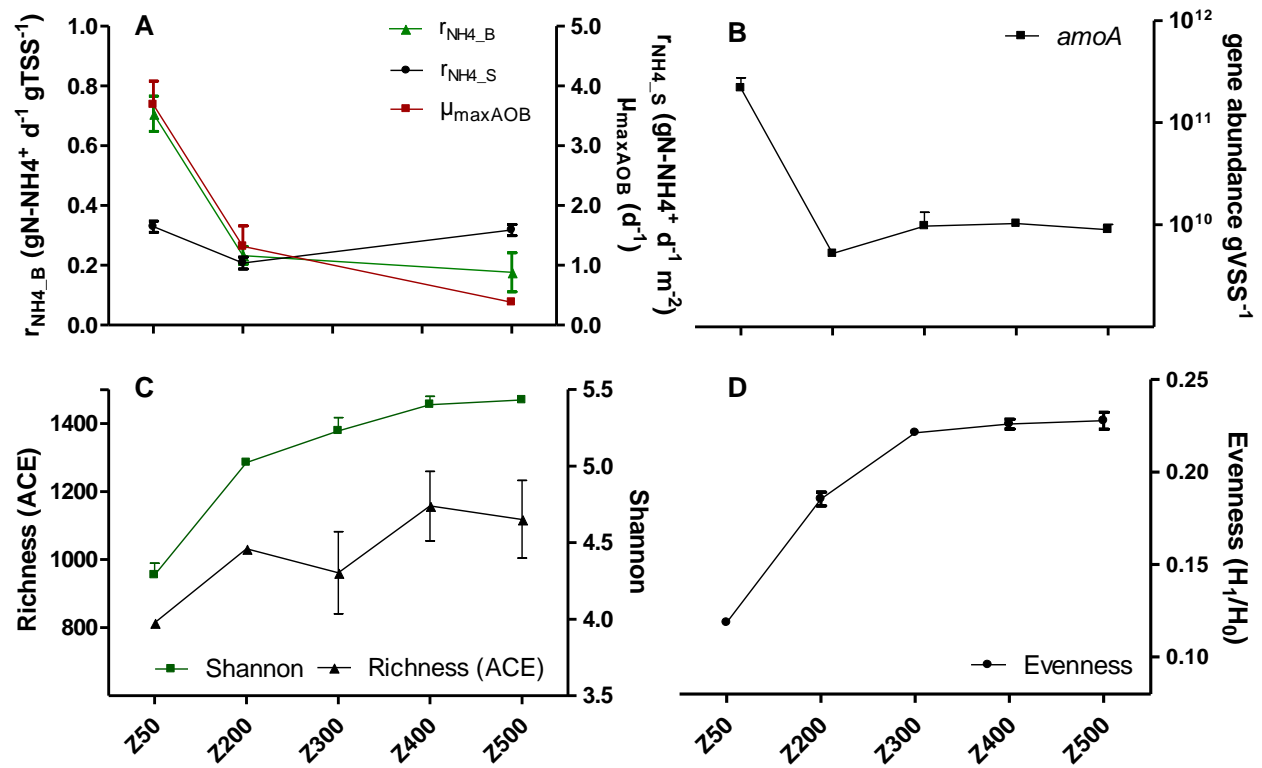


Figure 1. Nitrification rates ($r_{\text{NH}_4_B}$) and specific growth rates of AOB (μ_{maxAOB}) for Z50, Z200 and Z500 (A); *amoA* gene abundance (B); extrapolated taxonomic richness (ACE), Shannon biodiversity (C) and evenness indices (D) estimated for the 5 Z-carriers (x-axis). Errors bars show standard deviation.

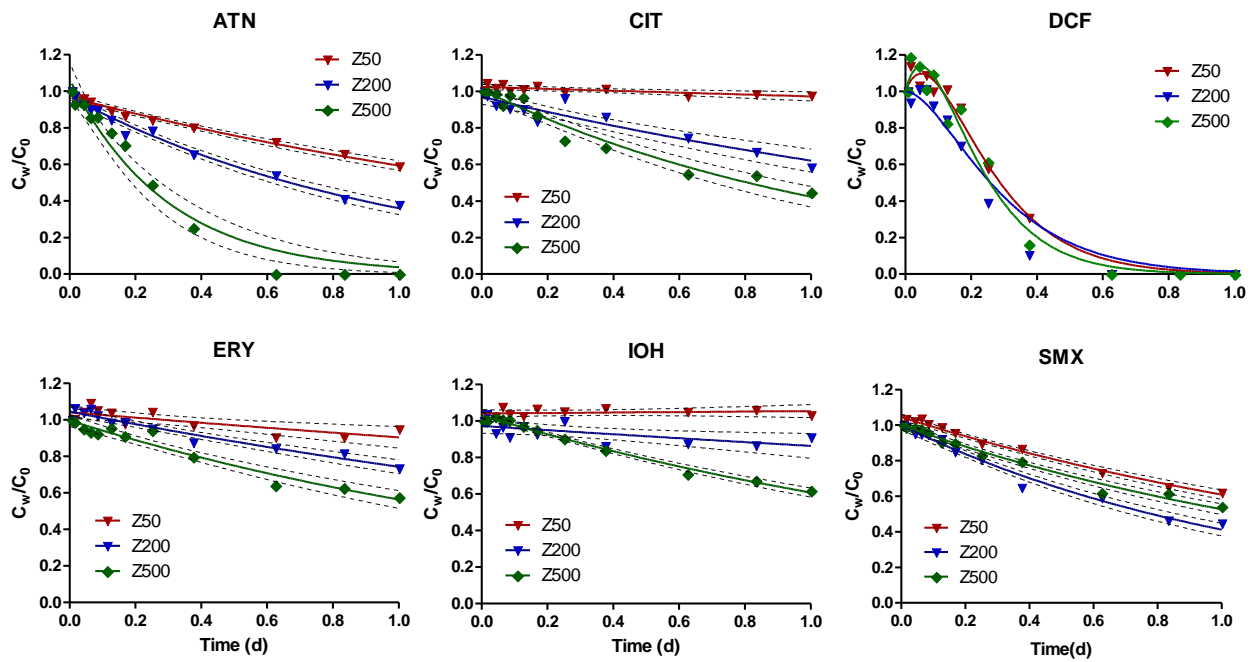


Figure 2. Measured and modelled relative concentration of six representative spiked micropollutants during batch experiment. C_w and C_0 denote the aqueous measured and initial concentration of the spiked chemicals. Dotted lines denote the 95% confidence interval.

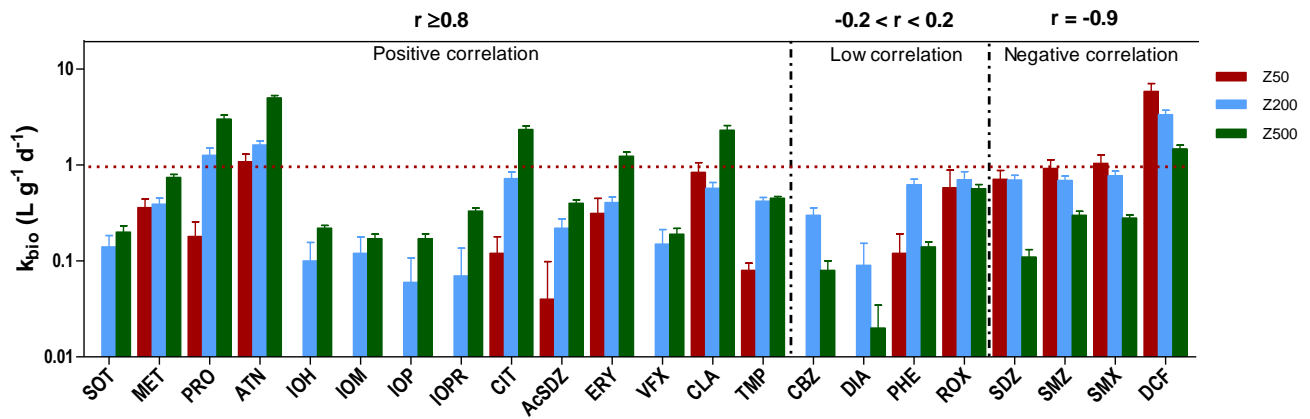


Figure 3. Biotransformation rates (k_{bio}) estimated for 22 micropollutants for 3 Z-carriers. The fast removal of ibuprofen prevented the estimation of k_{bio} . Pearson's coefficient r was used to measure the correlation between k_{bio} and biofilm thickness.

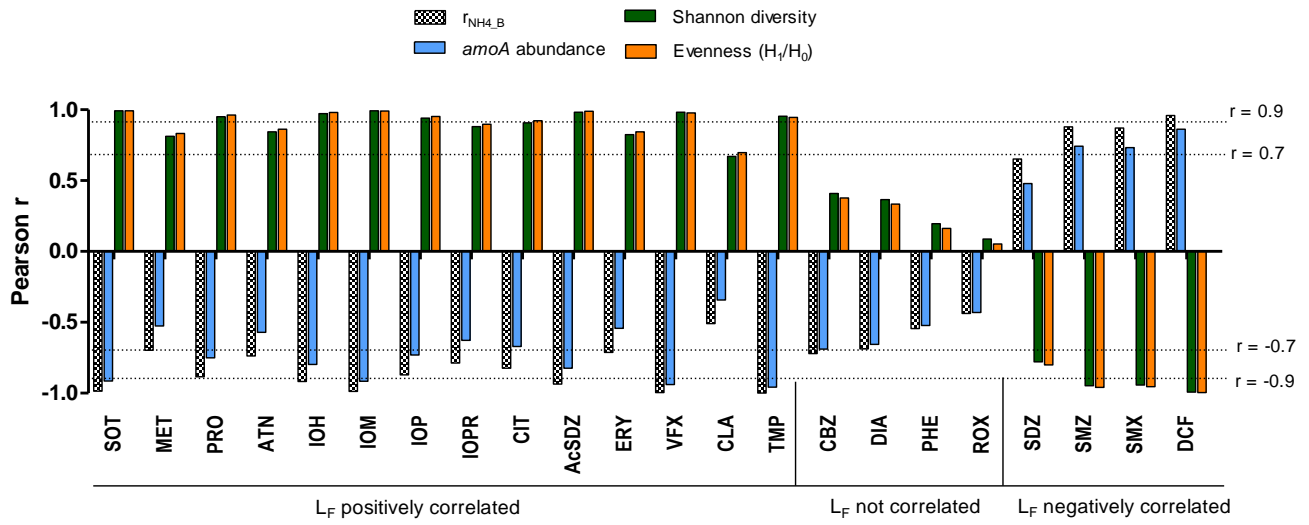


Figure 4. Correlation between estimated biotransformation rate constants k_{bio} with nitrification rate $r_{NH_4_B}$, *amoA* abundance, Shannon diversity and evenness indices. L_F indicates biofilm thickness.