Repeated introduction of micropollutants enhances microbial succession despite stable degradation patterns

Running title: Ecological feedback between microbe-micropollutants

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26 **Abstract**

27 The increasing-volume release of micropollutants into natural surface waters has raised
28 great concern due to their environmental accumulation. Persisting micropollutants can
29 impact multiple generations of organisms, but their microbially-mediated degradation
30 and their influence on community assembly remain understudied. Here, freshwater
31 microbes were treated with several common micropollutants, alone or in combination,
32 and then transferred every 5 days to fresh medium containing the same micropollutants
33 to mimic the repeated exposure of microbes. Metabarcoding of 16S rRNA gene makers
34 was chosen to study the succession of bacterial assemblages following micropollutant
35 exposure. The removal rates of micropollutants were then measured to assess
36 degradation capacity of the associated communities. The degradation of
37 micropollutants did not accelerate over time but altered the microbial community
38 composition. Community assembly was dominated by stochastic processes during early
39 exposure, via random community changes and emergence of seedbanks, and
40 deterministic processes later in the exposure, via advanced community succession.
41 Early exposure stages were characterized by the presence of sensitive microorganisms
42 such as Actinobacteria and Planctomycetes, which were then replaced by more tolerant
43 bacteria such as Bacteroidetes and Gammaproteobacteria. Our findings have important

implication for ecological feedback between microbe-micropollutants under anthropogenic climate change scenarios.

Keywords: Micropollutant transformation; press disturbance; community turnover; stochastic assembly; deterministic assembly

Introduction

The rapid exploitation and widespread use of emerging organic chemicals have resulted in environmental damage and human health issues during the last several decades [1–3]. These organic chemicals include pharmaceuticals and personal care products, hormones, and industrial chemicals, and are typically classified as micropollutants, because of their occurrence at a low to very low concentration of μg and ng per litter in the receiving waters [4, 5]. The majority of micropollutants originate from direct and indirect inputs of organic contaminants that are released via the effluents of wastewater treatments plants [1, 6–8]. The micropollutants gradually accumulate at sites of their release and reach concentrations high enough to exert disruptive effects on aquatic organisms such as fish, invertebrates, and microbes [9–13]. Nonetheless, little is known about the mechanisms by which micropollutants impact the turnover of aquatic microbial communities [7, 14, 15], especially those of freshwater bacteria [16, 17], and the role of microbes on trace micropollutant degradation.

How microbial assemblages respond to micropollutants can be classified as: subsidy responses and stress responses. The subsidy responses is mainly via biodegradation, in which the microbes are able to utilize the micropollutant compounds as carbon sources, while the stress responses is relevant to the growth and survival of microorganisms inhibited [18, 19]. For instance, bisphenol A (BPA) and its substituted

67 derivatives (e.g., bisphenol S, BPS) [20] are typical subsidy responses, as their
68 relatively simple chemical structure and low toxicity allow both to be utilized as carbon
69 sources by microbial isolates [19, 21]. Stress responses are elicited by pharmaceutical
70 and personal care micropollutants such as triclosan (TCS) and its substituted derivatives
71 (e.g., triclocarban, TCC) [22], because both inhibit the growth of several microbial taxa,
72 consistent with their resemblance to antimicrobials [18, 23]. Both BPA and TCS in the
73 field strongly influence microbial diversity, especially that of the central species and
74 module communities comprising the microbial co-occurrence network [24].

75 Microbial responses to chemical pollutants can be modified by the exposure legacy
76 of the community. For example, microbial communities with a history of containment
77 exposure may differ from contaminant-naïve communities in the rate and extent of
78 chemical degradation [25–27]. They may also differ in their plasticity [14] upon
79 subsequent exposure to similar contaminants. However, the ecological drivers of
80 community succession following repeated micropollutant exposure are not well-
81 understood. Studies of community dynamics over multiple generations and a time
82 period relevant for community stability could provide insights into the ecological traits
83 that determine taxon sensitivity and tolerance, and therefore the ability of microbial
84 community members to degrade micropollutants.

85 Press disturbances in natural environments can impact multiple generations of
86 organisms [28] by altering the affected ecosystems beyond the possibility of recovery
87 or by causing a shift to a community with a new stable state [29]. Following a stress
88 event such as repeated introduction of micropollutants in recipient environments,
89 communities rely on reassembly from the local seedbanks such that succession
90 eventually results in a new steady state. During the early stages of community dynamics,
91 no-longer-dormant local species undergo a secondary succession characterized by new

92 abiotic and biotic interactions. The emergence of resuscitation from dormancy is
93 considered as stochastic processes that lead to divergent communities [30–32]. When
94 abiotic and biotic conditions allow a stabilization of the initial communities, community
95 formation at a later phase of a press disturbance is expected to converge, through the
96 succession of microbial species [33]. In addition, the plasticity of microbial taxa in
97 response to multiple micropollutant exposure has been shown to increase over multiple
98 generations [14], with later-assembled communities shifting to a new stable state that
99 differs from the one before the exposure.

100 Here, a press disturbance was simulated in a microcosm experiment in which a
101 reservoir bacterial community was transferred into fresh medium containing trace
102 concentrations of typical micropollutants that were refreshed every 5 days. Several
103 different micropollutants are typically present simultaneously within aquatic
104 environments, but whether their interactions lead to effects on microbial communities
105 that differ from those of single micropollutants is unclear [12]. Thus, we also
106 experimentally manipulated single and multiple micropollutants to determine their
107 impacts on taxon coexistence, the regularity of community assembly, and
108 micropollutant degradation ability. We hypothesized that because BPA elicits a subsidy
109 response and TCS a stress response, the removal rate of BPA would be higher than that
110 of TCS, with larger differences in the removal rates by microbial communities exposed
111 to these micropollutants individually rather than in combination with other
112 micropollutants. We also hypothesized that micropollutants degrade and alter the
113 microbial community compositions after exposure. Accordingly, the microbial
114 communities assembled at the initial and late phases of the micropollutant exposure
115 would differ substantially and are primarily driven by either stochastic or deterministic
116 assembly process.

Materials and Methods

Water collection

We selected the bacterial species pools from Shidou Reservoir, which is one of largest sources of drinking water in Xiamen, China [34] and is therefore under governmental protection from pollution originating from household drainages and a nearby dam (Fig. S1). Unlike other contaminated aquatic environments [18, 35, 36], the concentrations of BPA, BPS, TCS, and TCC in Shidou Reservoir (China) are very low (< 1 ng/L), such that the reservoir's waters provide an ideal system to investigate the influence of persistent micropollutants on an aquatic microbiome. Surface water (100 L) from Shidou Reservoir (24˚41'39"N, 118˚0'37"E) was collected at 0.5 m depth on June 1, 2017 (Fig. S1). Temperature, pH, conductivity, dissolved oxygen (DO), and dissolved oxygen saturation, measured *in situ* using a HACH HQ40d multi-parameter meter (HACH, Loveland, CO, USA), were 29.0°C, 8.90, 54.4 μs/cm, 8.84 mg/L and 116.7%, respectively. The concentrations of BPA, BPS, TCS, and TCC in the reservoir water were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (ABI 3200QTRAP, Framingham, MA, USA) with solid-phase extraction and all were < 1 ng/L at the time of sampling (details of the measurements are provided below). The collected water was transported from the field to the laboratory within 2 h after sampling.

Experimental setup

BPA, BPS, TCS, and TCC were dissolved in acetone at concentrations of 800, 800, 400, and 400 μg/L, respectively, and stored as stocks in glass bottles (HPLC grade). The micropollutants were previously shown to be chemically stable when dissolved in an organic solvent [36, 37]. From the 100 L of collected water, 15 L were filtered through

141 a 1.2-μm polycarbonate filter (Millipore, Ireland), to minimize top-down regulation by
142 planktonic grazers, and then used as the initial inoculum. The remaining unfiltered
143 water was filtered through a 0.22-μm polycarbonate filter (Millipore, Cork, Ireland) to
144 remove microorganisms and then stored in the dark for later use in the preparation of
145 incubation medium. Two treatments consisting of single micropollutants (BPA and
146 TCS) and two treatments consisting of micropollutant mixtures (MI = BPA + TCS) and
147 (MII = BPA + BPS + TCS + TCC) were established.

148 Time-serial microcosms comprising seven inoculation batches from batch 1
149 (named hereafter 'B1') through batch 7 ('B7') were established to investigate the
150 microbial community response to a press disturbance of micropollutants resulting from
151 a single short-term event but maintained for 35 days (Fig. 1). Prior to the experimental
152 initiation, the caps of the bottles containing the micropollutants were loosened for > 12
153 h to volatilize the acetone in a fume hood, as described in [38, 39]. In microcosm B1,
154 600 ml of the initial inoculum was added to bottles that already contained the
155 micropollutants and nutrients, such that the final concentration of each micropollutant
156 in the treatments was as follows: BPA: 1 μg/L BPA, TCS: 1 μg/L TCS, MI: 1 μg/L
157 BPA + 1 μg/L TCS, and MII: 1 μg/L BPA + 0.1 μg/L BPS + 1 μg/L TCS + 0.1 μg/L
158 TCC. BPS and TCC are the derivatives of the BPA and TCS, respectively, and the
159 concentrations of those analogues in the environment are one order of magnitude lower
160 than their chief compounds in the seawater [20, 40, 41]. The final concentration of each
161 micropollutant in the microcosms closely represented their ambient environmental
162 conditions as reported in literature [18, 42]. Further, given the low nutrient
163 concentrations [34, 43], we adjusted the concentrations of nitrogen and phosphorus in
164 the filtered water to 128.6 and 8.04 μM to promote microbial growth by the addition of
165 KNO_3 and KH_2PO_4, respectively [44]. After 5 days of incubation, 60 mL of the B1

166 inoculum was pipetted from each of the replicates and transferred to a sterile bottle
167 containing 540 mL of 0.22-μm-filtered water and freshly added micropollutants. This
168 newly inoculated microcosm served as batch 2 (named hereafter 'B2'). The
169 micropollutant treatments and controls were amended with 128.6 μM N and 8.04 μM P
170 after each inoculation to maintain similar amounts of these nutrients throughout the
171 experiment. The same inoculation procedure was applied at each 5-day interval to
172 initiate microcosms B3–B7, such that the experimental period was 35 days (Fig. 1).
173 Five days was chosen as the inoculation duration because previous unpublished
174 observation (Fig. S2) indicated that reservoir bacteria exposed to a micropollutant-
175 added medium had shown a high removal efficiency in the early growth stage, and that
176 was expected to lead up to stationary phase on day 5 of the culture. Microbial cells were
177 transferred between pairwise replicates, e.g., replicate 1 in B1 was the inoculum for
178 replicate 1 in B2, with the remainder used in the downstream analyses described below.
179 An additional set of experimental bottles without micropollutant addition served as the
180 controls. To maintain the same level of exposure to acetone in all microcosms, the
181 controls also received the same spiking of solvent acetone and were left for overnight
182 evaporation before each transfer. In total, there were five treatments, each with three
183 replicates, for a total of 15 microcosm replicates per batch. All microcosms, i.e., 105
184 experimental bottles were incubated in the dark at 28°C with shaking at 100 rpm.

185 Additional experiments were conducted to determine (i) micropollutant removal
186 rates in the absence of microorganisms, to rule out the gradual degradation of the
187 micropollutants in the treatments due to their chemical instability (Fig. S2), and (ii)
188 whether the ability of the microorganisms to transform micropollutants differed
189 depending on their pre-exposure history (Fig. S3). Please see details on these two
190 experiments in the Supplemental methods part 1.

191 **Micropollutant analysis**

192 For each microcosm batch, ~20 mL of the inoculum was removed from each bottle at
193 0, 2 and 5 days for micropollutant analysis. The four micropollutants BPA, BPS, TCS,
194 and TCC were concentrated and purified by a 12-port Visiprep-DL SPE vacuum
195 manifold (Supelco, Bellefonte, PA, USA) with Oasis HLB cartridges (500 mg, 6 mL;
196 Waters, Millford, MA, USA), and then were measured using LC-MS/MS (ABI
197 3200QTRAP, Framingham, MA, USA), as described in our previous work [37].
198 Chromatographic separation was performed by using a Kinetex C18 column (100 mm
199 × 4.6 mm, 2.6 μm; Phenomenex, Torrance, CA, USA) from Shimadzu LC system
200 (Shimadzu, Japan).

201 **DNA extraction and 16S rDNA amplicon high-throughput sequencing**

202 The remaining inoculum (~470 mL) of each replicate was filtered onto 0.22-μm
203 polycarbonate filters (Millipore, Ireland), which were stored at −80°C until used for
204 DNA extraction. DNA from the 105 samples was extracted using the FastDNA for soil
205 kit (MP Biomedical, Santa Ana, CA, USA) according to the manufacturer's protocol,
206 except that the beating speed was set to 6.0 m/s for 80 s. The universal primer pair 515F
207 (5'-GTG YCA GCM GCC GCG GTA-3') and 907R (5'-CCG YCA ATT YMT TTR
208 AGT TT-3') was used to amplify the V4-V5 region of the bacterial 16S rRNA genes.
209 The PCR products were purified, mixed in equal amounts, and sequenced on an
210 Illumina MiSeq platform with the 2 × 300 bp paired-end protocol at the Majorbio Bio-
211 Pharm Technology Co., Ltd. (Shanghai, China).

212 **Sequence processing**

213 The raw sequencing data were processed using the LotuS pipeline [45] with the
214 following criteria: i) average sequence quality > 27, ii) sequence length > 170 bp, iii)
215 no ambiguous bases, iv) no mismatches with the barcodes or primers, and v)
216 homopolymer length < 8 bp. Operational taxonomic units (OTUs) were clustered at a
217 97% similarity using UPARSE [46]. A representative sequence of each OTU was
218 taxonomically quantified using the RDP classifier with the Silva database v132 [47], at
219 a bootstrap cutoff of 80%. All samples were rarified to 36, 000 sequence reads (the
220 smallest library size). The subsampled OTU table contained 1375 OTUs. The α- and β-
221 diversity indices of bacterial communities were calculated from the rarified dataset
222 using QIIME v1.9.1 [48] with the script "core_diversity.py".

223 **Inferring the community growth phase from the16S rRNA gene copy number**

224 The 16S rRNA gene copy number among prokaryotes within a defined period indicates
225 the number of individuals within a community and reflects the growth phase of that
226 community [49]. In this study, the 16S rRNA gene copy number of each OTU found in
227 the samples was estimated using the rrnDB database v5.5 [49] and the weighted mean
228 rRNA gene copy number of all OTUs found in each sample was then calculated as
229 described previously [50] (Please see details on the use of rrnDB database and the
230 calculation of weighted mean rRNA gene copy number in the Supplemental methods
231 part 2). The abundance-weighted average gene copy number of a community was
232 plotted against the sampling time for each treatment. The similar overarching pattern
233 of the average rRNA gene copy numbers of bacterial communities in all microcosms
234 (Figs. S4A-F) indicated the similar growth trajectories of those communities over time.
235 A hierarchical cluster analysis with complete clustering criterion and Bray-Curtis
236 dissimilarity metric was used to group the similarity of average gene copy numbers

237 from all micropollutant-treated microcosms belonging to the same batch type, without
238 including the controls. The clustering analyses was done in the R package 'stats'
239 package v 3.6.3. Three growth progression phrases across all microcosm batches were
240 identified based on similar average gene copy numbers and the time continuity of the
241 inoculation (Fig. S4G): phase 1 (B1–B2), phase 2 (B3–B4), and phase 3 (B5–B7).

242 **Relative contribution of ecological processes**

243 According to a quantitative ecological framework [51, 52], community assembly can
244 be summarized by five major ecological processes: i) homogenizing selection for a
245 convergent composition between communities, ii) variable selection for a divergent
246 composition between communities, iii) dispersal limitation for a divergent composition
247 between communities due to limited dispersal events, iv) homogenizing dispersal for a
248 convergent composition between communities due to unlimited dispersal, and v)
249 ecological drift (referred to "undominated processes" in [52]. The phylogenetic
250 turnover among the communities was quantified by the weighted β-mean nearest taxon
251 distance (βMNTD), as described in [53]. The βNTI was calculated as the difference
252 between the observed βMNTD and the mean of the null distribution (999
253 randomizations). The criteria determining the fraction of each process were as follows:
254 i) homogenizing and variable selection: higher (> 2) and lower (< -2) βNTI,
255 respectively; ii) dispersal limitation and homogenizing dispersal: non-significant |βNTI|
256 (< 2) but $RC_{bray} > 0.95$ and < -0.95, respectively. iii) ecological drift: non-significant
257 |βNTI| value (< 2) and non-significant $|RC_{bray}|$ value (< 0.95) [52]. To infer the relative
258 contributions of the different community assembly processes to community turnover
259 over time, for each pair consisting of a micropollutant treatment and its control the

260 βNTI and RC_{bray} values were calculated separately for each of the three growth phrases
261 described above.

262 **Ecological grouping of microbial responses to micropollutants**

263 We determined ecological responses of bacterial taxa based on their relative
264 abundances at growth phases (phase 1: B1–B2, phase 2:B3–B4, and phase 3: B5–B7,
265 Fig. S4G) throughout repeated micropollutant exposure, using the 'DESeq2' R package
266 v 1.26.0 [54]. Significant values were corrected for multiple tests using the Benjamini-
267 Hochberg correction with an adjusted α value of 0.05. The responses were categorized
268 as: i) sensitive, when relative OTU abundances were significantly higher in phase 1
269 than in phases 2 and 3; ii) opportunistic, when relative abundances were significantly
270 higher in phase 2 than in phases 1 and 3; iii) tolerant, when relative abundances were
271 significantly higher in phase 3 than in phases 1 and 2. The ecological categories
272 comprising a group of bacterial members whose growth fitness optimal in response to
273 repeated exposure could be documented collectively. In addition, to visualize the
274 phylogenetic relationships of the OTUs assigned to the ecological groups,
275 representative sequences of those OTUs were used to construct a phylogenetic tree
276 using iTOL v5 [55].

277 **Micropollutant turnover and community analysis**

278 A Wilcoxon's test was used to assess the removal rate of BPA, TCS, BPS, and TCC in
279 consecutive microcosm batches for the single-micropollutant (BPA and TCA)
280 treatments and the mixed-micropollutant (MI and MII) treatments. Additionally, a
281 Kruskal-Wallis test was used to analyze the difference in the removal rates between the
282 single micropollutant treatments and between any two micropollutants for the mixture

283 treatments. In this case, the removal rate of the micropollutants from each microcosm
284 batch was used as replicates for the analysis.

285 Differences in the bacterial communities in all microcosms were explored using
286 non-metric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarity
287 metric and by fitting removal rates of micropollutants to the ordination. A permutational
288 multivariate analysis of variance (PERMANOVA) [56] was conducted to test the
289 effects of micropollutants, inoculation time (as indicated by the microcosm batch), and
290 their interactive effects on the taxonomic composition of the bacterial communities. A
291 pairwise PERMANOVA was performed to test for significant differences in
292 community composition between each treatment pair. The multivariate analyses were
293 performed using the R package 'vegan' [57]. Whether communities with the shared
294 taxa exhibit similar dynamics was assessed in a dissimilarity-overlap analysis [58].
295 Briefly, the relative abundance of each OTU in each community was calculated to
296 identify sets of OTUs within each pair of communities (i.e., within a treatment over
297 time). In the calculation of dissimilarity, only the shared OTUs were considered, and
298 the relative abundance of shared taxa was transformed to add up to 1. The dissimilarity
299 between renormalized vectors X and Y can be calculated using root Jensen–Shannon
300 divergence. This method and the relevant R script were modified from [59] Moreover,
301 the rate of community turnover in relative to the controls over time was quantified with
302 a linear regression model (see the Supplemental methods part 3 for details). A Wilcoxon
303 test was then applied to test for significant differences in community turnover between

304 microcosm B1 (day 5) and the other microcosm batches from later time points (B2–
305 B7), using the 'ggsignif' package [60].

306 **Results**

307 **Transformation of trace micropollutants**

308 There was no evidence that the removal rate of any of the micropollutants differed
309 between any of the consecutive microcosm batches (Fig. 2, Wilcoxon's test, all $P >$
310 0.05). In the microcosms treated with single micropollutant, most of BPA added to each
311 microcosm batch was degraded, achieving > 80% removal in the individual batches,
312 whereas the removal rate of TCS was less than 60% (Fig. 2). There was no evidence
313 that the removal of the BPA and TCS differed in the MI microcosms differed ($P > 0.05$)
314 (17.86 ± 15.17% and 25.11 ± 18.88 %, respectively; Fig. S5B). In the MII microcosms,
315 the percentage of removed BPA (65.83 ± 6.54%) was highest ($P < 0.05$), followed by
316 the TCS (13.76 ± 16.76%), BPS (5.93 ± 8.97%), and TCC (11.97 ± 14.08%), but the
317 differences were not significant (Fig. S5B).

318 **Community succession following micropollutant exposure**

319 The bacterial communities in the microcosms became taxonomically distinct both over
320 time and by micropollutant type (Fig. 3). There was very weak evidence that the
321 variation in the community composition among all microcosm batches correlated with
322 differences in the removal rates of BPA (P =0.06, Table S1), with high association in
323 its single treatments. However, there was strong evidence that the community variation
324 was correlated with TCS, with high association in TCS single and mixed treatments
325 (Fig. 3 and Table S1, P =0.001). The inoculation time (PERMANOVA, R^2 = 0.149),
326 micropollutant addition (R^2 = 0.11), and their interaction (R^2 = 0.055) significantly

327 influenced the community composition (all $P < 0.001$) (Table S2). Irrespective of the
328 inoculation time, the composition of the TCS, MI, and MII communities differed
329 significantly from that of the controls (pairwise PERMANOVA, $P < 0.01$; Table S2)
330 but not from each other. Conversely, the BPA communities significantly differed from
331 the other three treatments but not from the controls.

332 We also performed sequence processing with the use of amplicon sequence
333 variants (ASVs) from the DADA2 pipeline [61], and found that overarching patterns of
334 beta diversity were statistically indistinguishable from those observed using OTUs
335 defined at 97% sequence similarity by two separate tests (Fig. S6; Correlation in a
336 symmetric Procrustes M^2 value = 0.20, $P < 0.001$ on 999 permutations; Mantel
337 correlation = 0.92, $P < 0.001$ on 999 permutations). It has been suggested that
338 broadscale ecological patterns are robust in terms of the use of ASVs vs. OTUs [62].
339 Therefore, the patterns of microbial community dynamics following repeated exposure
340 to micropollutants can be considered robust regardless of whether 97% OTUs or ASVs
341 are selected.

342 Overall, the dissimilarity in the micropollutant-treated vs. control communities
343 significantly increased across microcosm batches, as each inoculation was replenished
344 with micropollutants (linear regression, all $P < 0.001$, Fig. S7). Furthermore, the
345 communities of the first inoculation (B1) impacted the response of some communities
346 inoculated at later time points (Fig. 4A). There was no evidence for the impact of the
347 B1 community composition on later communities across B2–B6 in the BPA-treated
348 microcosms and B2–B5 in the MII-treated microcosms, because of their community
349 resemblances (Wilcoxon test, $P > 0.05$; Fig. 4A). In both BPA and MII communities,
350 the correlation between dissimilarity and overlap (i.e., the number of shared OTUs
351 between the community pair) was negative ($P < 0.05$ Fig. 4B). This result was not

352 unexpected since the dispersal of microbial cells from one condition to another similar
353 environment may drive community dynamics towards convergence [63, 64]. However,
354 in the TCS and MI treatments, an impact of the initial communities on later ones
355 occurred only in B3, which is supported by the fact that the B3–B7 communities were
356 more dissimilar to B1 ($P < 0.05$; Fig. 4A). The negative correlation of the dissimilarity
357 and overlap analysis was nonsignificant for the TCS and MI communities such that the
358 high overlap did not result in a low dissimilarity ($P > 0.05$; Fig. 4B). This suggests that
359 there were differences in the dynamics of the shared taxa in those TCS and MI
360 communities over time compared to the communities in the other treatments.

361 **OTUs with distinct responses to micropollutant exposure**

362 In all treatments, a greater number of sensitive OTUs (41–64), a moderate number of
363 tolerant OTUs (6–15), and fewer opportunistic OTUs (0–3) were identified across
364 phases 1 (B1−B2), 2 (B3−B4), and 3(B5−B7) (Table 1; Fig. 5). The ecological groups
365 replaced one another over time, together accounting for a minimal relative abundance
366 of 0.5 % and a maximum of 60% in the individual samples (Fig. S8). Regardless of the
367 micropollutant type, members of Actinobacteria, Planctomycetes, and Verrucomicrobia
368 primarily consisted of sensitive OTUs (Table 1; Fig. 5). The tolerant OTUs were
369 primarily comprised of members affiliated with Alphaproteobacteria,
370 Gammaproteobacteria and Bacteroidetes. The majority of the tolerant OTUs were
371 unique to one treatment, with only two OTUs (OTU_48 and OTU_78) tolerating
372 micropollutant addition in all treatments (Fig. 5; Table S4). By contrast, many sensitive
373 OTUs (51) were shared between treatments, and at least between two treatments (Table
374 S4). Among the overlapping, 20 OTUs found in all treatments, maximal relative
375 abundance of which was mainly detected in the initial inocula or the controls, and most

376 were members of Actinobacteria (Table S4; Fig. 5). For instance, strictly sensitive taxa
377 Actinobacteria *ACK-M1*, found in both single and mixed micropollutant treatments,
378 exhibited its maximal relative abundance (15.94%) in the initial inocula. Whereas a
379 tolerant taxa Alphaproteobacteria *Novosphingobium*, shared between TCS and MI, had
380 its maximal relative abundance (30.48%) in the BPA treatments at late exposure batches
381 (B6), which has been reported as TCS-degrading bacteria in wastewater treatments and
382 agricultural soil [65, 66].

383 We further checked if the identification of those ecological categories is biased
384 towards the selection of bacterial taxa that were simply enriched in the controls. A large
385 fraction of the bacterial OTUs assigned with ecological strategies (> 67% of the total
386 OTUs surveyed) was unique to the micropollutants-added microcosms, which are not
387 shared in the controls when analyzed with the same filtering criteria as done for
388 ecological grouping (Fig. S9). This suggests that OTU ecological assignment was
389 robust when accounted for bias presented by composition data derived from the controls,
390 although there was little overlap in taxon responses between the controls and the
391 treatments.

392 **Partition of ecological processes on community variation**

393 Overall, homogenizing selection played a minor role in influencing community
394 variation across phases for each community pair (micropollutant-treated and control),
395 as it accounted for less than 8.33% of the ecological processes (Fig. 6). Instead, variable
396 selection (13.89–61.73%) and ecological drift (30.86–80.56%) dominated the
397 ecological processes across the three phases. These two processes exhibited
398 compensatory contributions, showing increasing variable selection but decreasing
399 ecological drift along the phases (Fig. 6). In the case of dispersal limitation, its relative

400 proportion was greater at phase 1 than the other phases for all treatments, especially
401 BPA and MII.

402 **Discussion**

403 We experimentally investigated the effects of persistent micropollutants on microbial
404 community dynamics to gain insights into community assembly mechanisms. Support
405 was mixed for our first hypothesis that differences in the removal rates of BPA vs. TCS
406 in environments exposed to single vs. mixed micropollutants. Although we observed a
407 certain degree of degradation for each of the four micropollutants, the removal rates of
408 each micropollutant differed depending on its presence alone or with other
409 micropollutants (Fig. 2 and Fig. S5). The BPA degradation was much greater than that
410 of TCS when the microbial communities were exposed to them separately (Fig. S5),
411 suggesting that the former elicits a subsidy response and the latter a stress response on
412 microorganisms [19, 21]. In contrast, these distinctive responses were counterbalanced
413 in the MI treatment, in which the microbial communities were exposed to a mixture of
414 BPA and TCS and there was no evidence found for the differences in their removal
415 rates. The presence of TCS may have inhibited the growth of bacteria that are capable
416 of degrading BPA. As such, the energy required by the microbial communities to
417 counteract the opposing responses to BPA and TCS in the MI treatment likely
418 accounted for the profound inhibition of BPA degradation. In MII, a significantly larger
419 fraction of BPA than of the three other micropollutants was removed. We hypothesize
420 that the presence of TCC reduced the inhibitory effect of TCS on BPA transformation
421 and thus degradation, even if the total concentration of TCC was 10-fold lower than
422 that of TCS in the microcosms. This could be tested by examining the removal
423 efficiency of BPA in the presence of TCC vs. that of both TCC and TCS with varying

424 quantity, but overall this finding highlights that the importance of micropollutant
425 characteristics in understanding the biodegradation potential performed by
426 microorganisms. Moreover, phenolic compounds (both BPA and BPS) act as a growth
427 medium for microorganisms by providing a source of carbon and energy. However,
428 they differ in degradability in the seawater (BPA > BPS)[67], as well as negative
429 impacts on biochemical and microbiological activity of soil (BPA < BPS) [68]. These
430 lines of reasoning explained a greater degradation of BPA than its analogue in MII (Fig.
431 2). The removal rates of micropollutants measured in our study are regarded as the
432 potential rather than absolute removal efficiency. This is because we cannot rule out a
433 potential role of labile carbon sources present in the 0.22-μm filtered medium in
434 influencing microbial capacity on degrading micropollutants. However, the starting
435 concentration of labile carbon sources present in all media was almost constant as they
436 originated from the same filtered freshwater. Thus, any effects of labile carbon sources
437 on the bacterial communities were most likely consistent in all the microcosms.

438 Our additional experiments showed that transformation of the micropollutants is
439 negligible in the absence of microorganisms and light (Fig. S2). Microorganisms pre-
440 exposed to low concentrations of BPA and TCS fully degraded higher concentrations
441 of those micropollutants during subsequent exposures and degradation occurred at a
442 faster rate (Fig. S3), but only when BPA and TCS were provided alone and not in
443 combination. Overall, pre-exposure to micropollutants can enhance the degradation
444 capacity of microbes later confronted with those or similar chemicals, which is
445 consistent with previous literature [14, 27].

446 Given that the ability of bacterial communities to transform the four
447 micropollutants surveyed, the questions remain: whether micropollutants accelerate
448 degradation through changes in bacterial communities, or the micropollutant degraded

449 and then altered the community composition? In our microcosms, each transfer was
450 random with respect to taxon identity, and such that no particular taxa were favoured
451 or disfavoured in one batch before being dispersed to the new batch. The variation
452 between the replicate batches was due to stochasticity in community assembly,
453 resulting in a slight but nonsignificant deviation in degradation patterns of
454 micropollutants over time. We found no evidence that the degradation of any
455 micropollutants differed across microcosm batches. Rather, there was strong evidence
456 on the differences in the bacterial communities between the micropollutant-added
457 microcosms vs. the controls, and that community turnover increased over time
458 following micropollutant exposure (Figs. 4A, S7). These results supported the second
459 hypothesis that the degradation of micropollutants alters the microbial communities.
460 Such community dynamics is mainly linked by the degradation of BPA and TCS in the
461 microcosms. We identified ecological groups based on changes in the relative
462 abundance of taxa following repeated micropollutant exposure, which integrates
463 population growth, death, survival, and reproduction over 35 days. Both sensitive and
464 tolerant taxa identified here may have affected micropollutant degradation either
465 directly or indirectly, such as by providing enzymatic repertoires to other community
466 members that enabled degradation, including interspecies interactions [69, 70]. The
467 larger proportion of sensitive than tolerant taxa in all treatments suggested that the
468 microbial community was not robust to the persistence of micropollutants in its
469 surroundings. Noteworthily, two strictly tolerant genera across treatments were
470 *Sphingomonas* and *Hydrogenophaga* (Table S4), which have been shown to be

471 enriched in the BPA or TCS-contaminated environments [68, 71, 72] and to produce
472 enzymes capable of degrading different pollutants [73].

473 Our results supported the third hypothesis that communities assembled at the early
474 stage of micropollutant exposure would be compositionally distinct from those that
475 formed at the later stage. Our findings generally corroborated previous studies showing
476 that the persistence of micropollutants even at low concentrations can influence
477 microbial community membership and abundance over time [24, 27, 74]. Despite a
478 substantial community variation between initial and late stages of the exposure,
479 community trajectory in the BPA and MII were driven by a set of shared taxa that
480 interacted similarly with the respective environments and other community members.
481 Conversely, the less pronounced negative dissimilarity-overlap relationships in the case
482 of the TCS and MI treatments indicated that the microbial taxa shared among different
483 inoculation batches within those treatments interacted differently with the same
484 environment over time. Hence, microbial communities assembled in identical habitats
485 can perceive those habitats differently such that their feedback on the surroundings and
486 other, coexisting members will differ as well, as noted elsewhere [59, 75].

487 We observed a gradual transition from stochastic to deterministic processes
488 towards the later stage of micropollutant exposure. In all treatments ecological drift
489 played a major role during phases 1 and 2 of community growth progression. This can
490 be explained by the random changes in abundances within microbial communities
491 exposed to micropollutants in confined microcosms, where dispersal is limited and
492 locally dormant cells are about to be awakened to colonize empty niches in the

493 environments upon disturbances [33, 76, 77]. In fact, sensitive taxa declined in the
494 relative abundances while tolerant taxa had negligible abundance (Fig. S8) during the
495 growth phases 1-2 (B1-B2 and B3-B4). Hence, tolerant taxa identified in this study
496 were recruited from rare member pool at the early stage of exposure and regrew to a
497 large population size (~ relative abundance of 10 %) at the later stage of exposure.
498 Moreover, during early exposure, microbial communities might have perceived no
499 differences in the environmental conditions between the controls and the treatments as
500 they were exposed under environmental levels of micropollutants. However, the
501 increasing contribution of variable selection to community assembly over time
502 suggested that the high phylogenetic turnover of the communities was driven by
503 heterogeneous abiotic and biotic conditions [52, 78]. The increasing effects of
504 micropollutants on the interactions among community members, such that the drivers
505 of community assembly no longer resembled those of the control and resulted in a high
506 rate of community turnover. Our findings suggest that the increasing differences in the
507 abiotic and biotic conditions imposed by the repeated introduction of micropollutants
508 across the microcosms led to higher species selection over time and thus enhance
509 microbial succession.

510 We also considered the potential role of priority effects on community assembly,
511 independent of the ecological process model used in our study. Priority effects emerge
512 when early colonizers exhaust or modify available resources, thus influencing the
513 establishment success of later colonizers. A strong priority effect in the case of TCS
514 and MI was evidenced by the significant difference in the composition of the initially

515 inoculated vs. any of the later inoculated communities (Fig. 4A). Once they were re-
516 inoculated, taxa that had thrived in the B1 batch modified the environments of later
517 batches, such that some taxa (presumably activated from a seed bank, given that no
518 source of new taxa was externally introduced into the systems) were less or more
519 favorable in later batches. However, for the BPA and MII treatments, the effects
520 occurred at later stage of the press disturbance, suggesting the increasing strength of
521 priority effects with increasing establishment time, as noted elsewhere [79].

522 Although our microcosms represent an ideal system which is removed from *in situ*
523 conditions, our experimental findings are valuable in at least three aspects, that is : i)
524 the prediction of microbial responses to persistent micropollutants, ii) the harnessing
525 of ecological principles and microbiomes in pollution management [80–82], and (iii)
526 the development of a conceptual model to be used to predict when and how stochastic
527 and deterministic processes contribute to community assembly after the exposure of
528 persistent micropollutants. Our results suggest that strong ecological drift drives
529 random community changes at the early stage of the exposure (Fig. 7). Prolonged
530 ecological drift, variable selection resulting from the effects of micropollutants, and the
531 abiotic and biotic conditions experienced differently by different microbes then
532 determine community succession at the mid-stage of the exposure. Additionally, the
533 high environmental heterogeneity together with the low densities of the communities
534 permitted the emergence of priority effects, in which the early establishment of taxa in
535 micropollutant-containing environments allowed a faster community succession. This
536 was supported by the decrease in the average gene copy number at the intermediate

537 phase (Fig. S4), when community assembly was driven by various ecological drivers
538 [83]. Finally, strong variable selection accompanied by increased priority effects likely
539 set the communities on distinct trajectories, with multiple equilibria at the late stage of
540 the exposure. In fact, more advanced community succession and multiple equilibria
541 characterized the end of the press disturbance, and both played a larger role in the TCS
542 and MII (and to a lesser extent in the BPA) communities than in the MI community
543 (Fig. 7), suggesting convergent recovery of those freshwater bacterial communities to
544 repeated micropollutant exposure.

545 In summary, we evidenced that micropollutants degrade and drive microbial
546 succession following repeated exposure. Mobile genetic elements (MGEs) function as
547 genetic rearrangement in microorganisms, thus allowing microbial communities to
548 tolerate or adapt to environmental disturbance, such as the presence of organic
549 pollutants [84, 85]. Future work that considers quantification of MGEs using qPCR at
550 successive time points when a microbial community is exposed to micropollutants,
551 would help assess the contribution of MGEs expression to changes in microbial
552 composition and degradation capacity. From the perspectives of microbiome
553 engineering, this study demonstrates the importance of the microbiome's preexposure
554 history in the design of long-term experiments that include artificial selection to direct
555 the evolution [81, 86] of a target microbiome highly optimized for pollutant removal in
556 an aquatic environment.

557 **Data and code availability**

558 The sequencing data were deposited in the NCBI SRA database with the Bioproject
559 number PRJNA497759. Our R scripts for statistics, data visualization and computing
560 notes are available on the Github repository (https://github.com/AnyiHu/Impacts-of-
561 micropollutants-on-freshwater-microbiome) and citable using
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570 **Authors' contributions**
571 AH and CPY conceived and designed the study. LS and TL performed the experiments
572 and measurement analyses. YL and QS provided the assistance for the LC-MS/MS
573 analysis. DIS, LS, JW and AH performed the data analyses and wrote the manuscript.
574 All authors discussed the results and commented on the manuscript.

575 **Competing interests**
576 The authors declare no competing interests.

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826 **Table 1.** Number of OTUs assigned to the ecological categories opportunistic, sensitive,
827 and tolerant in the four micropollutant treatments: 'BPA' microcosms containing
828 bisphenol A, 'TCS' microcosms containing triclosan, 'MI' microcosms containing a
829 mixture of bisphenol A and triclosan, 'MII' microcosms containing a mixture of
830 bisphenol A, triclosan, bisphenol S and triclocarban.

	Number of OTUs in each ecological category		
Treatment	Opportunistic	Sensitive	Tolerant
BPA	1	64	6
TCS	1	47	15
MI	3	51	12
MII	0	41	10

831

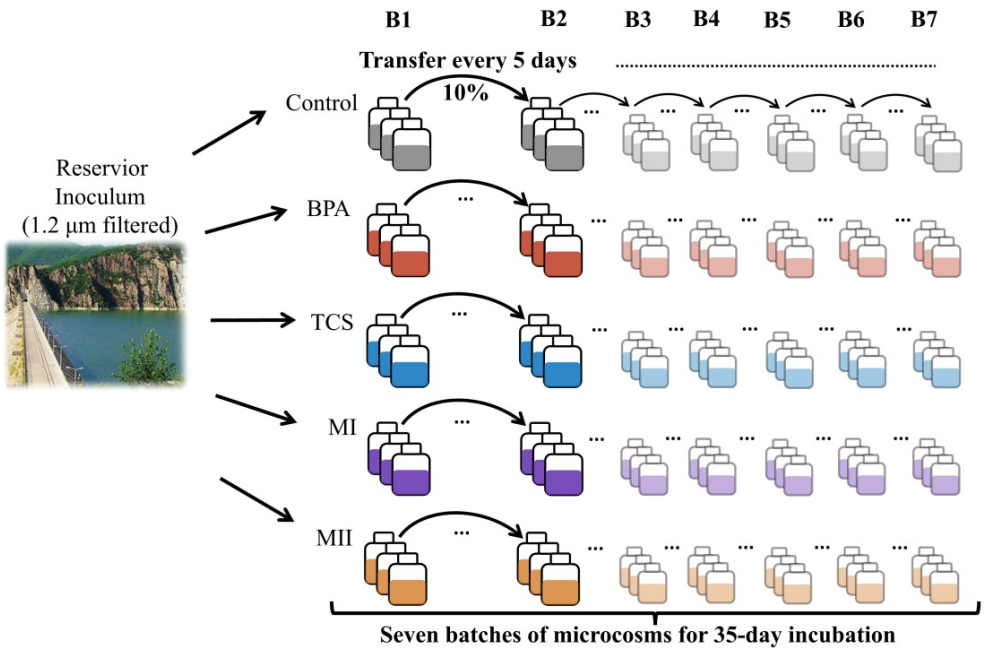

Figure 1. Experimental setup. In microcosm batch 1 (B1), 600 ml of the initial inoculum was added to bottles containing the micropollutants. Starting from the B1, 60 mL (10%) of the inoculum was pipetted from each of the replicates and transferred to fresh medium containing 540 mL of 0.22-µm-filtered water and freshly added micropollutants every 5 days. This procedure was carried out in the seven batches of microcosms from batch 1 (B1) through batch 7 (B7). Samples for bacterial community characterization and micropollutant removal were collected prior to the next inoculation. Treatments are color-coded: 'Control' is (without micropollutant addition, grey), 'BPA' (microcosms added with bisphenol A, red), 'TCS' (microcosms added with triclosan, blue), 'MI' (microcosms added with a mixture containing bisphenol A and triclosan, purple), 'MII' (microcosms added with a mixture containing bisphenol A and triclosan, bisphenol S and triclocarban, yellow).

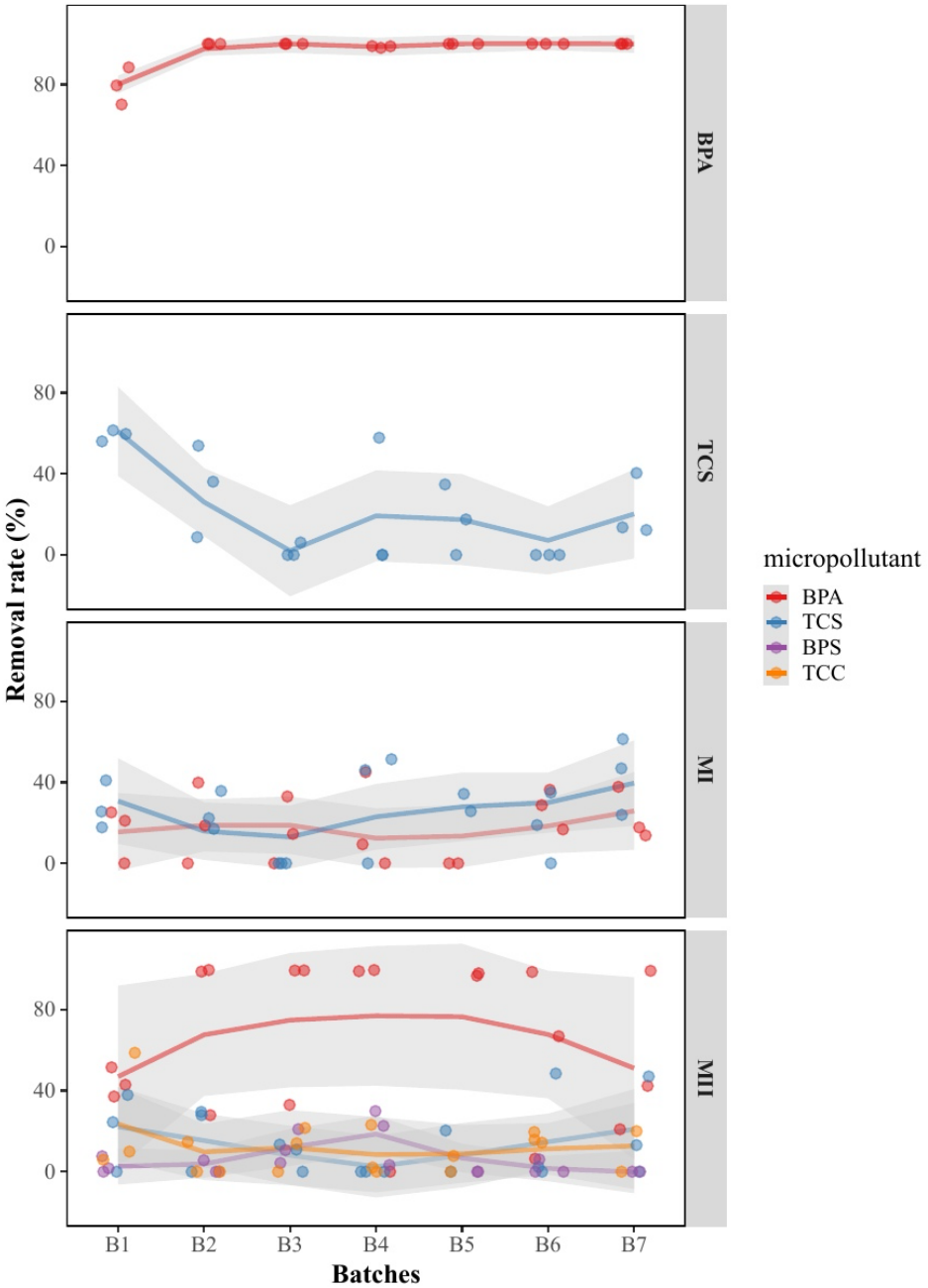

Figure 2. Percent removal of bisphenol A (BPA), triclosan (TCS), bisphenol S (BPS), and triclocarbon (TCC) in all microcosm batches for the single-micropollutant (BPA and TCA) treatments and in the mixed-micropollutant (MI and MII) treatments. Removal was defined as the decrease in the concentration of the chemical relative to the total concentration within each batch of microcosms. 'B1-B7' represent the number of microcosm batch that corresponds to every 5-day inoculation.

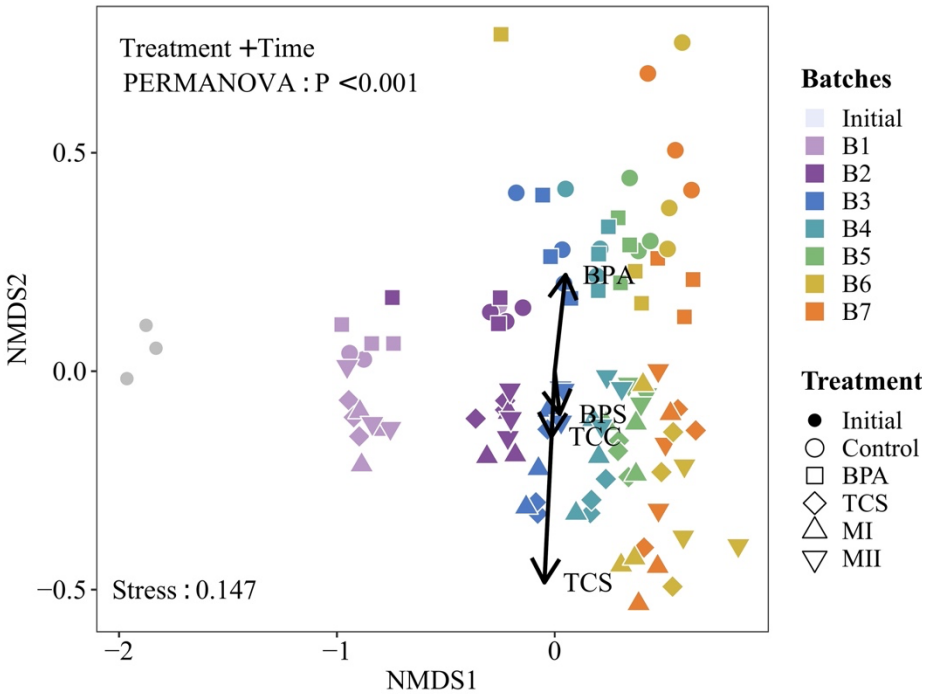

Figure 3. Difference in the bacterial communities in all microcosms batches as determined by non-metric multidimensional scaling (NMDS) ordination. Different treatments are represented by different symbols, and microcosms batches are color-coded. Explanatory environmental variables fitting the ordination are shown with solid arrows. Explanatory values of the environmental variables to differences in the communities are displayed in Supplementary Table S1. Treatment IDs are: 'Initial' containing the starting bacterial communities before the experimental implementation; 'Con' microcosms containing no micropollutant additions and serve as the controls; 'BPA' microcosms containing bisphenol A; 'TCS' microcosms containing triclosan; 'MI' microcosms containing a mixture of bisphenol A and triclosan; 'MII' microcosms containing a mixture of bisphenol A, triclosan, bisphenol-S and triclocarban. 'B1-B7' represent the number of microcosm batch that corresponds to every 5-day inoculation. Explanatory values of the environmental variables to differences in the communities are presented in Supplementary Table S1.

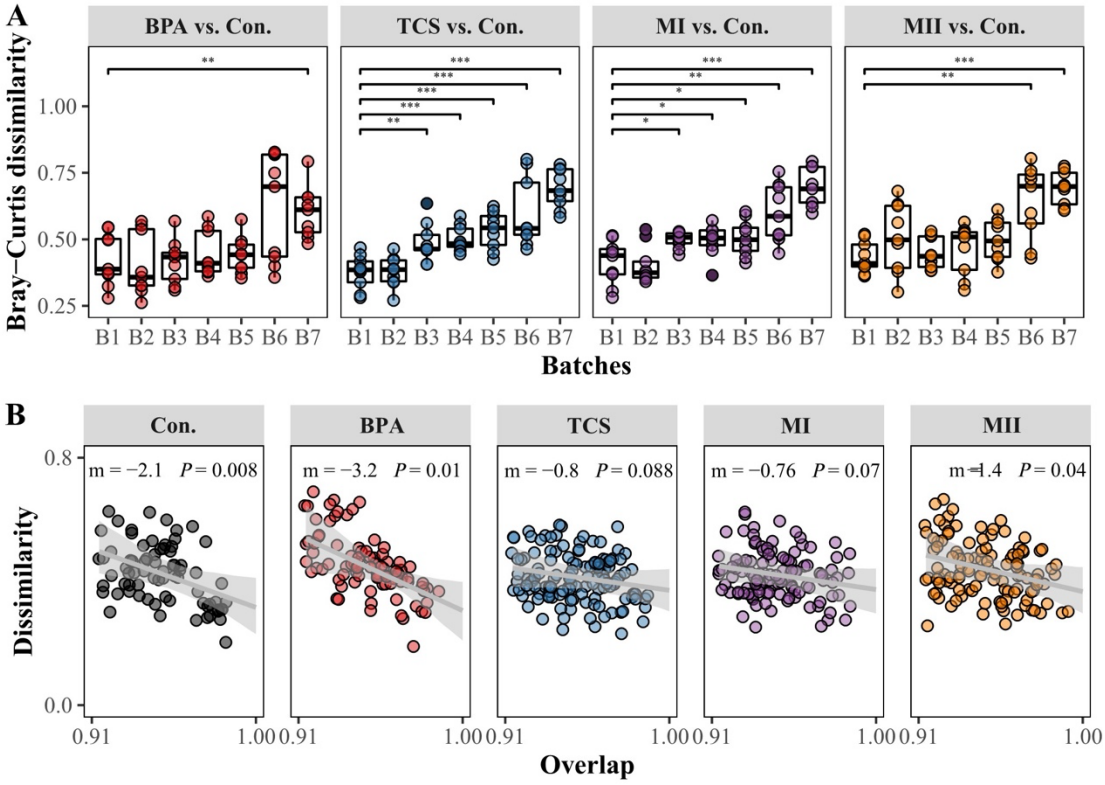

Figure 4. Bacterial community dissimilarity in the micropollutant treatments vs. the controls (i.e., 'Con.') over time (A) and the dynamic regularity within treatments (B). Significant differences in the Bray-Curtis dissimilarity for each pairwise comparison of the treatment and control (B1 microcosms vs. later microcosms in (A) were determined in a Wilcoxon test: ***, $P < 0.001$; **, $0.001 < P < 0.01$; *, $0.01 < P < 0.05$. The dissimilarity-overlap analysis within each treatment is shown in (B); out of total 210 sample pairs, only the sample pairs that had median overlap above cutoff = 0.913 were shown in the plots. The gray lines indicate the linear regression with the slope (m), and the P value (the fraction of bootstrap realizations in which the slope was negative). Gray shaded areas indicate the 95% confidence intervals of the linear regression. Significance level was determined at $P < 0.05$. 'B1-B7' represent the number of microcosm batch that corresponds to every 5-day inoculation.

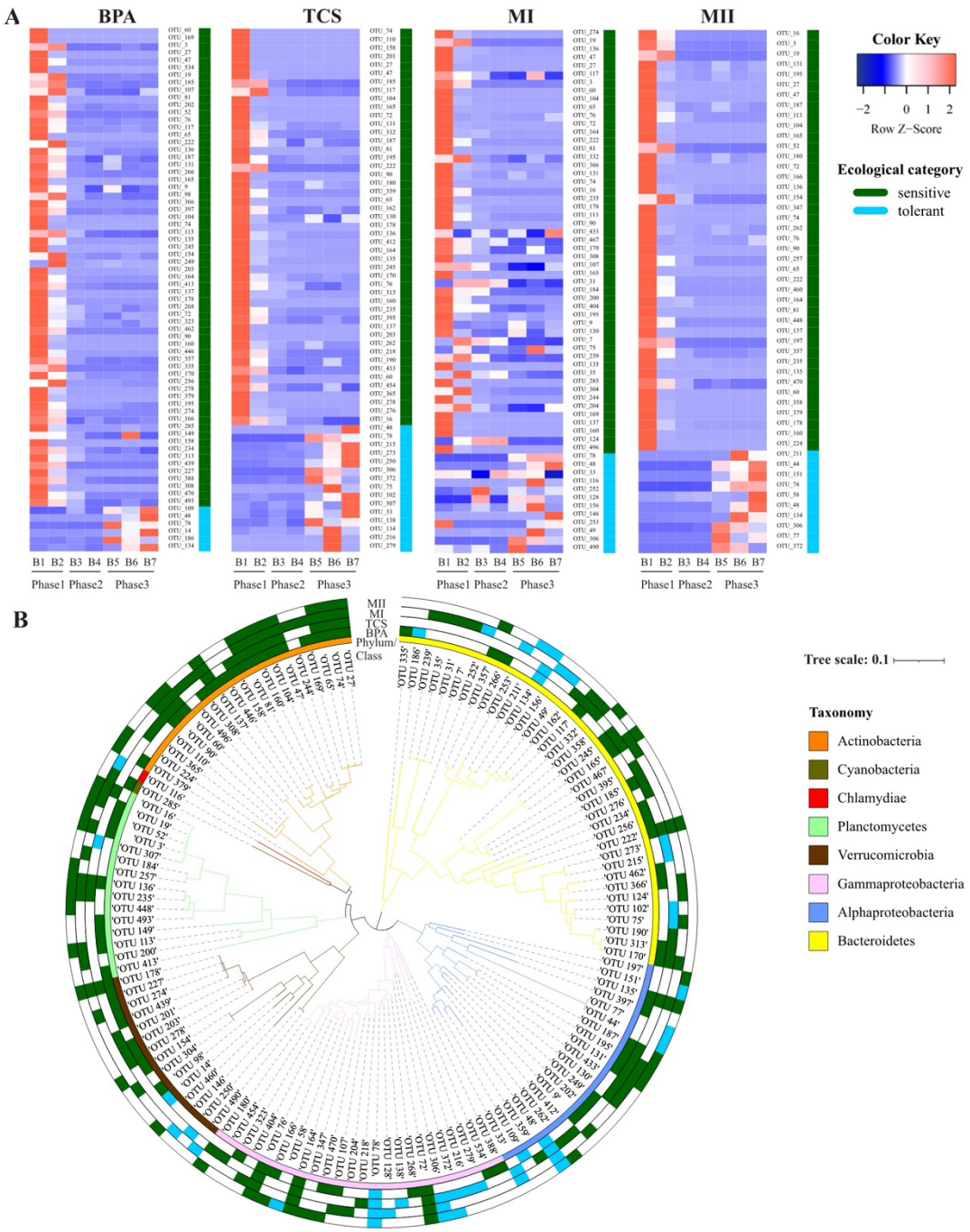

Figure 5. Changes in the relative abundances (rows of heatmap) of the OTUs assigned to the ecological categories tolerant and sensitive across the three growth phases (columns of heatmap) (A) and their phylogenetic distribution (B). In (A), color gradients of heatmap represent the relative abundances of individual by column with warm colors (toward red) indicating high abundance and cold colors (toward blue indicating low abundance within the sample. Column labels are batch IDs and the grouping of growth phases, and row labels the OTU IDs. Tolerant and sensitive OTUs were denoted in green and blue colors, respectively. In (B), the innermost circle

888 indicates the taxonomy (phylum/class) of the OTUs. The outer circles indicate the
889 ecological group of OTUs in each treatment group (BPA, TCS, MI, MII from inside to
890 outside, respectively).

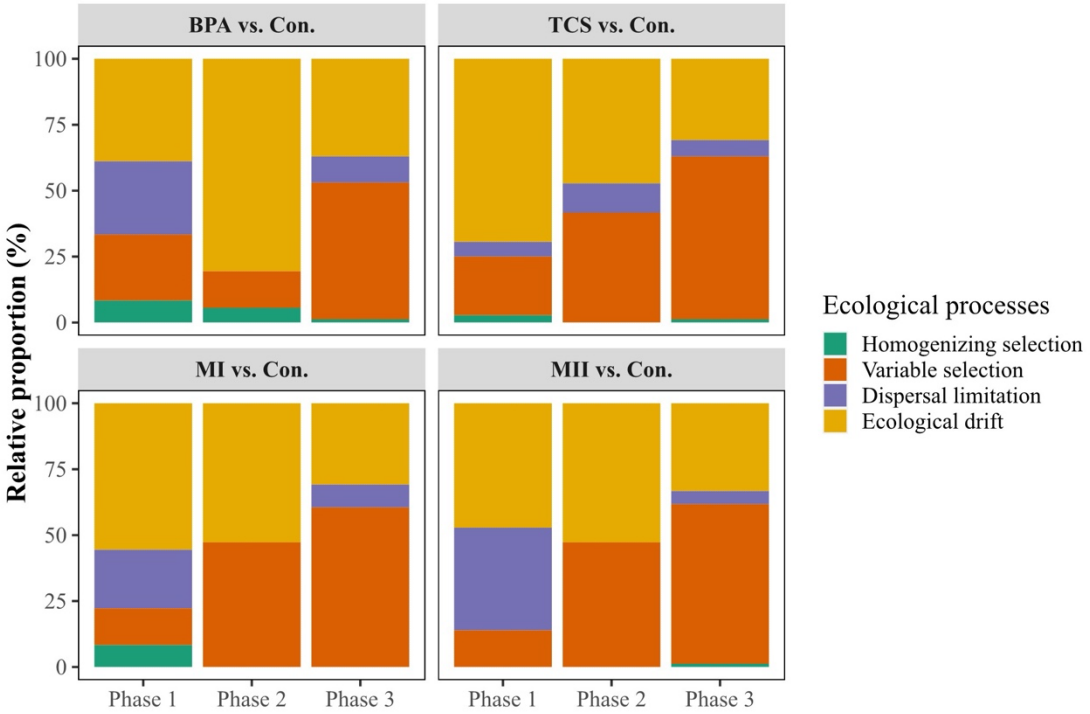

Figure 6. Relative proportion of the ecological processes (homogeneous selection, variable selection dispersal limitation, and ecological drift) contributing to the communities assembled in the micropollutant treatments (BPA, TCS, MI or MII) vs. the controls (i.e., 'Con.'). The relative contribution was expressed as the portion of pairwise sample assembly that could be attributed to each ecological process. Each of the three phases (corresponding to the early, mid, and late stages of the press disturbance) was classified according to the similarity in the average 16S rRNA gene copy numbers as an indicator of community growth progression. Three growth phrases across microcosm batches were: phase 1 (B1–B2), phase 2 (B3–B4), and phase 3 (B5–B7), as shown in Fig. S4G.

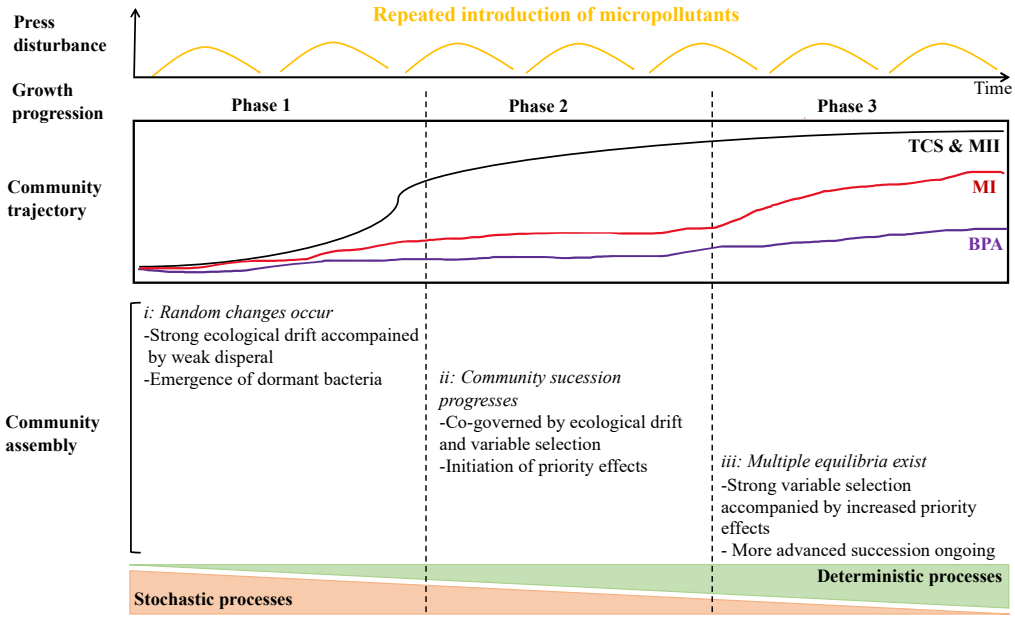

Figure 7 Conceptual model of microbial community assembly processes following a press disturbance. Considering the time continuity of the batch inoculation, the three phases of community growth progression (1, 2, and 3) also correspond to the early, mid, and late stages of the 35-day disturbance. This conceptual model illustrates when and how stochastic/deterministic processes contribute to the dynamics of microbial communities repeatedly exposed to micropollutants. Changes in community trajectory over time in the TCS or the MII treatment are indicated by the black line, MI treatment by the red line, and the BPA treatment by the purple line. Overall, stochastic and deterministic processes act in concert during community dynamics: stochastic assembly dominates at the early stage of a press disturbance/community development, and deterministic assembly at the later stage. Major community assembly mechanisms are delineated for each phase. Community succession accelerates at phase 2 such that multiple equilibria occur at phase 3. Community succession and multiple equilibria are not exclusive, but community turnover is high towards the end of the disturbance.