Microbial succession on degrading micropollutants under recurrent exposure

Running title: Ecological feedback between microbe-micropollutants

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Abstract

The increasing-volume release of micropollutants into natural surface waters has raised great concern due to their environmental accumulation. Persisting micropollutants can impact multiple generations of organisms via recurrent exposure, but their microbially-mediated degradation and the influence on community assembly remain understudied. Here, freshwater microbes were treated with four common micropollutants, alone or in combination, and then transferred to fresh medium containing the same level of micropollutants every 5-day to mimic the recurrent exposure of microbes for 35 days. The degradation of micropollutants and the associated communities were surveyed for each transfer. Even though the degradation of micropollutants did not accelerate over time, the degradation *per se* altered the microbial community composition. Community assembly was dominated by stochastic processes during early exposure, via random community changes and emergence of seedbanks, and deterministic processes later in the exposure, via advanced community succession. Early exposure stages were characterized by the presence of sensitive microorganisms such as Actinobacteria and Planctomycetes, which were then replaced by more tolerant bacteria such as Bacteroidetes and Gammaproteobacteria. This succession was tightly linked to a high degradation of bisphenol A and a lower one for triclosan. We hypothesize that microbes are not robust to micropollutant persistence but have an intrinsic capacity to recover

from that disturbance by acquiring carbon and energy from micropollutant degradation. 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 (Deblonde et al., 2011; Schwarzenbach et al., 2006). 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 (Luo et al., 2014; Z. Wang et al., 2015). For instance, the majority of micropollutants originate from direct and indirect inputs of organic contaminants that are released via the effluents of wastewater treatments plants (da Silva et al., 2020; Eggen et al., 2014; Schwarzenbach et al., 2006; Vila-Costa et al., 2020). 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 (Kuroda et al., 2012; Liu et al., 2015; Roberts et al., 2016; Rodea-Palomares et al., 2016; Yang et al., 2013). Nonetheless, little is known about the mechanisms by which micropollutants impact the turnover of aquatic microbial communities (Cerro-Gálvez et al., 2021; Martinez-Varela et al., 2021; Vila-Costa et al., 2020), especially those of freshwater bacteria (Adyari et al., 2020; Tlili et al., 2020), 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 (Chalew & Halden, 2009; Zhang et al., 2013). For instance, bisphenol A (BPA) and its substituted derivatives (e.g., bisphenol S, BPS) (Staples et al., 1998) are typical subsidy responses, as their relatively simple chemical structure and low toxicity allow both to be utilized as carbon sources by microbial isolates (Choi & Lee, 2017; Zhang et al., 2013). Stress responses are elicited by pharmaceutical and personal care micropollutants such as triclosan (TCS) and its substituted derivatives (e.g., triclocarban, TCC) (McMurry et al., 1998), because both inhibit the growth of several microbial taxa, consistent with their resemblance to antimicrobials (Cabana et al., 2007; Chalew & Halden, 2009). Both BPA and TCS in the field strongly influence microbial diversity, especially that of the central species and module communities comprising the microbial co-occurrence network (Hu et al., 2017).

Microbial responses to chemical pollutants can be modified by the exposure legacy of the community. For example, microbial communities with a history of containment exposure may differ from contaminant-naïve communities in the rate and extent of chemical degradation (Boyd et al., 2008; Coll et al., 2020; Okere et al., 2017). They may also differ in their plasticity (Cerro-Gálvez et al., 2021) upon subsequent exposure to similar contaminants. However, the ecological drivers of community succession following repeated micropollutant exposure are not well-understood. Studies of community dynamics over multiple generations and a time period relevant for community stability could provide insights into the ecological traits that determine

92 taxon sensitivity and tolerance, and therefore the ability of microbial community
93 members to degrade micropollutants.

94 Press disturbances in natural environments can impact multiple generations of
95 organisms (Edward A. Bender, 1984) by altering the affected ecosystems beyond the
96 possibility of recovery or by causing a shift to a community with a new stable state
97 (Shade et al., 2012). Following a stress event such as recurrent micropollutant exposure,
98 communities rely on reassembly from the local seedbanks such that succession
99 eventually results in a new steady state. During the early stages of community dynamics,
100 no-longer-dormant local species undergo a secondary succession characterized by new
101 abiotic and biotic interactions. The emergence of resuscitation from dormancy is
102 considered as stochastic processes that lead to divergent communities (Buerger et al.,
103 2012; Lee et al., 2017; Lennon et al., 2021). When abiotic and biotic conditions allow
104 a stabilization of the initial communities, community formation at a later phase of a
105 press disturbance is expected to converge, through the succession of microbial species
106 (Philippot et al., 2021). In addition, the plasticity of microbial taxa in response to
107 recurrent micropollutant exposure has been shown to increase over multiple generations
108 (Cerro-Gálvez et al., 2021), with later-assembled communities shifting to a new stable
109 state that differs from the one before the exposure.

110 Here, a press disturbance was simulated in a microcosm experiment in which a
111 reservoir bacterial community was transferred into fresh medium containing trace
112 concentrations of typical micropollutants that were refreshed with recurrent
113 micropollutant exposure every 5 days. Several different micropollutants are typically
114 present simultaneously within aquatic environments, but whether their interactions lead
115 to effects on microbial communities that differ from those of single micropollutants is
116 unclear (Rodea-Palomares et al., 2016). Thus, we also experimentally manipulated

117 single and multiple micropollutants to determine their impacts on taxon coexistence,
118 the regularity of community assembly, and micropollutant degradation ability. We
119 hypothesized that because BPA elicits a subsidy response and TCS a stress response,
120 the removal rate of BPA would be higher than that of TCS, with larger differences in
121 the removal rates by microbial communities exposed to these micropollutants
122 individually rather than in combination with other micropollutants. We also
123 hypothesized that micropollutants degrade and alter the microbial community
124 compositions after exposure. Accordingly, the microbial communities assembled at the
125 initial and late phases of the micropollutant exposure would differ substantially and are
126 primarily driven by either stochastic or deterministic assembly process.

127 **Materials and Methods**

128 **Water collection**

129 We selected the bacterial species pools from Shidou Reservoir, which is one of largest
130 sources of drinking water in Xiamen, China (Hu et al., 2018) and is therefore under
131 governmental protection from pollution originating from household drainages and a
132 nearby dam (Fig. S1). Unlike other contaminated aquatic environments (Chalew &
133 Halden, 2009; Im & Löffler, 2016; Sun, Li, Ma, et al., 2016), the concentrations of BPA,
134 BPS, TCS, and TCC in Shidou Reservoir (China) are very low (< 1 ng/L), such that the
135 reservoir's waters provide an ideal system to investigate the influence of persistent
136 micropollutants on an aquatic microbiome. Surface water (100 L) from Shidou
137 Reservoir (24˚41'39"N, 118˚0'37"E) was collected at 0.5 m depth on June 1, 2017 (Fig.
138 S1). Temperature, pH, conductivity, dissolved oxygen (DO), and dissolved oxygen
139 saturation, measured *in situ* using a HACH HQ40d multi-parameter meter (HACH,
140 Loveland, CO, USA), were 29.0°C, 8.90, 54.4 μs/cm, 8.84 mg/L and 116.7%,

141 respectively. The concentrations of BPA, BPS, TCS, and TCC in the reservoir water
142 were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS)
143 (ABI 3200QTRAP, Framingham, MA, USA) with solid-phase extraction and all were
144 < 1 ng/L at the time of sampling (details of the measurements are provided below). The
145 collected water was transported from the field to the laboratory within 2 h after
146 sampling.

147 **Experimental setup**

148 BPA, BPS, TCS, and TCC were dissolved in acetone at concentrations of 800, 800, 400,
149 and 400 μg/L, respectively, and stored as stocks in glass bottles (HPLC grade). The
150 micropollutants were previously shown to be chemically stable when dissolved in an
151 organic solvent (Sun et al., 2017; Sun, Li, Ma, et al., 2016). From the 100 L of collected
152 water, 15 L were filtered through a 1.2-μm polycarbonate filter (Millipore, Ireland), to
153 minimize top-down regulation by planktonic grazers, and then used as the initial
154 inoculum. The remaining unfiltered water was filtered through a 0.22-μm
155 polycarbonate filter (Millipore, Cork, Ireland) to remove microorganisms and then
156 stored in the dark for later use in the preparation of incubation medium. Two treatments
157 consisting of single micropollutants (BPA and TCS) and two treatments consisting of
158 micropollutant mixtures (MI = BPA + TCS) and (MII = BPA + BPS + TCS + TCC)
159 were established.

160 Time-serial microcosms comprising seven inoculation batches from batch 1
161 (named hereafter 'B1') through batch 7 ('B7') were established to investigate the
162 microbial community response to a press disturbance of micropollutants resulting from
163 a single short-term event but maintained for 35 days (Fig. 1). Prior to the experimental
164 initiation, the caps of the bottles containing the micropollutants were loosened for > 12

165 h to volatilize the acetone to minimize its influence on the microbial responses. In
166 microcosm B1, 600 ml of the initial inoculum was added to bottles that already
167 contained the micropollutants and nutrients, such that the final concentration of each
168 micropollutant in the treatments was as follows: BPA: 1 μg/L BPA, TCS: 1 μg/L TCS,
169 MI: 1 μg/L BPA + 1 μg/L TCS, and MII: 1 μg/L BPA + 0.1 μg/L BPS + 1 μg/L TCS +
170 0.1 μg/L TCC. BPS and TCC are the derivatives of the BPA and TCS, respectively, and
171 the concentrations of those analogues in the environment are one order of magnitude
172 lower than their chief compounds in the seawater (Staples et al., 1998; McMurry et al.,
173 1998; Xie et al., 2022). The final concentration of each micropollutant in the
174 microcosms closely represented their ambient environmental conditions as reported in
175 literature (Chalew & Halden, 2009; Yamazaki et al., 2015). Further, given the low
176 nutrient concentrations (Hu et al., 2018; Kalyuzhny & Shnerb, 2017), we adjusted the
177 concentrations of nitrogen and phosphorus in the filtered water to 128.6 and 8.04 μM
178 to promote microbial growth by the addition of KNO_3 and KH_2PO_4, respectively (Wang
179 et al., 2016). After 5 days of incubation, 60 mL of the B1 inoculum was pipetted from
180 each of the replicates and transferred to a sterile bottle containing 540 mL of 0.22-μm-
181 filtered water and freshly added micropollutants. This newly inoculated microcosm
182 served as batch 2 (named hereafter 'B2'). The micropollutant treatments and controls
183 were amended with 128.6 μM N and 8.04 μM P after each inoculation to maintain
184 similar amounts of these nutrients throughout the experiment. The same inoculation
185 procedure was applied at each 5-day interval to initiate microcosms B3–B7, such that
186 the experimental period was 35 days (Fig. 1). Microbial cells were transferred between
187 pairwise replicates, with the remainder used in the downstream analyses described
188 below. An additional set of experimental bottles without micropollutant addition served
189 as the controls. In total, there were five treatments, each with three replicates, for a total

190 of 15 microcosm replicates per batch. All microcosms, i.e., 105 experimental bottles
191 during the 35 days, were incubated in the dark at 28°C with shaking at 100 rpm.

192 **Additional experiments to test micropollutant degradation**

193 Additional experiments were conducted to determine (i) micropollutant removal rates
194 in the absence of microorganisms, to rule out the gradual degradation of the
195 micropollutants in the treatments due to their chemical instability, and (ii) how the
196 ability of the microorganisms to transform micropollutants differed depending on
197 whether they have previous exposures or not. For the first additional experiment,
198 reservoir water was filtered through 0.22-μm filters and then amended with a mixture
199 consisting of 1 μg BPA/L, 0.1 μg BPS/L, 1 μg TCS/L, and 0.1 μg TCC/L as a blank
200 control. A second additional experiment was conducted to assess whether the ability of
201 bacterial assemblages to degrade BPA and TCS was enhanced by the evolving
202 community succession following micropollutant addition. Thus, 40 ml of the initial
203 inoculum and 40 ml of the bacterial assemblage at the end of the experiment (after
204 incubation of the B7 microcosms) were each amended with 160 ml of BPA, 160 ml of
205 TCS, or a mixture thereof, with each micropollutant present at a final concentration of
206 1 mg/L. The removal rates of BPA and TCS in these treatments after 7 days were
207 measured and the rates in the initial inocula vs. those in the B7 microcosms were
208 compared. The incubation conditions were the same for each treatment and the
209 experiment was conducted in triplicate.

210 **Micropollutant analysis**

211 For each microcosm batch, ~20 mL of the inoculum was removed from each bottle at
212 0, 2 and 5 days for micropollutant analysis. The four micropollutants BPA ($C_{15}H_{16}O_2$,

213 BPS ($C_{12}H_{10}O_4S$), TCS($C_{12}H_7Cl_{13}O_2$), and TCC ($C_{13}H_9Cl_3N_2O$) were concentrated
214 and purified by a 12-port Visiprep-DL SPE vacuum manifold (Supelco, Bellefonte, PA,
215 USA) with Oasis HLB cartridges (500 mg, 6 mL; Waters, Millford, MA, USA), and
216 then were measured using LC-MS/MS (ABI 3200QTRAP, Framingham, MA, USA),
217 as described in our previous work (Sun et al., 2017). Chromatographic separation was
218 performed by using a Kinetex C18 column (100 mm × 4.6 mm, 2.6 μm; Phenomenex,
219 Torrance, CA, USA) from Shimadzu LC system (Shimadzu, Japan).

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

221 The remaining inoculum (~470 mL) of each replicate was filtered onto 0.22-μm
222 polycarbonate filters (Millipore, Ireland), which were stored at −80°C until used for
223 DNA extraction. DNA from the 105 samples was extracted using the FastDNA for soil
224 kit (MP Biomedical, Santa Ana, CA, USA) according to the manufacturer's protocol,
225 except that the beating speed was set to 6.0 m/s for 80 s. The universal primer pair 515F
226 (5'-GTG YCA GCM GCC GCG GTA-3') and 907R (5'-CCG YCA ATT YMT TTR
227 AGT TT-3') was used to amplify the V4-V5 region of the bacterial 16S rRNA genes.
228 The PCR products were purified, mixed in equal amounts, and sequenced on an
229 Illumina MiSeq platform with the 2 × 300 bp paired-end protocol at the Majorbio Bio-
230 Pharm Technology Co., Ltd. (Shanghai, China).

231 **Sequence processing**

232 The raw sequencing data were processed using the LotuS pipeline (Hildebrand et al.,
233 2014) with the following criteria: i) average sequence quality > 27, ii) sequence length >
234 170 bp, iii) no ambiguous bases, iv) no mismatches with the barcodes or primers, and
235 v) homopolymer length < 8 bp. Operational taxonomic units (OTUs) were clustered at

236 a 97% similarity using UPARSE (Edgar, 2013). A representative sequence of each OTU
237 was taxonomically quantified using the RDP classifier with the Silva database v132
238 (Quast et al., 2013), at a bootstrap cutoff of 80%. All samples were rarified to 36, 000
239 sequence reads (the smallest library size). The subsampled OTU table contained 1375
240 OTUs. The α- and β-diversity indices of bacterial communities were calculated from
241 the rarified dataset using QIIME v1.9.1 (Caporaso et al., 2010) with the script
242 "core_diversity.py".

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

244 The 16S rRNA gene copy number among prokaryotes within a defined period indicates
245 the number of individuals within a community and reflects the growth phase of that
246 community (Klappenbach et al., 2000). In this study, the 16S rRNA gene copy number
247 of each OTU found in the samples was estimated using the rrnDB database v5.5
248 (Klappenbach et al., 2001) and the weighted mean rRNA gene copy number of all OTUs
249 found in each sample was then calculated as described previously (Wu et al., 2016).
250 The abundance-weighted average gene copy number of a community was plotted
251 against the sampling time for each treatment. The similar overarching pattern of the
252 average rRNA gene copy numbers of the bacterial communities in all microcosms over
253 time (Figs. S2A-F) indicated the similar growth trajectories of those communities. In
254 addition, a hierarchical cluster analysis with complete clustering criterion was used to
255 cluster the similarity of average gene copy numbers from all micropollutant-treated
256 microcosms belonging to the same batch type, with an exclusion of the controls (Fig.
257 S2G). Three growth progression phrases across all microcosm batches were identified

258 based on similar average gene copy numbers and the time continuity of the inoculation
259 (Fig. S2G): phase 1 (B1–B2), phase 2 (B3–B4), and phase 3 (B5–B7).

260 **Relative contribution of ecological processes**

261 According to a quantitative ecological framework (Stegen et al., 2013, 2015),
262 community assembly can be summarized by five major ecological processes: i)
263 homogenizing selection for a convergent composition between communities, ii)
264 variable selection for a divergent composition between communities, iii) dispersal
265 limitation for a divergent composition between communities due to limited dispersal
266 events, iv) homogenizing dispersal for a convergent composition between communities
267 due to unlimited dispersal, and v) ecological drift (referred to "undominated processes"
268 in (Stegen et al., 2015)). The phylogenetic turnover among the communities was
269 quantified by the weighted β-mean nearest taxon distance (βMNTD). The βNTI was
270 calculated as the difference between the observed βMNTD and the mean of the null
271 distribution (999 randomizations). The criteria determining the fraction of each process
272 were as follows: i) homogenizing and variable selection: higher (> 2) and lower (< −2)
273 βNTI, respectively; ii) dispersal limitation and homogenizing dispersal: non-significant
274 |βNTI| (< 2) but RC_{bray} > 0.95 and < −0.95, respectively. iii) ecological drift: non-
275 significant |βNTI| value (< 2) and non-significant $|RC_{bray}|$ value (< 0.95) (Stegen et al.,
276 2015). To infer the relative contributions of the different community assembly
277 processes to community turnover over time, for each pair consisting of a micropollutant
278 treatment and its control the βNTI and RC_{bray} values were calculated separately for each
279 of the three growth phrases. By comparing the community assembly process in the
280 treatment vs. the controls, we were able to minimize confounding effects that may have
281 obscured community variation and thus better predict the treatment effect. The relative

282 contribution was expressed as the portion of pairwise sample assembly that could be
283 attributed to each ecological process.

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

285 The responses of individual OTUs to micropollutant addition were identified by
286 analyzing the differences in the relative abundance of each OTU during the three
287 growth phases for each treatment, using the 'DESeq2' R package v 1.26.0 (Love et al.,
288 2014). Significant values were corrected for multiple tests using the Benjamini-
289 Hochberg correction with an adjusted α value of 0.05. The responses were categorized
290 as: i) sensitive, when relative OTU abundances were significantly higher in phase 1
291 than in phases 2 and 3; ii) opportunistic, when relative abundances were significantly
292 higher in phase 2 than in phases 1 and 3; iii) tolerant, when relative abundances were
293 significantly higher in phase 3 than in phases 1 and 2. The ecological categories
294 represented a group of bacterial members whose growth fitness optimal in response to
295 repeated exposure could be documented collectively. In addition, to visualize the
296 phylogenetic relationships of the OTUs assigned to the ecological groups,
297 representative sequences of those OTUs were used to construct a phylogenetic tree
298 using iTOL v5 (Letunic & Bork, 2019).

299 **Micropollutant turnover and community analysis**

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

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

307 Differences in the bacterial communities in all microcosms were explored using
308 non-metric multidimensional scaling (NMDS) based on the Bray-Curtis dissimilarity
309 metric and by fitting removal rates of micropollutants to the ordination. This allowed
310 us to assess whether the degradation of micropollutants was tied to the microbial
311 succession. A permutational multivariate analysis of variance (PERMANOVA)
312 (Anderson, 2001) was conducted to test the effects of micropollutants, inoculation time
313 (as indicated by the microcosm batch), and their interactive effects on the taxonomic
314 composition of the bacterial communities. A pairwise PERMANOVA was performed
315 to test for significant differences in community composition between each treatment
316 pair. The multivariate analyses were performed using the R package 'vegan' (Oksanen
317 et al., 2019). Whether communities with the shared taxa exhibit similar dynamics was
318 assessed in a dissimilarity-overlap analysis (Bashan et al., 2016). Briefly, the relative
319 abundance of each OTU in each community was calculated to identify sets of OTUs
320 within each pair of communities (i.e., within a treatment over time). In the calculation
321 of dissimilarity, only the shared OTUs were considered, and the relative abundance of
322 shared taxa was transformed to add up to 1. The dissimilarity between renormalized
323 vectors X and Y can be calculated using root Jensen–Shannon divergence. Based on
324 the dissimilarity-overlap analysis, 15 pairwise comparisons were obtained in this study.
325 This method and the relevant R script were modified from Vila et al. (2020). Moreover,
326 the rate of community turnover in relative to the controls over time was quantified with

327 a linear regression model (see the Supplemental methods for details). A Wilcoxon test
328 was then applied to test for significant differences in community turnover between
329 microcosm B1 (day 5) and the other microcosm batches from later time points (B2–
330 B7), using the 'ggsignif' package (Ahlmann-Eltze & Patil, 2021). The data obtained in
331 this study were visualized using the R package 'ggplot2'.

332 **Results**

333 **Transformation of trace micropollutants**

334 There was no evidence that the removal rate of any of the micropollutants differed
335 between any of the consecutive microcosm batches (Fig. 2, Wilcoxon's test, all $P >$
336 0.05). In the microcosms treated with single micropollutant, most of BPA added to each
337 microcosm batch was degraded, achieving > 80% removal in the individual batches,
338 whereas the removal rate of TCS was less than 60% (Fig. 2). When comparing the
339 removal rates of BPA and TCS in the MI microcosms, there was no evidence that the
340 removal of the two micropollutants from the MI microcosms differed ($P > 0.05$) (17.86
341 ± 15.17% for BPA and 25.11 ± 18.88 % for TCS; Fig. S3B). In the MII microcosms,
342 the percentage of removed BPA (65.83 ± 6.54%) was highest ($P < 0.05$), followed by
343 the TCS (13.76 ± 16.76%), BPS (5.93 ± 8.97%), and TCC (11.97 ± 14.08%), but the
344 differences were not significant (Fig. S3B).

345 The removal proportion of the four micropollutants in the bacteria-free controls
346 was stable during the 7-day incubation, as shown in the first additional experiment (Fig.
347 S4). This therefore suggests that transformation of the micropollutants is negligible in
348 the absence of microorganisms and light. The second experiment showed that
349 microorganisms pre-exposed to low concentrations of BPA and TCS fully degraded
350 higher concentrations of those micropollutants during subsequent exposures and

351 degradation occurred at a faster rate (Fig. S5), but only when BPA and TCS were
352 provided alone and not in combination.

353 **Community succession following micropollutant exposure**

354 The bacterial communities in the microcosms became taxonomically distinct both over
355 time and by micropollutant type (Fig. 3). There was very weak evidence that the
356 variation in the community composition among all microcosm batches correlated with
357 differences in the removal rates of BPA (P =0.06, Table S1), with high association in
358 its single treatments. However, there was strong evidence that the community variation
359 was correlated with TCS, with high association in TCS single and mixed treatments
360 (Fig. 3 and Table S1, P =0.001). The inoculation time (PERMANOVA, R^2 = 0.149),
361 micropollutant addition (R^2 = 0.11), and their interaction (R^2 = 0.055) significantly
362 influenced the community composition (all $P < 0.001$) (Table S2). Irrespective of the
363 inoculation time, the composition of the TCS, MI, and MII communities differed
364 significantly from that of the controls (pairwise PERMANOVA, $P < 0.01$; Table S2)
365 but not from each other. Conversely, the BPA communities significantly differed from
366 the other three treatments but not from the controls.

367 Overall, the dissimilarity in the micropollutant-treated vs. control communities
368 significantly increased across microcosm batches, as each inoculation was replenished
369 with micropollutants (linear regression, all $P < 0.001$, Fig. S6). Furthermore, the
370 communities of the first inoculation (B1) impacted the response of some communities
371 inoculated at later time points (Fig. 4A). There was no evidence for the impact of the
372 B1 community composition on later communities across B2–B6 in the BPA-treated
373 microcosms and B2–B5 in the MII-treated microcosms, because of their community
374 resemblances (Wilcoxon test, $P > 0.05$; Fig. 4A). In both BPA and MII communities,

375 the correlation between dissimilarity and overlap (i.e., the number of shared OTUs
376 between the community pair) was negative ($P < 0.05$ Fig. 4B). This result was not
377 unexpected since the dispersal of microbial cells from one condition to another similar
378 environment may drive community dynamics towards convergence (Lindström &
379 Östman, 2011; Shen et al., 2018). However, in the TCS and MI treatments, an impact
380 of the initial communities on later ones occurred only in B3, which is supported by the
381 fact that the B3–B7 communities were more dissimilar to B1 ($P < 0.05$; Fig. 4A). The
382 negative correlation of the dissimilarity and overlap analysis was nonsignificant for the
383 TCS and MI communities such that the high overlap did not result in a low dissimilarity
384 ($P > 0.05$; Fig. 4B). This suggests that there were differences in the dynamics of the
385 shared taxa in those TCS and MI communities over time compared to the communities
386 in the other treatments.

387 **OTUs with distinct responses to micropollutant addition**

388 In all treatments, a greater number of sensitive OTUs (41–64), a moderate number of
389 tolerant OTUs (6–15), and fewer opportunistic OTUs (0–3) were identified across
390 phases 1 (B1−B2), 2 (B3−B4), and 3(B5−B7) (Table 1 and Fig. 5). The ecological
391 groups replaced one another over time, together accounting for a minimal relative
392 abundance of 0.5 % and a maximum of 60% in the individual samples (Fig. S7).
393 Regardless of the micropollutant type, members of Actinobacteria, Planctomycetes, and
394 Verrucomicrobia primarily consisted of sensitive OTUs (Table 1; Fig. 5). The tolerant
395 OTUs were primarily comprised of members affiliated with Alphaproteobacteria,
396 Gammaproteobacteria and Bacteroidetes. The majority of the tolerant OTUs were
397 unique to one treatment, with only two OTUs (OTU_48 and OTU_78) tolerating
398 micropollutant addition in all treatments (Fig. 5 and Table S4). By contrast, many

399 sensitive OTUs (51) were shared between treatments, and at least between two
400 treatments (Table S4). Among the overlapping, 20 OTUs found in all treatments,
401 maximal relative abundance of which was mainly detected in the initial inocula or the
402 controls, and most were members of Actinobacteria (Table S4; Fig. 5). For instance, a
403 strictly sensitive taxa Actinobacteria *ACK-M1*, found in both single and mixed
404 micropollutant treatments, exhibited its maximal relative abundance (15.94%) in the
405 initial inocula. Whereas a tolerant taxa Alphaproteobacteria *Novosphingobium*, shared
406 between TCS and MI, had its maximal relative abundance (30.48%) in the BPA
407 treatments at late exposure batches (B6), which has been reported as TCS-degrading
408 bacteria in wastewater treatments and agricultural soil (Zhou et al., 2013; Thelusmond
409 et al., 2019).

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

411 Overall, homogenizing selection played a minor role in influencing community
412 variation across phases for each community pair (micropollutant-treated and control),
413 as it accounted for less than 8.33% of the ecological processes (Fig. 6). Instead, variable
414 selection (13.89–61.73%) and ecological drift (30.86–80.56%) dominated the
415 ecological processes across the three phases. These two processes exhibited
416 compensatory contributions, showing increasing variable selection but decreasing
417 ecological drift along the phases (Fig. 6). In the case of dispersal limitation, its relative
418 proportion was greater at phase 1 than the other phases for all treatments, especially
419 BPA and MII.

420 **Discussion**

421 We experimentally investigated the effects of persistent micropollutants on microbial

422 community dynamics to gain insights into community assembly mechanisms. Our first
423 hypothesis that differences in the removal rates of BPA vs. TCS in environments
424 exposed to single vs. mixed micropollutants was supported. he energy required by the
425 microbial communities to counteract the opposing responses to BPA and TCS in the MI
426 treatment (Choi & Lee, 2017; Zhang et al., 2013) likely accounted for the profound
427 inhibition of BPA degradation. In MII, a significantly larger fraction of BPA than of the
428 three other micropollutants was removed, indicating that the presence of TCC reduced
429 the inhibitory effect of TCS on BPA transformation and thus degradation. The
430 mechanism underlying such effect is beyond the scope of this study. Phenolic
431 compounds (both BPA and BPS) act as a growth medium for microorganisms by
432 providing a source of carbon and energy. However, they differ in degradability in the
433 seawater (BPA > BPS, Danzl et al., 2009), as well as negative impacts on biochemical
434 and microbiological activity of soil (BPA < BPS), Zaborowska et al., 2020). These lines
435 of reasoning explained a greater degradation of BPA than its analogue in MII (Fig. 2).
436 Altogether, our findings obtained from all three experiments demonstrate that
437 degradation of micropollutants is a microbially-mediated rather than an ordinary
438 chemical process. Pre-exposure to micropollutants can enhance the degradation
439 capacity of microbes later confronted with those or similar chemicals, which is
440 consistent with previous literature (Cerro-Gálvez et al., 2021; Coll et al., 2020).

441 Given that the ability of bacterial communities to transform the four
442 micropollutants surveyed, the questions remain: whether micropollutants accelerate
443 degradation through changes in bacterial communities, or the micropollutant degraded
444 and then altered the community composition? We found no evidence that the
445 degradation of any micropollutants differed across microcosm batches. Rather, there
446 was strong evidence on the differences in the bacterial communities between the

447 micropollutant-added microcosms vs. the controls, and that community turnover
448 increased over time following micropollutant exposure (Figs. 4A, S6). These results
449 supported the second hypothesis that the degradation of micropollutants alter the
450 microbial communities. Such community dynamics is mainly linked by the degradation
451 of BPA and TCS in the microcosms. We identified ecological groups based on changes
452 in the relative abundance of taxa following repeated micropollutant exposure, which
453 integrates population growth, death, survival, and reproduction over 35 days. Both
454 sensitive and tolerant taxa identified here may have affected micropollutant degradation
455 either directly or indirectly, such as by providing enzymatic repertoires to other
456 community members that enabled degradation, including interspecies interactions
457 (Freilich et al., 2011; Pacheco et al., 2019). The larger proportion of sensitive than
458 tolerant taxa in all treatments suggested that the microbial community was not robust
459 to the persistence of micropollutants in its surroundings. Noteworthily, two strictly
460 tolerant genera across treatments were *Sphingomonas* and *Hydrogenophaga* (Table S4),
461 which have been shown to be enriched in the BPA or TCS-contaminated environments
462 (Oh et al., 2019; Zaborowska et al., 2020; Hagberg et al., 2021) and to produce enzymes
463 capable of degrading different pollutants (Gao et al., 2019).

464 Our results supported the third hypothesis, that communities assembled at the early
465 stage of micropollutant exposure would be compositionally distinct from those that
466 formed at the later stage. Our findings generally corroborated previous studies showing
467 that the persistence of micropollutants even at low concentrations can influence
468 microbial community membership and abundance over time (Coll et al., 2020; Hu et
469 al., 2017; Wolff et al., 2018). Despite a substantial community variation between initial
470 and late stages of the exposure, community trajectory in the BPA and MII were driven
471 by a set of shared taxa that interacted similarly with the respective environments and

472 other community members. Conversely, the less pronounced negative dissimilarity-
473 overlap relationships in the case of the TCS and MI treatments indicated that the
474 microbial taxa shared among different inoculation batches within those treatments
475 interacted differently with the same environment over time. Hence, microbial
476 communities assembled in identical habitats can perceive those habitats differently such
477 that their feedback on the surroundings and other, coexisting members will differ as
478 well, as noted elsewhere (Bajić et al., 2018; Vila et al., 2020).

479 We observed a gradual transition from stochastic to deterministic processes
480 towards the later stage of micropollutant exposure. In all treatments ecological drift
481 played a major role during phases 1 and 2 of community growth progression. This can
482 be explained by the random changes in abundances within microbial communities
483 exposed to micropollutants in confined microcosms, where dispersal is limited and
484 locally dormant cells are about to be awakened to colonize empty niches in the
485 environments upon disturbances (Nemergut et al., 2013; Philippot et al., 2021; Zhou &
486 Ning, 2017). In fact, sensitive taxa declined in the relative abundances while tolerant
487 taxa had negligible abundance (Fig. S7) during the growth phases 1-2 (B1-B2 and B3-
488 B4). Hence, tolerant taxa identified in this study were recruited from rare member pool
489 at the early stage of exposure and regrew to a large population size (~ relative
490 abundance of 10 %) at the later stage of exposure. However, the increasing contribution
491 of variable selection to community assembly over time suggested that the high
492 phylogenetic turnover of the communities was driven by heterogenous abiotic and
493 biotic conditions (Stegen et al., 2015; Vellend, 2010). The increasing effects of the
494 micropollutants on the communities over time led to higher species selection and an

495 elevated impact on the interactions among those species within the community, such
496 that the drivers of community assembly no longer resembled those of the control and
497 resulted in a high rate of community turnover. Our findings suggest that the repeated
498 introduction of a micropollutant enhances succession in microbial communities (Jiao et
499 al., 2017).

500 We also considered the potential role of priority effects on community assembly,
501 independent of the ecological process model used in our study. Priority effects emerge
502 when early colonizers exhaust or modify available resources, thus influencing the
503 establishment success of later colonizers (Fukami, 2015). A strong priority effect in the
504 case of TCS and MI was evidenced by the significant difference in the composition of
505 the initially inoculated vs. any of the later inoculated communities (Fig. 4A). Once they
506 were re-inoculated, taxa that had thrived in the B1 batch modified the environments of
507 later batches, such that some taxa (presumably activated from a seed bank, given that
508 no source of new taxa was externally introduced into the systems) were less or more
509 favorable in later batches. However, for the BPA and MII treatments, the effects
510 occurred at later stage of the press disturbance, suggesting the increasing strength of
511 priority effects with increasing establishment time, as noted elsewhere (Svoboda et al.,
512 2018).

513 Although our microcosms represent an ideal system which is removed from *in situ*
514 conditions, our experimental findings are valuable in at least three aspects, that is : i)
515 the prediction of microbial responses to persistent micropollutants, ii) the harnessing
516 of ecological principles and microbiomes in pollution management (Bernstein, 2019;

517 Borchert et al., 2021; Rocca et al., 2021), and (iii) the development of a conceptual
518 model to be used to predict when and how stochastic and deterministic processes
519 contribute to community assembly after the exposure of persistent micropollutants. Our
520 results suggest that strong ecological drift drives random community changes at the
521 early stage of the exposure (Fig. 7). Prolonged ecological drift, variable selection
522 resulting from the effects of micropollutants, and the abiotic and biotic conditions
523 experienced differently by different microbes then determine community succession at
524 the mid-stage of the exposure. Additionally, the high environmental heterogeneity
525 together with the low densities of the communities permitted the emergence of priority
526 effects, in which the early establishment of taxa in micropollutant-containing
527 environments allowed a faster community succession. This was supported by the
528 decrease in the average gene copy number at the intermediate phase (Fig. S2), when
529 community assembly was driven by various ecological drivers (Nemergut et al., 2016).
530 Finally, strong variable selection accompanied by increased priority effects likely set
531 the communities on distinct trajectories, with multiple equilibria at the late stage of the
532 exposure. In fact, more advanced community succession and multiple equilibria
533 characterized the end of the press disturbance, and both played a larger role in the TCS
534 and MII (and to a lesser extent in the BPA) communities than in the MI community
535 (Fig. 7), suggesting convergent recovery of those freshwater bacterial communities to
536 repeated micropollutant exposure.

537 **Conclusions**

538 In summary, we evidenced that micropollutants degrade and drive microbial succession
539 following repeated exposure. This work can aid in microbe-pollution management by
540 providing insights into micropollutant removal as well as microbial assembly
541 mechanisms and dynamics. From the perspectives of microbiome engineering, this
542 study demonstrates the importance of the microbiome's preexposure history in the
543 design of long-term experiments that include artificial selection to direct the evolution
544 (Borchert et al., 2021; Chang et al., 2021) of a target microbiome highly optimized for
545 pollutant removal in an aquatic environment.

546 **Availability of data and material**

547 The sequencing data were deposited in the NCBI SRA database with the Bioproject
548 number PRJNA497759. Our R scripts for statistics, data visualization and computing
549 notes are available on a zenodo archive (https://doi.org/10.5281/zenodo.5242686)
550 of the Github repository (https://github.com/AnyiHu/Impacts-of-micropollutants-on-
551 freshwater-microbiome)

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878 **Table 1.** Number of OTUs assigned to the ecological categories opportunistic, sensitive,
879 and tolerant in the four micropollutant treatments: 'BPA' microcosms containing
880 bisphenol A, 'TCS' microcosms containing triclosan, 'MI' microcosms containing a
881 mixture of bisphenol A and triclosan, 'MII' microcosms containing a mixture of
882 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

883

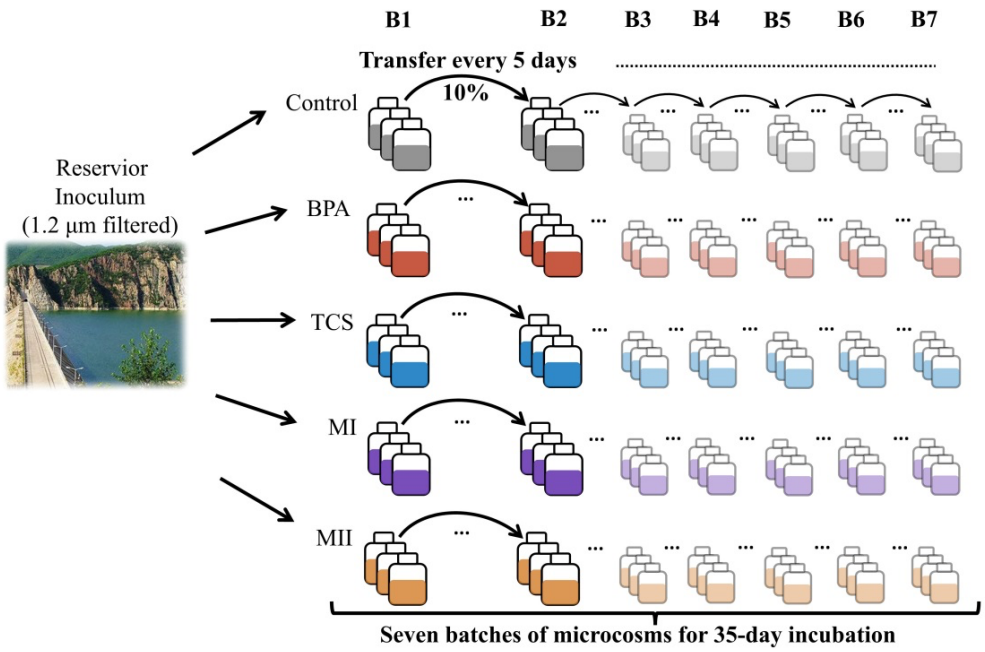

Figure 1. Experimental setup. In microcosm batch 1 (B1), 600 ml of the initial inoculum was added to bottles containing the micropollutants. Starting from the B1, 60 mL (10%) of the inoculum was pipetted from each of the replicates and transferred to fresh medium containing 540 mL of 0.22-µm-filtered 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).

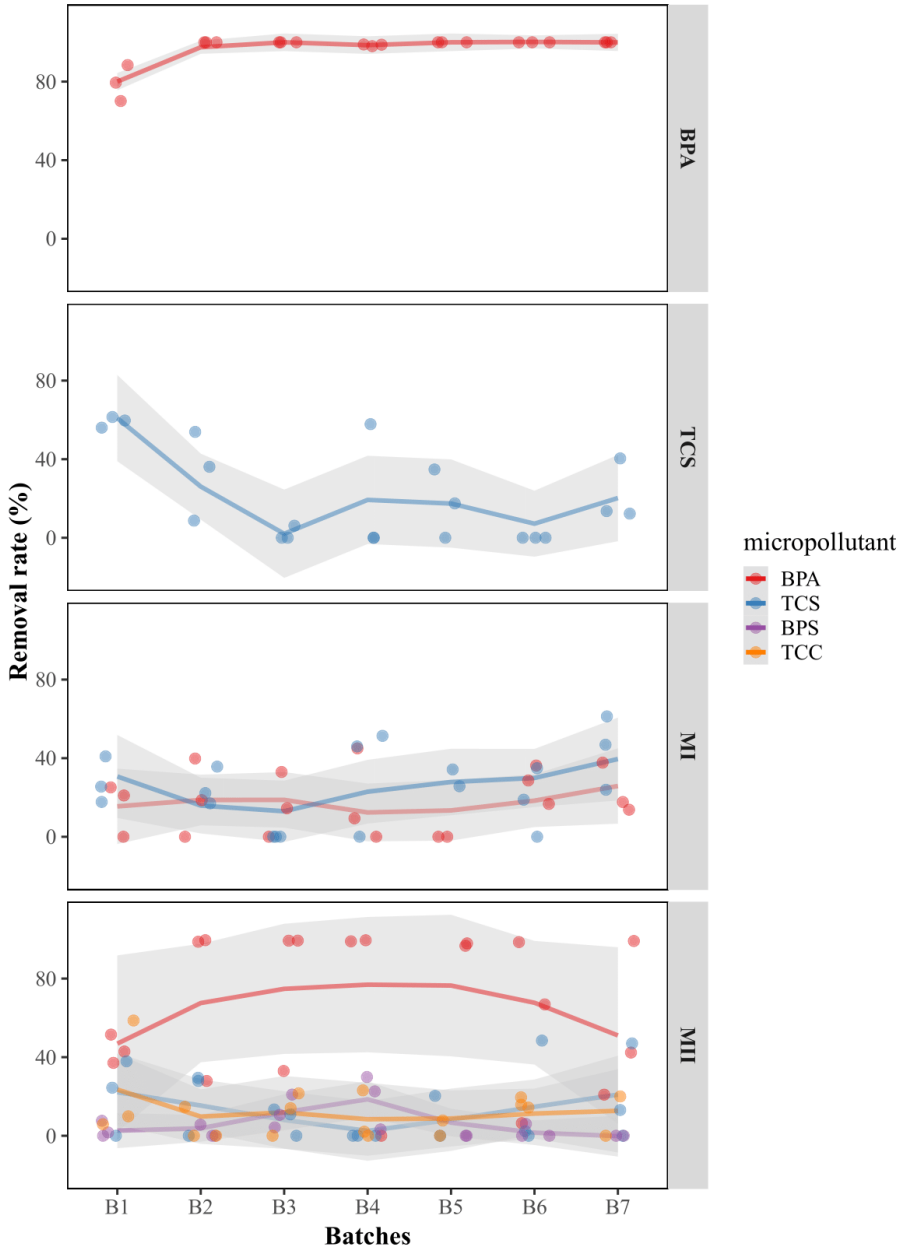

Figure 2. Percent removal of bisphenol A (BPA), triclosan (TCS), bisphenol S (BPS), and triclocarbon (TCC) in all microcosm batches for the single-micropollutant (BPA and TCA) treatments and in the mixed-micropollutant (MI and MII) treatments. Removal was defined as the decrease in the concentration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

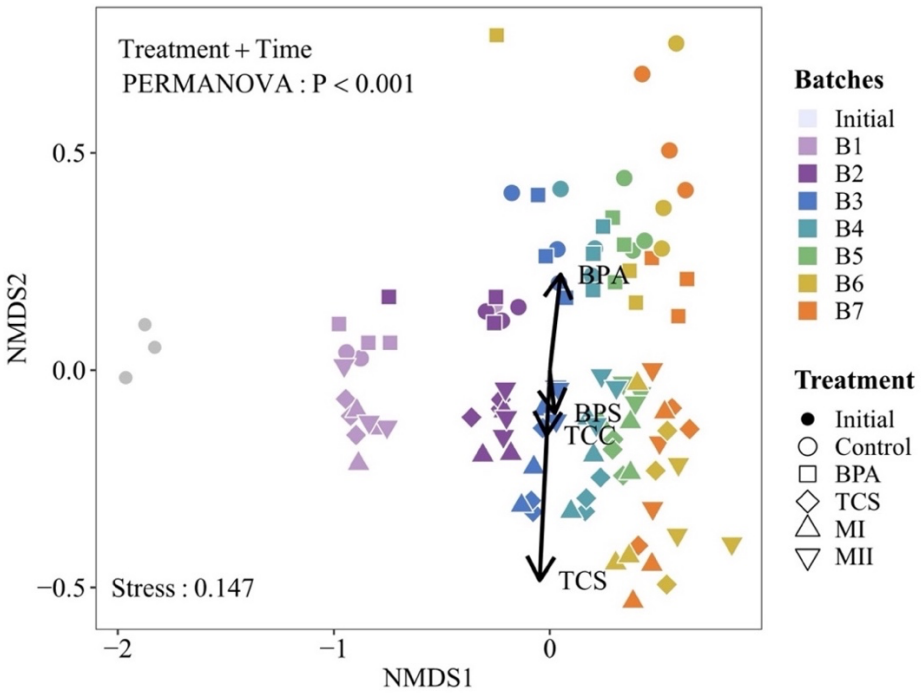

Figure 3. Difference in the bacterial communities in all microcosms batches as determined by non-metric multidimensional scaling (NMDS) ordination. Different treatments are represented by different symbols, and microcosms batches are color-coded. Explanatory environmental variables fitting the ordination 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.

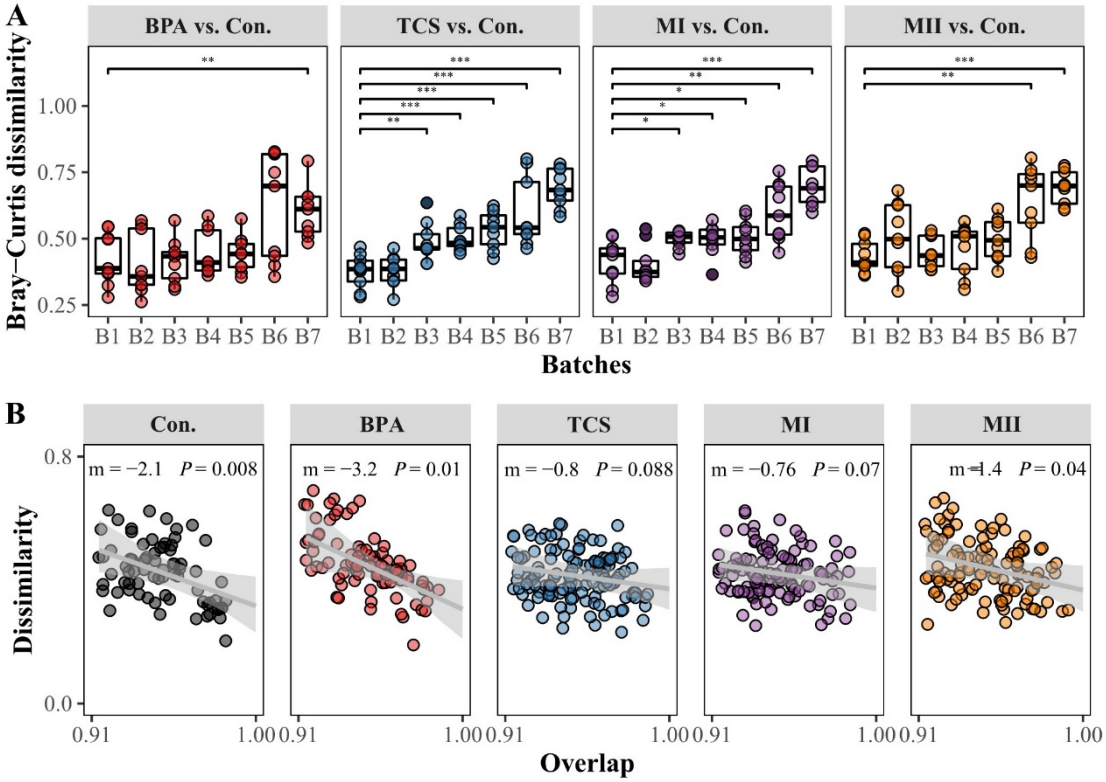

Figure 4. Bacterial community dissimilarity in the micropollutant treatments vs. the controls (i.e., 'Con.') over time (A) and the dynamic regularity within treatments (B). Significant differences in the Bray-Curtis dissimilarity for each pairwise comparison of the treatment and control (B1 microcosms 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.

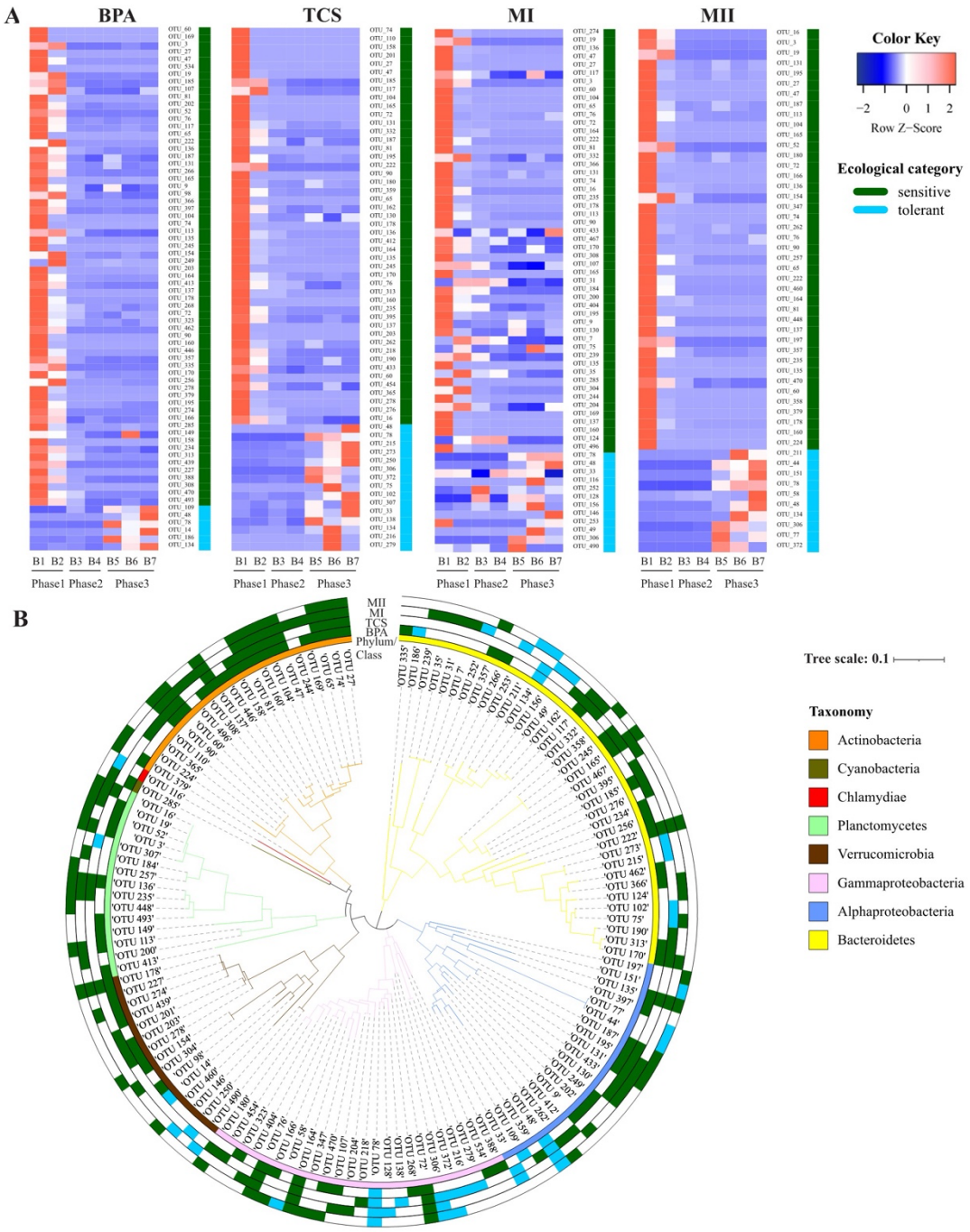

Figure 5. Changes in the relative abundances (rows of heatmap) of the OTUs assigned to the ecological categories tolerant and sensitive across the three growth phases (columns of heatmap) (A) and their phylogenetic distribution (B). In (A), color gradients of heatmap represent the relative abundances 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

939 were denoted in green and blue colors, respectively. In (B), the innermost circle
940 indicates the taxonomy (phylum/class) of the OTUs. The outer circles indicate the
941 ecological group of OTUs in each treatment group (BPA, TCS, MI, MII from inside to
942 outside, respectively).

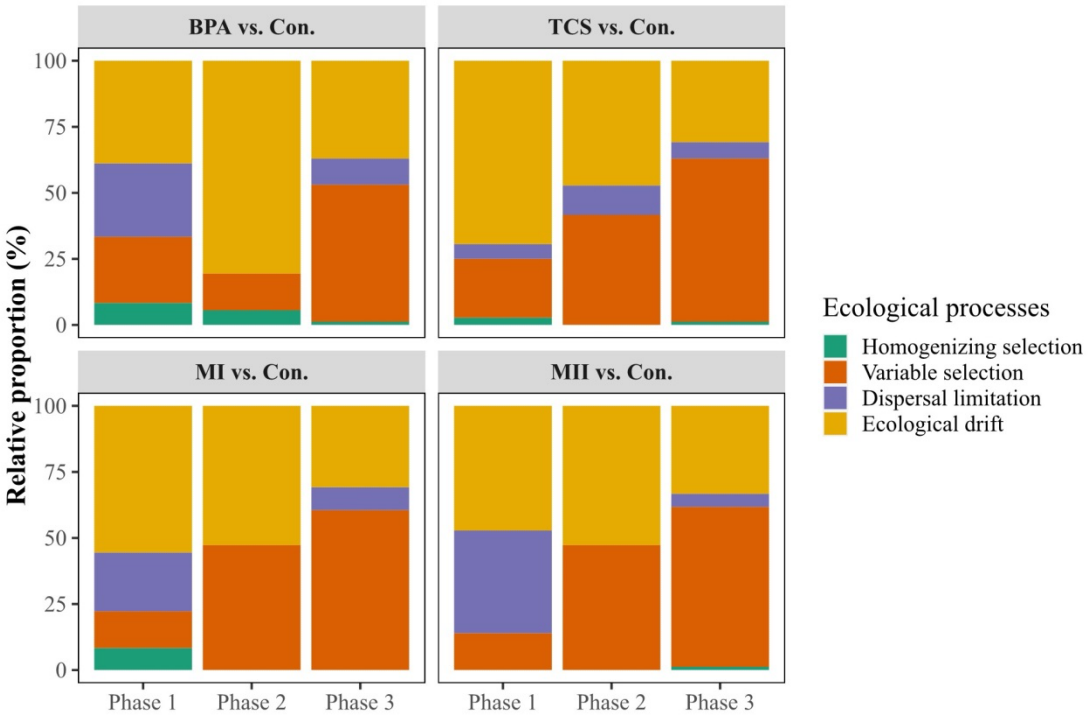

Figure 6. Relative proportion of the ecological processes (homogeneous selection, variable selection dispersal limitation, and ecological drift) contributing to the communities assembled in the micropollutant treatments (BPA, TCS, MI or MII) vs. the controls (i.e., 'Con.'). Each of the three phases (corresponding 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. S2G.

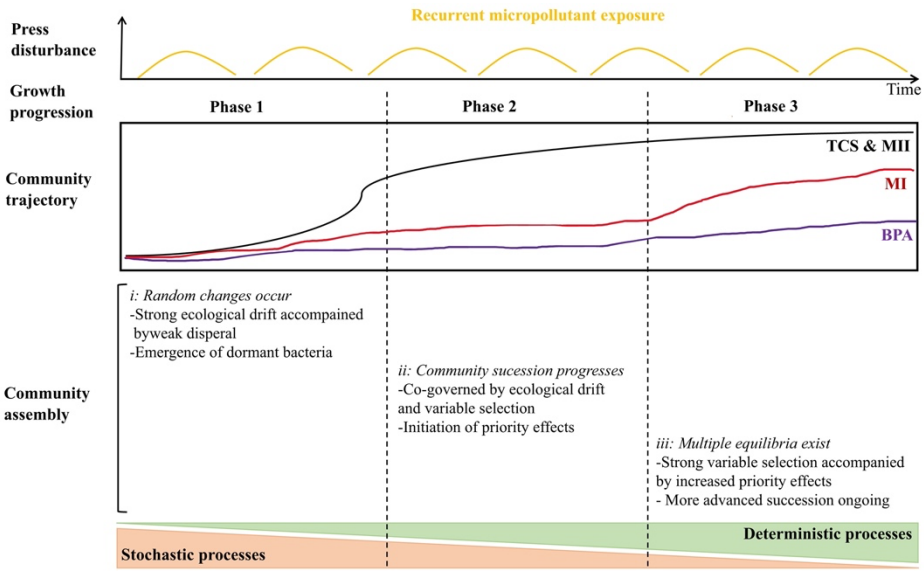

Figure 7 Conceptual model of microbial community assembly processes following a press disturbance. Considering the time continuity of the batch inoculation, the three phases of community growth progression (1, 2, and 3) also correspond to the early, mid, and late stages of the 35-day disturbance. This 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. Note that community succession and multiple equilibria are not exclusive, but community turnover is high towards the end of the disturbance.