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1 The sensitivity of aquatic microbial communities to a complex agricultural contaminant depends on
2 previous drought conditions

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4

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8

9 Abstract:

10 In intermittent rivers, which represent a prominent part of worldwide rivers, aquatic organisms are
11 exposed to sequential disturbances including flow cessation, potentially associated with water
12 warming, desiccation process and flow resumption. At flow resumption, pollutants stored in soil and
13 washed by rainfalls can reach fresh waters. The interaction between contamination and river
14 intermittency is poorly understood. In this study, we aimed at understanding in what extent the
15 intensity of dry period combined or not to water warming drives the sensitivity of aquatic
16 communities to a complex agricultural run-off (ARO) during rewetting. Phototrophic biofilms, at the
17 basis of freshwater food webs, were chosen as a model of community. Biofilms grown in laboratory
18 were first exposed to a disturbance crossing two temperature conditions (not warmed, 22°C or
19 warmed, 32°C) and three dry periods (no drying, short (3 days), or long (3 months)). Then they were
20 exposed to a chemical mix of nitrates, copper and 3 pesticides at 6 gradual concentrations. Various
21 descriptors associated with biofilm structure and function were assessed one week after ARO
22 addition. When undisturbed biofilms were exposed to ARO, they shifted toward a more
23 heterotrophic state as they lost algal richness and diversity, and gross primary production tended to
24 decrease. Warming alone only slightly modified the sensitivity of biofilms to ARO, with lower effects

25 on algal richness and a trend to increase the effect on gross primary production. In contrast, the
26 association of warming and a dry period strongly modified the sensitivity to ARO, certainly due to the
27 selection of generalist species and/or physiological acclimation inducted by the first disturbance. This
28 study emphasizes the importance of considering water intermittency in the management of the
29 ecological risk of chemicals in aquatic ecosystems.

30

31

32 Keywords: global change, desiccation process, agricultural runoff, sequential disturbance,
33 phototrophic biofilms

34

35

36 **1. Introduction**

37 Intermittent rivers represent more than 50% of rivers worldwide (Datry, Larned, and Tockner, 2014),
38 they can be found on every continent, within every climate (Messenger et al., 2021). A river is
39 considered intermittent or nonperennial if its flow ceases and the riverbed dries out a part of the
40 year (Datry et al., 2017a). Even if this phenomenon is frequent and naturally occurs in arid and
41 semiarid regions because of low precipitation events and strong direct evaporation, it is exacerbated
42 by climate change and human uses and is expected to increase both in space and duration (Sabater
43 et al., 2016). The prevalence of intermittency requires to adjust the classical model of river for a
44 more accurate understanding and prediction of river functioning, biodiversity and vulnerability.

45 Intermittency is a complex phenomenon, which covers contrasted situations. During the desiccation
46 process, the water flow first decreases and the system undergoes a slow transition from a lotic
47 toward a lentic system (Datry et al., 2017b). The decrease of water level induces modifications of
48 water quality, depending notably on the hydrological drivers and mass balance in the system
49 (Mosley, 2015). For instance, temperature increase has been observed in many studies during
50 drought, up to 7 degrees in a regulated river (Ha et al., 1999). After this first step, the riverbed
51 completely dries out and there is a shift toward terrestrial habitat for some days to several months
52 before rewetting occurs (Sabater et al., 2016). Depending on land use practices, agricultural
53 contaminants stored in soils during drought can be leached by the precipitation events and reach
54 fresh waters through runoff, exposing aquatic communities to a cocktail of pollutants during flow
55 resumption. A post-drought increase in nitrate concentration was thus observed in several studies
56 (see Mosley, 2015). In this context, flow intermittency can be considered as a sequence of different
57 disturbances occurring before, during and after the dry period, that can affect all the river biocenosis.

58 Benthic microbial communities which cannot circumvent such disturbances are particularly exposed
59 to the consequences of river intermittency. Phototrophic biofilms are benthic microbial communities
60 made up of both phototrophic and heterotrophic cells embedded in an extracellular polymeric

61 matrix. These communities are of major importance in ecosystem functioning as they are key
62 primary producers in many rivers, and are involved in organic matter and nutrient cycling (Battin et
63 al., 2016). Their short generation time makes them “early warning systems” to many stressors
64 (Sabater et al., 2007), especially to chemical stressors from agriculture practices like fertilizers, trace
65 metals such as copper widely used for vineyard culture, but also organic pesticides (herbicides,
66 insecticides, fungicides). Biofilm structural and functional responses to independent chemical
67 stressors have been well studied. For instance, nutrients enhance biofilm growth rate and
68 functioning but can also induce significant changes in community structure by favouring filamentous
69 green algae (Dalton, Boutin, and Pick, 2015). Compared to planktonic algae, biofilms exhibit an
70 important tolerance to copper contamination with usually few effects on functioning even at high
71 concentration (2 mg.L^{-1}) (Zhong, Zhao, and Song, 2020). In contrast, they tend to be negatively
72 impacted by organic herbicides, although the effect of combinations of pesticides are more difficult
73 to predict (Halstead et al., 2014). More generally, the chemical complexity of agricultural run-off
74 makes difficult to predict its effects on biofilm, as the interactions of stressors do not result in a
75 simple addition of stressor effects (Pesce, Bouchez, and Montuelle, 2011).

76 Warming and desiccation are the main effects of river flow drying-up. With global change, intensity
77 (i.e. duration) of dry periods is expected to increase, and increasing temperatures intensify the
78 severity of drought (Chiang, Mazdiyasi, and AghaKouchak, 2021). When temperature increases,
79 metabolic activities are enhanced. For instance, under warming, biofilms use more carbon substrates
80 (Ylla, Canhoto, and Romani, 2014) and basal fluorescence and enzymatic activity are enhanced
81 (Romero et al., 2018). Conversely, desiccation appears to have negative impacts on biofilm structure
82 and functioning. During desiccation, photosynthetic processes weaken regardless of the disturbance
83 duration, while heterotrophic activities and community respiration could persist longer depending on
84 the humidity degree remaining in biofilm (Acuña et al., 2015). Besides those functional responses, it
85 has been shown that desiccation tends to induce changes in both algal and bacterial communities
86 (McKew et al., 2011; Sabater et al., 2016).

87 Some studies have investigated the response of microbial communities to a stressor after a prior
88 exposure to another disturbance, but with contrasted results. In some cases, the community was
89 then more affected (e.g. Tobor-Kapton et al., 2006) while in others, the exposure to a first
90 disturbance decreased the effects of the second one, the microbial community showing antagonistic
91 responses to the successive disturbances (e.g. Sjöstedt et al., 2018). Antagonistic responses can be
92 explained by different mechanisms. First, some species in the community can exhibit co-tolerance to
93 both disturbances (Vinebrooke et al., 2004) with the selection of generalist species that are usually
94 less sensitive to environmental changes (Chen et al., 2021). Acquired stress resistance can also be
95 implied: after a first disturbance, communities have been prepared to resist a second one (Rillig et
96 al., 2015). On the opposite, additive or synergic effects can be observed when there is a negative
97 correlation between species tolerance to each disturbance (Vinebrooke et al., 2004). Therefore, the
98 outcome of the disturbance sequence for a microbial community strongly depends on the order of
99 disturbances. This was experimentally demonstrated in soil microbial communities by manipulating
100 the sequence of three different disturbances (heat, freeze-thaw and anoxia; Calderon et al., 2018).

101 In intermittent rivers, rewetting appears to be a hot biogeochemical moment (McClain et al., 2003),
102 i.e. shortly after the water returns, biogeochemical processes are reactivated and all biological
103 activities are exacerbated, including primary production and respiration (Timoner et al., 2012). It
104 appears important to understand in which extent phototrophic biofilms, that are critical for the
105 whole river functioning, could be sensitive to contaminants leached by precipitations during
106 rewetting. Some studies may provide a partial response. Proia et al. (2013) have shown that flow
107 reduction and interruption amplified the effects of one contaminant, the triclosan, on river biofilms.
108 Corcoll et al. (2015) highlighted the cumulative effects of the exposure to a mixture of
109 pharmaceuticals followed by a dry period. However, these previous studies do not allow to predict
110 the sensitivity to a complex agricultural run-off (ARO) of biofilms that have experienced a complete
111 drying. Such a complex ARO, whose components (nutrients, herbicide, insecticide, fungicide, heavy

112 metal) may have antagonistic effects, has been already shown to induce either positive or neutral
113 effects on biofilm biomass (Allen et al., 2021).

114 This study aims at quantifying how the sensitivity of phototrophic biofilms to a complex agricultural
115 runoff is modulated by river intermittency history. We conducted a microcosm experiment where we
116 first manipulated both water warming and the duration of the dry period and then, we exposed
117 produced biofilms to a gradient of ARO concentrations during rewetting. The objective was to
118 understand in what extent the first disturbance (dry period and/or warming) drives the structural
119 and functional biofilm response to the exposure to contaminant. Our hypothesis was that the first
120 disturbance will decrease biofilm sensitivity to contamination through the selection of co-tolerant
121 species.

122

123 **2. Material and methods**

124 Complex phototrophic biofilms were grown in the laboratory. They were next exposed to a typical
125 dryness sequence that consisted in a period of no flow (NF) followed by a short dry period (S) or a
126 long dry period (L) combined or not with warming (NW or W) (Fig. 1). Next, biofilms were exposed to
127 ARO-enriched water at gradual concentrations to mimic rewetting with gradually contaminated
128 water.

129

130 **2.1 Experimental design**

131 **2.1.1 Biofilm growth**

132 Natural biofilms were sampled in February 2020 in the river Garonne (43.396635, 1.300394, South-
133 West France). Biofilms were scrapped with a toothbrush on field and were stored in river water at
134 4°C. Back to the laboratory, biofilm suspension was filtered on a 500- μ m sieve to remove large
135 mineral particles, and was used to inoculate artificial mini-channels designed for biofilm culture

136 (Loustau et al., 2019). The temperature in the room was 22°C, and the flow velocity was 0.15 m³.min⁻¹,
137 ¹, light intensity was 70 μmol.m⁻².s⁻¹ (16h/8h light/dark periods). Biofilm were grown on ceramic
138 coupons (5.76 cm²). Volvic® water was used as culture medium. Twice a week, in addition to a supply
139 with demineralized water to compensate evaporation, nutrients were added to maintain
140 concentrations of the Volvic® water (final concentration in channel tanks: N-NO₃⁻: 1.64 mg.L⁻¹, P-PO₄³⁻
141 : 30 μg.L⁻¹ and SiO₂: 32 mg.L⁻¹).

142 2.1.2 First disturbance

143 After 16 days of growth, biofilms experienced a no-flow period for one week either at 22°C (NW) or
144 32°C (W) to mimic the first step of dryness process. Colonised ceramic coupons were thus dispatched
145 in 36 crystallizers (15 coupons per crystalliser) filled with 600 ml of Volvic® water in 2 distinct
146 thermostatic chambers (ventilation 100%) in the same lighting conditions than during growth. After
147 this first week, produced biofilms were exposed to contrasted hydric stress at both temperatures:
148 one third of coupons were dried up for a short period (3 days), one third were dried up for a long (84
149 days) period before contamination, and the remaining third was not dried up and directly exposed to
150 contamination. Dry period durations applied are in the range of dry durations, extending from 1 to
151 150 days, observed in Mediterranean streams (Colls et al., 2019), and within the range of what can
152 be observed in French intermittent rivers (Snelder et al., 2013).

153 2.1.3 Second disturbance

154 The contaminant cocktail used was the same as in Allen et al. (2021). It consisted in a mix of nitrates
155 (KNO₃), copper (CuSO₄) that is a metallic trace element used as algicide, and three organic pesticides
156 (PESTANAL®analytical standards, Merck, Darmstadt, Germany): an insecticide inhibiting
157 acetylcholinesterase (Pirimicarb, CAS number 23103-98-2), an herbicide inhibiting photosynthesis
158 (Terbuthylazine, CAS number 5915-41-3) and a fungicide inhibiting sterol synthesis (Tebuconazole,
159 CAS number 107534-96-3). The pulse of contaminant was applied in crystallizer water at six
160 concentrations of 0, 1, 10, 50, 100 and 250 times the lowest concentration (Table SI1). Maximal

161 concentrations (250x) were: N-NO₃⁻ 750 mg.L⁻¹, Cu 3.5 mg.L⁻¹, PC 1.25 mg.L⁻¹, TBA 0.25 mg.L⁻¹ and TCZ
162 7.5 mg.L⁻¹. Stock solutions were prepared in demineralized water for KNO₃ and CuSO₄ and in dimethyl
163 sulfoxide (DMSO, CAS 67-68-5, Merck) for organic pesticides. DMSO (0.01 mg.L⁻¹) alone was added to
164 uncontaminated crystallizers. After one week of exposure to contaminant, coupons were removed
165 and scrapped in Volvic® water (4 mL per coupon). The obtained biofilm suspension was used for the
166 measure of all the descriptors.

167

168 **2.2 Measured descriptors**

169 18 descriptors were measured on biofilm suspension to assess the structure of algal and bacterial
170 communities and their functioning: the chlorophyll *a* content (Chl *a*), the autotrophic index (AI), the
171 pigment content (main primary producer groups, pigment richness and diversity), the bacterial
172 density, the stoichiometric composition (Carbon, Nitrogen and Phosphorus %), the quantity of
173 extracellular polymeric substances (EPS), the carbohydrate-to-protein ratio in EPS to bring out
174 changes in EPS composition depending on environmental conditions (Fang et al., 2014), oxygen
175 fluxes to assess primary production and respiration, and the algal and bacterial cell physiological
176 states (maximal quantum yield of photosystem II and % of live bacteria).

177 Biofilm dry mass was assessed to normalise other descriptors. One ml of each biofilm suspension was
178 centrifuged (10 000 g, 5 min) in pre-weighed tubes. Then supernatant was removed and the pellet
179 was oven-dried (65°C, 24h) before being weighed.

180 **2.2.1 Pigment analysis**

181 1.5 mL of biofilm suspensions were centrifuged (10 000 g, 5 min at 4°C) and pellets were stored at -
182 80°C until pigment extraction. After freeze-drying, pellets were extracted in 2 mL of methanol (2% of
183 ammonium acetate 1 M) (Capdeville et al., 2019). After sonication during 3 min, samples were
184 incubated at -20°C for 15 min before another sonication step. After centrifugation (10 000 g, 5 min at
185 4°C), supernatants were stored at 4°C. This extraction was repeated 1 to 3 times, as long as the

186 supernatant was still coloured. Then 1 mL of each extract were filtered with a 0.2 µm PTFE
187 membrane syringe filter (VWR International, United States). Each pigmental composition was
188 analysed using HPLC (LC1200 series, Agilent Technologies, United States). Specific pigment
189 concentration was quantified by comparing each peak area of the chromatogram with those of
190 known standards using the ChemStation software (Agilent technologies). Specific pigments were
191 summed to obtain the proportion of each primary producer group: lutein, chlorophyll *b*, violaxanthin,
192 neoxanthin and astaxanthin for green algae; fucoxanthin, diadinoxanthin, chlorophylls *c1* and *c2* and
193 diatoxanthin for diatoms; myxoxanthophyll, echinenone and zeaxanthin for cyanobacteria (Majdi et
194 al., 2011; Schlüter et al., 2006). In addition to Chl *a* quantification (reported by coupon area) used for
195 assessing the algal biomass, a relative autotrophic index (AI) was calculated, as the ratio between Chl
196 *a* concentration and biofilm dry weight. Finally, we calculated pigment richness (number of pigments
197 detected) and Shannon pigment diversity index: $S = -\sum [P_i \ln(P_i)]$ with P_i the proportion of each
198 pigment.

199 2.2.2 Extracellular polymeric substances

200 Extracellular polymeric substances (EPS) were assessed by summing both proteins and carbohydrates
201 content. EPS were extracted on fresh samples as described by Loustau et al. (2019). After a washing
202 step, extraction was first carried out in 1x PBS with a 37% formamide treatment. Following
203 incubation for 60 min at 4°C at slow shaking (150 rpm), tubes were centrifugated (8 000 g, 5 min, 4°C)
204 and supernatant stored in 2-mL tubes. The pellets were then resuspended in 1x PBS with 50 g.gDW⁻¹
205 of cation exchange resin (CER, Dowex Marathon). Another incubation of 90 min was carried out as
206 described in the first step. After centrifugation, supernatants were extracted, and pellets were
207 washed another time in PBS to ensure good extraction. Supernatants tubes were stored at -20°C
208 before quantification. The PierceTM BCA protein assay kit (N° 23223, Sigma Aldrich) was used to
209 quantify proteins content in EPS (Smith et al., 1985). Bovine serum albumin (BSA) was used as a
210 standard, with 10 concentrations ranging from 0 to 1 g.L⁻¹. To increase sensitivity, microplates were
211 incubated for 30 minutes at 60°C (Walker, 2009). After 20 min of cooling, optical density (OD) was

212 measured at 540 nm (Synergy Mx Biotek). The anthrone method was used to quantify carbohydrates
213 (Gaudy, 1962), using glucose as standard with concentrations ranging from 0 to 0.1 g.L⁻¹. After the
214 same incubation and cooling times than for proteins, OD was measured at 620 nm. Results expressed
215 in standard equivalents were normalised to sample biomass.

216 2.2.3 Flow cytometry

217 We used the Live/Dead BacLight viability kit (ThermoFisher) to discriminate between both living and
218 dead bacterial cells and to estimate total bacterial density (number of cells by coupon area).
219 Propidium iodide only enters bacteria with damaged membranes, while Syto9 stain labels all bacteria
220 in green, those with intact membranes and those with damaged membranes. One mL of fresh
221 samples was filtered on a 40- μ m pore size mesh after sonication and diluted 6 times before
222 coloration in NaCl at 0.85%. Dying was applied directly in 96-well microplates. The final volume was
223 200 μ L per well. After a 15-min incubation in the dark, bacterial counts were performed in a
224 microplate using a Guava[®] EasyCyte™ cytometer at a flow rate of 0.59 μ L.s⁻¹. We recorded a
225 maximum of 5000 events per sample. The green and red fluorescence were used to discriminate live
226 and dead cells. Finally, the percentage of live bacterial cells was quantified using the guavaSoft
227 software (version 3.3).

228 2.2.4 Stoichiometric composition

229 Total carbon, nitrogen and phosphorus contents in biofilms were measured in 1-mL biofilm
230 suspensions oven-dried at 60°C for 24h. Carbon and nitrogen contents were determined by
231 elemental analysis using a CHN analyser (Flash 2000, Thermo Scientific). For phosphorus
232 quantification, samples were previously oxidized with potassium persulfate in an acid environment in
233 autoclave to mineralize matter, and phosphorus content was determined following the molybdate-
234 blue method (Koroleff, 1983) using a spectrophotometer at 880 nm (Uvi Light XT5, SECOCAM, Alès,
235 France).

236 2.2.5 Primary production and respiration

237 Biofilm primary production and respiration were analysed by measuring changes in oxygen
238 concentration over time using FireSting oxygen sensors (PyroScience). Measurements were
239 conducted under agitation in 10-mL transparent vials containing 1 mL of biofilm suspension and 9 mL
240 of Volvic water, and closed with a gas tight lid. Dissolved oxygen concentration within each vial was
241 monitored every 5 seconds over 10 minutes using oxygen logger software. Net primary production
242 (NPP) ($\text{mgO}_2 \cdot \text{h}^{-1} \cdot \text{mgDW}^{-1}$) was determined by measuring dissolved oxygen changes over a 5-min
243 period of daylight (neon lamps, $90 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$). The rate of community (autotrophic and
244 heterotrophic) respiration (R) was then determined as the change in oxygen concentration over a 5-
245 min incubation period in the dark. The GPP (gross primary production) was calculated by summing
246 NPP and R.

247 2.2.6 Fluorescence measurement

248 PhytoPAM measurements (Phytoplankton Analyzer, Heinz Walz GMBH, Effeltrich, Germany) have
249 been carried out on 1/10 diluted 200- μL biofilm suspensions. The maximal quantum yield of
250 photosystem II (PSII) or ΦPSII was used to assess algal physiological state. This metric depends on the
251 algal species present in the biofilm, but also on the physiological state of cells as it decreases when
252 algae are stressed or damaged (Roháček and Barták, 1999). Measurements of ΦPSII were done under
253 dark-adapted state (Baker, 2008). ΦPSII ($0 < \Phi\text{PSII} < 1$) corresponds to the ratio F_V / F_M , where $F_V = F_M - F_0$
254 with F_M the maximal fluorescence and F_0 the minimal fluorescence of PSII (Roháček and Barták,
255 1999).

256

257 2.3 Data analysis

258 All analyses were performed using R software version 3.6.1 (R Development Core Team). For all
259 response variables, normality was tested, and log-transformed if necessary. A PCA was performed in
260 order to visualize the effects of the first disturbance and contamination on biofilm global structure
261 and functioning (Fig. 2). Effects of dry period duration (D) and warming (W) were tested with

262 PERMANOVAs. The two first component (PC1 and PC2) of the PCA were extracted and coordinates
263 were used as summary variables to describe the response of biofilms. Two-ways ANOVAs (D x W) and
264 Tukey Post-hoc tests were performed on each variable as well as on PC1 and PC2.

265 In a second time, we assessed biofilm sensitivity to ARO by comparing the slopes of biofilm response
266 to contaminant concentrations depending on the first disturbance biofilms were exposed to.
267 Therefore, linear regressions were conducted on every variable as a function of contaminant
268 concentrations. Variables were standardised using z-score to enable comparison between effects. A
269 significant regression (t-test) indicates a significant effect of the increasing contaminant
270 concentration on the variable. Regression slopes, as well as standard deviations, were extracted for
271 each first disturbance treatment, and plotted together to compare the effects.

272

273 **3. Results**

274 **3.1 Biofilm response to dry period and warming**

275 PERMANOVA analysis demonstrated a significant effect of dry period duration ($p < 0.001$) on the
276 global biofilm response. Such an effect was clearly described by the first PCA component (Fig. 2). The
277 longer the dry period was, the more biofilm shifted toward heterotrophy (Fig. 3, Table SI2). These
278 results were consistent with those observed on univariate analyses performed on each variable (Fig.
279 3). There was a significant negative effect of dry period duration on phototrophic descriptors (Table
280 SI2): Chl *a* and AI gradually decreased with increasing dry period duration (ANOVA, $p < 0.001$, Fig. 3).
281 Both pigment richness and diversity significantly decreased in dried biofilms ($p < 0.001$, Fig. 3). Green
282 algae proportion increased in biofilms having experienced the long dry period (NW+L and W+L) ($p =$
283 0.004 , Fig. 3, Table SI2), to the detriment of cyanobacteria ($p = 0.01$, Fig. 3, Table SI2). This
284 corresponds to the effects observed on PC2 (Fig. 2, Fig. 3). Biofilm EPS quantity was significantly
285 higher in previously undisturbed biofilm than in dried biofilms ($p < 0.001$, Fig. 3) with a lower
286 carbohydrate-to-protein ratio in EPS compared to biofilms exposed to the long dry period ($p < 0.001$,

287 Fig. 3, Table SI2). Bacterial concentration was highly enhanced in long dry period duration treatments
288 ($p < 0.001$, Fig. 3). Heterotrophic activity represented by respiration (R) and the % of live bacteria
289 increased while phototrophic activity represented by GPP and NPP sharply decreased with increasing
290 dry period duration (ANOVA, $p < 0.001$, Fig. 2, Fig. 3, Table SI2). Consistently, the proportion of live
291 bacteria significantly increased in dried treatments, and this effect was significantly reduced in
292 warmed treatments (Table SI2, Fig. 3). In terms of biofilm stoichiometry, increasing dry period
293 duration induced significant decreases in C, N, and P proportions ($p < 0.001$, Table SI2), while
294 significant differences between undisturbed and dried biofilms could only be observed on nitrogen
295 content in W+L and NW+L treatments (post-hoc analysis, Fig. 3).

296 In addition, a significant effect of the interaction term between dry period duration and warming was
297 observed on PC1 and PC2 (ANOVA, $p < 0.04$, Table SI2). Diatoms exhibited a negative response to
298 warming with significantly less diatom-specific pigments after exposure to warming (Fig. 3, $p < 0.001$,
299 Table SI2). A negative effect of warming was observed on bacterial density after the longest dry
300 period (Fig. 3, $p < 0.001$, Table SI2).

301

302 **3.2. Biofilm sensitivity to ARO contamination**

303 The contaminant used to mimic ARO consisted in a mix of nitrates (KNO_3), copper (CuSO_4) and three
304 organic pesticides (an herbicide, a fungicide and an insecticide). The response of the different
305 descriptors to contamination was assessed by analysing the estimated regression slopes between
306 each variable and the contaminant concentrations when the regression was significant (Table SI3,
307 Fig. SI1).

308 ARO contamination induced a significant increase in PC1 value in the treatments NW-FN, W-NF and
309 W-L (Fig. 4), which indicates an increase in biofilm heterotrophy (Fig. 2). Contamination had no
310 impact on PC1 values of the other treatments. The effect of contaminant on PC2 was significant for
311 biofilms that were previously dried for a short time and those subjected to warming alone (NF-NW, p

312 < 0.05). Similar slope values were observed for all the other treatments except for W-L.
313 Contamination had no impact on the algal compartment, except sporadic positive ones on biofilm Chl
314 *a* in biofilms previously exposed to a short dry period without warming (NW-S treatment, Fig. 4, $t =$
315 2.797, $p < 0.05$) and on AI in undisturbed biofilms (NF-NW, Fig. 4, $t = 5.016$, $p < 0.01$). Contamination
316 increased EPS production in biofilms that had been subjected to a short dry period (NW-S and W-S,
317 Fig. 4, $t < 3.77$, $p < 0.02$). In previously undisturbed biofilms, pigment richness (Fig. 4, $t = -4.937$, $p <$
318 0.01) and the Shannon diversity index based on pigments (Fig. 4, $t < -3.265$, $p < 0.05$) significantly
319 decreased with contaminant concentration, while there was no significant response in previously
320 disturbed biofilms. Biofilms previously subjected to a long dry period with warming exhibited a shift
321 from green algae to cyanobacteria (Fig. 4). This response to ARO contamination was confirmed on
322 the second PCA axis (Fig. 2): low-ARO contaminated (0, 1x, 10x) samples dominated by green algae
323 are clearly distinct from high ARO contaminated (50x, 100x, 250x) samples dominated by
324 cyanobacteria.

325 When biofilms were previously exposed to warming, the total carbon content decreased with
326 contamination (Fig. 4, $t < -2.771$, $p < 0.05$), but this sensitivity to contamination diminished with
327 increasing dry period duration. The response of bacterial viability to contamination was also
328 modulated by previous warming (Fig. 4) with bacterial viability increasing with contamination in W-
329 NF ($t = 10.10$, $p < 0.001$) and W-L ($t = 3.618$, $p = 0.05$) treatments. Respiration also tended to be
330 stimulated by the contaminant when biofilms had been previously exposed to warming (Fig. 4). F_V/F_M
331 decreased with contamination in biofilms previously dried (Fig. 4, $t < -2.734$, $p < 0.05$). On the
332 contrary, gross primary production (Fig. 4) and net primary production (Fig. 4) tended to decrease
333 with contamination only when biofilms were not previously dried, though it was significant only for
334 the W-NF treatment ($t < -3.582$, $p < 0.023$).

335

336 4. Discussion

337 The prevalence of intermittency in world rivers (Messenger et al., 2021) makes necessary to take into
338 account their specificities in the assessment of aquatic ecosystem vulnerability to anthropogenic
339 stressors. The aim of this study was to assess if intermittency would alter the sensitivity of a river
340 microbial community to a complex contamination. In addition to dryness, we considered the effect of
341 water warming associated to water flow cessation. Though the response of biofilm to a synthetic
342 agricultural-run off (ARO) was moderate, our results show that it is strongly driven by biofilm history
343 of disturbance.

344

345 **Response to ARO in previously undisturbed biofilms**

346 The biofilms produced for the experiment were mainly dominated by cyanobacteria and in a lesser
347 extent by diatoms, and were characterized by a positive net primary production. When exposed to
348 increasing ARO contamination, such phototrophic communities lost algal richness and diversity, and
349 GPP tended to decrease, while heterotrophy was enhanced (Fig. 4, PC1). Loss in pigment richness
350 and diversity was likely linked to the removal of species sensitive to ARO, notably to copper and
351 herbicide that were expected to negatively affect microalgae (Sousa, Chow, and Pompêo, 2019).
352 Despite this effect, previously undisturbed biofilms globally exhibited rather low sensitivity to ARO,
353 given the very high concentrations applied, with values reaching 750 mg.L^{-1} of N-NO_3 , 3.5 mg.L^{-1} of Cu
354 and $250 \text{ }\mu\text{g.L}^{-1}$ of terbuthylazine.

355 Different reasons, such as ARO complexity, biofilm abilities to resist, and experimental timing may
356 explain such rather weak effects. First, the pesticides toxic effects could be counterbalanced by
357 positive effects of N inputs at lowest ARO concentrations, as shown by Aristi et al., (2016), and
358 therefore could mitigate pesticides impact. Additionally, N inputs may have increased heterotrophy
359 by providing an alternative electron acceptor that may promote heterotrophic activities
360 (denitrification) in the anoxic deeper layers of biofilms. Such a compensation between negative and
361 positive effects of ARO components depending on ARO concentration is also consistent with the
362 negative impact we observed on GPP at the highest ARO concentrations. Resistance to organic

363 pesticides and heavy metals is notably linked to the EPS matrix which adsorbs those pollutants
364 (Flemming et al., 2016), and to biofilm thickness that limits contact between microbial cells and
365 pesticides for the deeper layers (Davies, 2003). The biofilm biomasses reached in this study
366 correspond to those of mature natural biofilms (Boulêtreau et al., 2006) and EPS production is similar
367 to the one of classic biofilms (Kroll et al., 2014), so that such resistance mechanisms may occur.
368 Finally, biofilms were likely to exhibit a partial recovery since they were sampled one week after ARO
369 addition, and algae can recover very fast from herbicide exposure, as already shown for atrazine, a
370 photosynthesis inhibitor like terbuthylazine (Laviale et al., 2011).

371

372 **Response to ARO in biofilms pre-exposed to warming**

373 Water temperature can dramatically increase over flow cessation, desiccation and fragmentation
374 into isolated pools (Gómez et al., 2017). In accordance with field personal observations, we
375 experimented how a +10°C heatwave could modulate ARO sensitivity. Despite the high intensity of
376 the heatwave, biofilm response to ARO contamination was not strongly impacted by warming. The
377 response of warmed but undried biofilms to ARO was globally similar to the one of previously
378 undisturbed biofilms. However, pigments richness decrease was weaker, respiration tended to be
379 enhanced and GPP was inhibited by ARO in biofilms pre-exposed to warming. This indicates that a
380 warming event before contamination would tend to mitigate algal simplification but would amplify
381 biofilm functioning shift toward heterotrophy. Switches to heterotrophy due to warming have been
382 observed in natural biofilms in intermittent rivers (Acuña et al., 2015). This is partly explained by the
383 higher metabolic response of respiration than photosynthesis to temperature (Boulêtreau et al.,
384 2012). Weak effects of warming observed on biofilm pigment composition do not rule out possible
385 replacements of algal or bacterial species according to their thermal preferences, since the
386 taxonomic composition of the communities was not assessed here.

387 Many studies have considered the effect of contaminants depending on temperature, but in most
388 cases the stressors were applied simultaneously rather than sequentially. Nutrient supply, combined

389 with warming, induced changes in structure with enhanced bacterial growth (Degerman et al., 2013).
390 In this study, authors have shown that increasing nutrient concentration enhanced growth, with a
391 stronger positive effect when the temperature exposure was higher (+4°C). Increasing temperature
392 (+3, +6 and +10°C) also reduced the negative effects of toxic organic pollutants (Larras et al., 2013).
393 When applied before contamination, warming could select species that are tolerant to heavy metal
394 exposure and reduce the effects of the latter (Pesce et al., 2018). On the opposite, Lambert et al.,
395 (2017) noticed that the sensitivity of phototrophic biofilms to copper increased after warming. These
396 contradictory observations all fit with the theory of co-tolerance developed by Vinebrooke et al.
397 (2004). When two disturbances are applied successively, the first disturbance can either increase the
398 tolerance to the second disturbance through antagonist effects (Rillig et al., 2015; Li et al., 2014) or
399 increase the sensitivity through synergic effects. The lower decrease in pigment richness compared
400 to undisturbed biofilms and the weak global warming effect we observed on contaminated biofilms
401 pre-exposed to warming could be explained by a positive correlation between warming and
402 contamination effects on species tolerance. Species more tolerant to ARO could have been already
403 selected by warming pre-exposure. All these experimental results suggest that the outcome of
404 successive disturbances is far from easy to predict depending on the initial community composition,
405 species properties, and the nature and chronology of the disturbances.

406

407 **Response to ARO in previously dried biofilms**

408 Non-perennial rivers and streams cover a wide spectrum of flow conditions from rarely intermittent
409 rivers periodically experiencing flow cessation during a few days to ephemeral streams that only
410 temporally flow after intense rainfall. Consistently, we exposed biofilms to two contrasted durations,
411 a short dry period of 3 days and a long dry period of 3 months. Dry period is a very strong
412 disturbance for the aquatic organisms forming phototrophic biofilms, inducing a high mortality rate
413 in algae (Barthès et al., 2015a; Souffreau et al., 2010). However, even after quite long dry periods,
414 biofilms are able to quickly recover and resume their functioning (e.g. Timoner et al., 2012). Fast

415 recovery is partly due to the ability of some species to form resting spores to resist dry period
416 (Hargraves and French, 1983) and to become active again within hours and days of flow resumption
417 (Robson et al., 2008). High functional resilience is often coupled with a low structural resilience, with
418 marked modification of the composition of both algal and bacterial components (Barthès et al.,
419 2015b; Gottlieb, Richards, and Gaiser, 2005). In our experiment, biofilms exposed to a dry period
420 showed deep modifications of their composition, well visible on the PCA first component, with less
421 chlorophyll *a* biomass, less EPS, lower pigment diversity, and more bacteria. Dry period also resulted
422 in changes in biofilm productivity, with a consistent decrease in GPP. This trend toward heterotrophy
423 is likely also promoted by the release of nutrients by dead algal cells.

424 In previously dried biofilms, there was almost no effect of ARO contamination visible on the PCA first
425 component. The contamination tended to mitigate the effect of the first disturbance on EPS
426 production: EPS were reduced by half with dryness, whatever its duration, but slightly increased with
427 ARO contamination. This makes sense since EPS are known to play a role in algal protection against
428 heavy metals (Flemming et al., 2016). Dryness also led to lower ARO sensitivity of pigment richness
429 and diversity, similarly to what was observed for biofilms pre-exposed to warming. This can be
430 explained by the selection of co-tolerant species, as hypothesized for warming. It may also be linked
431 to the fact that dry period had already a very strong effect on pigment diversity. The maximum
432 quantum yield of PSII (F_V/F_M) was negatively impacted by ARO in dried biofilms in contrast with
433 undisturbed or biofilms pre-exposed to warming. F_V/F_M value can reflect the nature of the main algal
434 groups present in biofilm but ARO did not induce clear changes in green algae, cyanobacteria and
435 diatom proportions. Therefore, the observed decrease in F_V/F_M suggests dry period would amplify
436 the deterioration of the physiological state of algae (Laviale, 2008) probably due to pesticide
437 exposition (Tiam et al., 2015). In terms of functioning, previously dried biofilms exhibited a global
438 lower sensitivity to contaminant than undisturbed biofilms, with notably NPP and GPP less affected
439 by ARO. This apparent reduced sensitivity is consistent with some previous studies. For instance,
440 Calapez et al., (2020) found that after a combined exposure to no-flow and to sewage contamination,

441 both the negative effect of contaminant on algae and the positive effect on bacteria were less
442 pronounced than after contaminant exposure alone. Similarly, it has been shown that after
443 intermittency, biofilms were able to maintain biodegradation of a certain pharmaceutical when
444 exposed to emerging contaminants, while biofilms exposed to permanent flow were not (Rožman et
445 al., 2018). It should be noted that in these two studies, biofilms were exposed to complex
446 contaminants. On the contrary, Proia et al. (2013) observed that flow intermittency increased biofilm
447 sensitivity to triclosan, suggesting that the toxicity of pollutants may be exacerbated following a
448 drought. In the present study, the regressions point out that the physical disturbance induced by the
449 dry period had the highest impact on biofilms and that the chemical stressor did not significantly
450 intensify drought-induced changes. A similar observation emerged from the meta-analysis performed
451 by Arenas-Sánchez et al. (2016) on studies focusing on the co-occurring effects of water scarcity and
452 chemical pollution on micro- and macro-organisms in aquatic ecosystems. This highlights the
453 necessity to prioritize at least minimal water support in river management.

454

455 **Response to ARO in biofilms previously exposed to both disturbances**

456 As seen on the PCA, the combination of warming and dry period induced a strong modification of
457 biofilm algal composition: green algae replaced cyanobacteria and diatoms which dominated
458 undisturbed biofilm. This modification of algal composition may explain why those biofilms exhibited
459 highest F_V/F_M than undisturbed biofilms, since green algae have highest F_V/F_M than cyanobacteria
460 (Suggett et al., 2009). When these previously disturbed biofilms were exposed to ARO, the
461 proportion of green algae (and F_V/F_M) significantly decreased and cyanobacteria proportion
462 increased. This stronger response to ARO, quite visible on the PCA plot may be explained by a higher
463 sensitivity of green algae to ARO (Ma, 2005).

464

465 **Conclusion**

466 As a whole, these results show very clearly that previous warming and/or dryness modulate biofilm
467 response to a complex contaminant added during rewetting. We hypothesized that the first
468 disturbance would decrease biofilm sensitivity to contamination. This was only partially observed
469 since warming decreased the ARO effects on biofilm composition but amplified them on biofilm
470 functioning. Dry periods significantly reduced the contaminant effects. Our findings show that the
471 first disturbance was the one impacting the most biofilms. The low sensitivity of dried biofilm to ARO
472 is certainly due to the selection of generalist species or the induction of physiological responses by
473 the first disturbance that allow biofilm to resist contamination during rewetting. Our study
474 emphasizes the importance of considering the hydrological history of a microbial community when
475 assessing its sensitivity to a contaminant, and more generally, the requirement to consider water
476 scarcity in the risk assessment of chemicals in aquatic ecosystems.

477

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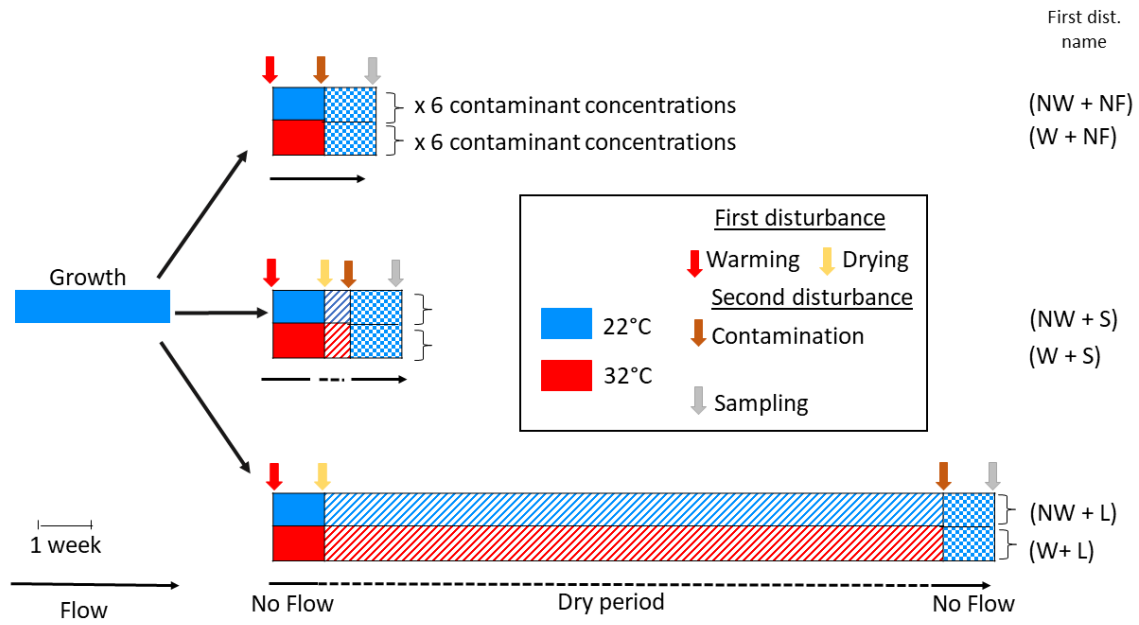


Figure 1: Experimental design (n=36). After a 2-week growth in normal flow conditions, biofilms were exposed to no-flow (NF) for a week at 22°C (NW, blue) or 32°C (W, red) (filled cases). Then, some biofilms were kept dried (hatched cases) for 3 days (short period, S) or 12 weeks (long period, L) at the same temperature than previously, before rewetting and addition of contaminant (chequered cases). Biofilm sampling and measurements were performed 1 week after contamination.

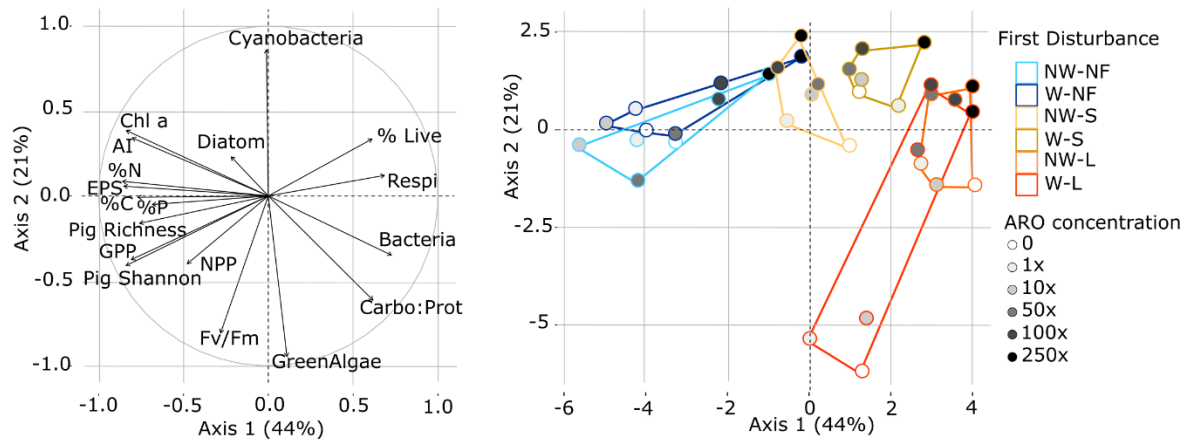


Figure 2: PCA representation of biofilm descriptors. Sample plots are grouped depending on dryness treatments and are coloured depending on ARO concentration samples were exposed to. 18 descriptors have been used to conduct the PCA analysis. Chl α = chlorophyll α , AI = autotrophic index, EPS = extrapolymeric substances, Pig Shannon = Shannon index of pigments, Pig Richness = pigments richness, Fv/Fm = maximal quantum yield of photosystem II, GPP = gross primary production, NPP= net primary production, Respi = respiration.

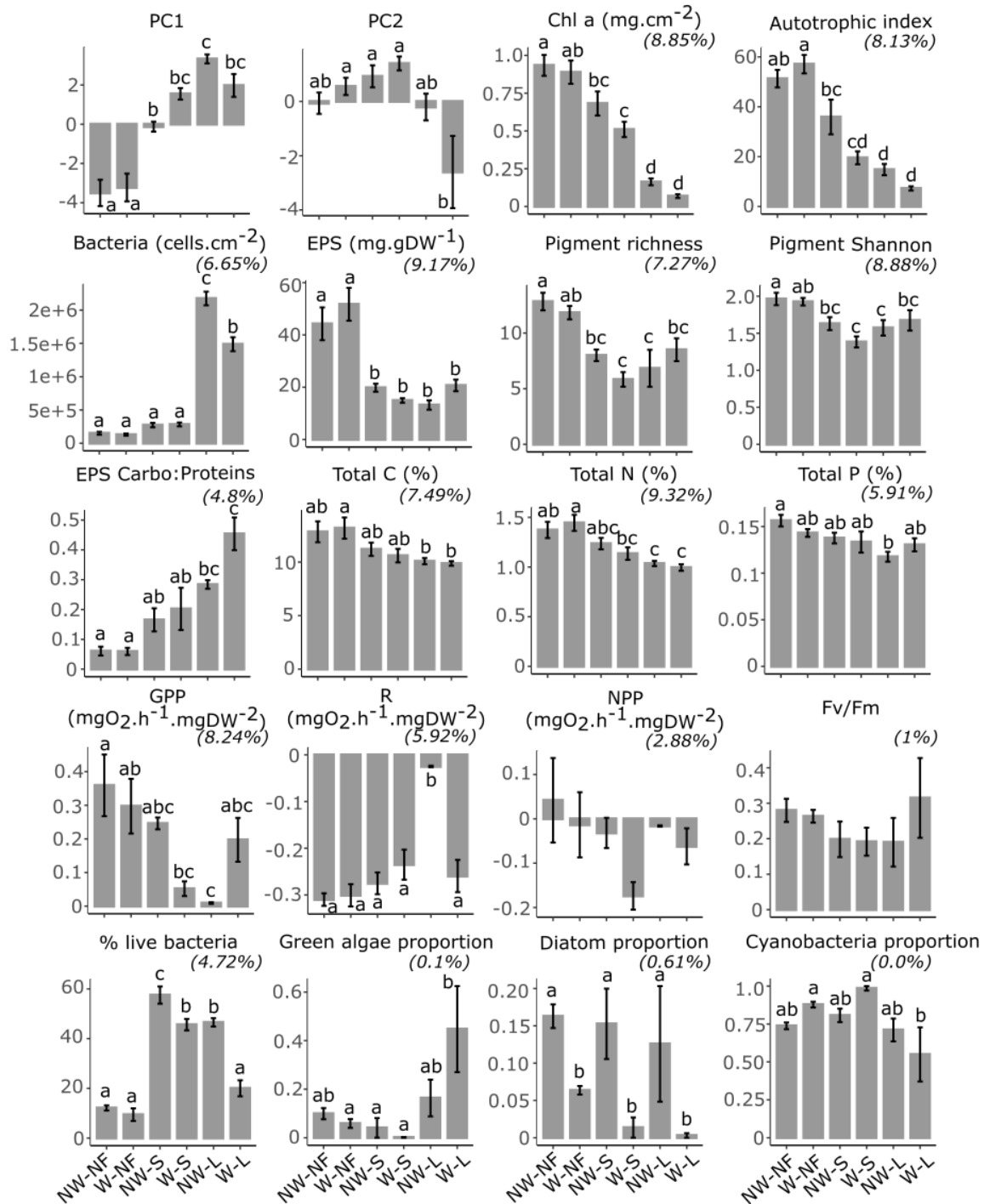


Figure 3: Barplot representations of the effects of the first disturbance on biofilm descriptors. Values represent the mean ± standard error (n = 6) PC1 and PC2 coordinates of the PCA respectively, and of each variable according to the 6 first conditions of dry period duration (NF, S or L) and warming (NW or W). Different letters mean significant statistical differences (level = 0.05). Percentage in parentheses represents the absolute contribution of the variable to the PC1.

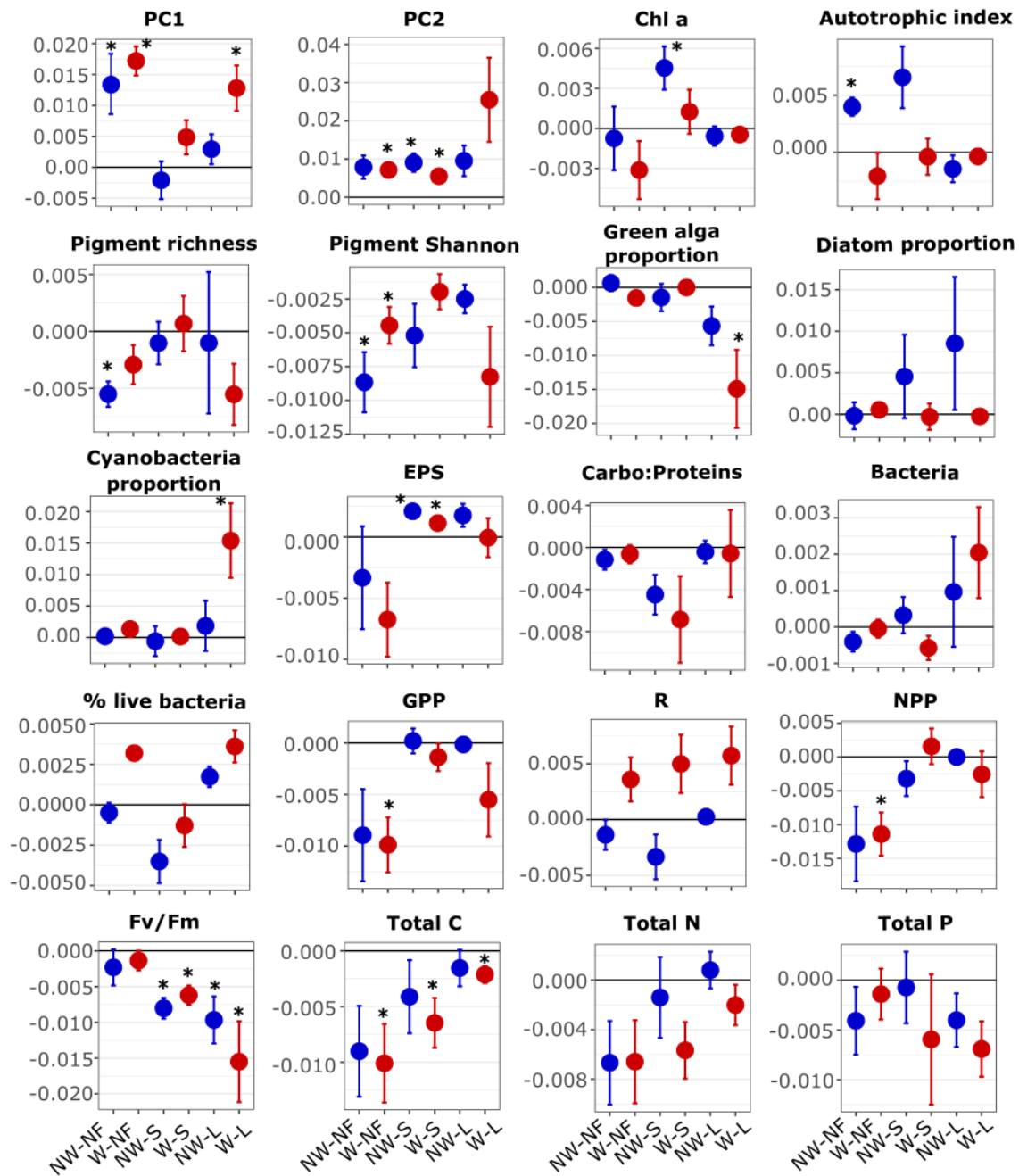


Figure 4: Biofilm sensitivity to ARO contamination depending on the first disturbance. Sensitivity to contamination is represented by the slope of regressions between measured parameter and contaminant concentrations for the 6 first conditions of dry period duration (NF, S or L) and warming (NW or W). It represents the mean (\pm standard error, $n = 6$) slopes of regressions between each parameter and contaminant concentration. No warmed and warmed treatments are represented by blue and red circles, respectively. Asterisks are for significant statistical differences (level = 0.05)

