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1 Chlordecone-contaminated epilithic biofilms show increased adsorption capacities

2

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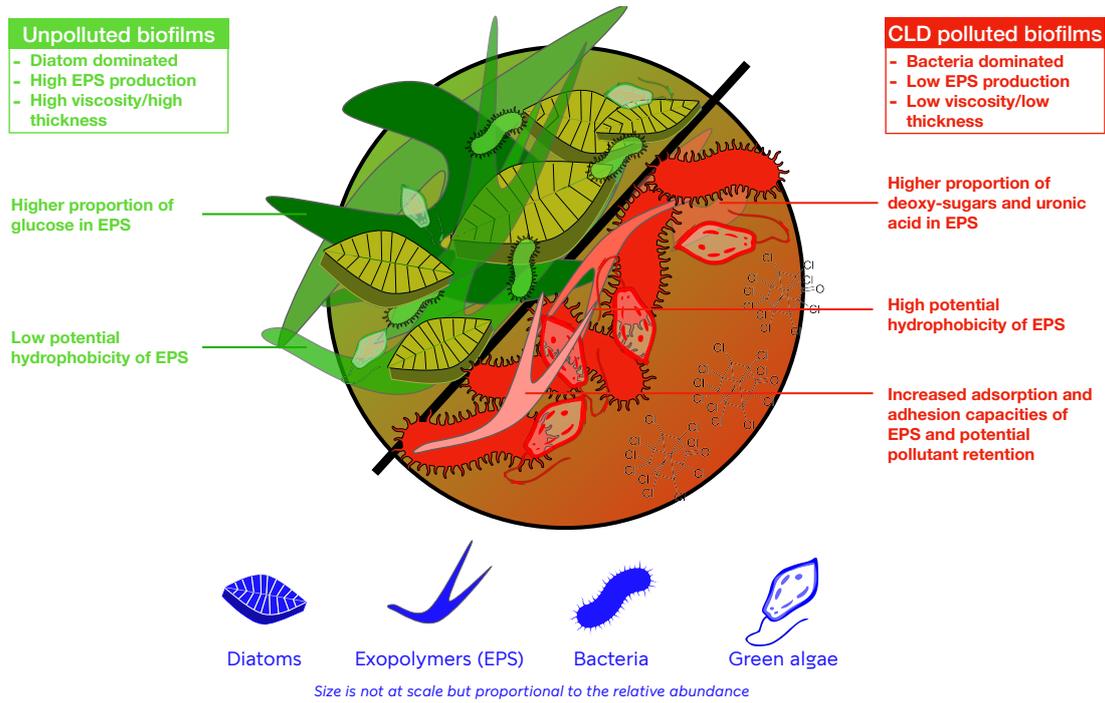
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26

27 **Graphical abstract**

28



30

31 **Abstract**

32

33 The rivers of Guadeloupe and Martinique (French West Indies) show high levels of chlordecone
34 (CLD) contamination. This persistent molecule has a dramatic impact on both aquatic ecosystems
35 and human health. In these rivers, epilithic biofilms are the main endogenous primary producers and
36 represent a central food source for fish and crustaceans. Recently, their viscoelastic properties have
37 been shown to be effective in bio-assessing pollution in tropical environments. As these properties
38 are closely related to the biochemical composition of the biofilms, biochemical (fatty acids,
39 pigments, extracellular polymeric substances (EPS) monosaccharides) and molecular markers (T-
40 RFLP fingerprints of bacteria, archaea and eukaryotes) were investigated. Strong links between
41 CLD pollution and both biofilm biochemistry and microbial community composition were found. In
42 particular, high levels of CLD were linked with modified exo-polysaccharides corresponding to
43 carbohydrates with enhanced adsorption and adhesion properties. The observed change probably
44 resulted from a preferential interaction between CLD and sugars and/or a differential microbial
45 secretion of EPS in response to the pollutant. These changes were expected to impact viscoelastic
46 properties of epilithic biofilms highlighting the effect of CLD pollution on biofilm EPS matrix.
47 They also suggested that microorganisms implement a CLD scavenging strategy, providing new
48 insights on the role of EPS in the adaptation of microorganisms to CLD-polluted environments.

49

50 Keywords: epilithic biofilms; chlordecone; extracellular polymeric substances; microbial
51 communities; T-RFLP; fatty acids ; monosaccharides, lipophilic pigments ; Caribbean

52

53

54 Introduction

55

56 At the beginning of the 2000's, high chlordecone (C₁₀Cl₁₀O; CAS number 143-50-0 | Kepone®)
57 contamination levels have been reported in Guadeloupe and Martinique (French West Indies) rivers.
58 Though chlordecone (CLD) usage was banned in the 1990's, and despite reported potential
59 degradability similar to other organochlorine (OC) pesticides (Dolfing et al., 2012), CLD, was
60 found to be highly present in soils, reaching 35 mg·kg⁻¹ ten years after the last agricultural
61 spreading (Martin-laurent et al., 2013). The widespread use of CLD for the intensive cropping of
62 bananas had caused ecological as well as a public-health disaster in the French West Indies. Indeed,
63 despite being reported as a possible human carcinogen in late 1970's (Reuber, 1978), CLD was yet
64 used for about 20 years and afterwards identified as the main cause of the increased prevalence of
65 prostate cancer in Martinique (Belpomme and Irigaray, 2011; Multigner et al., 2010).

66

67 To date most studies have focused on microbial ecology of CLD-contaminated soil (andosols,
68 ferralsols, and nitisols), evidencing that CLD changed soil microbial communities according to the
69 contamination level and physical-chemical soil characteristics (Mercier et al., 2013; Merlin, 2015).
70 Though evidences in soil microcosms confirmed the existence of potentially CLD-respiring or -
71 fermenting microorganisms, CLD-degradation metabolites have yet to be detected in soils
72 (Fernández-Bayo et al., 2013). Also, the reported CLD mineralisation rates are well below those
73 necessary to remove completely the CLD (Fernández-Bayo et al., 2013). In contrast, microbial
74 communities of French West Indies rivers have been seldom studied so far despite the recognized
75 contamination, at varying levels, of numerous aquatic systems (Crabit et al., 2016). This is
76 surprising since during the massive CLD-contamination of the Saint James River (Virginia, USA) in
77 the 1970's (Huggett, 1989) the disruptive effects of CLD on survival and growth of a significant
78 fraction of bacteria inhabiting the water column and the sediment were demonstrated (Orndorff and
79 Colwell, 1980). In Guadeloupe, due to turbulent flows and high-water velocity, autochthonous river
80 primary production is mainly supported by epilithic biofilms growing on riverbed stones. These
81 biofilms are one of the few food sources that are accessible to higher trophic levels in this habitat
82 (Coat et al., 2009; Lefrançois et al., 2011). Recent studies have shown a very low level of essential
83 fatty acids (eicosapentaenoic acid, EPA) in aquatic species living in the rivers of Guadeloupe, and
84 as this molecule is contained in the epilithic biofilm consumed by these species, this suggests that
85 dietary EPA may be a limiting factor for growth or survival (Frotté et al., 2021).

86

87 Biofilms are complex and highly organized assemblages characterized by a great diversity of
88 microorganisms (algae, fungi, protozoa, bacteria and archaea) embedded in a matrix of extracellular
89 polymeric substances (EPS) produced by the microorganisms . The EPS are ubiquitous component
90 of epilithic biofilms primarily composed of high molecular weight polymers, such as carbohydrates
91 and proteins, containing charged functional groups (Decho, 2000; Decho et al., 2010). These
92 functional groups provide binding sites that serve as natural adsorptive and adhesive supports for
93 charged particles/molecules, including pollutants (Bhaskar and Bhosle, 2006), then available for
94 bioaccumulation in aquatic food webs (Decho, 2000; Decho et al., 2010).
95 More recently, the relationships between viscoelastic properties of epilithic biofilms and their EPS
96 production were pointed out in relation to CLD pollution (Monti et al., 2020). However, the CLD
97 effect on EPS chemistry was not addressed.

98

99 In the present study, we characterized the EPS composition of epilithic biofilms in relation to CLD
100 pollution combining a set of chemotaxonomic biomarkers (fatty acids, lipophilic pigments), EPS
101 biochemical markers (monosaccharides), as well as molecular fingerprints of prokaryotic and
102 eukaryotic communities based on 16S- 18S rRNA genes, respectively. The main objective of the
103 study was to examine changes in the EPS matrix as well as in the microbial composition of the
104 biofilm in response to CLD pollution to better understand the changes previously observed in the
105 viscoelastic properties of epilithic biofilms.

106 **Materials and methods**

107

108 **Sampling site and experimental design:** Biofilms were obtained from 6 rivers in February 2013.
109 Rivers were located in the western-half of Guadeloupe (i.e. Basse-Terre island, figure 1) and were
110 chosen according to their content in CLD and their ability to contain pristine waters upstream from
111 the banana plantations and contaminated waters downstream from the banana fields (i.e. one
112 sampling site above banana fields and one sampling site below for each river: 12 sampling sites in
113 total). All the rivers belong to the same hydroecoregion, a homogenous zone regarding geology,
114 climate and landscape. Additional information about the sampling sites is available in Monti et al.
115 (2020, i.e. first sampling campaign, February 2013). Biofilms were obtained by immersing glass
116 slides (76x26 mm) which were then collected in triplicates 7, 14 and 21 days after immersion
117 (hereafter named T07, T14 and T21) as described by these authors. Glass slides were carefully
118 wrapped in foil after sampling and kept cold until return to laboratory. They were then stored at –
119 20°C until analysis. In each river, water samples were taken (100 mL) to measure the CLD
120 concentration, at each sampling date.

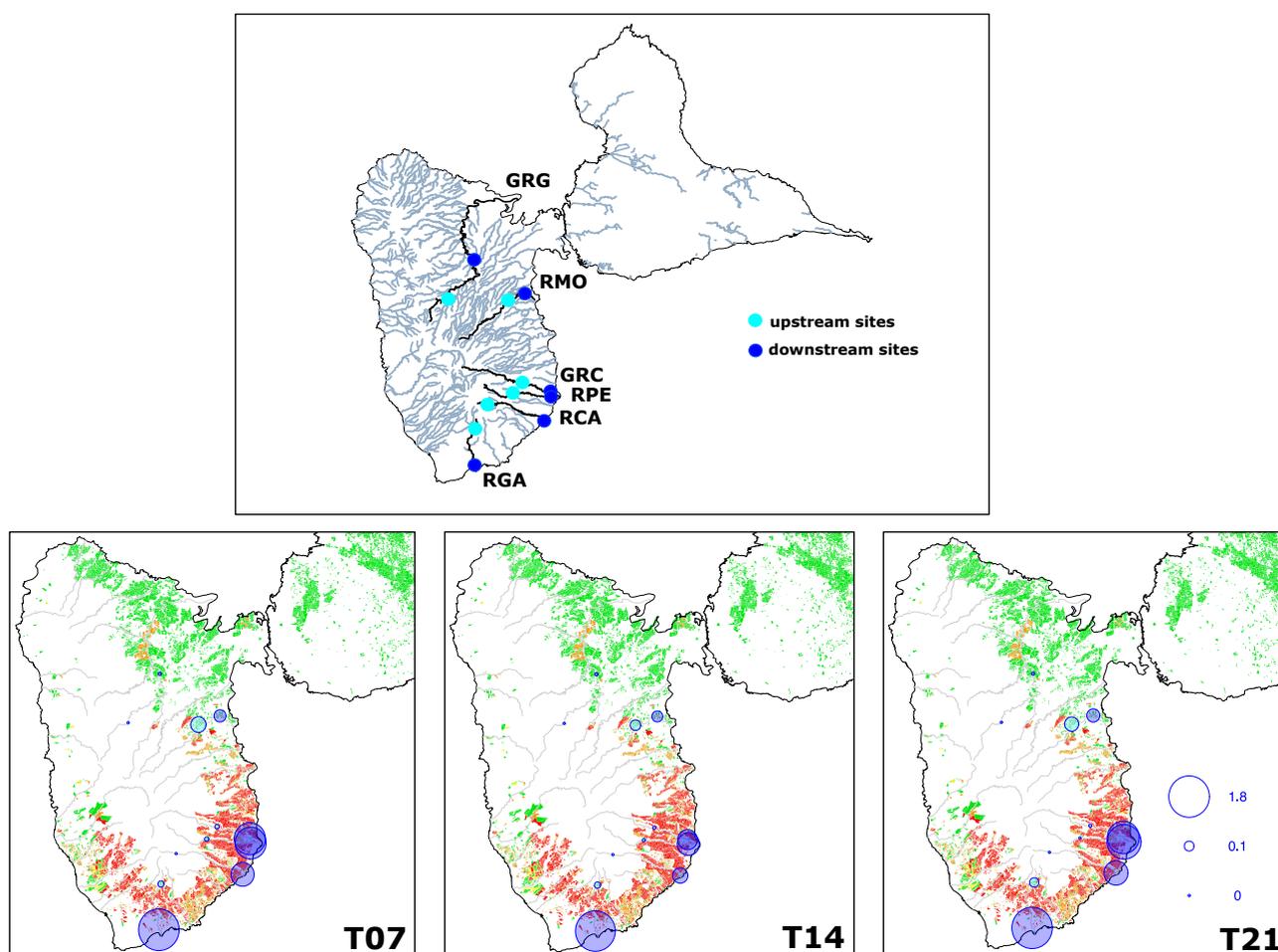


Figure 1: Description of the sampling site. Upper panel: Localisation of the upstream (light-blue) and downstream (darkblue) sampling sites in 6 different rivers. Grande rivière à Goyaves River: GRG, Moustique River: RMO, Grande rivière de Capesterre River: GRC, Pérou River: RPE, Carbet River: RCA, Grande Anse River: RGA. Bottom panel: vector based mapping of the potential CLD pollution according to the French DAAF (Direction de l'Alimentation, de l'Agriculture et de la Forêt de Guadeloupe) regulation (calculated from satellite imaging and archives data of banana plantations). Red = severe, orange = high, yellow = low, Green = minor. The size of the light-blue and dark-blue circles is proportional to the CLD concentrations (in $\mu\text{g}\cdot\text{L}^{-1}$) measured during the February 2013 campaign in river waters. From left to right: Levels of CLD pollution in river waters 7, 14 and 21 days after immersion of the glass slides. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

122 **Chlordecone extraction and analysis:** Water samples collected into glass bottles were stored at -
 123 20°C until analysis. They were thawed before extraction, and processed without filtration step
 124 because of the low quantity of suspended matter presents in the samples. Water samples of 100 mL
 125 were spiked with internal standard solution (chlordecone ^{13}C) and were then extracted with 10 mL
 126 of dichloromethane three times. These extracts were dried on sodium sulphate (Na_2SO_4) and then
 127 evaporated under a gentle flow of nitrogen. The final extract was transferred into 100 μL of
 128 acetonitrile for injection. The extracts were analyzed by HPLC-ESI-MS-MS (UPLC-Quattro
 129 premier Waters). Separation was achieved using a BEH-C18 column (50 mm x 2.1 mm; 1.7 μm) at

130 a temperature of 35°C. Acetonitrile and water (ultrapure deionized water) were used as the mobile
131 phases. The injection was carried out using 5 µL of sample and the flow rate was 0.6 mL·min⁻¹. The
132 gradient composition of the mobile phase started with 0% of acetonitrile and increased up to 100%
133 in 3 min. This composition was held for 0.5 min and then decreased down to 0% in 0.5 min and was
134 held at 0% until the end of the analysis. The spectrometer was operated with a negative electrospray
135 ion source (ESI) and multiple reaction monitoring mode (MRM) using nitrogen as the collision gaz.
136 Quantification were made with internal standard. Analytical method was validated in terms of
137 calibration linearity, specificity, extraction recoveries (93 ± 8%), and limits of quantifications (2 pg
138 injected or 1 ng·L⁻¹). For each series of analysis, blank experiments (complete procedure but
139 without matrix) were performed. Recoveries of three samples of fortified mineral water (50 ng·L⁻¹)
140 were evaluated for water analysis (from 90 to 105%). Control calibrating standards (0.1 à 100 ng·g⁻¹)
141 were also injected every 15 samples and analytical blanks were performed. All solvents for
142 chemicals analysis, dichloromethane (DCM) and acetonitrile (ACN) (HPLC reagent grade,
143 Scharlau) were purchased from ICS (Belin Beliet, France). Analytical standards of CLD were
144 purchased from LGC Standards (Molsheim, France)

145

146 **Fatty acid composition:** Fatty Acids (FA) were extracted following the method of Bligh and Dyer
147 (1959). Total lipids were extracted by scraping the glass slides with a pre-combusted GF/F filter
148 saturated with 2 mL of methanol (MeOH). Lipids were extracted with a 20 min ultrasonication
149 (sonication bath, 80 kHz, Fisherbrand™) in a mixture of distilled water, chloroform and methanol
150 in ratio 1:1:2 (v:v:v, in mL). An internal standard (23:0) was added to every sample for
151 quantification purpose (0.5 mg·mL⁻¹). Lipids were concentrated under N₂ flux, and saponified, in
152 order to separate FA, with a mixture of NaOH (2 mol·L⁻¹) and methanol (1:2, v:v, in mL) at 90°C
153 during 90 min. Saponification was stopped with 400 µL hydrochloric acid. Samples were then
154 incubated with BF₃-methanol at 90°C during 10 min to transform free FA into fatty acids methyl
155 esters (FAME), which were isolated and kept frozen in chloroform. Just before analysis, samples
156 were dried under N₂ flux and transferred to hexane. One µL of the mixture was injected in a gas
157 chromatograph (GC, Varian CP-3800 equipped with flame ionization detector), which allowed
158 separation and quantification of FAME. Separation was performed with a Supelco® OMEGAWAX
159 320 column (30 m × 0.32 mm i.d., 0.25 µm film thickness) with He as carrier gas. The following
160 temperature program was used: 60 °C for 1 min, then raise to 150 °C at 40 °C·min⁻¹ (held 3 min),
161 then raise to 240 °C at 3 °C·min⁻¹ (held 7 min) at 1 mL·min⁻¹. FAME Peaks were identified by
162 comparison of the retention time with analytical standards. Additional identification of the samples
163 was performed using a gas chromatograph coupled to mass spectrometer (GC-MS, Varian 450GC
164 with Varian 220-MS). Compounds annotation was performed by comparing mass spectra with NIST

165 2017 library. Fatty acids were quantified using the FID detector and the internal standard.
166 Corresponding fatty acids are designated as X:Yn-Z, where X is the number of carbons, Y the
167 number of double bonds and Z the position of the ultimate double bond from the terminal methyl.
168

169 **Pigment composition:** Lipophilic pigments were extracted by scraping the glass slides with a GF/F
170 filter saturated with 2mL of MeOH. Filters were placed in a sterile tube and crushed in 2 mL of 95
171 % cold buffered MeOH (2 % ammonium acetate) for 4h at 4°C, in the dark. Samples were sonicated
172 (37 kHz) for 30 s prior to extraction. Extracts were then filtered (0.2 µm) immediately before High
173 Performance Liquid Chromatography (HPLC) analysis according to Brotas and Plante-Cuny
174 (2003). Pigment extracts were analysed using an Agilent 1260 Infinity HPLC composed of a
175 quaternary pump (VL 400bar), a UV-VIS photodiode array detector (DAD 1260 VL, 190 to 950
176 nm), and a 100 µL sample manual injection loop (overfilled with 250 µL). Chromatographic
177 separation was carried out using a C18 column for reverse phase chromatography (Supelcosil, 25
178 cm long, 4.6 mm inner diameter). The solvents used were A: 0.5 M ammonium acetate in methanol
179 and water (85:15, v:v), B: acetonitrile and water (90:10, v:v), and C: 100 % ethyl acetate with a
180 flow rate of 0.5 mL·min⁻¹. Identification and calibration of the HPLC peaks was performed with
181 chlorophyll *a*, astaxanthin, β-carotene, chlorophyll *c*, fucoxanthin standards. Pigments were
182 identified by their absorption spectra and relative retention times. Quantification was performed by
183 repeated injections of standards over a range of dilutions to determine the relationship between peak
184 area and standard concentrations. The relative abundance of each pigment (%) was calculated from
185 their respective concentration (µg·cm⁻²).

186

187 **Monosaccharide composition of exopolymers:** Colloidal and bound exopolymers (EPS) of the
188 biofilms were extracted by rotating the glass slides in a mixture of distilled water and ion-exchange
189 resin (Dowex Marathon C, sodium, Sigma) for 1.5 h at 4°C. Glass slides were placed in 50 ml tubes
190 filled with the mixture so that they were immersed during the agitation procedure. The exopolymer
191 solution was then retrieved and freeze-dried. Monosaccharide composition was determined using
192 gas chromatography (GC), following Passarelli et al. (2015). EPS were hydrolysed in 2 mol·L⁻¹
193 HCl, and heated 4 h at 90°C to release individual monosaccharides prior their derivatization
194 (silylation), which was performed with a mixture of BSTFA:TMCS (N,O-
195 bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane, 100:1, Sigma) and pyridine (1:1,
196 v:v), for 2 h at room temperature. After the silylation, 1 µL of the sample was injected in GC (GC,
197 Varian CP-3800 equipped with flame ionization detector), which allowed separation and relative
198 quantification of monosaccharides. Separation was performed with an Agilent Technologies VF-
199 1701ms column (30 m × 0.32 mm i.d., 0.25 µm film thickness) with He as carrier gas. The

200 following temperature program was used: start at 150 °C, raise to 200 °C at 7 °C·mi⁻¹ (held 5 min).
201 Injector temperature was set at 250 °C. Peaks of monosaccharides were identified by comparison of
202 the retention time with analytical standards (rhamnose, fucose, xylose, mannose, galactose, glucose,
203 scyllo- and myo-inositol, galacturonic and glucuronic acid), which had been prepared (silylation
204 and injection only) as samples.

205

206 **DNA extraction and T-RFLP analysis:** For each sampling point epilithic biofilms were removed
207 from three slides with a sterile toothbrush in 50 mL of sterile ultrapure water. Collected water was
208 then filtered onto a 0.22 µm sterile cellulose nitrate membrane filter (Sartorius, Goettingen,
209 Germany) and filters were stored at -80°C until DNA extraction.

210 The Power Water DNA Isolation Kit (Mo Bio Laboratories, Solana Beach, CA, USA) was used to
211 extract total biofilm DNA according to the manufacturer's instructions. The quantity and quality of
212 the DNA extracts were checked by 1 % agarose gel electrophoresis, ethidium bromide staining and
213 UV-translumination. The Bacteria universal primers 357F (5'-CCTACGGGAGGCAGCAG-3')
214 (Teske et al., 1996) and 926R (5'-CCGTCAATTCMTTTRAGT-3') (Lane, 1991) and the Archaea
215 universal primers 349F (5'-GYGCASCAGKCGMGAAW-3') and 806R (5'-
216 GGACTACNNGGGTATCTAAT-3') (Takai and Horihoshi, 2000) were used to amplify the 16S
217 rRNA gene. The Eukarya primers targeting 18S rRNA genes were HEX uk-1A-F (5'-
218 CTGGTTGATCCTGCCAG-3') (Sogin and Gunderson, 1987) and Euk-516-GC-R (5'-ACC AGA
219 CTT GCC CTC C-3') (Amann et al., 1990). Forward primers were 5' labelled with
220 carboxyfluorescein (FAM) for prokaryotes and with hexa-chloro-fluorescein-phosphoramidite
221 (HEX) for eukaryotes. An initial denaturation (98°C for 30 s) followed by 30 cycles of denaturation
222 (98°C for 10 s), annealing (58°C for 30 s), and extension (72°C for 30 s) and a terminal extension at
223 72°C for 10 min were used for PCR reactions. The reaction mixture was as followed: 0.5 µM of
224 each primer, 12.5 µL of AmpliTaq Gold® 360 Master Mix (Applied Biosystems, Carlsbad, CA, US)
225 and 1 µL of DNA template. Sterile distilled water was added up to 25 µL of final volume. PCR
226 products were visualized on agarose gel and then purified with the PCR purification kit (GE
227 Healthcare, Velizy-Villacoublay, France). After purification the amplicons (100 ng per sample) were
228 digested by 3 U of AluI restriction enzyme (New England Biolabs) for 3 h at 37°C. The digested
229 products (1 µL) were mixed with 8.75 µL of deionized formamide and 0.25 µL of the Genescan
230 ROX 500 size standard (Applied Biosystems Carlsbad, CA, US). Fluorescently labelled fragments
231 were separated with ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA,
232 US). Raw T-RFLP profiles obtained through GENEMAPPER software (version 1.4, Applied
233 Biosystems, Carlsbad, CA, US) were normalized and analysed using T-REX to produce the final
234 terminal fragments (T-RFs) data matrix (Culman et al., 2009). Only T-RFs with size ranging from

235 35 bp to 500 bp and with height > 30 fluorescence units were considered for analysis as described
236 in Volant et al. (2014).

237

238 **Statistical analyses:** All statistical analyses were performed using R studio Version 1.2.5033 and
239 packages ggplot2 (Wickham, 2016), scales (Wickham and Seidel, 2020), corrplot (Wei and Simko,
240 2021) and cowplot (Wilke, 2019) for data processing and visualisation. Raw data file together with
241 in-house R script for data processing, univariate and multivariate statistics as well as figures are
242 available at github repository: <https://github.com/Hubas-prog/CLD-Biofilm>. All maps were drawn
243 using the Open Source Geographic Information System QGIS 2.10.1. For univariate comparisons,
244 normality and homogeneity of variance were systematically checked and statistical tests chosen
245 accordingly.

246 For multivariate comparisons, permutational multivariate analysis of variance (Permanova) was
247 calculated using the Bray-Curtis dissimilarity index and 999 permutations. Homogeneity of
248 multivariate dispersion was systematically checked prior to Permanova.

249 Multiple Factor Analyses (MFA) were performed according to Escofier and Pagès (1994) using the
250 package ade4 in order to find common structures in several groups of variables defined on the same
251 set of individuals. MFA analysis is a specific case of principal component analysis (PCA) applied to
252 a table for which several groups of variables can be identified and for which each column of group i
253 is weighted by the inverse of the first PCA eigenvalue of group i . The biochemical markers were
254 all measured on the same set of statistical individuals (i.e., on the same glass slides). The same
255 applies to the molecular markers. However, it was not possible to perform a unique MFA using all
256 these markers together as molecular and biochemical markers were not measured on the same glass
257 slides. One MFA was thus performed using the three groups of biochemical markers (fatty acids,
258 lipophilic pigments and EPS monosaccharides) and another MFA was performed using the three
259 groups of molecular markers (targeting Bacteria, Archaea and Eukaryotes)

260 Additionally, in order to explore the weight of a given factor, we performed a supervised Between
261 Class Analysis (BCA) by decomposing the total inertia of the MFA dataset according to a given
262 instrumental variable (i.e., CLD pollution context). The final result is a supervised MFA called BC-
263 MFA. Percentage of total inertia explained by the instrumental variable was calculated and a Monte
264 Carlo test was performed to test the significance of the BC-MFA ordination. The above-mentioned
265 statistical procedure was previously detailed elsewhere (Lavergne et al., 2017) and can be
266 downloaded at the following repository: <https://github.com/Hubas-prog/BC-MFA> (doi:
267 [10.5281/zenodo.4603552](https://doi.org/10.5281/zenodo.4603552)). A threshold based on the square cosine of the coordinates of the
268 variables was applied for highlighting the most discriminating variables of the BC-MFA.

269 Chlordecone effect was also tested by fitting the CLD concentrations to the first and second axis of
270 the MFA ordination.

271 **Results**

272

273 **Chlordecone contamination in river water:**

274

275 The water in upstream sites was significantly less impacted (or not affected at all) by CLD than in
276 downstream sites (Wilcoxon signed rank test, $p=0.001$) with an average of 0.05 ± 0.07 and $0.68 \pm$
277 $0.61 \mu\text{g}\cdot\text{L}^{-1}$, respectively (Fig. 1). The only exception being the RMO River, where the upstream site
278 had similar CLD concentrations as the downstream site (Wilcoxon signed rank test, $p=0.5$). CLD
279 pollution changed significantly over time: on average, CLD concentrations significantly decreased
280 from T07 to T14 (paired t test, $df=5$, $p=0.028$), then significantly increased from T14 to T21 (paired
281 t test, $df=5$, $p=0.028$)

282 As pointed out previously by Monti et al. (2020), CLD measurements revealed three pollution
283 contexts: a very limited or unpolluted context (UnP: between undetectable to $0.1 \mu\text{g}/\text{L}$), a medium
284 polluted context where values are comparable with those currently observed in the rivers of
285 Guadeloupe and Martinique (Med: from 0.1 to $1 \mu\text{g}\cdot\text{L}^{-1}$), and extreme situations (Ext: $> 1 \mu\text{g}\cdot\text{L}^{-1}$
286 and up to $2.76 \mu\text{g}\cdot\text{L}^{-1}$), downstream from the banana plantations. Focusing on downstream sites,
287 CLD concentrations were thus further categorized according to Monti et al., (2020) as unpolluted
288 (UnP), moderately polluted (Med) and extremely polluted (Ext). The BC-MFA were performed
289 using these categories as an instrumental variable.

290

291 **Biochemical fingerprinting of epilithic biofilms:**

292

293 **Fatty acid composition:** On average, the highest concentrations of total, saturated and mono-
294 unsaturated FA were found in the upstream section of the RMO River (total: $15.18 \pm 14.03 \mu\text{g}\cdot\text{cm}^{-2}$,
295 saturated FAs: $7.64 \pm 7.48 \mu\text{g}\cdot\text{cm}^{-2}$, mono-unsaturated FA: $4.40 \pm 4.61 \mu\text{g}\cdot\text{cm}^{-2}$) and the lowest in
296 the upstream section of the RPE River (total: $1.99 \pm 2.58 \mu\text{g}\cdot\text{cm}^{-2}$, saturated FAs: $1.88 \pm 2.50 \mu\text{g}\cdot\text{cm}^{-2}$,
297 mono-unsaturated FA: $0.07 \pm 0.07 \mu\text{g}\cdot\text{cm}^{-2}$). The highest concentrations of poly-unsaturated FA
298 were however registered in the downstream section of the GRG River ($3.38 \pm 4.14 \mu\text{g}\cdot\text{cm}^{-2}$)
299 whereas the lowest were found in the upstream section of the RPE River ($0.03 \pm 0.02 \mu\text{g}\cdot\text{cm}^{-2}$).
300 Palmitic acid (16:0) was on average the most abundant fatty acid followed by stearic acid (18:0),
301 palmitoleic acid (16:1n-7) and eicosapentaenoic acid (20:5n-3, Fig 2).

302 In downstream sites, eicosapentaenoic acid (EPA) and arachidonic acid (20:4n-6) were significantly
 303 negatively correlated with CLD concentration (respectively: $r=-0.54$, $p=0.0268$ and $r=-0.51$,
 304 $p=0.0355$). Saturated fatty acids were significantly positively correlated with CLD concentration
 305 ($r=0.52$, $p=0.0340$). All correlations between CLD and fatty acids are reported in Supp. Mat. Table
 306 1.
 307

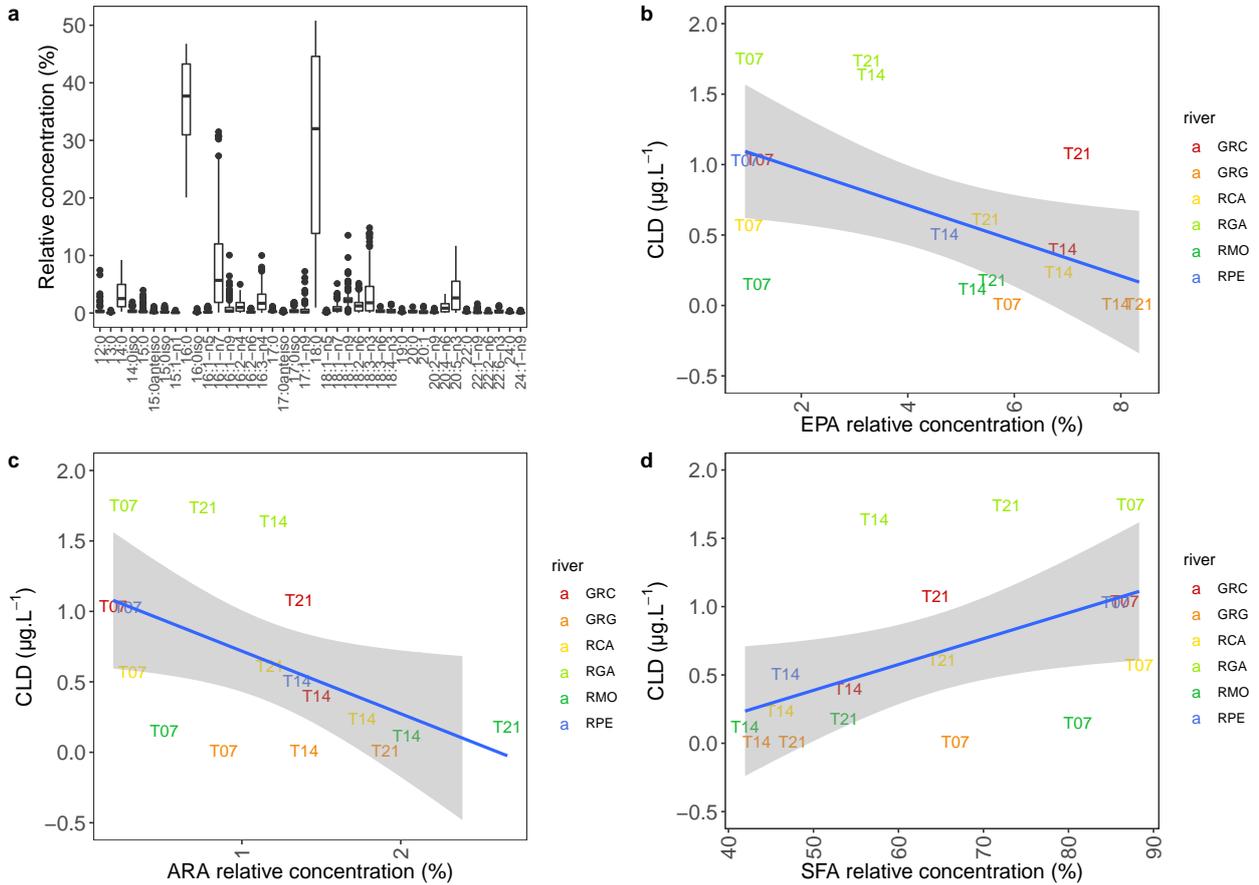


Figure 2: **a**: Relative concentration (in %) of fatty acids at all sites, all rivers and all sampling times. **b**, **c** and **d**: Relationships between eicosapentaenoic acid (**b**: EPA=20:5n-3 ; F test, adjusted $R^2=0.24$, $df_1=1$, $df_2=15$, $p=0.0268$), arachidonic acid (**c**: ARA=20:4n-6 ; ; F test, adjusted $R^2=0.27$, $df_1=1$, $df_2=15$, $p=0.0355$) or saturated fatty acids (**d**: SFA ; F test, adjusted $R^2=0.27$, $df_1=1$, $df_2=15$, $p=0.0340$) relative concentrations and CLD concentrations in downstream sites. For each site, the sampling date is indicated (i.e. T7, T14 and T21 days after immersion of the glass slides).

309 **Pigment composition:** The highest mean concentrations of chlorophyll *a*, chlorophyll *b*,
 310 xanthophylls and carotenes were always recorded in the downstream sites. Chlorophyll *a* was on
 311 average the most abundant pigment followed by fucoxanthin, chlorophyll *a* allomer and chlorophyll
 312 *b* (Fig 3).

313 In downstream sites, the proportion of fucoxanthin (a dominant and essential pigment of diatoms,
 314 Kuczynska et al., 2015) was significantly negatively correlated with the CLD concentration ($r=-$

315 0.54, $df=15$, $p=0.02429$). In addition, CLD concentration was also significantly correlated to
 316 Chlorophyll *a*, *c*2 and *c*3, lutein, pheophytin *a* and violaxanthin (respectively $r=0.63$, -0.56 , -0.74 , $-$
 317 0.54 , 0.61 , -0.65 and 0.56 , $p=0.007$, 0.020 , 0.001 , 0.010 , 0.005 and 0.021). All correlations between
 318 CLD and fatty acids are reported in Supp. Mat. Table 2.

319

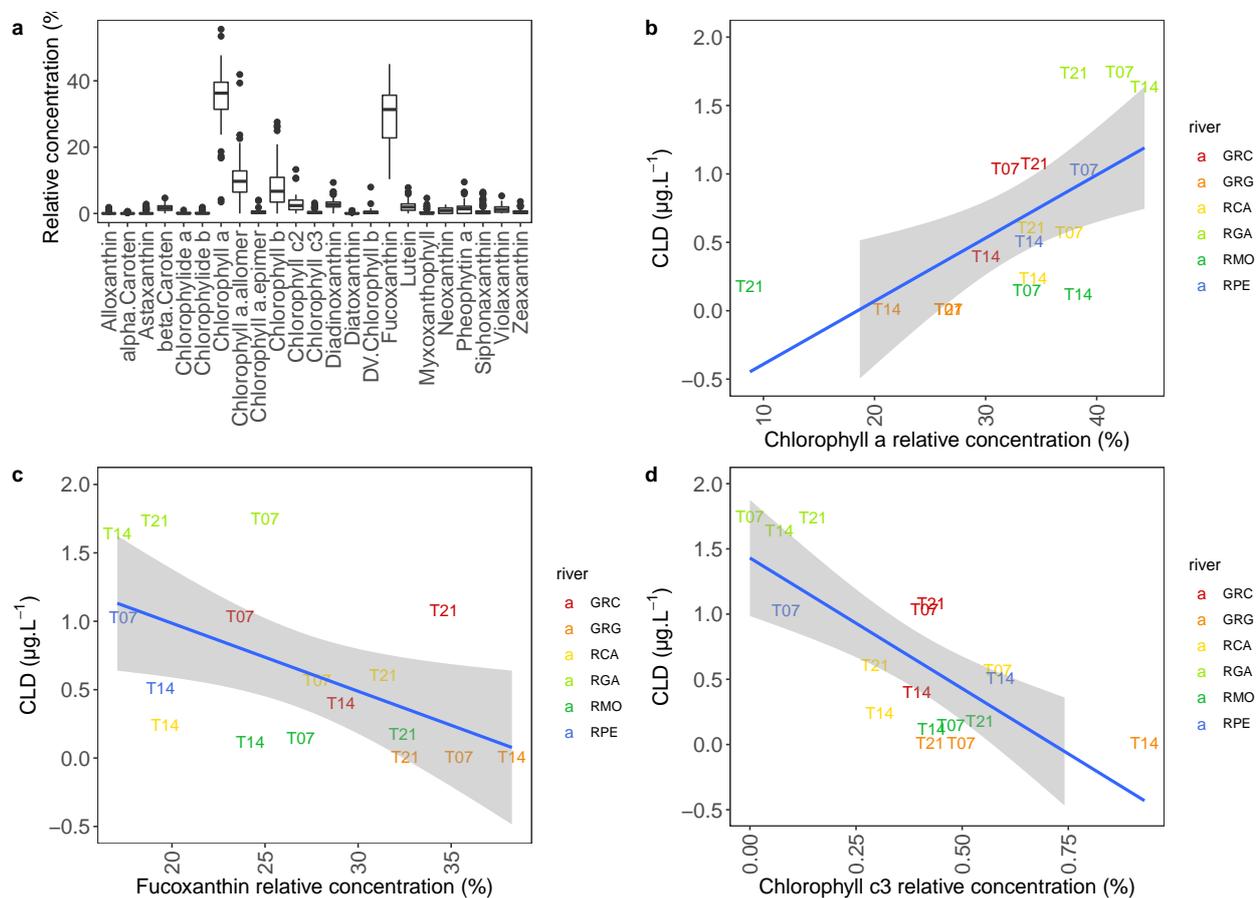


Figure 3: **a**: Relative concentration (in %) of lipophilic pigments at all sites, all rivers and all sampling times. **b**, **c** and **d**: Relationships between chlorophyll *a* (**b**: *F* test, adjusted $R^2=0.36$, $df_1=1$, $df_2=15$, $p=0.0067$), fucoxanthin (**c**: *F* test, adjusted $R^2=0.25$, $df_1=1$, $df_2=15$, $p=0.0243$) or chlorophyll *c*3 (**d**: *F* test, adjusted $R^2=0.52$, $df_1=1$, $df_2=15$, $p=0.0006$) relative concentrations and CLD concentrations in downstream sites. For each site, the sampling date is indicated (i.e. T7, T14 and T21 days after immersion of the glass slides).

321 **EPS monosaccharide composition:** On average, the highest concentrations of EPS
 322 monosaccharides were found in the downstream section of GRG and the lowest in the upstream
 323 section of GRG. Glucose was in average the most abundant sugar followed by mannose, and
 324 galactose (Fig 4).

325 CLD concentration was only significantly correlated to log transformed myo-inositol, scyllo-
 326 inositol and glucuronic acid (respectively $r = -0.74$, 0.58 and 0.56 , $p = 0.0007$, 0.015 and 0.019). In
 327 addition it was also significantly correlated to the logarithm of the myo- to scyllo-Inositol and myo-
 328 to glucuronic acid ratio (respectively $r=-0.70$ and -0.59 , $p=0.002$ and 0.014) (Fig. 4). These ratios

329 were also significantly higher in the downstream sites than in the upstream sites (permutation Welch
 330 t test, $p=0.002$, Fig. 4). All correlations between CLD and monosaccharides are reported in Supp.
 331 Mat. Table 3.
 332

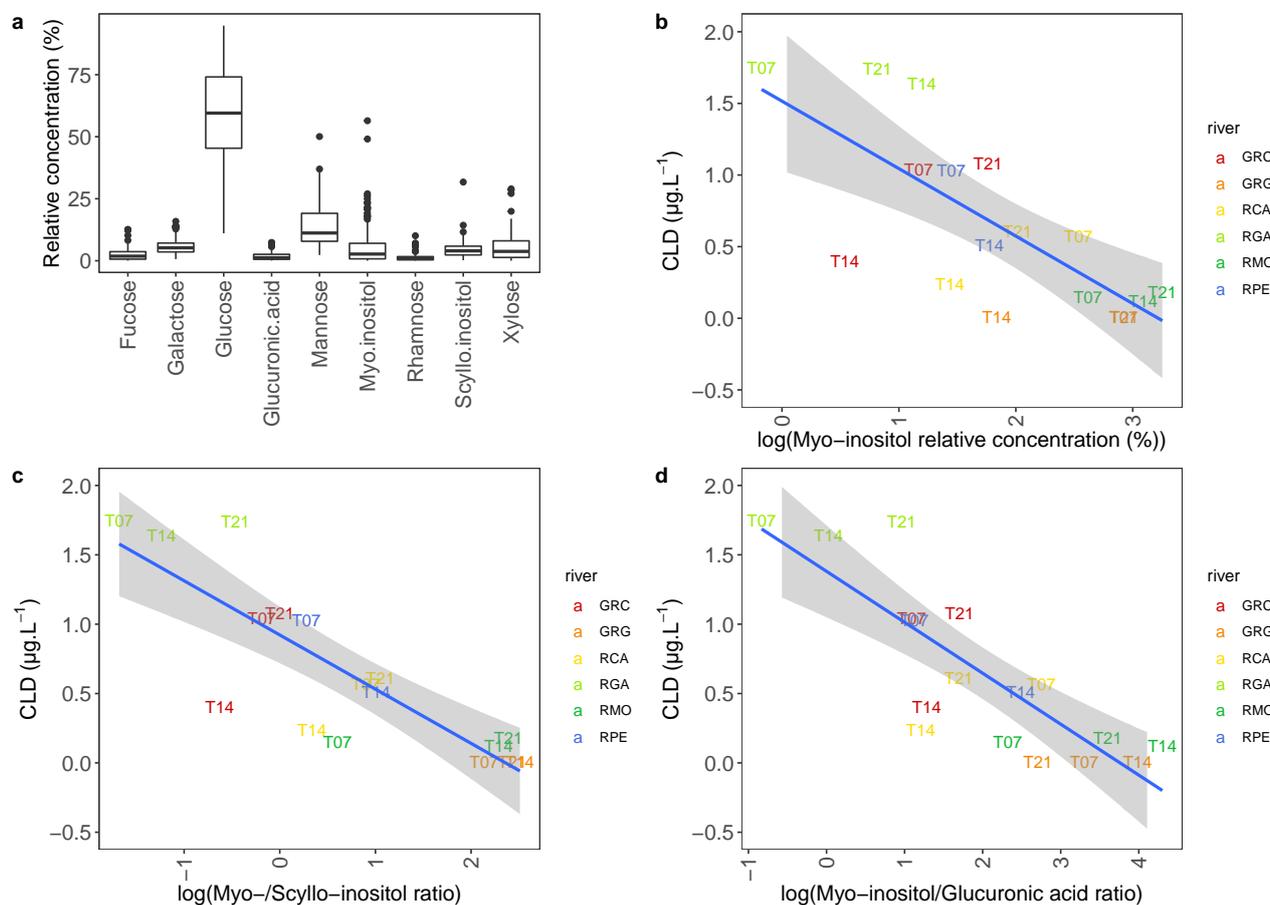


Figure 4: **a**: Relative concentration (in %) of monosaccharides at all sites, all rivers and all sampling times. **b**, **c** and **d**: Relationships between log transformed myo-inositol percentage (**b**: F test, adjusted $R^2=0.52$, $df_1=1$, $df_2=15$, $p=0.0007$), myo- to scyllo-Inositol ratio (**c**: F test, adjusted $R^2=0.68$, $df_1=1$, $df_2=15$, $p<0.0001$) or myo-Inositol to glucuronic acid ratio (**d**: F tests, adjusted $R^2=0.66$, $df_1=1$, $df_2=15$, $p<0.0001$) and CLD concentrations in downstream sites. For each site, the sampling date is indicated (i.e. T7, T14 and T21 days after immersion of the glass slides).

334 **Combination of biochemical fingerprints:** The two first axes of the MFA analysis (Fig. 5a)
 335 explained 47% of the total inertia. Pigments contributions to axis 1 and 2 were respectively 37 and
 336 17%, monosaccharides contributions were 38 and 9%, fatty acids contributions were 25 and 75%.
 337 The first axis separated the different sites and represented an axis of CLD pollution (Pearson
 338 correlation between MFA axis 1 and CLD concentrations: $r=0.62$, $p=0.0080$).
 339 By decreasing order, the main variables displaying a contribution higher than the bulk average
 340 contribution were: Scyllo-inositol, Mannose, Glucuronic acid, Xylose, Fucose, Glucose,

341 Chlorophyll *c*3, Fucoxanthin, Chlorophyll *c*2, Lutein, Rhamnose, Neoxanthin, Galactose,
342 Diadinoxanthin, Chlorophyll *a*, Violaxanthin, β - β caroten, Phaeophytin *a*, β - ϵ caroten,
343 Diatoxanthin, 18:1n-9, 12:0, Chlorophyll *b*, 17:0anteiso, 22:1n-9 and 17:0. These variables
344 explained 79% of the inertia of axis1. The main variables explaining most axis2 inertia (83%) were:
345 16:2n-6, 14:0iso, 16:0, 20:4n-6, 18:1n-7, 18:0, Galactose, 16:0iso, 18:2n-6, 15:1n-1, β - ϵ caroten,
346 18:1n-5, 22:2n-6, 15:0iso, 17:0iso, 14:0, 16:1n-5, 15:0anteiso, 18:3n-3, 20:0, Myo-inositol,
347 Phaeophytin *a*, Astaxanthin, 16:3n-4, 19:0, 16:1n-9, 16:2n-4, Alloxanthin, Glucose, 20:5n-3, 15:0,
348 18:4n-3, Chlorophyll *a*, 17:1n-9.

349 Our results showed that FA, pigments, EPS monosaccharides were significantly different between
350 sites and across time (Permanova $p=0.001$ for site and time effect and interaction).

351 The BC-MFA performed using CLD pollution context as an instrumental variable (Fig.5b) showed
352 that CLD was clearly distributed along axis 1 (Pearson correlation between BC-MFA axis 1 and
353 CLD concentrations: $r=0.81$, $p<0.0001$) and explained 21% of total inertia of the whole biochemical
354 dataset (Monte-Carlo test based on 999 replicates, $p=0.016$). The most important variables in
355 explaining CLD categories were fucoxanthin, diadinoxanthin and lutein (for pigments, with
356 respectively a correlation coefficient with axis 1: $r = 0.64$, 0.63 and -0.63), myo-inositol, scyllo-
357 inositol and glucuronic acid (for monosaccharides, with $r = 0.56$, -0.56 and 0.50) and 16:2n-4,
358 16:1n-7 and 16:3n-4 (for fatty acids, with $r = 0.70$, 0.68 and 0.63).

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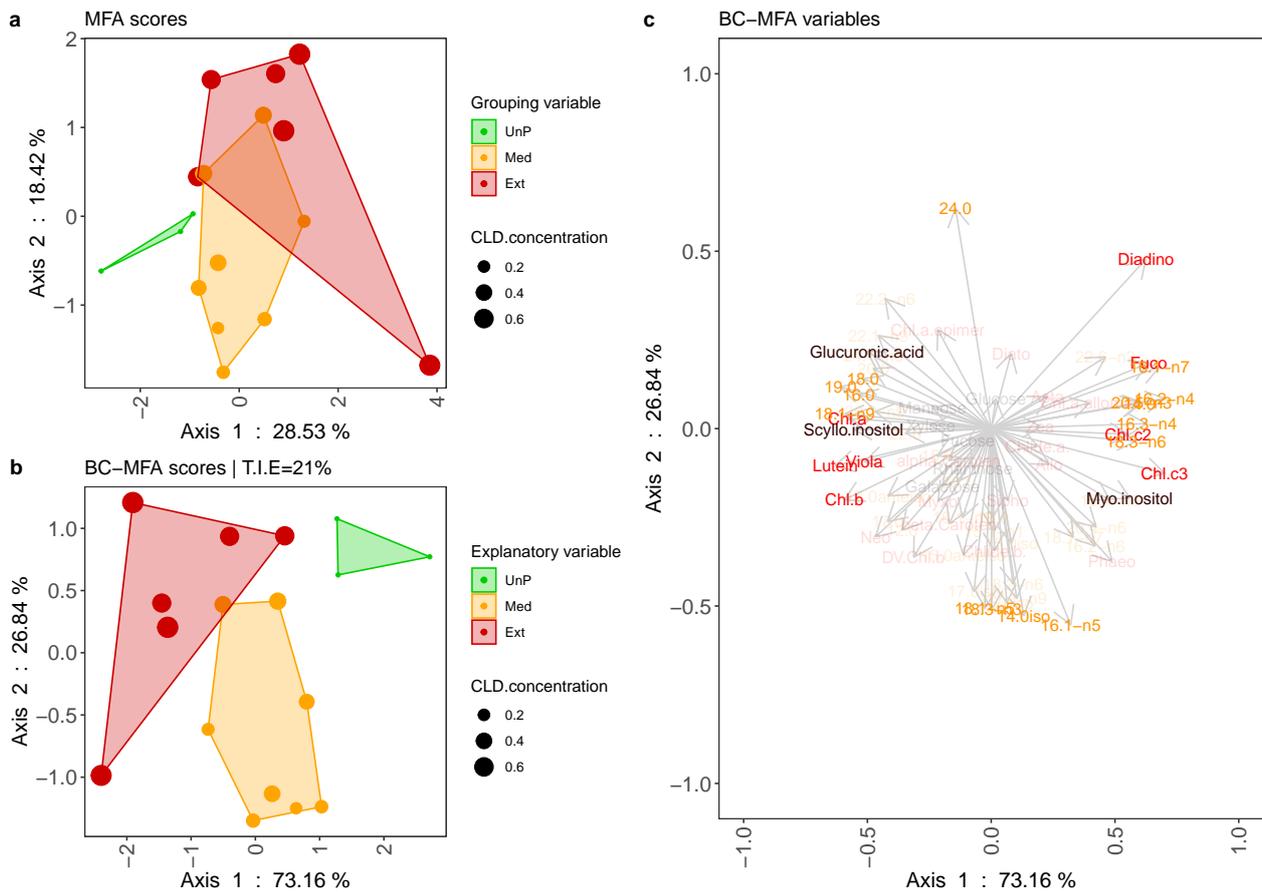


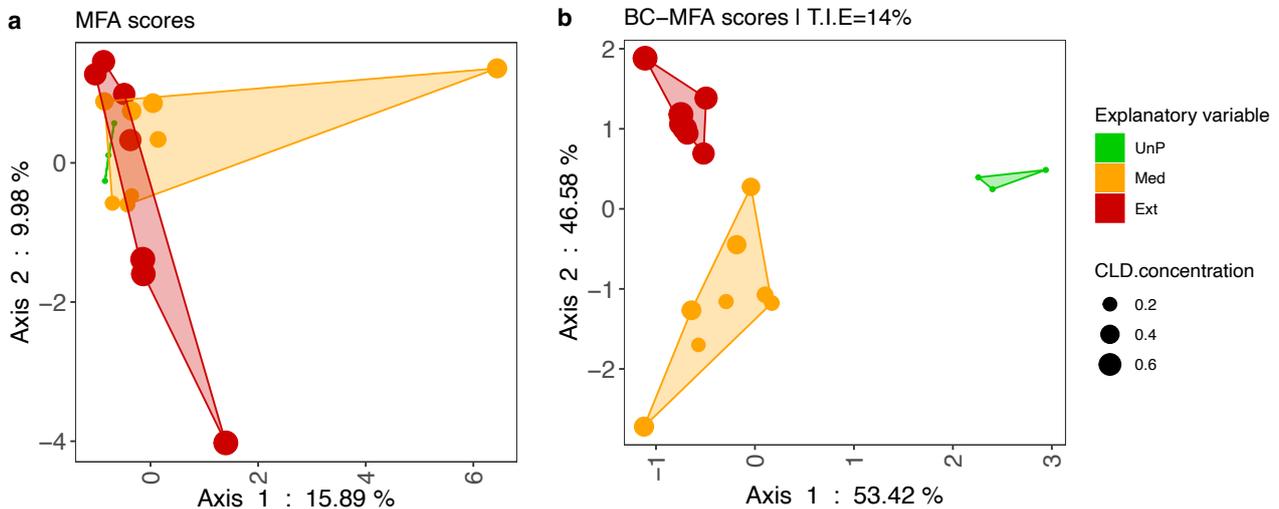
Figure 5: Results of the BC-MFA procedure using pigments, fatty acids and EPS monosaccharides of epilithic biofilms of the downstream sites. UnP = Unpolluted context ($<0.1 \mu\text{g.L}^{-1}$), Med = Medium pollution (from 0.1 to $1 \mu\text{g.L}^{-1}$) and Ext = extreme pollution context ($<1 \mu\text{g.L}^{-1}$) according to Monti et al., (2020) **a**: Multiple Factor Analysis scores using CLD pollution context as a grouping variable. **b**: Interclass analysis based on the results of the MFA and using the CLD pollution context as an explanatory variable (i.e. BC-MFA). Total Inertial Explained (T.I.E) by the explanatory variable was 21% (Monte-Carlo test based on 999 replicates, $p=0.016$). For both MFA and BC-MFA scores, points are proportional to the CLD concentration values (in $\mu\text{g.L}^{-1}$) **c**: coordinates of the variables according to the BC-MFA ordination. A cos^2 threshold of 0.25 was applied to highlight the most important variables in the construction of the BC-MFA axes.

365 **Molecular fingerprinting of epilithic biofilms:**

366

367 The MFA, based on T-RFLP fingerprints explained 26% of the total inertia. Archaea contributions
 368 to axis 1 and 2 were respectively 34 and 28%, Bacteria contributions were 31% and 47%, Eukarya
 369 contributions were 35% and 25% (Fig. 6a). Axis 1 and 2 did not allow a separation of the different
 370 sites according to the pollution contexts (Pearson correlation between MFA axis 1 and CLD
 371 concentrations: $r=0.12$, $p=0.6386$). However, our results showed that T-RFLP fingerprints were
 372 significantly different between sites and time (Permanova $p=0.001$ for site and time effect and
 373 interaction).

374 When using CLD category as an instrumental variable (Fig.6b), CLD was clearly distributed along
 375 axis 1 of the BC-MFA (Pearson correlation between BC-MFA axis 1 and CLD concentrations:
 376 $r=0.62$, $p=0.0056$) and explained 14% of total inertia of the whole molecular dataset (Monte-Carlo
 377 test based on 999 replicates, $p=0.044$).
 378



1 *Figure 6: Results of the BC-MFA procedure using T-RFLP data of the downstream sites. UnP =*
 2 *Unpolluted context ($<0.1 \mu\text{g}\cdot\text{L}^{-1}$), Med = Medium pollution (from 0.1 to $1 \mu\text{g}\cdot\text{L}^{-1}$) and Ext = extreme*
 3 *pollution context ($<1 \mu\text{g}\cdot\text{L}^{-1}$) after Monti et al., (2020) a: Multiple Factor Analysis scores using*
 4 *CLD pollution context as a grouping variable. b: Interclass analysis based on the results of the MFA*
 5 *and using the CLD pollution context as an explanatory variable (i.e. BC-MFA). Total Inertial*
 6 *Explained (T.I.E) by the explanatory variable was 14% (Monte-Carlo test based on 999 replicates,*
 7 *$p=0.044$). For both MFA and BC-MFA scores, points are proportional to the CLD concentration*
 8 *values (in $\mu\text{g}\cdot\text{L}^{-1}$).*

380 Discussion

381

382 CLD pollution and general context of the study:

383

384 CLD concentrations in river water were highly variable between the sampling sites but were in
 385 agreement with the mapping of the potential CLD pollution risk (Fig. 1). Indeed, the most impacted
 386 rivers were in the southern part of the island where the use of the CLD was the greatest,
 387 representing a “severe” risk of contamination as reported by the French DAAF (Cabidoche et al.,
 388 2006). Our results confirmed that upstream sites were not impacted by the CLD pollution except for
 389 the RMO River.

390 It is unclear, however, why the upstream site of this river displayed CLD contamination levels
 391 similar to the downstream site. In a previous study, this site also showed inconsistent EPS and CLD
 392 concentration values (compared to other upstream sites), as well as viscoelastic properties that led

393 the authors to categorize it as "moderately polluted" a posteriori (Monti et al., 2020). Nevertheless,
394 by focusing on the downstream sites, we showed contrasting CLD concentrations (in average
395 RGA>RPE>GRC>RCA>RMO>GRG) in agreement with Monti et al. (2020). Multivariate analyses
396 (Fig. 5 & 6) allowed the separation of the different downstream sampling sites according to the
397 CLD pollution.

398 Microbial communities between sites were significantly different, as revealed by both
399 chemotaxonomic and molecular fingerprints. Such differences can be explained by multiple
400 confounding factors that could differentially influence microbial attachment and biofilm
401 development, but this is very unlikely in the present study. Indeed, it was previously shown that the
402 sampling sites^{1/} were located in water masses of similar temperature, oxygenation and pH, in
403 identical calm facies, ^{2/} that they differed primarily in the type of agricultural land use near their
404 banks and ^{3/} that a meta-analysis of 2069 pesticides, obtained on the same sampling sites at the
405 same period, confirmed the large prevalence of the CLD pollutant in the sampled rivers (Monti et
406 al., 2020).

407

408 **Changes in microbial assemblages induced by CLD pollution:**

409

410 We showed that rivers characterized by contrasting CLD pollution (i.e. downstream RGA and
411 GRG), had biofilms exhibiting different chemistry and microbial community composition. In
412 addition, multiple factor analysis also showed that CLD pollution contexts were relatively well
413 described by at least one axis which suggested a gradual change in epilithic biofilms in response to
414 CLD pollution.

415 Biochemical fingerprints (such as fatty acids and pigments) allowed to further characterize the
416 relationships between CLD concentration and biofilm communities. Unpolluted (UnP) rivers were
417 marked by a higher proportion of fucoxanthin, chlorophyll *c*, and diadinoxanthin. These pigments,
418 though not specific, are commonly used as indicators of diatoms in natural biofilms. Especially,
419 diatoxanthin and diadinoxanthin are representative of diatom antennary complexes and, in stream
420 periphyton, the high proportion of these pigments was related by microscopic observation to the
421 presence of diatoms (Laviale et al., 2009). In addition, the higher contribution of several fatty acids
422 such as 16:1n-7 and 20:5n-3, commonly used as indicators of diatoms (Dalsgaard et al., 2003;
423 Kharlamenko et al., 1995), to UnP sites (and their co-occurrence with the above mentioned
424 pigments within the MFA ordination) further indicates that diatoms were probably dominant within
425 these biofilms.

426 Extremely polluted (Ext) sites were however characterized by a higher proportion of chlorophyll *b*,
427 and lutein. These pigments are commonly used as indicators of Chlorophyceae and are considered

428 as unambiguous markers of green algae (Jeffrey, 1976; Lee, 2008). Their presence in Ext sites (and
429 the relative absence of fucoxanthin, and diato- diadino-xanthin) indicated that the phototrophic
430 communities were mainly dominated by Chlorophyceae. In addition, Ext biofilms were also
431 characterized by a higher proportion of 18:1n-9, a compound commonly extracted from
432 Chlorophyceae together with lutein and chlorophyll *b* (e.g. Wiltshire et al., 2000) but also
433 particularly abundant in cyanobacteria (Abed et al., 2008), bacteria (Véra et al., 2001), and protozoa
434 such as heterotrophic flagellates and amoebae (Erwin, 1973; Véra et al., 2001). Ext sites were also
435 marked by 22:1n-9 that contributed significantly to the first axis of the MFA ordination. This fatty
436 acid occurs naturally (but in small amounts) in animal tissues but has never been reported in
437 significant amount in either Chlorophyceae or cyanobacteria. In mycobacterium, it was suggested
438 that 22:1n-9 originate mainly from 18:1n-9 followed by subsequent C-2 chain elongation (Hung and
439 Walker, 1970). Thus, 18:1n-9 and 22:1n-9 might have a bacterial origin at these sites. This was
440 confirmed by the prevalence of saturated fatty acids (18:0, 19:0 and 16:0) and a higher proportion
441 of 17:0 and 17:0anteiso fatty acids. These branched fatty acids are particularly abundant in bacteria
442 and thereby commonly used as biomarkers for these groups (Kaneda, 1991).
443 Together, these results suggest that CLD pollution hindered diatom development and/or promoted
444 bacterial proliferation. This is consistent with previous micro-indentation measurements showing
445 that the CLD-polluted biofilm consisted mainly of bacteria embedded in a thin layer of low-
446 viscosity EPS, whereas the unpolluted biofilms contained large diatoms and/or a more complex or
447 mature EPS network with a high surface viscosity (Monti et al., 2020).

448 449 **Changes in microbial EPS monosaccharides according to CLD pollution:**

450
451 Microbial exopolysaccharides were further investigated by measuring EPS monosaccharide
452 compositions of epilithic biofilms. EPS have already been associated with numerous ecosystem
453 functions, including stabilization, food source and photosynthesis (Bellinger et al., 2009; Passarelli
454 et al., 2015; Zhou et al., 1998). The MFA analysis revealed that the most contaminated sites (Ext
455 sites) were characterized by a higher proportion of deoxy-sugars such as fucose and rhamnose, and
456 pentose (xylose), which are known to promote sediment biostabilization by enhancing biofilm
457 hydrophobicity (Zhou et al., 1998). The monosaccharide composition may depend, as well, on the
458 ability of different bacterial groups to degrade EPS carbohydrates (Taylor et al., 2013). For instance,
459 glucose may act as weak links, or access points, for bacterial degradation, as opposed to fucose- or
460 rhamnose-rich exopolymers. The higher proportion of deoxy-sugar over glucose in contaminated
461 sites may result from a higher bacterial glucosidase activity (Giroldo et al., 2003) and further
462 confirmed the bacterial dominance at these sites.

463

464 **CLD polluted biofilms EPS with increased adsorption and adhesion properties**

465

466 In the present study, CLD was significantly related to EPS inositols and glucuronic acid.
467 Monosaccharides have relatively similar numbers of CH and OH groups, and the hydration
468 behaviour of monosaccharide is related to the orientation (i.e. equatorial vs. axial group) of the
469 hydroxyl group (Kabayama and Patterson, 1958). Hydrogen bonds between sugar hydroxyl groups
470 and adjacent water molecules in aqueous solution give the saccharide its hydrophobic character.
471 Even if this is considerably weak, different monosaccharides will display contrasting hydrophobic,
472 and therefore stabilizing and absorptive capacities (Miyajima et al., 1988).

473 Uronic acids, such as glucuronic acid, introduce additional carboxylate groups to the biofilm, which
474 facilitate the fixation of cells via polyvalent metal ions (e.g. Ca^{2+} , Fe^{2+}) cross links (Bellinger et al.,
475 2009; Poulsen et al., 2014). These charged functional groups serve as natural adsorptive and
476 adhesive source, and provide binding sites for charged particles/molecules including several
477 pollutants (Bhaskar and Bhosle, 2006) such as CLD. Uronic acid rich polysaccharides are major
478 constituents of the adhesive biofilms of bacteria and several diatom species (Poulsen et al., 2014;
479 Sutherland, 2001)

480 The origin of the scyllo-inositol in epilithic biofilms is unclear because stereoisomers, other than the
481 widely distributed myo-inositol, are rare in biological tissues (L'Annunziata, 2007). Scyllo-inositol
482 may originate from inositol phosphates, which are quantitatively important in soils. Indeed, the
483 second most abundant inositol phosphate isomer is scyllo-inositol and has been found in large
484 quantities in soils, sometimes exceeding myo-inositol (Turner, 2007). However, despite this
485 widespread occurrence in soils, the scyllo-inositol (and other isomers than myo-) has been detected
486 rarely elsewhere in nature and its origin in soil is still unknown. It has been suggested that microbes
487 play a key role in the synthesis of this compound to protect phosphorus from uptake by nearby
488 competing organisms, although it has never been detected in any soil organism (Turner, 2007). Also,
489 it has been observed that amongst stereoisomeric inositol phosphates, scyllo-inositol is extremely
490 resistant to hydrolysis in comparison to myo-inositol (Turner, 2007). This feature (i.e. active but
491 biologically recalcitrant and hence nutritionally useless) makes it a good protection against erosion
492 by stabilizing clay-metal-humic complexes or alternatively against pollution by forming precipitates
493 with metals.

494 In our study, a causal link cannot be established between glucuronic acid or scyllo-inositol and
495 CLD. However, the enrichment of biofilms, in polluted sites, with compounds exhibiting increased
496 adsorption and adhesion capacities suggested that microbial assemblages might respond to CLD
497 pollution by developing pollutant scavenging strategies.

498 Altogether, our results pave the way for the hitherto unsuspected role of exopolymeric substances in
499 tropical epilithic biofilms and encourage further studies to focus on these extracellular molecules in
500 order to develop bioindicators of pollution. For instance, simple quantitative bioassay methods for
501 the uronic and glucuronic acid content of biofilm EPS (Mojica et al., 2007) may represent potential
502 bioindicators for monitoring and report environmental health status in tropical freshwater
503 ecosystems.

504 **Author contributions**

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508 Supervision: D.M. (PI), C.H., R.D., B.L., T.M., H.B. (WP leaders);

509 Project administration: D.M., B.L., C.H.;

510 Formal analysis: C.H., J.M.M., N.Thiney. (biochemical analyses), H.B., N. Tapie, S.A., P.D., T.R.
511 (chemical analyses of chlordecone), A.C., T.R., S.K., B.L. (T-RFLP analyses);

512 Data curation: C.H.;

513 Writing – original draft: C.H.;

514 Writing – review & editing: C.H., D.M., R.D., T.M., J.M.M., B.L.;

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527

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532

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696 **Supp. Material**

697

698 Table 1: Correlations between CLD and fatty acids

Fatty acids	Pearson coefficient	P-value
12.0	0.27	0.3014
13.0	-0.06	0.8221
14.0	-0.46	0.0640
14.0iso	-0.26	0.3086
15.0	0.28	0.2790
15.0anteiso	0.01	0.9814
15.0iso	-0.20	0.4382
15:1-n1	-0.02	0.9282
16.0	0.51	0.0382
16.0iso	-0.22	0.3984
16:1-n5	-0.51	0.0372
16:1-n7	-0.53	0.0277
16:1-n9	0.11	0.6711
16:2-n4	-0.63	0.0071
16:2-n6	-0.52	0.0307
16:3-n4	-0.58	0.0145
17.0	0.32	0.2078
17.0anteiso	0.43	0.0815
17.0iso	-0.06	0.8309
17:1-n9	-0.34	0.1752
18.0	0.49	0.0435
18:1-n5	-0.14	0.5990
18:1-n7	-0.40	0.1073
18:1-n9	0.68	0.0027
18:2-n6	-0.26	0.3102
18:3-n3	-0.17	0.5033
18:3-n6	-0.55	0.0223
18:4-n3	-0.54	0.0253

19.0	0.56	0.0188
20.0	0.44	0.0786
20.1	0.12	0.6415
20:2-n9	0.53	0.0285
20:4-n6	-0.51	0.0355
20:5-n3	-0.54	0.0268
22.0	0.29	0.2646
22:1-n9	0.71	0.0015
22:2-n6	0.61	0.0088
22:6-n3	-0.28	0.2721
24.0	0.44	0.0761
24:1-n9	0.59	0.0125
Saturated FA	0.52	0.0340

699

700

701 Table 2: Correlations between CLD and pigments

Pigments	Pearson coefficient	P-value
Alloxanthin	-0.30	0.2455
alpha.Caroten	0.16	0.5512
Asta	-0.14	0.6044
beta.Caroten	0.21	0.4223
Chl.a	0.63	0.0067
Chl.a.allomer	-0.41	0.0999
Chl.a.epimer	0.31	0.2214
Chl.b	0.43	0.0814
Chl.c2	-0.56	0.0195
Chl.c3	-0.74	0.0006
Chlide.a.	-0.28	0.2712
Chlide.b.	-0.15	0.5653
Diadino	-0.46	0.0619
Diato	-0.20	0.4449
DV.Chl.b	0.05	0.8369
Fuco	-0.54	0.0243
Lutein	0.61	0.0096
Myxo	0.23	0.3775
Neo	0.37	0.1383
Sipho	-0.11	0.6821
Phaeo	-0.65	0.0050
Viola	0.56	0.0206
Zea	-0.22	0.3928

702

703

704 Table 3: Correlations between CLD and the logarithm of monosaccharides

Monosaccharides	Pearson coefficient	P-value
Glucuronic.acid	0.56	0.0185
Fucose	0.17	0.5058
Galactose	0.16	0.5490
Mannose	0.31	0.2318
Myo.inositol	-0.74	0.0007
Rhamnose	0.12	0.6388

	Scyllo.inositol	0.58	0.0150
	Xylose	0.30	0.2371
	Glucose	-0.07	0.7834
705			
706			