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1 Importance of ecological interactions during wastewater treatment using High Rate Algal
2 Ponds under different temperate climates

3

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20 Microalgae; predators; bacteria; urban effluents; ammonia and phosphate removal

21

22

23 **Abstract**

24 Several studies focused on wastewater treatment in High Rate Algal Ponds (HRAP) suggest
25 that highly variable climatic conditions cause large variations of microalgal biomass
26 productivity. In the present study, we show that similar carbon, nitrogen and phosphorus
27 removal efficiencies were reached in different HRAPs treating urban wastewaters located in
28 two different temperate (Mediterranean and oceanic) climates. Furthermore, similar
29 ecological successions were observed in these HRAPs. During the start-up phase, the
30 consumption of organic matter by detritivores, already present in the wastewater, appears to
31 be necessary for the microalgae to grow within two weeks in spring. The growth of the rapid-
32 growing species, *Chlorella* sp, followed by the grazing-resilient species, *Scenedesmus* sp,
33 combined with nitrifying and denitrifying bacterial activity, removed most the ammonia. The
34 resulting exhaustion of ammonia would limit the complete removal of dissolved COD by
35 bacteria and phosphate by microalgae in the HRAPs. This study shows that similar biological
36 and environmental constraints were applied on the HRAPs, making the process efficiency
37 highly reproducible under different temperate latitudes.

38

39 1. Introduction

40

41 The use of intensive microalgae cultivation systems such as High Rate Algal Ponds (HRAPs)
 42 has been found to be more efficient than conventional oxidation ponds in the treatment of
 43 organic and inorganic wastewater [1, 2]. However, several studies using pilot plants have
 44 pointed out that the highly variable environmental conditions in HRAPs can cause large
 45 variations in microalgal biomass productivity under real-life operating conditions [3, 4, 5, 6].
 46 The presence of diverse grazer communities, the variable characteristics of the wastewater
 47 influent, and rapid changes in climatic conditions are all invoked to explain the changes in
 48 microalgae diversity and the resultant changes in their performance for nutrient removal [7, 8,
 49 9, 10]. However, little is known about the effects of the interactions between microorganisms
 50 already present in the wastewater and those that are growing in the HRAPs on carbon,
 51 nitrogen and phosphorus removal under real-life operating conditions. The association of
 52 microalgae and bacteria is usually proposed as a source of O₂ and a CO₂ supplementation,
 53 respectively, although they may compete for nutrients [10]. A recent study pointed out the
 54 importance of the presence, in the wastewater, of microalgae that may strongly influence both
 55 the diversity of the microalgal community growing in the HRAP and its productivity [7]. Cho
 56 et al. [9] also suggest that the high diversity within the microalgae community developing
 57 naturally in engineered system would help to sustain the whole community from different
 58 climatic conditions and predatory pressures. The objective of the present study is in perfect
 59 line with these findings. Indeed, the study was designed to assess if similar carbon, nitrogen
 60 and phosphorus removal efficiencies can be achieved between different geographical sites, the
 61 underlying reason being that indigenous bacterial and microalgal communities growing from
 62 wastewaters are the most adapted to local abiotic and biotic pressures. Under such natural

63 conditions, we hypothesize that the optimal removal efficiencies will depend more on trophic
64 interactions than on changes in climatic conditions

65

66 The present study investigated the dynamics of the bacterial, microalgal, protozoan
67 and metazoan communities of two HRAPs continuously fed with wastewater, one located in
68 the North of France under a typical oceanic climate and the other in the South of France under
69 a typical Mediterranean climate. The biological succession observed is discussed together
70 with the carbon, nitrogen and phosphorus removal performance of the HRAP and the biomass
71 production during the first five months of operation.

72

73

74 2. Materiel and Methods

75

76 2.1. HRAP operation and sample collection

77 Two similar high rate algal pond (HRAPs) systems were connected to two different urban
78 wastewater treatment plants, one in the North of France (Lat. 49° 8' 2" N, Long. 0° 10' 13" W)
79 and the other in the South of France (lat. 43° 47' 26" N, Long. 4° 20' 27" W). The HRAP
80 comprised a single-loop transparent polycarbonate raceway with an operating depth
81 determined setting the outflow weir at a height of 400 mm giving a total treatment volume of
82 1.9 m³. The HRAPs were initially filled with pre-treated wastewater (after screening and
83 removing grit, sand and grease) without adding any microalgal inoculum. A pump, with a
84 flow rate of 1 to 5 m³.h⁻¹ and a 2.2 mm filter, recirculated the water through the HRAP. The
85 HRAPs were run in continuous mode. The HRAPs were continuously topped up with
86 wastewater at 315 L per day (equivalent to 2.1 persons equivalent in France) and the same
87 volume of HRAP water was simultaneously discharged through the outflow weir, in order to

88 keep the HRAP volume constant. A hydraulic retention time of 6 days was thus achieved. A
89 buffer tank was installed before the HRAP at the northern site one month after the beginning
90 of experiment. This tank was used to smooth potential abrupt fluctuations of the suspended
91 solids in the wastewater at the northern site but it was not necessary for the southern site.

92 The HRAP at the northern site was in operation from the 28th April 2015 to the 15th
93 September 2015. The HRAP at the southern site was in operation from the 20th January 2016
94 to the 29th September 2016 (first experimental run) and from the 12th April 2017 to the 19th
95 May 2017 (second experimental run) after completely draining and cleaning the HRAP.

96 Environmental variables were measured using a radiometer and an air temperature
97 sensor (Electrona, Parlite radiometer) for the northern site and a weather station (Vantage
98 Pro2 Plus, Davis Instruments) for the southern site, located near the HRAPs recording
99 rainfall, incident radiation and air temperature every 30 min. The water temperature in the
100 HRAPs was measured every 30 min using the temperature sensor in a pH immersion probe
101 (pHD, Hach Lange). During the second experimental run in the southern site, the light
102 attenuation was measured daily within the HRAP. Samples for chemical analyses (total and
103 dissolved chemical oxygen demand, total and dissolved nitrogen, ammonia, nitrite, nitrate,
104 total and dissolved phosphorus, phosphate, pH, total alkalinity, total suspended solids) were
105 taken from the HRAP and from the wastewater daily, for the whole experimental run at the
106 northern site and daily for the first 3 months and then weekly for both experimental runs at
107 the southern site. The carbon, nitrogen, phosphorus and chlorophyll *a* contents in the biomass
108 in the HRAPs were measured daily during the period when the microalgal biomass was
109 growing and then every week. Samples for bacterial diversity analysis were taken weekly
110 from the HRAPs and samples for microalgal, protozoan and metazoan analysis were taken
111 monthly from the HRAPs (first run only for the southern site).

112

113 2.2. Physical and chemical properties

114 *Water chemistry*

115 Hach Lang cuvette test kits were used to assay total and dissolved chemical oxygen demand
116 (COD, disCOD, kit LCK 314), total and dissolved nitrogen (N_{tot}, disN, kit LCK 338), nitrite
117 (NO₂⁻, kit LCK 342), nitrate (NO₃⁻, kit LCK 339) and total and dissolved phosphorus (P_{tot},
118 disP, kit LCK 350) with a Hach Titralab analyser and a Biogas Titration Manager, according
119 to the manufacturer's instructions. If required, the samples were diluted to be within the
120 measurement range. Samples of dissolved compounds were prepared by first filtering through
121 17-30 µm folded filters, then filtering through 0.7 µm GF-F filters using a Büchner funnel.

122 Ammonia (NH₄⁺) concentration was determined using Nessler's reagent and phosphate
123 (PO₄³⁻) concentration was determined using the ISO 6878 method. Total alkalinity (TA) was
124 determined using the ISO 9963-1 method. Total suspended solids (TSS) were determined
125 using the EN 872:2005 method. pH was measured using a double-junction Ag/AgCl pH
126 electrode (Sentix 41, WTW, for the northern site and PHC 201, Hach, for the southern site).

127

128 *Carbon, nitrogen, phosphorus content in the biomass*

129 10-mL samples were filtered onto precombusted Whatman GF/F filters and stored at -80°C
130 until analysis. The filters for particulate organic carbon (POC) and nitrogen (PON) analysis
131 were dried at 60°C for 24 h, pelleted and analyzed using an ANCA mass spectrometer
132 (Europa Scientific). The filters for particulate organic phosphorus (POP) were processed by
133 persulphate wet-oxidation in weak alkaline conditions to convert the organic phosphorus to
134 phosphate [11]. The phosphate concentration was then determined using colorimetry [12] and
135 modified by Koroleff [13].

136

137 *Chlorophyll a in the biomass*

138 10 ml samples for chlorophyll *a* determination were filtrated onto 13 mm GF/F glassfiber
139 filters. Filters were stored frozen at -20°C until extraction with 5 ml of absolute ethanol,
140 consisting in a step of ultrasonication in ice bath followed by overnight extraction at 4°C,
141 filtration and absorbance measurement of the extract at 665 and 750 nm on a Hitachi U3000
142 zeroed with absolute ethanol. Chlorophyll *a* concentration was calculated using Ritchie [14]
143 equation:

$$144 [\text{chl } a] (\mu\text{g.mL}^{-1}) = 11,904 * (\text{Abs665} - \text{Abs750}) * v/V$$

145 With *v* the volume of the extract (ml), *V* the filtrated volume (mL), Abs665 the absorbance
146 read at 665 nm and Abs750 the absorbance read at 750 nm.

147

148 *Light attenuation*

149 Discrete measurements of underwater light intensity were performed around zenithal time, in
150 order to minimize bias (planar shape of the sensor, potential shading from surroundings
151 structures and proportion of underwater light scattering), at 5 depths (sub-surface, 10, 20, 30
152 and 40 cm) from 30/03/2017 to 21/05/2017 using 5 waterproof light and temperature
153 dataloggers (UA-002-64 HOBO, Onset). Despite their planar and wide spectrum sensor, these
154 simple light loggers can reliably be a good alternative to cosine or scalar sensors as tested by
155 Long et al [15] for relative intensity changes. Vertical attenuation coefficient for light was
156 calculated from the irradiance exponential decrease with depth using Kirk [16] equation :

$$157 E_d(z) = E_d(0) \exp(-K_d * z)$$

158 With $E_d(z)$ the light intensity at depth *z* in lux, $E_d(0)$ the light intensity just below the surface
159 in lux, *z* the depth in m and K_d the vertical attenuation coefficient for light in m^{-1} .

160

161 2.3. Biological properties

162 *DNA extraction and bacterial 16S rDNA sequencing*

163 Bacterial 16S rDNA was extracted from a 10-mL sample filtered onto a 0.2 μ m membrane
164 (PALL Supor® 200 PES), using the DNeasy PowerWater Kit (Qiagen) according to the
165 manufacturer's instructions. The V4-V5 region of the 16S rRNA gene was amplified over 30
166 amplification cycles at an annealing temperature of 65°C, with the forward primer (5'
167 CTTTCCCTACACGACGCTCTTCCGATCTGTGYCAGCMGCCGCGGTA-3') and the
168 reverse primer
169 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTCCCCGYCAATTCMTTTRAGT-3') with
170 their respective linkers. The resulting products were purified and loaded onto the Illumina
171 MiSeq cartridge for sequencing of paired 300 bp reads following the manufacturer's
172 instructions (v3 chemistry). The Genotoul Lifescience Network Genome and Transcriptome
173 Core Facility in Toulouse, France (get.genotoul.fr) carried out the library preparation and
174 sequencing. A modified version of the standard operation procedure for MiSeq data [17] in
175 Mothur version 1.35.0 [18] was used for alignment and as a taxonomic outline.
176 Representative sequences of bacterial operational taxonomic units (OTUs) were identified at
177 the 3% level using Mothur.

178

179 *Quantification of total bacteria using quantitative real-time PCR*

180 We used qPCR to quantify total bacteria with forward primer 5'-
181 ACTCCTACGGGAGGCAG-3', reverse primer 5'-GACTACCAGGGTATCTAATCC-3''
182 and TaqMan probe 5'Yakima Yellow-TGCCAGCAGCCGCGGTAATAC-TAMRA-3' to
183 quantify total bacteria [19].

184 qPCR assays were carried out in triplicate using 96-well real-time PCR plates (Eppendorf)
185 and a Mastercycler® ep Realplex (Eppendorf). The reaction mixture consisted of 12.5 μ L of
186 EXPRESS SYBR GreenER qPCR mix with premixed ROX (Invitrogen), 5 μ L of DNA, 1 μ L of
187 primers and 1 μ L of probe and water to a final volume of 25 μ L. An initial incubation of 20s at

188 95°C was followed by 40 cycles of denaturation (15s at 95°C) and hybridization/elongation
 189 (60s at 60°C). A standard curve was generated for each assay by using 10-fold dilutions of
 190 pEX-A plasmids (Eurofins MWG Operon) containing the targeted gene sequences. Two
 191 different dilutions of each DNA sample were amplified and the calculated initial
 192 concentrations were compared to detect PCR inhibition. Inhibited PCR reactions produced
 193 lower values which were eliminated from the analysis. The quantification limit, defined using
 194 the lowest concentration of the linear range of the standard curve, was 10 copies per qPCR
 195 reaction for all qPCR systems. The amplification efficiency of the standard curves was
 196 between 98% and 100%, with a regression coefficient value (R^2) systematically above 0.99.
 197 Four copies of the 16S rRNA gene were considered to be equivalent to a single cell [20]. A
 198 carbon conversion factor of 30 fgC.cell⁻¹ was used to convert bacterial cell abundance into
 199 bacterial C biomass [21].

200

201 *Microalgal diversity, abundance, biovolume and carbon biomass*

202 To quantify the phytoplankton communities, 125-mL water samples were fixed with formalin
 203 (2% final concentration). The phytoplankton was enumerated in 1-mL subsamples in a
 204 sedimentation chamber [22, 23] using an IX70 inverted microscope (Olympus Life Science,
 205 Hamburg, Germany) equipped with a MoticamPro digital camera (Moticam, Hong Kong,
 206 China). Units (cells or colonies) were counted and sized in random fields under 40×
 207 magnification until at least 400 units of the dominant species had been enumerated [24]. The
 208 biovolume was calculated for each species according to Hillebrand et al. [25] and Sun and Liu
 209 [26] and converted into carbon biomass (C Biomass) using a conversion factor of 220 fg C
 210 μm⁻³ [27].

211

212 *Protozoan and metazoan diversity, abundance, biovolume and carbon biomass*

213 For protozoan and metazoan abundance and identification, 2-L water samples were filtered
214 through a 100 μm filter and then through a 35 μm filter to separate 35 to 100 μm and >100
215 μm size fractions, which were then stored in neutralized formalin (4% final concentration).
216 The protozoan and metazoan taxa in the 35 to 100 μm fraction were identified, sized and
217 enumerated using an Olympus AX70 dissecting microscope with a hemocytometer. Protozoan
218 and metazoan taxa in the >100 μm fraction were identified and enumerated using a Olympus
219 Stereomicroscope SZX7 with a Bogorov counting chamber. Conversion factors for carbon
220 biomass (C biomass) were taken from the literature for ciliates, rotifers and nematodes [28,
221 29, 30].

222

223 2.4. Statistical tests

224 A t test was used to test differences between geographical sites for each variable of the dataset
225 consisting of the environmental factors (daily incident light dose, water temperature, rainfall),
226 the microbial biomass (concentration of chlorophyll *a* as a proxy of algal biomass and
227 bacterial biomass), and the removal efficiencies (COD, NH_4^+ , PO_4^{3-}) measured weekly
228 ($n=35$), from the period when microalgae biomass (chlorophyll *a*) was reached the maximal
229 value (4mg L^{-1}). A correlation-based principal component analysis (PCA) was applied for this
230 weekly samples dataset in order to detect significant correlations between the environmental
231 and microbial variables and the removal efficiencies measured in the two geographical sites
232 after the period of the rapid microalgae growth. All the statistical tests were performed with
233 PAST version 3.22. [31].

234

235

236

237 3. Results

238

239 3.1. Climatic conditions

240 The maximum incident light and daily dose received at both sites ranged from 500 to 2000
241 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and from 2 to 57 $\text{mE}\cdot\text{m}^{-2}\cdot\text{d}^{-1}$, respectively, with the lowest values in winter (S1-
242 Figure). The water temperature ranged between 10-15°C and 30°C in the HRAPs with the
243 lowest values in winter and highest values in summer (S1-Figure). In general, the water
244 temperature was about 5°C higher than the air temperature (data not shown). The highest rain
245 values were occasionally measured in August in the Northern site and in October (57 mm)
246 and in October (52 mm) in the southern site. The proportion of days without rainfall during
247 the studied periods was 33% and 76% for northern and southern site, respectively (S1-
248 Figure).

249

250 3.2. Chemical and biological properties

251 Over the study period, the characteristics of the wastewater were similar for the two sites, but
252 there were some significant differences (Table 1). The average ratio of ammonium to
253 phosphate (mol:mol) was significantly higher in the northern wastewater (44:1) than in the
254 southern wastewater (34:1). However, the phosphorus contents (dissolved and total) were
255 much higher at the northern site indicating a higher contribution of organic phosphorus in the
256 northern wastewater than in the southern wastewater (Table 1) where the wastewater is
257 partially diluted with rainwater drainage.