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1 **Individual metabolism and behaviour as complementary endpoints to**
2 **better understand mangrove crab community variations linked to**
3 **wastewater inputs**

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15

16 **Abstract**

17 Mangrove forests are impacted by a large range of anthropogenic activities that challenge
18 their functioning. For example, domestic wastewater (WW) discharges are known to increase
19 vegetation growth but recent studies indicate that they have negative effects on benthic
20 macrofauna, especially on mangrove crabs, these ecosystem engineers playing a key role on
21 the functioning of the mangrove. In experimental areas regularly receiving WW at low tide
22 (Mayotte Island, Indian Ocean), a drastic decrease in burrowing crab density has been
23 reported. In this context, the individual behavioural and physiological responses of the fiddler
24 crab *Paraleptuca chlorophthalmus* exposed to short-term (6h) pulse of WW and ammonia-N
25 (as a potential proxy of WW) were investigated. This species is one of the most sensitive to
26 WW within the mangrove crab community. For the behavioural experiment, crabs could
27 choose between the aquatic and aerial environment. Individual metabolic rate (O₂
28 consumption) was monitored after 6h of exposure in WW or ammonia-N. Aerobic and

29 anaerobic metabolic markers (citrate synthase and lactate dehydrogenase activities,
30 respectively) were also evaluated. Results indicate that crabs exposed to WW are more active
31 and mobile than controls after 3 hours. Crabs actively emerged from WW and reduced their
32 activity and mobility after 6 hours. A higher metabolic rate in WW occurred immediately (t =
33 0h), 3 and 6 hours after WW exposure, with also, a burst in aerobic bacterial consumption in
34 WW, but no effect of ammonia-N. No effect of WW or ammonia-N was observed on
35 enzymatic aerobic and anaerobic metabolic markers. Therefore, short-term pulses with
36 domestic polluted wastewater trigger quick behavioural and metabolic responses that could be
37 deleterious if prolonged. These results could contribute to the understanding of the
38 community-scale changes observed in benthic macrofauna after several years of regular
39 domestic pollution pulses.

40

41 **Keywords**

42 Crustacea

43 Pulse exposure

44 Behavioural endpoints

45 Metabolism

46 Wastewater

47

48 **1. Introduction**

49 Mangroves are forests that grow at the interface between terrestrial and marine ecosystems in
50 tropical and subtropical areas (Xiang et al., 2020) and are regularly exposed to anthropogenic
51 pressure. This is due to their proximity with urban areas, aquatic farms, ports, that caused
52 contrasted effects on vegetation, benthos and macrofauna (negative, neutral, even positive)
53 (Capdeville et al., 2018). Because of their capacity to absorb nutrients and their filtering
54 properties (amongst others), they have been widely mentioned as bioremediation sites for
55 domestic wastewater (WW) treatment (Capdeville et al., 2018; Kristensen, 2008; Tam and
56 Wong, 1995). Potential effects of sewage discharge on vegetation, benthos and macrofauna
57 have been studied in parallel (Herteman et al., 2011; Wong et al., 1997).

58 In this context of bioremediation, a pilot site has been set up in Mayotte Island (Indian Ocean)
59 in 2008, with regular pulses of WW discharge (5-6h duration : two times / day) occurring on
60 chosen natural mangrove areas where both vegetation and macrofauna were monitored
61 (Capdeville et al., 2018; Herteman et al., 2011). Among results of this project, effects on the
62 assemblages of macrofauna communities have been observed in WW impacted areas with
63 diminution or even disappearance of some species of burrowing mangrove crabs
64 (Ocypodidae), against an increase of some Sesarmidae species (Capdeville et al., 2019, 2018).
65 Globally, a reduction in the number of burrows has been observed (Theuerkauff et al., 2020).
66 This could be due to a decrease in burrowing crabs abundance, but also to a modification of
67 their bioturbation activity (foraging and burrowing) that could modify the mangrove
68 ecosystem functioning (Cannicci et al., 2008). The study of burrowing crabs such as fiddler
69 crabs that can have ecological engineering impacts on mangrove functioning is promising to
70 improve our understanding of mangroves and the monitoring of their health state (Xiang et
71 al., 2020).

72 Worldwide, effects of domestic effluent discharges (controlled or not) on the assemblages of
73 crab communities have been observed in several different mangroves with very contrasted
74 results regarding diversity and density of crab communities (see Capdeville et al. (2018), for a
75 review). In the pilot experimental site of Mayotte, the prediction that small species like
76 Ocypodidae should dominate the mangrove areas enriched by organic matter (Pearson and
77 Rosenberg, 1978) was not confirmed by Capdeville et al (2018).

78 To complete these field observations, recent studies were conducted in controlled conditions
79 in order to investigate the effects of WW on mangrove crab physiology. Burrowing mangrove
80 crabs *N. africanum* were exposed to short (5h) but ecologically relevant WW pulses (similar
81 to the discharge generated on the field experimental site of Mayotte). A burst in O₂
82 consumption, histological changes (osmoregulatory gills and hepatopancreas), and disturbed
83 antioxidant defences were observed (Theuerkauff et al., 2018, Mégevand et al., 2021). These
84 effects partially explain the modifications of crab communities observed *in situ* as they unveil,
85 at different integrated levels, several energy compromises and adaptations to maintain the
86 crabs global metabolism. They also reveal the physiological flexibility of this species and a
87 lack of knowledge about the short-term survival strategies they may develop, including
88 behavioural responses.

89 In this context, could fine-scale behavioural and metabolic responses of burrowing mangrove
90 crabs exposed to WW could contribute to the understanding of large-scale changes in
91 communities observed in the field?

92 Careau et al. (2008) formulated the existing link between physiology and behaviour, these two
93 disciplines having long been perceived as complementary in the field of ecology and
94 evolution. Behaviour is readily seen as a powerful way to cope with environmental challenges
95 by physiologists (see, for example, Aimon et al., 2021; Urbina et al., 2011). Also, behavioural

96 ecologists recognize the importance of energetics in the context of behavioural decisions and
97 the evolution of life-history strategies.

98 In this study, we focus on a fiddler crab *Paraleptuca chlorophthalmus* distributed among
99 Indo-pacific mangroves, that usually inhabits muddy banks and flats of mangrove estuaries,
100 near high-tide levels of mangrove forests (Crane, 1975). This species is one of the declining
101 decapod species in the WW impacted parcels of the pilot site of Malamani (Capdeville et
102 al., 2018). Behavioural observations coupled with physiological analyses can provide a more
103 complete understanding of the impact of an external stimulus on an organism, a population or
104 a species (Filiciotto et al., 2018). For fiddler crabs and other crustaceans, altered behaviour
105 can be considered as an early warning biomarker of chemical exposure (Ungherese and
106 Ugolini, 2009). In fiddler crabs, different escaping behaviours have been studied *in situ*, often
107 resulting in a rapid home run towards the burrows in case of predation (Hemmi, 2005) or,
108 inversely, by emerging from their burrows, with for example crabs exposed to thermal stress
109 (Halal et al., 2020). Emersion behaviour has been proved as a useful escaping strategy, but
110 this has to be in a limited time period as crabs become more exposed to predation or
111 dehydration (Luppi et al., 2013). Moreover, the elimination of CO₂ and ammonia is
112 deprecated in the aerial environment (Luquet et al., 1998), which brings them regularly into
113 the water.

114 As intertidal crab biological timing follows circadian and circatidal rhythms (Naylor, 1997;
115 Thurman, 2004), behavioural and physiological responses are expected to vary during the
116 time of the day in environmental conditions, as it has been proved in ghost shrimps
117 *Neotrypaea uncinata* (Leiva et al., 2016). Pollutants may interfere with such a regulation of
118 crab's activity according to the patterns of tides and day (Azpeitia et al., 2013; Stillman and
119 Barnwell, 2004).

120 By investigating several behavioural endpoints and assessing metabolic state (O₂ consumption
121 and metabolic enzymes activities) of the fiddler crab *P. chlorophthalmus* exposed to WW and
122 ammonia-N as a proxy of WW in microcosm, we sought to better understand ecological
123 observations in the field through laboratory experiments.

124 We thus hypothesized that (1) crabs exposed to pollutants would show a higher activity state
125 (escape response) coupled with an emersion behaviour corresponding to short-term response
126 to contaminated water; (2) Both WW and Ammonia-N exposure would impact crab
127 physiology leading to energy compromises in order to maintain their global metabolism.

128 O₂ consumption was measured as resting metabolic rate, with crabs placed in metabolic
129 chambers that limited their movements. This measure was considered as physiologically
130 relevant (Borges et al., 2018) to assess the potential effects of contaminated water on crabs,
131 independently from a potential locomotor or escape behaviour. Then, to reveal underlying
132 metabolic trade-offs resulting from WW and ammonia-N exposure, we measured citrate
133 synthase (CS) activity as a marker of aerobic metabolism through mitochondrial efficiency,
134 and lactate dehydrogenase (LDH) activity that would maybe show a shift to anaerobic
135 metabolism in order to cope with the energy needs of the animals.

136

137 **2. Materials & Methods**

138 **2.1 Animal collection and acclimatisation**

139 Male *P. chlorophthalmus* were collected in February and March 2020 in the mangrove of
140 Malamani (Boueni Bay, Mayotte -12,923155, 45.154053) from a population extending along
141 both sides of the stream of Malamani, in the *Avicennia marina* and *Cerriops tagal* vegetation
142 belt (above the *Rhizophora mucronata* belt). This open habitat presents no canopy cover, a
143 large temperature gradient and regular water immersion/emersion, depending on the season,
144 the tides and time of the day.

145 All animals were hand collected at low tide when crabs are active and out of their burrows.
146 They were placed into individual boxes for transportation in order to minimize stress and
147 fights. These males were then directly transported to the University Centre of Mayotte
148 (CUFR, Dembeni, France) and placed for three days for acclimation in tanks containing
149 natural seawater ($\sim 33\%$ salinity; $1050 \text{ mOsmol kg}^{-1}$), under a natural photoperiod (12h light:
150 12h dark) and without feeding prior to experimentation. These crabs (140) were acclimated to
151 an aerial-aquatic environment (crabs could move freely between these two environments).
152 The acclimation microcosms were the same as those used for the behavioural experiment.
153 For behavioural analyses, 60 crabs were studied. For the respirometry experiment, 40 crabs
154 were studied. For the biochemical analysis on metabolic markers (citrate synthase, CS and
155 lactate dehydrogenase, LDH enzyme activity), 40 crabs were studied. No mortality was
156 observed during acclimation and experimental processes.

157

158 **2.2 Wastewater sampling and conservation**

159 Samples of raw wastewater effluent (WW) were directly collected from the Dembeni
160 wastewater treatment plant. In order to minimize any possible variation in WW composition
161 during the experiments, 100 litres were collected on a single day. WW was then distributed in
162 1 litre containers and stored at -20°C for the duration of the experiments (up to 40 days).
163 in order to stop bacterial proliferation and to ensure a better conservation of nitrogen
164 compounds. This WW treatment plant was chosen because basic water parameters were
165 routinely monitored due to the European water framework directive (WFD). In addition,
166 frozen samples of collected WW were sent to CHROME laboratory (University of Nîmes,
167 France) to assess NO_3^- , NO_2^- , NH_4^+ , PO_4^3 concentrations and additional parameters on
168 Metrohm ion chromatography system (Metrohm, Switzerland) with conductivity detection
169 (see Table 1).

170
171
172
173
174
175

Chemical species	Concentration ($\mu\text{mol/Kg}$)
Na ⁺	3207
NH ₄ ⁺	2313
K ⁺	364
Mg ²⁺	488
Ca ²⁺	286
F ⁻	5.8
Acetate	190
Formate	<l.d.
Cl ⁻	2238.2
NO ₂ ⁻	<l.d.
Br ⁻	3.1
NO ₃ ⁻	0.6
Benzoate	<l.d.
PO ₄ ³⁻	68.5
SO ₄ ²⁻	253.9
Oxalate	<l.d.

176

177 **Table 1.** Physical and chemical characterization ($\mu\text{mol/Kg}$) of undiluted WW (pH = 8.3) collected in
178 Dembeni treatment plant system during the rainy season (March 2020). Samples were stored at -20°C
179 prior analyses in CHROME Laboratory (Nîmes, France).

180

181 **2.3 Ammonia-N exposure and experimental conditions**

182 Four different experimental conditions were performed: some crabs were maintained in
183 seawater ($\sim 33\%$ salinity), others were transferred to diluted seawater ($\sim 5\%$ salinity) or to 10
184 mg.l^{-1} ammonia-N solution. This solution was prepared by adding 1 mmol of ammonium

185 chloride (Sigma, USA) to diluted seawater (~5‰ salinity). WW was also adjusted to ~5‰
186 salinity by adding Instant Ocean® Sea Salt.

187 Ammonia-N (called N in our experimental design) was chosen as a second experimental
188 treatment because the ionized form of ammonia-N (NH_4^+) is the major component of WW
189 (Table 1). This condition referred to total ammonia (ammonia-N), which represents the sum
190 of unionized ammonia NH_3 and ionized ammonia NH_4^+ (Lemarié et al., 2004). Finally,
191 focusing on single ammonia-N effects in a separate experimental condition allow us to avoid
192 pollutant cocktail and potential hypoxic effects (aerobic bacteria that require oxygen to
193 degrade the substrate) (Gerardi, 2006) caused by WW on crabs physiology.

194 The ammonia-N concentration was chosen on the basis of concentrations that may be released
195 by treatment plants in Mayotte (Herteman, 2010). It corresponds to a sub-lethal concentration
196 for a fiddler crab species such as *U. princeps* with adults sharing similar size and morphology
197 with *P. chlorophthalmus* (Azpeitia et al., 2013).

198 In order to take into account crabs biological timing and potential handling stress, three time
199 steps were considered for the behavioural and respirometry measurements: 0h (start of
200 exposure), 3h, 6h. In order to assess the activity of CS and LDH, crabs were exposed during
201 6h to the same experimental conditions before euthanasia and sampling. All the experiments
202 started at 10 am, in order to minimize the potential response variations due to circadian
203 rhythms of the animals.

204

205 **2.4 Behavioural endpoints: locomotor activity, agitation and emersion choice**

206 Behavioural endpoints of *P. chlorophthalmus* were assessed using four independent
207 28x22x16cm microcosms for each exposure condition, placed on the floor on a polystyrene
208 plate to minimize any potential vibration. Microcosms were divided into two communicating
209 environments: aquatic and aerial. Crabs could move freely between those environments with

210 this binary-choice setup. Aerial part was made of a surelevated plastic grid platform
211 accessible through a gentle slope avoiding crabs from slipping. Prior to each experimentation,
212 exposure solutions were quickly poured in the microcosms and oxygenated during twenty
213 minutes, in order to start the experiment with, at least, 90% air saturated water.

214 Then, crabs were individually placed in the microcosms (N = 15 per condition), in the aquatic
215 environment. The 0h trials started 5 minutes after the crabs were placed in the microcosms, in
216 order to minimize the potential stress due to handling. Videos were recorded at 0h, 3h and 6h
217 hours during 15 minutes with a Sony Handycam® CX900E attached ~210 cm above
218 microcosms. Individual crab movements were analysed using a multiple-arena module by
219 EthoVision® XT 15.0 software (Noldus Information Technology, Wageningen, Netherlands),
220 with a defined sampling threshold of 5 frames per second.

221 Four parameters were evaluated: ‘travelled distance’ (cm), ‘mobility’ (a crab may be
222 considered as “mobile” even if it does not travel a distance), ‘agitation’ (based on the
223 “activity” parameter of EthoVision® with a threshold defined at 30), and, the ‘percentage of
224 time spent in the aerial environment’ of the microcosms. The agitation parameter focuses on
225 the detection of the movements of the crabs’ legs only. A non-moving, or non-mobile crab, is
226 not necessarily a quiet or undisturbed crab. Crab leg movement has been used as a stress
227 indicator among crustaceans (Urban, 2015).

228 All parameters except ‘travelled distance’ are expressed as % of time. At the end of each
229 experiment, animals were weighed and put back in the aerial/aquatic aquariums before being
230 released into the mangrove.

231

232 **2.5 Oxygen consumption rate**

233

234 The experimental design consisted in a static, intermittent flow-through respirometry system
235 based on Clark et al (2013). Crabs were individually placed into 125 ml chambers allowing
236 them to make sporadic movements considering their size, and at the same time ensure
237 accurate measurements of O₂ consumption. Following procedures described in Killen (2014),
238 O₂ measurements were performed using a 4-channel fiber-optic system with contactless O₂
239 sensor spots (FireSting O₂, PyroScience, GmbH, Aachen, Germany) where water oxygen
240 content was quantified once every 5 seconds.

241 Water-mixing within the chambers was achieved with magnetic stirrers located under 1 mm²
242 mesh grid to avoid too much disturbance (Rivera-Ingraham et al., 2016). Sensors were
243 calibrated to 100% (using air-saturated water) and 0% air saturation. Chambers were filled
244 with a tubing system providing control SW or contaminated water from aerated, filtered and
245 temperature-controlled tanks.

246 Crabs were gently introduced in the chambers and left to acclimate for one hour in aerated
247 seawater prior experiment.

248 Following acclimation, the system was filled with the exposure solution. An automated flush
249 pump was switched on for 20 minutes to ensure proper mixing and was cut off during 40
250 minutes. During that time, the chambers were sealed. The decrease in oxygen content could
251 be analysed to indicate the rate of oxygen uptake. After the 40-minute cycle, the pump was
252 turned on to flush the metabolic chambers with aerated seawater (or aerated WW or diluted
253 seawater enriched with ammonia-N) during 20 minutes. Due to the presence of aerobic and
254 anaerobic microorganisms capable of degrading organic compounds and consuming O₂
255 (Shchegolkova et al., 2016), a 20 minutes flushing was run to ensure 99% air saturation. The
256 40 minutes measurements allowed accurate O₂ measurements without falling under the
257 threshold of 70% air saturation in order to maintain aerobic metabolism and to avoid hypoxic
258 stress (Rodgers et al., 2016).

259 Similarly to the behavioural experiment, O₂ measurements were recorded at 0h, 3h and 6h for
260 all individuals (N = 10 per condition). The same procedure was applied for blanks chambers
261 for each condition (N = 8 per condition) and then subtracted from the MO₂ per individual
262 At the end of each experiment, animals were weighed and put back in aerial/aquatic
263 aquariums before being released into the mangrove.

264

265 **2.6 Sampling and dissections**

266 In order to perform the CS and LDH activity assays, crabs were anesthetized and euthanized
267 on ice for tissue sampling of the muscle of the big chelipeds, anterior and posterior gills.
268 Samples were transferred in 1.5 ml tubes and flash frozen in liquid nitrogen then conserved
269 separately at -80°C for enzymatic assays.

270

271 **2.7 Metabolic markers**

272 Citrate synthase (CS) and lactate dehydrogenase activity (LDH) activities were determined to
273 assess the potential physiological effect of WW and Ammonia-N exposures. Indeed, they may
274 be useful to determine what sort of locomotor behaviour the animals are most likely to rely on
275 due to the expression of aerobic versus anaerobic enzymes found on the tissue (Ombres et al.,
276 2011).

277 A different batch of animals was chosen for the investigation on metabolic markers. Crabs
278 were maintained in the same microcosms used as for the behavioural experiments, but without
279 the aerial platform. Crabs could move freely but stayed totally immersed (no “aerial bias”
280 resulting from the potential time spent in air between individuals).

281 Citrate synthase activity was used as a marker of mitochondrial content. CS was measured
282 using a kit from Sigma Aldrich (#MAK193) following the manufacturer's instructions.
283 Briefly, 2-8 mg of anterior gill, posterior gill, and muscle of the big cheliped were

284 homogenized in 100 μ L of icecold CS Assay Buffer. Then, samples were centrifuged at
285 10,000 g for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. Fifty
286 microlitres of sample were added to wells of 96-well plates in duplicate with appropriate
287 reaction mixes (CS assay buffer, developer and substrate mix). A standard curve was obtained
288 with serial dilutions of GSH solution (0-40 nmol/well). The plate was incubated for 3 minutes
289 at 25°C and the absorbance was recorded at 412 nm every 5 minutes for 40 minutes. The
290 colorimetric product (GSH) was proportional to the enzymatic activity of CS and normalized
291 to the quantity of tissue.

292 Lactate dehydrogenase activity was measured using a kit from Sigma Aldrich (Oakville, ON,
293 Canada, #MAK066) following the manufacturer's instruction. Briefly, 2-8mg of anterior gill,
294 posterior gill, and muscle of the big cheliped were homogenized in 200 μ L of ice-cold LDH
295 Assay Buffer. Then, the samples were centrifuged at 10,000 g for 15 minutes at 4°C and the
296 supernatant was transferred to a fresh tube. Fifty microlitres of sample were added to wells of
297 96-well plate in duplicate with appropriate reaction mixes (LDH assay buffer, developer and
298 LDH substrate Mix). A standard-curve was obtained with serial dilutions of NADH solution
299 (0 to 12.5 nmol/well). The plate was incubated at 37°C and the absorbance was recorded at
300 450 nm every 5 minutes for 30 minutes. The colorimetric product (NADH) was proportional
301 to the enzymatic activity of LDH and normalized to the quantity of tissue.

302

303 **3. Statistical analyses**

304 Statistical analyses were performed in R version 3.5.2 with Rstudio version 0.99.491
305 (Rstudio, Inc), with statistical significance being assigned at $\alpha = 0.05$.

306 To test for potential effects of treatment and time on behavioural endpoints, we used
307 generalized mixed-effects modelling (Zuur et al., 2013) using the package glmmTMB

308 (Brooks et al., 2017). The time allocated to each behaviour was first translated into
309 percentage, except for the distance moved, expressed in centimetres.

310 Three behavioural endpoints (distance moved, mobility, agitation) were modelled in function
311 of treatment (SW, DSW, N, WW) (four-level fixed factor) and exposure time (0h, 3h, 6h)
312 (three-level fixed factor).

313 Individual differences and autocorrelation due to repeated measurements were controlled via a
314 random intercept of crab ID. Since data followed a compound Poisson-gamma distribution
315 while recording a lot of exact zeroes not being missing values, a Tweedie distribution error
316 was applied to the models (Candy, 2004).

317 To explore the magnitude of the effects of each fixed factor (treatment and time) and their
318 interaction, a type II ANOVA (Wald chi-square test) was applied using Anova function in
319 CAR package (Fox and Weisberg, 2019). When significant interactions between treatment
320 and time were detected –which occurred for the three behavioural endpoints- they were
321 investigated through multiple pairwise comparisons using lsmeans package (Lenth, 2016).
322 Bonferroni correction was applied to all p-values resulting from this analysis.

323 For one of the behavioural endpoints (time spent in the aerial environment), GLMM was not
324 found suitable. Therefore, we focused on the variability of individual responses between
325 treatment groups. We investigated, for each time step, the coefficient of variation (CV) of
326 each group and used Krishnamoorthy and Lee's (2014) modified signed-likelihood ratio test
327 from the R package cvequality (Marwick and Krishnamoorthy, 2019) to compare the
328 variability between individuals.

329 For O₂ oxygen consumption, a generalized linear mixed model approach was taken assuming
330 Gaussian distribution error with log link. As with above, time (0h, 3h, 6h) and treatment
331 group (SW, DSW, N, WW) were considered as fixed factors.

332 Crab ID was set as random factor nested within experimental tank (two tanks of exposure
333 solution per treatment, N = 4 per tank), which are nested within treatment group (SW, DSW,
334 N or WW). Interaction terms were examined using the approach outlined above, using type II
335 ANOVA and multiple pairwise comparisons followed by Bonferroni correction. The same
336 statistical analysis was used for aerobic O₂ consumption, with metabolic chamber ID set as
337 random factor.

338 For metabolic markers (CS and LDH), residuals were evaluated using Shapiro-Wilk and
339 Levene's test respectively in order to test the data normal distribution behaviour and variance
340 homogeneity. When parametric assumptions were not met, data were log-transformed. One-
341 way ANOVAs were performed separately for anterior gills, posterior gills and muscles to
342 assess potential differences of enzymatic activities of CS and LDH between treatments (SW,
343 DSW, N, WW) after 6h exposure. All values are represented as average \pm SEM.

344

345 **4. Results**

346 **4.1 Behavioural endpoints**

347 Results of the behavioural analyses are depicted in Fig.1 and Fig.2, which include temporal
348 tracking responses of 0h, 3h, 6h for the distance moved (Fig.1A), mobility (Fig.1B), agitation
349 (Fig.1C) and emersion time parameters (Fig.2).

350 For the distance moved (cm), differences across treatments and exposure times accounted for
351 significant ($p < 0.05$) effects of treatment, exposure time, and their interaction (Table 2,
352 Fig.1A).

353 Exposure to WW and Ammonia-N resulted in a significant increase in the distance moved by
354 crabs at 3h compared with those exposed to DSW following multiple comparison test with
355 Bonferroni correction.

356 For 0h and 6h, no significant difference in distance moved between the four treatments was
357 observed. Considering exposure time as factor level, crabs exposed to DSW showed a
358 significantly reduced distance moved at 3h compared with the first time point (0h).
359 A significant effect ($p < 0.005$) of treatment and exposure time on mobility (% time) was
360 observed. Their interaction was also significant (Table 2).
361 Post-hoc multiple comparisons on the interaction terms showed that crabs exposed to WW
362 were more mobile than crabs exposed to DSW and SW at 3h and that crabs exposed to
363 Ammonia-N were more mobile than crabs exposed to DSW at the same time point (3h)
364 (Fig.1B). For 0h and 6h, no significant difference in mobility between treatments was
365 observed. Considering exposure time factor level, crabs exposed to DSW showed a
366 significantly reduced mobility at 3h compared with the first time point (0h), similarly to
367 distance moved.

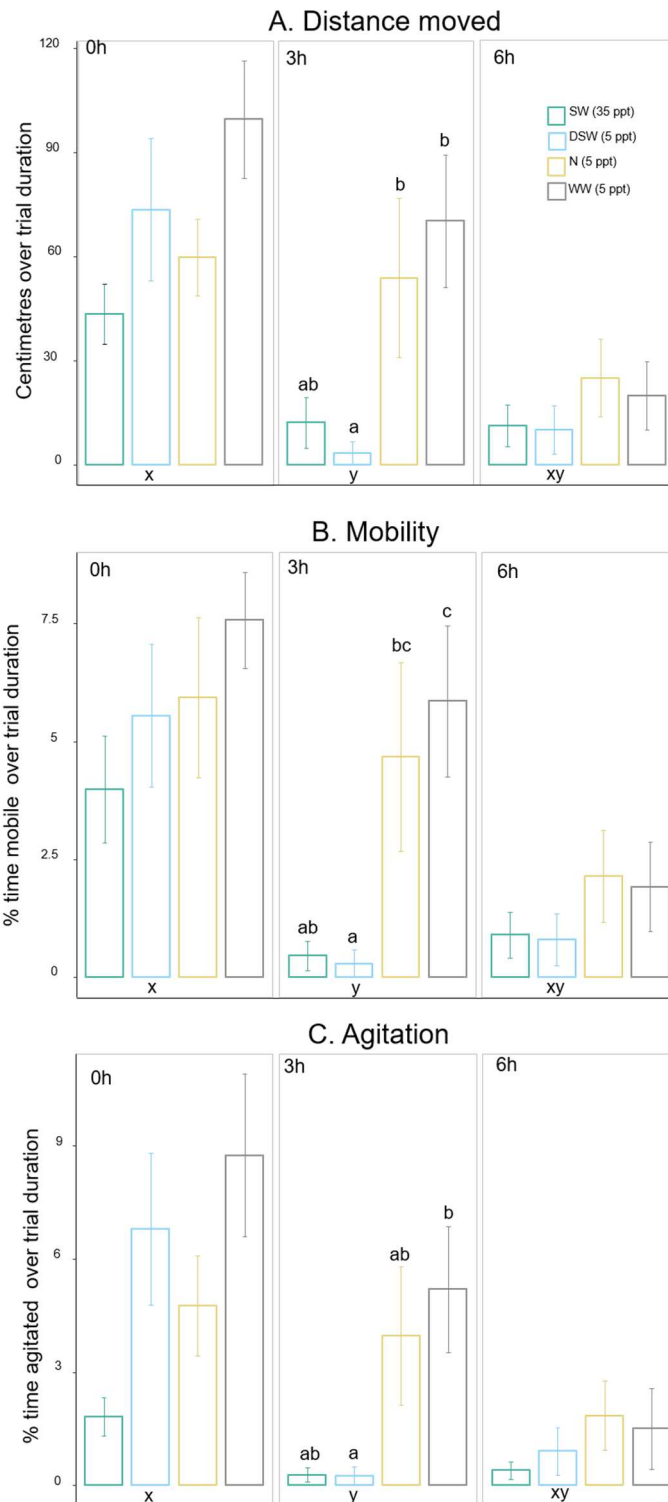


Figure 1. Averages of (A) distance moved

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370

371

372

by crabs, (B) percentage of time where crabs were mobile, and (C) percentage of time where crabs were agitated at 0h, 3h and 6h exposure to SW, DSW, N and WW. Different colours represent treatments (see legend). Values are mean \pm SE. N = 15 per treatment. Different letters on the top (a,b,c) and at the bottom (x,y,) of bar plots indicate significant two-way interaction effects of treatment

373 and time respectively after Wald Chi-square test and pairwise multiple comparisons (Bonferroni
 374 corrected).

375 Crabs were also significantly more agitated when exposed to WW compared with DSW at 3h
 376 only (Fig.1C). In the same way as for the two previous parameters, crabs exposed to DSW
 377 were significantly less agitated at 3h than at 0h considering the exposure time factor level.
 378 These results accounted for significant ($p < 0.005$) treatment, exposure time and interaction
 379 (Table 2).

380

A		Distance moved		
Factor	DF	χ^2	P	
Treatment	3	9.91	0.019	
Time	2	25.72	< 0.001	
Treatment X Time	6	2.85	0.045	

B		Mobility		
Factor	DF	χ^2	P	
Treatment	3	26.25	0.002	
Time	2	15	<0.001	
Treatment X Time	6	13.79	0.032	

C		Agitation		
Factor	DF	χ^2	P	
Treatment	3	21.11	<0.001	
Time	2	27.63	<0.001	
Treatment X Time	6	13.2	0.04	

381

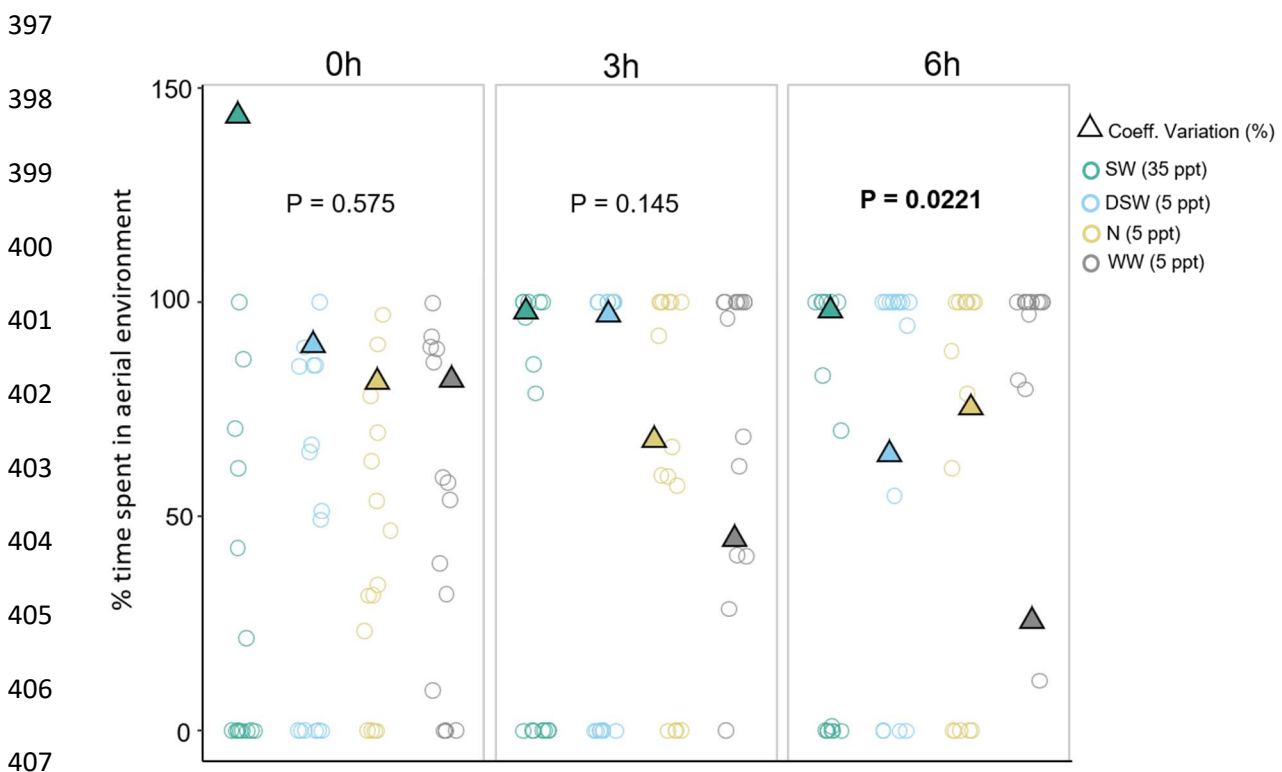
382 **Table 2.** Behavioural responses of fiddler crabs as a function of treatment and time.

383 Analysis of deviance with Wald Chi-square tests are performed for testing the significant differences
 384 of fixed categorical variables in GLMMs (N = 15 per treatment for 4 treatment groups and 3 time
 385 steps). Autocorrelation and non-independence were controlled with crab ID as random factor.

386

387 As depicted in Fig.2, time spent in aerial environment varied a lot between individuals for all
 388 treatment groups at 0h with no clear preference between the aerial or aquatic environment
 389 (test statistic = 1.98, $p = 0.56$, CV: SW = 143.58, DSW = 89.64, N = 81.26, WW = 81.65). At

390 3h, coefficients of variation of N and WW exposed crabs slightly decreased without bringing
 391 significant difference between treatment group coefficient of variations (test statistic =
 392 5.3719, $p = 0.146$, CV: SW = 97.51, DSW = 96.82, N = 67.83, WW = 44.38). At 6h,
 393 individual variations significantly differed between treatment groups, as 93% of the crabs
 394 exposed to WW spent at least 95% in the aerial environment (test statistic = 9.62, $p = 0.02$,
 395 CV: SW = 97.85, DSW = 64.54, N = 75.17 WW = 25.22) whereas other treatments showed
 396 similar variations.



408 **Figure 2.** Percentage of time that crabs exposed to DSW, SW, N and WW spent in aerial environment
 409 at 0h, 3h, and 6h (individual values). Different colours represent treatments (see legend). Circles
 410 represent individuals (N = 15 per treatment). Triangles represent the coefficient of variation by
 411 treatment and time step. P values indicate if there are significant differences between treatments after
 412 modified signed-likelihood ratio test.

413

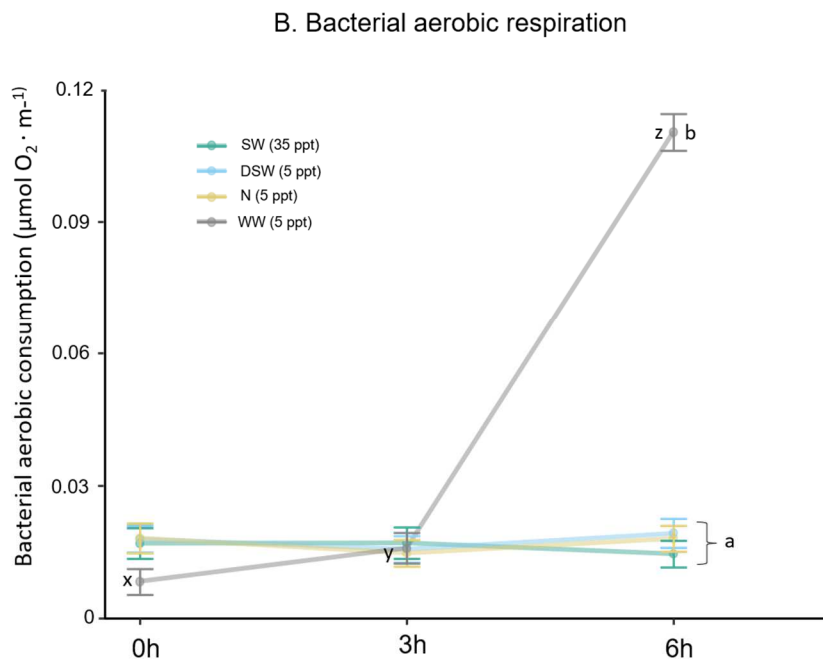
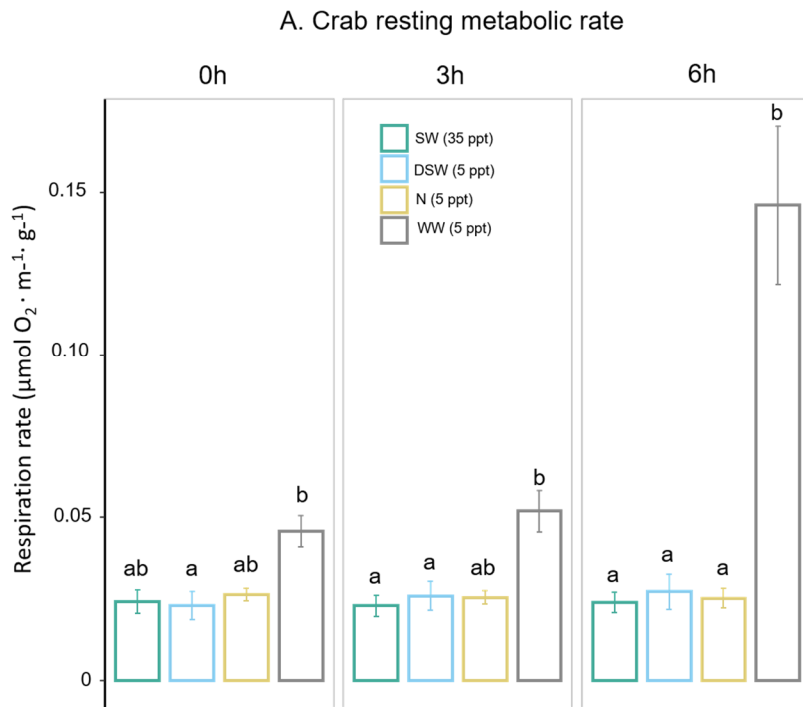
414 **4.2 Oxygen consumption**

415 Results of the respiration rate measurements are depicted in Fig.3A, which includes temporal
416 responses for each treatment at 0h, 3h, 6h.

417 A significant effect ($p < 0.001$) of treatment and exposure time on O_2 consumption ($\mu\text{mol } O_2 \cdot$
418 $\text{m}^{-1} \cdot \text{g}^{-1}$) was observed. Their interaction was also significant (Table 3).

419 Post hoc multiple comparisons on the interaction terms showed that crabs exposed to WW
420 consumed significantly more O_2 than crabs exposed to DSW and at the first-time step (0h)
421 (Fig.3A). At 3h, WW exposed crabs consumed significantly more O_2 than DSW and SW
422 exposed crabs. At 6h, a burst in O_2 consumption in crabs exposed to WW generated
423 significant differences in respiration rate with the three other treatment groups (DSW, SW and
424 N).

425 Results of the consumption of O_2 by aerobic bacteria are depicted in Fig.3B, including
426 temporal responses for each treatment at 0h, 3h, 6h. A significant effect ($p < 0.01$) of
427 treatment and exposure time on O_2 consumption ($\mu\text{mol } O_2 \cdot \text{min}^{-1}$) was observed (Table 3).
428 Their interaction was also significant (Wald chi-square test). Post hoc multiple comparisons
429 on the interaction terms showed that bacterial O_2 consumption in WW increased significantly
430 between 0h and 3h, and 3h and 6h, with a clear burst in consumption at 6h (Fig.3B). Bacterial
431 O_2 consumption in WW was significantly higher at 6h compared with all the other conditions
432 (DSW, SW and N).



433

434 **Figure 3.** Resting metabolic rate of *P. chlorophthalmus* (A, N = 10 per treatment) and O₂ consumption
 435 by aerobic bacteria (B, N = 8 per treatment) in SW, DSW, N and WW at 0h, 3h, and 6h exposure.
 436 Values are mean ± SE. Different letters on the top (a,b,c) and at the bottom (x,y,) of barplots indicate
 437 significant two-way interaction effects of treatment and time respectively after Wald Chi-square test
 438 and pairwise multiple comparisons (Bonferroni corrected).

A		O ₂ crab consumption		
Factor	DF	χ^2	P	
Treatment	3	48.33	< 0.001	
Time	2	32.89	< 0.001	
Treatment X Time	6	80.98	< 0.001	

B		O ₂ bacterial consumption		
Factor	DF	χ^2	P	
Treatment	3	14.42	< 0.01	
Time	2	363.46	< 0.001	
Treatment X Time	6	1030.44	< 0.001	

439

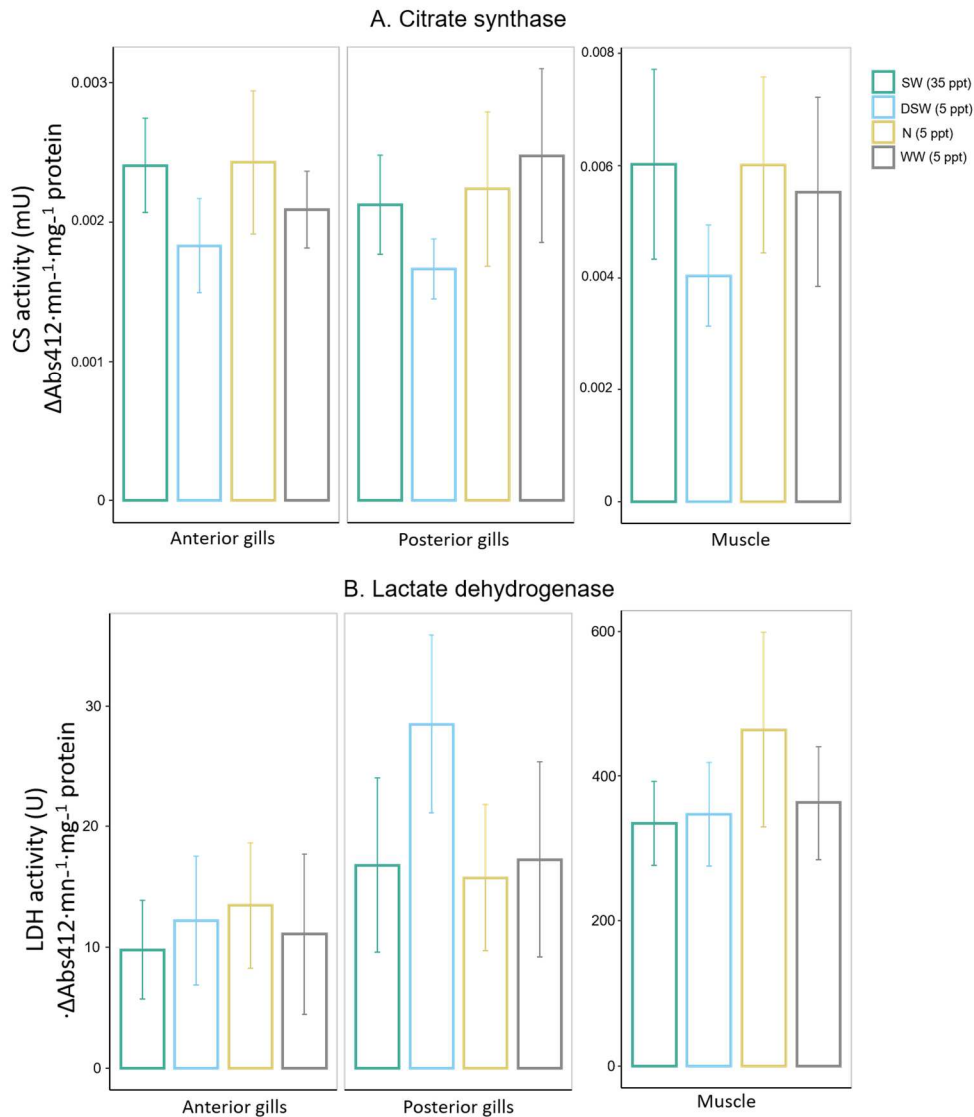
440 **Table 3.** *P. chlorophthalmus* resting metabolic rate and aerobic bacteria consumption as a function of
 441 treatment and time. Analysis of deviance with Wald Chi-square tests are performed for testing the
 442 significant differences of fixed categorical variables in GLMM (N = 8 per treatment for 4 treatment
 443 groups and 3 time steps)

444

445 **4.3 Metabolic markers**

446 Mean enzyme (CS and LDH) activities in anterior gills, posterior gills and muscles for the
 447 four treatments are shown respectively in Fig.4A and 4B. Activities are expressed as μmol
 448 substrate converted to product per minute (U) per mg of protein ($\text{U}\cdot\text{mg}^{-1}\cdot\text{protein}$).

449 No significant differences were found between treatments in CS activity for anterior gills
 450 (one-way ANOVA, $F(3,35) = 0.59$, $p = 0.62$), posterior gills (one-way ANOVA, $F(3,31) =$
 451 0.23 , $p = 0.87$) and muscles (one-way ANOVA, $F(3,33) = 0.063$, $p = 0.979$). WW and
 452 Ammonia-N did not affect either LDH activities of anterior gills (one-way ANOVA, $F(3,34)$
 453 $= 0.38$, $p = 0.77$), posterior gills (one-way ANOVA, $F(3,30) = 1.27$, $p = 0.30$) and muscles
 454 (Kruskal-Wallis test, $H = 0.05$, $df = 3$, $P = 0.99$).



455

456 **Figure 4.** Activity of citrate synthase (A) and lactate dehydrogenase (B) in anterior gill, posterior gill
 457 and cheliped muscles of *P. chlorophthalmus* exposed to DSW, SW, N and WW for 6h. N = 10 per
 458 treatment. Values are mean \pm SE and different colours represent treatments (see legend).

459

460 **5. Discussion**

461

462 **5.1 Locomotor behaviour and agitation: an escape response to WW pollution?**

463 In this research, crabs exposed to WW and ammonia-N exhibited a stimulation of their
 464 locomotor behaviour (distance moved and time spent mobile) that became significantly
 465 different from DSW and SW controls after 3h. At the beginning of the exposure (0h), no

466 significant difference was found between the treatments and most of the animals exhibited
467 erratic behaviours (Fig.1 A, B, C). This could be explained by a potential stress coupled with
468 an exploring activity of crabs in their new environment. After 3h exposure, DSW exposed
469 crabs decreased significantly their locomotor behaviour and agitation at a very low rate in
470 contrast to 0h (Table 2). This species has been described as "lethargic" (Crane, 1975), mostly
471 feeding while standing in place unlike other fiddler crab species such as *G. vocans* and *G.*
472 *tetragonon* that are more active (wandering, waving, fighting) (Weis and Weis, 2004). The
473 behaviour that was observed in crabs in DSW and SW conditions after 3h could, thus, be
474 interpreted as an acclimation to the microcosms, handling and experimental conditions.
475 This is not the case for WW and ammonia-N exposed crabs, which maintained a high
476 locomotor and mobility rate at 3h with significant differences compared to DSW and SW
477 exposed crabs. This peak activity in crabs exposed to wastewater has also been observed in
478 mesocosms after 3h exposure, in two fiddler crab species (Bartolini et al., 2009). This frenetic
479 wandering, without any apparent purpose, was interpreted as an escape response from
480 stressful conditions and the direct sewage impact such as lower salinity or pH alteration.
481 Other studies focusing on amphipod species exposed to effluent also manifested compulsion
482 to escape as a general increase in activity, at least in short time periods (Love et al., 2020). In
483 our study, we may rule out the salinity factor since crabs in the DSW control condition
484 exhibited no apparent stress. Also, they mainly inhabit stream banks that can have reduced
485 salinities. Furthermore, avoiding freshwater could be deleterious (predation risk). However,
486 other factors such as high ammonia-N concentrations, or pollutant cocktails may be noxious
487 to these organisms.

488 Ammonia-N is known to disrupt physiological processes such as osmoregulation,
489 immunology, acid/base balance and gas exchange in Decapod crustaceans (Romano and
490 Zeng, 2013; Weihrauch et al., 2004), especially at low salinities. On the other hand, domestic

491 effluents are complex and inherently inconsistent, for that reason the impact of individual
492 components of WW is difficult to ascertain (Love et al., 2020).
493 Our results showed a **different intensity** in the behavioural responses between crabs exposed
494 to ammonia-N only and to WW. Responses appear more acute when crabs are exposed to the
495 latest, particularly concerning the mobility and agitation parameters (Fig. 1B,C). In our study,
496 the locomotor activity and agitation of both ammonia-N and WW exposed crabs decreased
497 after 6h exposure, in such a way that there was no significant difference with control
498 treatments anymore. This decrease in activity could be an acclimation behaviour regarding the
499 pollutant, with an initial burst in velocity due to handling stress (T0H), an acute response that
500 could be an avoidance behaviour (T3H), and an acclimation to contaminated water with a
501 decrease in activity and dissipation of avoidance behaviour (T6H). Such responses have
502 already been observed in amphipods exposed to WW, with an activity peak noticed at short-
503 term, generally followed by a decrease in activity (Love et al., 2020). In the present research,
504 crabs exposed to ammonia-N acted the same way while remaining in the water. However,
505 those exposed to WW emersed themselves from the water after 6h of exposure.

506

507 **5.2 Emersion behaviour: an escape strategy driven by physiological compromises under** 508 **pollutant stress**

509 Some crustaceans (amongst them, Ocypode crabs) called bimodal breathers, are able to
510 perform gas exchange in both aquatic and aerial environments through gills and lungs (Henry
511 and Wheatly, 1992). This bimodal life strategy where organisms can move facultatively
512 between aerial and aquatic media, can be seen as an adaptation to efficiently face the
513 heterogeneity of intertidal habitats (Fusi et al., 2016). Our results showed that after 3h of
514 exposure, crabs exposed to SW and DSW controls were mostly resting (Fig.1) indifferently
515 between aerial and aquatic environment (Fig.2). Bimodal breathers need to maintain contact

516 with an aqueous environment even under terrestrial conditions and keep a supply of water in
517 the branchial chamber, bathing the gills due to morphological and physiological adaptations,
518 such as releasing carbon dioxide (CO₂) and ammonia (NH₃) (Henry and Wheatly, 1992;
519 Taylor and Butler, 1973). However, several studies showed that whole body emersion
520 behaviour happens in crabs facing environmental stress such as exposure to contaminants,
521 temperature increase or hypoxia (nuanced with some species-specific responses) (de Lima et
522 al., 2021; Taylor et al., 1973). Our results showed that crabs exposed to WW spend
523 substantially more time in air as an escape behaviour with a clear preference for this
524 environment (see Fig.2), since they are almost all emerged after 6h exposure, and they all
525 emerged themselves at least once.

526 This could represent a prolonged avoidance behaviour with crabs finally relying on emersion
527 after a peak of activity. This behaviour is not observed in animals facing ammonia-N
528 exposure, which decrease their activity after 6h without choosing any particular media,
529 similarly to control treatment exposed crabs.

530 Acute exposure to high environmental ammonia (HEA) triggered emersion responses in the
531 green shore crab *Carcinus maenas* at high NH₄ concentrations (4 and 10 mmol.L⁻¹ against 1
532 mmol.L⁻¹ in the present study) (Zimmer and Wood, 2017). However, intertidal crabs such as
533 *P. chlorophthalmus* are usually exposed to lower levels of ammonia-N. In this regard, a recent
534 study demonstrated the involvement of the fiddler crab holobiont in the nitrogen cycle around
535 the animals, and the importance of considering invertebrate-bacteria associations in
536 understanding biogeochemical processes in mangroves (Zilius et al., 2020). The authors
537 suggest that in the presence of eutrophic conditions, such as regular WW discharges, the
538 composition of the holobiont biofilm could potentially be modified and have an impact on the
539 biogeochemical conditions of the environment near the animals, and thus potentially on their
540 ecology.

541 Weihrauch et al. (1999) proposed that the high ammonia environment (1-2 mmol.L⁻¹) in the
542 sediment surrounding crab burrows may have driven the evolution of active ammonia
543 excretion mechanism in crabs with no need to escape or emerge from the water.
544 This could explain why crabs exposed to ammonia-N in the present study exhibited an escape
545 behaviour with an increase in locomotion at first (until at least 3h of exposure) but did not
546 show a full emersion response since excretion of nitrogen products must occur in water
547 (Luquet et al., 1998).

548

549 **5.3 Oxygen consumption and enzymatic markers: the metabolic cost of contaminant** 550 **exposure**

551 In aquatic organisms, exposure to wastewater effluents causes metabolic and behavioural
552 effects, often demonstrated by an increase in metabolic rate with behavioural modifications.
553 These changes are often associated with synergistic effects of contaminants with other factors,
554 which are difficult to discriminate (Du et al., 2018) . The resting metabolic rate represents an
555 integrated way of assessing a potential metabolic cost of contaminant exposure at the
556 organism level, this time without behavioural responses. Research conducted on fish species
557 showed that animals exposed during 21 days to WW increase their metabolic rate and that the
558 contaminants impact the oxygen cascade through various alterations: increased morphological
559 capacity of the gills for gas exchange, modulation of haemoglobin-O₂ binding affinity of the
560 blood, modification of mitochondrial functions (Du et al., 2018).

561 In the present study, crabs totally immersed in WW drastically increased their resting
562 metabolic rate with a burst in O₂ consumption observed after 6h in order to supply their
563 energy requirements, as expected (hypothesis 2). The increase in O₂ consumption, recorded in
564 metabolic chambers means that the crabs in this study still have scope to extract O₂ from
565 water, with no change in the CS activity as a marker of maximum aerobic potential (Bishop et

566 al., 2004. But it is important to remember that the water was regularly oxygenated for the
567 metabolic rate measurements experiments, unlike WW from the behavioural and enzymatic
568 marker experiments. Thus, in parallel with multiple stressors, a potential hypoxia stress could
569 occur when crabs are exposed to WW in these experiments: we observed (Fig 3.B) that
570 bacterial and algal consumption increases sharply with WW exposure, which is however
571 regularly aerated.

572 Exposure to environmental stressors such as hypoxia can induce physiological and
573 behavioural trade-offs (Killen et al., 2012). Generally, organisms use two initial metabolic
574 strategies when exposed to hypoxic conditions: an overall reduction in metabolic rate, and a
575 shift in the aerobic and anaerobic contributions to total metabolism (Cooper et al., 2002),
576 which was not the case for the crabs in our study. The assessment of LDH enzymatic activity
577 (indicator of glycolytic potential) in gills and cheliped muscles confirms this hypothesis
578 (Fig.4B) at a lower integration level. It suggests that crabs could rely on other metabolic
579 strategies in coping with a potentially hypoxic WW, sometimes by trapping an air bubble
580 inside while sealing their burrows during high tide, providing them with sufficient oxygen
581 until emersion (Penha-Lopes et al., 2009). They could also rely on the capacity to live in an
582 aerial environment rather than to process more complex physiological changes.

583 Again, no effect of isolated ammonia-N treatment was observed (Fig.3A, Fig.4), between
584 treatments and time, in resting metabolic rate and metabolic marker experiments, supporting
585 the fact that WW impacts result in a combination of stress factors such as multiple
586 contaminants and, perhaps, progressive hypoxia. The interactions between multiple stressors
587 have been discussed and are likely to be more complex than a neutral additive interaction
588 where the effects of multiple stress would be equal to the sum of each isolated stress (here,
589 ammonia-N, possible other contaminants, and, a potential hypoxic stress) (Henry et al., 2017).
590 For example, decreased O₂ concentrations can hinder nitrification and promote reduction of

591 nitrate to ammonia by micro-organisms, increasing ammonia concentration particularly when
592 nitrate concentration is high (Henry et al., 2017), which is the case in WW. These combined,
593 synergistic effects could explain the acute behavioural and metabolic responses observed in
594 crabs exposed to a short-term (6h) WW pulse.

595

596 **6. Conclusion**

597 Behaviour and swimming performances are now well recognized indicators for aquatic and
598 intertidal animals, of their individual ability to respond to environmental stressors (Amiard-
599 Triquet, 2009; Aimon et al., 2021). The study of the effects of complex sewage effluents in
600 intertidal environments on semi-terrestrial crustaceans induces additional considerations such
601 as nutrient enrichment, potential hypoxic condition and tidal rhythm that introduce
602 complexity in assessing the whole-effluent toxicity (Melvin, 2016) at different biological
603 levels.

604 Our study showed that pulse exposures with WW pollution induce an increase in the
605 locomotor behaviour and an emersion behaviour in a fiddler crab species, associated with a
606 drastic increase in metabolic rate. Crabs facing nutrient-enriched water (ammonia-N) as a key
607 characteristic of WW also increase their locomotor behaviour, although to a lesser extent, and
608 did not display emersion behaviour, nor did they increase their metabolic rate. Behavioural
609 analyses offer many benefits as ecotoxicological endpoints and are a more sensitive indicator
610 of toxicity than mortality (Love et al., 2020). Our study also indicates that behavioural
611 endpoints could allow the expression of several degrees of intensity in individual responses to
612 different stressors. In both Ammonia-N and WW, no change in metabolic enzyme pathways
613 have been observed (CS and LDH), implying that no switch in energy supplies occurs when
614 crabs cope with short-term pollution pulses. Finally, fine-scale metabolic and behavioural
615 responses observed in WW are based on a short-term behavioural and physiological flexibility

616 that could be deleterious when repeated in the chronic exposure context of a bioremediation
617 site. Within their flexible behavioural repertoire used to adapt to changing conditions, crabs
618 have already been observed abandoning their burrows in “herds” to find better living
619 conditions when predation risk is too high, when food lacks or in case of prolonged
620 harassment by neighbours (Zeil and Hemmi, 2006). The repetition of emersion behaviour, and
621 the chronic polluted conditions due to regular pulses of WW, could greatly diminish the living
622 conditions within these burrows while increasing the risk of predation and dehydration. Such
623 disturbances in the established rhythms in crab societies could lead to massive wandering in
624 search of new burrows and territory. This could partly explain why regular anthropogenic
625 pollution discharges such as WW can lead to changes in crab communities *in situ* within a
626 few years.

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