# Individual metabolism and behaviour as complementary endpoints to better understand mangrove crab community variations linked to wastewater inputs

Laura Megevand, Pauline Kreienbuhl, Dimitri Theuerkauff, Jehan-Herve Lignot, Elliott Sucre

# Individual metabolism and behaviour as complementary endpoints to better understand mangrove crab community variations linked to wastewater inputs

**Laura Mégevand[a*], Pauline Kreienbühl[b], Dimitri Theuerkauff[c], Jehan-Hervé Lignot[a] & Elliott Sucré[a, b]**

[a] UMR9190-MARBEC, Université de Montpellier, Place Eugène Bataillon, 34095 Montpellier, France

[b] Centre Universitaire de Formation et de Recherche de Mayotte (CUFR), 97660 Dembeni, Mayotte, France

[c] Station de Recherche Océanographiques et Sous-marines STARESO, Punta Revellata, 20260 Calvi, France

* Corresponding author at: UMR MARBEC (University of Montpellier, CNRS, IFREMER, IRD), Montpellier, France. E-mail address: laura.megevand@umontpellier.fr (L. Mégevand).

## Abstract

Mangrove forests are impacted by a large range of anthropogenic activities that challenge their functioning. For example, domestic wastewater (WW) discharges are known to increase vegetation growth but recent studies indicate that they have negative effects on benthic macrofauna, especially on mangrove crabs, these ecosystem engineers playing a key role on the functioning of the mangrove. In experimental areas regularly receiving WW at low tide (Mayotte Island, Indian Ocean), a drastic decrease in burrowing crab density has been reported. In this context, the individual behavioural and physiological responses of the fiddler crab *Paraleptuca chlorophthalmus* exposed to short-term (6h) pulse of WW and ammonia-N (as a potential proxy of WW) were investigated. This species is one of the most sensitive to WW within the mangrove crab community. For the behavioural experiment, crabs could choose between the aquatic and aerial environment. Individual metabolic rate ($O_2$ consumption) was monitored after 6h of exposure in WW or ammonia-N. Aerobic and

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

## Keywords

Crustacea

Pulse exposure

Behavioural endpoints

Metabolism

Wastewater

# 1. Introduction

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

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

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

To complete these field observations, recent studies were conducted in controlled conditions in order to investigate the effects of WW on mangrove crab physiology. Burrowing mangrove crabs *N. africanum* were exposed to short (5h) but ecologically relevant WW pulses (similar to the discharge generated on the field experimental site of Mayotte). A burst in $O_2$ consumption, histological changes (osmoregulatory gills and hepatopancreas), and disturbed antioxidant defences were observed (Theuerkauff et al., 2018, Mégevand et al., 2021). These effects partially explain the modifications of crab communities observed *in situ* as they unveil, at different integrated levels, several energy compromises and adaptations to maintain the crabs global metabolism. They also reveal the physiological flexibility of this species and a lack of knowledge about the short-term survival strategies they may develop, including behavioural responses.

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

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

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

In this study, we focus on a fiddler crab *Paraleptuca chlorophthalmus* distributed among Indo-pacific mangroves, that usually inhabits muddy banks and flats of mangrove estuaries, near high-tide levels of mangrove forests (Crane, 1975). This species is one of the declining ocypodidae species in the WW impacted parcels of the pilot site of Malamani (Capdeville et al., 2018). Behavioural observations coupled with physiological analyses can provide a more complete understanding of the impact of an external stimulus on an organism, a population or a species (Filiciotto et al., 2018). For fiddler crabs and other crustaceans, altered behaviour can be considered as an early warning biomarker of chemical exposure (Ungherese and Ugolini, 2009). In fiddler crabs, different escaping behaviours have been studied *in situ*, often resulting in a rapid home run towards the burrows in case of predation (Hemmi, 2005) or, inversely, by emerging from their burrows, with for example crabs exposed to thermal stress (Halal et al., 2020). Emersion behaviour has been proved as a useful escaping strategy, but this has to be in a limited time period as crabs become more exposed to predation or dehydration (Luppi et al., 2013). Moreover, the elimination of $CO_2$ and ammonia is deprecated in the aerial environment (Luquet et al., 1998), which brings them regularly into the water.

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

By investigating several behavioural endpoints and assessing metabolic state ($O_2$ consumption and metabolic enzymes activities) of the fiddler crab *P. chlorophthalmus* exposed to WW and ammonia-N as a proxy of WW in microcosm, we sought to better understand ecological observations in the field through laboratory experiments.

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

$O_2$ consumption was measured as resting metabolic rate, with crabs placed in metabolic chambers that limited their movements. This measure was considered as physiologically relevant (Borges et al., 2018) to assess the potential effects of contaminated water on crabs, independently from a potential locomotor or escape behaviour. Then, to reveal underlying metabolic trade-offs resulting from WW and ammonia-N exposure, we measured citrate synthase (CS) activity as a marker of aerobic metabolism through mitochondrial efficiency, and lactate dehydrogenase (LDH) activity that would maybe show a shift to anaerobic metabolism in order to cope with the energy needs of the animals.

## 2. Materials & Methods

### 2.1 Animal collection and acclimatisation

Male *P. chlorophthalmus* were collected in February and March 2020 in the mangrove of Malamani (Boueni Bay, Mayotte -12,923155, 45.154053) from a population extending along both sides of the stream of Malamani, in the *Avicennia marina* and *Ceriops tagal* vegetation belt (above the *Rhizophora mucronata* belt). This open habitat presents no canopy cover, a large temperature gradient and regular water immersion/emersion, depending on the season, 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 (~33‰ salinity; 1050 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).

| Chemical species | Concentration (µmol/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. |

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

### 2.3 Ammonia-N exposure and experimental conditions

Four different experimental conditions were performed: some crabs were maintained in seawater (~33‰ salinity), others were transferred to diluted seawater (~5‰ salinity) or to 10 $mg.l^{-1}$ 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 ($NH4^+$) 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 NH3 and ionized ammonia $NH4^+$ (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

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

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

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

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

**2.5 Oxygen consumption rate**

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 $O_2$ consumption. Following procedures described in Killen (2014),
238 $O_2$ measurements were performed using a 4-channel fiber-optic system with contactless $O_2$
239 sensor spots (FireSting $O_2$, 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 $mm^2$
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 $O_2$
255 (Shchegolkova et al., 2016), a 20 minutes flushing was run to ensure 99% air saturation. The
256 40 minutes measurements allowed accurate $O_2$ 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, $O_2$ 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 $MO_2$ 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

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

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

## 3. Statistical analyses

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

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

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

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

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

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

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

For $O_2$ oxygen consumption, a generalized linear mixed model approach was taken assuming Gaussian distribution error with log link. As with above, time (0h, 3h, 6h) and treatment group (SW, DSW, N, WW) were considered as fixed factors.

Crab ID was set as random factor nested within experimental tank (two tanks of exposure solution per treatment, N = 4 per tank), which are nested within treatment group (SW, DSW, N or WW). Interaction terms were examined using the approach outlined above, using type II ANOVA and multiple pairwise comparisons followed by Bonferroni correction. The same statistical analysis was used for aerobic $O_2$ consumption, with metabolic chamber ID set as random factor.

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

# 4. Results

## 4.1 Behavioural endpoints

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

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

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

For 0h and 6h, no significant difference in distance moved between the four treatments was observed. Considering exposure time as factor level, crabs exposed to DSW showed a significantly reduced distanced moved at 3h compared with the first time point (0h).

A significant effect ($p < 0.005$) of treatment and exposure time on mobility (% time) was observed. Their interaction was also significant (Table 2).

Post-hoc multiple comparisons on the interaction terms showed that crabs exposed to WW were more mobile than crabs exposed to DSW and SW at 3h and that crabs exposed to Ammonia-N were more mobile than crabs exposed to DSW at the same time point (3h) (Fig.1B). For 0h and 6h, no significant difference in mobility between treatments was observed. Considering exposure time factor level, crabs exposed to DSW showed a significantly reduced mobility at 3h compared with the first time point (0h), similarly to distance moved.

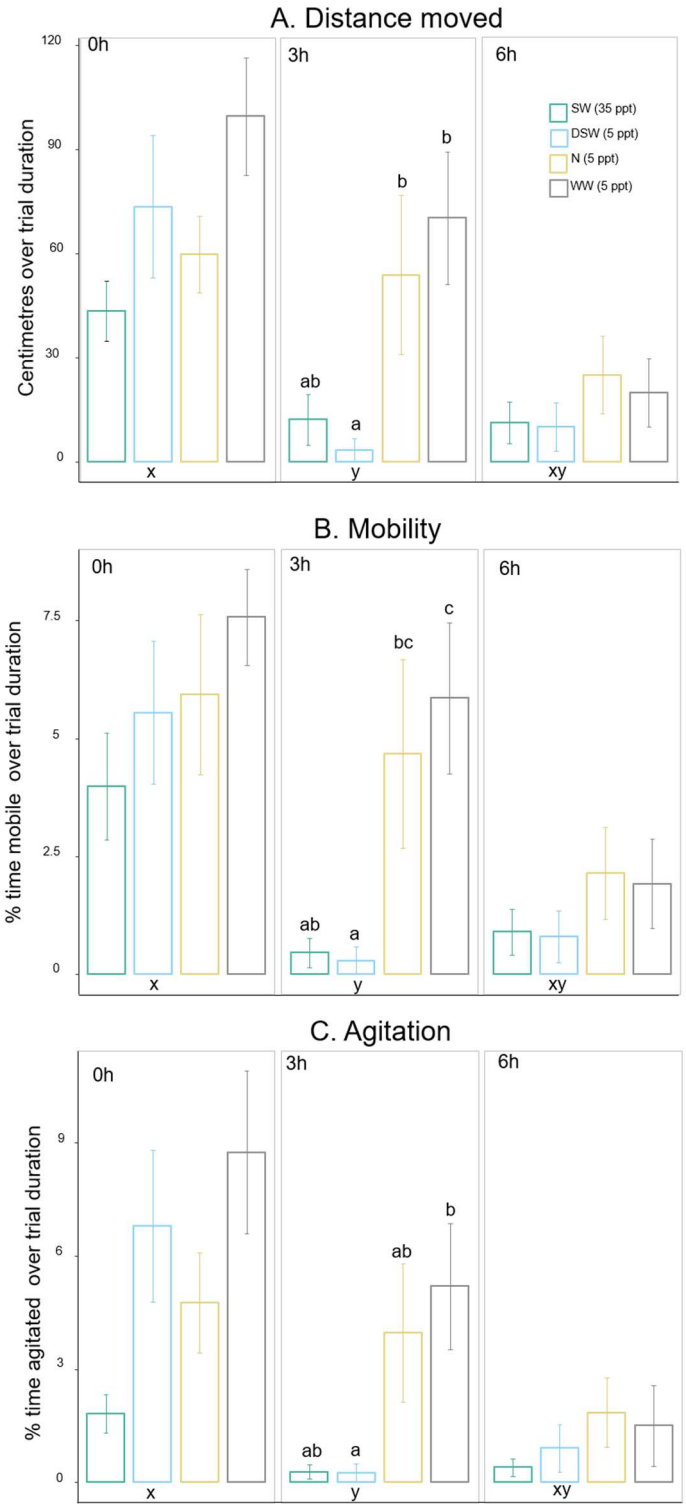



**Figure 1**. Averages of (A) distance moved by crabs, (B) percentage of time where crabs were mobile, and (C) percentage of time where crabs were agitated at 0h, 3h and 6h exposure to SW, DSW, N and WW. Different colours represent treatments (see legend). Values are mean ± SE. N = 15 per treatment. 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 | $\chi^2$ | 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 | $\chi^2$ | P |
| Treatment | 3 | 26.25 | **0.002** |
| Time | 2 | 15 | **<0.001** |
| Treatment X Time | 6 | 13.79 | **0.032** |

| C | Agitation | | |
|---|---|---|---|
| Factor | DF | $\chi^2$ | 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

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

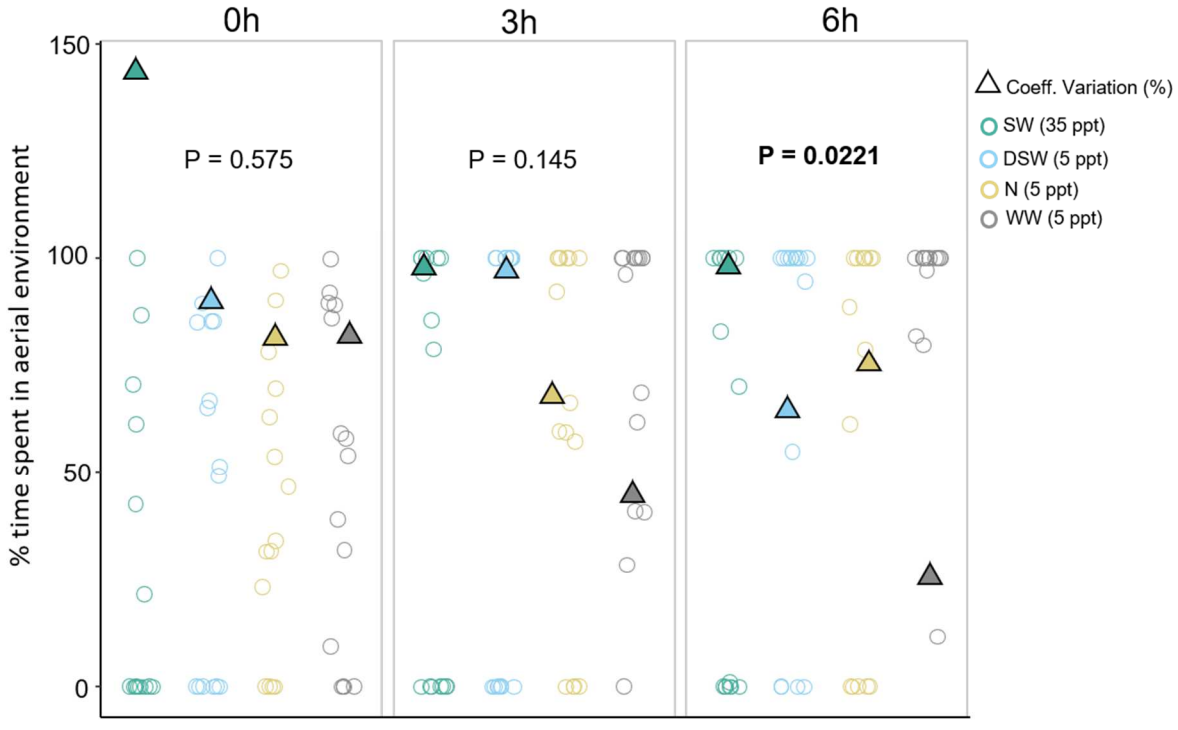



**Figure 2.** Percentage of time that crabs exposed to DSW, SW, N and WW spent in aerial environment at 0h, 3h, and 6h (individual values). Different colours represent treatments (see legend). Circles represent individuals (N = 15 per treatment). Triangles represent the coefficient of variation by treatment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

## 4.2 Oxygen consumption

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

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

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

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

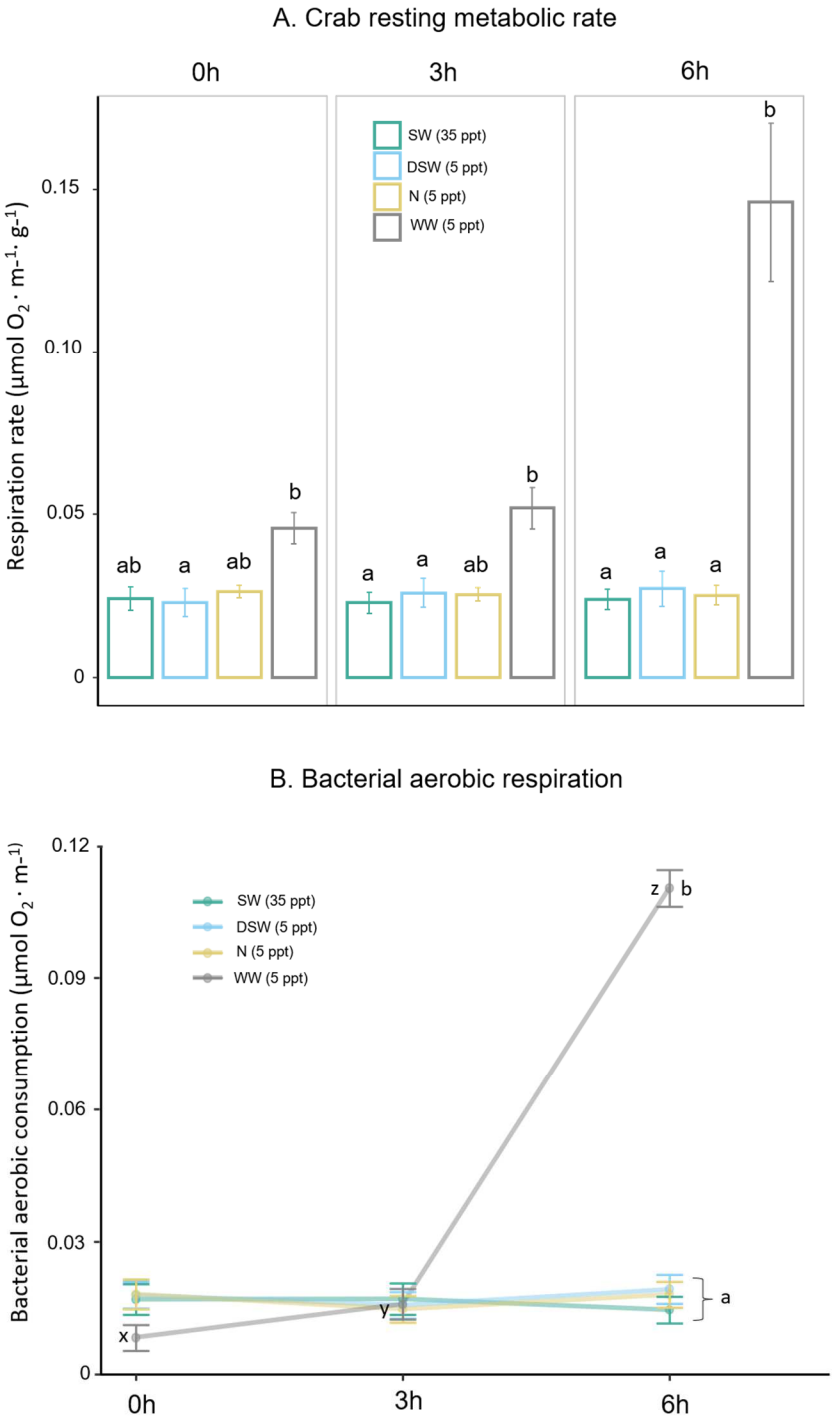



**Figure 3.** Resting metabolic rate of *P. chlorophthalmus* (A, N = 10 per treatment) and $O_2$ consumption by aerobic bacteria (B, N = 8 per treatment) in SW, DSW, N and WW at 0h, 3h, and 6h exposure. Values are mean ± SE. Different letters on the top (a,b,c) and at the bottom (x,y,) of barplots indicate significant two-way interaction effects of treatment and time respectively after Wald Chi-square test and pairwise multiple comparisons (Bonferroni corrected).

| A | $O_2$ crab consumption | | |
|---|---|---|---|
| Factor | DF | $\chi^2$ | 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 | $\chi^2$ | P |
| Treatment | 3 | 14.42 | **< 0.01** |
| Time | 2 | 363.46 | **<0.001** |
| Treatment X Time | 6 | 1030.44 | **<0.001** |

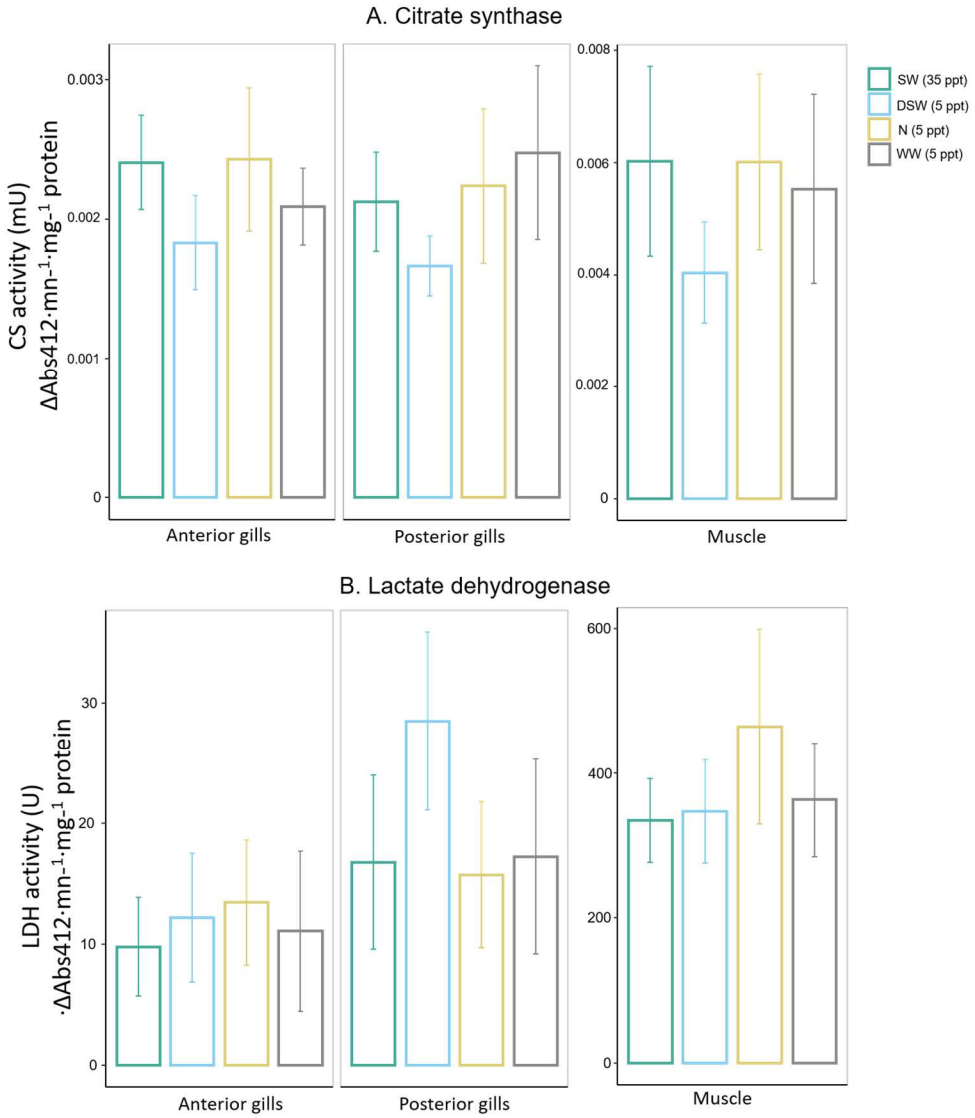
439

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 ($U \cdot mg^{-1} \cdot protein$).
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 ± 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

significant difference was found between the treatments and most of the animals exhibited erratic behaviours (Fig.1 A, B, C). This could be explained by a potential stress coupled with an exploring activity of crabs in their new environment. After 3h exposure, DSW exposed crabs decreased significantly their locomotor behaviour and agitation at a very low rate in contrast to 0h (Table 2). This species has been described as ”lethargic” (Crane, 1975), mostly feeding while standing in place unlike other fiddler crab species such as *G. vocans* and *G. tetragonon* that are more active (wandering, waving, fighting) (Weis and Weis, 2004). The behaviour that was observed in crabs in DSW and SW conditions after 3h could, thus, be interpreted as an acclimation to the microcosms, handling and experimental conditions.

This is not the case for WW and ammonia-N exposed crabs, which maintained a high locomotor and mobility rate at 3h with significant differences compared to DSW and SW exposed crabs. This peak activity in crabs exposed to wastewater has also been observed in mesocosms after 3h exposure, in two fiddler crab species (Bartolini et al., 2009). This frenetic wandering, without any apparent purpose, was interpreted as an escape response from stressful conditions and the direct sewage impact such as lower salinity or pH alteration.

Other studies focusing on amphipod species exposed to effluent also manifested compulsion to escape as a general increase in activity, at least in short time periods (Love et al., 2020). In our study, we may rule out the salinity factor since crabs in the DSW control condition exhibited no apparent stress. Also, they mainly inhabit stream banks that can have reduced salinities. Furthermore, avoiding freshwater could be deleterious (predation risk). However, other factors such as high ammonia-N concentrations, or pollutant cocktails may be noxious to these organisms.

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

effluents are complex and inherently inconsistent, for that reason the impact of individual components of WW is difficult to ascertain (Love et al., 2020).

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

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

Some crustaceans (amongst them, Ocypode crabs) called bimodal breathers, are able to perform gas exchange in both aquatic and aerial environments through gills and lungs (Henry and Wheatly, 1992). This bimodal life strategy where organisms can move facultatively between aerial and aquatic media, can be seen as an adaptation to efficiently face the heterogeneity of intertidal habitats (Fusi et al., 2016). Our results showed that after 3h of exposure, crabs exposed to SW and DSW controls were mostly resting (Fig.1) indifferently 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 ($CO_2$) and ammonia ($NH_3$) (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 $NH_4$ 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.

Weihrauch et al. (1999) proposed that the high ammonia environment (1-2 mmol.$L^{-1}$) in the sediment surrounding crab burrows may have driven the evolution of active ammonia excretion mechanism in crabs with no need to escape or emerge from the water.

This could explain why crabs exposed to ammonia-N in the present study exhibited an escape behaviour with an increase in locomotion at first (until at least 3h of exposure) but did not show a full emersion response since excretion of nitrogen products must occur in water (Luquet et al., 1998).

### 5.3 Oxygen consumption and enzymatic markers: the metabolic cost of contaminant exposure

In aquatic organisms, exposure to wastewater effluents causes metabolic and behavioural effects, often demonstrated by an increase in metabolic rate with behavioural modifications. These changes are often associated with synergistic effects of contaminants with other factors, which are difficult to discriminate (Du et al., 2018) . The resting metabolic rate represents an integrated way of assessing a potential metabolic cost of contaminant exposure at the organism level, this time without behavioural responses. Research conducted on fish species showed that animals exposed during 21 days to WW increase their metabolic rate and that the contaminants impact the oxygen cascade through various alterations: increased morphological capacity of the gills for gas exchange, modulation of haemoglobin-$O_2$ binding affinity of the blood, modification of mitochondrial functions (Du et al., 2018).

In the present study, crabs totally immersed in WW drastically increased their resting metabolic rate with a burst in $O_2$ consumption observed after 6h in order to supply their energy requirements, as expected (hypothesis 2). The increase in $O_2$ consumption, recorded in metabolic chambers means that the crabs in this study still have scope to extract $O_2$ from water, with no change in the CS activity as a marker of maximum aerobic potential (Bishop et

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

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

Again, no effect of isolated ammonia-N treatment was observed (Fig.3A, Fig.4), between treatments and time, in resting metabolic rate and metabolic marker experiments, supporting the fact that WW impacts result in a combination of stress factors such as multiple contaminants and, perhaps, progressive hypoxia. The interactions between multiple stressors have been discussed and are likely to be more complex than a neutral additive interaction where the effects of multiple stress would be equal to the sum of each isolated stress (here, ammonia-N, possible other contaminants, and, a potential hypoxic stress) (Henry et al., 2017). For example, decreased $O_2$ 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

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

**Acknowledgements**

The authors are grateful to Emilie Farcy (MARBEC, University of Montpellier) for making the use of Ethovision® XT possible, Guus Bongers (Ethovision, Nantes) for his precious help in setting up the behavioural experiments and Ethovision settings, Axelle Cadière and Julien Rigaud (CHROME laboratory, Nîmes, France) for the physico-chemical analyses of the wastewater samples, to Thibaut l'Honoré (CUFR, Mayotte) for his help in laboratory experiments, to the staff of the wastewater treatment plant of Tsararano for the collection of wastewater and to Sophie Czich and Justine Courboulès for their help for the graphical abstract.

641 **REFERENCES**


642 Aimon, C., Simpson, S.D., Hazelwood, R.A., Bruintjes, R., Urbina, M.A., 2021.
643 Anthropogenic underwater vibrations are sensed and stressful for the shore crab
644 *Carcinus maenas*. Environ. Pollut. 285, 117148.
645 https://doi.org/10.1016/j.envpol.2021.117148

646 Amiard-Triquet, C., 2009. Behavioral disturbances: The missing link between sub-organismal
647 and supra-organismal responses to stress? Prospects based on aquatic research. Hum.
648 Ecol. Risk Assess. 15, 87–110. https://doi.org/10.1080/10807030802615543

649 Azpeitia, E., Vanegas-Pérez, C., Moreno-Sáenz, E., Betancourt-Lozano, M., Miranda-Anaya,
650 M., 2013. Effect of chronic ammonia exposure on locomotor activity in the fiddler crab
651 *Uca princeps* upon artificial tides and light cycles. Biol. Rhythm Res. 44, 113–123.
652 https://doi.org/10.1080/09291016.2011.652864

653 Bartolini, F., Penha-Lopes, G., Limbu, S., Paula, J., Cannicci, S., 2009. Behavioural responses
654 of the mangrove fiddler crabs (*Uca annulipes* and *U. inversa*) to urban sewage loadings:
655 Results of a mesocosm approach. Mar. Pollut. Bull. 58, 1860–1867.
656 https://doi.org/10.1016/j.marpolbul.2009.07.019

657 Bishop, R.E., Kakuk, B., Torres, J.J., 2004. Life in the hypoxic and anoxic zones: Metabolism
658 and proximate composition of Caribbean troglobitic crustaceans with observations on the
659 water chemistry of two anchialine caves. J. Crustac. Biol. 24, 379–392.
660 https://doi.org/10.1651/C-2459

661 Borges, F.O., Sampaio, E., Figueiredo, C., Rosa, R., Grilo, T.F., 2018. Hypercapnia-induced
662 disruption of long-distance mate-detection and reduction of energy expenditure in a
663 coastal keystone crustacean. Physiol. Behav. 195, 69–75.
664 https://doi.org/10.1016/j.physbeh.2018.07.023

665 Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A.,
666 Skaug, H.J., Mächler, M., Bolker, B.M., 2017. glmmTMB balances speed and flexibility
667 among packages for zero-inflated generalized linear mixed modeling. R J. 9, 378–400.
668 https://doi.org/10.32614/rj-2017-066

669 Candy, S.G., 2004. Modelling catch and effort data using generalised linear models, the
670 tweedie distribution, random vessel effects and random stratum-by-year effects.
671 CCAMLR Sci. 11, 59–80.

672 Cannicci, S., Burrows, D., Fratini, S., Smith, T.J., Offenberg, J., Dahdouh-Guebas, F., 2008.
673 Faunal impact on vegetation structure and ecosystem function in mangrove forests: A
674 review. Aquat. Bot. 89, 186–200. https://doi.org/10.1016/j.aquabot.2008.01.009

675 Capdeville, C., Abdallah, K., Buffan-Dubau, E., Lin, C., Azemar, F., Lambs, L., Fromard, F.,
676 Rols, J.L., Leflaive, J., 2018. Limited impact of several years of pretreated wastewater
677 discharge on fauna and vegetation in a mangrove ecosystem. Mar. Pollut. Bull. 129,
678 379–391. https://doi.org/10.1016/j.marpolbul.2018.02.035

679 Capdeville, C., Abdallah, K., Walcker, R., Rols, J.L., Fromard, F., Leflaive, J., 2019.
680 Contrasted resistance and resilience of two mangrove forests after exposure to long-term
681 and short-term anthropic disturbances. Mar. Environ. Res. 146, 12–23.
682 https://doi.org/10.1016/j.marenvres.2019.03.002

683 Cooper, R.U., Clough, L.M., Farwell, M.A., West, T.L., 2002. Hypoxia-induced metabolic
684 and antioxidant enzymatic activities in the estuarine fish *Leiostomus xanthurus*. J. Exp.
685 Mar. Bio. Ecol. 279, 1–20. https://doi.org/10.1016/S0022-0981(02)00329-5

686 Crane, J., 1975. Fiddler crabs of the world: Ocypididae: Genus Uca. Princeton University
687 press, New Jersey. https://doi.org/10.1016/0003-3472(78)90130-6

688 de Lima, T.M., de Ramos, B., de Souza Tavares, M., Leidens, D., Ayres, B.S., Maciel, F.E.,
689 Nery, L.E.M., 2021. Emersion behavior of the semi-terrestrial crab Neohelice granulata
690 during hypoxic conditions: Lactate as a trigger. Comp. Biochem. Physiol. -Part A Mol.
691 Integr. Physiol. 252, 2–7. https://doi.org/10.1016/j.cbpa.2020.110835

692 Du, S.N.N., McCallum, E.S., Vaseghi-Shanjani, M., Choi, J.A., Warriner, T.R., Balshine, S.,
693 Scott, G.R., 2018. Metabolic Costs of Exposure to Wastewater Effluent Lead to
694 Compensatory Adjustments in Respiratory Physiology in Bluegill Sunfish. Environ. Sci.
695 Technol. 52, 801–811. https://doi.org/10.1021/acs.est.7b03745

696 Filiciotto, F., Sal Moyano, M.P., de Vincenzi, G., Hidalgo, F., Sciacca, V., Bazterrica, M.C.,
697 Corrias, V., Lorusso, M., Mazzola, S., Buscaino, G., Gavio, M.A., 2018. Are semi-
698 terrestrial crabs threatened by human noise? Assessment of behavioural and biochemical
699 responses of *Neohelice granulata* (Brachyura, Varunidae) in tank. Mar. Pollut. Bull. 137,
700 24–34. https://doi.org/10.1016/j.marpolbul.2018.07.023

701 Fox, J., Weisberg, S., 2019. An R Companion to Applied Regression, Third. ed. Sage,
702 Thousand Oaks CA.

703 Fusi, M., Cannicci, S., Daffonchio, D., Mostert, B., Pörtner, H.O., Giomi, F., 2016. The trade-
704 off between heat tolerance and metabolic cost drives the bimodal life strategy at the air-
705 water interface. Sci. Rep. 6, 1–8. https://doi.org/10.1038/srep19158

706 Gerardi, M.H., 2006. Bacterial Growth, in: Wastewater Bacteria. John Wiley & Sons, Ltd, pp.
707 65–73. https://doi.org/10.1002/0471979910.ch9

708 Halal, L.M., Ayres, B.S., Lima, T.M., Maciel, F.E., Nery, L.E.M., 2020. High temperature
709 acclimation alters the emersion behavior in the crab *Neohelice granulata*. J. Therm. Biol.
710 91, 102617. https://doi.org/10.1016/j.jtherbio.2020.102617

711 Hemmi, J.M., 2005. Predator avoidance in fiddler crabs: 1. Escape decisions in relation to the
712 risk of predation. Anim. Behav. 69, 603–614.
713 https://doi.org/10.1016/j.anbehav.2004.06.018

714 Henry, R.P., Wheatly, M.G., 1992. Interaction of respiration, ion regulation, and acid-base
715 balance in the everyday life of aquatic crustaceans. Integr. Comp. Biol. 32, 407–416.
716 https://doi.org/10.1093/icb/32.3.407

717 Henry, Y., Piscart, C., Charles, S., Colinet, H., 2017. Combined effect of temperature and
718 ammonia on molecular response and survival of the freshwater crustacean *Gammarus*
719 *pulex* . Ecotoxicol. Environ. Saf. 137, 42–48.
720 https://doi.org/10.1016/j.ecoenv.2016.11.011

721 Herteman, M., Fromard, F., Lambs, L., 2011. Effects of pretreated domestic wastewater
722 supplies on leaf pigment content, photosynthesis rate and growth of mangrove trees: A
723 field study from Mayotte Island, SW Indian Ocean. Ecol. Eng. 37, 1283–1291.
724 https://doi.org/10.1016/j.ecoleng.2011.03.027

725 Killen, S.S., 2014. Growth trajectory influences temperature preference in fish through an
726 effect on metabolic rate. J. Anim. Ecol. 83, 1513–1522. https://doi.org/10.1111/1365-
727 2656.12244

728 Killen, S.S., Marras, S., Ryan, M.R., Domenici, P., Mckenzie, D.J., 2012. A relationship
729 between metabolic rate and risk-taking behaviour is revealed during hypoxia in juvenile
730 European sea bass. Funct. Ecol. 26, 134–143. https://doi.org/10.1111/j.1365-
731 2435.2011.01920.x

732 Krishnamoorthy, K., Lee, M., 2014. Improved tests for the equality of normal coefficients of

733 variation. Comput. Stat. 29, 215–232. https://doi.org/10.1007/s00180-013-0445-2

734 Kristensen, E., 2008. Mangrove crabs as ecosystem engineers; with emphasis on sediment
735 processes. J. Sea Res. 59, 30–43. https://doi.org/10.1016/j.seares.2007.05.004

736 Lefrançois, C., Ferrari, R.S., Moreira Da Silva, J., Domenici, P., 2009. The effect of
737 progressive hypoxia on spontaneous activity in single and shoaling golden grey mullet
738 *Liza aurata*. J. Fish Biol. 75, 1615–1625. https://doi.org/10.1111/j.1095-
739 8649.2009.02387.x

740 Leiva, F.P., Niklitschek, E.J., Paschke, K., Gebauer, P., Urbina, M.A., 2016. Tide-related
741 biological rhythm in the oxygen consumption rate of ghost shrimp (*Neotrypaea*
742 *uncinata*). J. Exp. Biol. 219, 1957–1960. https://doi.org/10.1242/jeb.133785

743 Lemarié, G., Dosdat, A., Covès, D., Dutto, G., Gasset, E., Person-Le Ruyet, J., 2004. Effect of
744 chronic ammonia exposure on growth of European seabass (*Dicentrarchus labrax*)
745 juveniles. Aquaculture 229, 479–491. https://doi.org/10.1016/S0044-8486(03)00392-2

746 Lenth, R. V., 2016. Least-squares means: The R package lsmeans. J. Stat. Softw. 69.
747 https://doi.org/10.18637/jss.v069.i01

748 Love, A.C., Crooks, N., Ford, A.T., 2020. The effects of wastewater effluent on multiple
749 behaviours in the amphipod, Gammarus pulex. Environ. Pollut. 267, 115386.
750 https://doi.org/10.1016/j.envpol.2020.115386

751 Luppi, T., Bas, C., Méndez Casariego, A., Albano, M., Lancia, J., Kittlein, M., Rosenthal, A.,
752 Farías, N., Spivak, E., Iribarne, O., 2013. The influence of habitat, season and tidal
753 regime in the activity of the intertidal crab *Neohelice (=Chasmagnathus) granulata*.
754 Helgol. Mar. Res. 67, 1–15. https://doi.org/10.1007/s10152-012-0300-9

755 Luquet, C.M., Cervino, C.O., Ansaldo, M., Pereyra, V.C., Kocmur, S., Dezi, R.E., 1998.
756 Physiological response to emersion in the amphibious crab *Chasmagnathus granulata*
757 *Dana* (Decapoda Grapsidae): Biochemical and ventilatory adaptations. Comp. Biochem.
758 Physiol. - A Mol. Integr. Physiol. 121, 385–393. https://doi.org/10.1016/S1095-
759 6433(98)10148-4

760 Melvin, S.D., 2016. Short-term exposure to municipal wastewater influences energy, growth,
761 and swimming performance in juvenile Empire Gudgeons (*Hypseleotris compressa*).
762 Aquat. Toxicol. 170, 271–278. https://doi.org/10.1016/j.aquatox.2015.06.003

763 Naylor, E., 1997. Crab clocks rewound. Chronobiol. Int. 14, 427–430.
764 https://doi.org/10.3109/07420529709001462

765 Ombres, E.H., Donnelly, J., Clarke, M.E., Harms, J.H., Torres, J.J., 2011. Aerobic and
766 anaerobic enzyme assays in Southern California Rockfish: Proxies for physiological and
767 ecological data. J. Exp. Mar. Bio. Ecol. 399, 201–207.
768 https://doi.org/10.1016/j.jembe.2010.11.007

769 Pearson, T.H., Rosenberg, R., 1978. Macrobenthic succession in relation to organic
770 enrichment and pollution of the marine environment. Oceanogr. Mar. Biol. An Annu.
771 Rev. 16, 229–311.

772 Penha-Lopes, G., Bartolini, F., Limbu, S., Cannicci, S., Kristensen, E., Paula, J., 2009. Are
773 fiddler crabs potentially useful ecosystem engineers in mangrove wastewater wetlands?
774 Mar. Pollut. Bull. 58, 1694–1703. https://doi.org/10.1016/j.marpolbul.2009.06.015

775 Rivera-Ingraham, G.A., Barri, K., Boël, M., Farcy, E., Charles, A.L., Geny, B., Lignot, J.H.,
776 2016. Osmoregulation and salinity-induced oxidative stress: Is oxidative adaptation
777 determined by gill function? J. Exp. Biol. 219, 80–89. https://doi.org/10.1242/jeb.128595

778 Rodgers, G.G., Tenzing, P., Clark, T.D., 2016. Experimental methods in aquatic respirometry:
779 The importance of mixing devices and accounting for background respiration. J. Fish
780 Biol. 88, 65–80. https://doi.org/10.1111/jfb.12848

781 Romano, N., Zeng, C., 2013. Toxic Effects of Ammonia, Nitrite, and Nitrate to Decapod
782 Crustaceans: A Review on Factors Influencing their Toxicity, Physiological
783 Consequences, and Coping Mechanisms. Rev. Fish. Sci. 21, 1–21.
784 https://doi.org/10.1080/10641262.2012.753404

785 Shchegolkova, N.M., Krasnov, G.S., Belova, A.A., Dmitriev, A.A., Kharitonov, S.L.,
786 Klimina, K.M., Melnikova, N. V., Kudryavtseva, A. V., 2016. Microbial community
787 structure of activated sludge in treatment plants with different wastewater compositions.
788 Front. Microbiol. 7, 1–15. https://doi.org/10.3389/fmicb.2016.00090

789 Stillman, J.H., Barnwell, F.H., 2004. Relationship of daily and circatidal activity rhythms of
790 the fiddler crab, *Uca princeps*, to the harmonic structure of semidiurnal and mixed tides.
791 Mar. Biol. 144, 473–482. https://doi.org/10.1007/s00227-003-1213-6

792 Tam, N.F.Y., Wong, Y.S., 1995. Mangrove soils as sinks for wastewater-borne pollutants.

Hydrobiologia 295, 231–241. https://doi.org/10.1007/BF00029130

Theuerkauff, D., Rivera-Ingraham, G.A., Lambert, S., Mercky, Y., Lejeune, M., Lignot, J.H., Sucré, E., 2020. Wastewater bioremediation by mangrove ecosystems impacts crab ecophysiology: In-situ caging experiment. Aquat. Toxicol. 218, 105358. https://doi.org/10.1016/j.aquatox.2019.105358

Theuerkauff, D., Rivera-Ingraham, G.A., Mercky, Y., Lejeune, M., Lignot, J.H., Sucré, E., 2018. Effects of domestic effluent discharges on mangrove crab physiology: Integrated energetic, osmoregulatory and redox balances of a key engineer species. Aquat. Toxicol. 196, 90–103. https://doi.org/10.1016/j.aquatox.2018.01.003

Thurman, C.L., 2004. Unravelling the ecological significance of endogenous rhythms in intertidal crabs. Biol. Rhythm Res. 35, 43–67. https://doi.org/10.1080/09291010412331313232

Ungherese, G., Ugolini, A., 2009. Sandhopper solar orientation as a behavioural biomarker of trace metals contamination. Environ. Pollut. 157, 1360–1364. https://doi.org/10.1016/j.envpol.2008.11.038

Urbina, M.A., Forster, M.E., Glover, C.N., 2011. Physiology & Behavior Leap of faith : Voluntary emersion behaviour and physiological adaptations to aerial exposure in a non-aestivating freshwater fish in response to aquatic hypoxia. Physiol. Behav. 103, 240–247. https://doi.org/10.1016/j.physbeh.2011.02.009

Weihrauch, D., Becker, W., Postel, U., Luck-Kopp, S., Siebers, D., 1999. Potential of active excretion of ammonia in three different haline species of crabs. J. Comp. Physiol. - B Biochem. Syst. Environ. Physiol. 169, 25–37. https://doi.org/10.1007/s003600050190

Weis, J.S., Weis, P., 2004. Behavior of four species of fiddler crabs, genus Uca, in southeast Sulawesi, Indonesia. Hydrobiologia 523, 47–58. https://doi.org/10.1023/B:HYDR.0000033093.84155.1d

Wong, Y.S., Tam, N.F.Y., Lan, C.Y., 1997. Mangrove wetlands as wastewater treatment facility: A field trial. Hydrobiologia 352, 49–59. https://doi.org/10.1007/978-94-011-5234-1_6

Xiang, H., Li, K., Cao, L., Zhang, Z., Yang, H., 2020. Impacts of pollution, sex, and tide on the time allocations to behaviours of *Uca arcuata* in mangroves. Sci. Total Environ. 742,

140609. https://doi.org/10.1016/j.scitotenv.2020.140609

Zeil, J., Hemmi, J.M., 2006. The visual ecology of fiddler crabs. J. Comp. Physiol. A Neuroethol. Sensory, Neural, Behav. Physiol. 192, 1–25. https://doi.org/10.1007/s00359-005-0048-7

Zilius, M., Bonaglia, S., Broman, E., Chiozzini, V.G., Samuiloviene, A., Nascimento, F.J.A., Cardini, U., Bartoli, M., 2020. N2 fixation dominates nitrogen cycling in a mangrove fiddler crab holobiont. Sci. Rep. 10, 1–14. https://doi.org/10.1038/s41598-020-70834-0

Zimmer, A.M., Wood, C.M., 2017. Acute exposure to high environmental ammonia (HEA) triggers the emersion response in the green shore crab. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 204, 65–75. https://doi.org/10.1016/j.cbpa.2016.11.008

Zuur, A.F., Hilbe, J.M., Ieno, E.N., 2013. A Beginner's Guide to GLM and GLMM with R, Beginner's Guide Series. Highland Statistics Limited.