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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] MARBEC (MARine Biodiversity, Exploitation and Conservation), Univ Montpellier, CNRS, Ifremer, IRD, 34000 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

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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 (6 h) 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 6 h 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 h. Crabs actively emersed from WW and reduced their activity and mobility after 6 h. A higher metabolic rate in WW occurred immediately (t = 0 h), 3 and 6 h 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.

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 (5–6 h duration: two times / day) occurring on chosen natural mangrove areas where both vegetation and macrofauna were monitored

* Correspondence to: MARBEC (MARine Biodiversity, Exploitation and Conservation), Univ Montpellier, CNRS, Ifremer, IRD.
E-mail addresses: laura.megevand@umontpellier.fr (L. Mégevand), pauline.kreienbuhl@gmail.com (P. Kreienbühl), dimitri.theuerkauff@stareso.com (D. Theuerkauff), jehan-herve.lignot@umontpellier.fr (J.-H. Lignot), elliott.sucre@univ-mayotte.fr (E. Sucré).

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(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 (5 h) 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 recognise 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 hypothesised 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.

All animals were hand collected at low tide when crabs are active and out of their burrows. They were placed into individual boxes for transportation in order to minimise stress and fights. These males were then directly transported to the University Centre of Mayotte (CUFR, Dembeni, France) and placed for three days for acclimation in tanks containing natural seawater (~33‰ salinity; 1050 mOsmol kg^{-1}), under a natural photoperiod (12 h light: 12 h dark) and without feeding prior to experimentation. These crabs (140) were acclimated to an aerial-aquatic environment (crabs could move freely between these two environments). The acclimation microcosms were the same as those used for the behavioural experiment.

For behavioural analyses, 60 crabs were studied. For the respirometry experiment, 40 crabs were studied. For the biochemical analysis on metabolic markers (citrate synthase, CS and lactate dehydrogenase, LDH enzyme activity), 40 crabs were studied. No mortality was observed during acclimation and experimental processes.

2.2. Wastewater sampling and conservation

Samples of raw wastewater effluent (WW) were directly collected from the Dembeni wastewater treatment plant. In order to minimise any possible variation in WW composition during the experiments, 100 litres were collected on a single day. WW was then distributed in 1 litre containers and stored at −20 °C for the duration of the experiments (up

to 40 days) in order to stop bacterial proliferation and to ensure a better conservation of nitrogen compounds. This WW treatment plant was chosen because basic water parameters were routinely monitored due to the European water framework directive (WFD). In addition, frozen samples of collected WW were sent to CHROME laboratory (University of Nîmes, France) to assess NO_3^-, NO_2^-, NH_4^+, PO_4^{3} concentrations and additional parameters on Metrohm ion chromatography system (Metrohm, Switzerland) with conductivity detection (see Table 1).

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

Ammonia-N (called N in our experimental design) was chosen as a second experimental treatment because the ionised form of ammonia-N ($NH4^+$) is the major component of WW (Table 1). This condition referred to total ammonia (ammonia-N), which represents the sum of unionised ammonia NH3 and ionised ammonia $NH4^+$ (Lemarié et al., 2004). Finally, focusing on single ammonia-N effects in a separate experimental condition allow us to avoid pollutant cocktail and potential hypoxic effects (aerobic bacteria that require oxygen to degrade the substrate) (Gerardi, 2006) caused by WW on crabs physiology.

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

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

2.4. Behavioural endpoints: locomotor activity, agitation and emersion choice

Behavioural endpoints of *P. chlorophthalmus* were assessed using four independent 28 × 22 × 16 cm microcosms for each exposure condition, placed on the floor on a polystyrene plate to minimise any potential vibration. Microcosms were divided into two communicating 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 0 h trials started 5 min after the crabs were placed in the microcosms, in order to minimise the potential stress due to handling. Videos were recorded at 0 h, 3 h and 6 h hours during 15 min 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.

Table 1
Physical and chemical characterisation (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).

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.

2.5. Oxygen consumption rate

The experimental design consisted in a static, intermittent flow-through respirometry system based on Clark et al. (2013). Crabs were individually placed into 125 ml chambers allowing them to make sporadic movements considering their size, and at the same time ensure accurate measurements of O_2 consumption. Following procedures described in Killen (2014), O_2 measurements were performed using a 4-channel fiber-optic system with contactless O_2 sensor spots (FireSting O_2, PyroScience, GmbH, Aachen, Germany) where water oxygen content was quantified once every 5 s

Water-mixing within the chambers was achieved with magnetic stirrers located under 1 mm^2 mesh grid to avoid too much disturbance (Rivera-Ingraham et al., 2016). Sensors were calibrated to 100% (using air-saturated water) and 0% air saturation. Chambers were filled with a tubing system providing control SW or contaminated water from aerated, filtered and temperature-controlled tanks.

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

Following acclimation, the system was filled with the exposure solution. An automated flush pump was switched on for 20 min to ensure proper mixing and was cut off during 40 min. During that time, the chambers were sealed. The decrease in oxygen content could be analysed to indicate the rate of oxygen uptake. After the 40-minute cycle, the pump was turned on to flush the metabolic chambers with aerated seawater (or aerated WW or diluted seawater enriched with ammonia-N) during 20 min. Due to the presence of aerobic and anaerobic microorganisms capable of degrading organic compounds and consuming O_2 (Shchegolkova et al., 2016), a 20 min flushing was run to ensure 99% air saturation. The 40 min measurements allowed accurate O_2 measurements without falling under the threshold of 70% air saturation in order to maintain aerobic metabolism and to avoid hypoxic stress (Rodgers et al., 2016).

Similarly to the behavioural experiment, O_2 measurements were recorded at 0 h, 3 h and 6 h for all individuals (N = 10 per condition).

The same procedure was applied for blanks chambers for each condition (N = 8 per condition) and then subtracted from the MO_2 per individual.

At the end of each experiment, animals were weighed and put back in aerial/aquatic aquariums before being released into the mangrove.

2.6. Sampling and dissections

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

2.7. Metabolic markers

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

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

Citrate synthase activity was used as a marker of mitochondrial content. CS was measured using a kit from Sigma Aldrich (#MAK193) following the manufacturer's instructions. Briefly, 2–8 mg of anterior gill, posterior gill, and muscle of the big cheliped were homogenised in 100 μL of icecold CS Assay Buffer. Then, samples were centrifuged at 10,000 g for 5 min 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 min at 25 °C and the absorbance was recorded at 412 nm every 5 min for 40 min. The colorimetric product (GSH) was proportional to the enzymatic activity of CS and normalised 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, 2–8 mg of anterior gill, posterior gill, and muscle of the big cheliped were homogenised in 200 μL of ice-cold LDH Assay Buffer. Then, the samples were centrifuged at 10,000 g for 15 min 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 (0–12.5 nmol/well). The plate was incubated at 37 °C and the absorbance was recorded at 450 nm every 5 min for 30 min. The colorimetric product (NADH) was proportional to the enzymatic activity of LDH and normalised 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 generalised 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 (0 h, 3 h, 6 h) (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 generalised linear mixed model approach was taken assuming Gaussian distribution error with log link. As with above, time (0 h, 3 h, 6 h) 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 6 h exposure. All values are represented as average ± SEM.

4. Results

4.1. Behavioural endpoints

Results of the behavioural analyses are depicted in Figs. 1 and 2, which include temporal tracking responses of 0 h, 3 h, 6 h 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 3 h compared with those exposed to DSW following multiple comparison test with Bonferroni correction.

For 0 h and 6 h, 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 3 h compared with the first time point (0 h).

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 3 h and that crabs exposed to Ammonia-N were more mobile than crabs exposed to DSW at the same time point (3 h) (Fig. 1B). For 0 h and 6 h, no significant difference in mobility between treatments was

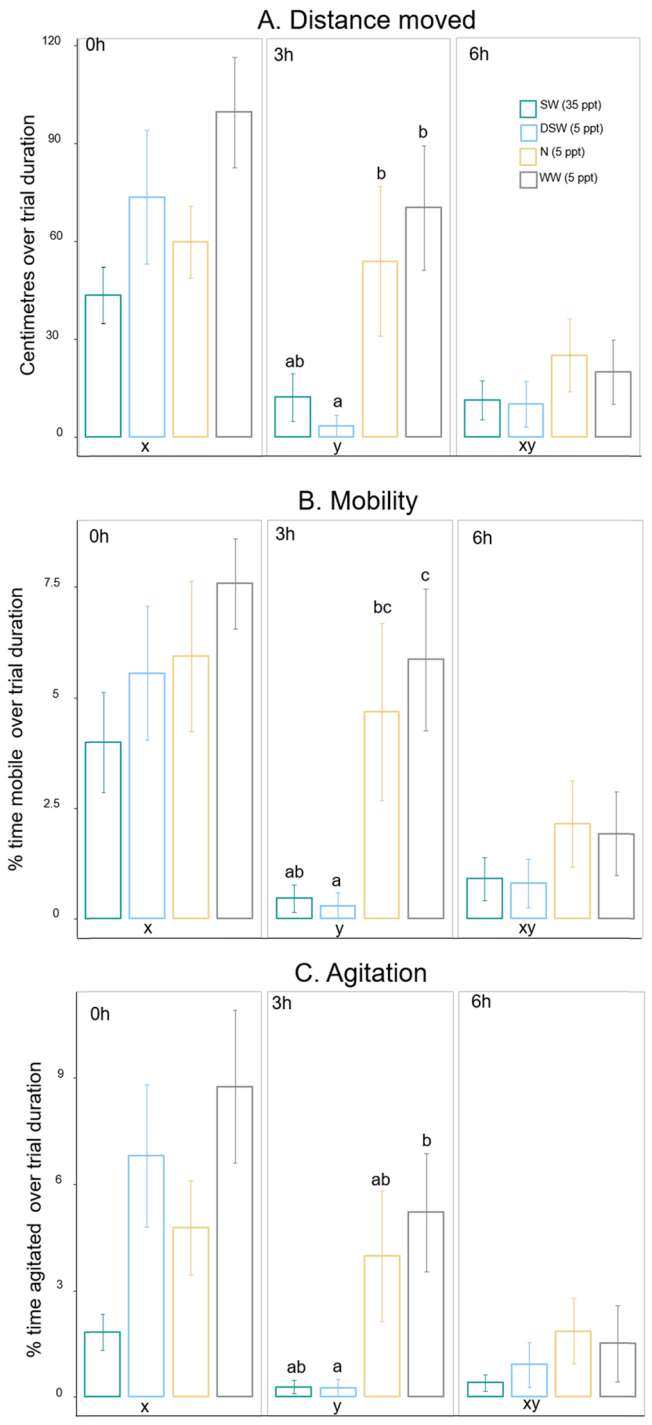

Fig. 1. Averages of (A) distance moved by crabs, (B) percentage of time where crabs were mobile, and (C) percentage of time where crabs were agitated at 0 h, 3 h and 6 h exposure to SW, DSW, N and WW. Different colours represent treatments (see legend). Values are mean ± SE. N = 15 per treatment. Different letters on the top (a,b,c) and at the bottom (x,y,) of bar plots indicate significant two-way interaction effects of treatment and time respectively after Wald Chi-square test and pairwise multiple comparisons (Bonferroni corrected).

observed. Considering exposure time factor level, crabs exposed to DSW showed a significantly reduced mobility at 3 h compared with the first time point (0 h), similarly to distance moved.

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

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

As depicted in Fig. 2, time spent in aerial environment varied a lot between individuals for all treatment groups at 0 h with no clear preference between the aerial or aquatic environment (test statistic = 1.98, p = 0.56, CV: SW = 143.58, DSW = 89.64, N = 81.26, WW = 81.65). At 3 h, 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 6 h, 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.

4.2. Oxygen consumption

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

A significant effect ($p < 0.001$) of treatment and exposure time on O_2 consumption (μmol $O_2 \cdot m^{-1} \cdot g^{-1}$) 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 (0 h) (Fig. 3A). At 3 h, WW exposed crabs consumed significantly more O_2 than DSW and SW exposed crabs. At 6 h, 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 0 h, 3 h, 6 h. A significant effect ($p < 0.01$) of treatment and exposure time on O_2 consumption (μmol $O_2 \cdot min^{1}$) 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 0 h and 3 h, and 3 h and 6 h, with a clear burst in consumption at 6 h (Fig. 3B). Bacterial O_2 consumption in WW was significantly higher at 6 h compared with all the other conditions (DSW, SW and N).

4.3. Metabolic markers

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

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

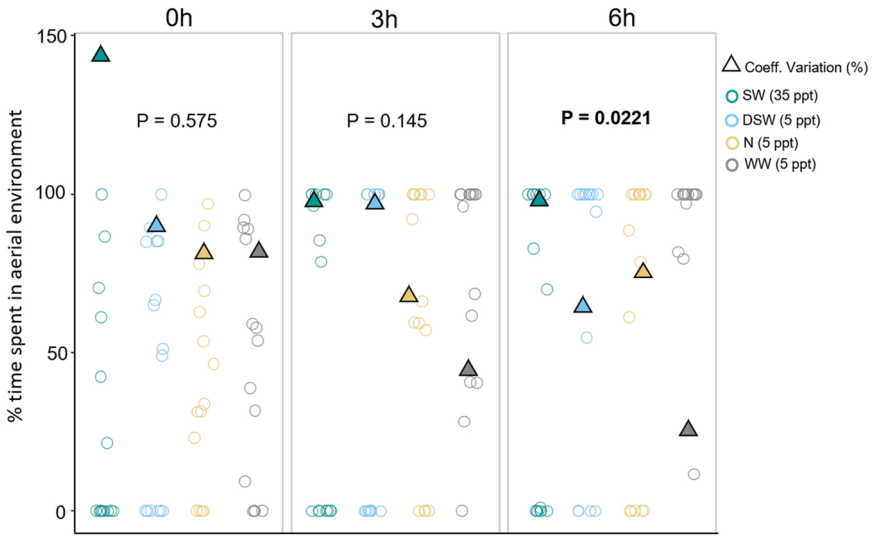

Fig. 2. Percentage of time that crabs exposed to DSW, SW, N and WW spent in aerial environment at 0 h, 3 h, and 6 h (individual values). Different colours represent treatments (see legend). Circles represent individuals (N = 15 per treatment). Triangles represent the coefficient of variation by treatment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

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

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**

5. Discussion

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

In this research, crabs exposed to WW and ammonia-N exhibited a stimulation of their locomotor behaviour (distance moved and time spent mobile) that became significantly different from DSW and SW controls after 3 h. At the beginning of the exposure (0 h), no significant difference was found between the treatments and most of the animals exhibited erratic behaviours (Fig. 1A, B, C). This could be explained by a potential stress coupled with an exploring activity of crabs in their new environment. After 3 h exposure, DSW exposed crabs decreased significantly their locomotor behaviour and agitation at a very low rate in contrast to 0 h (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 3 h 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 3 h 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 3 h 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 6 h 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 6 h of exposure.

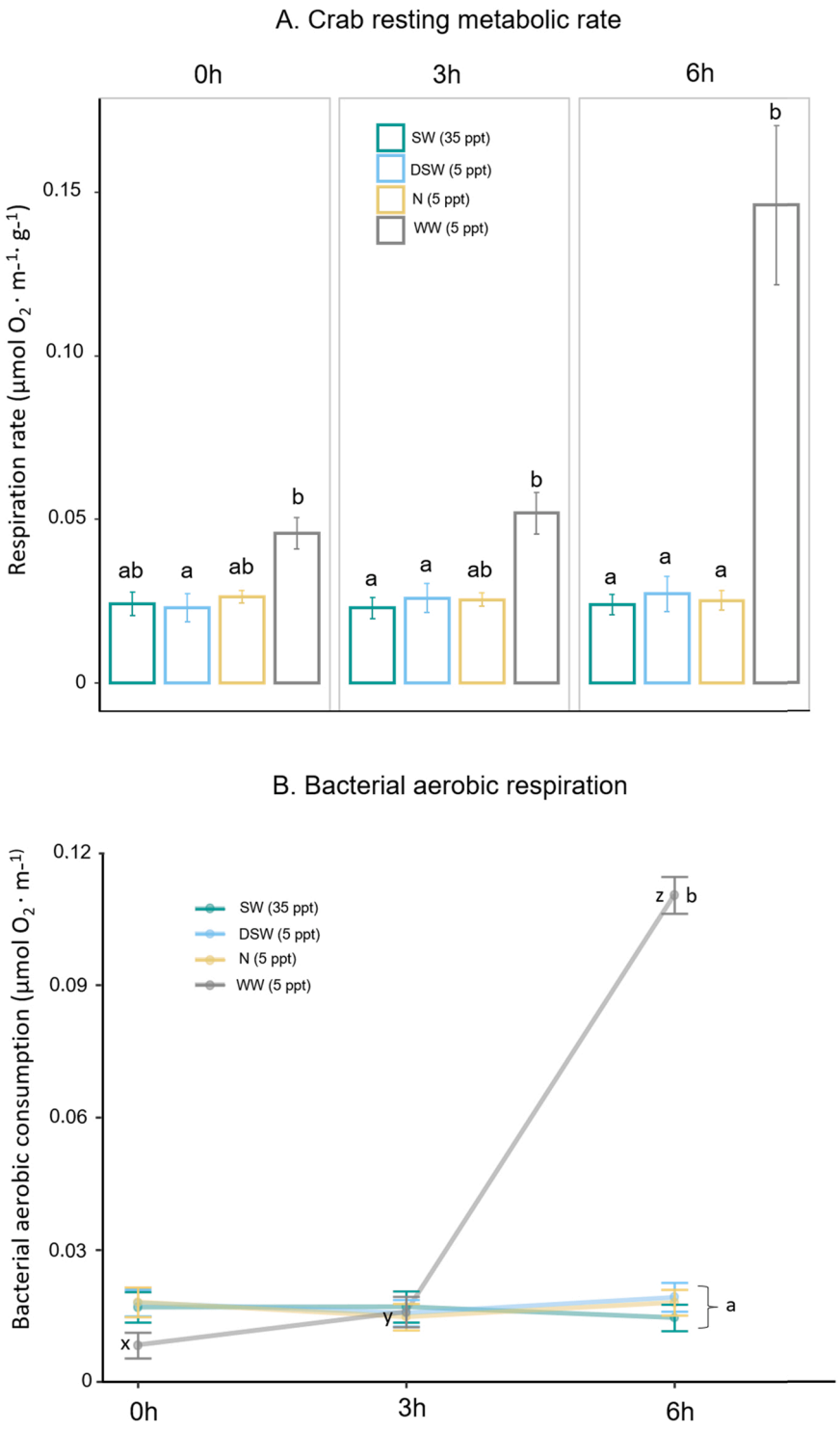

Fig. 3. Resting metabolic rate of *P. chlorophthalmus* (A, N = 10 per treatment) and O_2 consumption by aerobic bacteria (B, N = 8 per treatment) in SW, DSW, N and WW at 0 h, 3 h, and 6 h exposure. Values are mean ± SE. Different letters on the top (a,b,c) and at the bottom (x,y,) of barplots indicate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

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 3 h 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 with an aqueous environment even under terrestrial conditions and keep a supply of water in the branchial chamber, bathing the gills due to morphological and physiological adaptations, such as releasing carbon dioxide (CO_2) and ammonia (NH_3) (Henry and Wheatly, 1992; Taylor

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

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**

and Butler, 1973). However, several studies showed that whole body emersion behaviour happens in crabs facing environmental stress such as exposure to contaminants, temperature increase or hypoxia (nuanced with some species-specific responses) (de Lima et al., 2021; Taylor et al., 1973). Our results showed that crabs exposed to WW spend substantially more time in air as an escape behaviour with a clear preference for this environment (see Fig. 2), since they are almost all emerged after 6 h exposure, and they all emerged themselves at least once.

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

Acute exposure to high environmental ammonia (HEA) triggered emersion responses in the green shore crab *Carcinus maenas* at high NH_4 concentrations (4 and 10 mmol.L^{-1} against 1 mmol.L^{-1} in the present study) (Zimmer and Wood, 2017). However, intertidal crabs such as *P. chlorophthalmus* are usually exposed to lower levels of ammonia-N. In this regard, a recent study demonstrated the involvement of the fiddler crab holobiont in the nitrogen cycle around the animals, and the importance of considering invertebrate-bacteria associations in understanding biogeochemical processes in mangroves (Zilius et al., 2020). The authors suggest that in the presence of eutrophic conditions, such as regular WW discharges, the composition of the holobiont biofilm could potentially be modified and have an impact on the biogeochemical conditions of the environment near the animals, and thus potentially on their 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 3 h of exposure) but did not show a full emersion response since excretion of nitrogen products must occur in water (Luquet et al., 1998).

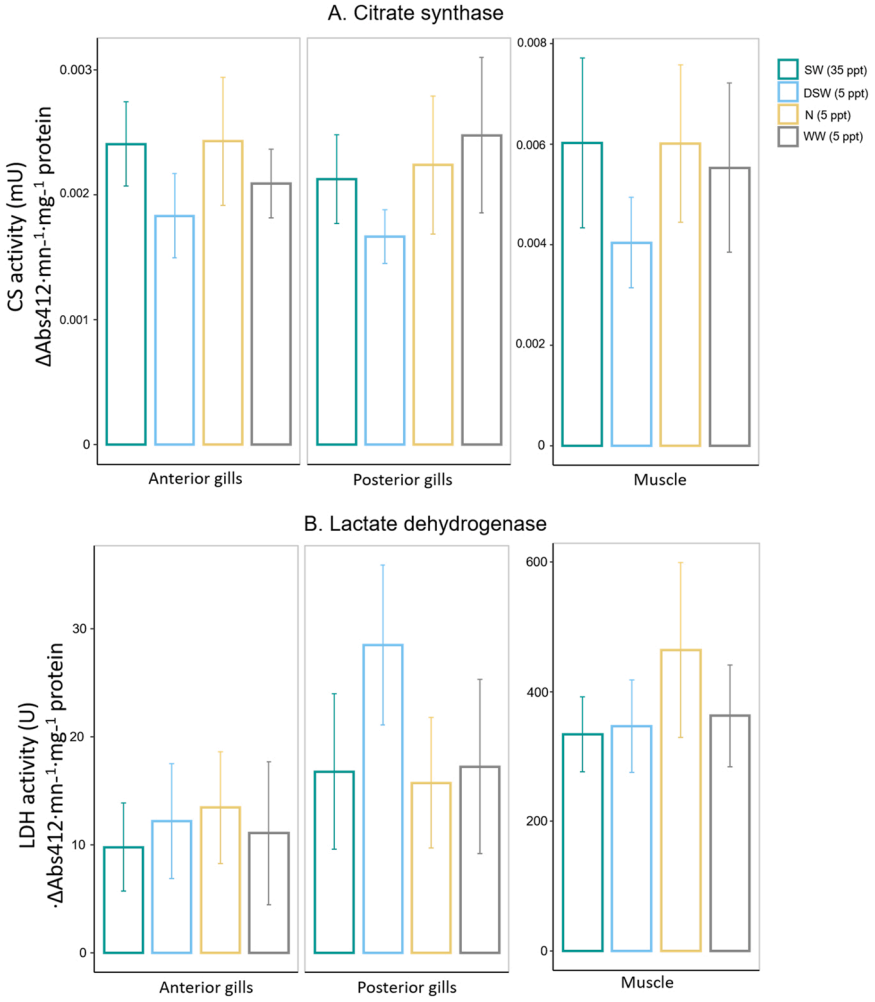

Fig. 4. Activity of citrate synthase (A) and lactate dehydrogenase (B) in anterior gill, posterior gill and cheliped muscles of *P. chlorophthalmus* exposed to DSW, SW, N and WW for 6 h. N = 10 per treatment. Values are mean ± SE and different colours represent treatments (see legend).

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 6 h 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 (Figs. 3A, 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 nitrate to ammonia by micro-organisms, increasing ammonia concentration particularly when nitrate concentration is high (Henry et al., 2017), which is the case in WW. These combined, synergistic effects could explain the acute behavioural and metabolic responses observed in crabs exposed to a short-term (6 h) WW pulse.

6. Conclusion

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

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

CRediT authorship contribution statement

Laura Mégevand: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. **Pauline Kreienbühl:** Conceptualization, Methodology, Investigation, Formal analysis. **Dimitri Theuerkauff:** Methodology, Investigation, Writing – review & editing. **Elliott Sucré:** Supervision, Conceptualization, Investigation, Project administration, Writing – review & editing **Jehan-Hervé Lignot:** Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

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

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

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

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

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

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

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

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

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

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

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

Careau, V., Thomas, D., Humphries, M.M., Réale, D., 2008. Energy metabolism and animal personality. Oikos. https://doi.org/10.1111/j.0030-1299.2008.16513.x.

Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of climate change: Respirometry, relevance and recommendations. J. Exp. Biol. 216, 2771–2782. https://doi.org/10.1242/jeb.084251.

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

Crane, J., 1975. Fiddler Crabs of the World: Ocypididae: Genus Uca. Princeton University Press, New Jersey. https://doi.org/10.1016/0003-3472(78)90130-6.

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

Du, S.N.N., McCallum, E.S., Vaseghi-Shanjani, M., Choi, J.A., Warriner, T.R., Balshine, S., Scott, G.R., 2018. Metabolic costs of exposure to wastewater effluent lead to compensatory adjustments in respiratory physiology in Bluegill sunfish. Environ. Sci. Technol. 52, 801–811. https://doi.org/10.1021/acs.est.7b03745.

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

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

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

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

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

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

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

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

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

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

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

Krishnamoorthy, K., Lee, M., 2014. Improved tests for the equality of normal coefficients of variation. Comput. Stat. 29, 215–232. https://doi.org/10.1007/s00180-013-0445-2.

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

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

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

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

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

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

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

Marwick, B., Krishnamoorthy, K.. cvequality: Tests for the Equality of Coefficients of Variation from Multiple Groups. R package version 0.1.3. https://cran.r-project.org/web/packages/cvequality/.

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

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

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

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

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

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

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

Romano, N., Zeng, C., 2013. Toxic effects of ammonia, nitrite, and nitrate to decapod crustaceans: a review on factors influencing their toxicity, physiological consequences, and coping mechanisms. Rev. Fish. Sci. 21, 1–21. https://doi.org/10.1080/10641262.2012.753404.

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

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

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.

Taylor, EW., Butler, P., 1973. The behaviour and physiological reponses of the shore crab *Carcinus maenas* during changes in environmental oxygen tension. J. Sea Res. 505, 496–505.

Taylor, E.W., Butler, P.J., Sherlock, P.J., 1973. The respiratory and cardiovascular changes associated with the emersion response of *Carcinus maenas* (L.) during environmental hypoxia, at three different temperatures. J. Comp. Physiol. 86, 95–115. https://doi.org/10.1007/BF00702531.

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. Sens. 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.