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Wastewater bioremediation by mangrove ecosystems impacts crab ecophysiology: *in-situ* caging experiment

Dimitri Theuerkauff[1,2*], Georgina A. Rivera-Ingraham[1†], Sophia Lambert[2], Yann Mercky[1,2], Mathilde Lejeune[1,2], Jehan-Hervé Lignot[1] and Elliott Sucré[1,2]

[1)] UMR MARBEC (University of Montpellier, CNRS, IFREMER, IRD), Montpellier, France

[2)] Centre Universitaire de Mayotte, Route Nationale 3, BP 53, 97660 Dembeni, Mayotte, France

[†]Present address: Laboratoire Environnement de Petit Saut, Hydreco-Guyane. BP 823, 97388. Kourou, French Guiana

[*)] Address for correspondence: dimitri.theuerkauff@univ-mayotte.fr

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Abstract

Mangroves are tidal wetlands that are often under strong anthropogenic pressures, despite the numerous ecosystem services they provide. Pollution from urban runoffs is one such threats, yet some mangroves are used as a bioremediation tool for wastewater (WW) treatment. This practice can impact mangrove crabs, which are key engineer species of the ecosystem. Using an experimental area with controlled WW releases, this study aimed to determine from an ecological and ecotoxicological perspective, the effects of WW on the red mangrove crab *Neosarmatium africanum*. Burrow density and salinity levels (used as a proxy of WW dispersion) were recorded, and a 3-week caging experiment was performed. Hemolymph osmolality, gill Na^+/K^+-ATPase (NKA) activity and gill redox balance were assessed in anterior and posterior gills of *N. africanum*. Burrow density decreased according to salinity decreases around the discharged area. Crabs from the impacted area had a lower osmoregulatory capacity despite gill NKA activity remaining undisturbed. The decrease of the superoxide dismutase activity indicates changes in redox metabolism. However, both catalase activity and oxidative damage remained unchanged in both areas but were higher in posterior gills. These results indicate that WW release may induce osmoregulatory and redox imbalances, potentially explaining the decrease in crab density. Based on these results we conclude that WW release should be carefully monitored as crabs are key players involved in the bioremediation process.

Keywords: Mangrove, Bioturbation, Wastewater, Ecophysiology, Oxidative stress, Osmoregulation

1 Introduction

Mangroves are critically threatened by human activities worldwide (Duke et al., 2007; Polidoro et al., 2010) although their ecological and socioeconomic importance are now well documented (Lee et al., 2014). They cover 137,760 km^2 in 118 tropical and subtropical countries and territories (Giri et al., 2011) and are characterized by being a unique coastal forest between land and sea.

Among the different possible threats, mangroves receive nutrients and pollutants from urban runoffs (Fusi et al., 2016; Lesirma, 2016; MacDonnell et al., 2017). However, mangrove trees are capable of absorbing excess in nutrient load (Reef et al., 2010) and are thus used in many tropical countries as a bioremediation tool for wastewater treatment (Ouyang and Guo, 2018). Whether natural or constructed mangrove wetlands (mesocosm), they are used for treating WW from aquaculture, sewage or others sources (Leung et al., 2016; Ouyang and Guo, 2016). WW is often a complex mixture of many pollutants, the most notable of which are the excess of organic matter and nutrients (mainly ammonium and phosphorus) but may also contain pharmaceutical residues, pesticides, heavy metals, etc. Moreover, recent studies in China showed that natural mangroves are the optimal paradigm under three scenarios of municipal WW treatment (Ouyang and Guo, 2018). In addition to eliminating excess nutrient and organic load, mangrove plants may bioaccumulate metals (Analuddin et al., 2017), and even immobilize some wastewater-borne pollutants, such as polycyclic aromatic hydrocarbons and polybrominated diphenyl ethers in the shape of iron plaques formed on their roots (Pi et al., 2017). They can also even mitigate different antibiotics (Li et al., 2016a; Liu et al., 2016). While most of these studies focus on plant or microbial communities, previous studies looking at the impact of WW on mangrove macrofauna showed contradictory effects (Bartolini et al., 2009; Cannicci et al., 2009; Capdeville et al., 2018; Fusi et al., 2016; Penha-Lopes et al., 2009b; Yu

et al., 1997). Thus, further focusing on the specific physiological consequences for macrofauna is still needed.

Among the macrofauna, burrowing mangrove crabs such as *Neosarmatium africanum* (Sesarmidae, de Man, 1887), previously known as *Neosarmatium meinerti* in Mayotte (Theuerkauff et al., 2018a), are engineer species playing a key role in the mangrove ecosystem: their bioturbation and organic matter degradation activities are crucial in element transfer within the matter cycle and allow the maintenance of sediment biochemical heterogeneity (Emmerson and McGwynne, 1992; Kristensen, 2008; Lee, 1998). They are also involved in the bioremediation process since their burrows allow WW to enter the sediment and to be absorbed by mangrove trees. Crab burrows also increase the air-sediment layer up to 400% and thus, impact microbial and geochemical processes (Kristensen and Kostka, 2013).

In the context of mangrove use for biofiltering, it is therefore crucial, to determine if these crabs are impacted by WW discharge. If this is the case, it could induce in the long term profound and global modifications of the ecological functioning of the mangrove ecosystem. Regular wastewater discharges may decrease crab abundance as shown for juvenile blue crabs *Portunus pelagicus* along a pollution gradient from the outfall of a secondary treated effluent (Wear and Tanner, 2007). Moreover, previous studies showed that WW may also impact crab community structures (Capdeville et al., 2018) or even their bioturbation activities (Bartolini et al., 2011). Previous results obtained by our group under laboratory conditions showed that WW exposure induces a burst of crab oxygen consumption as well as osmotic and redox imbalances (Theuerkauff et al., 2018a). This study aims to compare the aforementioned laboratory results (in which crabs were directly exposed to domestic WW for 5h) with field experimentation using the same WW discharge. To do this, we here merge the use of ecological markers (e.g. crab burrow density) with physiological indicators of cell homeostasis (e.g. osmoregulation and

energy-redox parameters) carried out in encaged crabs during 3 weeks to adequately evaluate the efficiency and challenges of using mangroves as biofilters.

2 Materials and methods

2.1 Study area

To assess the impact of WW on mangrove crabs, two areas were selected in the Malamani mangrove (Boueni's bay, Mayotte island, 12°55′1″S, 45°9′23″E) located in the Comoros Archipelago (Fig. 1A, B, C). This is an experimental site which has been used since 2008 to evaluate the capacities of the mangrove ecosystem for natural WW treatment (Herteman et al., 2011). Briefly, the WW produced by the nearby village of Malamani (400 population equivalent), is collected, stored and decanted in a pre-treatment (Imhoff) tank. About 10 m^3 of this pretreated WW is discharged daily 1h before low tide approximately every 24h (i.e. a low tide on two) through pierced pipes (Fig. 1E) on three 45m x 15m plots. In this study, only the plot dominated by *Ceriops tagal* and oriented perpendicular to the main coastline was considered (Fig. 1B,C). This area was designated as the "impacted area". A second area that was not subjected to WW discharge was used as a control site (Fig. 1 C). This area is located in the same mangrove belt and is also dominated by *Ceriops tagal* and was chosen as it did not differ from the impacted area before the start of WW discharge (Herteman, 2010). The chemical composition of the pretreated WW, surface water and porewater from impacted and control areas are reported in Table 1. More *in-situ* physicochemical parameters are reported in Capdeville (2018).

2.2 Salinity and crab burrow mapping

116 A field survey was conducted in March 2015 to collect data on WW dispersion and crab burrow
117 density in the Malamani mangrove. Four 80 m-long transects covering the impacted area as
118 well as the control zone were established parallel to the shore. For each transect, 8 plots (1 m^2,
119 located approximately 10 m from each other) were monitored after one day without rain. Thirty
120 two plots were divided in 3 areas: one subjected to WW release and two control areas (one
121 northern and one southern area, located respectively on each side of the impacted area). These
122 plots were in all cases established on flat bottoms only, since it is where WW flows and
123 infiltrates the sediment through the crab burrows. The geographical coordinates of these plots
124 were recorded using a GPS system (Garmin GPSMAR®78S). Osmolality variation (used as a
125 measure of salinity) around the WW discharge was used as a proxy of WW dispersion (as WW
126 salinity is close to freshwater), ensuring that control areas were not impacted by wastewater
127 release. Salinity was measured from 1.5 ml samples collected with a 3 ml plastic pipette from
128 the interstitial water in burrows. The osmotic pressure (OP) of these samples was measured in
129 duplicate by freezing point depression osmometry using an Advanced ™ Micro-Osmometer,
130 model 3300 (Advanced Instruments, Inc.) using 20 µl per sample. The number of crab burrows
131 in a 1 m^2 quadrat was also counted for each plot and used as a an estimation of the crab density
132 (Mouton and Felder, 1996).
133
134 *2.3 Animal collection, caging and sampling*
135 Twenty adult *N. africanum* (Fig. 2) in intermolt stage C3 (as confirmed through epipodite
136 examination) were collected in September 2015 from an undisturbed area of the Malamani
137 mangrove at low tide. Specimens identified before as *N. meinerti* in Mayotte belong in fact to
138 *N. africanum* species (Ragionieri et al., 2012), as recently confirmed by DNA sequencing
139 (Ragionieri, pers. com.). Immediately after collection, ten crabs were individually introduced
140 in artificial burrows (Fig. 1D) in the impacted and control area (Fig. 1C). We chose this caging

141 approach due to the fact that long-term tagging of individuals is not possible in crustaceans due
142 to molting. Each artificial burrow consisted of a closed grid cylinder of 6 cm wide and 50 cm
143 long inserted into a perforated PVC pipe ensuring adequate water flow. These artificial burrows
144 were partially buried in the sediment with an angle of 45° (Fig. 1D). After 3 weeks, in October
145 2015, the crab and some water from each burrow were collected during low tide outside of the
146 WW discharge period and was individually taken to the field laboratory located about 350 m
147 (4-5 minutes walk) from the experimental site. For each crab, a hemolymph sample (without
148 any anticoagulant) was obtained using a 0.5-ml syringe by inserting the needle between the
149 cephalothorax and the first pereiopod. This sample was directly used for OP determination
150 using 20 µl per sample in duplicate as described above. Crabs were then weighed and
151 euthanized on ice for gill sampling. Gill pairs were divided in 2 groups according to their
152 function (Neufeld et al., 1980; Theuerkauff et al., 2018b): anterior pairs (1 to 4) being
153 respiratory gills, and posterior pairs (5 to 8) with both a respiratory and osmoregulatory
154 function. Anterior and posterior gills were flash frozen in liquid nitrogen, stored separately into
155 a container filled with liquid nitrogen (Voyageur 5, Air liquide). After sampling completion,
156 frozen samples were transported to the University Center of Mayotte and then preserved at -80
157 ºC until further analyses (see details in sections 2.4 and 2.5). Crab molting stages were re-
158 checked *post-mortem* to avoid handling-associated stress and only animals in intermolt stage
159 (C3) were considered.
160
161 *2.4 Assessments of antioxidant defenses and oxidative damage in gills*
162 For each crab, antioxidant defenses were quantified as the activities of superoxide dismutase
163 (SOD) and catalase (CAT) and measured spectrophotometrically in anterior and posterior gills
164 (right-sided gills 3 and 5). All frozen samples were diluted in a 50 mmol l^{-1} KPi buffer with 120
165 mmol l^{-1} KCl (pH=7.0) to 1:40 and 1:10 (w:v) for SOD and CAT measurements, respectively.

166 Tissue homogenization was achieved using 3 steel balls (Retsch, n°. FR0120) in a Mixer Mill
167 MM 400 (Retsch GmbH, Haan, Germany; 30s at 30 beats s^{-1}). SOD activity was determined in
168 supernatants after centrifugation (3min, 13000 rpm, 4°C) using the protocol of McCord and
169 Fridovich (1969) and modified by Livingstone et al. (1992). CAT activity was measured in
170 supernatants following the decomposition of H_2O_2 in a 50 mmol l^{-1} KPi buffer according to the
171 assay developed by Aebi (1984). All measurements were carried out at least in triplicate using
172 a microplate reader (Tecan Infinite M200, TECAN, Männendorf, Switzerland). Samples were
173 preserved on ice at all times and results were expressed per mg of proteins and body weight.
174 Protein were quantified in triplicates according to Bradford (1976) with BSA (Sigma-Aldrich,
175 St Louis, MO, USA) as standard. Oxidative damage was estimated as the concentration of
176 malondialdehyde (MDA), a product resulting from lipid peroxidation, in right-sided anterior
177 and posterior gills (gills 4 and 6, respectively). MDA was quantified through measurements of
178 thiobarbituric acid reactive substances (TBARS) using the protocol originally described by
179 Uchiyama and Mihara (1978) and further modified by Abele et al. (2002).
180
181 *2.5 Assessment of Na^+/K^+-ATPase (NKA) activity*
182 NKA activity was determined on right-sided anterior and posterior gills (2 and 7, respectively)
183 by homogenizing tissues separately as described above in a buffer composed of 250 mM
184 sucrose, 5 mM $MgCl_2$ (pH= 7.4) in a 1:6 (w:v) ratio. After centrifugation (3min, 13000 rpm,
185 4°C), protein concentration in supernatants was determined as described above, and all samples
186 were diluted to achieve equal protein concentrations. The specific NKA ouabain-sensitive
187 activity in these supernatants, was measured using a protocol originally by Flik et al. (1983)
188 and adapted from Lorin-Nebel et al. (2013) and Tsai and Lin (2007). Briefly, specific NKA
189 activity was measured in triplicate as the difference of ATP hydrolysis in two different media:
190 both were composed of 100 mM NaCl, 30 mM imidazole, 0,1 mM Na_2EDTA and 5 mM $MgCl_2$,

191 3 mM Na-ATP and pH 7.4 but while one contained 14 mM KCl, the second contained 2.8 mM
192 ouabain. After a 30 min incubation period at 37°C, the reaction was stopped by adding an ice-
193 cold solution composed of TCA 5%, 0.33 mM $FeSO_4$, 0.66 M H_2SO_4 and 9.2 mM ammonium
194 molybdate. The amount of inorganic phosphates released in presence or absence of oubain was
195 colorimetrically measured after 10 min and using Na_2HPO_4 as standard (Sigma, France). The
196 enzyme specific activity was expressed in µmol Pi mg protein^{-1} h^{-1}.
197
198 *2.6 Statistics*
199 All statistical analyses were performed in R version 3.3.2 (R Core Team, 2015) with RStudio
200 Version 0.99.891 (RStudio, Inc). The Shapiro-Wilk test was used to test normality and the
201 Bartlett test was used to test the homogeneity of variances. If data did not meet the assumptions
202 for parametric statistical techniques, they were log transformed. A one-way ANOVA was
203 performed on crab density and a Kruskal-Wallis test followed by a Nemenyi *post hoc*
204 comparison test on surface water salinity. The relationship between crab burrow salinity and
205 crab burrow density was determined using a linear regression. A two-way ANOVA was
206 performed on crab body weight (sex and treatment), oxidative damage, antioxidant defenses
207 and NKA activities according to gill position and treatment. All factors were considered as
208 orthogonal and fixed. All ANOVA tests were followed by a Tukey's HSD *post hoc* comparison
209 test and significance level was $p < 0.05$. Hemolymph osmotic pressures are reported with the
210 osmotic curve previously published for *N. africanum* (Theuerkauff et al., 2018b). The residuals
211 between hemolymph osmotic pressures and those given by the osmotic curve are compared by
212 a Student t-test.
213
214 **3 Results**
215

3.1 Surface water salinity and crab burrow density

Salinity values of the crab burrow residual water ranged from 64 $mOsm \cdot kg^{-1}$ to 1360 $mOsm \cdot kg^{-1}$. Due to the effect of the WW effluent, salinity values significantly decreased in the impacted area (Kruskal-Wallis test, $p < 0.001$) (Fig. 3A). The crab burrow density demonstrates a similar pattern since lower density was found in the impacted area (Fig. 3B; one-way ANOVA; df error = 29; $F = 45.5$; $p < 0.001$) and the variations in crab density are correlated (Fig. 3C) with the variations in salinity ($r^2 = 0.58$, $p < 0.001$).

3.2 Crab size

Crab body weight was significantly different between males and females (47.4g ± 8.9g and 31.1g ± 2.6g, respectively) but not between treatments (two-way ANOVA; df error = 16; sex $F = 34.2$; df = 1; $p < 0.001$; treatment $F = 1.3$; df = 1; interaction: $F = 2.9$; df = 1). Except for weight, no sex-related differences were recorded and thus, only pooled results are shown. No mortality was recorded throughout the experiment.

3.3 Osmotic pressure and NKA activity

Hemolymph OP of crabs collected from the impacted area was significantly lower compared to the OP collected from crabs of the control area (Fig. 4A, Student t-test, $p < 0.01$). OP of the water collected from the artificial burrows was also lower in the impacted area (Student t-test, $p < 0.01$). However, when considering the residuals (Fig.3 B) between hemolymph OP observed on the field and hemolymph OP (predicted at the same salinity using the osmotic curve published by Theuerkauff et al., 2018), a significant decrease is observed for crabs collected from the impacted area (one-sided Student t-test, $p < 0.01$). However, differences were not significant for those crabs caged in the control area (2-sided Student t-test, $p = 0.94$) showing that there is not only an effect of decreased salinity but also of WW exposure. Gill NKA activity

241 (Fig. 4C) showed no significant difference between crabs of the two areas but NKA activity
242 was 2.8 higher in posterior gills compared to anterior gills (two-way ANOVA, table 2A).
243
244 *3.4 Oxidative stress*
245 SOD activity (Fig. 5A) did not differ between anterior and posterior gills (two-way ANOVA,
246 table 2B) unlike CAT activity (Fig. 5B, two-way ANOVA, table 2C). However, for both gill
247 types, SOD activity was significantly lower in crabs collected from the impacted area compared
248 to crabs from the control area. Branchial MDA concentration (Fig. 5C) was the same for
249 impacted and control crabs but differed according to gill type (two-way ANOVA, table 2D).
250 For both impacted and control animals, the MDA content of posterior gills was 1.9 times higher
251 compared to the anterior gills (two -way ANOVA, table 2D).
252
253 **4 Discussion**
254 To our knowledge, this study corresponds to the first *in-situ* ecotoxicological assessment of the
255 effects of regular and controlled urban WW discharges on crab physiology in a natural
256 mangrove. This new approach using a caging experimental protocol in a natural mangrove
257 forest is complementary to previous studies that focused on artificial mesocosms (Bartolini et
258 al., 2009; Penha-Lopes et al., 2012, 2009a) or to *in-situ* studies using sampled crabs in polluted
259 mangroves (Amaral et al., 2009; Penha-Lopes et al., 2009b). As already discussed in Capdeville
260 (2018), the response of the crab community and the individual physiological response may be
261 linked to WW discharge but also to the induced environmental modifications. Indeed, different
262 feedback effects are possible since WW discharges induce major changes in mangrove plants
263 (Herteman et al., 2011) and microbial communities (Bouchez et al., 2013), which are the food
264 source for most of the mangrove crab species.
265

4.1 Decrease of crab burrow density as a consequence of WW release

As already demonstrated, eutrophication occurs in the impacted area (Bouchez et al., 2013; Capdeville et al., 2018; Herteman et al., 2011). However, this increase in nutrient concentration is highly variable spatially and is mainly due to the unequal dispersion of the WW in the impacted area (different microrelief conditions). WW flows on the soil according to surface roughness and infiltrates the sediment through the crab burrows. Therefore, flat bottoms are highly exposed to WW runoffs, while mounds surrounding mangrove trees are likely to be preserved from direct exposure to the effluent water. Consequently, in this study, visual countings only considered the flat bottom areas. This methodological choice may explain why a clear decrease in crab density was observed in the impacted area, differing from previous results provided by Capdeville (2018). In their study, they did not record differences in crab abundance between impacted and control areas in 2012 and 2014, but crab burrows were counted from 1 m^2 areas containing 50% of flat bottoms and 50% tree mounds (Capdeville, pers. com.). Even if crabs could take refuge on the tree mounds, the decrease in crab burrow density reported for flat bottoms should limit WW infiltration and alter the bioremediation processes as well as their engineering role. This decrease in burrow density strongly correlates with the salinity decrease and is consistent with previous observations (Herteman, 2010) showing that WW dispersion induces a decrease in salinity around the impacted area. Our results contrast with those obtained in other similar mangrove forests in Kenya and Mozambique by Cannicci et al. (2009) and Bartolini et al. (2011). In these studies, an increase in sesarmid and fiddler crab biomass was observed in a peri-urban mangrove receiving WW. However, these authors also found a negative effect of the effluent on ecosystem functionality marked by a decrease in crab bioturbation activity, which is a cryptic ecological degradation according to Dahdouh-Guebas et al. (2005). Nevertheless, it is difficult to compare these

290 different studies since WW composition is highly variable both in time and space (local
291 dispersion and WW origin).
292
293 *4.2 Osmoregulatory disruption*
294 In natural conditions, *N. africanum* is a hypo-osmoregulator in SW but the osmotic pressure of
295 the hemolymph slightly decreases with decreased salinity (Theuerkauff et al., 2018b; this
296 work). These values were significantly lower in caged crabs in the impacted area, thus
297 experiencing a decreased osmoregulatory capacity even if gill NKA activities remained
298 unmodified. This has already been observed to occur in other mangrove crab species
299 maintained in laboratory conditions and exposed for 5h to the same WW effluent (Theuerkauff
300 et al., 2018a). This decrease in osmoregulatory capacity is representative of the physiological
301 condition of the animal (Lignot et al., 2000) and could be due to histological damage in the gill
302 epithelia (Theuerkauff et al., 2018a). Such morphological damage has already been described
303 when considering the toxic effect of ammonia (Leone et al., 2017), which can reach up to 3800
304 µM in the WW discharged in our study area (Capdeville, 2018). Given that NKA pumps are
305 also involved in the excretion of ammonia-N, as already discussed in Theuerkauff et al. (2018a),
306 this pattern may also reveal a trade-off between osmoregulation and ammonia excretion since
307 NH_4^+ can substitute and compete with K^+ in NKA and other K^+ channels (Weihrauch and
308 Donnell, 2017). Moreover, other pollutants present in the WW with a potential additive or
309 synergic effect (e.g. with salinity variation) may have also contributed to this osmoregulatory
310 disturbance. This could be the case of high nitrite or nitrate concentrations (Romano and Zeng,
311 2013), which can reach up to 3.7 and 22 µM in the wastewater from Malamani (Herteman,
312 2010). Since WW composition is highly variable, it is important to note that these potential
313 additive, synergic or antagonist responses to stressors may also vary. Therefore, WW
314 bioremediation in natural mangroves may require specific field impact studies and case-by-case

follow-ups by the competent authorities. It would also be very interesting to study the resilience of the ecosystem and especially if crabs may recover physiological health when exposure stops.

4.3 Oxidative stress modulation

As previously observed in laboratory conditions, posterior gills maintain higher CAT activities and MDA levels compared to anterior gills (Theuerkauff et al., 2018a). This pattern also occurs in *Carcinus aestuarii* (Rivera-Ingraham et al., 2016) and is probably linked to the osmoregulatory function of these gills (Rivera-Ingraham and Lignot, 2017). Osmoregulation is an energy-demanding process as ATPase pumps are key players for active ion transport. Therefore, in decapod crabs, posterior gills exhibit numerous mitochondria and a high abundance and activity of NKA (Copeland and Fitzjarrell, 1968; Lignot and Charmantier, 2015; Pequeux, 1995). This is also the case for *N. africanum* (Theuerkauff et al., 2018b; this work). These pumps are located in specialized ionocites (aka mitochondria-rich cells), and mitochondria are considered as the major ROS producers in aquatic animals (Abele et al., 2007). This may explain the higher MDA levels recorded in the posterior gills. Also, compared to anterior gills, their high CAT activities may be an adaptation to maintain redox homeostasis in these gills (Rivera-Ingraham et al., 2016). Long-term exposure under field conditions did not induce oxidative damage in either anterior or posterior gills but a marked decrease in SOD activity is observed in both tissues. This redox imbalance may be explained by a wide variety of different stressor and/or molecules contained in WW (see reviews by Abele et al., 2012; Lushchak, 2011). For example, such decrease has already been reported after exposure to heavy metals (Jasinska et al., 2015) or even infections (Neves et al., 2000; Rameshthangam and Ramasamy, 2006). Moreover, such changes may work in synergy with environmental factors, such as temperature, as observed for the Cape River crab after exposure to silver nanoparticles AgNP (Walters et al., 2016) or low levels of oxygen (Li et al., 2016b).

340

341 *4.4 Oxidative stress and gender*

342 Gender-related variability in the oxidative stress response has already been reported in the shore
343 crab *Carcinus maenas* (Pereira et al., 2009) and other invertebrates (Correia et al., 2003;
344 Espinosa and Rivera-Ingraham, 2016; Radhika et al., 1998), even if most papers on aquatic
345 organisms lack information about gender (Abele et al., 2012). Previous laboratory conditions
346 reported a gender difference in response to WW exposure (Theuerkauff et al., 2018a), which
347 could potentially lead to sex-dependent tolerance to a pathological environment (Fanjul-Moles
348 and Gonsebatt, 2012). However, these differences were not reported in this field study probably
349 because waters in the field are temporally and spatially diluted. Nevertheless, these gender-
350 related differences should be considered as an important factor when studying other species or
351 stress intensities.

352

353 **5 Conclusion**

354 This study demonstrates that mangrove crab abundance is significantly decreased in WW-
355 impacted areas, and encaged crabs forced to remain in such areas show both osmoregulatory
356 and redox disturbances. Many different compounds present in the WW, such as ammonium or
357 nitrite, could be responsible for the aforementioned results, and these compounds may
358 furthermore have potential additive, synergic or antagonist effects. Therefore, WW
359 bioremediation by natural mangroves should require specific field impact studies and case-by-
360 case follow-ups by competent authorities. This work emphasizes the need for a proper WW
361 management based on results from both laboratory and field analyses. Moreover, if the
362 observed effects on osmoregulation and oxidative stress could explain the decrease in crab
363 burrow density, crabs may also escape and/or avoid the discharged area. More behavioral
364 studies on crabs under WW discharge are so needed. Further studies should also look at the

365 digestive tract and especially the hepatopancreas which is involved in absorption and nutrient
366 storage, enzyme synthesis, lipid and carbohydrate metabolism, detoxification and absorption
367 processes (Wang et al., 2014). Finally, this study clearly indicates that mangrove crabs are
368 affected by WW exposure and are most likely impacted by uncontrolled WW discharges that
369 occur in mangrove systems in tropical countries across the world.
370

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Table 1: Chemical characterization of the (undiluted) WW as well as the surface water and porewater for both the impacted and control sites (partly modified from Capdeville, 2018 and 2019). N.A. = data not available. For WW, mean values (± SE) were obtained from a total of 4 measurements made on two consecutive days. Surface water (residuals pools) and porewater (piezometers installed at approximately 1 m depth) data was collected at low tide and mean values (± SE) were obtained from two-day measurements under similar hydrological conditions, in the upper and lower parts of the areas (total of 4 measurements).

Table 2: Results for the two-way ANOVAs on NKA activity (A), SOD activity (B), CAT activity (C) and TBARS concentration (D). Factors, df (degrees of freedom), MS (variance) and value of F ratio are shown. Statistically significant effects are indicated by asterisks: * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$).

Figure legends

Figure 1: Location of the Comoros archipelago and the study site in Mayotte (A); aerial view of the experimental setup in the mangrove of Malamani (B) and of the control and impacted areas (C); artificial burrow (D); pierced pipes discharging WW in the impacted area (E). *Neosarmatium africanum* as well as water samples used for chemical analyses were collected within the *Avicenia marina* and *Ceriops tagal* belts in non-impacted sites. In B, the experimental parcels 1 and 2 corresponds to the *Ceriops tagal* and *Avicenia marina* belts, respectively. 3 corresponds to the overflow discharging site. Only the parcel 1 was considered in this study. In C, artificial burrows (n=20) are indicated by red (impacted, n=10) and yellow (control north only, n=10) circles. In B and C, grey lines represent the WW transporting pipes and the white lines represent the WW discharging drains.

Figure 2: Dorsal (A, C) and ventral (B, D) view of *Neosarmatium africanum* female (A, B) and male (C, D). Scale bars: A = 2.6 cm; B = 1.7 cm; C = 2.3 cm and D = 2.5 cm.

Figure 3: Surface water salinity (A), crab burrows density (B) and relationship between crab density and salinity (C). Control south (CS, green, n = 11), Impacted area (I, red, n = 10), Control north (CN, green, n = 11). Different letters represent statistically significant differences at $p < 0.05$ according to Kruskal-Wallis test followed by a Nemenyi post hoc comparison test (A) or a one-way ANOVA followed by Tukey's HSD test (B). Boxplots show medians (central crossbars), 25^{th} and 75^{th} percentiles (boxes) and whiskers which extent to the most extreme data point which is no more than 1.5 times the interquartile, data point outside this range are represented by an open circle.

Figure 4: Hemolymph osmotic pressure according to water (from artificial burrow) osmotic pressure (A); OP residuals (B) and Na^+/K^+-ATPase activity in anterior (A, light green/red) and posterior (P, dark green/red) gills (C). A: Black symbols and dotted lines correspond to 95% confidence interval of the osmotic curve of *N. africanum* (see Theuerkauff et al., 2018). Different letters represent statistically significant differences at $p < 0.05$ from two-way ANOVA followed by Tukey's HSD test. Boxplots show medians (central crossbars), 25^{th} and 75^{th} percentiles (boxes) and whiskers which extent to the most extreme data point which is no more than 1.5 times the interquartile, data point outside this range are represented by an open circle.

Figure 5: Redox parameters in anterior (A, light green/red) and posterior (P, dark green/red) gills of *N. africanum*: SOD activity (A); CAT activity (B) and oxidative damage (C) measured by TBARS concentration. Units (U). Different letters represent statistically significant

differences at $p < 0.05$ according to a two-way ANOVA followed by Tukey's HSD test. Boxplots show medians (central crossbars), 25^{th} and 75^{th} percentiles (boxes) and whiskers which extent to the most extreme data point which is no more than 1.5 times the interquartile, data point outside this range are represented by an open circle.

Table 1

	Wastewater	Control area		Impacted area	
		Surface Water	Porewater	Surface Water	Porewater
NH_4-N (µm)	3834 ± 134	37.97 ± 20.28	0.38 ± 0.23	294 ± 147	0.14 ± 0.09
NO_3^- (µm)	0.46 ± 0.03	1.05 ± 0.21	0.35 ± 0.20	1.28 ± 0.64	0.39 ± 0.22
NO_2^- (µm)	0.64 ± 0.25	0.61 ± 0.11	0.06 ± 0.01	4.20 ± 0.08	0.07 ± 0.00
PO_4^{3-} (µm)	186 ± 0.1	0.83 ± 0.01	5.93 ± 1.28	0.37 ± 0.15	10.79 ± 2.1
Temperature (°C)	28.1	23.2 ± 1.2	24.8 ± 0.2	22.8 ± 0.2	25.0 ± 0.0
Salinity (psu)	0.39 ± 0.02	41.1 ± 1.3	39.5 ± 1.7	35.3 ± 2.0	45.8 ± 0.2
pH	7.23 ± 0.00	7.58 ± 0.01	n.a.	7.45 ± 0.01	n.a.

Table 2

	A	NKA activity		B	SOD activity		C	CAT activity		D	TBARS	
Factors	df	MS	F	df	MS	F	df	MS	F	df	MS	F
Gill	1	25.3	**40.20*** **	1	0.86	3.4778	1	1601	**27.5*** **	1	119780	**27.6*** **
Treatment	1	0.71	1.13	1	6.15	**24.6805*** **	1	54	0.94	1	7133	1.64
Gill x Treatment	1	0.035	0.057	1	0.018	0.0749	1	28	0.49	1	121	0.028
Error	36	0.62		36	0.24		33	58		27	4330	

df: degrees of freedom; MS: variance; Statistically significant effects are indicated by asterisks: *** ($p < 0.001$).

Figure 1

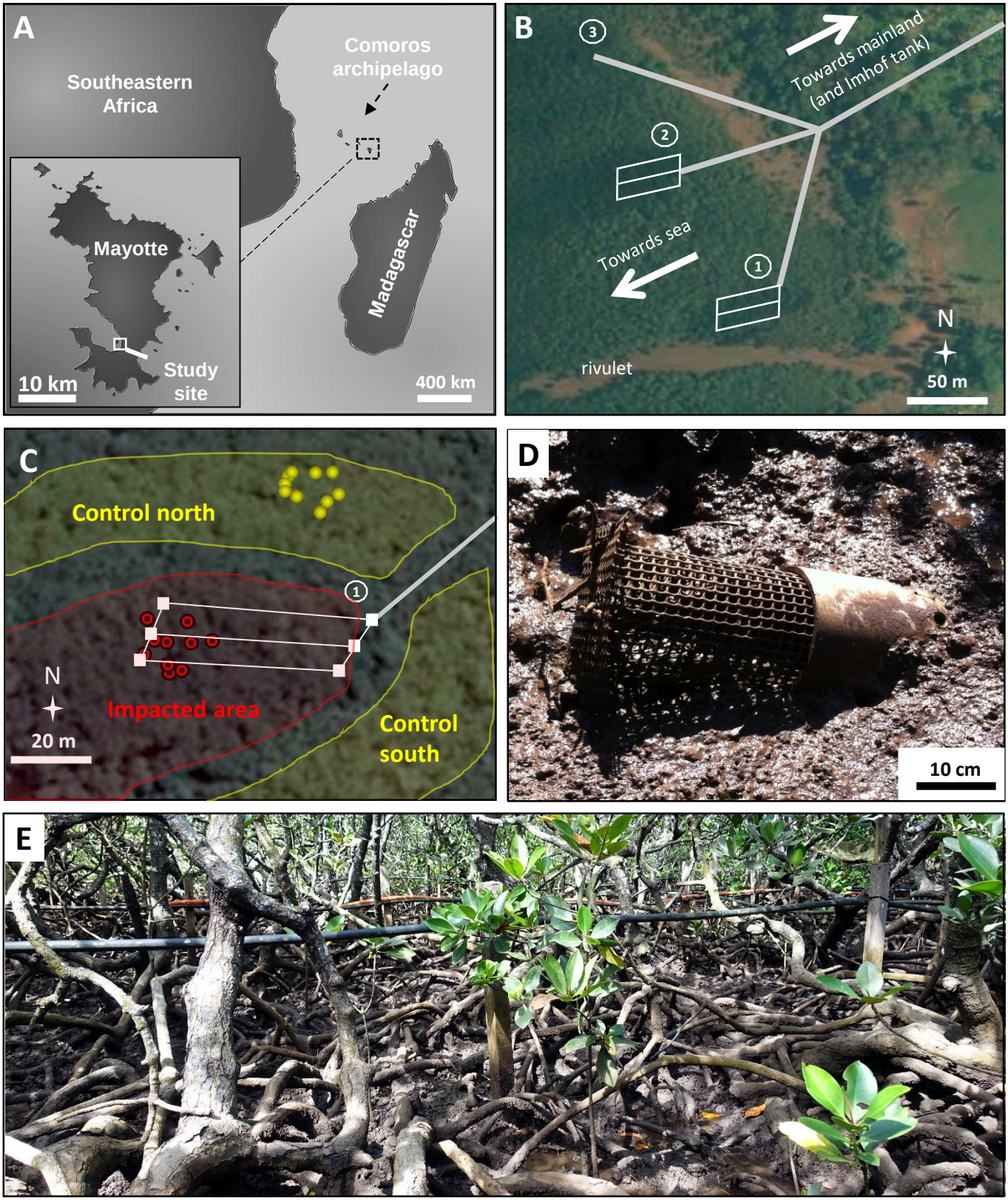

A
Southeastern Africa
Comoros archipelago
Mayotte
Madagascar
Study site
10 km
400 km
B
3
2
1
Towards mainland (and Imhof tank)
Towards sea
rivulet
N
50 m
C
Control north
Impacted area
Control south
N
20 m
D
10 cm
E

Figure 2

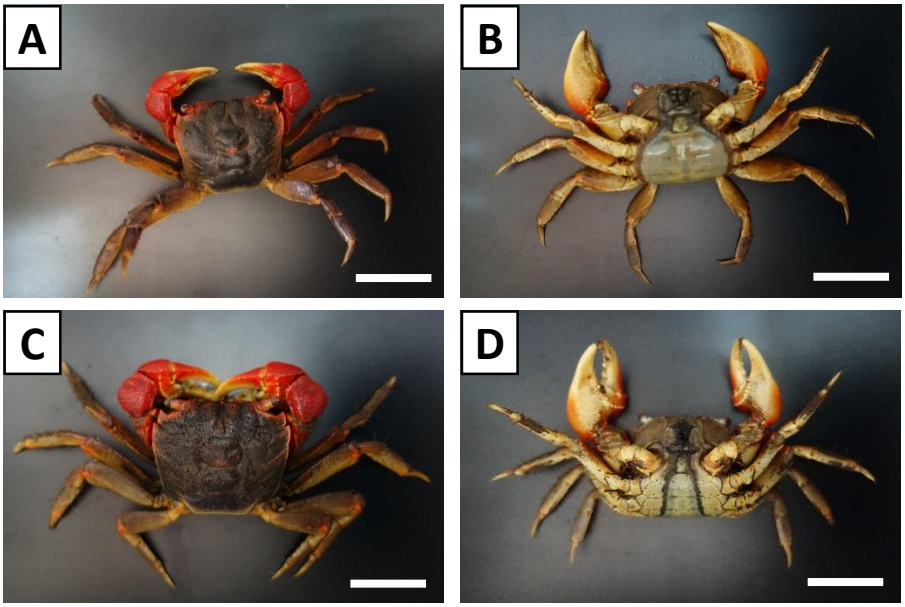


Figure 3

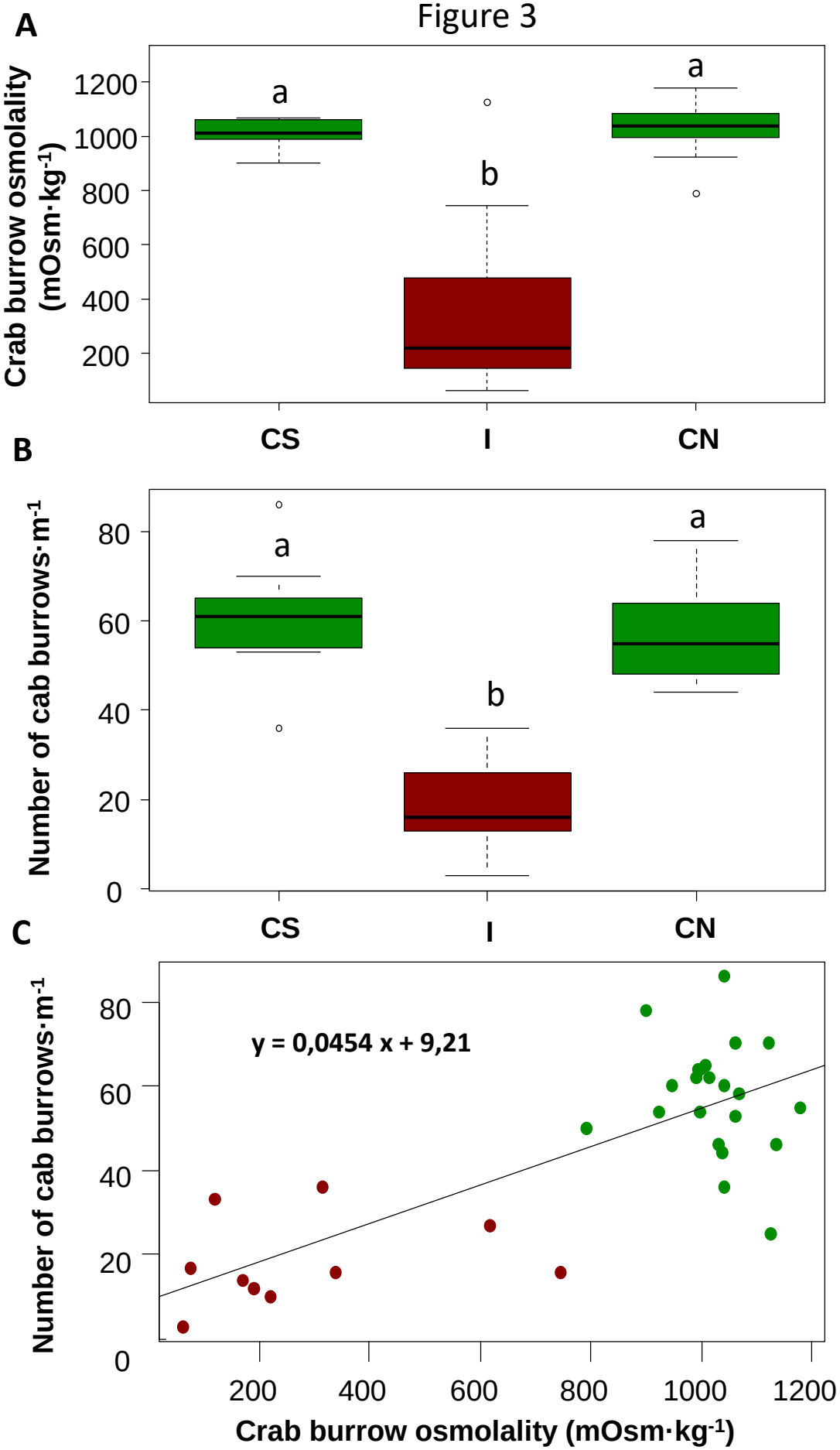
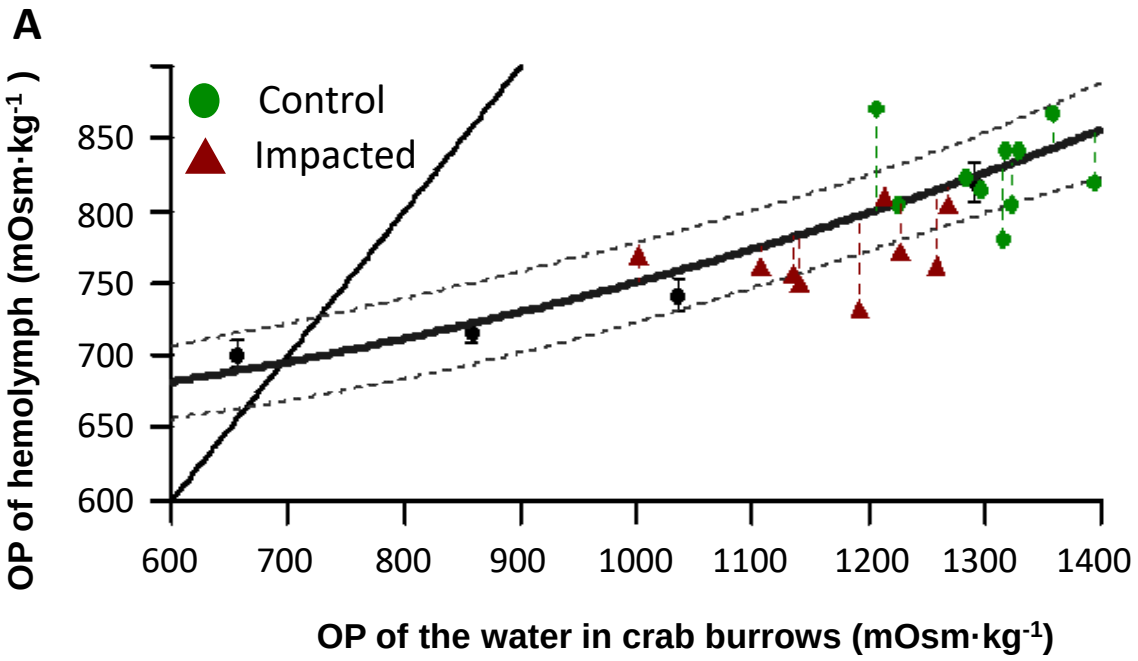
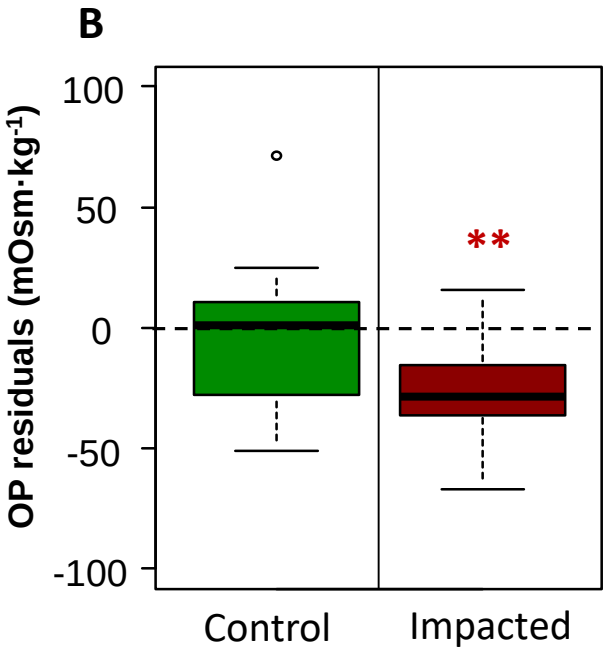

A
Crab burrow osmolality (mOsm·kg-1)
1200
1000
800
600
400
200
a
b
a
CS
I
CN
B
Number of cab burrows·m-1
80
60
40
20
0
a
b
a
CS
I
CN
C
Number of cab burrows·m-1
80
60
40
20
0
y = 0,0454 x + 9,21
200
400
600
800
1000
1200
Crab burrow osmolality (mOsm·kg-1)

Figure 4


A
Control
Impacted
OP of hemolymph (mOsm·kg-1)
850
800
750
700
650
600
600
700
800
900
1000
1100
1200
1300
1400
OP of the water in crab burrows (mOsm·kg-1)


B
OP residuals (mOsm·kg-1)
100
50
0
-50
-100
°
**
Control
Impacted

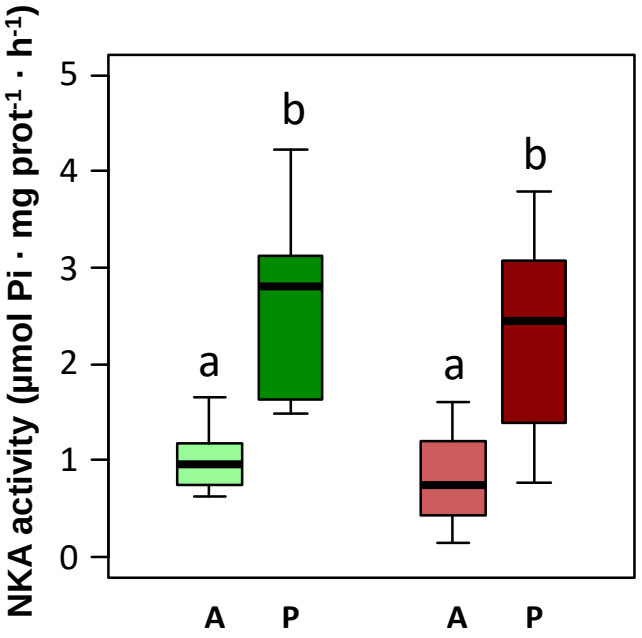

C
NKA activity (µmol Pi · mg prot-1 · h-1)
5
4
3
2
1
0
a
b
a
b
A
P
A
P

Figure 5

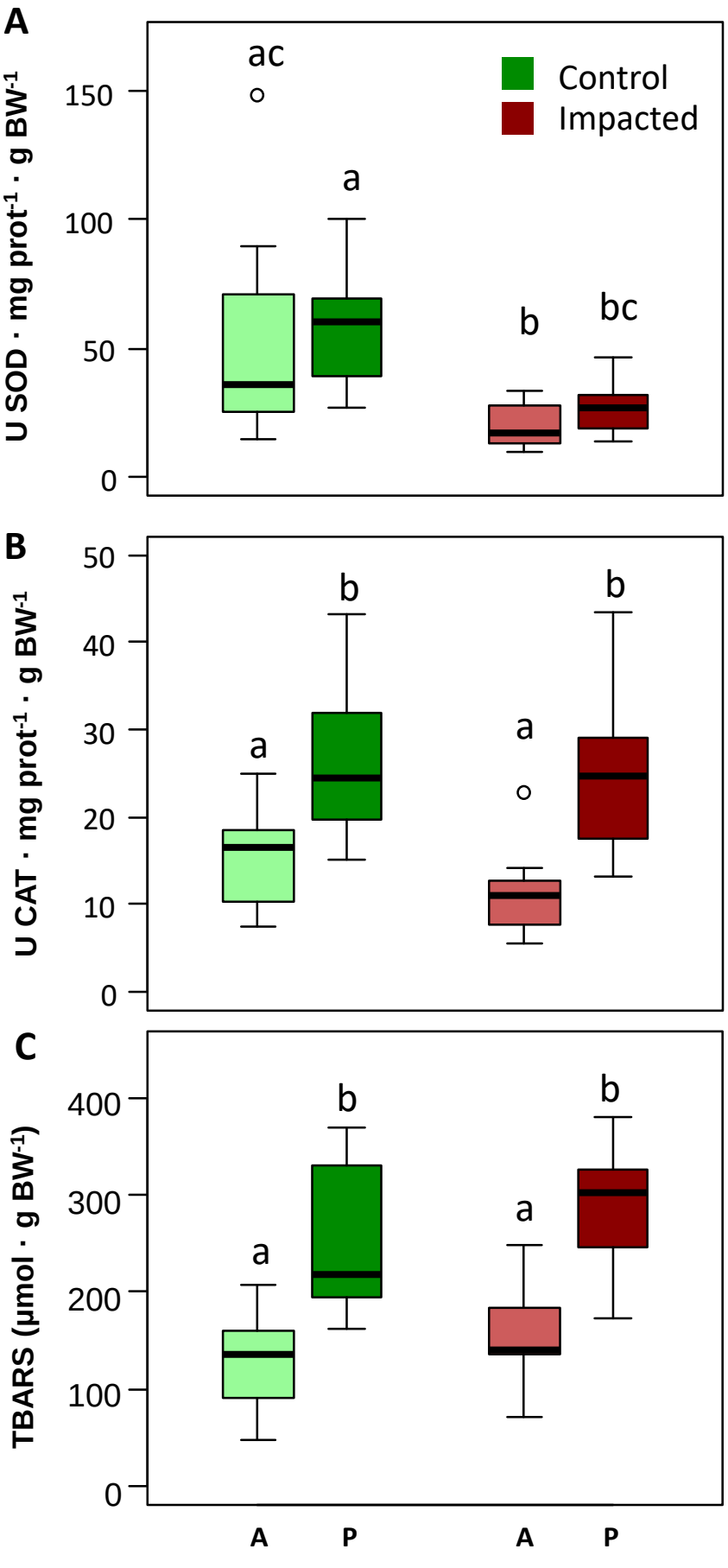

A
B
C
Control
Impacted
U SOD · mg prot-1 · g BW-1
U CAT · mg prot-1 · g BW-1
TBARS (µmol · g BW-1)
ac
a
b
bc
a
b
a
b
a
b
a
b
a
b
0
50
100
150
0
10
20
30
40
50
0
100
200
300
400
A
P
A
P

Table 1

	Wastewater	Control area		Impacted area	
		Surface Water	Porewater	Surface Water	Porewater
NH_4-N (µm)	3834 ± 134	37.97 ± 20.28	0.38 ± 0.23	294 ± 147	0.14 ± 0.09
NO_3^- (µm)	0.46 ± 0.03	1.05 ± 0.21	0.35 ± 0.20	1.28 ± 0.64	0.39 ± 0.22
NO_2^- (µm)	0.64 ± 0.25	0.61 ± 0.11	0.06 ± 0.01	4.20 ± 0.08	0.07 ± 0.00
PO_4^{3-} (µm)	186 ± 0.1	0.83 ± 0.01	5.93 ± 1.28	0.37 ± 0.15	10.79 ± 2.1
Temperature (°C)	28.1	23.2 ± 1.2	24.8 ± 0.2	22.8 ± 0.2	25.0 ± 0.0
Salinity (psu)	0.39 ± 0.02	41.1 ± 1.3	39.5 ± 1.7	35.3 ± 2.0	45.8 ± 0.2
pH	7.23 ± 0.00	7.58 ± 0.01	n.a.	7.45 ± 0.01	n.a.

Table 2

	A	**NKA activity**		**B**	**SOD activity**		**C**	**CAT activity**		**D**	**TBARS**	
Factors	df	MS	F	df	MS	F	df	MS	F	df	MS	F
Gill	1	25.3	**40.20*** **	1	0.86	3.48	1	1601	**27.5*** **	1	119780	**27.6*** **
Treatment	1	0.71	1.13	1	6.15	**24.68*** **	1	54	0.94	1	7133	1.64
Gill x Treatment	1	0.035	0.057	1	0.018	0.075	1	28	0.49	1	121	0.028
Error	36	0.62		36	0.24		33	58		27	4330	

df: degrees of freedom; MS: variance; Statistically significant effects are indicated by asterisks: *** ($p < 0.001$).