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Role of crab holobionts in benthic N cycling in mangroves with different trophic status

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ABSTRACT: Microbial dinitrogen (N_2) fixation and intense bioturbation by macrofauna can contribute to reduce nitrogen (N) limitation in mangrove systems. In particular, crabs are important ecosystem engineers that rework sediments, redistribute organic matter, accelerate nutrient cycling and shape microbial communities in mangrove sediments. Hosting functionally diverse microbial communities, crabs form a discrete ecological unit (a holobiont). In this study, we report rates of respiration, inorganic N fluxes, net N_2 fixation, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) measured in the fiddler crab *Leptuca thayeri* and sesarmid crab *Armases rubripes* holobionts, which are dominant in oligotrophic and eutrophic mangrove systems of southeast Brazil, respectively. We measured lower biomass-specific rates of respiration and ammonium (NH_4^+) production for the larger *L. thayeri* compared to *A. rubripes*, with very different molar ratios of O_2 respiration to NH_4^+ production (152:1 vs. 20:1, respectively). This suggests a size–metabolism relationship, different food quality or different coupling of N excretion and assimilation by the crab holobionts in the 2 systems. Both crab holobionts contributed to net denitrification and DNRA, with faster N cycling in *A. rubripes* in the eutrophic system. Net N_2 fixation was also detected, with nearly 4-fold higher rates in *A. rubripes* compared to *L. thayeri*. Overall, our results illustrate active and complex N cycling associated with the 2 dominant crab holobionts and highlight their potential and overlooked role as important conduits of fixed N, which may double N_2 fixation rates in the mangrove's rhizosphere.

KEY WORDS: Fiddler crab · Sesarmid crab · Holobionts · Mangroves · Nitrogen cycle · N_2 fixation

1. INTRODUCTION

Mangrove forests are intertidal ecosystems dominating tropical and subtropical coastlines. Heavily threatened by human pressures and climate change, they show a decline of ~0.7 to 3% yr^{-1} on a global scale (Polidoro et al. 2010, Duarte et al. 2013, Goldberg et al. 2020). Mangrove forests are among the world's most productive ecosystems, playing an important role in global carbon (C) and nitrogen (N) cycles (Bouillon et al. 2008, Kristensen et al. 2008, Alongi 2020). Despite their high productivity, mangroves are primarily nutrient-limited (Reef et al. 2010). This is due to the refractory nature of the

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organic matter that they produce and retain (Woolfe et al. 1995, Dittmar et al. 2006) and to their large C outwelling, possibly exceeding burial (Cabral et al. 2021, Santos et al. 2021). Limitation of nutrients, in particular, N, may be alleviated by microbial dinitrogen (N_2) fixation and intense bioturbation by macrofauna (Woitchik et al. 1997, Cannicci et al. 2008, Reis et al. 2017). Bioturbating organisms affect nutrient availability and microbial processes through sediment reworking, burrow construction and bioirrigation, feeding and excretion (Kristensen et al. 2012, Stief 2013). Additionally, macrofauna can alleviate N limitation by priming the remineralization of refractory N, reducing plant–microbe competition (Laverock et al. 2011, Gilbertson et al. 2012).

Sesarmid (Grapsidae) and fiddler (Ocypodidae) crabs are dominant macrofaunal organisms in mangrove ecosystems in terms of both abundance and biomass (Ribeiro & Bezerra 2006, Kristensen 2008, Nagelkerken et al. 2008). Both crabs are herbivores and important ecosystem engineers that build and maintain intricate burrows in mangrove sediments (Kristensen 2008). Through their bioturbation, these crabs shape microbial community composition and activity, processing organic matter and redistributing electron acceptors and nutrients in the intertidal muddy banks of mangroves (Thongtham & Kristensen 2003, Kristensen & Alongi 2006, Cannicci et al. 2008, Quintana et al. 2015, Booth et al. 2019, An et al. 2022). Notwithstanding, most studies on crabs have addressed aspects of population biology, behaviour and physiology, while their contribution to sediment reworking and nutrient cycling has been comparatively understudied (Thongtham & Kristensen 2003, Nordhaus et al. 2009, Marochi et al. 2018, Rodrigues et al. 2021, An et al. 2022). Recent studies have highlighted that crabs host diverse microbial communities, forming discrete ecological units, i.e. holobionts (Cuellar-Gempeler & Leibold 2018, Zilius et al. 2020, Tongununui et al. 2021), which have a large potential to affect N cycling and its supply to the mangrove ecosystem (Zilius et al. 2020).

The diversity and community composition of the microbial community colonising the crab exterior and interior largely depend on the living and resting habitats (Cuellar-Gempeler & Leibold 2018). There are differences between fiddler crab and sesarmid crab ecology. Sesarmid crabs feed on leaf litter (Gao & Lee 2022), whereas fiddler crabs are surface feeders that mainly rely on microphytobenthos and bacteria and do not feed on litter (France 1998, Reinsel 2004). Since the benthic fauna in mangrove forests is sually dominated by burrowing sesarmid and fiddler crabs, the analysis of the effect of these 2 holobionts on nutrient dynamics is important to understand mangrove ecosystem functioning in the face of local and global changes (Ribeiro & Bezerra 2006, Lee et al. 2017). Indeed, an increasing proportion of mangrove systems is subject to ever-increasing human pressure, resulting in larger inputs of reactive N that can potentially offset the dependency of mangroves on N_2 fixation and crab bioturbation.

In the present study, we aimed to elucidate the contribution of crab holobionts to nutrient turnover, with a special focus on different N-cycling pathways, in 2 species: the fiddler crab *Leptuca thayeri* (Rathbun, 1900) and the sesarmid crab *Armases rubripes* (Rathbun, 1897). We hypothesised that crabs act as a mobile biogeochemical reactor, and thus can deliver nutrients needed for mangrove ecosystem functioning. In a previous study, we showed that crabs have a large potential to affect N cycling and its supply to the mangrove ecosystem (Zilius et al. 2020). However, that study was limited to a single crab species and habitat and mostly addressed the genetic potential of crab-associated microbiome in N cycling. Here, we quantified solute fluxes and microbial N cycling (N_2 fixation, denitrification and dissimilatory nitrate reduction to ammonium [DNRA]) associated with the 2 crab species. These processes were analysed in 2 mangrove systems, located on the southeast coast of Brazil, that differ in background nutrient availability and dominant crab species. The Cananéia-Iguape estuarine system (hereafter Cananéia) is a pristine and oligotrophic mangrove area dominated by the fiddler crab *L. thayeri*, whereas the Bertioga estuarine system is heavily anthropized and displays higher nutrient levels. We expected a different contribution of the crab holobionts in benthic N cycling, reflecting the different degrees of N limitation. In particular, we expected increasing denitrification potential in Bertioga, along with the higher trophic status and nitrate (NO_3^-) concentration in the water (Asmala et al. 2017). At this site, we also expected a lower ratio between denitrification and DNRA, as eutrophication and labile organic matter accumulation favour the onset of chemically reduced conditions, which promote N recycling over N losses (Bonaglia et al. 2014). We also hypothesized higher rates of N_2 fixation in the pristine Cananéia site due to stronger N limitation and low nutritional quality of the available organic matter. Findings are discussed considering how differences in microbial processes and rates can result from the interplay among the background nutrient levels and the 2 crab species' physiological or behavioural features.

2. MATERIALS AND METHODS

2.1. Study sites and sampling

Sampling was carried out in 2 estuarine systems, Cananéia-Iguape and Bertioga, along the southeast coast of Brazil. The Cananéia (~200 km^2) is a pristine estuarine system situated on a sandy coastal plain adjacent to the South Atlantic Ocean comprising an intricate set of channels and interconnected coastal lagoons. Water circulation in the channels is driven by tides (mean semidiurnal amplitude: 0.82 m; Mahiques et al. 2009) and variable seasonal inflow of freshwater from continental drainage of several rivers (Mahiques et al. 2009). The adjacent mangrove forest is primarily dominated by the smooth cordgrass *Spartina alterniflora* and the mangrove species *Rhizophora mangle*, *Laguncularia acemose* and *Avicennia schaueriana* (Cunha-Lignon et al. 2011). In contrast to Cananéia, the Bertioga system is located near the industrial Baixada Santista Metropolitan Region, where large-scale petrochemical industry, port activities and urban areas exert a large pressure on the coast. The site suffers from sludge discharge, urban waste and accidental oil spills, which lead to ecosystem deterioration. Channels are under a microtidal regime, predominantly semidiurnal, of moderate amplitude (Bernardes & Miranda 2001). As a consequence of these differences, nutrient concentrations at the 2 sites are markedly different (see Table 1).

Different crab species were found at the 2 study sites, with the dominant being the mangrove fiddler crab *Leptuca thayeri* in Cananéia and the sesarmid crab *Armases rubripes* in Bertioga. *L. thayeri* is a key species in Brazilian estuaries due to its abundance and distribution (Melo 1996, Masunari 2006, Ribeiro & Bezerra 2006). This species prefers muddy banks in shaded areas of the mangrove forest (Masunari 2006, Gusmão-Junior et al. 2012), whereas the smaller *A. rubripes* primarily inhabits the roots and vegetation stems (e.g. *S. alterniflora*) as well as cracks and substratum cavities (Melo 1996). In contrast to sesarmid crabs, *L. thayeri* builds a relatively large sedimentary structure in the form of a chimney surrounding its burrow (Gusmão-Junior et al. 2012). Given the different living habitat, behaviour and diet of *L. thayeri* and *A. rubripes*, it is likely that the crab-associated microbiome is also adapted to both their habitat and feeding preferences, with consequences on their species-specific contribution to nutrient cycling in mangrove ecosystems (Thongtham & Kristensen 2003).

Specimens of *L. thayeri* and *A. rubripes* (n = 30 per site) were collected on 18 and 21 August 2018 in their burrows on the bank of small secondary channels of Cananéia (25° 2′ 55.50″ S, 47° 58′ 31.24″ W) and in a mangrove of Bertioga in Santos Complex (23° 51′ 22.47″ S, 46° 9′ 6.00″ W) during low tide (Fig. 1). Crabs were carefully collected from their burrows with minimal handling interaction to minimize disturbance of the biofilm on their exoskeleton. The carapace length (CL), carapace width (CW) and dry weight (DW) of the collected species were measured using a Vernier calliper and an analytical scale, respectively. The mean DW of a single *L. thayeri* was 1010.7 ± 95.6 mg (n = 10) and CW ranged from 18.1–22.6 mm, whereas *A. rubripes* was much smaller with a mean DW of 44.2 ± 5.9 mg (n = 10) and CW of 6.4–10.8 mm. The average carapace surface area (= π × [CL / 2] × [CW / 2]), a proxy for the area where biofilm can grow, was 179.1 ± 10.2 and 25.3 ± 3.9 mm^2 on *L. thayeri* in *A. rubripes*, respectively. The ratio between the carapace surface and DW differed by a factor of 2.5 between the 2 species (65 ± 6 and 162 ± 15 mm^2 mg^{-1} DW for *L. thayeri* and *A. rubripes*, respectively). At the crab collection sites, intact sediment cores were also sampled by hand in triplicate with Plexiglas transparent tubes (inner diameter 4 cm, height 20 cm) to measure dissolved oxygen (O_2), inorganic N and dissimilatory nitrate (NO_3^-) reduction rates (see details in Section 2.2). In addition, 50 l of surface water was collected at each site in the channel in proximity of the crab habitat for subsequent experiments.

2.2. Biogeochemical experiments

In the laboratory, crabs were left overnight for acclimation in 2 different 20 l tanks, filled with ambient water and under continuous aeration and constant temperature (19°C), for later gas and inorganic N fluxes and NO_3^- reduction measurements. The next day, crab individuals were carefully transferred into separate Plexiglas microcosms with unfiltered water (n = 5, volume: 227 ± 3 ml) to measure gas and nutrient fluxes between the water phase and crabs (Expt 1). In addition, 3 microcosms with water alone were prepared to serve as controls. Experimental design and setup are described in detail in Zilius et al. (2020). Briefly, all microcosms were equipped with a stirring magnet for continuous water mixing (25 rpm) during incubation (~6 h) with gas-tight lids. At the beginning (from the incubation tank, in triplicate) and end of the incubations (from each microcosm), 50 ml aliquots were transferred to 12 ml exetainers (Labco) and fixed with 100 µl of 7 M $ZnCl_2$ for later N_2:Argon

Fig. 1. (a) The sedimentary system within the Cananéia study area at low tide, (b) a close-up of a crab burrow, (c) a fiddler crab, and (d) an intact sediment core collected for benthic flux measurements. The sedimentary environment at Bertioga (not shown) was similar, but the nutrient level in the water was higher (see Section 2.1)

(Ar) and O_2:Ar measurements. An additional aliquot of 20 ml was filtered (Frisanette GF/F filters) into 50 ml centrifuge tubes and stored frozen for later dissolved inorganic nitrogen analyses. After incubation, crabs from all microcosms were removed and anesthetized by cooling for 10 min in a freezer before being measured, dried at 60°C for 48 h and weighed.

Expt 2 was carried out following a similar setup to quantify NO_3^- reduction rates (denitrification and DNRA) associated with the crab holobionts, using the isotope pairing technique (IPT; Nielsen 1992). Briefly, 6 microcosms (5 replicates containing 1–2 ind. and 1 control with only filtered water) were incubated. ^{15}N labelled NO_3^- from a stock solution (20 mM $Na^{15}NO_3$, 98 atom % ^{15}N, Sigma Aldrich) was added to the water column of each microcosm to a final concentration of 1.0 and 8.2 µM in Cananéia and Bertioga, respectively, reflecting different *in situ* NO_3^- concentrations. The microcosms were then capped and incubated in the dark as in Expt 1. Before incubations, water in the microcosms was filtered (MCE filters, 142 mm diameter, pore size 0.22 µm, MF-Millipore™) to remove phytoplankton, suspended particles and microorganisms so that metabolic rates measured in the incubation could be solely attributed to the microbiota living inside the crabs (e.g. in the digestive system) or as a biofilm outside the crabs (e.g. on the exoskeleton). The incubations lasted ~6 and ~25 h for the 2 species, respectively. Water aliquots (40 ml) were collected from each microcosm replicate at t = 0, 2.5 and 6 h for *L. thayeri* and at t = 0, 10 and 25 h for *A. rubripes*. Collected aliquots were then in part transferred without headspace into 12 ml exetainer (Labco) with 100 µl of 7 M $ZnCl_2$ for $^{29}N_2$ and $^{30}N_2$ analysis and in part filtered (0.22 µm) and transferred into PE test tubes for ammonium pool ($^{14}NH_4^+$ + $^{15}NH_4^+$) analysis (see Section 2.3 for details). The subsampled aliquots were replaced with the same amount of filtered water from the initial stock. Slopes of the linear regression of $^{29}N_2$ and $^{30}N_2$ concentrations versus time were used to calculate rates of denitrification, using the equations from Nielsen (1992). The slope of the linear regression of $^{15}NH_4^+$ concentration versus time was used to calcu-

late rates of DNRA according to Bonaglia et al. (2016). NO_3^- reduction rates were then calculated as a function of biomass DW. Rates in the microcosms with crabs were corrected by subtracting rates measured in the control.

In addition to animal incubation, intact sediment cores underwent 2 sequential dark incubations to determine fluxes and NO_3^- dissimilatory processes as described by Politi et al. (2021) and Zilius et al. (2022). The first incubation aimed at the measurement of net dissolved O_2 and inorganic N fluxes, whereas the second incubation measured dissimilatory NO_3^- reduction via r-IPT (Robertson et al. 2019). The obtained rates were used to evaluate and compare the role of crab holobionts in N turnover.

2.3. Analytical methods

The concentrations of NO_2^-, and NO_x^- were measured on a continuous flow analyser (Technicon AutoAnalyzer II, SEAL Analytical) using colorimetric methods by Tréguer & Le Corre (1975). NO_3^- concentration was calculated as the difference between NO_x^- and NO_2^-. Dissolved NH_4^+ was analysed spectrophotometrically using the method of Tréguer & Le Corre (1975). Dissolved N_2 and O_2 were quantified from N_2:Ar and O_2:Ar ratios measured with a membrane inlet mass spectrometer (Bay Instruments; Kana et al. 1994) and corrected for Ar concentration and solubility based on temperature and salinity (Colt 2012). Isotopic samples for $^{29}N_2$ and $^{30}N_2$ production were analysed by gas chromatography–isotopic ratio mass spectrometry (GC–IRMS, Thermo Delta V Plus, Thermo Scientific) at the University of Southern Denmark following the protocol described by De Brabandere et al. (2015). Samples for $^{15}NH_4^+$ production were analysed by the same GC–IRMS after conversion of NH_4^+ to N_2 by the addition of alkaline hypobromite reagent (Warembourg 1993).

2.4. Statistical analyses

A *t*-test was used to check differences in solute fluxes, their molar ratio, and NO_3^- reduction rates between the 2 crab holobionts. Assumptions of normality and homogeneity of variance were checked using Shapiro-Wilk and Cochran's tests, respectively. In the case of heteroscedasticity, data were square-root transformed. The significance level was set at $\alpha = 0.05$. Results are given as average values and standard errors. All statistical analyses were performed using the SigmaPlot 14.0 software.

3. RESULTS

3.1. O_2 respiration and NH_4^+ excretion by crab holobionts

On a per-individual basis, *Leptuca thayeri* respired ~154 µmol O_2 ind.$^{-1}$ d^{-1}, whereas *Armases rubripes* consumed ~43 µmol O_2 ind.$^{-1}$ d^{-1}. Biomass-normal-

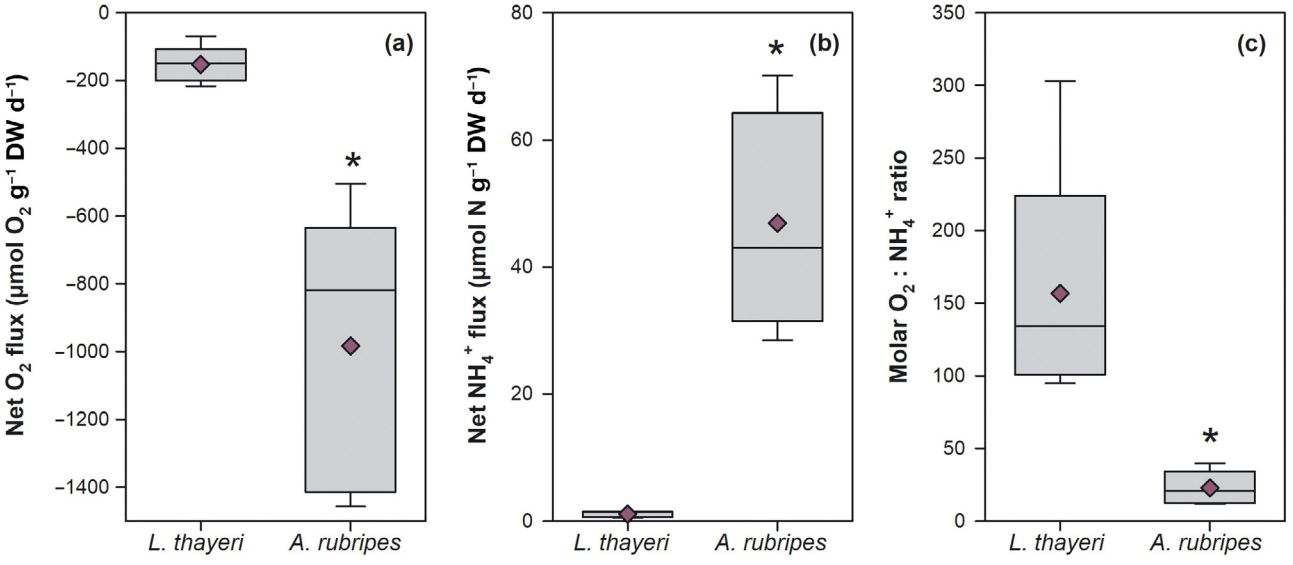

Fig. 2. Net fluxes of (a) dissolved oxygen and (b) ammonium, and (c) their molar ratios associated with the holobionts of *Leptuca thayeri* and *Armases rubripes*. Data range (whiskers), upper and lower quartiles (edges), the median (horizontal line), and the mean (diamond) are represented (n = 5). Asterisks indicate significant differences in rates between crabs (*t*-test, $p < 0.05$)

ized respiration rates suggest that the smaller *A. rubripes* respired nearly 6.5 times more O_2 than the larger *L. thayeri* (983.5 ± 184.4 vs. 152.9 ± 24.4 µmol O_2 crab g^{-1} DW d^{-1}, respectively) (Fig. 2a).

Both crab holobionts actively released NH_4^+, with rates significantly higher in *A. rubripes* ($t = -6.0$, $p < 0.001$; Fig. 2b). We measured an NH_4^+ release of ~1 and ~2 µmol N $ind.^{-1}$ d^{-1} for *L. thayeri* and *A. rubripes*, respectively, with a biomass-normalized lower NH_4^+ release by *L. thayeri* consistent with its lower O_2 respiration. The molar ratio between O_2 respiration and net NH_4^+ release for the crab holobionts was significantly different ($t = 5.2$, $p < 0.001$) with ratios of 152:1 and 20:1 for *L. thayeri* and *A. rubripes*, respectively (Fig. 2c).

3.2. Crab holobiont-mediated N transformations

Net NO_x^- fluxes in the *L. thayeri* holobiont were negligible, suggesting an equilibrium between NO_2^- and NO_3^- producing and consuming processes (nitrification and denitrification or DNRA) or limited microbial activity in the oligotrophic water column of Cananéia (Table 1, Fig. 3a). In contrast, the *A. rubripes* holobiont showed significantly higher rates of NO_x^- consumption ($t = 3.5$, $p < 0.05$), partially offsetting (–64%) net NH_4^+ production. Net uptake of N_2 was detected in all microcosms with *L. thayeri* (–13.0 ± 2.9 µmol N crab g^{-1} DW d^{-1} or ca. –1 µmol N $ind.^{-1}$ d^{-1}) and *A. rubripes* (–465.0 ± 59.7 µmol N crab g^{-1} DW d^{-1} or ca. –20 µmol N $ind.^{-1}$ d^{-1}) (Fig. 3b), suggesting the dominance of N_2 fixation over N_2 production processes in both crab holobionts and stressing significantly higher rates in the organism living at the more eutrophic site.

Incubations with $^{15}N\text{-}NO_3^-$ revealed that denitrification and DNRA co-occurred in both crab holobionts (Fig. 3c,d), for the first time reporting NO_3^- reduction directly associated with crabs. Denitrification ranged between 6.6 and 59.8 nmol crab N g^{-1} DW d^{-1}, with rates 60% higher in *A. rubripes* ($t = -3.2$, $p < 0.05$). DNRA rates varied greatly, from 2.4–478.1 nmol N crab g^{-1} DW d^{-1}, with the large variability masking significant differences between the 2 holobionts despite higher average rates in *A. rubripes* ($t = -1.8$, $p > 0.05$). The ratio of denitrification to DNRA was very different in the 2 crab holobionts, with denitrification dominating in *L. thayeri* (66% of total NO_3^- reduction through denitrification and DNRA) and DNRA dominating in *A. rubripes* (81% of total NO_3^- reduction).

Table 1. Salinity, nutrient concentrations and their stoichiometric ratio in the water column measured in the main channel of the study sites during low tide. Average ± SE are given (n = 3). DIN: dissolved inorganic nitrogen; DIP: dissolved inorganic phosphorus; DSi: dissolved silica

Water chemistry	Cananéia (oligotrophic)	Bertioga (eutrophic)
Salinity	26	25
NH_4^+ (µM)	1.39 ± 0.12	8.59 ± 0.22
NO_2^- (µM)	0.09 ± 0.00	0.82 ± 0.01
NO_3^- (µM)	0.28 ± 0.01	9.97 ± 0.31
DIN (µM)	1.76 ± 0.11	19.34 ± 0.54
DIP (µM)	0.25 ± 0.03	0.99 ± 0.12
DSi (µM)	13.48 ± 0.86	68.08 ± 9.17
DIN:DSi:DIP (molar)	7:54:1	20:70:1

4. DISCUSSION

4.1. Drivers of O_2 and NH_4^+ fluxes in the 2 crab holobionts

Both *Leptuca thayeri* and *Armases rubripes* migrate between water and land. Their sedimentary habitat can be exposed for many hours to the atmosphere or, conversely, be completely submerged during high tides. In this study, we measured O_2 respiration by the 2 crab holobionts when they were submerged to investigate their ability to extract O_2 from water (Jimenez & Bennett 2005). Results from unpublished, preliminary experiments where we incubated biofilm-covered crabs with a polished carapace suggest that the share of microbial respiration to the total crab holobiont O_2 consumption is low for the 2 species. However, as we discuss below, such tests need to be properly designed and realised to quantify the microbial and macrofaunal contributions to the holobiont metabolism. Respiration rates of *A. rubripes* exceeded by a factor of 5 the rates measured at higher temperatures for the larger sesarmid species *Neoepisarma versicolor* by Thongtham & Kristensen (2003). Higher O_2 consumption in the smaller *A. rubripes* as compared to the larger *L. thayeri* or *N. versicolor* aligns with the typical inverse relationship between metabolic rates and biomass (e.g. Brey 2010). However, a factor of 5–6 between respiration rates might also indicate species-specific metabolism, shaped by the environment where the species live (surface sediment or burrows) or by other ecological constraints. The higher ability of *A. rubripes* compared to *L. thayeri* to use dissolved O_2 can be, for instance, an adaptation to a life predominantly spent in terrestrial habitats (O'Mahoney & Full 1984). As *L. thayeri* spends more

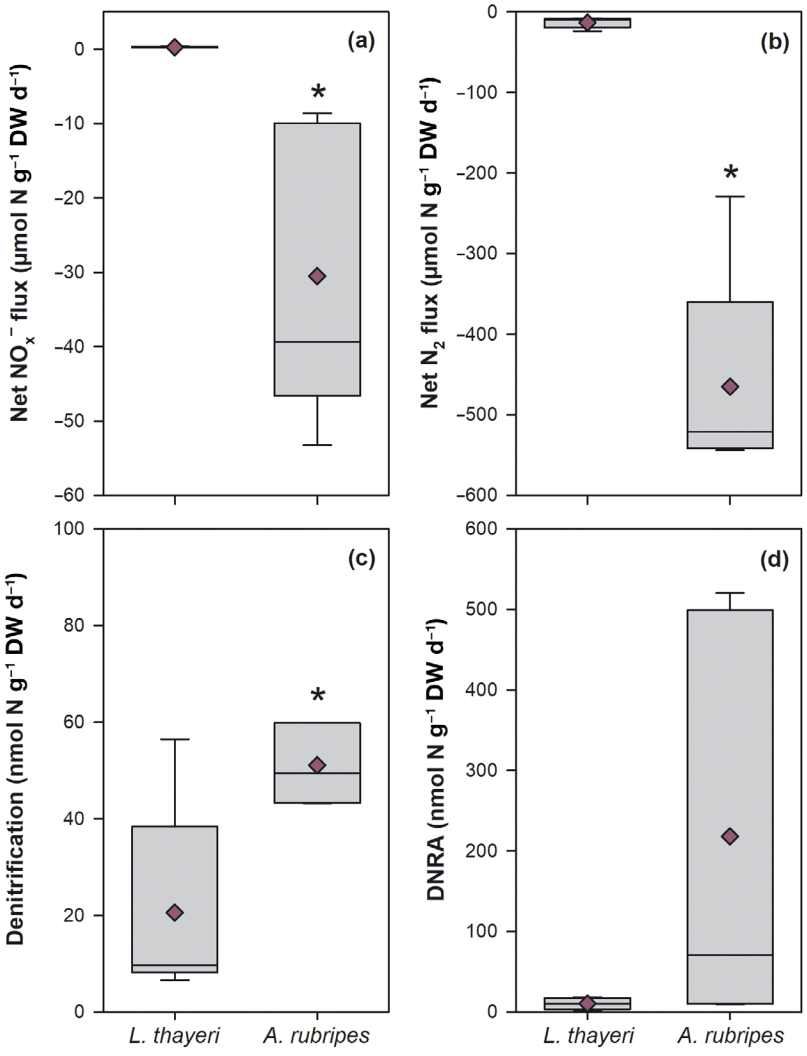

Fig. 3. Net fluxes of (a) nitrite and nitrate (NO_x^-), (b) molecular nitrogen, (c) denitrification, and (d) dissimilatory nitrate reduction to ammonium (DNRA) in holobionts of *Leptuca thayeri* and *Armases rubripes* incubated sequentially with (a,b) unfiltered and (c,d) 0.22 µm filtered *in situ* water added with $^{15}NO_3^-$. Data range (whiskers), upper and lower quartiles (edges), the median (horizontal line), and the mean (diamond) are represented (n = 5). Asterisks indicate significant differences in rates between crabs (*t*-test, $p < 0.05$). DNRA: dissimilatory nitrate reduction to ammonium

time in sediment burrows compared to *A. rubripes*, low biomass-specific respiration can be advantageous to survive in a niche with limited O_2 availability.

In most aquatic animals, excretion generally occurs in the form of NH_3/NH_4^+, and in crabs, it primarily occurs during submersion periods, substantially decreasing during air exposure (Weihrauch et al. 2004). Since our incubations allow measurement of net fluxes from the crab holobionts to the surrounding water, we cannot rule out that part of the released NH_4^+ was immediately re-assimilated by the biofilm growing on *L. thayeri* crabs, which would result in very high O_2 respiration to net NH_4^+ release ratios. Such a mechanism would prevent nutrient losses from the crab holobionts, which, in an oligotrophic habitat such as the Cananéia estuarine system, likely include N-limited primary producers and microbes. At the high-nutrient site, the smaller *A. rubripes* holobiont released proportionally more N to the surrounding environment. There are 2 possible explanations for this difference. First, at Bertioga, higher nutrient availability can favour the growth of macrophytes and opportunistic algae that can supplement the crab diet. Indeed, the C:N molar ratio of epiphytic algae, microphytobenthos and cordgrass can vary between ~6 and ~20 (Sundbäck et al. 2011, Zhang et al. 2020), which is consistent with the respiration to excretion ratio calculated for *A. rubripes* and suggests a food source alternative to mangrove leaves. Future studies should focus on the diet of the 2 crabs to provide insights on their role in recycling organic matter and nutrients in mangrove habitats. Besides excretion, another source of NH_4^+ by crab holobionts is represented by microbial N_2 fixation and DNRA, as discussed in Section 4.2.

4.2. *A. rubripes* holobionts fix and recycle more N than *L. thayeri*

Our measured net N_2 fluxes were always negative (i.e. from the water to the crab holobionts) and suggest active N_2 fixation in both holobionts exceeding N_2-producing processes. These results confirm our previous findings on the presence and activity of a diazotroph community growing on the carapace of fiddler crabs (Zilius et al. 2020) and stress the need to explore whether N_2 fixation in crab holobionts is a relevant process in different species and across habitats. Based on mass balance estimations per individual, Thongtham & Kristensen (2003) suggested that N_2-fixing bacteria living in association with leaf-eating sesarmid crabs did not directly support the growth of their host. However, the relevance of N_2 fixation might vary according to the overall availability of N.

Along with the decomposition of refractory organic matter, the initially high C:N ratios tend to decrease due to microbial N inputs that increase the nutritional value of the litter material (Palmia et al. 2019). As such, N_2 fixers may contribute to increasing the nutritional quality of the food on which the crabs feed.

Net NO_x^- consumption by the *A. rubripes* holobiont may be driven by assimilative and dissimilatory processes that likely coexist in the crab holobionts. Genes encoding dissimilatory NO_3^-/NO_2^- reduction pathways are typically transcribed under hypoxic to anoxic conditions (Härtig & Zumft 1999), which can develop both in the host gut or in biofilms on macrofauna exterior surfaces (Stief et al. 2009).

Weight-specific denitrification rates were relatively low compared to rates reported for other benthic macrofauna holobionts from estuarine environments (Table 2). Conversely, despite large variability in the measurements, DNRA rates in *A. rubripes* holobionts were among the highest found in benthic invertebrates such as bivalves and worms, indicating favourable conditions for DNRA bacteria in sesarmid crabs from Bertioga. The dominance of DNRA over denitrification in Bertioga can be explained by low NO_3^- availability relative to organic matter in mangrove ecosystems (Tiedje et al. 1983). In Cananéia, it is likely that both denitrification and DNRA are strongly limited by the low inorganic N availability, driving the effects of the crab holobionts on N cycling toward net import, efficient recycling and reuse and limited losses. In contrast, in the Bertioga system, N recycling via DNRA dominates over N losses via denitrification and promotes a positive feedback towards eutrophic conditions.

Overall, dissimilatory NO_3^- reduction pathways were of minor importance over net N_2 fixation rates in the 2 holobionts, with denitrification and DNRA representing <1% of the imported N regardless of the site. Future studies should include primary producers associated with the crabs and their activity as well as measurements of assimilative N pathways. Higher N_2 fixation and DNRA rates in *A. rubripes* holobionts have the potential to produce and eventually release to the surrounding environment larger amounts of NH_4^+ compared to the amounts produced by *L. thayeri*. However, the effect of the 2 crab holobionts at the ecosystem level ultimately depends on their local population size.

Table 2. Summary of NO_3^- reduction rates (nmol N g^{-1} DW d^{-1}) measured in benthic macrofauna across different estuarine systems (average ± SE; DW: dry weight). Reported references were selected on the basis of the same method used to measure rates (^{15}N labelling)

Macrofauna taxa	Denitrification	DNRA	Reference
Chironomus plumosus	38.6 ± 4.7	4.6 ± 0.8	Politi et al. (2021)
Dreissena polymorpha	1391.9 ± 694.9	749.1 ± 464.8	Marzocchi et al. (2021)
Limecola balthica	116.8 ± 52.7	5.0 ± 2.0	Zilius et al. (2022)
Marenzelleria spp.	123.2 ± 38.1	8.0 ± 1.5	Zilius et al. (2022)
Monoporeia affinis	2553.9 ± 1738.8	40.8 ± 11.6	Zilius et al. (2022)
Leptuca thayeri	20.6 ± 9.3	10.5 ± 3.1	This study
Armases rubripes	51.1 ± 3.7	217.9 ± 115.5	This study

4.3. Methodological limitations and future improvements

In the present study, we incubated crabs covered by biofilm, as our main aim was to highlight the contribution of crab holobionts to benthic N cycling in 2 distinct mangrove areas. Results of our incubations represent the net of a number of different co-occurring processes, including the metabolic activity of crabs and that of microbes growing inside and outside of the crabs. However, measured rates do not allow discrimination of the inventory of different contributions. For this reason, we reported rates on a per crab holobiont gram DW or a per crab individual basis in order to discuss how size can be important (emphasizing higher biomass-normalized rates in smaller organisms) and to upscale processes (individual basis, multiplied by the population size). We acknowledge that the colonizable surface to DW ratio is very different in small and large crabs, but in this study, we did not consider the crab surface as it is challenging to distinguish between processes occurring inside or outside the crabs. In future studies, we recommend performing another set of incubations on clean crabs (gently brushed to remove the exterior biofilms) to separate the contribution of microbes growing inside and outside the crabs and considering the surface to normalize and compare data from species of different sizes.

We also acknowledge that in the present study, we were not able to separate 2 factors such as 'species' and 'site' that simultaneously contribute to the differences we have discussed. Therefore, we only aimed to describe how 2 distinct crab holobionts contribute to benthic N cycling at 2 different sites and compare their role not in absolute terms, but rather in relation to the specific site in which they live.

4.4. Implication for the mangrove ecosystem

Fiddler and sesarmid crabs are the most abundant macrofauna species in Brazil's mangrove ecosystems. Their densities in the Cananéia area are reported to vary from 1–30 ind. m^{-2} (Ferreira et al. 2007, de Almeida Duarte et al. 2016). Similarly, crab densities in Bertioga vary from 0.8–94 ind. m^{-2} (de Almeida Duarte et al. 2016, Natálio et al. 2017). Considering a median crab density of 16 and 47 ind. m^{-2} in Cananéia and Bertioga, respectively, we upscaled our measured rates to the ecosystem level (Fig. 4) to

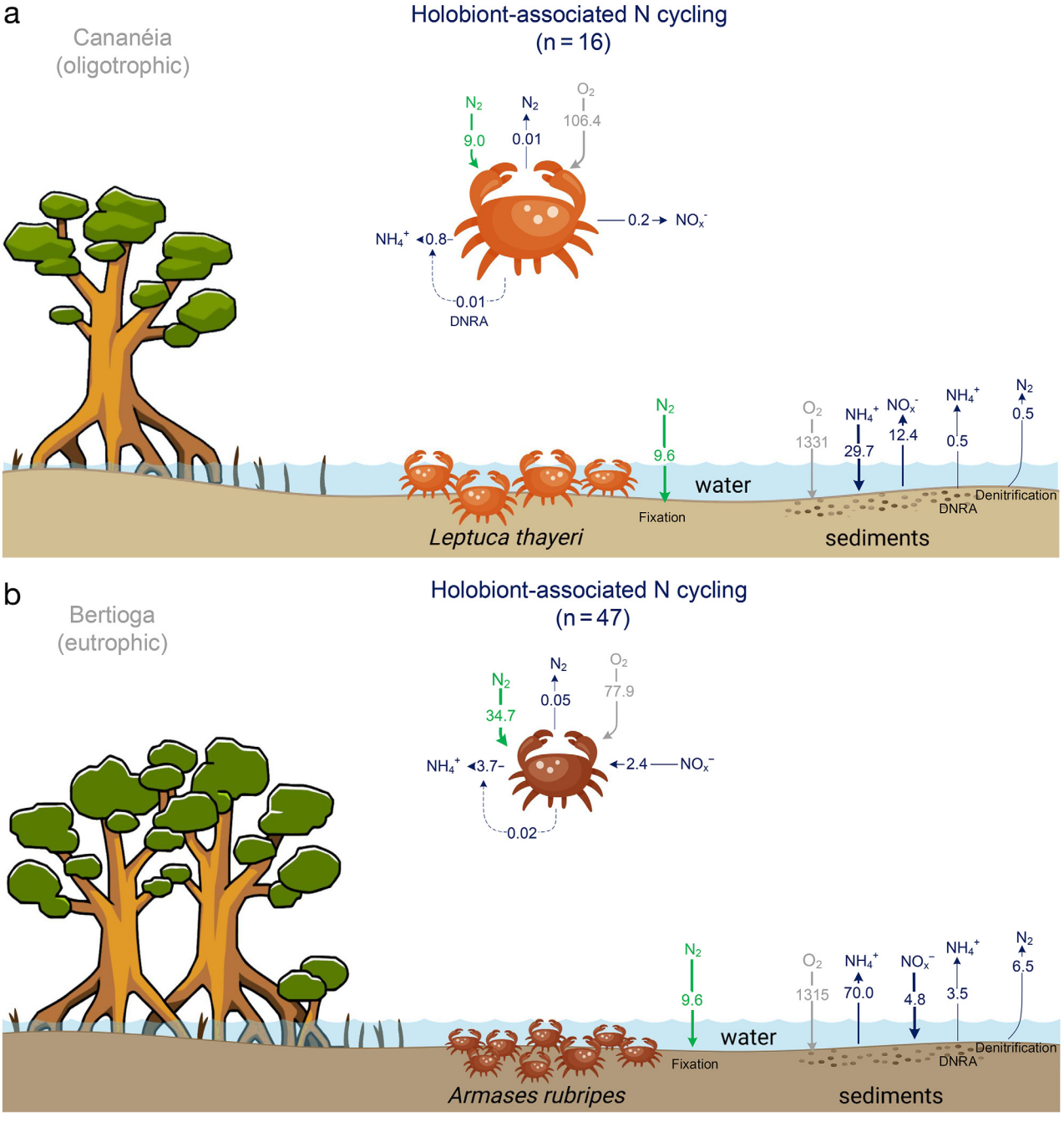

Fig. 4. Schematic representation of N cycling by (a) *Leptuca thayeri* and (b) *Armases rubripes* holobionts in mangrove forests. N cycling was reconstructed by combining data from holobiont-associated N-fluxes and processes, net fluxes and N-processes from the incubation of intact sediments collected in proximity to the crab habitats and from median global N_2 fixation rates in soil surface reported by Alongi (2020). Mean rates are multiplied by the median density of crab holobionts and expressed as µmol m^{-2} h^{-1}. DNRA: dissimilatory nitrate reduction to ammonium

analyse the role of crab holobionts (their metabolic rates) in the benthic metabolism (sediment–water net fluxes, N_2 fixation, denitrification and DNRA rates) of the 2 study areas. This is relevant to better understand the contribution of macrofauna to benthic N cycling in mangrove ecosystems, as suggested by Alongi (2020).

Notably, both crab populations accounted for a similar percentage (6–8%) of benthic areal O_2 respiration. NH_4^+ production measured in the *A. rubripes* and *L. thayeri* holobionts represented 5 and 3% of the net NH_4^+ fluxes at the sediment–water interface in the Cananéia and Bertioga systems, respectively. At both sites, regardless of different nutrient levels and metabolic differences between species, the 2 crab holobionts were marginal sources of NH_4^+ to the surrounding environment. At both sites, NH_4^+ production via DNRA by the crab holobionts accounted for <0.1% of the net sediment NH_4^+ fluxes. These findings confirm what we observed in other macrofauna species, where microbial NH_4^+ production in holobionts was negligible compared to NH_4^+ production by the host via excretion or by the surrounding sediments via ammonification and DNRA (Politi et al. 2021, Zilius et al. 2022).

The role of crab holobionts in NO_x^- turnover was different between the 2 mangrove systems (Fig. 4). At the oligotrophic Cananéia site, *L. thayeri* holobionts contributed little (1%) to the NO_x^- efflux from sediment. Conversely, *A. rubripes* holobionts significantly enhanced NO_x^- uptake (7%) at the benthic ecosystem level. However, crab holobionts contributed <0.1% of sediment NO_x^- reduction through denitrification. Denitrification measurements suggest that the role of crab holobionts in removing N from the ecosystem via this route was marginal in the investigated tropical mangrove forests. Crabs are strong bioturbators and it is likely that they indirectly stimulate denitrification rates by burrowing, enhancing the penetration of NO_3^- in sediments or favouring coupled nitrification–denitrification, amplifying the oxic–anoxic interface surface area in mangrove sediments (Kristensen 2008). We speculate that crabs exert a stronger stimulation of NO_3^- removal via burrowing at Bertioga due to higher NO_3^- concentration in the water (Table 1) and likely more labile organic C in sediments; however, specific studies should address this issue.

Most notably, N_2 fixation rates measured in *A. rubripes* and *L. thayeri* holobionts (Fig. 4) are comparable or even higher than those measured in surrounding mangrove soils and cyanobacterial mats (Alongi 2020). In a global context, our estimations show that mean N input via N_2 fixation by crab holobionts (nearly 20 µmol N m^{-2} h^{-1}) can account for ~90% of root + rhizome N_2 fixation (Alongi 2020), thus almost doubling this N input to mangrove ecosystems. Kristensen & Alongi (2006) argued that fiddler crabs can improve the growth of mangrove trees by oxidizing the sediment and detoxifying it from sulphides, while the crabs benefit from access to their food sources. In the present study, we show that crab holobionts also act as a conduit of key nutrients to their ecosystem via N_2 fixation by their associated microbiome. Interestingly, the rates measured here were higher in *A. rubripes* from the eutrophic site, further refuting the long-standing paradigm of N_2 fixation being regulated by the mere concentrations of inorganic N in the environment. Differences between rates of N transformations in the 2 crab holobionts can be ascribed to different environmental regulation (e.g. the quality of the organic matter at the study sites, the O_2 or nutrient levels, including oligoelements) or to different microbiomes. In a previous study, we analysed the microbial diversity of *L. thayeri* and demonstrated that the N_2-fixing community was dominated by the Cyanobacteria genus *Geitlerinema*, performing photosynthetic anoxygenic N_2 fixation in the oligotrophic Cananéia system (Zilius et al. 2020). We speculate that N_2 fixation in *A. rubripes* may be carried out by heterotrophic bacteria, which are not limited by the reductant in the eutrophic Bertioga system. Future work should examine the diversity of the *A. rubripes* holobiont to confirm or refute this hypothesis.

5. CONCLUSIONS

In the present study, we examined the overlooked role of fiddler and sesarmid crab holobionts on the N cycle in 2 mangrove systems differing in inorganic N availability and likely in the macromolecular quality of the available organic matter. The impact of crab holobionts was site- and species-specific, suggesting multiple control of microbial activity by environmental features and/or by differing crab physiologies and behaviours. Future studies should specifically address the crab species and trophic level factors that are not separated in the present work. However, we can conclude from this study that at an oligotrophic site, *Leptuca thayeri* holobionts contributed to increased reactive N availability in a nutrient-limited system producing refractory organic matter. At a eutrophic site, the *Armases rubripes* holobionts played a larger role in supplying N to the ecosystem, possi-

bly exacerbating eutrophication and favouring the growth of labile and opportunistic primary producers. Our findings suggest that the mangrove N cycle should be revised, including the contribution of benthic macrofauna to multiple N-cycling pathways and, in particular, to N_2 fixation.

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