

Monitoring nitrogen pollution in seasonally-pulsed coastal waters requires judicious choice of indicator species

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Abstract

We compared the sensitivity of algae and hermit crabs to seasonal shifts in the dominance of continuous sewage discharge vs. pulsed inputs of terrestrial material to a subtropical bay. During periods of low rainfall, when sewage was proportionately more important than diffuse loads from adjacent catchments, algae and crabs provided comparable information on the spatial distribution of N pollution. Conversely, during the wet season, when diffuse nitrogen loads from the catchment were of greater importance, the isotope signal of algae decoupled from that of crabs, indexing a greater magnitude of change and a more pronounced spatial gradient. Overall, algae better indexed the short-term impacts of anthropogenic nitrogen pollution whereas the signals provided by crabs provided a longer-term integrated measure of N inputs. Our results demonstrate the value of including multiple taxa with variable traits when monitoring the spatial and temporal extent of nitrogen inputs to coastal waters.

Keywords: wastewater, stable isotope, marine monitoring, sentinel, spatial subsidy

1. Introduction

Nitrogen contained in human sewage can be a major source of pollution in many coastal waters (McClelland et al., 1997; Schlacher et al., 2005). Elevated levels of the bioavailable forms of N discharged to near-shore waters can significantly impact productivity, biodiversity, organism health, habitat quality, food safety, and the delivery of ecosystems services (Cloern, 2001; Deegan et al., 2012; Diaz and Rosenberg, 2008; Schlacher et al., 2007). This makes reliable monitoring of the spatial and temporal signals of sewage pollution an important task in many environmental management settings (Costanzo et al., 2001).

Measuring stable isotope ratios ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) in the tissues of organisms in receiving waters has become a widely-used technique to trace the origin of inputs and map their distribution (Corbett et al., 2015; Costanzo et al., 2003; Gorman, 2009). While spatial signals in tissue $\delta^{15}\text{N}$ of organisms exposed to sewage reflect proximity to known point sources (Connolly et al., 2013; Oakes and Eyre, 2015), it is not widely known how temporal variation in upland nitrogen and carbon sources (e.g. with variation in rainfall or river discharge) affect isotope ratios in the tissues of indicator species (but see; Fertig et al., 2009; Schlacher and Connolly, 2009). For example, variation in $\delta^{15}\text{N}$ signals of algae have been reported over spatial (Orlandi et al., 2014), seasonal (Lemesle et al., 2016) and human -stress gradients (Calizza et al., 2015). Such variation is likely to be particularly important in systems where inputs from adjacent catchments are strongly pulsed, notably during quasi-predictable seasonal variation in peak rainfall that drives diffuse run-off and river discharge (Caffrey et al., 2007; Gorman et al., 2009; Schlacher et al., 2008; van de Merwe et al., 2016).

Choice of indicator taxa is a critical decision step in any environmental monitoring program (Schlacher et al., 2014). There are three important considerations for studies that use tissue- $\delta^{15}\text{N}$ of exposed organisms to monitor nitrogen pollution: a) source of uptake (inorganic N from water in plants or organic N via food in animals, b) tissue turnover rates which determine the period prior to sampling over which a taxon is likely to reflect ('integrate') a pollution signal; and c) organism mobility, which determines the spatial scale over which a pollution signal is integrated; not surprisingly then, a diverse range of taxa has been used to map sewage (e.g.

seagrass, algae, mussels, corals, clams, fishes; Gappa et al., 1990; Gartner et al., 2002; Jones et al., 2001; Reopanichkul et al., 2009; Schlacher et al., 2005; Watanabe et al., 2009).

Testing how the specific biological attributes or traits of indicators vary in their capacity to provide spatial and temporal information is vital for pollution monitoring studies. Here we compare two organisms; a sessile autotrophic green alga, and a mobile heterotrophic hermit crab, to assess their capacity to provide both singular and cumulative information about the nitrogen inputs to a coastal bay. Because sources of nitrogen to the study system are variable and comprise of a combination of continuous sewage release and a variety of riverine and terrestrial inputs driven by seasonal rainfall we expected considerable spatio-temporal variation in isotope signals. Our hypothesis was that spatial (across sites) and temporal (seasonal) change in the response of indicators (change in tissue $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) would be greater for marine algae than it would be for mobile benthic invertebrates. This type of information is particularly important for monitoring programs that require reliable and integrated measures of pollution sources that can change in response to a range of environmental factors. Our model system is a subtropical bay subjected to multiple forms of nitrogen input (sewage as a distinct point source vs. diffuse urban run-off and catchment inputs) and where regular seasonal cycles in rainfall lead to predictable seasonal peaks in the contribution of catchment-derived nitrogen inputs. We compare the alga *Ulva lactuca* (an organism that assimilates N from the water column) and the hermit crab *Clibanarius vittatus* (indirect assimilation from ingested food) with respect to; (a) the magnitude of change in isotope signals between seasons; and (b) the degree of spatial concordance of isotope signals between algae and crabs and whether this changes over time.

2. Methods

We tested the sensitivity of indicator species to seasonal variation in sewage-signals in Araçá Bay, São Paulo state (Brazil; Fig. 1). This is a relatively shallow bay (max. depth ~11 m at the eastern opening), located on the mainland-side of the São Sebastião Channel, the location of Brazil's largest oil shipping port. The area has a meso-tidal range of <2 m, and low to moderate wave exposure (Amaral et al., 2010). Water temperature in the channel typically ranges between 21°C in winter and 26°C in summer (Cerdeira and Castro, 2014). The main current flow within the

channel is from the south (Castro-Filho, 1990), but tidal oscillations give the site a characteristic counter-clockwise circulation pattern (Siegle et al., 2014). During the winter 'dry season' (April to September) storm mixing of the water column occurs, resulting in low but relatively constant inputs of nutrients. This contrasts summer 'wet season' (November to March) where rainfall-driven pulses of riverine inputs (from the Mae Isabel river) and restricted local upwelling events lead to a strong spatial gradient in nutrient concentrations within the bay (Dottori et al., 2015). The ratio of Dissolved Inorganic Nitrogen (DIN) inputs from the Mae Isabel river vs. outfall varied from ca. 1 : 1 during the dry season, to ca. 6 : 1 during the wet season (Carrilho, 2015; SABESP, 2015). Input of these materials results in a persistent enrichment of bay waters when compared to the São Sebastião Channel; observations validated by measures of mean inorganic nitrate (NO_3^-) concentrations, which are consistently greater within the bay when compared to channel (i.e., means: 23.6 vs. 9.9 mg/L over the same study period; Ciotti et al., 2014).

Araçá Bay has a long history of human modification, with the area incrementally reduced in size through consecutive expansions of an adjacent port since 1936 (Mani-Peres et al., 2016) and a population that has quadrupled over a similar period (SEADE, 2015). Primary-treated sewage (ca. $4.4 \times 10^6 \text{ m}^3$ -per year) is discharged via a 1 km long pipe at the bay's southern-most edge (Fig.1; CETESB, 2005). The bay also receives large volumes of diffuse surface runoff from urban and industrial areas (notably from the port of São Sebastião), and illegal dumping of commercial and domestic waste is common. While the flow of sewage into the bay is fairly constant (ca. $140 \text{ l}\cdot\text{s}^{-1}$), seasonal rainfall increases river flow from ca. $84 \text{ l}\cdot\text{s}^{-1}$ (dry season) to $>250 \text{ l}\cdot\text{s}^{-1}$ (wet season)(Carrilho, 2015).

The biological indicators assessed in this study were the green alga *Ulva lactuca* (Linnaeus 1753) and the hermit crab *Clibanarius vittatus* (Bosc 1802). Collections were made at 45 sites (24 during the dry season and 21 during the wet season) spread throughout the bay where both algae and crabs were found. While it was not possible to replicate the exact sampling locations (i.e., collection was done opportunistically) the spatial extent and depth range of sampling sites was concurrent for both periods. To capture seasonal signals related to changes in the amount of diffuse surface run-off driven by variations in rainfall, we sampled during two periods; (1) October 2013 at the end of the dry season (April to September) with mean daily rainfall of 1.67

± 0.35 (se) $\text{mm}\cdot\text{d}^{-1}$; and (2) March 2014 at the end of the wet season (November to March: 3.18 ± 0.80 (se) $\text{mm}\cdot\text{d}^{-1}$; source CEBIMar weather station). The rationale for sampling at the end of each distinct rainfall period was to allow organisms to reach isotopic equilibrium in their tissues if the input signals were to change.

For stable isotope analysis, we excised muscle tissue from the chelae of crabs (samples comprising material from 3 individuals pooled for each site \times time) and rinsed the entire thalli of algae (~5g of wet material) that had been cleaned of epiphytes by scraping with a scalpel. Given the small amount of material obtained from each crab specimen, we were unable to derive measures of variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ signals among individuals for any particular site. Stable isotope mass spectrometry was done at the University of California, Davis. Precision (standard deviation) was 0.09‰ for $\delta^{13}\text{C}$ and 0.05‰ for $\delta^{15}\text{N}$.

Data for each site and season were integrated into a Geographic Information System (ArcGIS version 10.1), where Kriging methods were used to produce continuous raster maps for each indicator, signal and season. The magnitude of seasonal change (‰) was calculated using the map algebra geoprocessing tool that calculated each pixel value using the equation $T2i - T1i$, (where i is the Kriging value for each point on the map). The function permitted the creation of spatially explicit maps describing the degree of seasonal change in the isotope signals of each indicator species.

We tested for seasonal differences in the mean value of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ with PERMANOVA and for differences in the dispersion of these signatures with PERMDISP (Anderson, 2001); both analyses were based on Euclidean distances. In this way, the experimental design compared both the spatial (i.e., replicate sites across the bay) and temporal (seasonal variation in relative importance of various inputs) variability in indicator species. We tested the general hypothesis that the spatial and temporal variation in indicator response (i.e., magnitude of difference % in $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ between the dry- and wet-seasons) would be greater for algae than it would be for hermit crabs. We also tested the consistency in spatial patterns by assessing variation in isotope signals with distance across the bay (i.e., using ANCOVA based on the distance from river and sewage inputs) and by testing the degree of spatio-temporal concordance between algae and crabs (i.e., do these indicators tell the same story across seasonal cycles).

3. Results

The $\delta^{15}\text{N}$ values of crab tissues were typically enriched by 1.92 ‰ (wet season) and 2.87 ‰ (dry season; Fig. 2) in relation to algae. Both algae and hermit crabs showed a significant shift towards heavier nitrogen isotope ratios after the wet season (Table 1a); this seasonal change was larger in algae (1.3×) than in crabs (1.1×; Table 1; Fig. 2). Spatial variability in $\delta^{15}\text{N}$ did not change significantly ($P = 0.426$) in hermit crabs between the dry and wet season (Table 1; Fig. 2). By contrast, $\delta^{15}\text{N}$ values of algae became significantly ($P = 0.019$) less variable after the wet season (Table 1; Fig. 2). Tissue $\delta^{13}\text{C}$ of algae did not change between seasons ($P = 0.871$) whilst hermit crabs became slightly ($\Delta \delta^{13}\text{C} = -0.96$) but significantly ($P = 0.028$) more depleted after rain (Table 1). Similar to $\delta^{15}\text{N}$, spatial variability in $\delta^{13}\text{C}$ of hermit crabs did not vary between season (Table 1; Fig. 2); but was significantly ($P = 0.019$) greater for algae after the wet season (Table 1; Fig. 2).

Nitrogen and carbon isotope signals showed spatial variation across Araçá Bay that differed between season and indicator (supplementary data). In general, signals demonstrated a north-to-south gradient across the bay, which was comparatively more pronounced for algae than it was for crabs. This gradient reflects depleted values in the area receiving discharge from the Mãe Isabel River, and enriched values adjacent the submarine outfall and channel entrance to the bay. These spatio-temporal trends become clearer when examining the degree of change (‰) between the two sampling periods (Fig. 3). In algae, tissue carbon and nitrogen became isotopically heavier after the wet season closer to the river mouth (i.e., changes of up to 5.5 ‰) but changed less at more distant sites. Change in hermit crab signals was generally below 1.5 ‰ and was more uniform across the Bay.

For nitrogen, the gradient of contrasts in $\delta^{15}\text{N}$ with increasing distance from the river mouth is significantly (ANCOVA, $P < 0.001$) steeper for algae (slope = -0.0061 ± 0.0007 se) compared with a less pronounced spatial decline in hermit crabs (slope = -0.0010 ± 0.0004 se; Fig. 4a). A similar pattern was observed for carbon isotopes, with algae showing a significantly (ANCOVA, $P < 0.001$) steeper gradient of temporal contrasts (slope = -0.0068 ± 0.0008 se) than hermit crabs (slope = -0.00168 ± 0.00021 se; Fig. 4b). While there was significant concordance between the

signatures of algae and crabs after the dry season when spatial variation in $\delta^{15}\text{N}$ of algae co-varied positively with that of crabs (Fig. 5) the signals of these two indicators became largely decoupled after the wet season (Fig. 5).

4. Discussion

Algae and hermit crabs indexed nitrogen inputs at different temporal resolutions, thus providing complementary information for pollution monitoring in systems where the relative influence of different nitrogen source types can vary according to catchment processes (Abrantes and Sheaves, 2008; Atwood et al., 2012). During periods when spatially distinct sewage inputs (i.e. point sources with heavy $\delta^{15}\text{N}$) dominated the nitrogen budget (dry season), algae and invertebrates showed a spatially concordant pattern (see also Connolly et al. 2013). By contrast, during times when diffuse inputs increased (i.e. with runoff and riverine inputs during the wet season) sewage signals became less detectable and more variable within algae. These general patterns reflect observed changes in average nutrient concentrations in the bay over the same period, which were ~ 1.3 times greater during the dry season than they were during the wet season (Ciotti et al., 2014).

The observed temporal variation in the response of indicator species to variations in the type of nitrogen inputs is likely to be driven by both physiology (particularly tissue turnover rates) as well as the route of nitrogen uptake (water column nutrients vs. food). Algae, and in particular *Ulva* spp., are fast integrators of water column nitrogen (i.e., replacing tissue constantly and adopting source signatures within days; Fernandes et al., 2012) and thus are able to respond faster to short-term changes in nitrogen inputs. This leads to predictable inter-annual changes in the $\delta^{15}\text{N}$ signals of opportunistic species such as *Ulva* spp. and *Porphyra* spp. (Lemesle et al., 2016). By contrast, animals integrate the signatures of the food they eat over longer time periods (ranging from weeks to months; Gaston and Suthers, 2004; Hesslein et al., 1993). The hermit crab *Clibanarius vittatus* has been described as a generalist feeder (Lartigue et al., 2003) that demonstrates aggregative behavior (Turra and Leite, 2000) and thus does not move large distances (typically only 10's meters; Turra and Leite, 2000), and therefore can provide spatially-explicit information on nitrogen inputs that reflect their diet over a longer time period.

Overall, the isotopic signatures of both algae and hermit crabs were within the range of other studies from impacted coastal areas (Connolly et al., 2013; Gartner et al., 2002). The maximum $\delta^{15}\text{N}$ value for algae (10.7‰) was lower than studies monitoring comparatively larger discharges from coastal cities (e.g., *Ulva australis* values up to 25‰; Connolly et al., 2013, and *Catenella nipae* values up to 24‰; Pitt et al., 2009), but higher than those expected in areas with few known pollution inputs (e.g., ca. 5.9‰; Gorman et al., 2009). Similarly, the maximum $\delta^{15}\text{N}$ value recorded for crabs (12.4 ‰) was higher than that of similar crustaceans collected from low-impact areas adjacent to Sao Sebastião (8.2‰; Corbisier et al., 2006). Although we lack data on the isotopic signal of effluent, a recent study has shown a stable gradient in mean $\delta^{15}\text{N}$ values of suspended particulate material (SPOM) across the study area, ranging from 6.48‰ close to the outfall, to 0.39‰ near the river mouth (Soares et al., 2015). The $\delta^{15}\text{N}$ enrichment in both taxa lends weight to our argument that the influence of sewage remains prominent in the outer part of the bay close to the outfall in both seasons, whilst a distinct signal typical of isotopically lighter $\delta^{15}\text{N}$ is detectable in algae (and to a lesser degree in crabs) near the river mouth after the rainy season.

Seasonal shifts in the relative influence of terrestrial and oceanic sources of N have been described in several tropical and subtropical regions receiving high rainfall (Abrantes and Sheaves, 2008; Atwood et al., 2012; Montes et al., 2013; Sarma et al., 2009). During low rainfall periods, effluent enters Araçá Bay directly from the sewage outfall in the form of an effluent plume (see Gartner et al., 2002); the footprint of which can be modified by hydrodynamics (i.e., treated waste water is more buoyant than seawater) and in some cases does not readily mix with the receiving water column (see also, Gaston et al., 2006; Schlacher and Connolly, 2009; Schlacher et al., 2008). Contrastingly, during the wet season pulsed inputs of river-born nitrates from the catchment (i.e., rainforest plants and soils having values of 3.7‰; Corbisier et al., 2006), raw untreated human effluent, and other urban and industrial inputs possibly including fertilisers and petrochemicals (Heaton, 1986) lead to a depletion in the signals of biota, especially at the upper intertidal margin. These patterns concur with numerous studies that show stormwater runoff can result in distinct source changes that influence the nitrogen dynamics of receiving waters (Bowen and Valiela, 2008; Hale et al., 2014; Yang and Toor, 2016).

These large subsidies of terrestrial material interact with the bay's circulation (see Siegle et al., 2014) to alter the isotope signals of indicator species, especially in the area directly receiving input from the Mãe Isabel River. In the vicinity of this area, the signals of both taxa demonstrated an isotopic depletion (i.e., shift to heavier isotopes) between the dry and wet seasons. This change however was far more pronounced for algae (i.e., sessile autotrophic indicator with rapid uptake) than it was for hermit crabs (i.e., mobile heterotrophic indicator with slow tissue turnover). Interestingly, the change in algal $\delta^{15}\text{N}$ (5.5 ‰) almost exactly mirrors the change in DIN inputs from 1:1 (dry season) to 6:1 (wet season). The seasonal increase in the importance of terrestrial inputs near the river was also supported by the $\delta^{13}\text{C}$ signals (again most notably for algae), which showed a marked change between the dry and wet seasons.

While concerns over treated effluent are often subordinate to those related to toxic organic chemicals and heavy metals in shallow marine bays (Martins et al., 2011; Rainbow, 1995), nutrient limitation within the Araçá region (Flores et al., 2015) makes it likely that sewage-derived nitrogen may impact local ecosystems. Accumulation of sewage in the western part of the bay, which borders local human communities, may also present a public health concern. Indeed, recent assessments have shown that the nutrient concentrations of bay waters are in the order of 2.3 – 2.5 times greater than the channel during the dry and wet seasons respectively (Ciotti et al., 2014). Given the recreational and economic value of the bay, including fishing, (i.e., fish and shellfish harvesting; Silva et al., 2014), upgrades to sewage treatment and improved management of land- and river-born effluents (e.g., from the Mãe Isabel River) should be important environmental management considerations.

The use of complementary indicators advocated in this study can be adapted to a broader range of situations. For example, it could be used to rapidly and cost-effectively (e.g., the costs of laboratory analysis in the present study were less than US\$ 1,000) measure nitrogen pollution over a range of coastal sites for which little or no information of input sources exists (a common situation throughout much of the developing world). It could also be used to evaluate the efficacy of upgrades to sewage treatment plants, or to gauge the consequences of changes to coastal land-use (e.g., urbanisation, land-use change in catchments, intensive agriculture). In these and other applications, both absolute (within species) and relative (between species)

changes offer complimentary information to better understand spatio-temporal patterns of nutrient inputs and sources and to better inform management interventions.

As coastal regions face increasing pressure from growing populations, understanding the capacity of different indicator taxa to reliably detect and map the types of nutrient inputs has, arguably, increased in importance. Our data suggests that choosing an effective indicator for pollution studies may depend on the relative importance of sewage vs. alternative diffuse inputs that may vary predictably with recurring weather and hydrodynamic processes. Thus, sewage monitoring programs previously developed for use in regions where persistent inputs do not vary substantially over time may not effectively capture the spatial and temporal dynamics of systems receiving strongly-pulsed inputs (see also, Connolly et al., 2009; Gaston et al., 2006; Schlacher and Connolly, 2009; Schlacher et al., 2008). In such situations it may be more sensible to employ multiple indicator species with different uptake properties and to use metrics such as the magnitude of signal change to adequately capture fluctuating input types in space and time.

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Table 1

Summary statistics (mean, confidence, t-statistic and significance value for the PERMANOVA) for temporal changes in stable isotope ratios ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) in algae (*Ulva lactuca*) and hermit crabs (*Clibanarius vittatus*) collected in Araçá Bay (Brazil) at the end of the dry season (T1 = October 2013) and at the end of the wet season (T2 = March 2014).

$\delta^{15}\text{N}$			T1 dry season	T2 wet season	t (perm)	P
<i>Means</i>						
	Algae	mean (95%CI)	6.57 (5.43 - 7.71)	8.49 (7.86 - 9.12)	2.94	0.004
	Hermit Crabs	mean (95%CI)	9.44 (8.79 - 10.09)	10.41 (9.79 - 11.03)	2.22	0.03
<i>Variability</i>						
	Algae	mean (95%CI)	2.17 (1.53-2.82)	1.01 (0.59-1.43)	3.03	0.019
	Hermit Crabs	mean (95%CI)	1.26 (0.90-1.62)	1.07 (0.69-1.44)	0.78	0.426
$\delta^{13}\text{C}$			T1 dry season	T2 wet season	t (perm)	P
<i>Means</i>						
	Algae	mean (95%CI)	-14.96 (-15.56 to -14.37)	-14.85 (-16.06 to -13.64)	0.03	0.871
	Hermit Crabs	mean (95%CI)	-12.52 (-13.18 to -11.87)	-13.48 (-14.01 to -12.94)	5.25	0.028
<i>Variability</i>						
	Algae	mean (95%CI)	1.166 (0.84 - 1.49)	2.316 (1.77 - 2.86)	15.20	0.002
	Hermit Crabs	mean (95%CI)	1.216 (0.83 - 1.60)	0.8384 (0.47 - 1.21)	2.11	0.177

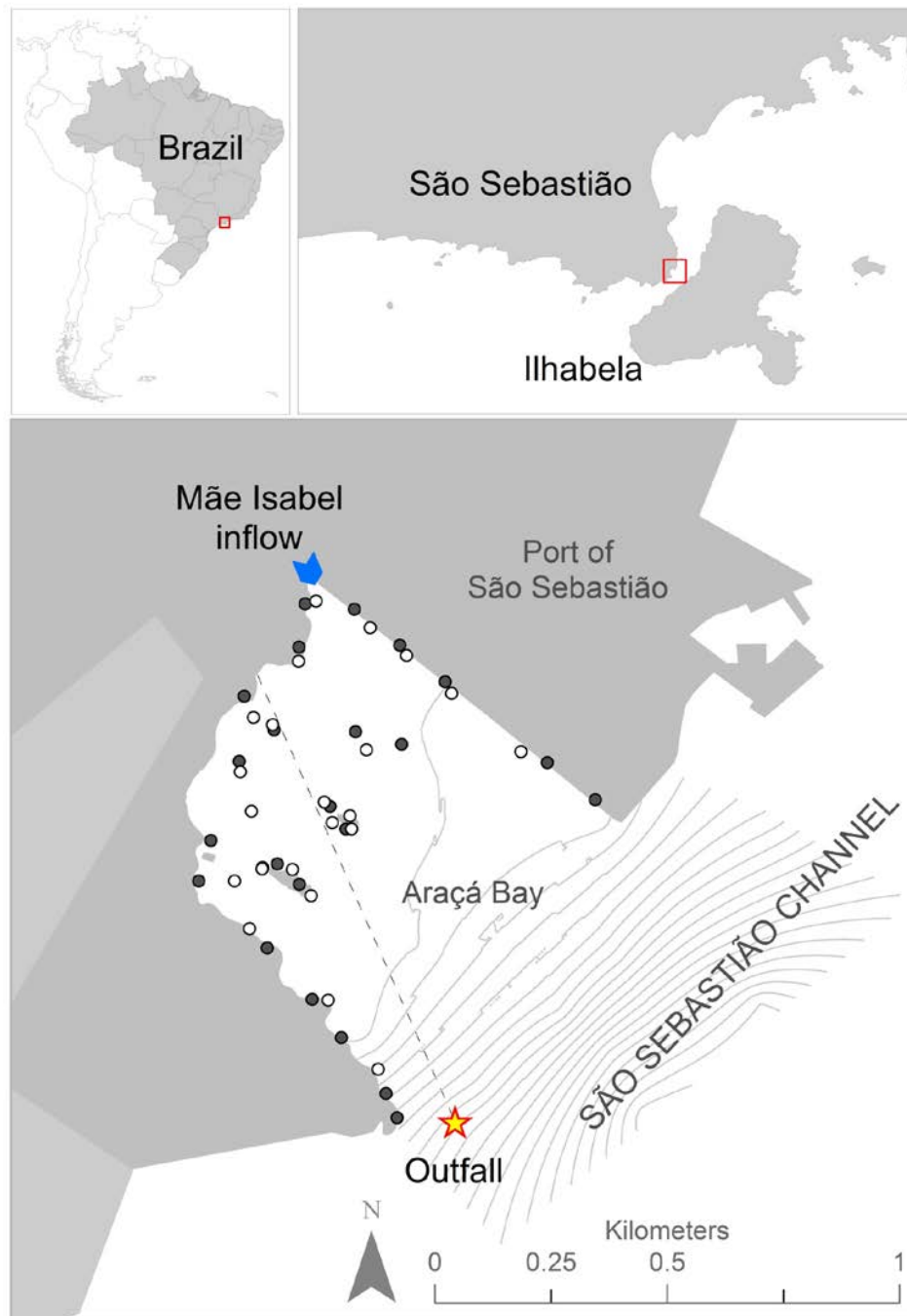


Fig. 1 The location of Araçá Bay (bottom panel) within the São Sebastião Channel (top right panel) in south-eastern Brazil (top left panel). The blue arrow denotes inputs from the Mãe Isabel River, the star symbol the location of the submarine sewage outfall, points are sampling locations and contours 1m depth intervals.

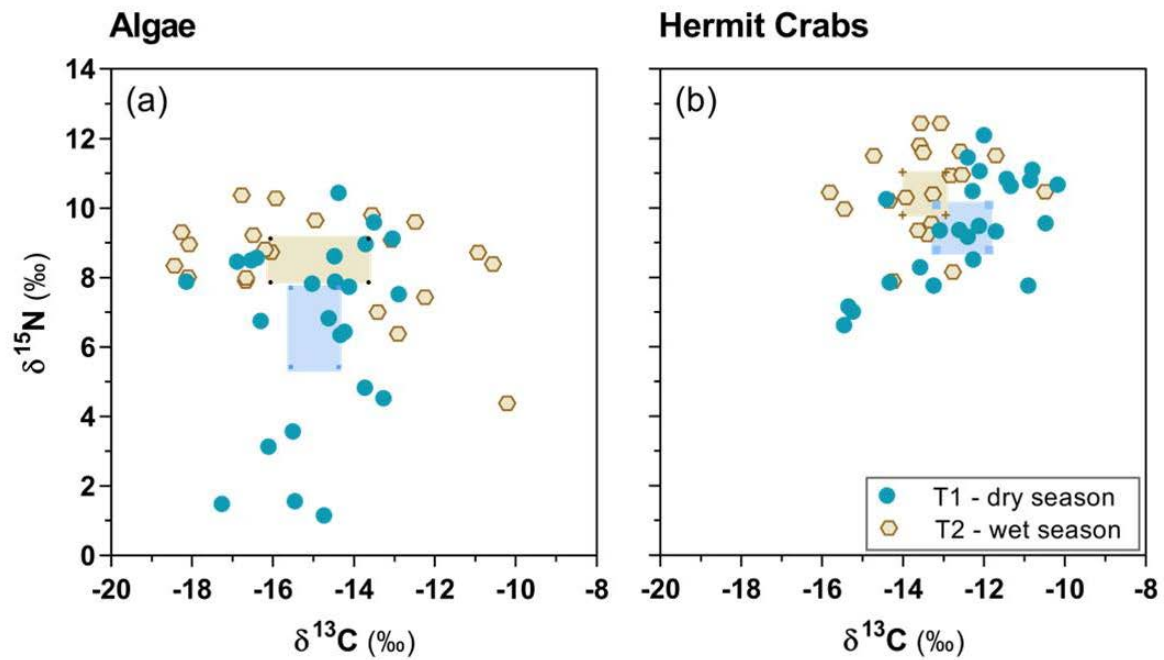


Fig. 2 Bi-plots illustrating temporal variation in carbon and nitrogen stable isotope signatures of (a) algae (*Ulva lactuca*), and (b) hermit crabs (*Clibanarius vittatus*) at the end of the dry season (T1 = October 2013) and the end of the wet season (T2 = March 2014) in Araçá Bay, south-eastern Brazil. The shaded boxes encompass the 95% confidence intervals.

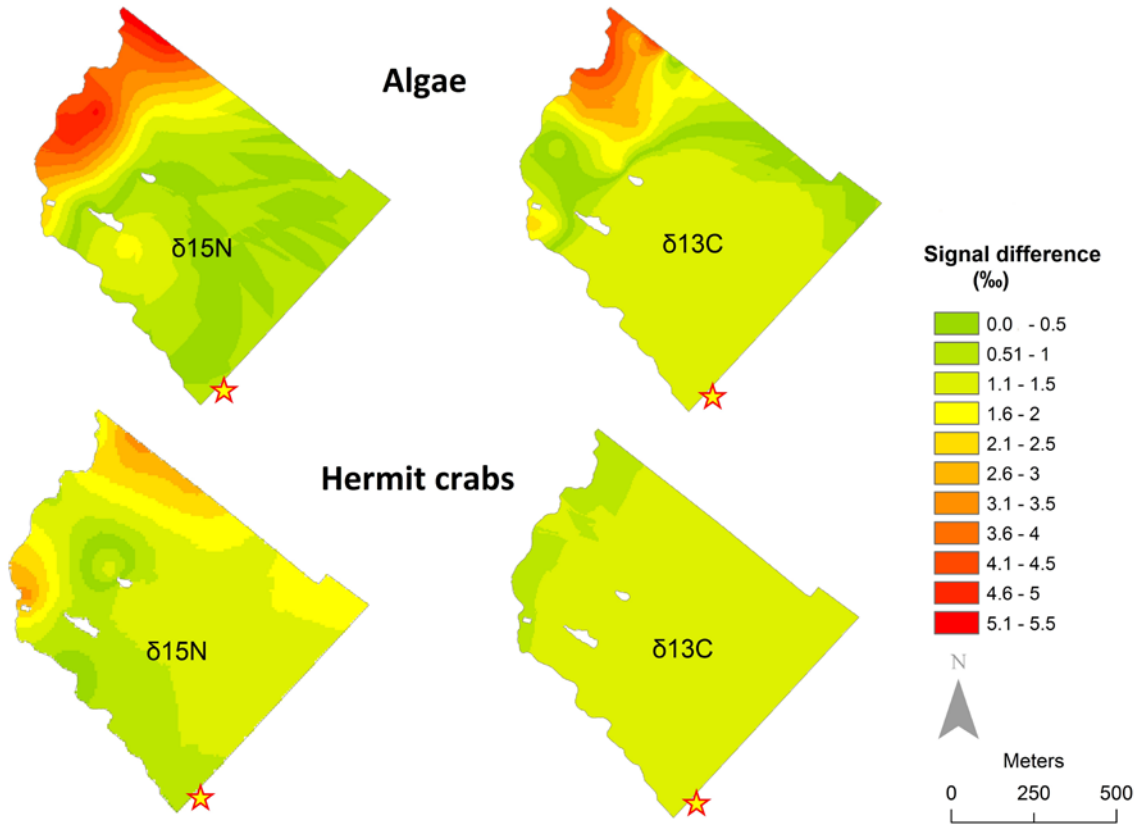


Fig. 3 Spatial variability in the magnitude of change (‰) in isotope signatures ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) of algae (*Ulva lactuca*) and hermit crabs (*Clibanarius vittatus*) sampled from replicate sites within Araçá Bay at the end of the dry season (T1=October 2013) and the end of the wet season (T2=March 2014). Note the change (T2-T1) reflects a general enrichment in isotopic values for both elements.

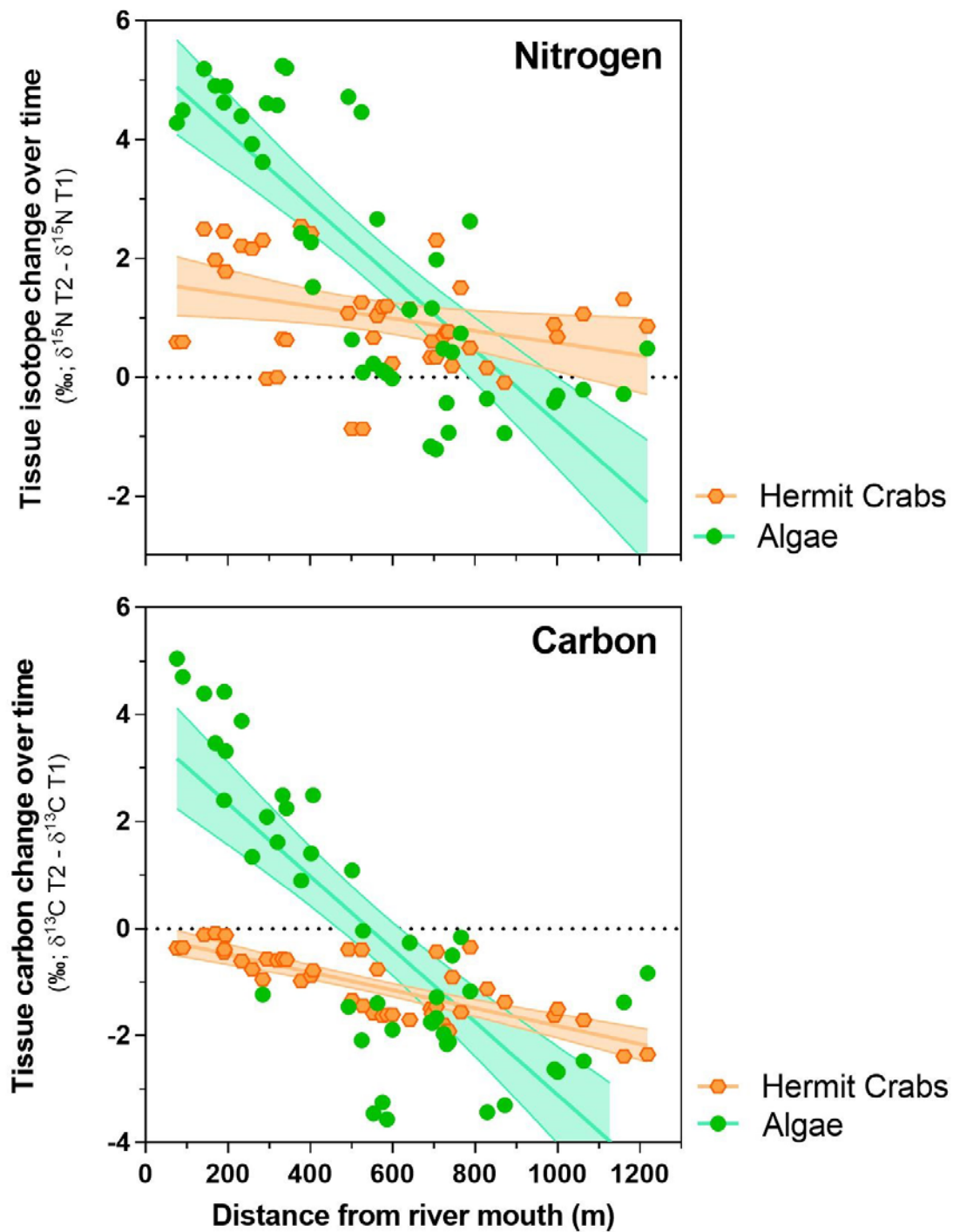


Fig. 4 Relationship between distance from river mouth and temporal change in isotope signals ($\delta^{15}\text{N}$ and $\delta^{13}\text{C}$) for the algae (*Ulva lactuca*) and hermit crabs (*Clibanarius vittatus*) in Araçá Bay, south-eastern Brazil.

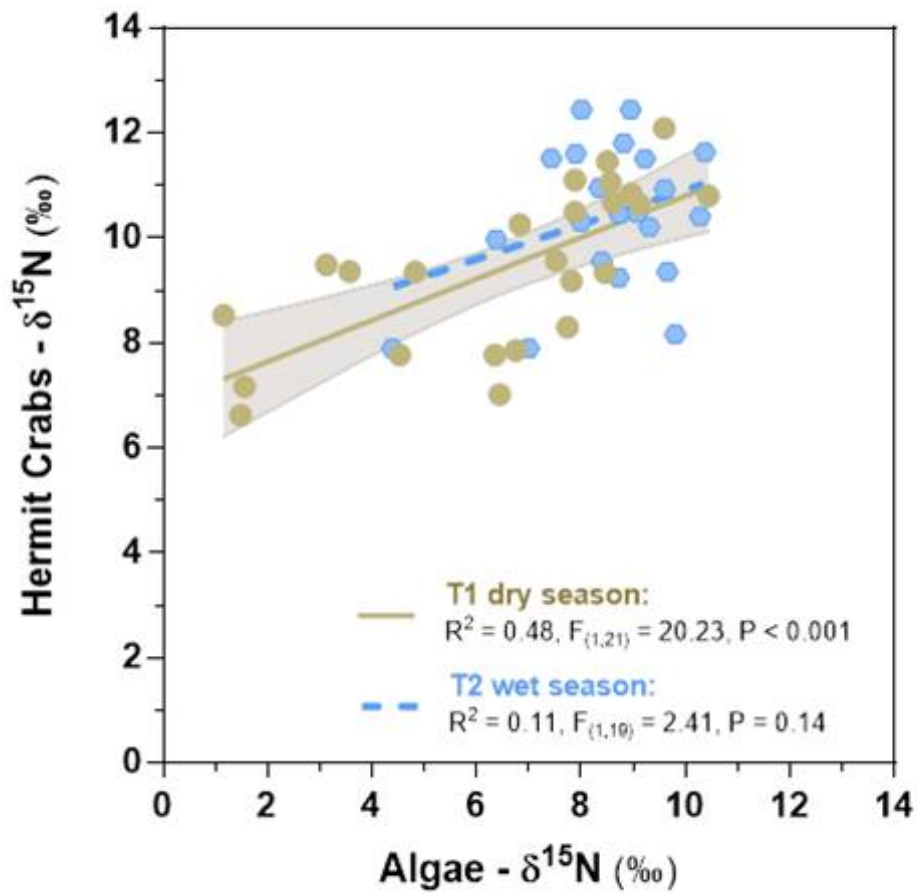


Fig. 5 Relationship between $\delta^{15}\text{N}$ signatures of algae (*Ulva lactuca*) and hermit crabs (*Clibanarius vittatus*) at matched sites sampled at the end of the dry season (T1 = October 2013) and the end of the wet season (T2 = March 2014), in Araçá Bay, south-eastern Brazil.