UNIVERSIDADE FEDERAL DO ESPIRITO SANTO
CENTRO DE CIENCIAS HUMANAS E NATURAIS
PROGRAMA DE PÓS GRADUAÇÃO EM CIENCIAS BIOLÓGICAS

DESEMPENHO DE ÍNDICES BIOTICOS DE QUALIDADE AMBIENTAL EM ESTUÁRIOS COM DISTINTAS CONDIÇOES DE URBANIZAÇÃO

Heliatrice Louise Hadlich

Vitória, ES
Novembro 2020

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Orientador: Dr. Angelo Fraga Bernardino

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RESUMO

Os estuários na América do Sul geralmente recebem efluentes não tratados de áreas metropolitanas próximas, demandando soluções de gerenciamento baseadas em ecossistemas para acessar os impactos dos diversos tipos de poluentes. Apesar da disseminação e do uso crescente de índices bênticos para a avaliação da qualidade de ambientes estuarinos alguns problemas relacionados à ambiguidade ainda necessitam de investigações detalhadas. Os índices bênticos podem responder tanto a distúrbios antropogênicos quanto aos naturais, podendo variar em distintas escalas espaciais e temporais, em virtude de diferentes processos interativos. Marcadores químicos como os esteróis são usados em paralelo aos índices biológicos para determinar a correspondência entre a contaminação do esgoto e os impactos ecológicos. A aplicação de índices que respondam ao enriquecimento orgânico ainda necessita de testes que detectem adequadamente os padrões de variabilidade com um delineamento amostral robusto. O presente trabalho teve como objetivo principal avaliar a qualidade ambiental de dois estuários situados na Ecorregião Marinha do Leste do Brasil, com distintas características de uso e conservação, baseada na determinação de múltiplos indicadores orgânicos (biopolímeros e esteróis) e inorgânicos (metais traço) de contaminação e da aplicação de índices bióticos. Para tanto, foram utilizadas as seguintes abordagens nos próximos dois capítulos: (i) uma aplicação multivariada foi usada para caracterizar o estado trófico de dois estuários (Baía de Vitória e Estuário dos rios Piraquê-Açú e Piraquê-Mirim) com distintas condições de urbanização, através dos indicadores químicos de contaminação; (ii) teste das respostas dos índices AMBI, M-AMBI, BENTIX e BO2A, usando um delineamento amostral hierárquico em dois estuários com distintos níveis de urbanização, com correlações aos proxies químicos de contaminação e análises de similaridade das respostas. No primeiro capítulo, os múltiplos biomarcadores orgânicos de qualidade sedimentar sugeriram que a Baía de Vitória, é predominantemente eutrófica ou hipertrófica dada uma alta entrada de esgoto não tratado no estuário, enquanto o estuário do rio Piraquê-Açu pode ser considerado um estuário prístino com indicação de contaminação baixa a moderada pela entrada de esgoto e a presença de sedimentos eutróficos a hipertróficos, não relacionados à contaminação fecal. Os resultados do segundo capítulo indicaram boa aplicabilidade dos índices bênticos. AMBI, M-AMBI, BENTIX e BO2A variaram significativamente na maior escala espacial ou na escala em que a poluição atua e foram principalmente correlacionados ao proxies químicos de contaminação, concordando com esses marcadores. No entanto, foram encontradas algumas ambigüidades ou inconsistências entre suas respostas, sugerindo que os índices AMBI e BO2A devem ser aplicados com cautela nas práticas de manejo, pois tendem a superestimar a qualidade ecológica das estações impactadas.

Palavras-chave: *índices bioticos, proxies químicos, coprostanol, qualidade ambiental, estuários.*

ABSTRACT

Estuaries in South America commonly receive untreated effluents from nearby metropolitan areas demanding ecosystem-based management solutions to access the different types of pollutant impacts. Despite the increased and widespread usage of benthic indices for environmental health assessment in estuarine areas some problems underlying ambiguous assessments still remain to be elucidated. The benthic indices may respond either to man-induced or natural disturbances and are likely to vary in space and time at many scales due to distinct interacting processes. Chemical markers such as sterols are used in parallel to biological indices in order to determine correspondence between sewage contamination and ecological impacts. The application of indexes that respond to organic enrichment still requires tests that adequately detect patterns of variability with a robust sample design. The main objective of this study was to assess the environmental quality of two estuaries located in the Eastern Brazil Marine Ecoregion, with different characteristics of use and conservation, based on determination of multiple organic (biopolymers and sterols) and inorganic (trace metals) indicators of contamination and use of the application of biotic indices. To this purpose, the following approaches were employed in the next two chapters: (i) a multivariate approach was used to characterize the trophic state of two estuaries (Vitória Bay and Piraquê-Açú estuarie) with different urbanization conditions, through the chemical indicators of contamination; (ii) testing the responses of AMBI, M-AMBI, BENTIX and BO2A indices, using a hierarchical sampling design in two estuaries with distinct levels of urbanization, with correlations to chemical proxies of contamination and analyzes of similarity of the responses. In the first chapter, the multiple organic biomarkers of sedimentary quality suggested that Vitória Bay is predominantly eutrophic or hypertrophic given a high entry of untreated sewage into the estuary, while the Piraquê-Açu estuary may be considered a pristine estuary with indication from low to moderate contamination by sewage input and the presence of eutrophic to hypertrophic sediments, not correlated to faecal contamination. The results of the second chapter indicated good applicability of benthic indices. AMBI, M-AMBI, BENTIX and BO2A did vary significantly at the largest spatial scale or the scale at which pollution acts and were mostly correlated to the chemical proxies of contamination, showing a congruence to the these markers. However, some ambiguities or inconsistencies were found between their responses, suggesting that the AMBI and BO2A indices should be applied with caution in management practices, as they tend to overestimate the ecological quality of the impacted stations.

Keywords: *biotic index, chemical proxies, coprostanol, environmental quality, estuaries.*

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CAPITULO 1

Poluentes orgânicos e efeitos no bentos estuarino na Ecoregião Marinha Leste do Brasil

The Eastern Brazil Marine Ecoregion (EME) extends for roughly 1200 km of coastline and over 50 estuaries from the states of Bahia and Espírito Santo on the Tropical Southwestern Atlantic province (11°28’S to 21°18’S; Spalding et al. 2007). The EME has atmospheric temperatures between 20 and 26 °C, with regional variability in mean annual rainfall values. The northern sector of the EME on the coast of Bahia state has higher annual rainfall (1600–2200 mm/year) when compared to the southern area on the Espírito Santo state (1000–1600 mm/year; Bernardino et al. 2015). During the last 40 years, there was a mean atmospheric temperature increase of 0.1 °C per decade along the EME (Bernardino et al. 2015).

Within the EME, there are several assessments of estuarine environmental quality near large urban areas, including the Todos os Santos and Vitória Bays located on the metropolitan areas of Salvador and Vitória, respectively. On the southern region of the EME, Vitória Bay (VB) is within a large metropolitan area and exhibits a marked salinity gradient from the main river (Santa Maria de Vitória) towards the ocean, with a significant presence of mangrove forests in the inner portions of the estuarine system (See Chapter 2). Several smaller estuaries within the southern region of the EME are located either near smaller towns or near marine protected areas on the coast. Two of these smaller estuaries have been relatively well sampled on the Espirito Santo state, including the Benevente (BE) and the Piraquê-Açu-Mirim (PAM) estuaries (Bissoli and Bernardino, 2018).

Estuaries and their associated habitats within the EME may provide significant ecological services to regional human populations. Typical ecosystem services provided by estuaries and their habitats include water quality (pollution retention), support to biodiversity and food provision, and coastal protection and climate regulation (Pendleton et al. 2012). Mangrove forests are an important habitat of estuaries along the EME, and likely provide important ecological services to the population within this region through pollutant retention and increasing water quality (Bernardino et al., 2020), support to biodiversity (Bernardino et al., 2018), and climate regulation (Servino et al. 2018). In a similar way, many traditional communities depend on services provided by smaller estuaries along the EME (Bissoli and Bernardino, 2018), and many conflicts on the management of those resources exist, including the lack of proper wastewater treatment and fisheries management. Habitat loss through mangrove removal or pollution is a major problem within most estuaries and even “visually preserved” ecosystems show some degree of contamination by sewage or industrial pollutants (Grilo et al. 2013; Bernardino et al. 2018; Varzim et al., 2019). These pressures impact the biodiversity associated with estuarine ecosystems (Barros et al. 2014) and likely put additional pressures on the services they provide (Lotze et al. 2006).

Human development leads to eutrophication, chemical pollution, chronic oil spilling, and clearance of vegetated habitats of estuaries for construction of ports, marinas, housing, and shrimp farms (Lotze et al. 2006). These impacts threaten the biodiversity and ecosystem services provided by estuaries. In the EME, these impacts reflect on benthic estuarine assemblages, which respond to acute and chronic impacts through changes in community abundance, composition, and diversity, which may lead to functional and important ecological changes (Varzim et al., 2019)

The Vitória Bay estuary is situated within the largest metropolitan area on the southern region of the EME and receives large inputs of untreated urban and industrial sewage (Grilo et al. 2013; Hadlich et al., 2018). As a result, some areas of Vitória Bay are contaminated with high concentrations (> 1.0 μg/g) of fecal lipids (e.g., coprostanol). In general, over 30% of VB are under high or severe contamination from untreated sewage, with negative effects on benthic diversity and assemblage composition. At the Piraquê-Açu-Mirim estuary, sewage contamination is an additional concern. In general, pollutants are released through untreated sewage, from direct impact of port activities or carried from the river basins and sequestered in estuarine sediments (Hadlich et al., 2018).

Identifying pollution impacts on estuarine ecosystems is therefore greatly important for management of ecosystem services provided by those areas. In addition to determining sources and sinks of pollutants, ecological indices are particularly useful to communicate scientific concepts in a simple format to the public opinion and managers, because they integrate numerous environmental factors of the system (Salas et al., 2006). According to Borja et al. (2009) the use of ecological indices is widely accepted for these ends. In the last few years, indices based on benthic assemblages have become a frequent tool to evaluate the environmental quality of marine and estuarine coastal zones (Muniz et al., 2005; Quintino et al., 2006; Borja et al., 2009), being widely applied globally and therefore very useful in comparing ecosystem quality between different estuaries globally (Borja et al., 2000; Simboura and Zenetos, 2002; Muniz et al., 2005; Muxica et al., 2005).

Benthic fauna have some features that make them potential candidates for evaluating the health of the coastal environment (Pearson and Rosenberg, 1978; McLusky and Elliott, 2004; Dauvin et al., 2010). Further, they play diverse roles in marine ecosystem functioning by being a primary food source for demersal fish and other benthic fauna. They also actively contribute to the marine biogeochemical cycling (Quintino et al., 2006). However, the macrobenthic community shows a spatio-temporal variation, a result of the natural functioning of the aquatic system. The marked change in the macrobenthic community pattern is reflected in the benthic indices that have often been the main criticism of its use (Reiss and Kröncke, 2005; Kröncke and Reiss, 2010).

The distinction between the responses of these indicators to natural and anthropogenic environmental sources of stress is often a challenge, especially in naturally stressed habitats such as estuaries (Dauvin, 2007; Elliott and Quintino, 2007). In these systems, the spatial variability can be either related to artificial organic enrichment, industrial waste, and other sources of pollution or to the natural detritus transport and salinity gradients, for instance. However, species or functional groups essentially responsive to organic enrichment and the associated reduction in oxygenation of sediments, properties that can vary naturally, might thus potentially confound any biotic response to anthropogenic disturbance (Tweedley et al., 2014; Brauko et al., 2015). Therefore, there is a demand for testing the reliability of different approaches when applying benthic indicators, using *in situ* data properly designed to segregate anthropogenically from naturally induced changes (Brauko et al., 2020). Improving our understanding of the natural dynamics of indices is of primary importance when considering the use of indices for environmental monitoring and assessment (Kröncke and Reiss, 2010). For this reason, benthic biotic indices may not be universally applicable, and

they should be tested in different geographic areas and perturbations (i.e., natural or anthropogenic).

In this context, this work then aimed to provide a first assessment of environmental pollution and habitat quality in an urbanized estuary (Vitoria Bay), and on the Piraquê-Açu-Mirim estuary located on a protected area without significant urbanization effects (Bissoli and Bernardino, 2018).

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CAPITULO 2

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Multiple biogeochemical indicators of environmental quality in tropical estuaries reveal contrasting conservation opportunities

Heliatrice LouiseHadlich[1]; NataliaVenturini[2]; Cesar C.Martins[c1]; VanessaHatje[d]; PolianaTinelli[1]; Luiz Eduardo de Oliveira Gomes[1]; Angelo FragaBernardino[1]

[1]Grupo de Ecologia Bentônica, Departamento de Oceanografia e Ecologia, Universidade Federal do Espírito Santo, Avenida Fernado Ferrari, 514, Goiabeiras, Vitoria, ES, 29075-910, Brazil

[2]Oceanografía y Ecología Marina, Instituto de Ecología y Ciencias Ambientales (IECA), Facultad de Ciencias, Universidad de la República (UdelaR), Iguá 4225, 11400 Montevideo, Uruguay

[3]Centro de Estudos do Mar, Universidade Federal do Paraná, Caixa Postal 61, 83255-976 Pontal do Paraná, PR, Brazil

[4]Centro Interdisciplinar de Energia e Ambiente (CIENAM), Instituto de Química, Universidade Federal da Bahia, Salvador, BA, Brazil

Abstract

Estuaries are under major impacts from pollution even when managed as conservation units. Here we used multiple biogeochemical indexes of contamination and trophic status, including faecal sterols, biopolymers and trace metals, to determine and compare environmental quality of two tropical estuaries with contrasting conservation status. In the metropolitan estuary, eutrophic/hypereutrophic conditions and high concentrations (> 1.0 μg g−1) of coprostanol were spatially correlated to sources of raw sewage input. Unexpected eutrophic sediments were also detected at the estuarine reserve, but with low sewage contamination indicating that high organic availability and burial predominated. The natural or polluted eutrophic sediments were determined by comparing multiple contamination indexes, which indicated sediment contamination within the metropolitan estuary. This study indicates that the long-term conservation of estuarine ecosystems on the Atlantic coast of Brazil are threatened by a typically poor sewage treatment and suggests that estuarine sediment quality need to be evaluated by multiple proxies before estuaries can be included in spatial conservation planning. Estuaries are ecosystems under major impacts from pollution and habitat loss. Here we evaluated sediment concentrations of fecal steroids, biopolimers and trace metals as markers of contamination in two tropical estuaries with contrasting conservation status. Within the Vitória Bay Estuarine System (VB), which receives sewage input from over 1 million habitants, eutrophic and hypereutrophic conditions indicated by organic polymers (BPC) and high concentrations (> 1.0 µg.g^{-1}) of fecal lipids (coprostanol). An unexpected indication of sewage contamination was also detected at an estuarine reserve 50 Km north of VB, which also had BPC values indicating eutrophic conditions and low sewage contamination. Trace metal contamination may also be a concern within VB as a result of industrial pollution. This study expands the low environmental quality assessment of a major metropolitan area and indicates that the long term conservation of estuarine ecosystems on the Eastern Brazil Marine Ecoregion are threatened by a poor sewage treatment within Brazil.

Keywords: Marine pollution, Estuaries, Organic Biopolymers, Sediments, Trace metals, Coprostanol.

1. Introduction

Estuaries worldwide have been heavily impacted by habitat transformation and pollution with severe risks to their ecological resilience (Lotze et al., 2006). The increasing pollution threaten the ecological services provided by estuaries, especially near urban centers where there is poor management of multiple impacts. In contrast to their low environmental quality, estuaries have become priority areas for conservation and to be managed as marine reserves (Ducrotoy and Elliott, 2006; Gilby et al., 2017). Given that estuarine sediments may concentrate pollutants (Dell'Anno et al., 2002; Baldock et al., 2004; Muniz et al., 2015), estuarine ecosystems need ecological indicators to ensure that protection is targeted to areas with higher environmental quality.

The sedimentary organic matter (OM) and pollutants on estuaries are important drivers of ecological changes observed on benthic assemblages and therefore biopolymers such as carbohydrates (CHO), lipids (LIP) and proteins (PRT), may be used as indicators for estuarine sediment quality (Nixon, 1995). The biopolymeric carbon (BPC) represent the OM labile fraction that is readily available for benthic organisms through remineralization (Aguiar et al., 2013), and therefore are highly sensitive to spatial and temporal changes in the benthic trophic status associated to both natural and human-induced environmental alterations (Dell'Anno et al., 2002; Pusceddu et al., 2003, 2009; Joseph et al., 2008). High concentrations of CHO with low nutritional value for benthic organisms may represent a degraded organic detritus (Joseph et al., 2008), whereas high PRT content, which is the main source of nitrogen for consumers, may reflect an increased fresh productivity (Danovaro et al., 1999). On the other hand, LIP have been used to indicate available energy to organisms and are indicators of detrital contributions derived from secondary production; furthermore, in polluted areas, LIP have been associated with anthropogenic sources of oil and sewage (Dell'Anno et al., 2002; Venturini et al., 2012). Therefore, the use of molecular markers in estuarine sediments are well established indicators of the benthic trophic status and identifying natural or human-induced eutrophication (Dell'Anno et al., 2008; Pusceddu et al., 2011; Venturini et al., 2012; Pita et al., 2017). The sedimentary trophic status is also largely applied to monitor long-term changes in estuarine environmental quality and management (Pusceddu et al., 2009), but their application with sewage and other OM biomarkers are still limited.

Sterols are used as molecular markers to characterize OM sources, either marine or terrigenous, and also to identify anthropogenic faecal contamination (Cordeiro et al., 2008; Martins et al., 2014; Cabral et al., 2018). Sterols are persistent in sediments, easily associate with particulate material and have resistance to anaerobic degradation (Muniz et al., 2015). Faecal sterols, including coprostanol and epicoprostanol, are used as tracers for human waste because they are present in human faecal and sewage effluents (Grimalt et al.,1990; Carreira et al., 2004; Montone et al., 2010; Venturini et al., 2015). Other sterols such as cholesterol and cholestanol are abundant in aquatic systems due to their presence in zooplankton and phytoplankton (Volkman, 2005; Loh et al., 2008). An additional marker of marine systems, trace elements have been increasingly released in estuarine ecosystems from anthropogenic inputs including mining, industry and diffuse sources such as agriculture, combustion, terrestrial and maritime transport, among others (Luiz-Silva et al., 2008; Gomes et al., 2017). Trace metals affect the ecosystem as a whole and human health through the processes of bioaccumulation and biomagnification and their determination in sediments may also support environmental quality assessments (Krull et al., 2014).

This study assessed the trophic status of sediments from two tropical estuarine systems with contrasting conservation and use on the Eastern Brazil Marine Ecoregion, based on determination of multiple organic (biopolymers and

sterols) and inorganic (trace metals) indicators. By contrasting a heavily urbanized estuary with another visually wellpreserved
ecosystem, we aimed to: (i) identify and quantify sources of the sedimentary OM and trace metals, and; (ii) assess the environmental quality of sediment from both estuarine systems in order to aid a basinwide management of both areas. Low environmental quality within the metropolitan estuary may be expected due to historical record of human activities, whereas the estuary located in a conservation reserve area would be less impacted by pollutants.

2. Materials and methods

2.1 Study area

The study was conducted in two estuaries located in the Eastern Brazil Marine Ecoregion, which has an average monthly rainfall of 145mm and temperatures from 24 to 26 °C (Bernardino et al., 2015, 2018a). The metropolitan estuary, Vitória bay (VB, 20°18′S, 40°20′W; Fig. 1) receives about 16,000m3 day−1 of untreated domestic and industrial
effluents from several tributaries (Bubu, Itanguá, Marinho, Aribiri rivers and Santa Maria da Vitoria city). VB has an important economic and touristic relevance to the region, but the historical record of degradation has not yet been used towards an improved management
of estuarine resources.

The Piraquê-Açú-Mirim estuary (PAM; 17°58′S, 40°00′W) is situated 50 km north of VB and has a well-preserved area with tidal flats and extensive mangrove forests (dominated by Rhizophora mangle and Avicennia schaueriana), with minor coastal development (Bernardino et al., 2018b; Bissoli and Bernardino, 2018). The PAM estuary is managed as a conservation unit with use of its natural resources by local traditional communities (Servino et al., 2018).

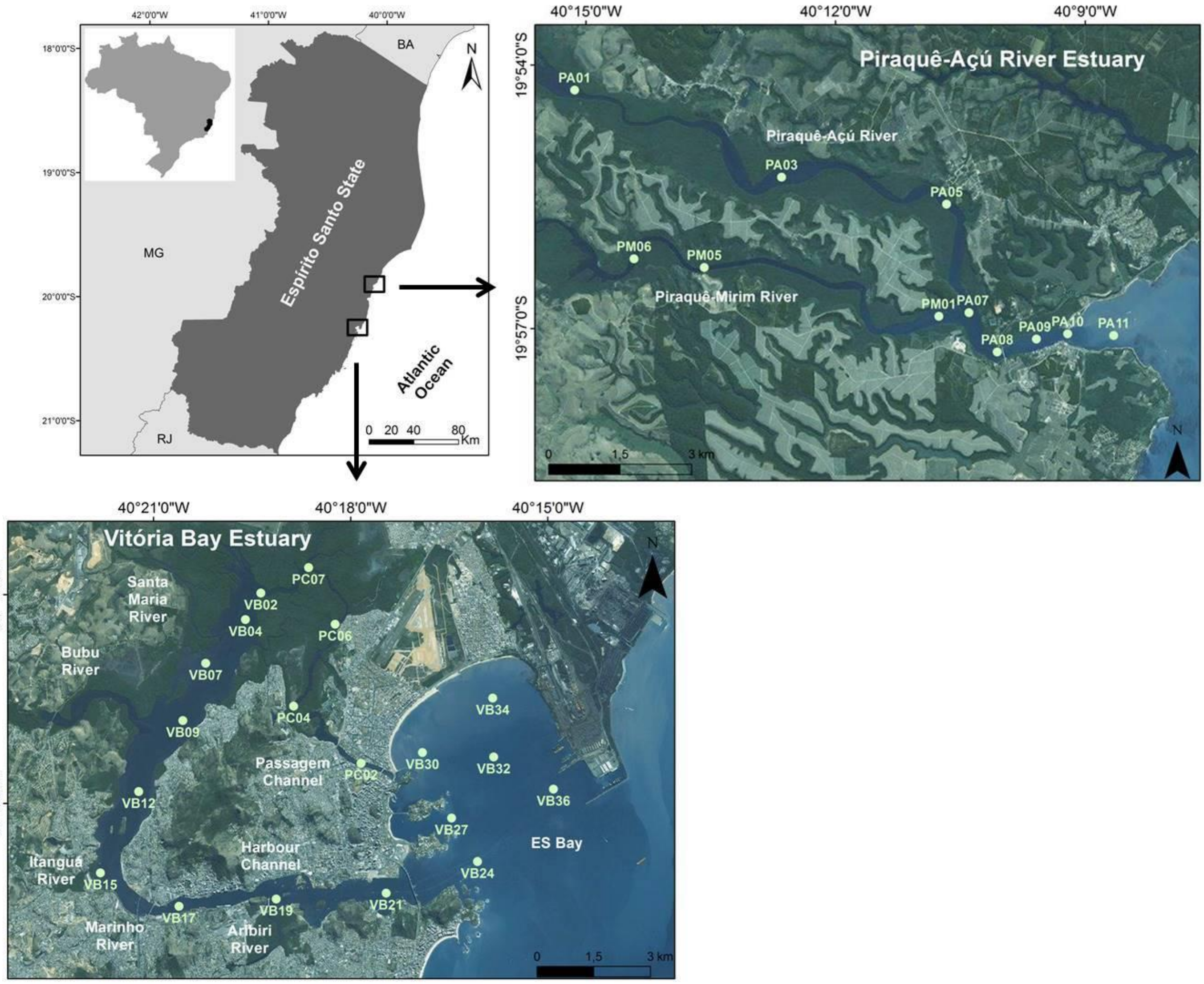

Figure 1. Study area showing the sediment sampling sites distributed along the Great Vitoria Estuarine System and Piraquê Açú-Mirim estuary in the Eastern Atlantic, Brazil.

2.2 Sampling

Sampling was carried in November 2014, with 19 sites in VB, and 11 sites in the PAM estuary (Fig. 1). Random stations were distributed across the estuaries, with VB02 to VB15 located in the inner VB estuary, VB17 to VB21 along the Vitória harbor channel and VB24 to VB36 in Espírito Santo Bay. In VB, four stations (PC 02, 04, 06, 07) were sampled along a secondary channel (Passagem Channel, PC) that connects the inner estuary to the Espirito Santo Bay. At the PAM estuary, stations

PA01 to PA05 were located in the northern Piraquê-Açu river, stations PM 01 to PM06 in the southern Piraquê-Mirim river, and stations PA07
to PA11 were sampled at the mouth of this estuary. Salinity was measured in situ with a calibrated SonTek CastAway CTD. Sediment samples (N=2 per station) were collected with a Day grab (0.1m2), and the top 3 cm of undisturbed surface sediment from two independent replicates were mixed into one composite sample for chemical and particle size analyses. For organic compounds, samples were stored in pre-cleaned aluminum container whereas surficial sediments for trace metal analysis were stored in pre-cleaned LDPE containers. Samples remained frozen (−20 °C) until laboratory procedures.

2.3. Laboratory procedures

2.3.1. Bulk sedimentary parameters

In laboratory, sediments were dry weighed and sieved (2 mm, 1 mm, 0.5 mm, 0.25 mm, 0.125mm and 0.63mm fractions) on a mechanical shaker, and the grain size was determined. Sub-samples of 1.5–2.0 g were analyzed for total organic matter (TOM) by weight loss on ignition at 550 °C for 4 h. Total organic carbon (TOC) and total nitrogen (TN) were analyzed by a LECO Elemental Analyser (Leco, TruSpec CNS). Accuracy of element analysis was checked employing a Soil CRMs (Thermo Soil Reference NCS, Italy and LECO Soil Calibration Sample, USA). Average recovery for TOC (2.36 ± 0.03%) and TN (0.185 ± 0.011%) were 99.8 and 101%, respectively.

2.3.2. Biochemical analysis of organic matter and sediment trophic status Categorization

Total protein (PRT) determination followed an extraction with NaOH (0.5 mol L^{-1}, 4 h) according to Hartree (1972), modified by Rice (1982). Total carbohydrates (CHO) were analyzed according to Gerchacov and Hatcher (1972) while total lipids (LIP) were extracted by ultrasonication (20 min) in 10 mL of chloroform:methanol (2:1 v/v) and analyzed following Marsh and Weinstein (1966). Blanks for each analysis were performed with pre-combusted sediments at 450 °C for 4 h. PRT, CHO and LIP concentrations were expressed as BSA, glucose and tripalmitine equivalents, respectively. All analyses were carried out in triplicate. Protein, carbohydrate and lipid concentrations were converted to carbon equivalents assuming a conversion factor of 0.49, 0.40 and 0.75 mg, respectively (Fabiano and Danovaro, 1994). The sum of PRT, LIP and CHO carbon equivalents was reported as the biopolymeric carbon (BPC) and used as a reliable estimative of the OM labile fraction (Fabiano et al., 1995). Based on BPC concentrations, estuarine areas were defined as: (i) hypertrophic (BPC≫5mg Cg^{-1}); (ii) eutrophic BPC (3–5mgCg^{-1}); (iii) mesotrophic (BPC=1–3mg C g^{-1}), and; (iv) oligotrophic BPC (< 1 mg Cg^{-1}) (Pusceddu et al., 2007, 2011). Also, the PRT:CHO and the CHO:LIP ratios were calculated and used as indicators of the status of biochemical degradation processes (Galois et al., 2000).

2.3.3. Sterols

Sterols were extracted from sediments with a Soxhlet apparatus for 8 h with 80 mL of n-hexane:dichloromethane (DCM) (1:1) after being spiked with surrogate standard (5α-androstanol) as described by Wisnieski et al. (2016). The extracts were concentrated to 1 mL using a rotary evaporator, purified and fractionated by silica and alumina liquid chromatographic column with an elution of 5 mL of ethanol/DCM (1:9, v/v), followed by 15 mL of ethanol. The purified extract fractions were dried, derivatized (BSTFA/TMCS (99:1) for 90 min at 70 °C), spiked with internal standard (5α -cholestane) before instrumental analyses. Sterols were analysed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC/FID) and an Agilent 19091J-015 capillary fused silica column coated with 5% phenylmethylsiloxane (50 m, 0.32mm ID and 0.17 μm film thickness). The oven temperature was programmed from 40 to 240 °C at 5 °C min^{-1}, then to 250 °C at 0.25 °C min^{-1} (holding for 5 min), then to 280 °C at 5 °C min^{-1} and to 300 °C at 20 °C min^{-1} (holding for 10 min). Calibration was based on external standard mixtures of sterols coprostanol, epicoprostanol, cholesterol, cholestanol, brassicasterol, campesterol, stigmasterol, sitosterol and sitostanol) at nine different concentrations between 0.25 and 15.0 ng μL^{-1}; $R^2 > 0.995$).

The concentrations of individual faecal sterols and the diagnostic ratios based on sterols from different origin were used to determine sewage contamination levels. The use of faecal sterols alone is not consensual for an accurate assessment of contamination (Writer et al., 1995; Gonzalez-Oreja and Saiz-Salinas, 1998); therefore, several authors has been applying ratios of coprostanol with other sterols to assess the sewage input in sediments (Grimalt et al., 1990; Cordeiro et al., 2008; Martins et al., 2010). A summary of sterol diagnostic indices and threshold levels applied to evaluation of sewage contamination were used in this study (Table 1).

Table 1. Summary of sterol diagnostic indices and threshold levels from Vitória Bay and Piraquê Açú-Mirim estuary, Eastern Brazil.

Sterol diagnostic indices	Threshold levels	Reference
Coprostanol	< 0.10 $\mu g.g^{-1}$: no contamination > 0.10 - 0.50 $\mu g.g^{-1}$: low sewage contribution > 0.50 $\mu g.g^{-1}$: moderate contamination > 1,00 $\mu g.g^{-1}$: high sewage	Writer et al. (1995) Gonzalez-Oreja and Saiz-Salinas (1998) Martins et al. (2007)
Ratio I: epicoprostanol/coprostanol	< 0,20: untreated sewage input > 0,80: treated sewage input	Mudge and Seguel (1999)
Ratio II: coprostanol / (coprostanol + cholestanol)	< 0,30: pristine environments > 0,50: sewage contamination	Grimalt et al.(1990) Leeming et al. (1998)
Ratio III: coprostanol / (coprostanol + cholesterol)	> 0,50: sewage contamination	Takada et al. (1994)
Ratio IV: coprostanol / (coprostanol + dinosterol)	> 0,50: sewage contamination	Venkatesan and Kaplan (1990)
Ratio V: % of faecal sterols/total sterols	< 5 %: no contamination > 50 %: high sewage contamination	Hatcher and McGillivary (1979)
Ratio VI: cholestanol/cholesterol	< 0,50: fresh organic matter input > 0.5: in situ reduction of cholesterol	Canuel and Martens (1993) Chalaux et al. (1995)

2.2.4 Trace and major elements

Sediments were extracted with 1.0 mol L^{-1} HCl (30%) according to Hatje et al. (2006). Concentrations of trace metals (Zn, V, Cr, Co, Cu and Pb) and major elements (Fe, Al and Mn) were measured with an inductively coupled plasma optical emission spectroscopy (ICP-OES PerkinElmer, Optima 7300 DV). Sediment acid extracts were carried out in triplicate and relative standard deviations were lower than 5%. Blanks and certified reference material (PACS-2, National Research Council of Canada, Canada) were utilized to assess the accuracy of the analytical procedure and results indicated good analytical precision, considering the incomplete digestion method. High levels of trace metals in sediments may affect marine biota and pose a risk to human health through the consumption of seafood (Siddique et al., 2009). To evaluate the ecological significance of trace metals, the concentrations obtained were compared to Threshold Effects Level (TEL), Probable Effects Level (PEL) and Apparent Effects Threshold (AET) (NOAA, 1999; Buchman, 2008). TEL is the limit under which no adverse effects on the biological community are observed while PEL is the probable level where adverse effects in the biological community would occur and AET is the contaminant concentration in sediment above which adverse effects are always expected for a particular biological indicator.

2.2.5 Data analyses

The distribution of molecular markers, metals, sedimentary bulk and OM biochemical parameters among sampling stations was compared between sites in each estuary. Inferences of sediment trophic status and anthropogenic inputs of contaminants to sediments were made based on the Principal Components Analysis (PCA), with abiotic variables log transformed for proportional data (Quintana et al., 2015). Person correlation coefficients (r) were calculated to evaluate the specific relationship between trace and major elements, organic markers and sediment variables. Multivariate analyses were performed using the software PRIMER v 6.0 (Clarke and Gorley, 2006).

3. Results

3.1 Ancillary data

Salinity ranged from 19 to 36 in VB and from 15 to 34 in the PAM estuary. The sediment composition in VB was typically a mixture of silt, clay and shells associated with coarse sand, likely as a response of variable river input and sediment transport. The VB harbor also had coarse sediments as a result of dredging along the harbor channel. Sediment grain size in the PAM estuary was predominantly of fine sand, silt and clay (Fig. 2, Table S1 in Supplementary Material). Sediments from VB and PAM exhibited wide ranges in TOC content (0.1–4.1% and 0.1–10.4%, respectively) and C:N ratios (3–67 and 4–23, respectively; Table S1).

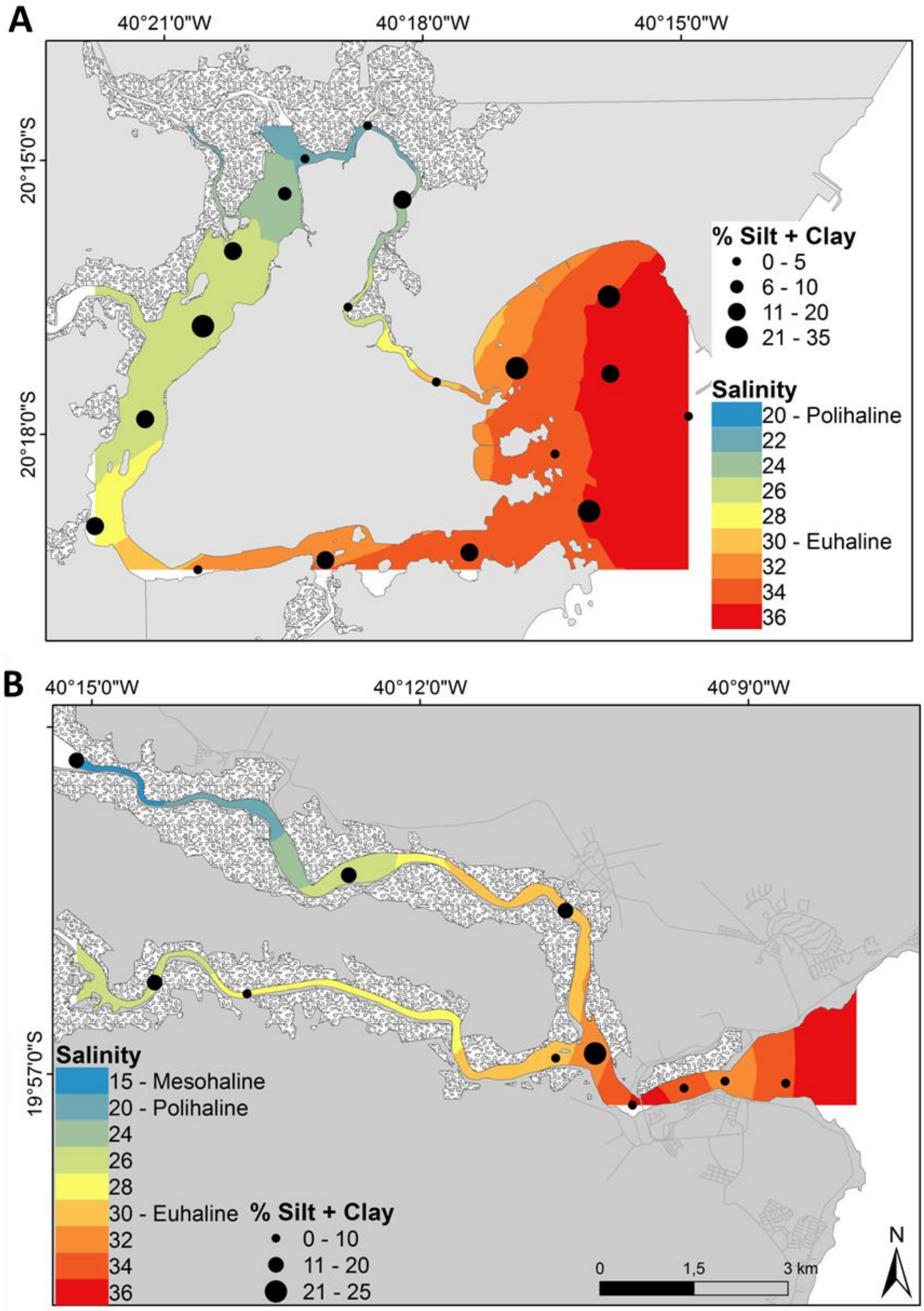

Fig. 2. Relative percentages of the fines sediment in Vitória Bay (A) and Piraquê Açú-Mirim estuaries (B). Legends in: green - sector mesohaline, red - sector polihaline and blue -sector euhaline.

3.2 Biochemical OM composition and sediment trophic status

The PRT, CHO and LIP concentrations were higher in the inner estuarine sites of VB and PAM and decreased towards coastal sites (Fig. 3). Sites near river tributaries in VB exhibited highest PRT, CHO and LIP concentrations suggesting sources of sewage input, contrary to the observed in the PAM estuary. Biopolymeric concentrations indicated that VB and PAM estuaries were typically from eutrophic to hypereutrophic conditions with BPC > 3 mg C g−1 (Fig. 3), with few sites in VB and PAM exhibiting an oligotrophic condition (Fig. 3, Table S2). BCP concentration ranged between 0.26 (VB27) to 11.3 mg C g−1 (VB15) and 0.99 (PA11) to 17.9 mg C g−1 (PM06) (Fig. 3).

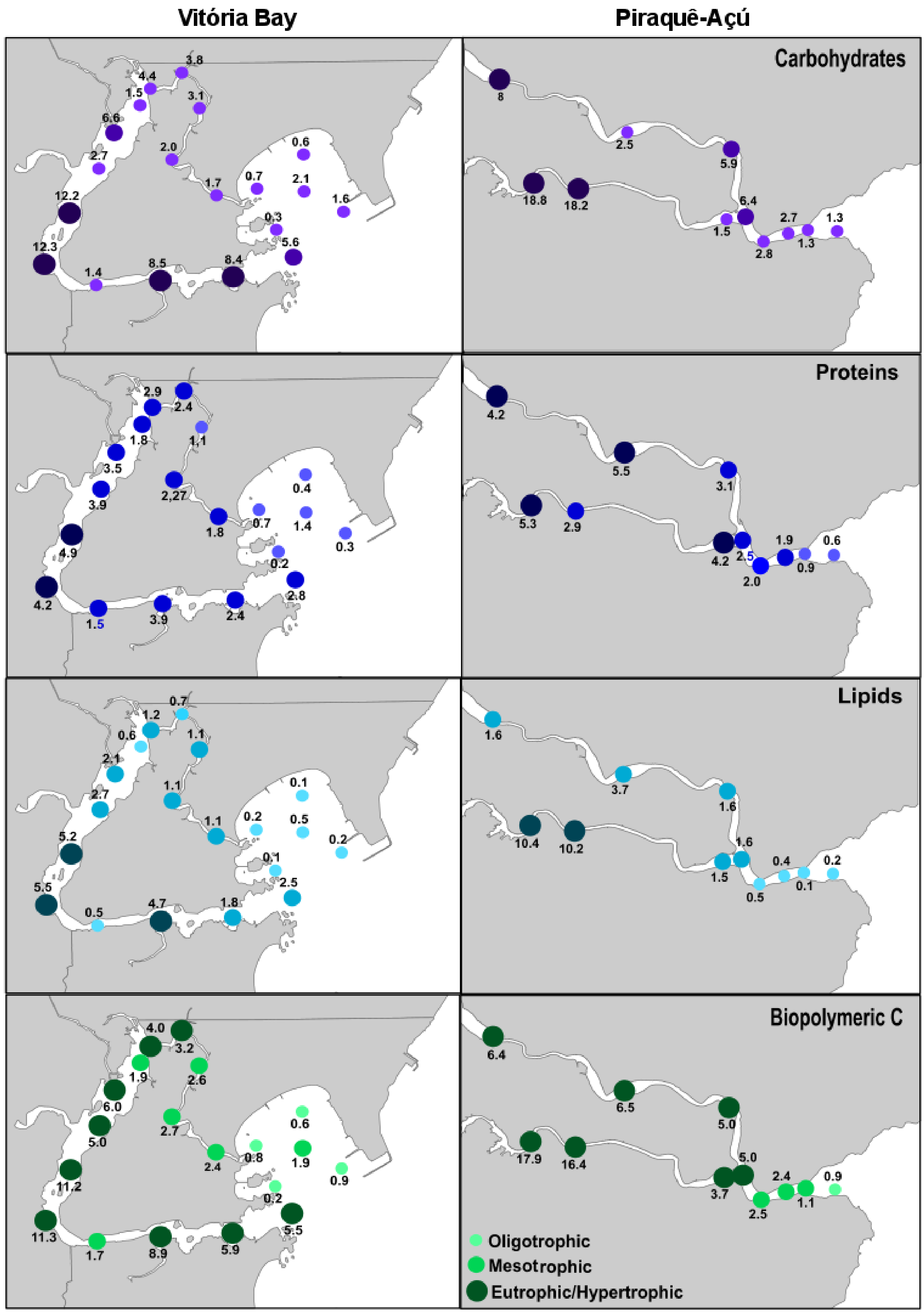

Fig. 3. Distribution of biopolymers in sediments from Vitória Bay (left) and Piraquê Açú-Mirim (right) estuaries. Circles represent mean concentrations of total proteins (in mg g−1), total carbohydrates (in mg g−1), total lipids (in mg g−1) and biopolymeric carbon (in mg Cg−1). Trophic classification followed Dell'Ano et al. (2002).

3.3 Distribution of fecal sterols

The following sterols: (i) faecal sterols: coprostanol (5β(H)-colestan-3β-ol) and epicoprostanol (5β(H)-colestan-3α-ol; (ii) C27 sterols: cholest-5en-3β-ol (cholesterol) and 5α-cholestan-3β-ol (cholestanol); (iii) C28 sterols 24-methylcholesta-5,22-E-dien-3β-ol (brassicasterol) and 24-methylcholesta-5-en-3β-ol (campesterol), (iv) C29 sterols: 24- ethylcholesta-5,22E-dien-3β-ol (stigmasterol), 24(α+β)-ethylcholest-5- en-3β-ol (sitosterol) and 24-ethylcholestan-3β-ol (sitostanol), and; (v) C30 sterol 4α,22,23-trimethylcholest-22E-en-3β-ol (dinosterol), were detected and quantified in the surface sediments of VB and PAM estuaries and indicated a variety of OM sources. The faecal sterols were detected in all samples, and stigmasterol represented about 34% and 43% of total sterols in VB and PAM, respectively. Sitosterol contributed to 30% of total sterols in the PAM estuary, whereas in VB, faecal sterols were 20% of all sterols. Coprostanol concentrations in VB ranged from 0.01 to 13.1 μg g−1, whereas epicoprostanol varied from<0.01 to 0.38 μg g−1 (Fig. 4; Table S2). Faecal sterols indicated a broad untreated sewage contamination of VB estuary based on values of Ratio I < 0.2 in at least 80% of the sampled sites (Fig. 4, Table S2). The PAM exhibited no evidence of faecal contamination in the Southern estuary (Piraquê-Mirim), whereas the Northern estuary (Piraquê-Açu) exhibited relative low coprostanol concentration (0.11–0.17 μg g−1) suggesting low sewage input (Fig. 4). Levels of epicoprostanol in the PAM estuary are close to detection limit (DL) ranged from<0.01 to 0.05 μg g−1 (Table S2), suggesting the PAM is under local sewage input (Fig. 5).

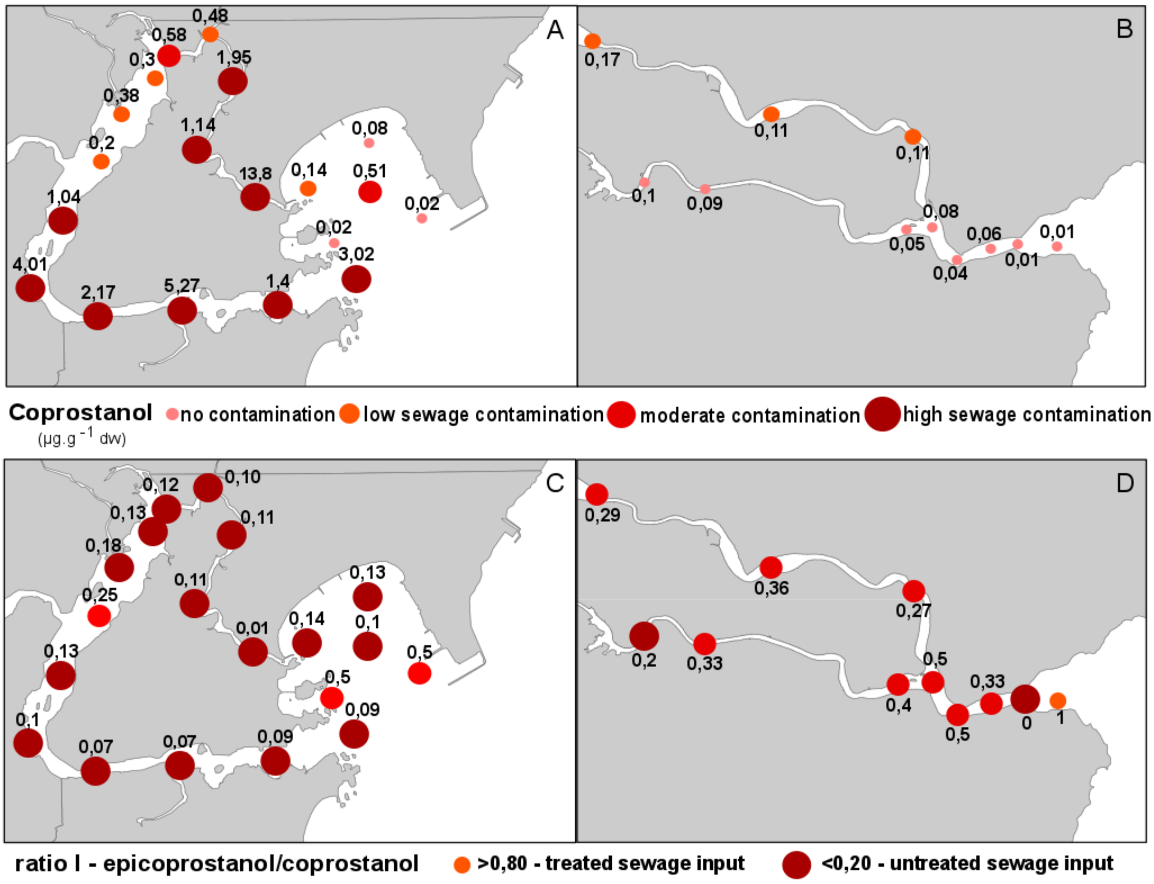

Fig. 4. Sediment sewage contamination based on coprostanol concentrations (µg g−1 dw) and the epicoprostanol/coprostanol ratio (Ratio I) in Vitória Bay (A, C) and Piraquê Açú-Mirim estuary (B, D).

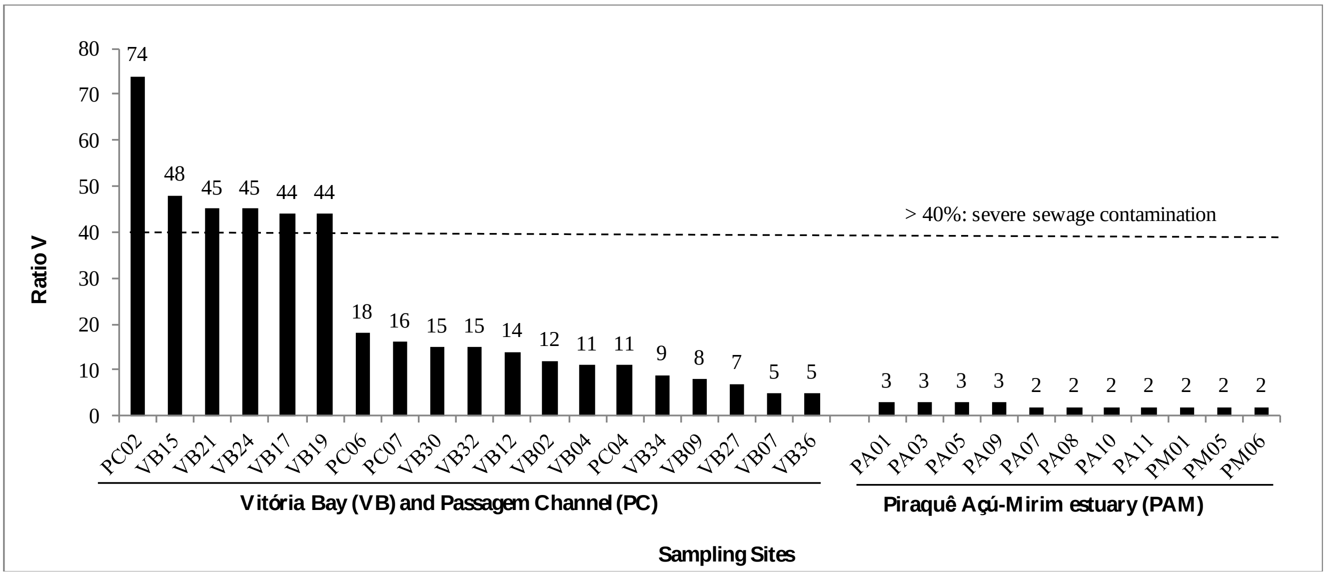

Fig 5. Percentage of faecal sterols in total sterols (Ratio V) in Vitória Bay (VB and PC stations) and Piraquê Açú-Mirim (PA and PM stations) estuary. Dashed line indicate extremely contaminated conditions, and values below 5% are considered not contaminated.

3.4 Trace metals and major elements

Levels of trace metals in superficial sediments from VB suggest intrinsic relation to anthropogenic sources from nearby rivers (Fig. 6, Table S3). In general, trace metals as Zn, Cr, Cu and Pb were detected in concentrations below PEL and TEL sediment quality reference values and suggest a low probability of adverse effects to the estuarine aquatic biota (Table 3). However, major elements (Mn, Fe and Al) and Co exhibited concentrations above AET at most stations in the VB and PAM estuaries, suggesting adverse effects in biota.

The spatial patterns of trace metals concentration in superficial sediments from VB showed typically highest values on the polyhaline sector, indicating potential sources of chemical discharges from nearby rivers (Fig 6; Table S3).

The TEL represents concentrations below which adverse biological effects were rarely observed, while the PEL represents concentrations above which effects were more frequently observed. All trace metals were detected below PEL and TEL reference values, and suggest a low probability of adverse effects to the local aquatic biota. Manganese, iron, aluminum and cobalt were the elements that presented concentrations above AET at most stations at VB and PAM, suggesting adverse effects in biota would be expected on these estuaries (Table 2).

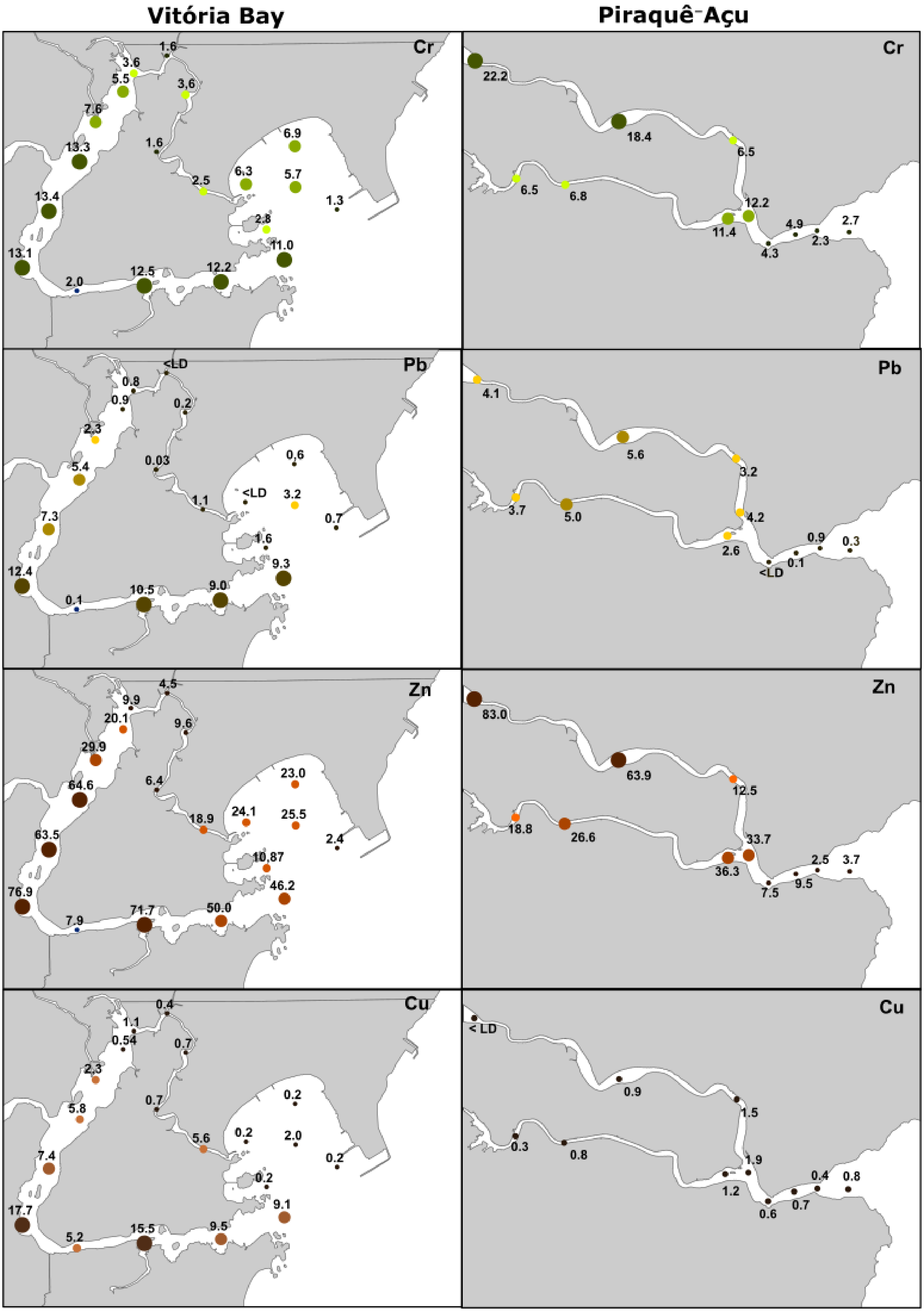

Fig. 6. Concentrations of Cr, Pb, Zn and Cu (in mg kg−1) in sediments of Vitória Bay and Piraquê Açú-Mirim estuaries.Table 2. Range concentration (mg.kg^{-1}) in surface sediment in estuaries near urban

areas or well-preserved areas around the world. * Fe and Al concentration in %. ** Fe and Al concentration in g.kg^{-1}. < below DL.

Place	**Country**	**Fe**	**Al**	**Mn**	**Zn**	**V**	**Cr**	**Co**	**Cu**	**Pb**	**References**
Piraquê Açú-Mirim estuary	Brazil	1735.8 - 17287.4	904.4 - 13251.6	16.1 - 267.8	2.5 - 83.0	2.4 - 29.9	2.4 - 22.2	4.7 - 62.5	< - 1.9	< - 5.6	This study
Vitória Bay	Brazil	956.8 - 15717.0	671.2 - 10571.0	< - 353.6	2.5 - 77.0	1.5 - 29.3	1.4 - 13.5	5.0 - 77.1	0.2 - 17.8	< - 12.4	This study
Rio Doce estuary before impact	Brazil	6122 - 11376	318.0 - 1425	84.9 - 438.4	1.3 – 2	0.2 - 0.9	1.0 - 7.3	< – 0.7	0.7 - 2.0	3.2 - 5.8	Gomes et al. (2017)
Rio Doce estuary after impact	Brazil	3682 - 45551	238.3 - 3941	151.0 - 506.4	0.5 - 5.8	0.3 – 1.1	1.0 - 27	< – 0.2	0.5 – 8.3	3.8 - 5.4	Gomes et al. (2017)
Todos os Santos Bay (BTS)	Brazil	-	-	89.90 - 936	37.2 - 877	-	4.45 - 145	-	10.4 - 49.9	10.9 - 363	Hatje et al. (2006a,b)
Laranjeiras Bay	Brazil	-	-	-	11 - 61	-	8.2 - 56	-	1.8 - 13	2.1 - 15	Martins et al. (2012)
Camamu Bay	Brazil	149 - 23325	-	1.5 - 518	0.44 - 77.5	< - 23.4	0.11 - 30	< - 9.92	0.13 - 2.5	0.26 - 24.8	Hatje et al. (2008)
Paranaguá Port	Brazil	-	-	-	27 - 81	-	5.5 - 40.50	-	0.10 - 4.47	< - 0.4	Sá et al. (2006)
Parnaíba River Delta	Brazil	* 0.3 - 2.5	-	145 - 1356	2.6 - 31	-	1.5 - 38	-	1.5 - 48	1.3 - 28	De Paula Filho et al. (2015)
Santos –	Brazil	1532.	1693.	11.0	5.81 -	2.2	2.75	-	0.70	1.29	Kim et al.

São Vicente Estuary		66 - 43798.2	82 - 42819.3	3-927.38	133.64	7-43.42	- 40.23		- 30.73	- 49.19	(2016)
Florida Bay	USA	347.2 – 3528.0	108.0 – 3240.0	11.6-62.6	0.06–3.99	-	2.96 – 18.04	-	0.4-2.03	0.39-5.34	Caccia et al. (2003)
Inner Masan Bay	Korea	*3.8-5.1	*8.9-12.4	650-849	83-97	-	35-41	-	13-27	24-27	Jeong et al. (2006)
Montevideo Harbour	Uruguay	-	-	-	120.23-508.70	-	35.97-145.83	-	51.26-243.39	17.45-180.22	Muniz et al. (2015)
Saint Louis Estuary	Senegal	**2.54-17.8	**32.5-77.9	-	8.98-88.5	-	47.8-105	-	21.8-121	63.9-1308	Diop et al. (2015)
Treshold effects level (TEL)		-	-	-	124	-	52.3	-	18.7	30.24	Buchman, M.F. (2008)
Probable effects level (PEL)		-	-	-	271	-	160	-	108	112	Buchman, M.F. (2008)
Apparent effects treshold (AET)		2200	1800	260	410	57	62	10	390	400	Buchman, M.F. (2008)

3.5 Multivariate analysis

The principal component analysis revealed a variable distribution of studied sites within the two estuaries, which were strongly related to concentrations of trace metals, faecal sterols and biopolymers with near 73% of total variation explained by the first two axes (Fig. 7). The PC1

axis explained 59.7% of the total variation, was represented by variability in faecal sterols and Co concentrations. Other trace metals, BPC values and fine sediments were negatively correlated to PC1, which clearly separated highly contaminated stations in VB that had evidence of severe sewage contamination. Sedimentary parameters including fine particles (silt+clay) and BPC were also related (PC2, 13.5% of explained variability), to the eutrophic conditions of some sites at the PAM and VB estuaries. The PC2 axis was also related with trace and major metals (Cr, Mn, Fe, V and Pb) and the distribution of stations along this axis were well correlated to a high to low gradient of impact on both estuaries.

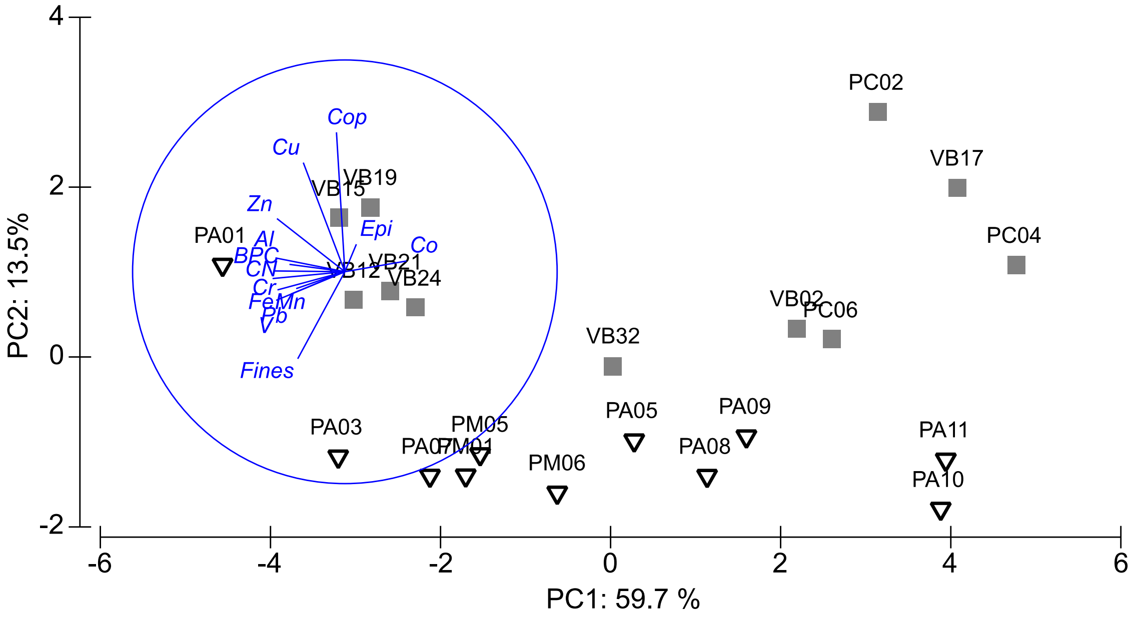

Fig. 7. Principal components analysis of the concentrations of trace metals, sterols, biopolymers and grain size in sediments from Vitória Bay and Piraquê Açú-Mirim estuaries.

4. Discussion

The high concentrations of biopolymers, faecal sterols and trace metals supported our hypothesis of environmental contamination within the Vitória Bay metropolitan estuary. Faecal sterols and trace metals were strongly concentrated near river tributaries in VB, and then distributed to nearby areas and to the outer estuary until dispersion along the coastal zone. On the contrary, our data support that the PAM estuary may be considered a pristine estuary with indication of low to moderate contamination by sewage input and the presence of eutrophic to hypertrophic sediments. The high hypertrophic status of PAM estuarine sediments is not correlated to faecal contamination and therefore we can assume that high sedimentation rates and natural sources of OM are driving the high availability of sedimentary BPC. On the other hand, VB clearly receives larger volumes of untreated sewage, resulting in broad eutrophic and polluted status (Costa et al., 2015).

The PRT distribution in both estuaries suggests a contamination gradient from tributaries to nearby areas as a result of extensive OM bacterial mineralization (Venturini et al., 2012). The high CHO concentrations and sewage in put in VB may support an increased OM production with dominance of aged detritus due to chemical contamination (Joseph et al., 2008; Pusceddu et al., 2009; Venturini et al., 2012). In contrast, the high CHO concentrations in the PAM estuary are likely associated to a high productivity and increased accumulation of organic detritus from terrestrial sources in that estuary. The high concentrations of CHO, PRT and LIP in some sites at VB are similar to those reported in intensely impacted urban estuaries worldwide and suggests a fresh sewage input (Pusceddu et al., 2009; Venturini et al., 2012; Aguiar et al., 2013). The accumulation of sewage in some sites may contribute to the prevalence of PRT in the sediments resulting in higher PRT:CHO ratios (Danovaro et al., 1993). However, the high LIP concentrations at the PAM seem to be related to autochthonous mangrove and plankton sources, which are assumed to be important carriers of lipids to marine sediments (Baldi et al., 2010).

In VB and PAM estuaries, PRT, CHO and LIP correlated significantly with faecal and total sterols, TOC and TN; suggesting that even apparently pristine estuaries may be under sewage contamination. The trophic status of estuarine sediments in both bays corresponded to an estuarine-marine gradient, but were distinctly impacted by sewage input. In both estuaries, BPC concentrations of>5 mg C g−1 indicated hypereutrophic sediments as proposed by Pusceddu et al. (2007), but only in VB these areas were clearly associated with areas receiving a high sewage discharge. High concentrations of faecal sterols (up to 13.9 μg g−1) near river tributaries revealed an acute sewage contamination in areas of VB with lower gradients of contamination on nearby coastal areas. The diagnostic ratios based on individual sterol concentrations were useful to indicate sewage input in both estuaries (Leeming et al., 1998; Takada and Eganhouse, 1998; Mudge and Duce, 2005). Additional diagnostic ratios based on coprostanol and "biogenic" sterols also evidenced that over 80% of sampled sites in VB had a strong sewage contamination (Ratios II, IV; Table 1; Table S2; Takada et al., 1994; Leeming et al., 1998; Takada and Eganhouse, 1998). The ratio between the percentage of faecal sterols and the total sterols (Ratio V, Table 1) should reflect the dispersion of sewage in a study area and help to eliminate the grain size variability and its effects on the accumulation of organic compounds (Venkatesan and Kaplan, 1990; Martins et al., 2002). For example, sites located in the sewage contaminated Passagem channel (PC02 to PC06) presented relatively high Ratio V compared with other low contaminated stations in the inner VB estuary and Espirito Santo Bay (Fig. 5). These results suggest that important sources of untreated sewage are located in the Passagem and Harbor channels. In general, coprostanol makes up 50–80% of the selected sterols in sewage effluents (Venkatesan and Kaplan, 1990), and therefore it is reasonable to assume that Ratio V values>40% indicates severe sewage contamination as evidenced in sites within both channels. In general, the combined sediment organic matter indices revealed that both estuaries are eutrophic and receive fresh organic matter from either anthropic or natural sources. The contamination of VB sediments by raw sewage is comparable to other chronically contaminated urban bays (Carreira et al., 2004, 2009, 2016; Martins et al., 2008a; Table 3), whereas the low sewage input (coprostanol < 0.18 μg g−1) in the environmentally protected PAM estuary suggests that it can still be considered pristine in relation to sewage impact.

Sedimentary trace metals also exhibited a strong positive correlation with each other and with TOC content, suggesting that their distribution is dependent on grain size, OM load, and also that trace metal contamination in VB may related to a common source. These correlations suggest that organic material is the main carrier of trace metals to the sediment through the formation of organic complexes and their flocculation (Zourarah et al., 2009). As a result, the tributaries that contaminate VB with untreated sewage are likely also receiving industrial, domestic and solid metallurgical waste (Costa et al., 2015; Grilo et al., 2013). The origin of contamination is also supported by the higher contamination levels of major elements including Fe, Al and trace metals (V, Cr and Pb) near the outflow of river tributaries. The heavy contamination of VB is typical of urban Brazilian estuaries that are poorly managed and that receive inefficient sewage treatment (Tables 2 and 3). Our study also suggests that even highly conserved estuaries supporting dense mangrove forests and no urban occupation can receive and accumulate trace metals, raising concerns of the sediment quality in many other smaller estuaries and bays. Given the very limited sewage treatment in Brazil, continental contaminants are potentially accumulating in estuarine sediments and threatening their environmental quality (Hatje et al., 2008; Martins et al., 2012; Kim et al., 2016). Although episodic events or environmental disasters immediately raise concerns of health effects from chronic metal pollution, estuarine sediments accumulate these contaminants which may decrease overall environmental quality on the long term (Muniz et al., 2006; Krull et al., 2014; Gomes et al., 2017; Queiroz et al., 2018).

Our study revealed that the combined use of indicators of sediment quality (organic and inorganic) offer great potential to identify and manage estuarine pollution. Given that contaminated sediments impact the biodiversity and functional attributes of benthic assemblages (Gusmao et al., 2016), biogeochemical indices add crucial information on ecosystem health that is easily understandable to policy managers. Although ecological indices are sensitive to pollution and offer a great solution for monitoring purposes, the common practice of untreated sewage discharge in Brazilian river tributaries may lead to long term accumulation of pollutants with sub-lethal effects and bioaccumulation. As a result, contaminated areas would likely be included into conservation planning of estuaries if based solely on biological traits or even on single biogeochemical indices as evidenced in this study. In the growing need to devise marine spatial planning for estuarine ecosystems (Gilby et al., 2017), increasing the representativeness of estuaries into marine reserves are clearly important to preserve these ecosystems, but here we show that even estuaries with good biological status may be under chronic pollution that threaten the long-term success of marine estuarine reserves.

Table 3. Concentration ranges of coprostanol from superficial sediments of selected estuaries and bays in Brazil. < DL: below detection limit (variable to each study).

Marine ecorregions of Brazil	**Local**	**Coprostanol ($\mu g.g^{-1}$)**	**References**
Amazonia	Guajará Estuary (PA)	0.06 - 7.93	Gomes et al., (2015)
Northeastern	Capibaribe estuary (PE)	0.52 - 7.30	Fernandes et al., (1999)
	Mundaú-Mangaba Estuary (AL)	<LD - 4.40	Ribeiro et al., (2011)
	São Francisco River Estuary	nd - 1.01	Frena et al, (2016)
Eastern	**Vitória Bay (ES)**	**0,02 - 13,8**	**This study**
	Piraquê Açú-Mirim (ES)	**0.01 - 0.17**	**This study**
	Passagem Channel (ES)	<LD - 86.09	Grilo et al, (2013)
Southeastern	Guanabara Bay (RJ)	0,33 - 40,0	Carreira et al., (2004)
	Sepetiba Bay (RJ)	0,77 - 9,24	Carreira et al., (2009)
	Ubatuba (SP)	<LD - 0.27	Muniz et al. (2006)
	Santos Bay and surroundings (SP)	<LD - 8.51	Martins et al., (2008a,b)
	Cubatão (SP)	4.21 - 8.32	Campos et al. (2012)
	Paranaguá Estuary (PR)	<LD - 2.22	Martins et al. (2010)
	Cotinga Channel (PR)	0,01 - 1,69	Abreu-Mota et al., (2014)
Rio Grande	Babitonga Bay	0.03 – 6.08	Martins et al., (2014)
	Florianópolis (SC)	<LD - 2.88	Mater et al. (2004)
	Patos Lagoon (RS)	<LD - 0.92	Martins et al. (2007)

<LD: below limit of detection (<0.01 g. g^{-1} dry weight). nd = not detected.

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Table S1. Trophic classification of the stations of Vitória Bay and Piraquê Açú-Mirim estuary according to Dell'Anno et al. (2002) and Pusceddu et al. (2011). Classification of the sampling stations is based on mean values. H = Hypertrophic; E = Eutrophic; MO = Meso-oligotrophic; M = Mesotrophic; PRT = total proteins; CHO = total carbohydrates; PRT:CHO = proteins to carbohydrates ratio; BPC = biopolymeric carbon. TOC and N = mean (SD).

Station	Sectors	PRT ($mg.g^{-1}$)	Trophic status	CHO ($mg.g^{-1}$)	Trophic status	PRT:CHO	Trophic status	BPC ($mg\ C\ g^{-1}$)	Trophic status	TOC (%)	N (%)
VB02	**Vitória Bay**	2.90	E	4.36	MO	0.67	MO	4.06	E	0.83 (0.01)	0.09 (0.00)
VB04		1.81	E	1.50	MO	1.21	H/E	1.94	M	0.42 (0.01)	0.06 (0.00)
VB07		3.58	E	6.62	E	0.54	MO	6.02	E	1.04 (0.01)	0.11 (0.01)
VB09		3.98	E	2.70	MO	1.47	H/E	5.05	E	2.41 (0.05)	0.20 (0.00)
VB12		4.99	H	12.16	H	0.41	MO	11.22	E	3.70 (0.01)	0.26 (0.01)
VB15		4.26	H	12.70	H	0.34	MO	11.28	E	4.13 (0.01)	0.29 (0.00)
VB17	**Vitoria Harbor channel**	1.54	E	1.41	MO	1.09	H/E	1.72	M	0.12 (0.01)	0.03 (0.00)
VB19		3.98	E	8.48	H	0.47	MO	8.92	E	3.84 (0.08)	0.26 (0.00)
VB21		2.40	E	8.40	H	0.29	MO	5.92	E	2.80 (0.02)	0.22 (0.01)
VB24	**Espírito Santo Bay**	2.85	E	5.58	E	0.51	MO	5.50	E	3.11 (0.01)	0.25 (0.01)
VB27		0.22	MO	0.28	MO	0.79	MO	0.26	O	0.09 (0.00)	0.03 (0.00)
VB30		0.75	MO	0.74	MO	1.01	H/E	0.78	O	2.35 (0.05)	0.04 (0.00)
VB32		1.39	MO	2.09	MO	0.67	MO	1.95	M	0.79 (0.00)	0.10 (0.01)
VB34		0.46	MO	0.63	MO	0.73	MO	0.56	O	2.69 (0.03)	0.04 (0.00)
VB36		0.29	MO	1.58	MO	0.18	MO	0.96	O	0.08 (0.00)	0.03 (0.00)
PC02	**Passagem Channel**	1.82	E	1.71	MO	1.07	H/E	2.41	M	0.39 (0.02)	0.06 (0.00)
PC04		2.27	E	2.01	MO	1.13	H/E	2.74	M	0.65 (0.02)	0.06 (0.00)
PC06		1.19	MO	3.09	MO	0.39	MO	2.64	M	0.97 (0.00)	0.08 (0.01)
PC07		2.48	E	3.81	MO	0.65	MO	3.25	E	0.44 (0.02)	0.05 (0.00)
PA01	**Piraquê-Açú river**	4.21	H	7.92	H	0.53	MO	6.46	E	1.85 (0.01)	0.13 (0.00)
PA03		5.58	H	2.49	MO	2.24	H/E	6.50	E	3.89 (0.04)	0.24 (0.00)
PA05		3.14	E	5.86	E	0.54	MO	5.08	E	1.55 (0.01)	0.11 (0.00)
PA07		2.48	E	6.46	E	0.38	MO	5.03	E	2.43 (0.03)	0.16 (0.01)
PA08	**PAM Confluence Zone**	2.03	E	2.80	MO	0.72	MO	2.47	M	0.93 (0.01)	0.04 (0.00)
PA09		1.99	E	2.70	MO	0.74	MO	2.37	M	1.12 (0.02)	0.06 (0.00)

PA10		0.98	MO	1.32	MO	0.75	MO	1.14	M	0.12 (0.01)	0.03 (0.00)
PA11		0.61	MO	1.38	MO	0.44	MO	0.99	O	0.13 (0.00)	0.03 (0.00)
PM01		4.19	H	1.47	MO	2.84	H/E	3.74	E	2.21 (0.01)	0.15 (0.01)
PM05	**Piraquê-Mirim river**	2.96	E	18.18	H	0.16	MO	16.37	E	10.41 (0.06)	0.50 (0.01)
PM06		5.31	H	18.80	H	0.28	MO	17.94	E	6.92(0.04)	0.33 (0.01)

Table S2. Concentrations of individual sterols, in μg.g^{-1}, and selected ratios in surface sediments from VB and PAM. Bold numbers indicate fecal pollution. Cop: coprostanol; e-cop: epicoprostanol; choles: cholesterol; cholest: cholestanol; brass: brassicasterol; campes: campesterol; stig: stigmasterol; sit: sitosterol; dino: dinosterol.< below DL.

Sterols/sites	Sectors	Cop	E-cop	Cholest	Cholest	Brass	Campes	Stig	Sit	Sitost	Dino	Fecal Sterols	Total Sterols	Ratio I	Ratio II	Ratio III	Ratio IV	Ratio V (%)
VB02	Vitória Bay	0,58	0,07	0,85	0,36	0,27	0,13	1,04	1,67	0,38	1,21	0,65	6,56	**0,12**	**0,62**	0,41	0,32	10
VB04		0,30	0,04	0,54	0,20	0,20	0,10	0,58	0,91	0,21	0,89	0,34	3,97	**0,13**	**0,60**	0,36	0,25	9
VB07		0,38	0,07	2,02	0,62	0,76	0,30	1,47	2,78	0,65	2,54	0,45	11,6	**0,18**	0,38	0,16	0,13	4
VB09		0,20	0,05	0,24	0,08	0,22	0,65	42,2	1,75	0,19	0,15	0,25	45,7	0,25	**0,71**	0,45	**0,57**	1
VB12		1,04	0,14	1,18	0,63	0,47	0,18	2,92	3,54	0,58	1,39	1,18	12,1	**0,13**	**0,62**	0,47	0,43	10
VB15		4,00	0,38	0,95	0,44	0,45	1,11	< LD	1,79	0,12	0,37	4,38	9,61	**0,10**	**0,90**	**0,81**	**0,92**	**46**
VB17	Vitoria Harbor channel	2,17	0,15	1,42	0,41	0,29	0,12	0,37	0,62	0,17	0,19	2,32	5,91	**0,07**	**0,84**	**0,60**	**0,92**	**39**
VB19		5,27	0,37	2,21	0,90	0,70	0,47	1,23	2,16	0,50	0,91	5,64	14,7	**0,07**	**0,85**	**0,70**	**0,85**	**38**
VB21		1,40	0,13	0,43	0,23	0,54	0,13	0,36	0,63	0,16	0,26	1,53	4,27	**0,09**	**0,86**	**0,77**	**0,84**	**36**
VB24	Espírito Santo Bay	3,02	0,28	1,25	0,50	1,64	0,41	0,82	1,13	0,32	0,35	3,30	9,72	**0,09**	**0,86**	**0,71**	**0,90**	**34**
VB27		0,02	0,01	0,11	0,04	0,06	0,03	0,06	0,08	0,04	0,13	0,03	0,58	0,50	0,33	0,15	0,13	5
VB30		0,14	0,02	0,29	0,12	0,14	0,08	0,15	0,19	0,09	0,15	0,16	1,37	**0,14**	**0,54**	0,33	0,48	12
VB32		0,51	0,05	1,05	0,33	0,65	0,17	0,47	0,75	0,23	0,56	0,56	4,77	**0,10**	**0,61**	0,33	0,48	12
VB34		0,08	0,01	0,26	0,12	0,09	0,07	0,16	0,20	0,09	0,13	0,09	1,21	**0,13**	0,40	0,24	0,38	7
VB36		0,02	0,01	0,20	0,04	0,08	0,04	0,08	0,15	0,05	0,04	0,03	0,71	0,50	0,33	0,09	0,33	4
PC02	Passagem Channel	13,8	0,09	0,54	0,07	0,58	3,08	11,3	0,54	0,35	0,25	13,9	30,6	**0,01**	**0,99**	**0,96**	**0,98**	**45**
PC04		1,14	0,12	1,48	0,66	0,34	0,32	1,33	2,83	0,71	4,27	1,26	13,2	**0,11**	**0,63**	0,44	0,21	10
PC06		1,95	0,22	1,60	0,82	0,43	0,23	1,26	2,34	0,70	4,12	2,17	13,7	**0,11**	**0,70**	**0,55**	0,32	16
PC07		0,48	0,05	0,47	0,27	0,16	0,09	0,67	0,97	0,25	0,74	0,53	4,15	**0,10**	**0,64**	**0,51**	0,39	12
PA01	Piraquê-Açú river	0,17	0,05	0,76	0,51	0,44	0,37	2,58	3,11	0,57	0,88	0,22	9,44	0,29	0,25	0,18	0,16	2
PA03		0,11	0,04	0,61	0,33	0,37	0,27	3,44	3,16	0,42	0,50	0,15	9,25	0,36	0,25	0,15	0,18	2
PA05		0,11	0,03	0,80	0,27	0,42	0,23	2,89	3,10	0,40	0,48	0,14	8,73	0,27	0,29	0,12	0,19	2
PA07		0,08	0,04	0,55	0,19	0,28	0,09	3,52	3,04	0,49	1,15	0,12	9,43	0,50	0,30	0,13	0,07	1
PA08	PAM Confluence Zone	0,04	0,02	0,85	0,13	0,23	0,13	0,79	1,22	0,19	0,26	0,06	3,86	0,50	0,24	0,04	0,13	2
PA09		0,06	0,02	0,59	0,15	0,19	0,13	0,98	1,48	0,22	0,23	0,08	4,05	0,33	0,29	0,09	0,21	2
PA10		0,01	BD	0,23	0,04	0,08	0,06	0,13	0,19	0,04	0,09	0,01	0,87	nc	0,20	0,04	0,10	1
PA11		0,01	0,01	0,54	0,08	0,14	0,06	0,20	0,32	0,05	0,08	0,02	1,49	1,00	0,11	0,02	0,11	1
PM01	Piraquê-Mirim	0,05	0,02	0,34	0,12	0,21	0,07	1,39	1,97	0,25	1,15	0,07	5,57	0,40	0,29	0,13	0,04	1
PM05		0,09	0,03	0,57	0,44	0,21	BD	4,63	4,45	0,54	0,38	0,12	11,3	0,33	0,17	0,14	0,19	1

PM06	**river**	0,10	0,02	0,26	0,07	0,17	0,12	17,1	4,24	0,27	0,33	0,12	22,7	0,20	**0,59**	0,28	0,23	1

Table S3. Concentrations of metals, in mg.kg^{-1}, in surface sediments collected in Vitória Bay and Piraquê Açú-Mirim estuary. <bellow DL.

Station	Sectors	Fe	Al	Mn	Zn	V	Cr	Co	Cu	Pb
VB02	**Vitória Bay**	4681.5 (53.7)	2291.4 (18.9)	<	10.0 (0.1)	5.5 (0.1)	3.6 (0.0)	50.5 (2.0)	1.1 (0.1)	0.8 (0.1)
VB04		11359.3 (352.2)	3722.2 (53.5)	35.2 (1.3)	20.1 (0.1)	5.7 (0.2)	5.5 (0.0)	71.2 (1.4)	0.5 (0.1)	0.9 (0.0)
VB07		11813.7 (28.8)	6408.6 (37.4)	25.6 (0.3)	29.9 (0.1)	11.4 (0.1)	7.7 (0.0)	30.3 (1.7)	2.3 (0.2)	2.3 (0.2)
VB09		15717.0 (563.9)	8879.1 (261.3)	133.0 (5.1)	64.6 (2.0)	21.3 (0.9)	13.4 (0.3)	63.0 (2.4)	5.8 (0.2)	5.4 (0.2)
VB12		14720.2 (75.7)	10571.0 (50.0)	137.7 (1.4)	63.5 (0.3)	24.5 (0.1)	13.5 (0.1)	20.4 (0.3)	7.4 (0.1)	7.3 (0.2)
VB15		10807.9 (110.1)	8268.5 (29.0)	178.6 (1.2)	77.0 (0.2)	29.3 (0.2)	13.1 (0.1)	6.7 (0.4)	17.8 (0.0)	12.4 (0.4)
VB17	**Vitoria Harbor channel**	1942.1 (141.6)	1121.1 (68.0)	<	8.0 (0.6)	1.5 (0.1)	2.0 (0.1)	41.1 (3.6)	5.2 (0.6)	0.1 (0.0)
VB19		10461.9 (338.4)	8083.1 (45.6)	199.7 (1.6)	71.8 (1.1)	28.0 (0.2)	12.5 (0.1)	7.5 (0.2)	15.6 (0.0)	10.5 (0.6)
VB21		9731.8 (75.2)	7829.3 (81.3)	274.3 (0.4)	50.0 (0.6)	27.7 (0.2)	12.2 (0.2)	5.5 (0.2)	9.6 (0.2)	9.0 (0.2)
VB24	**Espírito Santo Bay**	8541.0 (259.7)	6025.2 (158.8)	352.6 (10.8)	46.2 (1.3)	28.9 (0.8)	11.1 (0.3)	5.0 (0.1)	9.1 (0.3)	9.3 (0.7)
VB27		4846.7 (78.9)	1427.5 (10.8)	58.2 (0.8)	10.9 (0.3)	6.5 (0.1)	2.9 (0.0)	22.0 (0.6)	0.2 (0.0)	1.6 (0.1)
VB30		7318.1 (52.3)	3068.9 (54.9)	177.6 (0.7)	24.1 (0.1)	9.7 (0.1)	6.3 (0.1)	10.1(0.3)	0.2 (0.0)	<
VB32		5853.3 (70.9)	3731.8 (43.0)	239.0 (1.3)	25.6 (0.1)	13.5 (0.0)	5.7 (0.0)	7.2 (0.1)	2.0 (0.0)	3.2 (0.2)
VB34		7459.8 (203.5)	3724.5 (19.2)	187.5 (3.2)	23.1 (0.4)	11.5 (0.2)	6.9 (0.2)	12.2 (0.1)	0.2 (0.0)	0.6 (0.1)
VB36		5179.3 (110.5)	671.2 (10.6)	11.8 (0.6)	2.5 (0.1)	4.0 (0.1)	1.4 (0.0)	53.2 (2.8)	0.3 (0.0)	0.8 (0.1)
PC02	**Passagem Channel**	1430.4 (12.8)	1241.7 (29.8)	<	19.0 (0.5)	3.2 (0.0)	2.5 (0.0)	40.9 (0.7)	5.7 (0.1)	1.1 (0.1)
PC04		956.8 (4.2)	839.2 (5.1)	<	6.4 (0.2)	2.1 (0.1)	1.7 (0.0)	77.1 (4.3)	0.7 (0.1)	<
PC06		5430.0 (33.8)	1815.7 (36.5)	<	9.7 (0.1)	4.4 (0.1)	3.6 (0.1)	51.6 (1.3)	0.7 (0.0)	0.2 (0.1)
PC07		1049.1 (14.1)	882.0 (13.9)	<	4.5 (0.1)	1.6 (0.1)	1.6 (0.0)	63.1 (1.3)	0.5 (0.0)	<
PA01	**Piraquê-Açú river**	17287.4 (296.5)	13251.6 (487.5)	267.8 (10.2)	83.0 (0.7)	17.4 (0.6)	22.2 (0.9)	14.8 (0.6)	<	4.1 (0.4)
PA03		16696.5 (167.2)	9932.7 (65.4)	187.3 (2.7)	63.9 (1.1)	28.3 (0.2)	18.0 (0.2)	10.2 (0.2)	1.0 (0.1)	5.6 (0.4)
PA05		8023.8 (46.8)	3464.6 (26.0)	75.3 (1.4)	12.6 (0.1)	15.5 (0.3)	6.6 (0.1)	28.3 (0.7)	1.5 (0.1)	3.2 (0.2)

PA07		13965.0 (93.2)	6874.3 (8.7)	237.2 (2.9)	33.7 (0.4)	25.9 (0.3)	12.2 (0.1)	14.2 (0.2)	1.9 (0.1)	4.3 (0.2)
PA08		5544.7 (25.3)	2094.2 (16.9)	205.0 (1.7)	7.6 (0.0)	7.6 (0.1)	4.4 (0.0)	19.3 (1.1)	0.6 (0.0)	<
PA09	**PAM Confluence Zone**	6112.7 (57.8)	2567.4 (34.7)	210.5 (0.7)	9.5 (0.1)	9.1 (0.1)	4.9 (0.0)	15.7 (1.2)	0.7 (0.0)	0.1 (0.0)
PA10		1735.8 (3.0)	904.2 (5.5)	125.8 (0.9)	2.5 (0.0)	2.4 (0.1)	2.4 (0.0)	62.5 (6.9)	0.5 (0.0)	1.0 (0.2)
PA11		1870.6 (8.6)	955.0 (9.9)	90.7 (0.8)	3.7 (0.0)	3.5 (0.1)	2.7 (0.0)	48.1 (0.9)	0.9 (0.0)	0.4 (0.1)
PM01		14102.1 (126.0)	6406.7 (25.2)	185.0 (1.1)	36.3 (0.1)	22.9 (0.3)	11.0 (0.2)	7.9 (0.4)	1.2 (0.1)	2.7 (0.1)
PM05	**Piraquê-Mirim river**	7946.3 (125.0)	6844.0 (134.0)	43.8 (1.2)	26.7 (0.3)	29.9(0.3)	6.8 (0.1)	4.7 (0.1)	0.9 (0.0)	5.0 (0.2)
PM06		7383.3 (355.7)	4778.7 (273.2)	16.1 (2.5)	18.8 (1.2)	28.0 (1.6)	6.6 (0.3)	16.4 (0.4)	0.3 (0.0)	3.7 (0.3)

CAPITULO 3

Spatial variability of benthic indices for marine quality assessment in protected and urban estuaries of Southeast Brazil

Abstract

We applied the benthic indices AMBI, M-AMBI, BENTIX and BO2A, in a protected and urbanized Brazilian estuaries in order to: (i) assess the spatial variability of the indices using a hierarchical sampling design with three progressively smaller spatial scales; (ii) test for correlations with molecular markers of contamination (indices consistency as real-world tools); and (iii) evaluate the overall agreement among their responses (indices congruency among themselves). The presumed strong and marked variations related to the larger spatial scale (non-urbanized and urbanized estuaries) were detected by all indices, but BENTIX showed to be less variable at this spatial scale. Our results showed correlations between the worse values of the indices and increases in such chemical proxies towards the urbanized stations, especially to high concentrations of coprostanol. EGI (sensitive species) and EGII (indifferent species) were possibly responsible for most of the variability of AMBI and M-AMBI, the EGII (tolerant species) for BENTIX , and the opportunistic annelids for BO2A.

Keywords. macrobenthic fauna, environmental quality assessment, indicators, Vitoria Bay

Introduction

In recent decades, marine habitats around the world have demonstrated progressive degradation due to the increased pollution, habitat losses and climate change, affecting multiple ecosystem components (Borja et al., 2015). The consequences of rapid deterioration of estuarine ecosystems, due to urbanization and industrialization word over, have been debated and well established by a multitude of studies (Muniz et al., 2005; Borja and Dauer, 2008; Halpern et al., 2008). Among the various biological components, macrobenthic animals are considered effective indicators of pollution, as they show predictive responses to different levels of natural and anthropogenic impact, due to varied species composition with differential tolerances to stress, their sedentary lifestyle and longevity (Reiss and Kröncke, 2005, Pinto et al., 2009).

Several biotic indices have been developed and used as an important tool to evaluate the ecological quality and degree of disturbance of estuarine and marine ecosystems (Borja et al., 2000; Simboura and Zenetos, 2002; Muxica et al., 2007). The categorization of estuarine zones based on the ecological quality has been undertaken by employing suitable macrobenthic biotic indices (Muniz et al., 2005; Borja et al., 2007; Chainho et al., 2007), that can encapsulate complex scientific data into simple information to facilitate easy interpretation by stakeholders and managers alike and implementation of monitoring programs (Borja and Muxica, 2005; Valença and Santos, 2012).

The use of biotic indices shows limitations, however, mainly in coastal and estuarine ecosystems in tropical and subtropical regions, where basic information on ecological characteristics of soft-bottom macrobenthic communities remains scarce (Muniz et al., 2005). The study of the ecology of these macrofauna communities in estuarine systems on the Atlantic coast of South America is recent and there are no previous data or long-term monitoring programs (Pagliosa and Barbosa, 2006). Although a number of indices are available for marine/estuarine coastal environments, there is a lack of consensus about which is the most appropriate. More recent benthic indices were developed at certain coastal and estuarine areas of the United States and Europe (Borja et al., 2009). Similarly, most of them are based on the organic matter enrichment model of Pearson-Rosenberg (Pearson and Rosenberg, 1978), and thus, they should be validated for other type of perturbations as well (Quintino et al., 2006). For this reason, benthic biotic indices may not be universally applicable, and they should be tested in different geographic areas and perturbations (i.e., natural or anthropogenic) (Hutton et al., 2015). Since there is not a perfect index for evaluating the environmental quality, it is widely recommended to use several indices based on different approaches.

The biotic indices, for example, benthic opportunistic annelida amphipods index (BO_2A) (Dauvin and Ruellet , 2009), BENTIX (Simboura and Zenetos, 2002) and AZTI Marine Biotic Index (AMBI) (Borja et al., 2000), have been the most used indices when using macrobenthic data, and the multivariate AMBI (M-AMBI) (Muxika et al., 2007) is being widely used as multivariate index, both evaluate the benthic habitat health in the estuarine and coastal waters impacted by strong human disturbances.

In South America, Muniz et al. (2005, 2011, 2012), Valença and Santos (2012), Brauko et al., (2015, 2016), Hutton et al. (2015) and Checon et al. (2018) have tested the efficiency of some of these indices on estuaries and marine coastal areas of Brazil and Uruguay, where different degrees of anthropogenic pressure are well documented. As many other developing countries, Brazil has a wide densely populated coastline with intense industrial and port activities. Given this intense pressure, there is a high potential to the use of biotic indices, Some studies have reported taking into account both human pressures and natural variability in tropical estuaries on the Eastern Marine Ecoregion of Brazil (Hatje et al., 2006; Barros et al., 2008; Barros et al., 2012; Egres et al., 2019; Bernardino et al., 2018; Bissoli and Bernardino, 2018). However, assessments of the index in this region are still in early stages, and further studies are necessary to better establish the validity of the index in tropical estuaries of Atlantic South. In addition, estuaries are simultaneously subjected to multiple sources of natural and human disturbances driving confounding patterns of faunal structure that may, in turn, influence the performance of indices yet to be validated (Elliot and Quintino, 2007; Brauko et al., 2020).

In this study, we tested the spatial variability of four biotic indices to assess estuarine environmental health, all based on the different sensitivity of species groups to organic contamination, using a multivariate approach to address congruence and consistency patterns in a southern Brazilian tropical estuaries. We applied the indices AZTI's marine biotic index - AMBI (Borja et al., 2000) and its multivariate extension M-AMBI (Muxika et al., 2007), BENTIX (Simboura and Zenetos, 2002), and the Benthic Opportunistic Annelida Amphipods Index - BO2A (Dauvin and Ruellet, 2009), in protected and urban subtidal regions in order to: (i) assess the spatial variability of the indices using a hierarchical sampling design with three progressively smaller spatial scales; (ii) test for correlations with molecular markers of contamination (indices consistency as real-world tools); and (iii) evaluate the overall agreement among their responses (indices congruency among themselves). All analyses were performed along two estuaries corresponding to the larger spatial scale of the work, Vitoria bay, under the high influence of several anthropic impacts and Piraquê-Açú, relatively pristine and part of the municipal conservation unit. As indices are expected to respond to non-biological measures of contamination (Benyiet al., 2009), the direct correlation of indices to environmental molecular markers (e.g. faecal sterols) may indicate their suitability. Many studies have evaluated the relative performance of different indices but although less subjective, indices are not always clearly correlated with reliable abiotic markers of contamination (Brauko et al., 2015). Hence, suitable indices are expected to vary significantly at the larger spatial scale (Urban x Protected estuaries) despite the background variability inherent to the smaller scales. They are also expected to be highly correlated with sewage molecular markers over space and to display a high percentage of similarity among responses.

Materials and methods

Study area

The study was conducted in two estuaries located on the eastern coast of Brazil. The metropolitan estuary, Vitória bay (VB, 20°18’ S; 40°20’W; Figure 1) is the largest and most urbanized estuary in the region, with historical high levels of sewage contamination (Grilo et al., 2013; Bernardino et al., 2015; Varzim et al, 2019). Vitória Bay has an area of 18.2 km^2 and a catchment area of 1,728 km^2 with an average freshwater discharge of 18.7 m^3 s^{-1} (Lessa et al., 2019). The bay is connected to the coastal ocean through two channels; the southern port channel is wider (~160 m wide) and deeper (5-24 m) than the northern Passagem channel that is on average 35 m wide and 1 to 8 m deep. The channel bed morphology and an abundant mangrove vegetation in Vitoria Bay results in a strong tidal amplification inside the estuary and ebb flows, with higher sediment bed load transport out of the bay (Rigo, 2004; Lessa et al., 2019). On the areas near the coast both channels are highy impacted by urban development that resulted in loss of intertidal mangrove forests for the construction of an industrial port and local marinas. Vitória Bay also receives a significant amount of treated and untreated sewage from nearby cities through its river tributaries, resulting in a historical accumulation of organic and trace metal pollutants in estuarine sediments (Grilo et al., 2013; Costa et al., 2015; Hadlich et al., 2018). Vitoria bay has an important economic and touristic relevance to the region, but the historical record of degradation has not yet been used towards an improved management of estuarine resources.

The northernmost estuary, Piraquê-Açú-Mirim (PAM; 19°57′S; 40°09′W) is situated 50 km of Vitoria Bay. A polyhaline sector is typically observed within the estuary as is a euhaline sector near the sea. The estuary has a well preserved area with tidal flats and extensive mangrove forests (dominated by *Rhizophora mangle* and *Avicennia schaueriana*), with minor coastal development (Bernardino et al., 2017; Bissoli and Bernardino, 2018). The PAM estuary is part of the municipal conservation unit, where traditional fishermen and indigenous communities depend on its natural resources for subsistence.

The Vitória Bay and Piraquê Açú were sampled in November 2014 along a salinity gradient from the inner estuary towards the coastal ocean, which are all under influence of multiple pollution discharges into the bay (Figure 1). Based on vertical CTD casts during sampling, average salinity within the bay ranged between 23.1 and 36.2 PSU and the average temperature ranged between 22.1 to 27°C. The sampling sites 02, 06, 07, 09 and 15 exhibited polyhaline conditions and the other sites 17, 19, 21, 24, 30 and 34 were euhaline at the time of sampling. The PAM estuary was sampled at 21 stations and Vitoria Bay 43 stations.

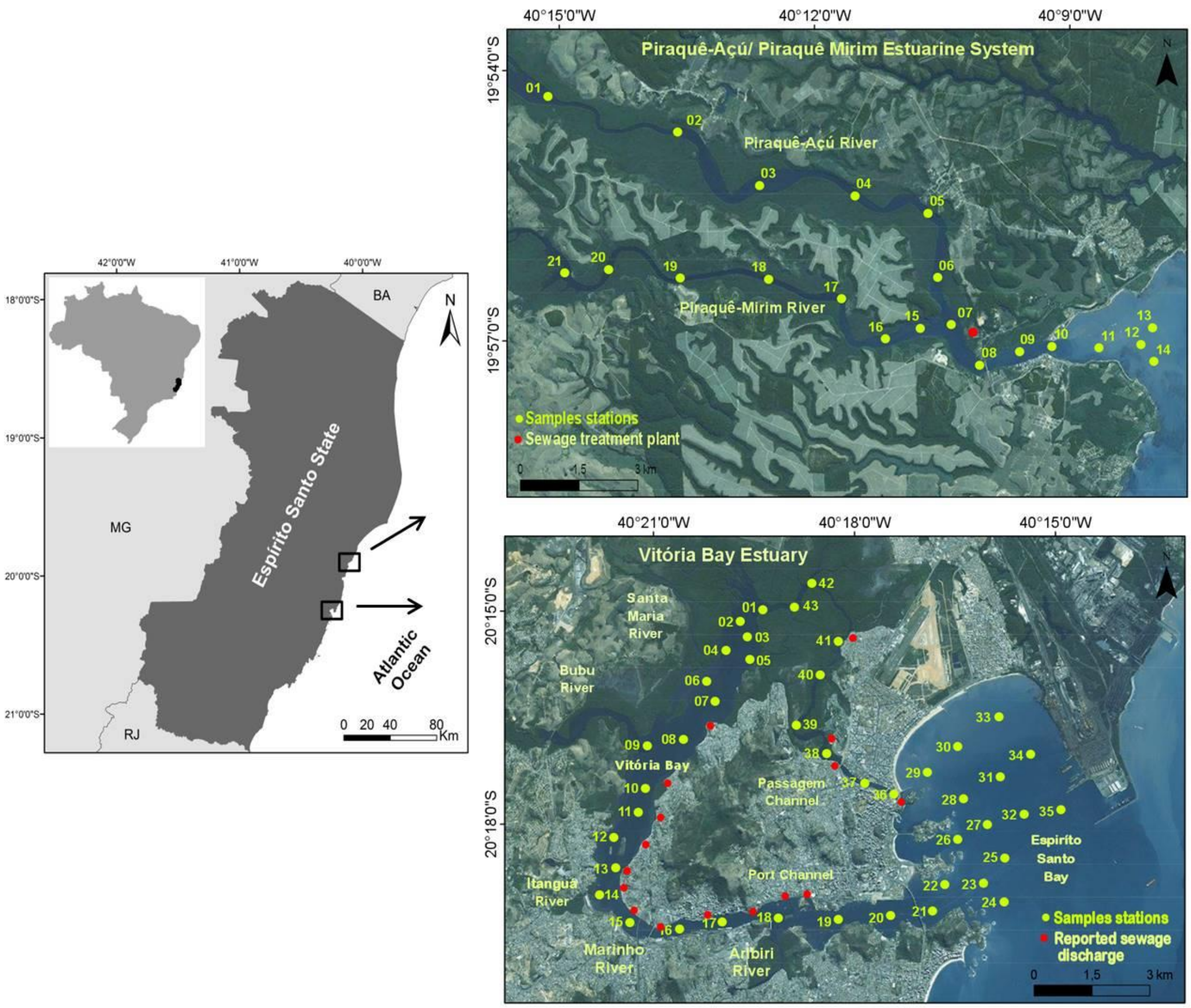

Fig. 1. Study area indicating sampling stations, urbanization and sewage discharges distributed along the Vitoria Bay Estuary and Piraquê Açú-Mirim Estuarine System in Eastern Brazil.

Sampling and laboratory analysis

Subtidal sampling was carried out in November 2014, random stations were distributed across the estuaries, with VB02 to VB15 located in the inner VB estuary, VB17 to VB21 along the Vitória harbor channel and VB24 and VB32 in Espírito Santo Bay. In VB, three stations (PC 02, 04, 06) were sampled along a secondary channel (Passagem Channel, PC) that connects the inner estuary to the Espirito Santo Bay. At the PAM estuary, stations PA01 to PA05 were located in the northern Piraquê-Açu river, stations PM 01 to

PM06 in the southern Piraquê-Mirim river, and stations PA07 to PA11 were sampled at the mouth of this estuary (Fig. 1). Salinity was measured in situ with a calibrated SonTek CastAway CTD. Sediment samples (N=2 per station) were collected with a Day grab ($0.1m^2$), and the top 3 cm of undisturbed surface sediment from two independent replicates were mixed into one composite sample for chemical proxies and particle size analyses. For organic compounds, samples were stored in pre-cleaned aluminum container whereas surficial sediments for trace metal analysis were stored in pre-cleaned LDPE containers. Samples remained frozen (−20 °C) until laboratory procedures.

Benthic macroinvertebrate were sampled from the remaining sediments from each independent replicate along the 22 stations. All faunal samples were sieved through a 1.0 mm mesh and fixed in 10% buffered formalin and transferred to 70% alcohol until laboratory analysis. The fauna was then sorted, counted and identified to the lowest possible taxonomic level under a microscope. The species names were corroborated using the World Register of Marine Species (http://www.marinespecies.org/index.php). The density was expressed as ind m^{-2}. All organic contaminant analysis and sediment size performed in this study were described in Hadlich et al., (2018).

Biotic indices and data analysis

In the present study, four biotic indices were employed for each station to assess the ecological quality status of the protected and urban estuaries: AMBI, M-AMBI, BENTIX, and BO2A (Table 1). AMBI and M-AMBI values were calculated using the software freely available at AZTI's web page (http://ambi.azti.es). The AMBI index is based on the abundance of five ecological groups according to their sensitivity/tolerance levels to organic enrichment, represented by species sensitive to disturbance (EGI), species indifferent to disturbance (EGII), species tolerant to disturbance (EGIII), second-order opportunistic species (EGIV) and first-order opportunistic species (EGV), already listed in the software (Borja et al., 2000). However, some species or taxa present at VB and PAM estuaries are not yet assigned into the AMBI list. To classify the species into each ecological group, we: (i) checked the literature to establish the sensitivity level of a taxon (e.g. Amaral et al., 2013; Nalesso et al., 2005) and (ii) assigned the taxon or species to the same genus present in the original AMBI list when their sensitivity could not be unequivocally determined. After assignment, *Anomalocardia flexuosa* was in GIII and *Sigambra sp*. in GIII, while *Dorvillea sp., Euclymene sp., Ophelina sp., Naineris setosa, Psionidens sp*., *Felaniella vilardeboana,* *Cyclostremiscus beauii and Leuocozonia nassa* remained unassigned for not being included in the software list.

The M-AMBI index was calculated by factorial analysis of AMBI, richness (as number of taxa) and Shannon–Wiener diversity values (for details, see Muxika et al., 2007). This index compares monitoring results with reference conditions by salinity stretch to derive an M-AMBI value. This value reflects the relationship between observed and reference condition values. At 'high' status, the M-AMBI value approaches one, where the reference condition can be regarded as an optimum. At 'bad' status, the M-AMBI approaches zero. As M-AMBI needs reference conditions to be calculated, and the Piraquê-Açú Estuary shows a salinity gradient, that can determine the benthic communities living at each salinity stretch (Muxika et al., 2007), each station was assigned to a salinity gradient to determine their reference conditions. In addition, as the estuary is not considered impacted by human activities, it is expected that the current structural parameters are suitable for M-AMBI reference conditions setting (Borja et al., 2012). Hence, the reference conditions were determined by the highest diversity and richness values of all replicates from PAM. As for the bad status, the references were based upon the azoic situation (diversity and richness equal to 0 and AMBI equal to 7).

The BENTIX (Simboura and Zenetos, 2002) is based on the same proposal as AMBI, but the taxa are categorized in three ecological groups, adapted as follows (Blanchet et al., 2007): group I of AMBI is group I of BENTIX; groups II and III of AMBI correspond to II of BENTIX, and groups IV and V of AMBI are group III of BENTIX. The BO2A (Dauvin and Ruellet, 2009) was also based on the ecological characteristics of specific taxonomic groups, comparing percentage ratios of opportunistic annelids (Polychaeta and Clitellata) and the percentage of amphipods (with exception to the opportunistic genus *Jassa* as recommended by the authors of the index). Each index was calculated for each replicate (N= 3) and averaged per station (Table 1).

Table 1. Calculated indices and their ecological status threshold values.

Indices		Environmental status				
		High	Good	Moderate	Poor	Bad
AMBI	[(0*%GI) + (1.5*%GII) + (3*%GIII) + (4.5*%GIV) + (6*%GV)]/100	0 - 1.2	1.2 - 3.3	3.3 - 4.3	4.3 - 5.5	5.5 - 7
M-AMBI		>0.82	0.82 - 0.62	0.61 - 0.41	0.4 - 0.2	<0.2
BENTIX	[6*%GI + 2*(%GII + %GIII)]/100	6 - 4.5	4.5 - 3.5	3.5 - 2.5	2.5 - 2	2 - 0
BO2A	Log[fP/fA+1)+1]	0 - 0.04576	0.04576 - 0.13966	0.13966 - 0.19382	0.19382 - 0.26761	0.26761-0.30103

Statistical analysis were conducted using the software PRIMER 6 with the PERMANOVA + add on (Anderson et al., 2008). The structural changes in macrobenthic assemblages in response to the distinct levels of urbanization were analyzed according to the four different benthic indices. The indices values were calculated for each replicate and their ecological status was therefore attributed as High, Good, Moderate, Poor and Bad (Table 1). Comparisons were carried out using multivariate techniques based on a two spatial scales design, including the factors: Estuary (fixed) and Stations (random, nested in Estuary). The spatial scales of variability were tested for each index using a permutacional analysis of variance, sometimes referred to as PERANOVA, based on the Euclidian distance. The test compares the homogeneity of multivariate dispersions among groups of a single factor and was carried out under 9999 permutations (Anderson, 2005). The data was log-transformed whenever necessary. The use of Euclidian distance as the measure of association makes this univariate test similar to a traditional ANOVA. To avoid the occurrence of type I error and to increase the robustness of our analysis we also calculated the components of variation to estimate the amount of variation attributed to each source, especially to the residuals. A Redundance Analysis (RDA) was then performed to correlate biotic indices to abiotic variables representing the potentially structuring drives operating in the estuaries, related to both natural and sewage derived processes. The RDA was conducted following Borcard et al. (2011). The statistical significance of the relationships was evaluated using Monte Carlo permutation tests under 9999 permutations. The degree of similarity was also calculated for each possible combination of indices, as the percentage of replicates having the same ecological status. Indices with a correlated response should have a high degree of similarity.

Finally, faunal differences between urbanization conditions and indices similarity were visualized with non-metric multidimensional scaling (nMDS) based on Bray–Curtis similarities, when using the species abundance as descriptor and Euclidian distance matrix, when using the indices values as descriptors.

Results

A wide range of values were observed for AMBI reflecting from ‘good’ to ‘bad’ ecological status for the Vitoria Bay stations. In VB, the ecological quality was in general worse and classifications were more variable amongst the indices. Stations closer to the open mouth of the estuary showed more good and high status, while the innermost stations attained the majority of the poor and bad status. Only four stations located in the inner part of VB showed the bad and poor status for all indices (VB16, VB39, VB40 and 41), except for BO2A, which classified VB39 as moderate (Fig. 2).

Based on the AMBI classification, of the 43 stations, only six attained the high status (preserved). About 70% of the sites could be ranked as good, and 14% as moderate to bad status, with values ranging from 0.8 (VB30 and VB34) to 6.2 (VB39) (Figs. 2 and 3). In the stations with poor and bad status, the ecological groups (EG) III, IV and V (tolerant and opportunistic species) dominated, with high proportion of EG V (represented mainly by the polychaete so called first order opportunist *Capitella sp.* and oligochaetes) and EG IV (represented by the polychaetes *Tharyx sp.* and *Paraprionospio sp.*) (Fig. 2 and 3). The opportunistic fauna of groups GIV and GV dominated VB36 to VB43, all located in the heavily urbanized and narrow Passagem Channel, and also in VB16 (immediately after the Vitória harbor).

The indices M-AMBI and BENTIX showed in general downgraded quality status in relation to AMBI. M-AMBI classified most of the stations as good and moderate (21% and 55% of the stations) and BENTIX as moderate to bad (40% and 21% of the stations), ranging from 0.09 to 0.87 (VB39 and VB11) and from 0.5 to 5.6 (VB39 and VB29), respectively (Fig 2).

In PAM, the protected estuary, all stations were in the high and good status by AMBI and BO2A. The M-AMBI and BENTIX indices classified the innermost stations as moderate (PA1, PA3, PA20). In AMBI and M-AMBI, the dominant ecological groups of fauna in all PAM stations were GI and GII, of sensitives and indifferent species, as expected (Fig. 3), represented mainly by the polychaetes *Magelona papilicornis, Lumbrineris sp.* and *Gimnonereis crosslandi.* These ecological groups of fauna were also heavily abundant in stations VB23 to VB35, all located in the Espirito Santo Bay, directly and widely open to the ocean. BENTIX followed similar patterns of faunal groups distribution, except for having a much larger proportion of opportunistic taxa represented by EG3 in all stations from both protected and urbanized estuaries (Fig. 4)

On the other hand, BO2A showed improved quality status in relation to the AMBI classifications for most stations in VB, and ranged from 0.01 to 0.27 values (VB32 and VB40, classifying most the VB and PAM stations as high and good (Fig. 2).

In BO2A, opportunistic Annelida was in general the dominant faunal group, especially in VB (Fig. 4). The stations from VB showed higher proportions of opportunistic Annelida in specific locations, such as from VB1 to VB11 (innermost portion of the estuary) and also from VB34 to VB43. Stations with higher amounts of amphipods were proportionately more concentrated in PAM.

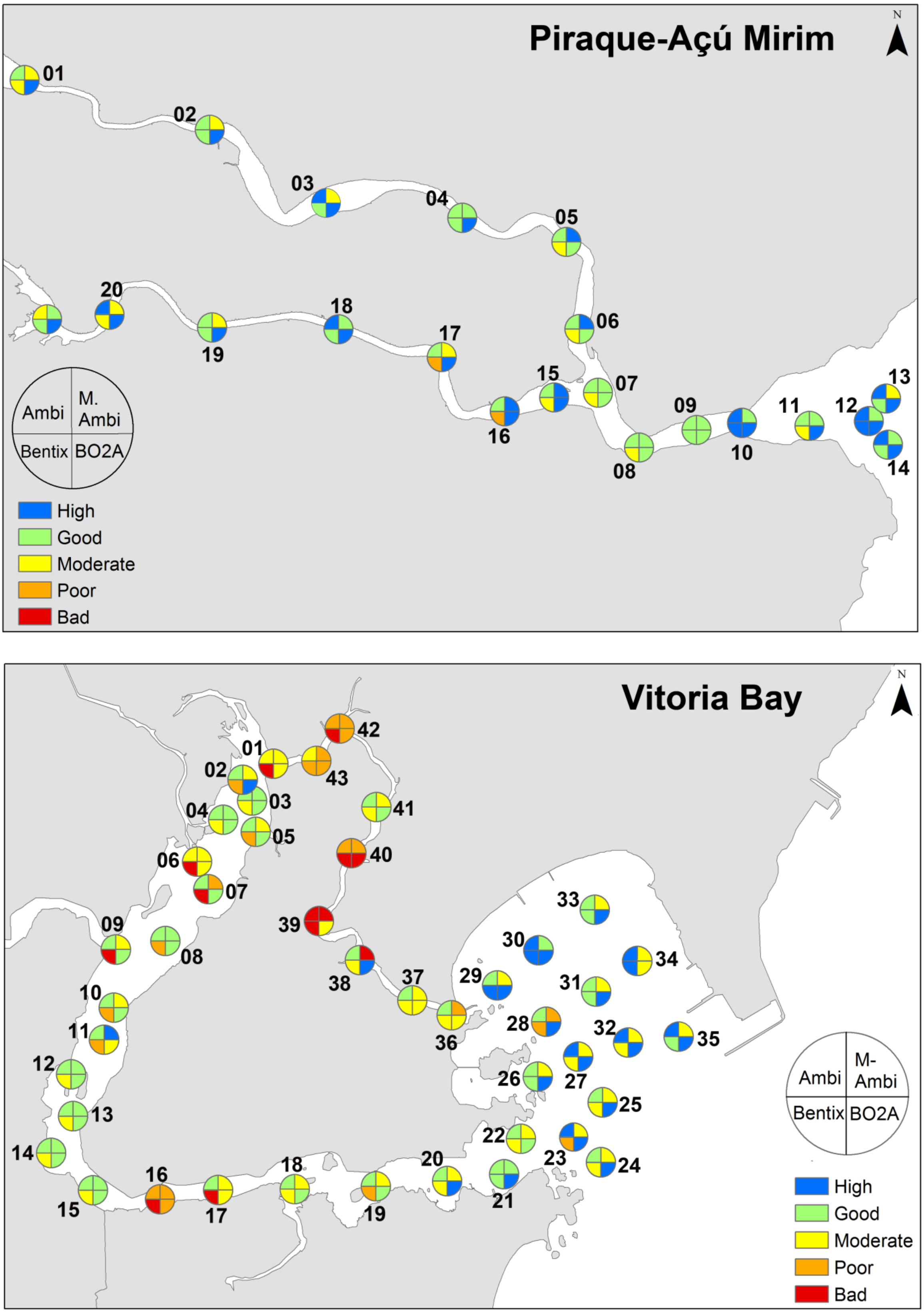

Fig. 2. Spatial distribution of the ecological quality classification obtained for each index along the Protected (PAM) and Urban (VB) estuaries. Only stations with matching indices and geochemical data were displayed.

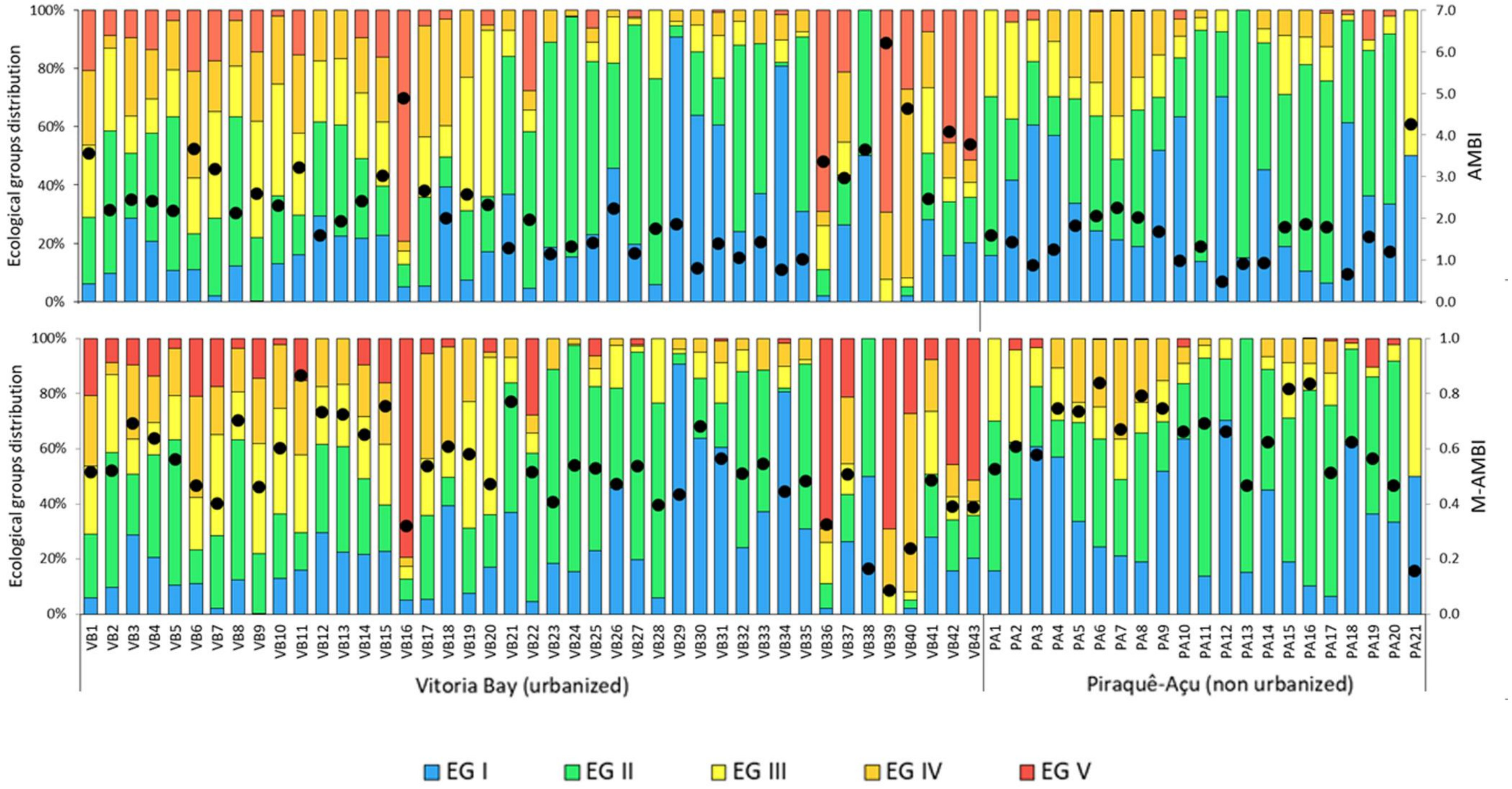

Fig. 3. Means AMBI and M-AMBI values (black dot, right y axis) and percentage (average across replicates) of macrobenthic ecological groups in subtidal stations, for urbanized and non-urbanized conditions. EGI: sensitive species to pollution; EGII: indifferent species; EGIII: tolerant species; EGIV: second-order opportunistic; EGV: first- order opportunistic.

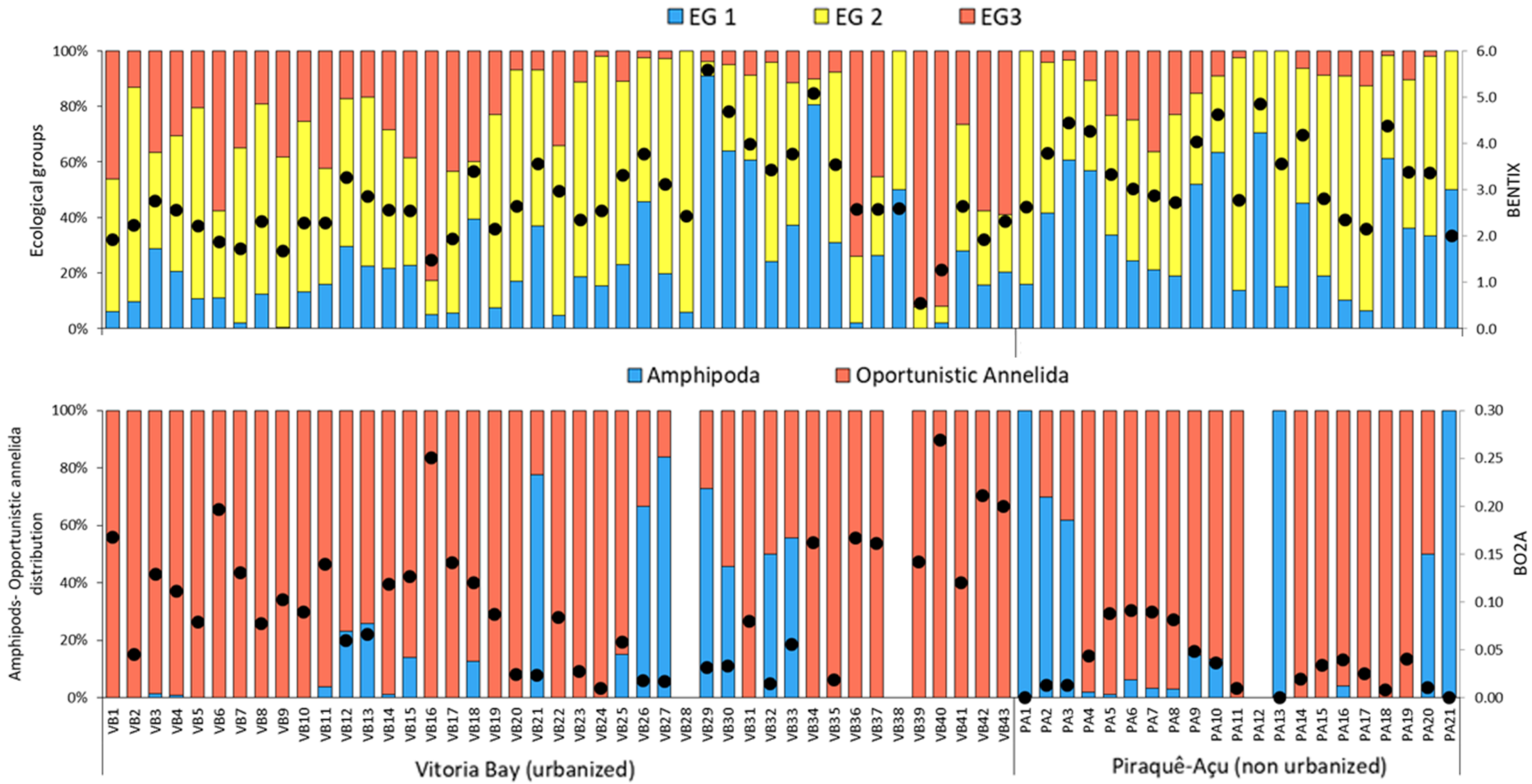

Fig. 4. Means BENTIX and BO2A values (black dot, right y axis) and percentage (average across replicates) of ecological groups and percentage of opportunistic annelid and amphipods in subtidal stations, for urbanized and non-urbanized conditions. EGI: sensitive species; EGII: indifferent and tolerant species; EGIII: second and first-order opportunistic.

PERMANOVA results showed consistent responses for all four indices, with very significant variability ($p<0.001$) at both spatial scales of Estuary (larger) and Station (smaller) (Table 2). The only exception was the Estuary spatial scale for BENTIX, which showed only significant variations ($p<0.05$).

Table 2. Summary of results from the permutational multivariate analysis of variance (PERMANOVA) according to the indices used to assess the benthic health of estuaries under distinct conditions of urbanization. Estuaries (Es) and Stations (St).

Source	**df**	**MS**	***F***
AMBI			
Es	1	2.224	32.8 **
St(Es)	62	0.182	2.7 **
Res	64	0.068	
M-AMBI			
Es	1	0.394	43.1 **
St(Es)	62	0.050	5.5 **
Res	64	0.009	
BENTIX			
Es	1	0.5474	5.4 *
St(Es)	62	0.3622	3.6 **
Res	64	0.101	
BO2A			
Es	1	0.5716	84.5 **
St(Es)	62	0.0298	4.4 **
Res	64	0.0068	

df, degrees of freedom; MS, mean squares; *$p < 0.05$, ** $p <0.001$.

In the partial redundancy analysis, the cumulative percentage of variance explained by the first two canonical axes accounted for 24.8% (22.4% and 2.4%, respectively, for the first and second axis) (Fig. 4). All environmental parameters (Mud, C/N ratio, Coprostanol and the metals Fe, Co, Cu, Cr, Mn) were significantly correlated with the first axis as evidenced by the Monte Carlo test ($p < 0.05$), and the test for all canonical axes was also significant ($p < 0.001$). The samples from protected (PAM) and from urban sites (VB) were oppositely grouped along axis 1 (Fig. 4). The mean values of the indices AMBI and BO2A increased as expected towards contaminated sites, and M-AMBI and BENTIX increased in the opposite side, towards the preserved ones. AMBI and BO2A were best correlated to increasing levels of contaminant proxyes of pollution coprostanol and Co at contaminated stations. The indices M-AMBI and BENTIX were in turn more strongly correlated to increasing contents of mud and Mn at the preserved stations.

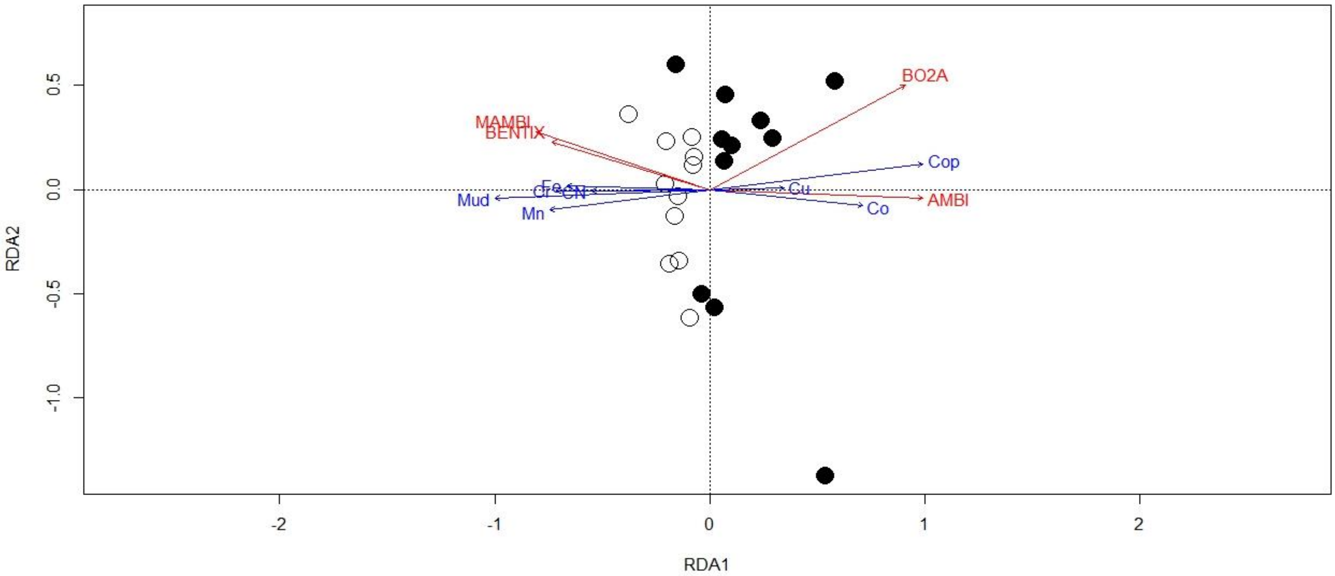

Fig 4. Redundancy analysis (RDA) of the relationships among biotic indices (red arrows), contamination/environmental variables (blue arrows) and sampling stations distribution (black circles – VB, white circles - PA). Mud: fines sediments, CN: C/N ratio; Cop: Coprostanol, and the metals Fe, Co, Cu, Cr, Mn. Black circles – urbanized (Vitória Bay); White circles - Non-urbanized (Piraquê-Açu). The arrows indicate the direction of increase for the variables studied. The angles between variables reflect their correlations (angles near 90° indicate no correlation, angles near 0° indicate high positive correlation and angles near 180° indicate high negative correlation). Only significant variables are shown for this model. Only stations with matching indices and geochemical data were used in the analysis.

The percentage of similarity or agreement among the four indices was in general higher at the non-urbanized stations (PAM), but was much lower and heterogeneous at the urbanized (VB) ones (Fig. 5). The highest agreement was found between AMBI and BO2A, which showed the highest proportion of bad, poor and moderate status, and between M-AMBI and BENTIX, with highest proportions of high and good status (Fig. 5).

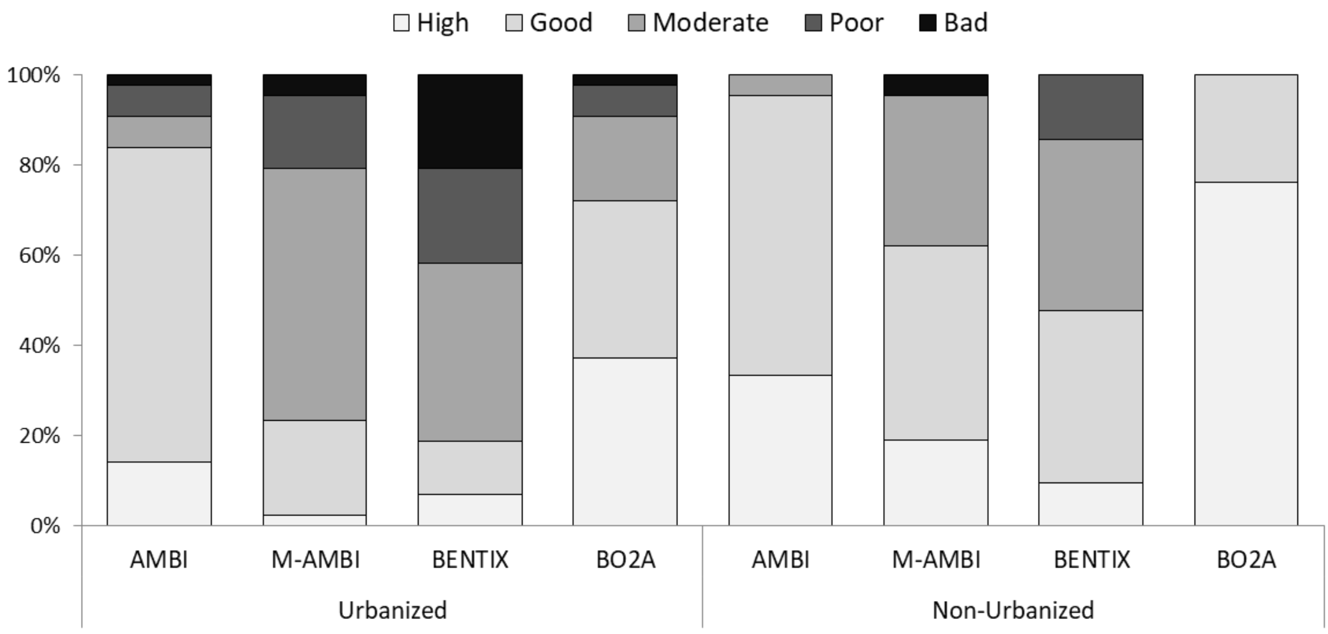

Fig. 5. Ecological status (%) derived from each index in all stations of Urbanized (Vitória Bay) and Non-urbanized (Piraquê-Açu river) conditions.

Discussion

Our tests to the applicability of the benthic indices was in general successful in VB and PAM estuaries, as AMBI, M-AMBI, BENTIX and BO2A did vary significantly at the largest spatial scale or the scale at which pollution acts and were mostly correlated to the chemical proxies of contamination, showing a congruence to the these markers. The only exception was BENTIX, which was not as strongly significant at the largest spatial scale as the other indices. In relation to the consistency of responses of the indices, the ecological status of the estuaries was largely unequal amongst them, with a higher general agreement between AMBI and BO2A and between BENTIX and M-AMBI.

The majority of diagnosis of the indices matched the ecological quality of the estuaries, as the relatively pristine areas of the protected Piraquê-Açu attained good to high ecological status and the urbanized stations in Vitória Bay estuary were largely classified as moderate, poor and bad. In the protected estuary, only stations 16 and 17 were assigned as poor by the BENTIX index, a possible reflex of abundance of the polychaetes from EG II (mainly

Dorvillea sp., Lumbrineris sp., *Gimnonereis crosslandi* and *Mellina sp.* classified as tolerant) and from EGIII (*Tharyx sp.* and *Paraprionspio sp*, classified as opportunist). The remaining stations within this estuary reflected macrobenthic communities typical of healthy habitats, with a more diverse mixture of species sensitive to environmental stress. Low levels of pollution chemical markers previously found in this estuary such as (coprostanol and other sterols makers of organic contamination, see chapter 2) corroborate the benthic indices assessment (Hadlich et al., 2018).

The urban estuary VB, on the other hand, attained more stations in the poor and bad status, mainly present at the confined Passagem Channel. This probably reflects the high levels of organic enrichment by multiple sources of contamination, mainly untreated sewage, and where mangrove vegetation abundance is low (Grilo et al., 2013), resulting in higher proportions of species that are indicative of worse environmental quality. These first and second order opportunistic faunal groups practically disappear in the PAM non-urbanized estuary. The remaining ecological groups of fauna showed a relatively homogeneous distribution between the protected and the urban estuaries for AMBI, M-AMBI and BENTIX indices. For BO2A, there was a massive dominance of opportunistic annelids in both estuaries, but this dominance pattern was more expressive in VB stations of more restricted water circulation. BO2A also did not classified the ecological status of three stations due to the absence of either opportunistic annelids or amphipods.

Efficient indices should respond to the local contamination gradient, which was clearly reflected at the largest spatial scale of our sampling design (of urban x protected estuaries). All indices varied at the pollution scale, but since BENTIX was the only index to show a weaker variability at this scale, it seems to be less suited for environmental quality assessment in the study area. The smaller spatial scale represented by stations was also significantly variable for all indices, a possible consequence of natural background forces acting simultaneously on the benthic communities of the estuaries. Previous studies have reported that organic inputs from mangroves adjacent to the estuarine areas can provide natural inputs of additional organic matter and nutrients that may stimulate opportunistic and tolerant species to flourish along with other sensitive species (Brauko et al., 2015; Valença and Santos, 2012, Salas et al., 2004). Therefore, the indices tend to show moderate environmental quality for pristine stations in estuaries, which occurred in many stations of the PAM protected estuary in this study, for instance. If this natural organic matter is released in smaller spatial scales, than it may represent an additional source of variability to the larger spatial scale represented in our PERMANOVA results.

The responses of the indices should also be congruent with the levels of pollution indicated by chemical markers of anthropogenic stress. Our results showed correlations between the worse values of the indices and increases in such chemical proxies towards the urbanized stations, especially to high concentrations of coprostanol. The correlations between the biotic indices to the contents of this highly stable fecal sterol is a strong and reliable indication that the indices did correspond to the conditions of contamination of the stations in the urban estuary (Albano et al., 2013; Hadlich et al., 2018).

Despite the satisfactory ability of the indices in distinguishing protected from urban estuaries and the congruence between biological and chemical variations, a clear mismatch among the ecological classifications provided by the four indices was found. In other words, if the indices were functioning equally, they should provide similar responses along protected and urban stations. However, the proportion of each ecological quality (from high to bad conditions) was very heterogeneous amongst indices, mainly in more impacted stations from VB. The indices diagnosis were more similar in the PAM estuary, suggesting that the dominant sensitive species were responsible for the conservative results found in this estuary. Oppositely, as indifferent and tolerant species become more abundant towards more polluted stations in VB estuary, the ecological quality given by the indices become much more variable. This happens because there is a shift in the grouping schemes of tolerance depending on the use of either AMBI and M-AMBI or BENTIX (Muniz et al., 2005; Quintino et al., 2006; Brauko et al., 2015). This is why BENTIX tended to downgrade the ecological quality of the stations. M-AMBI also showed the same tendency of BENTIX and was probably influenced by high values of diversity used in its calculations (Brauko et al., 2016). The patterns for AMBI and BO2A were of improved responses in relation to BENTIX and M-AMBI, which could be inadequate for the management of impacted coastal areas in real-world situations.

In summary, our results show that the four indices could be successfully applied in estuarine areas in the southern coast of Brazil, mainly because of their congruence to chemical proxies of pollution. However, we found some ambiguities or inconsistencies amongst their responses suggesting that indices such as AMBI and BO2A should be applied with caution in management practices as they tended to overestimate the ecological quality of impacted stations.

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