UNIVERSIDADE FEDERAL DO PARANÁ

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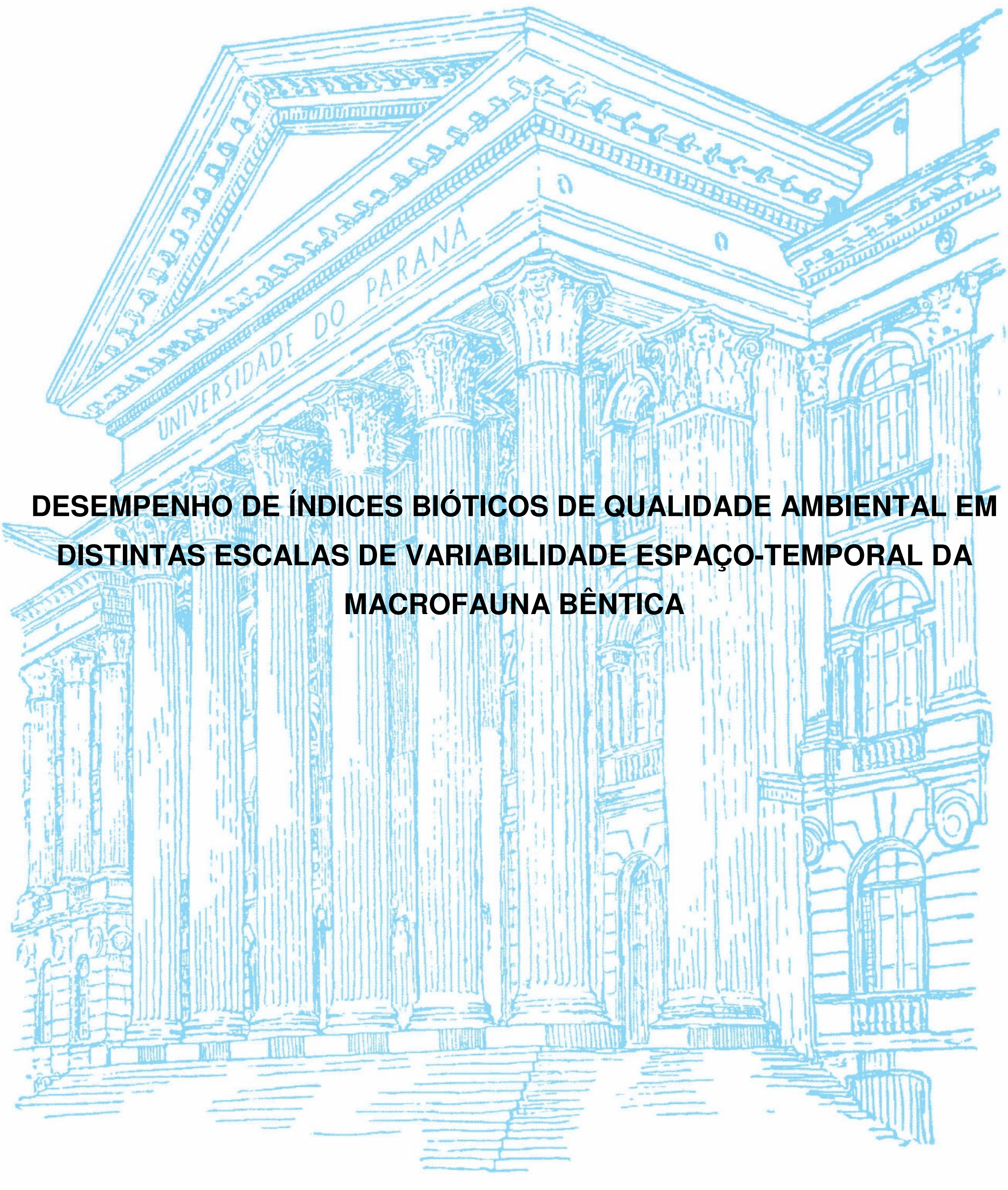

DESEMPENHO DE ÍNDICES BIÓTICOS DE QUALIDADE AMBIENTAL EM DISTINTAS ESCALAS DE VARIABILIDADE ESPAÇO-TEMPORAL DA MACROFAUNA BÊNTICA

CURITIBA
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Tese apresentada como requisito à obtenção do grau de Doutor em Zoologia, no Curso de Pós-Graduação em Zoologia, Setor de Ciências Biológicas da Universidade Federal do Paraná.

Orientador: Dr. Paulo da Cunha Lana
Co-orientador: Dr. Pablo Muniz

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RESUMO

Problemas relacionados à poluição por efluentes urbanos e anoxia são antigos, profundamente enraizados na sociedade e demandam urgente solução. Apesar da disseminação e do uso crescente de índices bênticos para avaliação da qualidade de ambientes marinhos e costeiros alguns problemas relacionados à ambiguidade ainda necessitam de investigações detalhadas. A estrutura trófica de associações bênticas pode igualmente integrar respostas funcionais ao enriquecimento orgânico. Entretanto, os índice bênticos e a estrutura trófica podem responder tanto a distúrbios antropogênicos quanto aos naturais, podendo variar em distintas escalas espaciais e temporais em virtude de diferentes processos interativos. A escolha de índices apropriados deve envolver técnicas de comparação o mais objetivas e integrativas o possível, de forma que variações temporais não sejam subestimadas. As respostas de indicadores à contaminação ainda necessitam de testes que adequadamente detectem os padrões de variabilidade com delineamentos amostrais robustos. O presente trabalho teve como objetivo principal avaliar a confiabilidade, congruência e variabilidade espaço-temporal das respostas de distintos indicadores de qualidade ambiental baseados na macrofauna bêntica. Para tanto, foram utilizadas as seguintes abordagens nos próximos quatro capítulos: (i) testes de congruência aos índices amplamente utilizados AMBI, M-AMBI e BENTIX, usando um delineamento hierárquico em um sub-estuário sujeito a distintos níveis de descargas de esgotos, com correlações a proxies químicos de contaminação e análises de similaridade de respostas; (ii) uma abordagem multivariada para a valiação da congruência e consistência dos índices ITI, BO2A, BENTIX, AMBI e M-AMBI, com correlações a marcadores químicos estáveis de contaminação e avaliação do grau de concordância entre diagnósticos ao longo de dois anos; (iii) avaliação da importância relativa de escalas temporais (quinzenas e estações ao longo de dois anos), espaciais (entre 10^1 e 10^3 m) e interativas na variabilidade que potencialmente afeta o desempenho dos índices AMBI, ITI e BO2A, assim como a contribuição relativa dos principais grupos ecológicos macrofaunais na explicação da variabilidade observada de cada índice; (iv) avaliação da consistência da estrutura trófica de comunidades expostas a diferentes níveis de contaminação por esgotos com o uso de dois métodos distintos para classificação de guildas tróficas a partir da abundância e biomassa como variáveis preditivas. Para tanto, um delineamento hierárquico foi novamente utilizado, com duas escalas espaciais (10^3 e 10^2m) e três temporais (Estações, Eventos e Quinzenas), em baixios entremarés da Baía de Paranaguá. No primeiro capítulo, os índices tiveram um baixo grau de similaridade de respostas em função da influência da variabilidade espacial em seu desempenho. Somente o AMBI variou na escala da contaminação (10^3 m) e foi congruente com os proxies físico-químicos. Respostas ambíguas refletiram efeitos de inputs naturais de matéria orgânica, e não a qualidade ambiental associada aos esgotos. Em adição, os resultados do segundo capítulo sugerem que apenas o ITI, AMBI e BO2A estão prontamente aptos à aplicação nas áreas estuarinas em termos de congruência de respostas e consistência com os proxies químicos de contaminação. Os piores graus de similaridade e correlação às variáveis da poluição envolveram os índices BENTIX e M-AMBI. Sua aplicação é, portanto, não recomendável antes de reajustes adequados dos limites numéricos das categorias de cada status ecológico (ou diagnósticas). Visto que uma avaliação mais detalhada das escalas de variabilidade de AMBI, ITI and BO2A ainda era necessária, no terceiro capítulo a consistência nos padrões de variação desses índices foi avaliada em diferentes graus de contaminação orgânica. As variações presumidamente fortes e marcadas relacionadas à mudança de estações não foi detectada por esses índices, apesar de variações interativas entre as menores escalas espaciais com escalas temporais de pequeno e longo prazo. Tais variações são uma provável consequência de distúrbios de *pulso* e *pressão* específicos. O input orgânico dos esgotos pode operar tanto na maior escala especial (10^3m) quanto na escala temporal de longo prazo (interanual). GI (espécies sensíveis) e suspensívoros tendenciaram grandemente os padrões de variabilidade do AMBI e ITI, assim como os anelídeos oportunistas para o BO2A. Ao contrário de testes equivocados (ou pseudoreplicados) de escalas espaciais, temporais e interativas de variação, nossos resultados foram consistentes à medida que foram avaliados com um delineamento hierárquico robusto e complexo e resultaram congruentes entre os três índices testados. A variabilidade interativa dos índices nas menores escalas não significa, necessariamente, que as respostas foram ambíguas ou inexpressivas. No quart capítulo, apesar da aplicação de distintos métodos de classificação de guildas tróficas da macrofauna, ambas as metodologias refletiram o estado trófico bêntico em escalas de variação espaço-temporais similares. A escala espacial da *condição* (10^3m) frequentemente interagiram temporalmente, o que significa que as diferenças entre áreas contaminadas e não-contaminadas não tiveram as mesmas magnitudes em todas as escalas temporais. As escalas restantes, de *baixios*, *quinzenas* e *eventos* também variaram, representando fatores estruturais adicionais ou secundários atuantes no sistema. O uso da biomassa como variável preditiva aumentou a consistência entre padrões de variabilidade em ambos os métodos de classificação de guildas tróficas. Independentemente do método utilizado, a detecção satisfatória de graus de poluição não depende somente do modo de alimentação da espécie e da qualidade e quantidade da matéria orgânica enriquecida, mas também do nível de tolerância a outros estressores ligados à poluição como a hipoxia. Os resultados enfatizam que os índices testados nos capítulos poderiam avaliar satisfatoriamente a saúde ambiental como ferramentas robustas de gestão, mas sua utilização ainda se beneficiará consideravelmente de avaliações das mudanças de níveis de tolerância de espécies indicadoras-chave. De forma semelhante, a estrutura trófica das associações ainda necessita de experimentos manipulativos que expliquem a complexidade dos fatores ecológicos estruturadores interativos. A utilização de invertebrados bênticos como indicadores, seja compondo índices ou em uma abordagem funcional com guildas tróficas, certamente contribuirá para a preservação da integridade das águas costeiras e para que as sociedades continuem a usufruir de seus bens e serviços.

Palavras-chave: *índices bióticos; indicadores; guildas tróficas; variabilidade; análise de qualidade ambiental.*

ABSTRACT

Problems related to pollution due to urban effluents and anoxia are ancient, deeply rooted in the society and demand urgent solution. Despite the increased and widespread usage of benthic indices for environmental health assessment in coastal and marine areas some problems underlying ambiguous assessments still remain to be elucidated. The trophic structure of benthic assemblages may as well integrate functional responses to organic enrichment. However, the benthic indices and the trophic structure of benthic assemblages 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. The choice of suited indicators must involve comparison techniques as objective and integrative as possible, so that temporal variations are as well outlined. The responses of indicators to disturbance remain to be adequately tested for the detection of spatial variability by robust sampling designs. The main objective of this study was to assess the reliability, conguence and spatiotemporal variation of the responses of distinct indicators of environmental health based on the macrobenthic fauna. To this purpose, the following approaches were employed in the next four chapters: (i) a congruence test to the widely used indices AMBI, M-AMBI and BENTIX using a hierarchical sampling design in a sub-estuary subjected to distinct levels of sewage discharges, with correlations to chemical proxies of contamination and an analysis of similarity of responses; (ii) a multivariate approach was used to address congruence and consistency patterns of the indices ITI, BO2A, BENTIX, AMBI and M-AMBI with correlations to stable chemical indicators of contamination and evaluation of the overall agreement among responses over two years; (iii) assessment of the relative importance of temporal (within fortnights and seasons along two years), spatial (at scales ranging from 10^1 to 10^3 m) and interactive variability affecting the performance of the biotic indices AMBI, ITI and BO2A, as well as the relative contribution of major macrofaunal ecological groups in explaining the observed variability of each index; (iv) evaluation of the consistency of trophic assemblages exposed to distinct levels of sewage contamination using two different methodological approaches for trophic guild assignment and both abundance and biomass as predictive variables. To this purpose we also used a hierarchical sampling design, nested at two spatial (10^3 and 10^2m) and three temporal scales (Seasons, Events and Fortnights) in non-vegetated tidal flats of the subtropical Paranaguá Bay. In the first chapter, we found a low degree of similarity among indices as an expression of the spatial variation of macrofaunal assemblages on their performances. Only AMBI varied at the contamination scale (10^3 m) and was congruent with physical-chemical proxies. Ambiguous responses indicated effects of natural inputs of organic matter rather than environmental quality associated to sewage. Furthermore, the results from the second chapter showed that only ITI, AMBI and BO2A seemed readily suited to assess the health condition of estuarine areas in terms of congruence among responses and consistency with chemical tracers of contamination. The worst levels of agreement and correlations to the pollution variables involved BENTIX and M-AMBI. We thereafter discouraged the application of BENTIX and M-AMBI prior to proper boundaries readjustments for such habitats. Since further detailed investigation of several scales of variability was still needed, in the third chapter AMBI, ITI and BO2A were consistently responsive to varying contamination levels. The presumed strong and marked variations related to seasons were not detected by these indices, although there was interactive variation between smaller spatial scales with short- and long-term temporal scales. Such variations are probably a consequence of specific pulse and press disturbances. The sewage input is likely to operate either at the largest spatial scale (thousands of meters), and at the long-term temporal scale (interannual). GI (sensitive species) and suspension feeders were possibly responsible for most of the variability of AMBI and ITI, as the opportunistic annelids for BO2A. Unlike biased tests of spatial, temporal and/or interactive scales of variation, our assumptions were consistent as they were both assessed with a robust and complex hierarchical sampling design and were congruent among all tested indices. Benthic indices varied at a variety of interactive scales, which does not necessarily mean ambiguous or meaningless responses. In the fourth chapter, regardless of applying a broader versus a narrower classification of trophic guilds, both methodologies were able to indicate the benthic trophic status at similar spatiotemporal scales of variation. The spatial scale of *condition* (10^3m) often interacted with time, meaning that the differences between contaminated and non-contaminated sites were not of similar magnitudes for all temporal scales. The remaining scales of *tidal flat*, *fortnight* and *event* also varied, representing additional or secondary structuring factors in the system. The use of biomass as a predictive variable increased the consistency between the patterns of variation in both methods of trophic guild assignment. Regardless of the method to trophic guild assignment, a successful application to pollution detection will not only depend on the feeding mode of the species and the quality or quantity of organic enriched material, but also on its level of tolerance to other pollution-stressors like hypoxia. We underline that all the tested indices could successfully assess benthic quality conditions as robust management tools but, a suitable application might still considerably benefit from additional investigation towards tolerance shifts of key indicator species. The structure of trophic assemblages also still need manipulative experiments to unravel the complex interplay of ecological structuring processes. The use of benthic invertebrates as indicators, either in the indices' composition or in functional approaches with trophic guilds, would certainly contribute to the conservation of the integrity of coastal waters, so that societies can still benefit from its goods and services.

Keywords: *biotic index; indicators; trophic guilds; variability; environmental quality assessment.*

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I. Spatial variability of three benthic indices for marine quality assessment in a subtropical estuary of Southern Brazil

Brauko, K.M., Souza, F.M., Muniz, P., Camargo, M.G., Lana, P.C., 2015. *Marine Pollution Bulletin* 91, 454 - 460.

II. Assessing the suitability of five benthic indices for environmental health assessment in a South American estuary

Brauko, K.M., Muniz, P., Martins, C.C., Lana, P.C. Manuscript to be submitted to *Ecological Indicators*.

III. Performance of benthic indices for environmental quality assessment at nested spatio-temporal scales: do time and space really matter?

Brauko, K.M., Souza, F.M., Muniz, P., Camargo, M., Gilbert, E.R., Lana, P.C. Manuscript to be submitted to *Marine Ecology Progress Series*.

IV. Consistency of responses of macrofaunal trophic guilds to sewage discharges at nested scales of variation in a subtropical estuary

Brauko, K.M., Dauer, D.M., Lana, P.C. Manuscript to be submitted to *Environmental Pollution*.

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INTRODUÇÃO GERAL

A conservação da qualidade da água e dos recursos vivos de ambientes costeiros aparece, na atualidade, como uma das mais urgentes demandas nos processos de planejamento e gestão ambientais (Dahms, 2014). Atividades antropogênicas como a eliminação de resíduos industriais e domésticos acarretam inevitavelmente modificações nas regiões costeiras. Estas intervenções exigem a implementação de programas de avaliação de impactos e de monitoramento ambiental como forma de balancear imperativos de crescimento sócio-econômico e de preservação.

A detecção de impactos por meio de mensurações de concentrações dos próprios contaminantes é muitas vezes dispendiosa. Mais ainda, estas concentrações tendem a ser pouco persistentes. Por outro lado, variáveis biológicas, configuradas como verdadeiros *bioindicadores*, representam diretamente as condições da biota, possibilitam a identificação de problemas não detectados ou subestimados por outros métodos e permitem a avaliação do progresso da recuperação dos ecossistemas envolvidos (Dauer, 1993). Neste sentido, as respostas da macrofauna bêntica são mais sensíveis e confiáveis do que medidas diretas da qualidade da água ou do sedimento, na medida em que processos como a perda da diversidade e a dominância de poucas espécies tolerantes em áreas poluídas podem modificar processos ecológicos e reduzir a complexidade da cadeia trófica a um nível irreversível (Lerberg *et al.*, 2000).

Muitas características qualificam a fauna bêntica como importante indicadora da saúde e condição de ecossistemas costeiros: (1) são animais relativamente sedentários, refletindo diretamente as condições do ambiente; (2) habitam a interface água-sedimento, onde a exposição a contaminantes e à anoxia ou hipoxia é mais freqüente; (3) possuem ciclos de vida relativamente longos e suas respostas integram alterações na qualidade ambiental ao longo do tempo; (4) incluem espécies com variados modos de vida e tolerância ao estresse, o que permite seu enquadramento em diferentes grupos funcionais; e (5) afetam o fluxo químico entre o sedimento e a coluna d'água através da bioturbação e atividades de alimentação, assumindo um papel vital na ciclagem de nutrientes (Dauer, 1993; Reiss & Kroncke, 2005).

Indicadores ecológicos são representações quantitativas das variáveis ambientais de um sistema e de respostas da biota a essas forçantes (Salas *et al.*, 2006). Índices bióticos marinhos, por sua vez, ilustram as respostas de comunidades frente a modificações naturais e antropogênicas na qualidade da água, e as integram em um único valor que expressa o estado da saúde de um ecossistema (Borja *et al.*, 2014). Esses índices são fundamentados na ideia de que comunidades biológicas são um reflexo do seu ambiente, e que diferentes organismos exibem variados graus de seletividade de habitats e tolerância à poluição. O valor numérico expresso sintetiza esta complexidade e pode ser ainda relacionado a uma ampla escala de medidas físicas, químicas e biológicas (Pinto *et al.*, 2009). Neste contexto, índices bióticos podem ser uma alternativa mais expedita, econômica e relevante para avaliar impactos sobre o ambiente.

Os índices bióticos marinhos têm sido tradicionalmente desenvolvidos e utilizados sob premissas e princípios específicos, em diversos ambientes costeiros da Europa e América do Norte, desde a década de 70 até um *boom* ocorrido na última década (Pinto *et al.*, 2009). Os índices mais comuns podem ser agrupados em três classes: *univariados* ou de medidas da estrutura de comunidades (por exemplo, diversidade de Shannon-Wiener), *índices multimétricos* combinando as variadas respostas das associações ao estresse e *índices ou estratégias multivariadas* que descrevem os padrões das associações de forma mais integrada a variáveis físico-químicas.

Uma outra classificação (Salas *et al.*, 2006) estabeleceu seis grupos de índices bênticos, de acordo com suas especificidades: *índices baseados em espécies indicadoras*, que consideram a presença/ausência de determinadas espécies indicadoras (ex.: AMBI, BENTIX), *índices baseados em estratégias* ecológicas, focados nas estratégias de vida dos organismos (ex.: Razão Polychaeta/Amphipoda, Índice de *r/k* estrategistas), *índices baseados em valores de diversidade* (ex.: diversidade de Margalef e Simpson), *indicadores baseados na biomassa ou abundância das espécies*, que consideram a variação energética do sistema através de variações na biomassa dos organismos (ex.: Curvas de Abundância-Biomassa), *indicadores termodinâmicos baseados em análise de rede,* que capturam informações do ecossistema em uma perspectiva mais holística (ex.: aplicação do conceito de exergia), e por último, *indicadores integrativos*, que tentam incluir toda a informação possível sobre o ambiente em um único valor (ex.: B-IBI).

Entretanto, é importante enfatizar que os índices bióticos possuem algumas limitações. A primeira é que resultam, inevitavelmente, da simplificação do ecossistema em questão e da redução de sua complexidade (Salas *et al*., 2006). Apesar de um único índice fornecer uma boa visão da saúde ambiental, um índice universal que funcione em todos os ambientes ou em ecossistemas semelhantes, no entanto, é impraticável, devido à enorme complexidade e diversidade das associações bênticas (Dauvin *et al*., 2006, Borja, 2014). Apesar disto, estes índices devem continuar a ser vistos como importantes ferramentas nos processos de tomada de decisão, uma vez que sintetizam a complexidade dos impactos e facilitam sua comunicação para os gestores e público não-especialista. Indicadores e índices, portanto, podem ser utilizados no direcionamento de estratégias de preservação ambiental após a validação ou avaliação de sua confiabilidade (Pinto *et al*., 2009).

Assim como quaisquer estruturas e processos biológicos, os índices também dependem estreitamente das escalas espaço-temporais de variação da comunidade considerada. As variações espaciais podem ocorrer de centímetros a centenas de quilômetros, condicionadas por diversos fatores como diferentes condições hidrodinâmicas, estações do ano, distúrbios físicos episódicos, mudanças nas características sedimentares, migração, recrutamento, competição, predação e produtividade (Morrissey *et al*., 1992a; Murphy et al., 2009). A variabilidade temporal é igualmente condicionadora dos padrões de variação, na medida em que flutuações podem depender da frequência de aquisição dos dados e da própria sazonalidade ou ciclicidade dos ambientes investigados. Alguns estudos sugerem a existência de variabilidade imprevisível e substancial em escalas temporais menores (i.e., dias, semanas e meses), o que pode mascarar padrões evidentes entre estações ou anos (Morrissey *et al*., 1992b; Olabaria & Chapman, 2001).

Padrões de distribuição da fauna bêntica podem ser mais evidentes em certas escalas e ausentes em outras, continuam pouco conhecidos, particularmente em regiões tropicais e subtropicais. Consequentemente, a heterogeneidade em qualquer escala entre as unidades amostrais (pequena escala) e os locais amostrais (grande escala) não é revelada ou dada *a priori*. Assim, as variações na ocorrência, distribuição e densidade da biota devem ser investigadas em múltiplas escalas, de forma a evitar estimativas e comparações impróprias e pseudoreplicação (Morrissey *et al.*, 1992a).

Um aspecto importante da variabilidade temporal normalmente negligenciado é a sua possível interação com escalas espaciais (p. ex., alterações na abundância e composição de um tempo para outro podem diferir entre locais). Neste sentido, a compreensão plena da estrutura e dinâmica das associações bênticas requer o conhecimento e investigação da interação entre variações temporais e espaciais nas mais diversas escalas (Murphy et al., 2009). Delineamentos amostrais hierarquizados têm sido amplamente utilizados para avaliar a variabilidade de escalas espaciais e temporais (Underwood *et al.,* 2000; Morrissey *et al.,* 1992b; Murphy et al., 2009). Por outro lado, estudos que avaliem o desempenho de diferentes índices bióticos frente à variabilidade espaço-temporal natural das associações utilizando delineamentos hierarquizados continuam escassos (Tattaranni & Lardicci, 2010; Muniz et al., 2012).

Apesar da ampla utilização de índices bióticos de qualidade ambiental, soluções para os problemas de padronização e validação são ainda provisórias ou pouco difundidas. A experimentação e avaliação da consistência dos índices já existentes é uma demanda mais urgente do que a criação de índices novos (Borja *et al.*, 2008). No Brasil, índices bênticos foram aplicados a poucas regiões costeiras (Muniz *et al.*, 2005, Omena et al., 2012, Valença & Santos, 2012), mas o seu grau de confiabilidade ou congruência de diagnósticos ainda devem ser testados. Os estudos disponíveis não chegaram a avaliar a resposta destes índices em diferentes escalas de variabilidade espacial e temporal combinadas.

No presente trabalho, procurou-se não apenas aplicar índices bênticos desenvolvidos originalmente em diferentes latitudes, mas avaliar criticamente sua confiabilidade e congruência de diagnósticos em uma região estuarina sul-americana. Para tanto, a tese foi estruturada em quatro capítulos, na língua inglesa e formatados como manuscritos para submissão em revistas científicas internacionais. O primeiro capítulo traz um exercício preliminar da aplicação de índices bênticos sobre dados-piloto amostrados com um delineamento amostral espacialmente hierarquizado na Baía de Paranaguá. O objetivo foi testar a congruência entre os índices AMBI, M-AMBI e BENTIX, a qual seria comprovada por: (i) fortes correlações com indicadores químicos de contaminação, (ii) alta similaridade de respostas, e (iii) variabilidade espacial significativa na maior escala espacial, ou a escala da contaminação. Este capítulo está apresentado no formato de manuscrito (para atender as formalidades deste documento), mas sua separata, publicada na revista *Marine Pollution Bulletin,* encontra-se em anexo no final da tese.

O segundo capítulo avaliou a adequação de cinco índices bênticos para avaliação da qualidade ambiental com uma abordagem multivariada na Baía de Paranaguá. Os índices foram simultaneamente correlacionados a marcadores orgânicos de contaminação altamente confiáveis, e depois tiveram o grau de similaridade de respostas testados e quantificados. Todas as análises foram realizadas em dois anos consecutivos de amostragens para que a consistência de padrões fosse avaliada em função do tempo. No terceiro capítulo, os índices que resultaram sistematicamente confiáveis (com respostas congruentes e correlações consistentes com os marcadores de contaminação ao longo dos dois anos) tiveram então sua variabilidade testada em função de três escalas espaciais e três temporais. Assim como no primeiro capítulo, as variações foram testadas com um delineamento hierárquico, mas desta vez com fatores espaciais e temporais interativos. Seguindo a mesma lógica hierarquizada, o quarto capítulo por fim avaliou a consistência entre duas abordagens de detecção de impactos, fundamentadas nas mudanças funcionais indicadas por guildas tróficas bênticas.

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 # Manuscript 1

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Spatial variability of three benthic indices for marine quality assessment in a subtropical estuary of Southern Brazil

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Abstract

Indices based on macrobenthic responses to disturbance remain to be adequately tested for the detection of spatial variability by robust sampling designs. We present herein a congruence test to real-world data of the widely used indices AMBI, M-AMBI and BENTIX in tidal flats of a subtropical estuary. We used a hierarchical sampling design to evaluate the spatial variability of the indices in response to distinct levels of sewage contamination. Indices were then tested for correlations with chemical proxies of contamination and for the similarity of responses. BENTIX and M-AMBI produced over- and underestimations of ecological status. We found a low degree of similarity among indices as an expression of the spatial variation of macrofaunal assemblages on their performances. Only AMBI varied at the contamination scale (10^3 m) and was congruent with physical-chemical proxies. Ambiguous responses indicated effects of natural inputs of organic matter rather than environmental quality associated to sewage.

Keywords: *hierarchical analysis; urban effluents; indicators, AMBI, BENTIX, M-AMBI.*

Introduction

A large array of ecological indicators is available for application to environmental health assessment. The number of accessible tools and techniques, such as biotic indices, is rapidly increasing. Macrobenthic animals are considered effective indicators of pollution stress, as they show predictive responses to different levels of natural and anthropogenic impact (Pinto et al., 2009). Therein lies a challenge for the future: to select appropriate monitoring designs and ecological indicators that will provide convincing scientific underpinnings for management and policy decisions on real-world problems (Niemi and McDonald, 2004).

Variation or patchiness in the distribution of benthic assemblages occurs at different spatial scales (Fraschetti et al., 2005; Morrisey et al., 1992). Real changes of the environmental quality associated to biotic indices are frequently confounded with such variability in the distribution of the macrobenthic assemblages (Tattaranni and Lardicci, 2010; Borja et al., 2008). Though patchiness patterns are evident at certain scales and absent at others, they are not adequately addressed in the literature due to a lack of appropriate spatial replication. There is evidence that biotic indices can likewise vary or respond to natural disturbances (Muniz et al., 2012). The efficiency of biotic indices or any inferences on their suitability requires some degree of congruence with criteria for degraded and undegraded sites based on nonbiological measures such as chemical proxies of contamination (Benyi et al. 2009).

Hierarchical sampling designs are considered an appropriate method to estimate the contribution of each spatial scale to the total variation among samples, and to discriminate between natural and human induced changes (Underwood and Chapman, 2013; Chapman et al., 2010; Murphy et al., 2009). The meaningful usage of biotic indices is strongly dependent on the quality and quantity of available data, to avoid erroneous classification of environmental health (Tattaranni and Lardicci, 2010). As yet, only two studies have assessed the variability of biotic indices using hierarchical sampling approaches (Muniz et al., 2012; Tattaranni and Lardicci, 2010), and no previous attempts have been conducted in tropical and subtropical coastal environments. The choice of appropriate biotic indices also involves understanding the association among physico-chemical and biological parameters. Despite the extensive amount of literature concerning the usage of biotic indices in subtidal areas, the actual application of such indices in intertidal areas have rarely been systematically examined using robust sampling designs. Desirable responses from indices involve the ability to detect quality trends across distinct environments found in both subtidal and intertidal systems (Borja et al., 2011).

We present herein a congruence test to real-world data of three of the most widespread macrobenthic community indices to assess environmental health (Forde et al., 2013; Wu et al., 2013; Munari and Mistri, 2010; Ponti et al., 2008), namely AMBI (AZTI marine biotic index), its multivariate extension M-AMBI and BENTIX in response to distinct levels of sewage contamination. The aim of this study was to assess the effects of spatial variation on the performance of these indices in non-vegetated tidal flats of a subtropical estuary in southern Brazil. We used a hierarchical sampling design to evaluate the variability of the indices in response to the distinct levels of sewage contamination of the tidal flats, at the scales of 10^3 (Conditions - Contaminated and Non-contaminated), 10^2 (Tidal flats) and 10^1m (Plots). Indices were then tested for correlations with chemical proxies of contamination levels, and for the percentage of similar responses. In this paper, the term congruence refers to the strength and significance within indices responses and their correlation with chemical proxies across a set of hierarchically distributed sites. In terms of strong congruence, we hypothesized that effective indices should preferably: (i) be highly correlated with chemical indicators of contamination; (ii) present a high percentage of similarity among responses and (iii) vary significantly at the largest spatial scale (10^3m), or the Conditon scale.

Materials and methods

Study area

The study was carried out at the Paranaguá Estuarine Complex (PEC) (25°03'S, 48°25'W), which covers an area of 612 km^2 and is one of the main estuaries on the southern coast of Brazil regarding port and tourist activities. The tidal regime is semi-diurnal with estimated average flushing times of three days in the wet season and of ten days in the dry season in average (Mantovanelli et al., 2004). The Cotinga sub-estuary extends for nearly 20 km and is located in the polyhaline sector, near the mouth of the estuary (Fig. 1). Mean neap and spring tidal heights are, respectively, 1.3 and 1.7 m, with a mean depth of 5.4 m (Lana et al., 2001, Marone and Jamiyanaa, 1997). About 34% of the surface area of the sub-estuary, strongly influenced by tidal currents, is covered by mangroves and marshes or remain non-vegetated (Noernberg et al., 2006).

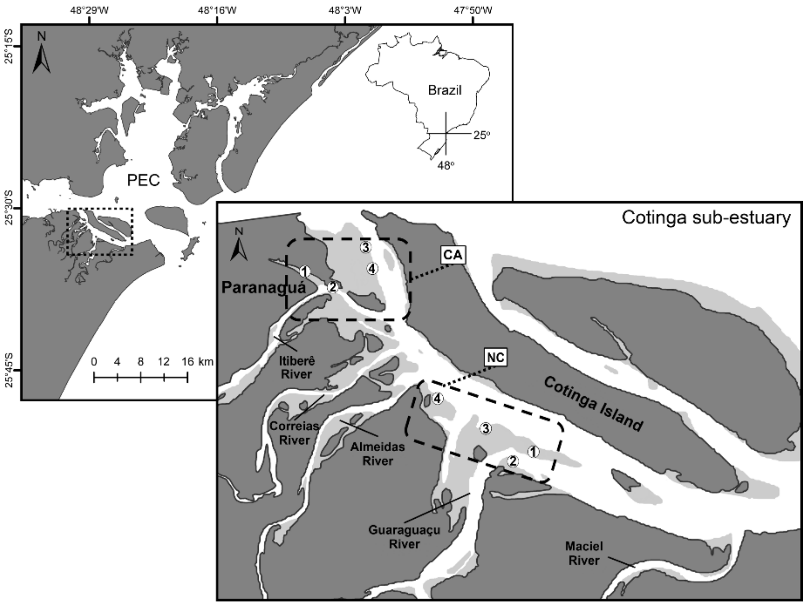

Fig. 1. Study area (modified from Souza et al., 2013). Paranaguá Estuarine Complex (PEC) and Cotinga Sub-estuary. Tidal flats 1, 2, 3 and 4 of the Contaminated area (CA) and the Non-Contaminated area (NC).

The Cotinga sub-estuary is the main dilution path for anthropogenic input of sedimentary organic matter, represented by sewage-derived material from Paranaguá city (Souza et al., 2013; Lana et al., 2000). Only 50% of the sewage output undergoes treatment, while the rest is released *in natura* to the sub-estuary (CAB-Águas de Paranaguá, 2010). *Escherichia coli* activity and concentrations of fecal steroids, highly stable organic markers, indicate a sharp and compressed gradient of domestic sewage contamination from the inner sector to the outer part of the sub-estuary (Barboza et al., 2013; Martins et al., 2010). However, strong sewage contamination indicated by coprostanol levels is confined to sites close to Paranaguá city (Martins et al., 2010). The sites near Paranaguá city can be considered contaminated by sewage inputs as average coprostanol concentrations above threshold limits (>0.5 µg g^{-1}) have been recently found, of up to 1.69 µg g^{-1}. As the distance from the sewage source increase these concentrations decrease, ranging from >DL (detection limit) up to only 0.14 µg g^{-1} (Abreu-Mota et al., 2014). Based on these evidences, we determined two contamination conditions, namely Contaminated and Non-contaminated. Our samplings were carried out in four tidal flats within each condition. All tidal flats corresponded to similar habitat types with no significant differences in salinity, granulometry, exposure to tides and slope (Souza et al., 2013, Noernberg et al., 2006).

We used a hierarchical sampling design to evaluate the variability of the indices in response to the distinct levels of sewage contamination of the tidal flats. The design incorporated three spatial scales, ranging from 10^0 m between replicate samples to 10^3 m between the two contamination conditions of Cotinga sub-estuary (Fig.2). The factors of the mixed linear model were: Conditions – fixed, with two levels (10^3 m); Tidal flats – random, with four levels (10^2 m), nested in Conditions; and Plots – random, with three levels (10^1 m), nested in Tidal flats, with three replicates each (10^0 m).

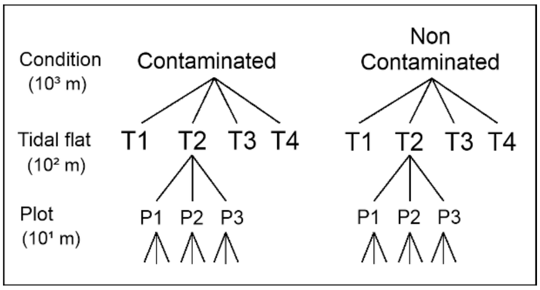

Fig. 2. Diagram of the experimental design (modified from Souza et al., 2013) and scales of spatial variability: Conditions (Contaminated and Non-contaminated); Tidal flats (T1, T2, T3 and T4); and Plots (P1, P2 and P3), with three replicate each.

Macrofauna was collected using plastic core tubes (10 cm diameter, 10 cm deep), and all plots were placed parallel to the water line, at similar tidal levels. All samples were sieved through a 0.5 mm mesh, fixed in 6% formaldehyde and preserved in 70% alcohol. In the laboratory, all organisms were counted and identified to the lowest possible taxonomic level.

Additional sediment samples were taken at each plot to determine total phosphorus (TP), total nitrogen (TN) and total organic carbon (TOC) contents. The concentrations of TN and TP were obtained according to the method described by Grasshoff et al. (1983), and the concentrations of TOC were measured with the oxidation method described by Strickland and Parsons (1972).

Biotic indices and data analysis

Three biotic indices were used to assess the ecological status of the Contaminated and Non-contaminated tidal flats (Table1). AMBI and M-AMBI values were calculated using the software available at AZTI's web page (http://ambi.azti.es). The AMBI index is based on the percentage of abundance of five ecological groups according to their sensitivity to organic pollution, already listed in the software (Borja et al., 2003, 2000). However, some species or taxa present at Paranaguá bay are not as 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 (Ferrando and Méndez, 2011; Boehs et al., 2008; Gamito, 2008; Nalesso et al., 2005; Palacios et al., 2005; Barnett, 1983) 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, *Sigambra* sp. in GIII, Tubificinae sp1. and Tubificinae sp2. were in GV, while the polychaete *Dorvillea* sp. remained unassigned.

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

Index		Classification				
		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

*Calculated by factorial analysis of AMBI, species richness and Shannon-Wienner diversity values.

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; Bald et al., 2005; Borja et al., 2004). 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. We defined *a priori* reference conditions by adapting the default values that determine the 'high' and the 'bad' ecological status. We used a different dataset previously obtained in samplings from the same locations in the Cotinga channel (Unpublished data). Afterwards, the index was derived in relation to these values. We used as the highest AMBI value ('Bad' reference conditions) the number derived from the most polluted site of the dataset. Conversely, "High" reference conditions were calculated from the pristine site.

The BENTIX is based on the same proposal as AMBI, but the taxa are categorized in three ecological groups (Simboura and Zenetos, 2002). We adapted the classification of AMBI as following (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 indices values were calculated for each replicate and their ecological status was therefore attributed as *High, Good, Moderate, Poor* and *Bad* (Table 1). The spatial scales of variability were evaluated using a mixed nested ANOVA model for each index. The analyses were conducted in the R environment (R Development Core Team R, 2009) using the package GAD (Sandrini-Neto and Camargo, 2011). Estimates of components of variation were also calculated to evaluate the amount of variation attributed to each source, and were analyzed together with the analysis of variance. All analyses were performed using untransformed data to provide variance components comparable across all data (Fraschetti et al., 2005).

Redundancy analysis (RDA), a constrained linear ordination method, was carried out to explore the relationships among the biotic indices, the chemical proxies of nutrient enrichment (TOC, TN and TP), and the variation on the distribution of sampling plots along the gradient of sewage contamination. 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.

Results

The three indices classified the majority of the sites as *poor* and *moderate* classes (Figs. 3 and 5).

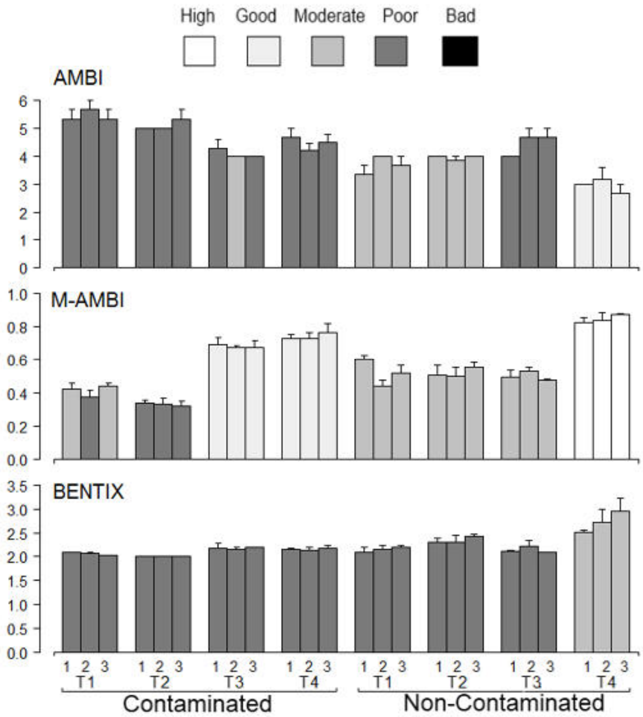

Fig. 3. Mean value (±SE) of the three biotic indices calculated for the Plots (1, 2 and 3) in each Tidal flat (T) of the Contaminated and Non-contaminated sites. Bar colors indicate the ecological status as defined by each index.

Based on the AMBI classification, Cotinga sub-estuary exhibited some degree of disturbance. No values attained the *high* status, ranging from 1.8 (Non-contaminated site, T4, P3) to 5.7 (Contaminated, T1, P2) (Fig. 3). About 44% and 7% of the sites could be ranked as *poor* and *bad*, mostly located in the inner part of the channel near Paranguá city. *Good* (13%) and *moderate* status (36%) were found throughout the Non-contaminated tidal flats (Fig. 3). As expected, ecological groups V and I (opportunistic and sensitive species) dominated, respectively, Contaminated and Non-contaminated sites. However, high proportions of ecological group IV (represented mainly by the gastropod *Heleobia australis*) were found on Non-contaminated sites (Table 3). Differences in mean AMBI values were observed at the Tidal flat spatial scale (10^2 m) (Table 2). Components of variation also showed consistent variability at the Contamination scale (10^3 m).

Table 2. Hierarchical nested ANOVA results. Plot (P) nested in Tidal flat (T), Tidal flat nested in Condition (Cond). Freedom degrees, mean square (MS), statistical value (F), *p* value (*p*) and components of variation (CV%) are presented. Significant differences are given in bold (*p*<0,05).

	Df	AMBI				M-AMBI				BENTIX			
		MS	F	*p*	CV %	MS	F	*p*	CV %	MS	F	*p*	CV %
Cond	1	19,107	5,6	0,0563	39	0,058	0,2	0,675681	0	1,038	2,9	0,142	27
T(Cond)	6	3,434	18,8	**<0,001**	35	0,302	75,8	**<0,001**	74	0,363	15	**<0,001**	39
P(T(Cond))	16	0,182	0,9	0,53471	0	0,004	1	0,469207	1	0,024	0,8	0,6567	0
Residual	48	0,195			26	0,004			25	0,029			34

The situation was worse for BENTIX, with status values varying from *moderate* to *poor* (10 and 90% of the plots) (Fig. 3). Since no values attained the *high*, *good* or *bad* status, no gradient could be identified. Plots with *moderate* status were located at the non-contaminated tidal flats. Ecological group III of BENTIX (equal to groups IV and V of AMBI) was made up by the so called second order opportunists *H. australis* and *Laeonereis culveri*, and by the first order opportunist Tubificinae sp1. (Table 3). The species that represented this ecological group were dominant at both Contaminated and Non-contaminated sites. Ecological groups I and II (sensitive and indifferent species) were also present, but with different proportions depending on the tidal flat. Significant spatial differences were only found at the Tidal flat scale (10^2 m), which was corroborated by the highest value of the component of variation (Table 2).

Table 3. Percentage of dominance of species within Non-contaminated sites, Contaminated sites and the Total. The respective Ecological Group following AMBI/M-AMBI (EGI, EGII, EGIII, EGIV and EGV) and BENTIX (EGI, EGII and EGIII) classifications are also shown.

	Ecological Group		Dominance (%)		
Species	AMBI / M-AMBI	BENTIX	Non-contaminated	Contaminated	Total
Tellina versicolor	I	I	2,59	0,48	0,92
Bulla striata	II	II	3,35	1,41	1,81
Sigambra sp.	III	II	8,16	3,65	4,59
Anomalocardia flexuosa	III	II	1,95	0,91	1,13
Streblospio benedicti	III	II	1,19	1,14	1,15
Heleobia australis	IV	III	47,5	7,19	15,61
Laeonereis culveri	IV	III	2,34	17,33	14,2
Tubificinae sp.1	V	III	14,86	46,21	39,66

The classification of sites by the M-AMBI index was less severe, with values ranging from 0.25 (Contaminated site, T2, P2) to 0.92 (Non-contaminated site, T4, P2) (Fig. 3). M-AMBI was the only index to assess the *high* ecological status (10% of the plots) in Non-contaminated tidal flats. No site was considered as *bad*, whereas 28% of plots of the Cotinga sub-estuary were classified as *good*, 42% as *moderate* and 21% as *poor*. This index includes the species richness and the Shannon-Wiener diversity measures, which were either similar or higher in contaminated tidal flats comparing to the Non-contaminated (see Souza et al., 2013 for details). M-AMBI was significantly variable at the scale of Tidal flats (10^2 m), a pattern equally important in terms of the percentage of the components of variation observed (Table 2).

The redundancy analysis considering the biotic indices and the chemical parameters of contamination displayed eigenvalues of 0.508 and 0.073 for axes 1 and 2, respectively (Fig. 4). The cumulative percentage of variance explained by the first two canonical axes accounted for 58.2% (50.82 and 7.4% respectively for the first and second axis) of indices data and 98.4% (86.0 and 12.4%) of index-environment relations.

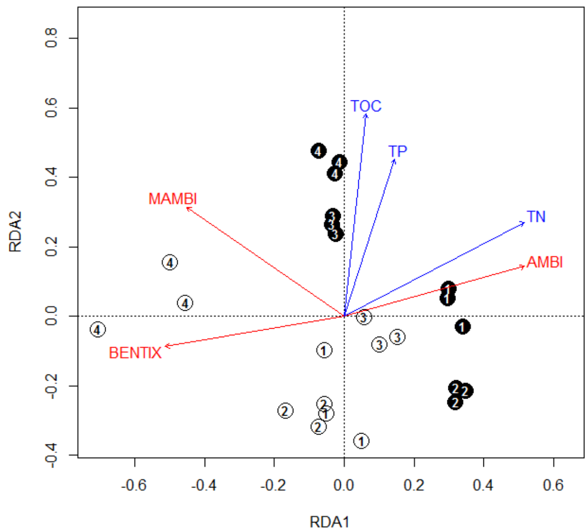

Fig. 4. Redundancy analysis (RDA) triplot of the relationships among biotic indices (red arrows), chemical indicators of contamination (blue arrows) and sampling plots distribution (circles). TOC – total organic carbon; TP – total phosphorus; TN – total nitrogen. Black circles - Contaminated tidal flats (T1 to T4); White circles - Non-contaminated tidal flats (T1 to T4). 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).

The environmental parameters were significantly correlated with the first axis as evidenced by the Monte Carlo test ($p < 0.001$), and the test for all canonical axes was also significant ($p < 0.001$). Chemical parameters which measure the contamination level at the study sites (total nitrogen - TN, phosphorus - TP and organic carbon - TOC) played an important role in the dispersion of the samples along the first axis. The samples from Non-contaminated and from contaminated sites were oppositely grouped along axis 1. However, T3 plots of the Non-contaminated site are closer to Contaminated plots. The mean values of the indices increased (AMBI) or decreased (M-AMBI and BENTIX) as expected from contaminated to Non-contaminated sites. AMBI was the only index positively correlated to all chemical variables, and the best correlation with the contamination proxies was for AMBI and total nitrogen (TN) in contaminated sites. M-AMBI was found among Non-contaminated and contaminated samples, and was negatively correlated to TN. However, among the Non-contaminated sites T4 was the tidal flat with lower TN. BENTIX was inversely correlated to all chemical parameters and related to Non-contaminated sites.

The percentage of similarity or agreement among the three indices was low (Fig. 5). A same ecological status was assigned to only 12.5% of all studied plots. The highest agreement was between AMBI and BENTIX (48.6%), followed by AMBI and M-AMBI (29.2%) and M-AMBI and BENTIX (22.2%).

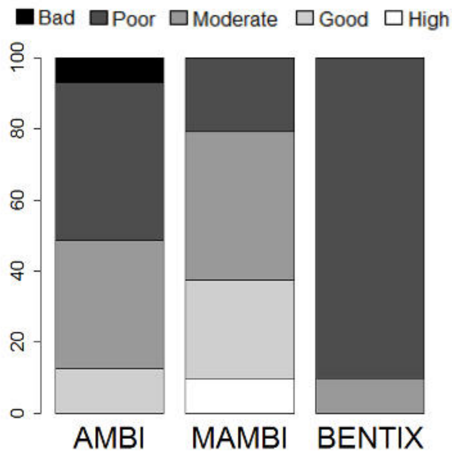

Fig. 5. Ecological status (%) derived from each index. Percentages were calculated for all tidal flats.

Discussion

A weak congruence was detected among the biotic indices, since they showed low correlations with the chemical proxies of contamination, their responses were of low similarity and significant spatial variability was not found at the *contamination* scale (10^3m). The only exception was AMBI, which was congruent with the contamination proxies and varied significantly at the largest spatial scale, or the pollution scale.

The responses of biotic indices to disturbance need to be minimally congruent with chemical signals of anthropogenic stress, represented by biogeochemical markers of contamination. Total organic carbon, nitrogen and phoshorus may be considered as proxies of sewage input, although not necessarily unequivocal indicators. Souza et al. (2013) showed significantly higher values of these proxies on the contaminated sites rather than Non-contaminated, which also sustain high background values of nutrients. Increased nutrient contents are a consequence of the massive sewage input to the contaminated sites near Paranaguá city. The local distribution of fecal steroids, much more conservative parameters than biological or other physico-chemical variables, support these assumptions (Martins et al., 2010).

However, our results and previous studies suggest a clear mismatch between the indices and the sewage impact (Souza et al., 2013), with the exception of AMBI. BENTIX overestimated the ecological status mainly in tidal flats classified by AMBI and M-AMBI as *high, good* and *moderate*. This index has been previously reported as less sensitive, overlapping two different intermediate responses into one quality status, since it classifies all species in only three ecological groups (Muniz et al., 2012; Dauvin et al., 2007). Conversely, the M-AMBI index produced an overestimation of the environmental status of the sites. The incorporation of diversity measures such as the Shannon diversity index and species richness, which are dependent on habitat type, sample size, seasonal variations and natural dominance of characteristic species, can lead to misinterpretations of M-AMBI (Simboura and Argyrou, 2010). The Pearson and Rosenberg (1978) paradigm predicts that benthic species richness or diversity should decrease with an increase in organic enrichment, above a certain threshold level. However, the highest records of diversity and richness at the contaminated sites of Cotinga channel are an unexpected pattern related to its moderate level of pollution (Souza et al., 2013). In these sites, the sewage load is constantly washed out by the tides but still provides enough organic matter to sustain a highly diverse community composed by tolerant and indifferent species, rather than leading to anoxia and habitat loss.

The three biotic indices varied at the hundreds of meters scale, or the tidal flat scale. Our findings are consistent with previous attempts to investigate the variability of indices using hierarchical sampling approaches, with evident patterns of variation at smaller spatial scales, from tens to hundreds of meters (Muniz et al., 2012; Tataranni and Lardicci, 2010). Patterns of distribution indicate how the ecological groups or key species defining the structure of the indices have responded to human pressure, directly influencing the performance of each index (Simboura and Argyrou, 2010). In intertidal systems species can be naturally more tolerant to a variety of stresses and the sewage effects could be minimalized during low tide levels, confounding indices assessments of the status of the benthic assemblages (Cowie et al., 2000, Dauer, 1984). Tidal flats may be exposed to low dissolved oxygen only at high tides, whether in low tides there is possible re-aeration of interstitial water from atmospheric diffusion. Nevertheless, the effects of contaminants can accumulate on the pore water and sediment, still selecting different patterns of occurrence and abundance of species according to the level of organic contamination. Efficient indices should respond to the contamination gradient, which is clearly reflected at our largest spatial scale (10^3 m). The only index to vary at the pollution scale was AMBI, which seems to be better suited for environmental quality assessment in the study area.

The responses of biotic indices at the scale of contamination may also be masked by natural organic inputs from mangroves near the Non-contaminated sites. Organic markers (low cholesterol/b-sitosterol ratios) have shown a greater contribution from organic matter of terrigenous origin in these sites (Barboza et al., 2013). This natural organic matter may represent an additional source of nutrients, somewhat simulating the sewage discharges at the Contaminated site. *Heleobia australis* dominated the Non-contaminated sites (see Table 3), though being classified as a second order opportunist by the indices, a category favoured in slight to pronounced pollution situations (Borja et al., 2000). The unexpected high abundance of *H. australis* is probably related to the high inputs of natural organic matter in the Non-contaminated tidal flats. T3 of the Non-contaminated site was also grouped closer to contaminated tidal flats according to the RDA results, as it shows high organic carbon content, however, probably derived from natural sources.

The sensitivity of marine species to certain stressors may change in different ecoregions, as their assignment into ecological groups (Borja et al., 2011). The shift in the numerically dominant *H. australis* sensitivity might influence the accuracy of the indices' responses. More effective indices would reflect the differences between Contaminated and Non-Contaminated sites, consequently leading to significant variations at the spatial scale of contamination. The inconsistent assignment of several species into appropriate ecological groups due to the lack of information on their ecological sensitivity additionally contributed to the weak congruence. These indices accurately assessed the ecological status of other geographical regions, as has been documented in previous reports (Borja et al., 2008; Simboura and Reizopoulou, 2008). However, their application in the southern Atlantic coast remain to be carefully investigated and validated. AMBI has been applied in coastal areas of NE and S Brazil, as in the heavily polluted Todos os Santos (Bahia) and Guanabara (Rio de Janeiro) bays, near oil and sewage discharges (Omena et al., 2012; Muniz et al., 2005). The sites from Paranaguá bay, also subjected to urban effluents (Souza et al., 2013; Martins et al., 2010), clearly display a better ecological status.

The unexpected significance of the tidal flats spatial scale had similar effects on the low similarity among the responses of all biotic indices (AMBI, BENTIX and M-AMBI). Equivalent responses should vary at the contamination spatial scale, meaning that the macrofauna assemblages are structured by sewage effluents rather than other natural processes. The discrepancies among responses could also denote a low congruence in the numerical boundaries of disturbance categories of each index (Muniz et al., 2012). The verbal classes (e.g. *bad* or *poor*) are determined by numerical threshold values, and a low correspondence possibly indicates that adjustments on the threshold values could improve the level of agreement or discrepancies in indices responses. The highest agreement between AMBI and BENTIX was expected, since they are based on similar concepts (species level of sensitivity to organic enrichment). The opposite relationship was observed between BENTIX and M-AMBI, which showed the lowest agreement as a consequence of the overestimation of results by M-AMBI and underestimation by BENTIX.

Our results highlight some degree of ambiguity in less congruent indices. BENTIX and M-AMBI produced over- and underestimations of the ecological status of the studied sites. Only AMBI varied at the “pollution” scale (10^3 m) and was congruent with physical-chemical proxies of contamination. We found a low degree of similarity among AMBI, M-AMBI and BENTIX, which may be an expression of the spatial variation of macrofaunal assemblages on the performance of indices. We emphasize the importance of establishing unequivocal spatial configurations of macrobenthic assemblages directly driven by sewage contamination. Incongruences in biotic indices assessments of benthic condition mean that indices reflect different attributes of the environment, not the contamination itself. The fauna of our Non-contaminated sites was influenced by the natural massive input of nutrients from the marginal vegetation. Therefore, the application of indices in such context may be meaningless, as their ambiguous responses indicate the effects of natural inputs instead of environmental quality associated to sewage. Regardless of the employed index, generalities on spatial variation should incorporate nested sampling designs. Temporal scales might also represent an important source of variability, and need to be included for a robust assessment of scales and processes. Information about variability can be used to develop models to predict the environmental health of the entire bay, applied in effective monitoring programs (Underwood and Chapman, 2013; Norén and Lindegarth, 2005).

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Manuscript 2

Assessing the suitability of five benthic indices for environmental health assessment in a South American estuary

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Abstract

Despite the increased and widespread usage of benthic indices for environmental health assessment some methodological ambiguities still remain to be elucidated. We tested the suitability of the indices ITI, BO2A, BENTIX, AMBI and M-AMBI using a multivariate approach to address congruence and consistency patterns in a southern Brazilian estuary (48º25'W, 25º30'S). Indices were applied to non-vegetated tidal flats subjected to increasing levels of sewage contamination in order to: (i) test for correlations with chemical indicators of contamination; and (ii) evaluate the overall agreement/similarity of their responses. Analyses were performed along two consecutive years, to assess the consistency of trends over time. Only ITI, AMBI and BO2A were adequate to assess the health condition of estuarine areas in terms of congruence among responses and consistency with chemical tracers of contamination. The worst levels of agreement and correlations to the pollution variables were displayed by BENTIX and M-AMBI. We thereafter discourage the application of BENTIX and M-AMBI before the readjustment of the boundaries for such habitats. Nevertheless, all indices seemed robust to assess interannual variation, although further detailed investigation of several scales of variability is still needed. Fecal sterols and nutrient contents supported the assessment and comparisons of environmental condition and are highly recomendable to future validation of benthic indices in other areas or habitats. Benthic indices can successfully assess benthic quality conditions as robust management tools but their suitable application may still benefit from further research on tolerance shifts of key indicator species.

Keywords: *macrobenthic fauna; organic enrichment; indices comparison, redundancy analysis; Paranaguá Bay.*

Introduction

Ecological indicators for marine health assessment such as benthic indices rely on the relationships between communities and pollution-induced changes (Pinto et al., 2009). Many indices based on macrobenthic faunal responses are currently employed as real-world tools for health assessment in coastal waters. Distinct indices should function and respond similarly, but the estimation of uncertainty of assessments still remain a challenge (Hering et al., 2010). Inconsistencies in indices' responses may be caused by the ambiguous indicative value of chosen species, erroneous index group assignment or simply non-adjusted boundaries of different indices (Gillet et al., 2015, Simboura and Reizopoulou, 2008). The adjustment of boundaries or intercalibration of indices is reached when the numerical interval of each ecological status (e.g. *good* or *poor*) of different indices is adjusted in order to achieve a maximum level of agreement.

Regardless of the nature of inconsistencies, the suitability of indices must be investigated prior to their application. Testing of indices is an exercise aiming not only to select the more appropriate for distinct habitats but also to assure that results are comparable to two or more indices (Simboura and Reizopoulou, 2008). Intercalibration of indices commonly rely on reference conditions, habitats that soundly correspond to good ecological status determined by complex and subjective criteria (Pinto et al., 2009). Nevertheless, as indices are expected to respond to non-biological measures of contamination (Benyi et al, 2009, Ranasinghe et al., 2002), the direct correlation of indices responses to chemical markers may be a simpler and more satisfactory approach to suitability assessment. Few studies have evaluated the relative performance of different indices (Brauko et al., 2015) and although less subjective, indices are not always subjected to clear correlations with reliable abiotic markers of pollution. Indices should be constantly tested for boundaries adjustments, metrics changes and algorithm enhancements towards simplification, stability and robustness (Sigovini et al., 2013, Borja et al., 2008, Muxica et al., 2007). As yet, no multi-integrative attempts using highly stable chemical markers of pollution have been conducted to assess the meaningful application of indices in South American coastal habitats.

Ideally, indices should also integrate linkages across different temporal scales, translated into robustness to natural temporal changes (Simboura et al., 2014, Rombouts et al., 2013, Tattarani and Lardicci, 2010). The capacity to distinguish human-induced and natural disturbance is an intrinsic feature of any index for quality assessment. In this sense, temporal variability could imply that the index is more responsive to natural variation instead of the variation attributed to pollution when the contamination source does not vary (Culhane et al., 2014). The choice of suited indices must involve comparison techniques as objective and integrative as possible (Dauvin et al., 2010) so that temporal variations are as well outlined.

In this study, we tested the suitability of five biotic indices to assess estuarine environmental health, all based on the varying sensitivity of species groups to organic pollution, using a multivariate approach to address congruence and consistency patterns in a southern Brazilian estuary. We applied the indices ITI (Infaunal trophic index), BO2A (Benthic Opportunistic Annelida Amphipods Index), BENTIX, AMBI (AZTI marine biotic index) and its multivariate extension M-AMBI in non-vegetated tidal flats subjected to distinct levels of sewage contamination in order to: (i) test for correlations with chemical indicators of contamination; and (ii) evaluate the overall agreement/similarity of their responses. All analyses were performed along two consecutive years, to assess the consistency of trends over time. Hence, suitable indices are expected to be highly correlated with chemical indicators of contamination and present congruent responses to increasing pollution levels over time and space.

Materials and methods

Study area

The Paranaguá Estuarine Complex (PEC) is one of the largest (612 km^2) and most preserved coastal areas in the southern coast of Brazil, despite of port and tourist activities (Fig. 1). The surveys were conducted in the Cotinga sub-estuary, of nearly 20 km long, close to the mouth of the estuary in its polyhaline sector. About 34% of the surface area of the sub-estuary, strongly influenced by tidal currents and freshwater discharges, is covered by mangroves and marshes or remain unvegetated (Noernberg et al., 2006). The inner sector of the sub-estuary receives most of the anthropogenic input of sedimentary organic matter or sewage-derived material from Paranaguá city (Souza et al., 2013; Lana et al., 2000). A compressed gradient of sewage contamination from the inner sector to the outer part of the sub-estuary was evidenced by *Escherichia coli* sediment concentrations and concentrations of fecal steroids, highly stable organic markers (Barboza et al., 2013; Martins et al., 2010). The strongest signals of sewage contamination indicated by coprostanol levels may vary from high to moderate, and are confined to sites close to Paranaguá city (Abreu-Mota et al., 2014; Martins et al., 2010).

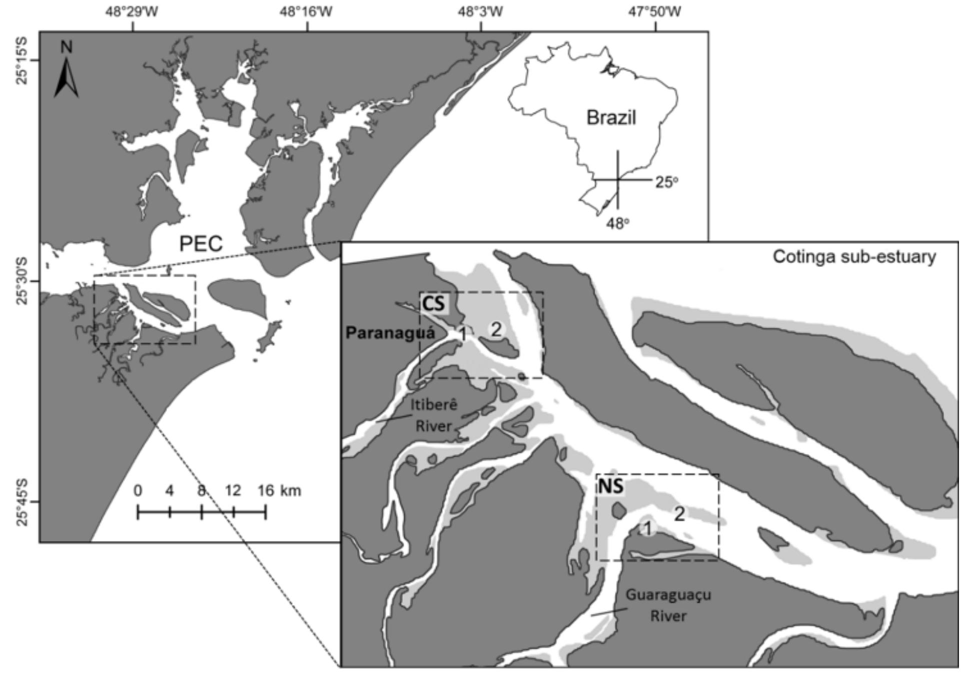

Fig. 1. Study sites in Paranaguá Estuarine Complex (PEC), Brazil. Indices were applied in 4 tidal flats within Contaminated sites (CS) and Non-contaminated sites (NS) of Cotinga sub-estuary in 2011 and 2012.

Sampling and laboratory procedures

The data used in this study correspond to twelve sampling surveys undertaken in 2011 and 2012. In all surveys four plots with three replicates each were sampled for macrofauna in each of four tidal flats (2 in the Non-contaminated and 2 in the Contaminated site), covering 96 plots/year. Benthic samples were collected during spring low tides using plastic core tubes (10 cm diameter, 10 cm deep), and all plots were placed parallel to the water line, at similar tidal levels. The corer size was adopted according to the results of pilot studies and previous studies carried out in tidal flats of Paranguá bay, in which richness and diversity did not increase with larger corers. Samples were sieved through a 0.5 mm mesh, fixed in 6% formaldehyde and preserved in 70% alcohol. In the laboratory, the organisms were counted and identified to the lowest possible taxonomic level.

Additional samples were taken at each plot to determine total phosphorus (TP), total nitrogen (TN) and total organic carbon (TOC) contents, as well as sediment parameters (mud content, grain size average, sorting, $CaCO_3$ and organic matter). The concentrations of TN and TP were obtained according to Grasshoff et al. (1983), and TOC was determined with the oxidation method described by Strickland and Parsons (1972). Sediment samples were processed according to Suguio (1973), and granulometric parameters were determined on the R software (R Development Core Team, 2013) using the package rysgran (Gilbert et al. 2012). Calcium carbonate ($CaCO_3$) and organic matter contents were determined using acid digestion and furnace combustion at 550°C for 1 hour, respectively.

In every survey, one sample from each tidal flat was also taken for fecal sterol analysis, with the method described by Kawakami and Montone (2002). Instrument specifications and calibration procedures are described by Montone et. al. (2010). The detection limits (DLs) were <0.01 µgg-1 for all analyzed compounds. Measured concentrations of target steroids in the IAEA-417 reference material were within 90–110% of the certified values provided by the International Atomic Energy Agency (IAEA).

Biotic indices and data analysis

ITI rely on Fauchald and Jumars (1979) and Word (1980), under the premises that feeding behaviour responds to organic material enrichment by shifting the dominance of suspended material feeders toward deposit feeders (Maurer et al., 1999). The four main trophic groups (TG) were: (TG1) suspension feeders, (TG2) detritus feeders (e.g., omnivorous and necrophagous), (TG3) surface deposit feeders and species that are both suspension and surface deposit feeders, and (TG4) subsurface deposit feeders that feed on sedimentary detritus and bacteria. BO2A (Dauvin and Ruellet, 2009) was also based on the ecological characteristics of specific taxonomic groups, and compares percentage ratios of opportunistic annelida (Polychaeta and Clitellata) to percentages of amphipods (with exception to the opportunistic genus *Jassa*).

AMBI and M-AMBI values were calculated using the software 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 to organic pollution, already listed in the software (Borja et al., 2003, 2000). However, some species or taxa present at Paranaguá bay are not as 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 (Ferrando and Méndez, 2011; Boehs et al., 2008; Gamito, 2008; Nalesso et al., 2005; Palacios et al., 2005; Barnett, 1983) 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, *Sigambra* sp. in GIII, Tubificinae sp1. and Tubificinae sp2. were in GV, while the polychaetes *Dorvillea* sp., *Ophelina* sp. and the bivalve *Macoma constricta* remained unassigned.

M-AMBI was calculated by factorial analysis of AMBI, richness and Shannon–Wiener diversity values (for details, see Muxika et al., 2007; Bald et al., 2005; Borja et al., 2004). 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. We defined *a priori* reference conditions by adapting the default values that determine the 'high' and the 'bad' ecological status. We used a different data set previously obtained in sampling surveys across several tidal flats along the Cotinga channel (Unpublished data). Afterwards, the index was derived in relation to these values. We used as the highest AMBI value ('Bad' reference conditions) the number derived from the most polluted site of the data set. Conversely, "High" reference conditions were calculated from the pristine site.

BENTIX is based on the same premises as AMBI, but the taxa are categorized in three ecological groups (Simboura and Zenetos, 2002). We adapted the classification of AMBI as following (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.

Indices values were calculated for each replicate and their ecological status was categorized as *High, Good, Moderate, Poor* and *Bad*. A weighted Kappa analysis (Cohen, 1960; Fleiss and Cohen, 1973) was then undertaken to assess the agreement among indices. The weights decrease importance of misclassification between close categories and increase importance between distant categories. The level of agreement is expressed by the following Kappa values: (i) Null < 0.05; (ii) Very low: 0.05–0.2; (iii) Low: 0.2–0.4; (iv) Moderate: 0.4–0.55; (v) Good: 0.55–0.7; (vi) Very Good: 0.7–0.85; (vii) Almost perfect: 0.85–0.99; and (viii) Perfect: 1 (Monserud and Leemans, 1992). The percentage of correspondence was also calculated for equivalent ecological status given by each combination of indices. Since ITI originally discriminate only four ecological status while the others discriminate five, we arbitrary defined the *bad* category by dividing the original *poor* threshold level (from 30 to 0) by two (new *poor* boundaries from 30 to 15 and *bad* from 15 to 0). Using these five ecological status, the proportion of sites where the indices agreed were quantified according to severity of disagreement, with more weight given to categories further apart (e.g., between high and moderate, or high and bad). Weighted Kappa was performed using IRR package (Gamer et al., 2013) in R software (R Development Core Team, 2013).

Partial Redundancy Analysis (pRDA), a form of variance decomposition, was used to evaluate the annual variation of the indices in relation to (i) chemical indicators of pollution and organic enrichment (coprostanol and cholesterol concentrations, coprostanol / coprostanol + cholestanol and coprostanol / cholesterol ratios, total organic carbon, total nitrogen and total phosphorus contents), and (ii) sediment characteristics (mud content, grain size average, sorting, $CaCO_3$ and organic matter). We partitioned the total variation of the data into (1) the unique or pure variation explained by chemical indicators of pollution after removing the (co)variation associated with the remaining sediment variables, (2) the unique variation explained by the remaining sediment variables after removing the (co)variation with chemical indicators of pollution, (3) the common or shared variation between pollution and granulometric variables, and (4) random error. The pRDA was carried out following Borcard et al (2011) and the indices were standardized prior to the analysis. First, redundancy analysis with no covariables was used to estimate the total amount of variance explained (as sum of canonical eigenvalues). In the next steps, the statistical significance of the model was evaluated using Monte Carlo permutation tests under 999 permutations and the variation inflation factor was used as the criteria to reduce covariation among abiotic variables (highly redundant variables indicated by VIF>10 were excluded). Separate pRDA were performed for both scalings 1 (focuses in the ordination of objects or sampling plots) and 2 (focuses in the ordination of response variables or indices vectors), using the vegan package (Oksanen et al. 2008) in R software (R Development Core Team 2013).

Results

The general patterns of environmental quality shown by mean values of the six pooled surveys indicate distinct ecological status according to the different regions along Cotinga sub-estuary, mostly of *moderate* to *poor* for all indices, except for BENTIX (Fig.2). The BENTIX index assigned a worse and lower range of quality classifications, as almost all studied plots were classified as *poor*. M-AMBI on the other hand detected a higher quality status with the highest proportions of *good* assignments. The overall environmental quality was worse in year two, as indicated by the five indices (Fig. 2). The proportion of ecological status *bad* and *poor* consistently increased in relation to year one, whereas the *high* and *good* status proportionately decreased.

According to the general trends shown by chemical proxies, the tidal flats from the inner sector close to Paranaguá city (CS1 and CS2) were more organically enriched by phosphorus, nitrogen and organic carbon than the tidal flats from the non-contaminated site (NS1 and NS2) (Table 2). The fecal sterol concentrations evidenced a general sewage derived contaminant gradient toward the outer sector of the sub-estuary. There were no evident or consistent temporal patterns of variation in the concentrations of chemical proxies.

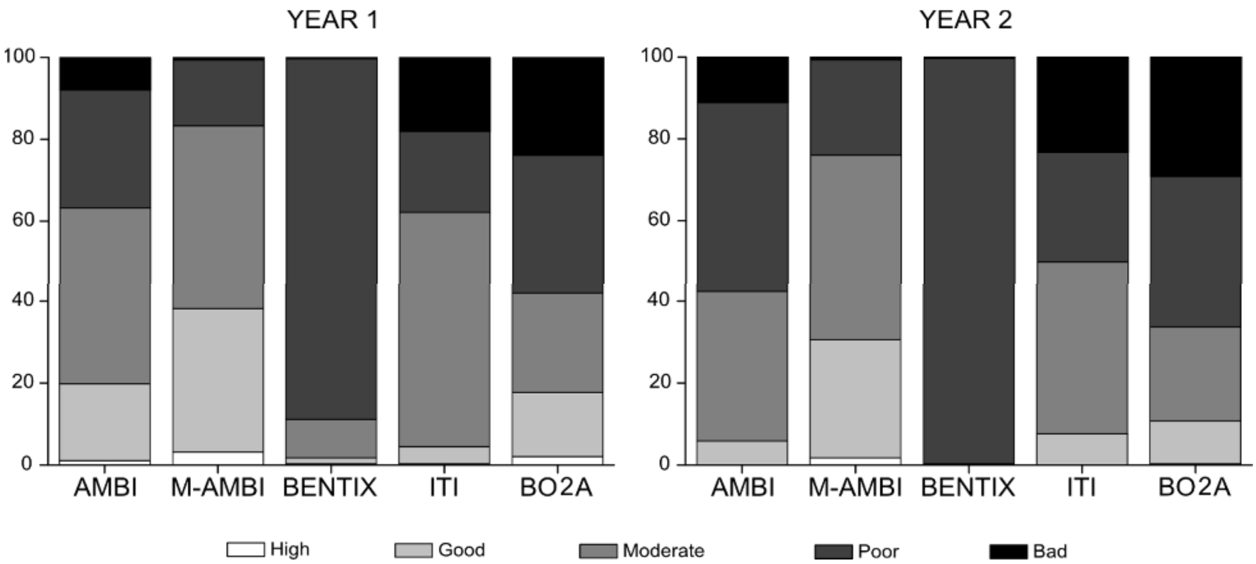

Fig.2. Percentage of each ecological status for AMBI, M-AMBI, BENTIX, ITI and BO2A in all plots of Cotinga sub-estuary, years 1 and 2.

Table 2. Fecal sterols, total organic carbon (C), total phosphorus (P) and total nitrogen (N) concentrations (mean ± SD) in the sediments of the four tidal flats (CS – contaminated site; NS – Non-contaminated site) of Cotinga sub-estuary.

		Coprostanol ($\mu g\ g^{-1}$)	SD	Cop/Cop+Chola (µg g-1)	SD	Cop/Cop+Choles (µg g-1)	SD	Cholesterol (µg g-1)	SD
Year 1	**CS1**	5.89	5.57	0.76	0.06	1.31	1.00	5.60	7.03
	CS2	0.96	0.62	0.56	0.08	0.25	0.09	3.83	2.08
	NS1	0.01	0.02	0.02	0.03	0.00	0.00	3.19	3.52
	NS2	0.21	0.22	0.16	0.10	0.06	0.06	2.89	1.01
Year 2	**CS1**	2.38	1.97	0.77	0.07	3.64	1.47	1.81	1.21
	CS2	1.04	0.80	0.59	0.21	1.96	1.35	1.33	0.52
	NS1	0.01	0.02	0.01	0.03	0.01	0.03	1.40	2.19
	NS2	0.01	0.03	0.06	0.13	0.08	0.20	1.09	1.73

		C (% µg g-1)	SD	P (% µg g-1)	SD	N (% µg g-1)	SD
Year 1	**CS1**	0.011	0.002	0.000015	0.000004	0.0027	0.0014
	CS2	0.009	0.003	0.000013	0.000006	0.0023	0.0015
	NS1	0.006	0.002	0.000006	0.000001	0.0011	0.0006
	NS2	0.011	0.003	0.000012	0.000004	0.0023	0.0016
Year 2	**CS1**	0.023	0.005	0.000021	0.000019	0.0015	0.0010
	CS2	0.025	0.002	0.000017	0.000008	0.0009	0.0006
	NS1	0.026	0.004	0.000009	0.000010	0.0005	0.0005
	NS2	0.026	0.003	0.000008	0.000006	0.0004	0.0004

The power of detection of indices was greatly different, but those differences were consistent over the years of study. According to the weighted Kappa analysis, a *very good* level of agreement was found among the responses of AMBI, ITI and BO2A, (Table 3). The percentage of match among these indices varied from 52.1% (AMBI and BO2A, year 1) to

72.9% (AMBI and ITI, year 2). The agreement between AMBI and ITI remained *very good* in both years of study, whereas it changed from *good* to *very good* between AMBI and BO2A, and BO2A and ITI. However, agreements of all indices with M-AMBI and BENTIX were much less satisfactory, of only *null* to *moderate* levels. The weakest Kappa values and percentages of match involved correlations of all indices with BENTIX.

Table 2. Kappa values, levels of agreement and percentage of match for the ecological status between all combinations of indices used in this study.

	Year 1			**Year 2**			**Total**		
	Kappa	**Level of agreement**	**% Match**	**Kappa**	**Level of agreement**	**% Match**	**Kappa**	**Level of agreement**	**% Match**
AMBI/MAMBI	0,48	Moderate	29,9	0,47	Moderate	36,5	0,49	Moderate	33,2
AMBI/BENTIX	0,25	Low	32,6	0,00	Null	46,5	0,18	Very low	39,6
AMBI/ITI	0,78	Very good	68,4	0,81	Very good	72,9	0,80	Very good	70,7
AMBI/BOPA	0,70	Good	52,1	0,75	Very good	61,5	0,73	Very good	56,8
MAMBI/BENTIX	0,06	Very low	20,1	0,00	Null	23,3	0,04	Very low	21,7
MAMBI/ITI	0,31	Low	22,9	0,47	Moderate	29,5	0,40	Low	26,2
MAMBI/BOPA	0,34	Low	21,2	0,35	Low	25,0	0,35	Low	23,1
BENTIX/ITI	0,15	Very low	26,0	0,00	Null	27,1	0,09	Very low	26,6
BENTIX/BOPA	0,24	Low	38,2	0,00	Null	37,5	0,15	Very low	37,8
ITI/BOPA	0,68	Good	54,5	0,82	Very good	68,4	0,75	Very good	61,5

In the partial redundancy analysis, 45.6% of the variability of indices was explained by all environmental variables (F-ratio = 6.1327; p-value < 0.001; Monte Carlo permutation test). The cumulative percentage of variance explained by the first two canonical axes accounted for 42.3% (39.1% and 3.2% respectively for the first and the second axis). The explained variance was partialled out within two groups of variables, of which 61% was exclusively explained by the chemical indicators of contamination. Sediment characteristics explained only 2% of the total variation of indices, and no variation was jointly explained by the two sets of variables. Among all chemical variables, cholesterol, and total carbon played a less important role in the dispersion of samples along axis 1 (fig.3). Samples from non-contaminated and contaminated sites were oppositely grouped along axis 1 (fig.3a). The group composed of samples of worse ecological classifications (higher AMBI and BO2A), collected in internal tidal flats near Paranaguá city, was related to higher concentrations of fecal sterols (coprostanol, coprostanol/coprostanol-cholestanol ratio and coprostanol/cholesterol ratio), total nitrogen and phosphorus. These variables were inversely related to ITI, which reached higher values in samples of better ecological status, located on the external section of the sub-estuary. Relationships involving M-AMBI and BENTIX were weak (Fig.3.a), more related to the second axis and total organic carbon and cholesterol, respectively. No temporal trends were evidenced by the dispersion of samples.

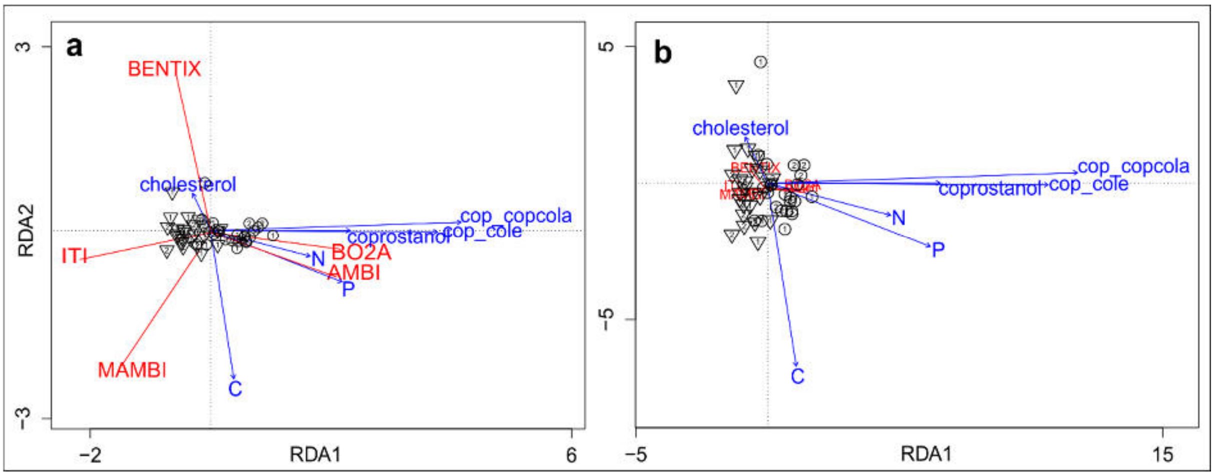

Fig. 3. pRDA triplots of scalings 1 (a) and 2 (b). Red arrows – indices. Blue arrows - Coprostanol, Cholesterol, Coprostanol/Cholesterol ratio (cop_cole), Coprostanol/Coprostanol+Cholestanol ratio (cop_copcola), total carbon (C), total phosphorus (P) and total nitrogen (N) contents. Triangles – samples of non-contaminated sites; circles - samples of contaminated sites of years 1 and 2. Arrows indicate the direction of increase in the studied variables. The angles between variables reflect their correlations (angles near 90° indicate no correlation, near 0° indicate high positive correlation and near 180° indicate high negative correlation). Only the variables that significantly explained the model are shown.

Discussion

Our results clearly evidenced significant differences in the quality status assignment among the tested indices. The level of agreement in determining benthic integrity or ecological status varied depending on the index, and not all indices were responsive to the distinct levels of sewage contamination of the tidal flats. However, BO2A, ITI and AMBI indeed reached higher levels of agreement and good correlations to chemical proxies of contamination.

The high agreement among ITI, AMBI and BO2A was certainly related to the species assignment into corresponding ecological or trophic groups of similar sensitivity to pollution: opportunistic annelids of BO2A belong to ecological groups IV and V of AMBI, and to trophic group 4 of ITI, all highly tolerant to pollution (see Annex for details). Conversely, most amphipods of BO2A belong to ecological group I of AMBI and to trophic groups 1 and 2 of ITI, all sensitive to contamination. The high level of agreement between AMBI and BO2A is consistent with northeastern Atlantic and Mediterranean comparisons (De-la-Ossa-Carretero and Dauvin, 2010). In South American estuarine areas the degree of similarity between AMBI and ITI was only of 53%, while AMBI and BENTIX displayed the highest level of agreement (Muniz et al., 2012). The high mismatch proportion of responses is commonly attributed to incompatible boundaries or threshold settings of the ecological status of indices (Borja et al., 2008). For Borja et al. (2007), the readjustment of boundaries of different methodologies indeed increased their level of agreement. The ecological status given by an index is a verbal class that correspond to a certain numerical interval in which the index value is inserted (e.g. AMBI values between 1.2 and 3.3 correspond to *good* status). Since our Kappa results were obtained from the correspondence among verbal classes of indices the adjustment of boundaries could, therefore, improve their level of agreement.

Inferences on the suitability of indices also require some degree of congruence with non-biological measures of impact such as chemical proxies of contamination or nutrient enrichment (Benyi et al. 2009). Furthermore, agreement on clear reference conditions is a requirement for critical comparison of indices performances and intercalibration methodologies (Hering et al, 2010). The results of our redundancy analysis were based on the numerical values of indices and doubtless showed a configuration of ecological status given by indices modeled by the contamination proxies, and not by environmental variables. Fecal sterols such as coprostanol and cholestanol have been often used as stable and source specific molecular tracers for sewage discharges along coastal areas (Abreu-Mota et al., 2014, Martins et al., 2012, Readman et al., 2005), and reliably pointed out the contamination degree of the studied sites. BO2A, ITI and AMBI had highly congruent quality assessment's and were satisfactory responsive to organic enrichment gradients, especially to some of the fecal sterols. These indices seem more likely to successfully assess the environmental health of estuaries in South America.

Nevertheless, BENTIX and M-AMBI had the weakest correlations to both the environmental gradient and the contamination proxies. They also showed remarkably incongruent status assessments. These outcomes are not only related to adjusting boundaries of verbal classes but also to the numerical outcome of BENTIX and M-AMBI. They may be related to the inadequacy of species assignments into the ecological groups list of each index, originally developed for European waters (Gillett et al., 2015). The accurate assignment of species may be compromised by species tolerance shifts in response to differences in latitude (and temperature), salinity, or even from sub- to intertidal habitats (Gillet et al., 2015; Simboura and Reizopoulou, 2008; Fitch and Crowe, 2010).

BENTIX showed a poor discriminating power and donwgraded the overall environmental health. This low level of sensibiliy has been attributed to the assignment of species into only three broad ecological groups (Muniz et al., 2012; Dauvin et al., 2007). In addition, our contaminated sites are in fact in a moderate state of eutrophication (Souza et al., 2013), condition in which BENTIX is less effective (Simboura and Reizopoulou, 2008). Similarly, M-AMBI overestimated the assignments, as a possible reflex of the incorporation of Shannon diversity index and species richness as metrics (Simboura and Argyrou, 2010). Diversity and species richness may be rather high in intermediate states of pollution, possibly suppressing and not highlighting the dominance of opportunistic species (Simboura and Reizopoulou, 2008). Another problem might be related to the use of factorial analysis as a mean to integrate the algorithm metrics, which was recently pointed out as not functional to M-AMBI (Sigovini et al., 2013).

Diagnostic discordances can determine important limits between acceptable and non-acceptable conditions of environmental health. Slight biological shifts could be detrimental if they determine the boundary between *moderate* and *good* quality status, a critical boundary for environmental managers and policymakers (Munari and Mistri, 2007). According to our results, the discordances involving both BENTIX and M-AMBI were surprisingly high. In the most severe cases of disagreement, some of the weighted Kappa values indicated null agreement for BENTIX, despite of 23% to 46% of match. This means that some responses indeed match, but the proportion of misclassification between categories further apart was higher (e.g. between *high* and *moderate*, or *good* and *poor* status). M-AMBI and BENTIX have been successfuly applied in European and North-American waters, during long-term monitoring assessments or even using higher taxonomic level data (Simboura et al., 2014, Forde et al., 2013). However, they might perform differently in South American estuaries, as a result of the local structure of the fauna.

The incongruences in indices responses remained the same from one year to another. Despite the differences and some inconsistencies with chemical tracers of contamination, all indices indicated overall worsening trends in the ecosystem health of year two, more or less severe depending on the index. As the structure of benthic assemblages tend to temporally vary (Underwood and Chapman, 2013), biotic indices could naturally follow these trends of variation. However, our results suggest a very discrete variation, which might imply that the indices did not respond to temporal background variability, a positive feature in terms of their ability to distinguish man-induced from natural disturbances (Salas et al., 2006; Bazairi et al., 2005). Although temporal variation in species presence and abundance is likely to occur, variability of ecological and trophic groups of the indices did not seem to be evident over the years. Although responses seem to be yearly consistent, these indices still need further investigation on large scale temporal variations.

Benthic indices have been applied, validated and compared in South American coastal habitats subjected to multiple stressors like urban effluents and oil spills (Omena et al., 2012; Muniz et al., 2012; Muniz et al., 2011; Muniz et al., 2005; Albano et al., 2013). Nevertheless, no previous study simultaneously tested the suitability of five multimetric benthic indices using a correlative and multivariate approach. In terms of congruence between responses and consistency to chemical proxies of contamination, only ITI, AMBI and BO2A seemed readily suited to the assessment of estuarine health in Southern Brasil. Thereafter, we discourage the application of BENTIX and M-AMBI prior to proper boundaries readjustments for such habitats. The indices seemed robust to eventual natural background variations not related to pollution from one year to another. Nevertheless, further detailed investigation integrating temporal and spatial scales of variability is still needed. Fecal sterols associated to nutrient contents fundamentally supported the assessment and comparisons of environmental condition and are more suitable to future validation processes of benthic indices in new geographical areas. We underline that these indices could successfully assess benthic quality conditions as robust management tools but their application may benefit from additional research on the tolerance shifts of key indicator species.

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Annex – Ecological groups of each taxa and total abundance in the tidal flats along years 1 (2011) and 2 (2012). CS = Contaminated site and NS = Non-contaminated site.

	Ecologic Group				Year 1				Year 2			
	AMBI/M-AMBI	BENTIX	ITI	BO2A	**CS1** 48°29'45"W 25°30'44"S	**CS2** 48°29'35"W 25°30'42"S	**NS1** 48°27'32"W 25°32'35"S	**NS2** 48°27'10"W 25°32'28"S	**CS1** 48°29'45"W 25°30'44"S	**CS2** 48°29'35"W 25°30'42"S	**NS1** 48°27'32"W 25°32'35"S	**NS2** 48°27'10"W 25°32'28"S
Tellina versicolor	I	1	TGIII (DEPSUP)	*	125	98	102	99	99	112	55	84
Lucina pectinata	I	1	TGI (SUS)	*	112	65	54	25	106	52	31	11
Scoloplos ohlini	I	1	TGIII (DEPSUP)	*	12	47	21	35	5	35	32	27
Sipuncula	I	1	TGIII (DEPSUP)	*	4	16	3	7	6	26	43	38
Diopatra aciculata	I	1	TGII (DETRI)	*	0	15	4	7	1	19	0	0
Aricidea catherinae	I	1	TGIII (DEPSUP)	*	0	3	15	4	1	0	4	1
Scoloplos sp	I	1	TGIII (DEPSUP)	*	0	0	1	2	0	0	4	14
Magelona papillicornis	I	1	TGII (DETRI)	*	1	0	3	1	0	1	6	6
Sabella sp	I	1	TGI (SUS)	*	3	3	1	0	0	0	0	0
Streblosoma sp	I	1	TGIII (DEPSUP)	*	1	2	0	4	0	0	0	0
Terebellides anguicomus	I	1	TGIII (DEPSUP)	*	0	1	1	4	0	0	0	0
Armandia hossfeldi	I	1	TGIV (DEPSUB)	*	0	0	3	0	0	0	1	1
Clymenella sp	I	1	TGII (DETRI)	*	0	0	1	1	0	0	0	0
Amphipoda	I	1	TGII (DETRI)	Amphipoda 1	0	0	0	0	1	0	6	5
Glycinde multidens	II	2	TGII (DETRI)	*	119	151	143	109	30	98	64	66
Bulla striata	II	2	TGII (DETRI)	*	14	107	91	61	39	24	62	142
Tagelus divisus	II	2	TGI (SUS)	*	47	107	84	108	19	59	60	37
Scoletoma tetraura	II	2	TGII (DETRI)	*	115	157	32	56	5	18	22	44
Isolda pulchella	II	2	TGIII (DEPSUP)	*	64	41	11	25	13	45	4	12
Sphenia fragilis	II	2	TGI (SUS)	*	3	12	14	39	0	15	20	99
Exogone sp	II	2	TGII (DETRI)	*	0	3	4	9	10	31	58	62
Polydora websteri	II	2	TGIII (DEPSUP)	*	51	20	4	20	1	5	0	74
Monokalliapseudes schubarti	II	2	TGII (DETRI)	*	46	7	7	3	16	1	0	10
Haminoea elegans	II	2	TGII (DETRI)	*	10	5	3	6	0	0	0	0
Phyllodoce sp	II	2	TGII (DETRI)	*	1	0	3	2	1	0	1	5
Paranaitis sp	II	2	TGII (DETRI)	*	7	2	2	1	0	1	0	0
Edwardsia fusca	II	2	TGII (DETRI)	*	1	3	4	1	0	2	1	1
Ceratonereis longicirrata	II	2	TGII (DETRI)	*	1	3	2	6	0	0	0	0
Nephtys fluviatilis	II	2	TGII (DETRI)	*	0	4	5	3	0	0	0	0
Kinbergonuphis difficilis	II	2	TGII (DETRI)	*	0	1	0	3	1	3	0	1
Eunoe serrata	II	2	TGII (DETRI)	*	3	1	1	4	0	0	0	0
Megalomma sp	II	2	TGIII (DEPSUP)	*	2	3	0	1	0	1	0	2
Hemipodia californiensis	II	2	TGII (DETRI)	*	4	0	4	1	0	0	0	0
Pholoe minuta	II	2	TGII (DETRI)	*	3	0	1	3	0	0	0	0
Hermundura tricuspis	II	2	TGII (DETRI)	*	3	0	2	0	1	0	0	0
Syllis sp	II	2	TGII (DETRI)	*	0	0	1	3	0	0	0	1
Owenia fusiformis	II	2	TGIII (DEPSUP)	*	0	0	2	2	2	0	0	0
Aglaophamus juvenalis	II	2	TGII (DETRI)	*	0	0	0	3	0	0	0	0
Ophiuroidea	II	2	TGII (DETRI)	*	0	0	0	2	0	0	0	0
Sthenelais limicola	II	2	TGII (DETRI)	*	0	0	0	1	1	0	0	0
Fimbriosthenelais marianae	II	2	TGII (DETRI)	*	0	0	0	1	0	0	0	0
Goniada maculata	II	2	TGII (DETRI)	*	1	0	0	0	0	0	0	0
Clibanarius vittatus	II	2	TGII (DETRI)	*	0	0	0	0	0	0	1	0
Sigambra sp	III	2	TGII (DETRI)	*	285	416	413	721	767	741	659	581
Anomalocardia flexuosa	III	2	TGI (SUS)	*	71	242	153	52	108	569	1141	983
Streblospio benedicti	III	2	TGIII (DEPSUP)	*	282	292	4	59	73	156	8	4
Mytella sp	III	2	TGI (SUS)	*	47	99	53	63	44	105	24	62
Monocorophium acherusicum	III	2	TGII (DETRI)	*	25	86	27	35	5	30	49	72
Spiophanes duplex	III	2	TGII (DETRI)	*	37	108	62	26	4	15	28	20
Nemertea	III	2	TGII (DETRI)	*	23	56	30	32	26	25	8	8
Alitta succinea	III	2	TGII (DETRI)	*	6	18	10	18	4	8	3	1
Spiochaetopterus costarum	III	2	TGIII (DEPSUP)	*	0	6	4	6	1	3	1	4
Spiophanes sp	III	2	TGII (DETRI)	*	0	1	4	5	0	0	2	0
Notomastus sp	III	2	TGIV (DEPSUB)	*	0	0	3	0	0	0	0	0
Neanthes sp	III	2	TGII (DETRI)	*	0	1	0	1	0	0	0	0
Sternaspis sp	III	2	TGIII (DEPSUP)	*	0	0	0	2	0	0	0	0
Laeonereis culveri	IV	3	TGIII (DEPSUP)	Polychaeta 1	4532	562	120	66	5052	1126	136	250
Heleobia australis	IV	3	TGIII (DEPSUP)	*	506	507	2150	933	646	702	900	1559
Heteromastus sp	IV	3	TGIV (DEPSUB)	Polychaeta 2	300	216	76	90	174	482	81	46
Prionospio heterobranchia	IV	3	TGIII (DEPSUP)	Polychaeta 3	6	33	57	66	3	53	121	134
Polydora sp	IV	3	TGIII (DEPSUP)	Polychaeta 4	19	1	6	7	16	1	13	7
Prionospio sp	IV	3	TGIII (DEPSUP)	Polychaeta 5	7	21	8	9	0	0	1	0
Polydora cornuta	IV	3	TGIII (DEPSUP)	Polychaeta 6	1	4	0	0	3	0	0	1
Tubificinae sp1	V	3	TGIV (DEPSUB)	Polychaeta 7	11412	2561	317	770	15857	3745	838	1343
Paranais cf. *frici*	V	3	TGIV (DEPSUB)	Polychaeta 8	3522	82	3	100	619	699	60	47
Capitella sp	V	3	TGIV (DEPSUB)	Polychaeta 9	930	143	1	13	2340	182	19	26

Manuscript 3

Performance of benthic indices for environmental quality assessment at nested spatio-temporal scales: do time and space really matter?

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Abstract

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. We assessed the relative importance of temporal (within fortnights and seasons along two years), spatial (at scales ranging from 10^1 to 10^3 m) and interactive variability affecting the performance of the biotic indices AMBI, ITI and BO2A in a subtropical estuary, using a hierarchical design. A six-factor model was used to test the indices' robustness in relation to seasonal background variation in tidal flats subjected to distinct degrees of sewage impact. We also assessed the relative contribution of major macrofaunal ecological groups in explaining the observed variability of each index. AMBI, ITI and BO2A were consistently responsive to varying contamination levels. The presumed strong and marked variations related to seasons were not detected by any index, although there was interactive variation between smaller spatial scales with short- and long-term temporal scales. Such variations are probably a consequence of specific pulse and press disturbances. The sewage input is likely to operate either at the largest spatial scale (thousands of meters), and at the long-term temporal scale (interannual). GI (sensitive species) and suspension feeders were possibly responsible for most of the variability of AMBI and ITI, as the opportunistic annelids for BO2A. Unlike biased tests of spatial, temporal and/or interactive scales of variation, our assumptions were consistent as they were both assessed with a robust and complex hierarchical sampling design and were congruent among all tested indices. Benthic indices varied at a variety of interactive scales, which does not necessarily mean ambiguous or meaningless responses.

Keywords: nested PERANOVA; sewage discharges; spatial and temporal scales, AMBI, ITI, BO2A.

Introduction

Multimetric benthic indices based on the responses of biological communities are currently recognized as effective and integrative ways to measure the quality of coastal waters (Hering et al., 2010). Natural and man-induced vectors of variation may combine in estuaries, resulting in a mosaic of heterogeneous habitats in which biological integrity assessments are more complex and frequently ambiguous (Dauvin, 2007; Elliott and Quintino, 2007). In such cases benthic indices may respond either to man-induced or natural disturbances (Muniz et al., 2012, Kröncke and Reiss, 2010). In addition, as indices reflect the faunal responses to the surrounding environment, the spatial configuration of health assessments is likely to change with time in presumably, but frequently underestimated scales of variation.

Methodological ambiguities and spatiotemporal variation are commonly associated to the confounding effect of small scales on larger scale comparisons (Underwood, 1997). In complex systems such as estuaries, a reliable way to guarantee that observed differences are indeed associated with the scale claimed (e.g., from a contaminated to a pristine site) is to demonstrate that differences at smaller scales are not as large (Morrisey et al., 1992). In this sense, determining precision of estimates and maximising power to detect impacts require care in the design, analysis and interpretation of the relevant data. The consequences of variation, the ensuing interactions and non-independent patterns must be taken into account in sampling designs (Underwood and Chapman, 2013).

Recent studies have increasingly employed hierarchical sampling designs to determine the spatial scales at which species and communities vary and to distinguish human from natural impacts (e.g. Morrisey et al., 1992, Noren and Lindegart, 2005, Murphy et al., 2009). In such designs, variation among the factors of interest are properly compared to the magnitudes of variation that occur within and among the spatial and temporal scales of interest.

Observed temporal trends, mainly the seasonal, are particularly likely to be quite spurious, as in most designs seasonal (or other temporal) patterns are not contrasted with temporal variation within each season, but against spatial variation (Underwood and Chapman, 2013). Hierarchical analysis is a powerful and unique framework for quantifying the proportion of the variation among samples that is unequivocally attributable to each spatial, temporal or interactive scale. Nevertheless, such approaches to evaluate the relationships between spatiotemporal patterns and processes on the performance of benthic indices are yet to be properly addressed using several interactive scales of interest (Muniz et al., 2012, Tattaranni and Lardicci, 2010).

In this study, we assessed the relative importance of temporal (within fortnights and seasons along two years), spatial (at scales ranging from 10^1 to 10^3 m) and interactive variability affecting the performance of the biotic indices AMBI, ITI and BO2A in a subtropical estuary, using a complex hierarchical linear model. The choice of indices was based on a previous assessment conducted in the area, in which AMBI, ITI and BO2A proved suitable in terms of high congruence of responses and consistency with reliable chemical markers of sewage contamination (Brauko et al., *in prep.*). The hierarchical multi-scale approach was used to test the indices robustness against the natural disturbance represented by the change of seasons in tidal flats subjected to distinct degrees of sewage impact. We expected indices to vary significantly at the spatial scale of contamination (10^3 m), interactive or not, despite the seasonal background variation. We also identified the role of macrofaunal ecological groups in explaining the observed variability of each index. The temporal variation was explicitly incorporated so that the presumed influence of temporal variability in spatial patterns of indices become accessible.

Materials and methods

The four tidal flats sampled in this study are located along the Cotinga sub-estuary within the Paranaguá Estuarine Complex (PEC), Southern Brazil, as described in Brauko et al. (2014). The sub-estuary is the dilution path for sewage discharges from Paranaguá city. Two of the sampled tidal flats were located in the inner *contaminated site* (CS) and the remaining two tidal flats were sampled in a *non-contaminated site* (NS), closer to the mouth of the estuary (Fig. 1). This contamination gradient was previously detected by Abreu-Mota et al. (2014) and Barboza et al. (2013).

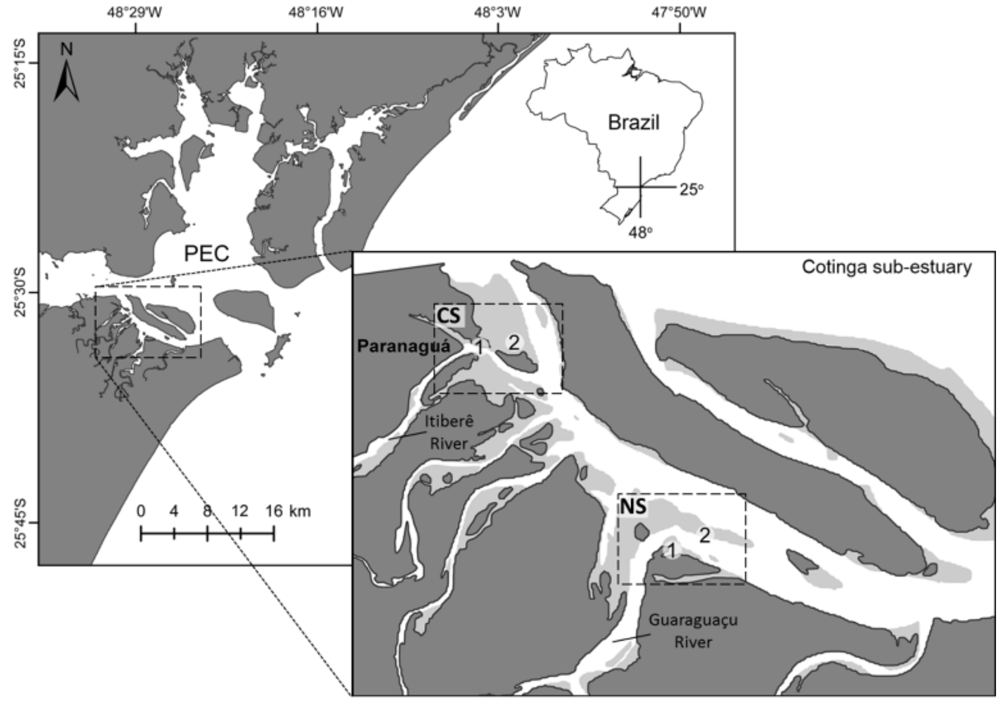

Figure 1: Paranaguá Estuarine Complex (PEC) and Cotinga sub-estuary. Tidal flats 1 and 2, sampled at Contaminated site (CS) and Non-contaminated site (NS).

We used a hierarchical sampling design to investigate the spatial and temporal variability of the indices in response to the distinct levels of sewage contamination of tidal flats. The sampling design was comprised of a six-factor linear model (three temporal and three spatial factors). The temporal factors included three consecutive *fortnights* (F - F1 to F3), nested in each of two sampling *events* (E - E1 and E2), within two *seasons* (S - summer and winter). The spatial factors included four sampling *plots* (P – P1 to P4 – at the scale of 10^1m) with three replicates each, nested in two *tidal flats* (TF – TF1 and TF2 – at the scale of 10^2m), within each of two *conditions* (C - contaminated and non-contaminated – at the scale of 10^3m). The temporal and spatial factors were orthogonally arranged (Fig. 2). Season (S) and condition (C) were fixed, and all other factors were random.

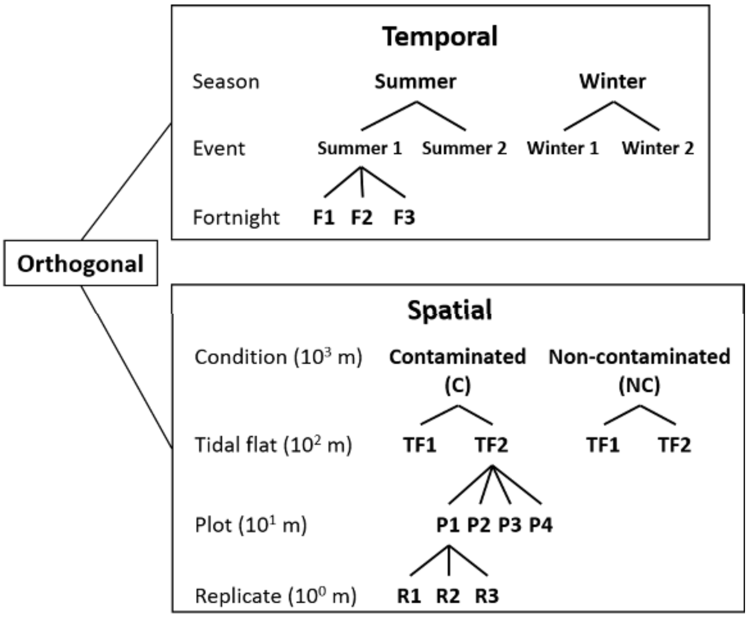

Figure 2: Sampling design diagram. Temporal and spatial scales correspond to the factors of the linear model. Two Seasons (S) were included (Summer and Winter), sampled in two Events each, with three consecutive Fortnights (F) per Event (F1, F2 and F3). In each Fortnight, two Conditions (C) were sampled (Contaminated and Non-Contaminated), with two Tidal flats (TF) per Condition (TF1 and TF2), four Plots (P) per Tidal flat (P1, P2, P3 e P4) and three replicates each.

Macrofauna was collected using plastic core tubes (10 cm diameter, 10 cm deep), and all plots were placed parallel to the water line, at similar tidal levels. All samples were sieved through a 0.5 mm mesh, fixed in 6% formaldehyde and preserved in 70% alcohol. In the laboratory, all organisms were counted and identified to the lowest possible taxonomic level. Samples were collected at low spring tides and plots were placed parallel to the waterline to avoid the occasional interference of macrofaunal zonation patterns due to steep.

The AMBI software is available online at AZTI's web page (http://ambi.azti.es). AMBI values are based on the proportion of species within five ecological groups according to their sensitivity to organic pollution (Borja et al., 2003, 2000). Some species or taxa found in this study were not yet assigned to the AMBI list. To classify the species into each ecological group, we: (i) checked the literature to establish the sensitivity level of a taxon (Ferrando and Méndez, 2011; Boehs et al., 2008; Gamito, 2008; Nalesso et al., 2005; Palacios et al., 2005; Barnett, 1983) 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, *Sigambra* sp. in GIII, Tubificinae sp 1 and Tubificinae sp 2 were in GV, while the polychaetes *Dorvillea* sp., *Ophelina* sp. and the bivalve *Macoma constricta* remained unassigned.

ITI rely on Fauchald and Jumars (1979) and Word (1980), under the premise that feeding behaviour responds to organic material enrichment by shifting the dominance of suspended material feeders toward deposit feeders (Maurer et al., 1999). The four main trophic groups (TG) were: (TG1) suspension feeders, (TG2) detritus feeders (e.g., omnivorous and necrophagous), (TG3) surface deposit feeders and species that are both suspension and surface deposit feeders, and (TG4) subsurface deposit feeders that feed on sedimentary detritus and bacteria. BO2A (Dauvin and Ruellet, 2009) was also based on the ecological characteristics of specific taxonomic groups, and compares percentage ratios of opportunistic annelids (polychaetes and clitellates) to percentages of amphipods (with exception of the opportunistic genus *Jassa*).The indices values were calculated for each replicate, and their ecological status was categorized as *High, Good, Moderate, Poor* and *Bad* (Table 1).

Table 1. Calculated indices and their ecological status threshold values. (GI= very sensitive to enrichment; GII= indifferent; GIII= tolerant; GIV= second-order opportunistic; GV= first-order opportunistic).

Indices		Environmental status				
		High	Good	Moderate	Poor	Bad
ITI	100-[33*1/3(0n1+1n2+2n3+3n4/n1+n2+n3+n4)]	100 - 80	80 - 60	60 - 30	30 - 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
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

Statistical analyses were conducted using the software Primer 6 (Clarke and Gorley, 2006) with the PERMANOVA+ add-on (Anderson et al. 2008) and the software R (R-Core-Team 2013). Accordingly, the linear mixed model to evaluate the spatial, and temporal variability becomes:

$$X = \mu + E_i + S(E)_{j(i)} + F(S(E))_{k(j(i))} + C_l + TF(C)_{m(l)} + P(TF(C))_{n(m(l))} + E_i{*}C_l + E_i{*}TF(C)_{m(l)} + E_i{*}P(TF(C))_{n(m(l))} + S(E)_{j(i)}{*}C_l + S(E)_{j(i)}{*}TF(C)_{m(l)} + S(E)_{j(i)}{*}P(TF(C))_{n(m(l))} + F(S(E))_{k(j(i))}{*}C_l + F(S(E))_{k(j(i))}{*}TF(C)_{m(l)} + F(S(E))_{k(j(i))}{*}P(TF(C))_{n(m(l))} + e_{o(n(m(l)))k(j(i))}$$

Where: “µ” = overall mean; “E” = Event; “S”= Season; “F” = Fortnight; “C”= Condition; “TF” = Tidal flat; “P” = Plot; “e” = error term or residual (equivalent to the variability within plots, or the replicates).

The linear model was tested using a permutational analysis of variance, sometimes referred to as PERANOVA as in Fanelli et al. (2011), Sweeting *et al.*(2009) and Ezgeta-Balić *et al.*(2011), based on the Euclidean distance (Anderson 2001). The use of Euclidean distance as the measure of association makes this univariate test similar to a traditional ANOVA (Anderson et al. 2008). The complexity of the design led to some non-testable terms, which were approximated using the linear combination of effects procedure described by Satterthwaite (1946), detailed by Blackwell et al. (1991) and implemented in the PERMANOVA+ package of the software Primer 6. The linear combination of effects may induce to an unknown F-distribution under a true null hypothesis. However, the permutation method avoids that problem so the P-value can be used for valid inference (Anderson et al. 2008). Separate PERANOVAs were performed using AMBI, ITI, BO2A and the relative abundance of the benthic groups that compose each index as dependent variables. 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. We used untransformed data to provide components of variation comparable to all data (Fraschetti et al., 2005), under 9999 permutations. Negative estimates of components of variation were set to zero and the proportion estimates of the remaining factors were recalculated.

We aimed not only to assess the significant differences attributed to the terms of our model but also to the proportion of total variance accounted for at each level of the nested design. The significance of a factor describes how likely (estimates the probability that) the patterns explained by the factor are simply due to random chance and thus serve no functional importance to the researcher. Significance is inherently dependent on sample size and is typically presented in the form of probability values (*P*-values). Conversely, determination of magnitude of effects is not probabilistic and not directly dependent on sample size, but rather is an estimate of the variance in a response variable that can be explained by the factor. Consequently, significance and magnitude of effects (measured as %CVs) do not necessarily co-vary and the most significant factors in a multi-factorial analysis are not guaranteed to also have the greatest fit (Graham et al., 2001). In this sense, estimates of significance and fit can be used to describe different aspects of statistical results. Therefore, given the likely presence of multiple significant factors of varied strength in our linear model, we enhanced the power of our ecological assumptions using both *P*-values (significance) and %CVs (fit) to describe and interpret the complexity of spatio-temporal variations.

Results

Consistent interdependence patterns were found between ecological and trophic benthic groups and indices quality status (Figs. 3, 4, 5). The overall trends were of worse assessments for contaminated tidal flats associated with high proportions of tolerant and/or opportunistic benthic groups. The sensitive groups did not numerically dominated the non-contaminated tidal flats though but were more abundant than in polluted sites. There were no evident relationships between intermediate benthic groups (less tolerant or indifferent to disturbance) and the choice of polluted or non-polluted sites.

Regarding AMBI, the lowest quality status was indeed assigned to contaminated sites and the highest status to the non-contaminated. The highest relative importance of the component of variation on the spatial scale of *contamination* (10^3m), of 39.8%, corroborate these assumptions (Fig.3, Table 2). The temporal scale of *fortnight* and spatiotemporal interactions at the scales of *event* with *tidal flat*, and *fortnight* with *plot* also varied significantly.

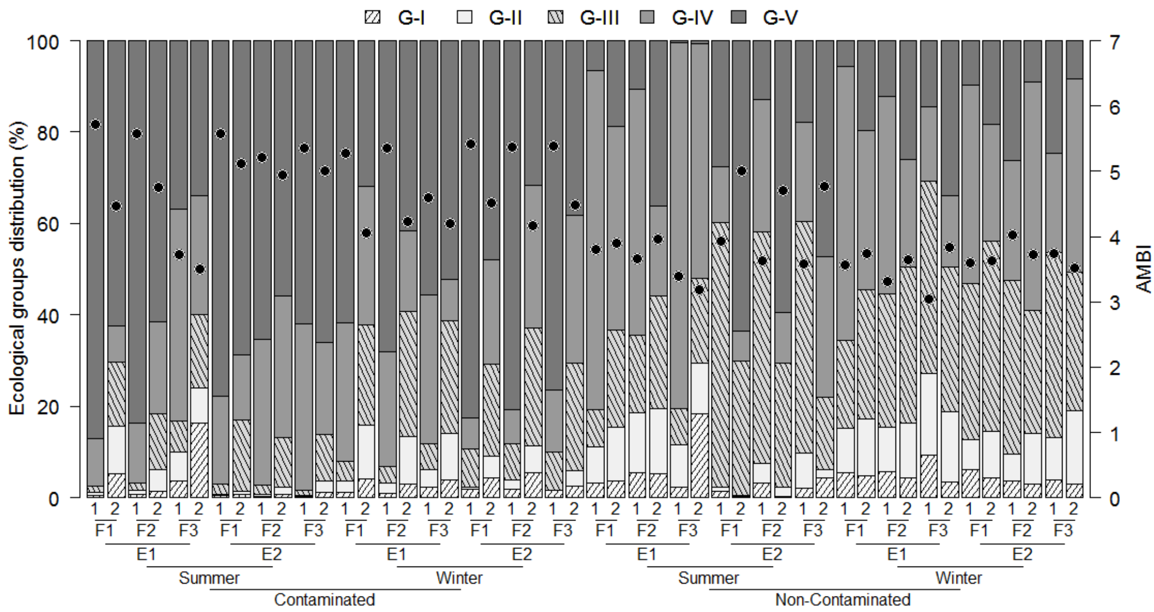

Fig. 3. Mean AMBI values (black dots, right *y* axis) and percentage of macrobenthic ecological groups in tidal flats 1 and 2 (bars, left *y* axis), nested in fortnights (F1 to F3), events (E1 and E2), and seasons (Summer and Winter), for contaminated and non-contaminated conditions. G-I: sensitive species; G-II: indifferent species; G-III: tolerant species; GIV: second-order opportunists; G-V: first-order opportunists.

PERANOVA components indicated different patterns for different ecological groups responsible for AMBI assessments. Only GI and GII (sensitive and indifferent species) significantly varied at the largest spatial scale or the scale of contamination, interactive or not (Fig.3, Table 2). With the exception of GV, the fortnight temporal scale was significant for the remaining groups. In addition, all ecological groups significantly varied at the interactive levels of *fortnight* x *tidal flat*, or, less often, between *fortnight* and *plot*, showing that small spatial scales (from 10^1m to 10^2m) changed differently within fortnights.

Table 2. PERANOVA results and components of variation (CV %) for AMBI and the corresponding ecological groups GI to GV. Significant differences are given in bold (p<0.05).

		AMBI			GI			GII		
Source	df	*Ps*-F	P(MC)	CV (%)	*Ps*-F	(MC)	CV (%)	*Ps*-F	P(MC)	CV (%)
S	1	0.70	0.56	0.0	19.52	**0.02**	**33.0**	6.75	0.06	22.5
C	1	6.34	0.12	39.8	14.43	**0.04**	3.0	0.11	0.98	0.0
E(S)	2	1.83	0.21	4.1	0.83	0.51	0.0	1.30	0.32	1.2
TF(C)	2	6.23	**0.04**	12.8	0.38	0.96	0.0	2.45	0.17	2.2
SxC	1	1.45	0.45	0.9	3.62	0.14	3.1	0.08	0.98	0.0
F(E(S))	8	8.86	**<0.001**	7.3	4.20	**0.01**	8.0	4.01	**0.01**	6.6
P(TF(C))	12	0.91	0.54	0.0	0.70	0.73	0.0	0.69	0.75	0.0
SxTF(C)	2	0.96	0.48	0.0	1.08	0.44	0.2	2.24	0.18	3.9
E(S)xC	2	0.13	1.00	0.0	0.81	0.64	0.0	5.39	**0.02**	22.5
SxP(TF(C))	12	0.68	0.76	0.0	0.66	0.77	0.0	0.78	0.66	0.0
E(S)xTF(C)	4	3.68	**0.01**	6.9	1.08	0.41	0.4	1.26	0.29	1.2

F(E(S))xC	8	2.40	0.07	2.6	0.77	0.63	0.0	1.79	0.15	3.5
E(S)xP(TF(C))	24	1.35	0.15	1.4	0.79	0.74	0.0	2.20	**<0.001**	3.6
F(E(S))xTF(C)	16	1.25	0.25	0.7	1.88	**0.03**	4.7	3.89	**<0.001**	6.5
F(E(S))xP(TF(C))	96	2.08	**<0.001**	6.2	1.60	**<0.001**	8.0	1.06	0.36	0.5
Res	384			**17.2**			**39.7**			25.6
Total	575									

		GIII			GIV			GV		
Source	df	*Ps*-F	P(MC)	CV (%)	*Ps*-F	P(MC)	CV (%)	*Ps*-F	P(MC)	CV (%)
S	1	5.68	0.10	37.5	1.46	0.38	1.7	0.39	0.82	0.0
C	1	0.25	0.80	0.0	0.90	0.47	0.0	1.91	0.29	19.3
E(S)	2	3.67	0.06	10.0	0.49	0.69	0.0	1.58	0.25	1.6
TF(C)	2	3.55	0.06	4.5	1.99	0.24	9.9	25.21	**<0.001**	38.4
SxC	1	0.16	0.96	0.0	0.59	0.72	0.0	0.53	0.74	0.0
F(E(S))	8	7.83	**<0.001**	7.2	3.41	**0.02**	6.5	1.46	0.25	1.4
P(TF(C))	12	1.70	0.13	1.3	1.47	0.20	0.5	2.28	**0.04**	0.6
SxTF(C)	2	2.12	0.17	3.2	0.25	0.83	0.0	0.15	0.98	0.0
E(S)xC	2	4.90	0.06	9.2	1.61	0.30	12.9	1.38	0.29	2.0
SxP(TF(C))	12	1.03	0.45	0.1	2.34	**0.03**	2.6	2.95	**0.01**	1.7
E(S)xTF(C)	4	1.54	0.19	1.7	8.60	**<0.001**	34.6	1.33	0.30	1.5
F(E(S))xC	8	0.55	0.81	0.0	1.04	0.46	0.2	1.19	0.37	1.2
E(S)xP(TF(C))	24	1.82	**0.02**	3.2	1.29	0.20	0.9	0.67	0.87	0.0
F(E(S))xTF(C)	16	1.42	0.15	1.3	4.71	**<0.001**	8.5	6.28	**<0.001**	10.3
F(E(S))xP(TF(C))	96	2.64	**<0.001**	7.4	1.44	**0.01**	2.8	1.09	0.28	0.7
Res	384			13.5			18.9			21.4
Total	575									

ITI was clearly responsive to the distinct levels of contamination of the sites as the quality status were mostly poor on polluted tidal flats and conversely good on the non-contaminated (Fig.4, Table 3). This high discriminating power was reflected in the highest component of variation in the spatial scale of *contamination* (10^3m), of 54.4%. We also found significant variation among *fortnights* and substantial interactive variability involving this temporal scale with the smaller spatial scales of *tidal flat* (10^2m) and *plot* (10^1m).

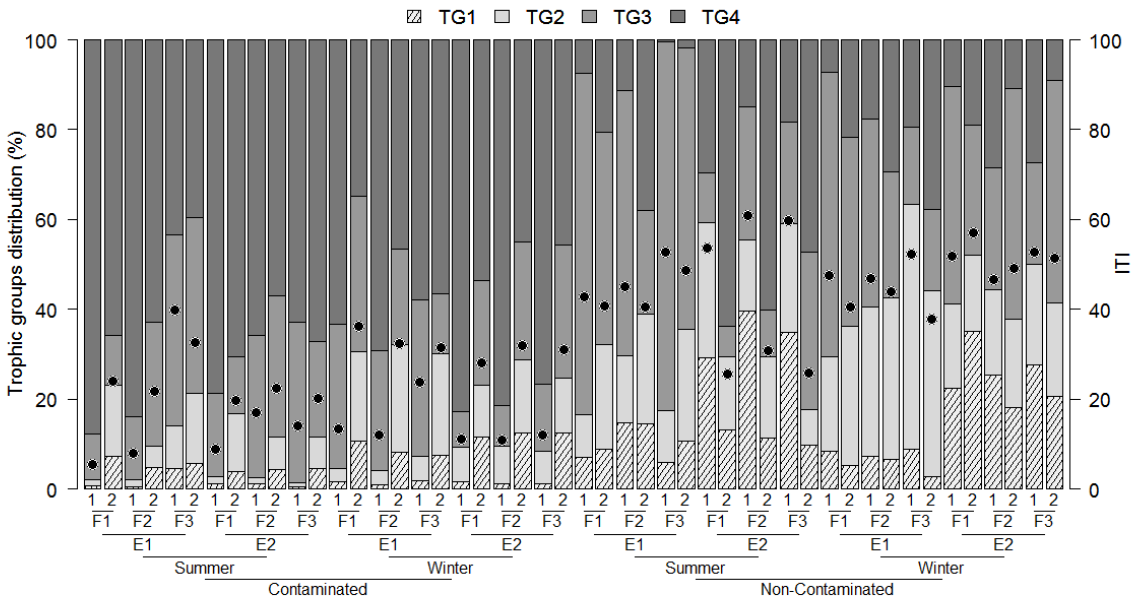

Fig. 4. Mean ITI values (black dots, right *y* axis) and percentage of macrobenthic trophic groups in tidal flats 1 and 2 (bars, left *y* axis), nested in fortnights (F1 to F3), events (E1 and E2), and seasons (Summer and Winter), for contaminated and non-contaminated conditions. TG1: suspension feeders; TG2: detritus feeders; TG3: surface deposit feeders; TG4: subsurface deposit feeders.

Different patterns of variability were found depending on the trophic group of the ITI index. TG1 (suspension feeders) did vary at the interactive scale of *event* x *condition* (10^3m), which means responsiveness to pollution along/depending on the years (Fig. 4, Table 3). The remaining variation of all trophic groups (from TG1 to TG4) was concentrated in: the *fortnight* scale; interactions between this temporal scale with the smallest spatial scales of *tidal flat* and *plot* (10^1m and 10^2m); and between *event* and *tidal flat*.

Table 3. PERANOVA results and components of variation (CV %) for ITI and the corresponding trophic groups TG1 to TG4. Significant differences are given in bold (p<0.05).

		ITI			TG1			TG2		
Source	df	Ps-F	P(MC)	CV(%)	Ps-F	P(MC)	CV(%)	Ps-F	P(MC)	CV(%)
S	1	1.24	0.44	0.5	1.19	0.37	2.8	73.81	**<0.001**	51.5
C	1	8.46	0.08	54.5	0.67	0.51	0.0	0.69	0.66	0.0
E(S)	2	0.26	0.90	0.0	7.14	**0.01**	21.3	0.15	1.00	0.0
TF(C)	2	4.66	0.08	10.5	2.33	0.18	3.2	1.30	0.36	0.8
SxC	1	0.51	0.74	0.0	0.11	0.98	0.0	1.40	0.39	0.7
F(E(S))	8	4.28	**0.01**	3.2	2.70	**0.04**	3.0	6.54	**<0.001**	8.4
P(TF(C))	12	1.38	0.24	0.3	1.29	0.28	0.5	0.72	0.72	0.0
SxTF(C)	2	1.44	0.33	2.4	2.60	0.16	7.1	0.62	0.68	0.0
E(S)xC	2	0.61	0.64	0.0	8.09	**0.03**	30.2	0.44	0.81	0.0
SxP(TF(C))	12	0.60	0.83	0.0	0.81	0.64	0.0	0.58	0.84	0.0
E(S)xTF(C)	4	5.22	**<0.001**	8.9	2.18	0.08	4.6	2.36	0.06	5.8
F(E(S))xC	8	1.04	0.44	0.1	0.40	0.90	0.0	1.15	0.38	0.4
E(S)xP(TF(C))	24	1.25	0.22	0.6	1.76	**0.03**	2.7	2.74	**<0.001**	5.7
F(E(S))xTF(C)	16	2.08	**0.02**	2.1	2.62	**<0.001**	4.3	2.47	**<0.001**	3.6
F(E(S))xP(TF(C))	96	1.64	**<0.001**	3.0	2.21	**<0.001**	5.8	1.51	**<0.001**	3.3

Res	384	13.9	14.5	19.5
Total	575			

		TG3			TG4		
Source	df	Ps-F	P(MC)	CV (%)	Ps-F	P(MC)	CV (%)
S	1	1.76	0.34	2.4	0.41	0.80	0.0
C	1	0.85	0.49	0.0	2.03	0.28	21.1
E(S)	2	0.48	0.70	0.0	1.48	0.27	1.4
TF(C)	2	2.06	0.23	9.8	22.41	**<0.001**	36.9
SxC	1	0.55	0.73	0.0	0.56	0.72	0.0
F(E(S))	8	3.66	**0.01**	7.2	1.49	0.23	1.5
P(TF(C))	12	1.58	0.17	0.5	2.22	0.05	0.5
SxTF(C)	2	0.18	0.89	0.0	0.19	0.94	0.0
E(S)xC	2	1.75	0.28	14.7	1.31	0.33	1.6
SxP(TF(C))	12	2.41	**0.04**	2.6	2.85	**0.01**	1.6
E(S)xTF(C)	4	7.93	**<0.001**	31.5	1.48	0.24	2.1
F(E(S))xC	8	1.04	0.45	0.2	1.20	0.35	1.2
E(S)xP(TF(C))	24	1.28	0.20	0.8	0.68	0.85	0.0
F(E(S))xTF(C)	16	5.03	**<0.001**	8.7	6.23	**<0.001**	10.1
F(E(S))xP(TF(C))	96	1.34	**0.03**	2.2	1.09	0.29	0.6
Res	384			19.3			21.2
Total	575						

Similarly to AMBI and ITI, most assignments of BO2A were of poor quality in contaminated sites, whereas a general better status was assigned in non-contaminated (Fig. 5). This consistency between BO2A diagnosis and pollution levels was reflected as the highest proportion of the component of variation (39.6%) at the contamination scale. BO2A additionally varied at the *fortnight* scale, the interactive levels of *event* x *tidal flat*, and between *fortnight* and *plot* (Table 4).

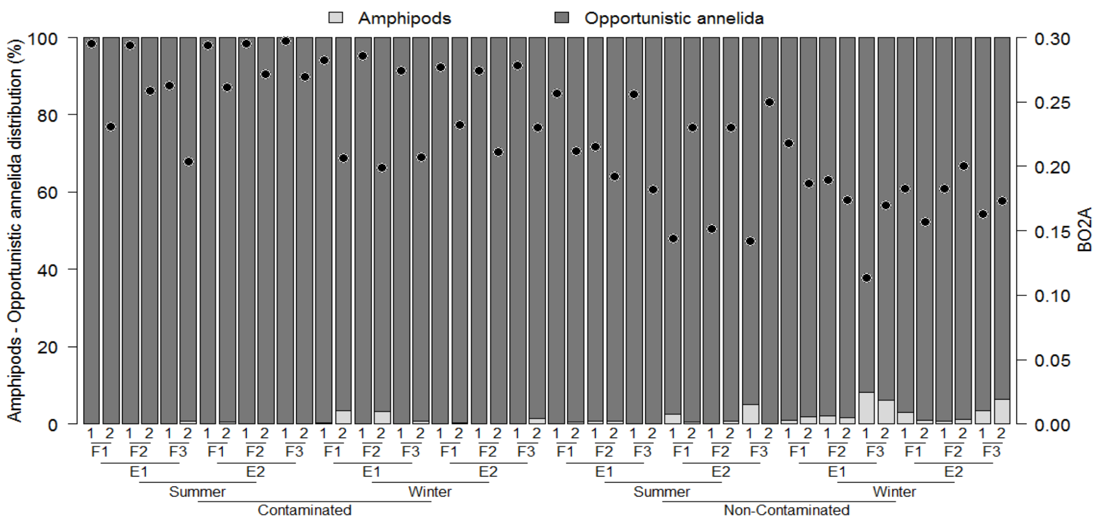

Fig.5. Mean ITI values (black dots, right *y* axis) and percentage of macrobenthic trophic groups in tidal flats 1 and 2 (bars, left *y* axis), nested in fortnights (F1 to F3), events (E1 and E2), and seasons (Summer and Winter), for contaminated and non-contaminated conditions. TG1: suspension feeders; TG2: detritus feeders; TG3: surface deposit feeders; TG4: subsurface deposit feeders.

Neither the opportunist annelids nor the amphipods of the BO2A index varied significantly at the spatial scale of *contamination*. Opportunist annelids were significant at the scale of *fortnight* and amphipods at the *tidal flat*. The groups simultaneously varied at the interactive *fortnight* x *tidal flat* scales.

Table 4. PERANOVA results and components of variation (CV %) for BO2A and the corresponding groups of opportunist annelids and amphipods. Significant differences in bold (p<0.05).

		BO2A			Opportunistic Annelida			Amphipoda		
Source	df	*Ps*-F	P(MC)	CV (%)	*Ps*-F	P(MC)	CV (%)	*Ps*-F	P(MC)	CV (%)
S	1	3.33	0.15	3.5	0.67	0.67	0.0	7.90	0.09	13.1
C	1	6.45	0.10	39.6	1.63	0.32	14.7	0.15	0.92	0.0
E(S)	2	0.44	0.72	0.0	1.06	0.42	0.3	0.35	0.97	0.0
TF(C)	2	3.01	0.12	9.0	11.41	**0.02**	36.9	2.82	0.11	2.3
SxC	1	1.72	0.31	1.6	0.55	0.74	0.0	0.08	0.99	0.0
F(E(S))	8	5.56	**<0.001**	4.1	2.01	0.11	2.7	2.91	**0.03**	7.3
P(TF(C))	12	2.13	0.05	1.5	2.12	0.05	0.3	2.18	0.05	1.0
SxTF(C)	2	0.30	0.85	0.0	0.17	0.90	0.0	3.13	0.10	5.0
E(S)xC	2	0.40	0.76	0.0	1.64	0.27	5.6	3.35	0.06	13.1
SxP(TF(C))	12	1.16	0.37	0.4	3.42	**0.01**	1.5	1.82	0.11	1.3
E(S)xTF(C)	4	6.38	**<0.001**	13.6	3.60	**0.02**	10.2	0.75	0.64	0.0
F(E(S))xC	8	0.99	0.48	0.0	1.05	0.45	0.3	1.55	0.22	4.2
E(S)xP(TF(C))	24	1.40	0.13	1.5	0.54	0.96	0.0	0.69	0.84	0.0
F(E(S))xTF(C)	16	1.27	0.23	0.8	6.38	**<0.001**	9.1	4.34	**<0.001**	11.8
F(E(S))xP(TF(C))	96	1.73	**<0.001**	4.8	1.16	0.16	1.0	1.05	0.38	0.7
Res	384			19.6			17.5			40.3
Total	575									

Discussion

Biological indices may be affected by man-made or natural disturbance (Wilson and Jeffrey, 1994). However, our results show that all tested indices responded consistently to contamination from urban discharges, operating at the largest spatial scale (10^3m). Surprisingly, the presumed strong natural background signal represented by the alternation between consecutive summers and winters was not significant to index variability. Alternatively, AMBI, ITI and BO2A responded to processes acting at interactive small spatial scales (from 10^1m to 10^2m) with both long-term temporal scale (events/years) and short-term (fortnights). Therefore, our results are a typical example of how the interaction of processes can lead to highly heterogeneous spatiotemporal patterns in estuaries (Dauvin, 2007, Elliott & Quintino 2007), and how these interactions can be separately measured. Such heterogeneity does not necessarily mean ambiguity of indices responses.

As expected, the indices did vary at the spatial scale of contamination, which is an effective signal of reliability for AMBI, ITI and BO2A. Contrasting results concerning seasonal variation have been reported depending on the index and ecoregion assessed (Reiss and Kröncke, 2005, Chainho et al., 2007). Nevertheless, so far no previous study enlightened the effects of interactive spatiotemporal variations on the performance of benthic indices of health assessment. Unlike seasonal variability, interactive variation of indices within small spatial scales along different fortnights and years were found to be significant. Such heterogeneous patterns mean that: (i) a large array of processes may interact with the system at distinct particular scales; (ii) one isolated process operate at several spatial and temporal scales or (iii) both occur simultaneously.

Benthic indices are likely to vary at smaller spatial scales, from tens to hundreds of meters, as a reflex of the variation of the structure of macrofaunal assemblages (Muniz et al., 2012; Tataranni and Lardicci, 2010). Our results showed that small-scale spatial variation (10^1m) interacted with short-term temporal variability (fortnight). Despite this significant interaction, the variation in the scale of fortnights itself was a recurrent pattern for all indices and almost all ecological/trophic macrofaunal groups. In most cases, this variation alone is much higher than its interactive variation with tidal flats and plots, an indicative that there is an important background signal at this scale regardless of the variation of spatial scales. Nevertheless, the fortnightly oscillations of indices and macrofaunal groups seem to increase inconsistently and decrease. This pattern is probably related to signals from pulse disturbances (Bender et al., 1984) which can either increase or decrease the population in small patches from one fortnight to another. Such pulse or short-lived events at the fortnight scale may operate unpredictably and with varying intensity, in the form of freshwater discharges from the rain or wind-driven variations of exposure by tides, for example.

Conversely, the interactive variability at the spatial scale of tidal flats (10^2m) with the long-term temporal variation (interannual) is more likely to relate to press disturbances. Long-term responses are typical of press events or disturbances that constantly persist for longer periods of time (Bender et al., 1984). The organic contamination in Cotinga sub-estuary is known to operate at the largest spatial scale, of 10^3m (Souza et al., 2012; Martins et al., 2010). Nevertheless, the contamination may simultaneously operate at the interannual significant scale. Local sewage discharges are chronic, but their quality and flow rate may vary at unknown temporal scales. If the sewage signal to the sub-estuary changed from one year to another, then it could alter the macrofaunal structure in the tidal flat scale, promoting the variation patterns detected simultaneously for AMBI, ITI and BO2A. Our results indeed showed an overall worsening of the environmental quality from one year to another. Variations of benthic indices at longer temporal scales, of tens of years, have shown contrasting responses depending on the ecoregion and indices assessed (Simboura et al., 2014, Kröncke and Reiss, 2010).

A biological meaning generally lies behind a benthic index; therefore, the significant scales of variation of indices are certainly triggered by ecological or trophic groups composed of species that vary on this same scale (for more details on species classification see Annex 1 and 2). Each group has distinct responses depending on the species levels of sensitivity to organic pollution (Dauvin and Ruellet, 2009, Borja et al., 2000, Word, 1980). According to our results only GI (sensitive species group) of AMBI and TG1 (suspension feeders) of ITI varied at the spatial scale of contamination. Despite their low abundance, GI and TG1 are responsible for the variation of AMBI and ITI at the scale of contamination, as suspension feeders of ITI are included in the sensitive group of AMBI. For BO2A, the opportunistic annelids were the only group to show a slightly important variation at the scale of condition, considering the total proportion. It seems that for this index some of the species included in the opportunistic annelids might not have responded to the contamination gradient, reflecting a noise to the significance of this scale. Nevertheless, unlike the results of Riera and Carretero (2013) the diagnostic power of BO2A did not decrease with the assignment of only two groups of sensitivity to pollution. The groups with intermediate sensitivity presented inconspicuous trends of spatiotemporal variation. The intermediate groups are composed of species that are either indifferent or only tolerant to slight pollution levels, which are not expected to clearly respond to pollution inputs, unlike sensitive or opportunists of extreme sensitivity.

We highlight that AMBI, ITI, and BO2A were successfully applied and were responsive to the distinct contamination levels. The presumed strong and marked variations related to seasons were not reflected in any index, although there was interactive variation between smaller spatial scales with short and long-term temporal scales. Such variations are probably a consequence of specific pulse and press disturbances. The sewage input is likely to operate either at the largest spatial scale (thousands of meters), and at the long-term temporal scale (interannual) as a press disturbance. Concerning the influence of ecological and trophic groups on the variation of indices, the sensitive and suspension feeder groups were possibly responsible for most of the variability of AMBI and ITI, as the opportunist annelids for BO2A. Unlike biased tests of spatial, temporal and/or interactive scales of variation, our assumptions were consistent as they were both tested with a robust and complex hierarchical sampling design and were congruent among all studied indices. The indices varied at several interactive scales, which does not necessarily mean ambiguous or meaningless responses.

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Annex 1 – Ecological groups of taxa and total abundance per tidal flat (TF – 1, 2) in each condition (C – CS, NS) along three fortnights (F – 1, 2, 3) sampled in two seasons (S – S, W) of ***2011*** (event 1 – E). (CS1: 48°29'45"W 25°30'44"S; CS2: 48°29'35"W 25°30'42"S; NS1: 48°27'32"W 25°32'35"S; NS2: 48°27'10"W 25°32'28"S).

					CS1S1	*CS2S1*	*CS1S2*	*CS2S2*	*CS1S3*	*CS2S3*	*NS1S1*	*NS2S1*	*NS1S2*	*NS2S2*	*NS1S3*	*NS2S3*	*CS1W1*	*CS2W1*	*CS1W2*	*CS2W2*	*CS1W3*	*CS2W3*	*NS1W1*	*NS2W1*	*NS1W2*	*NS2W2*	*NS1W3*	*NS2W3*	
				Condition (C)	CS	CS	CS	CS	CS	CS	NS	NS	NS	NS	NS	NS	CS	CS	CS	CS	CS	CS	NS	NS	NS	NS	NS	NS	
				Tidal flat (TF)	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
				Season (S)	S	S	S	S	S	S	S	S	S	S	S	S	W	W	W	W	W	W	W	W	W	W	W	W	
				Fortnight (F)	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	
	Ecological group																												
	AMBI M-AMBI	BENTIX	ITI	BO2A																									***Total***
Tellina versicolor	I	1	TGIII (DEPSUP)	*	6	3	10	5	15	24	6	13	24	15	3	17	39	51	33	10	22	5	51	29	11	20	7	5	424
Lucina pectinata	I	1	TGI (SUS)	*	11	0	12	1	12	1	10	2	1	6	3	10	23	22	29	23	25	18	16	4	15	3	9	0	256
Scoloplos ohlini	I	1	TGIII (DEPSUP)	*	0	15	2	6	0	13	4	9	3	7	0	3	3	5	5	4	2	4	5	6	5	3	4	7	115
Sipuncula	I	1	TGIII (DEPSUP)	*	0	7	0	2	0	0	3	4	0	0	0	0	3	7	1	0	0	0	0	3	0	0	0	0	30
Diopatra aciculata	I	1	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	5	0	3	2	5	2	2	0	0	26
Aricidea catherinae	I	1	TGIII (DEPSUP)	*	0	2	0	1	0	0	10	2	2	0	0	0	0	0	0	0	0	0	2	2	0	0	1	0	22
Scoloplos sp	I	1	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	3
Magelona papillicornis	I	1	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	0	0	1	1	5
Sabella sp	I	1	TGI (SUS)	*	1	0	0	0	2	2	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	7
Streblosoma sp	I	1	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	1	0	2	0	2	0	0	7
Terebellides anguicomus	I	1	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	1	1	0	1	6
Armandia hossfeldi	I	1	TGIV (DEPSUB)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	3
Clymenella sp	I	1	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	2
Amphipoda	I	1	TGII (DETRI)	Amphipoda 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Glycinde multidens	II	2	TGII (DETRI)	*	10	18	10	18	11	13	21	16	29	20	9	11	29	29	31	37	28	36	31	30	26	16	27	16	522
Bulla striata	II	2	TGII (DETRI)	*	0	6	0	0	0	0	22	25	0	1	0	0	11	62	3	25	0	14	55	20	13	13	1	2	273
Tagelus divisus	II	2	TGI (SUS)	*	8	17	4	26	3	4	28	47	38	50	6	5	13	32	10	14	9	14	6	3	2	1	4	2	346
Scoletoma tetraura	II	2	TGII (DETRI)	*	0	0	0	0	1	0	0	0	0	0	0	0	28	109	60	42	26	6	18	11	10	24	4	21	360
Isolda pulchella	II	2	TGIII (DEPSUP)	*	4	3	1	4	2	1	1	2	1	1	1	0	26	19	22	13	9	1	5	13	2	7	1	2	141
Sphenia fragilis	II	2	TGI (SUS)	*	0	0	1	0	1	0	0	0	1	0	0	0	0	4	1	6	0	2	7	19	4	19	2	1	68
Exogone sp	II	2	TGII (DETRI)	*	0	1	0	2	0	0	2	4	1	2	0	0	0	0	0	0	0	0	1	1	0	0	0	2	16
Polydora websteri	II	2	TGIII (DEPSUP)	*	0	1	0	0	0	0	0	0	0	0	0	0	21	11	21	5	9	3	3	14	1	3	0	3	95
Monokalliapseudes schubarti	II	2	TGII (DETRI)	*	6	3	5	0	31	1	0	1	0	0	6	2	1	1	3	2	0	0	1	0	0	0	0	0	63
Haminoea elegans	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	9	1	1	3	0	1	2	4	1	1	24
Phyllodoce sp	II	2	TGII (DETRI)	*	0	0	0	0	0	0	1	1	1	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	6
Paranaitis sp	II	2	TGII (DETRI)	*	1	0	0	0	0	0	2	1	0	0	0	0	3	1	3	0	0	1	0	0	0	0	0	0	12
Edwardsia fusca	II	2	TGII (DETRI)	*	0	2	0	1	0	0	2	0	1	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	9
Ceratonereis longicirrata	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	0	1	1	0	0	1	5	12
Nephtys fluviatilis	II	2	TGII (DETRI)	*	0	1	0	0	0	0	0	0	0	1	1	0	0	3	0	0	0	0	2	1	1	0	1	1	12
Kinbergonuphis difficilis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	3	0	0	0	0	4
Eunoe serrata	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	1	1	2	0	0	0	2	9
Megalomma sp	II	2	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	0	0	0	0	0	1	0	0	6
Hemipodia californiensis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	2	0	2	0	0	0	2	1	0	0	2	0	9
Pholoe minuta	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	1	2	0	0	0	1	7
Hermundura tricuspis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	1	0	0	0	0	0	1	0	5
Syllis sp	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	1	0	0	4
Owenia fusiformis	II	2	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	1	0	0	0	4
Aglaophamus juvenalis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	3
Ophiuroidea	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	1	0	0	2
Sthenelais limicola	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1
Fimbriosthenelais marianae	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1
Goniada maculata	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1
Clibanarius vittatus	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sigambra sp	III	2	TGII (DETRI)	*	45	42	25	27	29	21	41	134	44	99	13	28	50	131	92	125	44	70	135	209	118	176	62	75	1835
Anomalocardia flexuosa	III	2	TGI (SUS)	*	4	18	1	22	0	5	32	20	20	7	5	3	37	117	21	56	8	24	65	10	24	7	7	5	518
Streblospio benedicti	III	2	TGIII (DEPSUP)	*	4	2	14	75	4	8	0	6	1	3	1	0	91	75	118	83	51	49	2	23	0	21	0	6	637
Mytella sp	III	2	TGI (SUS)	*	3	2	0	2	17	2	2	6	23	14	1	0	20	73	7	18	0	2	26	20	1	20	0	3	262
Monochorophium acherusicum	III	2	TGII (DETRI)	*	0	0	0	1	0	1	0	3	3	2	0	0	18	51	6	29	1	4	9	11	8	6	7	13	173
Spiophanes duplex	III	2	TGII (DETRI)	*	0	3	0	0	0	0	5	7	2	3	0	0	10	39	19	46	8	20	21	5	20	5	14	6	233
Nemertea	III	2	TGII (DETRI)	*	1	3	3	1	1	1	0	1	1	0	0	0	5	14	7	20	6	17	8	9	8	11	13	11	141
Alitta succinea	III	2	TGII (DETRI)	*	0	0	1	0	0	1	0	0	0	0	0	0	2	8	3	6	0	3	3	8	4	6	3	4	52
Spiochaetopterus costarum	III	2	TGIII (DEPSUP)	*	0	0	0	0	0	0	1	2	0	1	0	0	0	0	0	2	0	4	0	3	2	0	1	0	16
Spiophanes sp	III	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	2	5	0	0	1	0	10
Notomastus sp	III	2	TGIV (DEPSUB)	*	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3
Neanthes succinea	III	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	2
Sternaspis sp	III	2	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	2
Laeonereis culveri	IV	3	TGIII (DEPSUP)	Polychaeta 1	366	13	346	155	293	49	20	4	28	16	11	10	1441	239	1449	86	637	20	28	22	26	13	7	1	5280
Heleobia australis	IV	3	TGIII (DEPSUP)	*	38	9	14	41	13	0	723	345	269	78	189	73	121	378	316	77	4	2	773	307	195	120	1	10	4096
Heteromastus sp	IV	3	TGIV (DEPSUB)	Polychaeta 2	23	17	9	16	50	14	6	15	3	10	0	2	81	66	85	69	52	34	23	23	32	25	12	15	682
Prionospio heterobranchia	IV	3	TGIII (DEPSUP)	Polychaeta 3	0	1	0	1	0	1	0	5	1	0	0	2	3	16	2	1	1	13	18	16	21	15	17	28	162
Polydora sp	IV	3	TGIII (DEPSUP)	Polychaeta 4	1	0	1	0	0	0	0	0	0	0	0	0	0	0	15	1	2	0	5	5	0	2	1	0	33
Prionospio sp	IV	3	TGIII (DEPSUP)	Polychaeta 5	0	0	0	0	0	0	0	2	1	0	0	0	1	2	6	16	0	3	3	0	0	0	4	7	45
Polydora cornuta	IV	3	TGIII (DEPSUP)	Polychaeta 6	0	0	1	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	5
Tubificinae sp1	V	3	TGIV (DEPSUB)	Polychaeta 7	3220	310	2168	597	258	12	66	156	59	187	1	0	2500	712	2608	555	658	375	78	207	77	148	36	72	15060
Paranais cf. frici	V	3	TGIV (DEPSUB)	Polychaeta 8	1	0	0	0	0	0	0	0	0	0	0	0	788	18	2329	29	404	35	1	1	1	41	1	58	3707
Capitella sp	V	3	TGIV (DEPSUB)	Polychaeta 9	384	2	167	51	26	71	0	0	1	3	0	1	73	11	160	7	120	1	0	3	0	3	0	3	1087

Annex 2 – Ecological groups of taxa and total abundance per tidal flat (TF – 1, 2) in each condition (C – CS, NS) along three fortnights (F – 1, 2, 3) sampled in two seasons (S – S, W) of ***2012*** (event 2 – E). (CS1: 48°29'45"W 25°30'44"S; CS2: 48°29'35"W 25°30'42"S; NS1: 48°27'32"W 25°32'35"S; NS2: 48°27'10"W 25°32'28"S).

					CS1S1	*CS2S1*	*CS1S2*	*CS2S2*	*CS1S3*	*CS2S3*	*NS1S1*	*NS2S1*	*NS1S2*	*NS2S2*	*NS1S3*	*NS2S3*	*CS1W1*	*CS2W1*	*CS1W2*	*CS2W2*	*CS1W3*	*CS2W3*	*NS1W1*	*NS2W1*	*NS1W2*	*NS2W2*	*NS1W3*	*NS2W3*	
				Condition (C)	CS	CS	CS	CS	CS	CS	NS	NS	NS	NS	NS	NS	CS	CS	CS	CS	CS	CS	NS	NS	NS	NS	NS	NS	
				Tidal flat (TF)	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	
				Season (S)	S	S	S	S	S	S	S	S	S	S	S	S	W	W	W	W	W	W	W	W	W	W	W	W	
				Fortnight (F)	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	1	1	2	2	3	3	
	Ecological group																												
	AMBI M-AMBI	BENTIX	ITI	BO2A																									***Total***
Tellina versicolor	I	1	TGIII (DEPSUP)	*	8	0	11	2	9	14	1	0	9	0	1	17	23	41	28	31	20	24	28	24	8	13	8	30	350
Lucina pectinata	I	1	TGI (SUS)	*	15	4	9	0	6	3	3	0	5	0	6	0	31	23	17	9	28	13	9	1	3	5	5	5	200
Scoloplos ohlini	I	1	TGIII (DEPSUP)	*	0	0	0	3	0	1	1	1	1	0	0	1	1	10	4	16	0	5	13	7	10	9	7	9	99
Sipuncula	I	1	TGIII (DEPSUP)	*	1	1	0	5	0	3	1	0	0	0	0	17	0	9	4	6	1	2	12	13	12	3	18	5	113
Diopatra aciculata	I	1	TGII (DETRI)	*	0	2	0	1	1	1	0	0	0	0	0	0	0	1	0	10	0	4	0	0	0	0	0	0	20
Aricidea catherinae	I	1	TGIII (DEPSUP)	*	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	4	0	6
Scoloplos sp	I	1	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	4	3	7	1	1	18
Magelona papillicornis	I	1	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	1	2	1	0	1	0	0	0	0	2	2	1	1	1	1	13
Sabella sp	I	1	TGI (SUS)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Streblosoma sp	I	1	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Terebellides anguicomus	I	1	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Armandia hossfeldi	I	1	TGIV (DEPSUB)	*	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	2
Clymenella sp	I	1	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amphipoda	I	1	TGII (DETRI)	Amphipoda 1	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	0	0	0	2	2	0	4	0	12
Glycinde multidens	II	2	TGII (DETRI)	*	6	7	1	3	6	17	2	1	6	4	7	8	11	30	6	23	0	18	27	15	13	22	9	16	258
Bulla striata	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	1	0	0	0	0	9	39	12	0	3	14	44	18	51	29	47	267
Tagelus divisus	II	2	TGI (SUS)	*	5	0	9	7	1	9	1	0	10	3	22	3	3	20	1	16	0	7	13	17	6	7	8	7	175
Scoletoma tetraura	II	2	TGII (DETRI)	*	1	0	0	2	0	2	1	1	0	0	1	0	2	7	2	5	0	2	4	15	7	1	9	27	89
Isolda pulchella	II	2	TGIII (DEPSUP)	*	1	1	0	8	6	8	0	0	2	0	2	0	0	8	5	9	1	11	0	1	0	5	0	6	74
Sphenia fragilis	II	2	TGI (SUS)	*	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	5	0	7	5	17	3	35	12	47	134
Exogone sp	II	2	TGII (DETRI)	*	2	1	3	0	4	4	0	0	0	1	0	2	0	5	1	7	0	14	5	8	14	23	39	28	161
Polydora websteri	II	2	TGIII (DEPSUP)	*	0	0	0	0	1	0	0	0	0	0	0	0	3	1	0	0	9	5	0	0	0	0	10	74	103
Monokalliapseudes schubarti	II	2	TGII (DETRI)	*	8	0	5	0	3	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	1	0	8	27
Haminoea elegans	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phyllodoce sp	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	2	0	0	1	1	7
Paranaitis sp	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1
Edwardsia fusca	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	1	4
Ceratonereis longicirrata	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Nephtys fluviatilis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kinbergonuphis difficilis	II	2	TGII (DETRI)	*	0	0	1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	1	0	0	5
Eunoe serrata	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Megalomma sp	II	2	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	3
Hemipodia californiensis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pholoe minuta	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Hermundura tricuspis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1
Syllis sp	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1
Owenia fusiformis	II	2	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Aglaophamus juvenalis	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ophiuroidea	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sthenelais limicola	II	2	TGII (DETRI)	*	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Fimbriosthenelais marianae	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Goniada maculata	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Clibanarius vittatus	II	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Sigambra sp	III	2	TGII (DETRI)	*	60	116	38	84	47	91	130	75	63	59	68	55	224	140	181	145	217	165	119	108	134	137	145	147	2748
Anomalocardia flexuosa	III	2	TGI (SUS)	*	39	32	30	45	8	45	132	65	163	39	120	80	13	157	15	116	3	174	194	371	245	168	287	260	2801
Streblospio benedicti	III	2	TGIII (DEPSUP)	*	0	1	1	1	5	14	0	0	0	0	2	0	21	49	22	48	24	43	4	0	0	2	2	2	241
Mytella sp	III	2	TGI (SUS)	*	9	4	14	6	14	18	0	0	0	0	2	1	1	19	2	19	4	39	14	12	2	24	6	25	235
Monochorophium acherusicum	III	2	TGII (DETRI)	*	4	4	0	0	0	0	5	2	0	0	9	0	0	5	0	1	1	20	18	3	2	10	15	57	156
Spiophanes duplex	III	2	TGII (DETRI)	*	3	2	0	1	0	0	0	0	0	0	14	0	0	5	1	6	0	1	6	2	3	9	5	9	67
Nemertea	III	2	TGII (DETRI)	*	11	3	9	5	3	3	0	2	1	1	2	1	1	2	1	4	1	8	1	0	0	2	4	2	67
Alitta succinea	III	2	TGII (DETRI)	*	0	0	2	0	2	0	0	0	1	0	0	0	0	5	0	3	0	0	0	1	2	0	0	0	16
Spiochaetopterus costarum	III	2	TGIII (DEPSUP)	*	0	1	1	1	0	0	0	1	0	0	1	0	0	0	0	0	0	1	0	0	0	1	0	2	9
Spiophanes sp	III	2	TGII (DETRI)	*	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Notomastus sp	III	2	TGIV (DEPSUB)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Neanthes succinea	III	2	TGII (DETRI)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sternaspis sp	III	2	TGIII (DEPSUP)	*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Laeonereis culveri	IV	3	TGIII (DEPSUP)	Polychaeta 1	886	126	1418	365	2336	281	31	4	8	10	14	9	48	122	115	97	249	135	23	13	22	39	38	175	6564
Heleobia australis	IV	3	TGIII (DEPSUP)	*	152	3	100	33	48	32	16	27	113	28	76	254	145	180	68	122	133	332	386	263	168	541	141	446	3807
Heteromastus sp	IV	3	TGIV (DEPSUB)	Polychaeta 2	60	19	27	14	52	21	9	1	9	3	2	0	9	107	20	175	6	146	7	7	24	22	30	13	783
Prionospio heterobranchia	IV	3	TGIII (DEPSUP)	Polychaeta 3	0	0	0	1	1	1	0	0	0	0	1	1	1	22	0	16	1	13	36	20	55	52	29	61	311
Polydora sp	IV	3	TGIII (DEPSUP)	Polychaeta 4	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	0	0	2	0	0	1	1	6	14
Prionospio sp	IV	3	TGIII (DEPSUP)	Polychaeta 5	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Polydora cornuta	IV	3	TGIII (DEPSUP)	Polychaeta 6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	1	4
Tubificinae sp1	V	3	TGIV (DEPSUB)	Polychaeta 7	3612	710	2579	707	3563	1065	123	314	57	218	76	405	2394	437	1972	347	1737	479	99	202	263	117	220	87	21783
Paranais cf. frici	V	3	TGIV (DEPSUB)	Polychaeta 8	0	0	0	0	0	0	0	0	0	0	0	0	40	411	209	45	370	243	0	0	2	0	58	47	1425
Capitella sp	V	3	TGIV (DEPSUB)	Polychaeta 9	812	7	581	39	609	34	5	0	1	0	1	4	108	64	58	26	172	12	3	17	3	2	6	3	2567

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Consistency of responses of macrofaunal trophic guilds to sewage discharges at nested scales of variation in a subtropical estuary

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Abstract

The trophic structure of benthic assemblages may integrate functional responses to organic enrichment. However, such responses may vary depending on prevailing environmental drivers, distinct methods to categorize trophic guilds or the use of abundance and biomass as predictive measures. This paper assesses the consistency of variation patterns of trophic guilds exposed to distinct levels of sewage contamination. We used: (i) two different methodological approaches for trophic group assignment and (ii) both abundance and biomass as predictive variables in each approach. We applied a hierarchical sampling design, nested at two spatial (10^3 and 10^2m) and three temporal scales (Seasons, Events and Fortnights) in non-vegetated tidal flats of the subtropical Paranaguá Bay (Southern Brazil). Regardless of applying a broader versus a narrower classification of trophic guilds, both methodologies were able to indicate the benthic trophic status at similar spatiotemporal scales of variation. The spatial scale of *condition* (10^3m) often interacted with time, meaning that the differences between contaminated and non-contaminated sites were not of similar magnitudes for all temporal scales. The remaining scales of *tidal flat*, *fortnight* and *event* also varied, representing additional or secondary structuring factors in the system. The use of biomass as a predictive variable increased the consistency between the patterns of variation in both methods of trophic guild assignment. Regardless of the method to trophic guild assignment, a successful application to pollution detection will not only depend on the feeding mode of the species and the quality or quantity of organic enriched material, but also on its level of tolerance to other pollution-stressors like hypoxia. Manipulative experiments are still needed to unravel the complex interplay of ecological processes that structure trophic assemblages.

Keywords: *trophic guilds; indicators; organic enrichment; hierarchical design; spatiotemporal variation.*

Introduction

The trophic structure of benthic assemblages may be used to integrate and assess functional responses of the biota along estuarine gradients (Brown et al., 2000). Macrobenthos are exposed to organic matter of varying quality, flux and texture that favor distinct feeding modes, and such responses can be quantified based on the dominant feeding strategies. Changes in the relative dominance of suspension and deposit-feeders may thus provide evidence of increasing particulate organic matter in the sediment (Word, 1978, Weston, 1990, Gaston, 1998).

Estuarine community composition is simultaneously influenced by non-anthropogenic or background drivers that may interact with anthropogenic processes, typically masking direct causal relationships (Dauvin, 2007, Elliot and Quintino, 2007). In such habitats, it is a major challenge to evaluate the relative influence and interaction of different potential drivers, including the effects of sewage discharges, on macrofaunal patterns (Brown et al., 2000). Such interactions lead to patchy spatial configurations that in turn are likely to change through time (Underwood and Chapman, 2014).

Seasonal variations of macrofaunal patches are frequent, yet much of our understanding of soft-sediment ecology comes from studies that have not properly separated seasonal variation from other temporal patterns because of poor replication (Murphy et al., 2009). Hierarchical sampling designs have been increasingly applied to determine the spatial scales at which species and communities vary and to distinguish human from natural disturbance (e.g. Morrisey et al., 1992, Noren and Lindegart, 2005). In such designs, variation among the factors of interest is properly compared to the magnitudes of variation that occur within and among the spatial and temporal scales of interest.

Different classifications of macrobenthic trophic guilds may result from different combinations of criteria that involve the food source and feeding apparatus (Jumars et al., 2015). Distinctc classifications may lead to varying interpretation of faunal variation patterns. In addition, assessments of the functional importance based on the relative abundance of individual species may be of poor ecological value, mainly in stressed areas where numerically dominant species tend to be small-bodied opportunists. Thus, species' biomass data might be a more relevant measure of the effects of organic enrichment. The super-abundance of one or a few dominants could also mask or confound the distinction between impacted and non-impacted sites (Warwick et al., 2002). The validity in assessing macrofaunal responses to organic pollution by combining different methods of trophic guild classification with both abundance and biomass measures along properly replicated scales of variability remains to be tested.

This paper assesses the consistency of spatiotemporal patterns of variation of macrobenthic trophic guilds exposed to distinct levels of sewage contamination by combining: (i) two different methodological approaches for groups' assignment and (ii) both abundance and biomass as predictive variables in each approach. We first examined the spatiotemporal variation in abundance and biomass of trophic guilds using the broad categories of ITI index (Word, 1980) versus a more detailed classification based on Jumars et al. (2015) and other authors. We applied a hierarchical sampling design, nested at two spatial (of 10^3 and 10^2m) and three temporal scales (Seasons, Events and Fortnights) in non-vegetated tidal flats of the subtropical Paranaguá Bay (Southern Brazil) subjected to distinct levels of sewage contamination. We expected to find significant differences between trophic guilds near and guilds far from the sewage source, namely our largest spatial scale (10^3m), or the *Condition* scale. If organic pollution is the main structuring force in the system, then significant differences between contaminated and non-contaminated (or the largest spatial scale) would be greater than the natural differences expressed by the alternation between summers and winters (or the temporal scale of *Seasons*). Failing to reject this hypothesis would validate the use of macrofaunal trophic guilds as reliable indicators of organic contamination.

We also assessed spatiotemporal variations of trophic guilds generated by the two methods. If there is a significant shift in the proportion of trophic guilds from the healthy to degraded environments, then the larger differences would also be detected at the scale of condition (10^3m). Consistent approaches to trophic guilds assignment would show similar patterns of spatiotemporal variation in terms of abundance and biomass. We then correlated the trophic guilds in both methodologies to a set of abiotic variables, and expected stronger correlations to chemical proxies of contamination.

Materials and methods

Study area

The tidal flats investigated in this study are located along the Cotinga sub-estuary within the Paranaguá Estuarine Complex (PEC), Southern Brazil, as described in Brauko et al. (2014). The sub-estuary is the dilution path for sewage discharges from Paranaguá city. This sharp and compressed gradient of contamination towards the mouth of the channel has been previously evidenced by other studies (Abreu-Mota et al., 2014, Barboza et al., 2013, Martins et al., 2010). Two tidal flats were sampled in the inner *contaminated site* (CS) and two tidal flats in the *non-contaminated site* (NS), distant from the source of sewage discharges (Fig. 1).

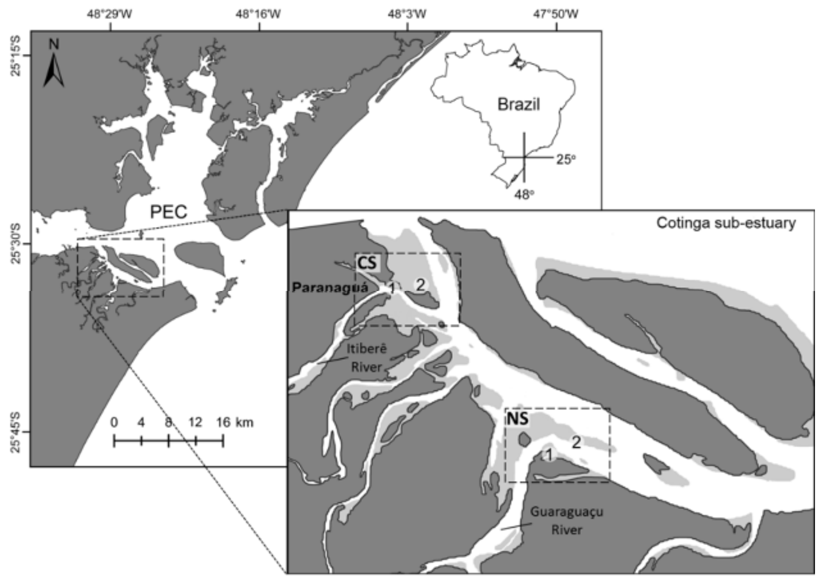

Figure 1: Paranaguá Estuarine Complex (PEC) and Cotinga sub-estuary. Tidal flats 1 and 2, sampled at Contaminated site (CS) and Non-contaminated site (NS).

Sampling design and sample processing

A hierarchical sampling design was used to assess the spatiotemporal variability of the macrofaunal trophic guilds between two distinct *conditions* in terms of sewage discharges (CO - Contaminated and Non-contaminated), distanced at the largest scale of 10^3m (Fig. 2). In each *condition,* the fauna was sampled in two non-vegetated *tidal flats* (TF - TF1 and TF2), distanced at the scale of 10^2m, with 12 replicates each, distanced at the scale of 10 m. Temporal variation was investigated by repeating the samplings in three consecutive *fortnights* (FO - F1 to F3), nested in each of two sampling *events* (EV - E1 and E2), within two *seasons* (SE - summer and winter). The five factors linear model comprised two spatial factors (CO, two levels; TF, two levels) orthogonal to three temporal factors (SE, two levels; EV, two levels; FO, three levels). Our top factors *season* and *condition* were fixed, and all other factors were random.

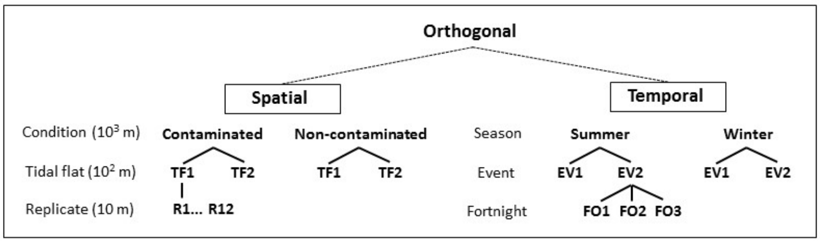

Figure 1: Sampling design for spatiotemporal variance in abundance and biomass of macrofaunal trophic guilds. Spatial factors TF1 and TF2 nested in Contaminated and Non-contaminated sites orthogonally arranged in temporal factors FO1, FO2 and FO3, nested in EV1 and EV2 within Summer and Winter. n = 12 replicates at each TF.

All samplings were performed during the spring low tide, and plots were placed parallel to the waterline, at similar tidal levels, to avoid the putative interference of macrofaunal zonation. Macrofauna was collected using plastic core tubes (10 cm diameter, 10 cm deep), and all plots were placed parallel to the water line, at similar tidal levels. All samples were sieved through a 0.5 mm mesh, fixed in 6% formaldehyde and preserved in 70% alcohol. In the laboratory, all organisms were counted and identified to the lowest possible taxonomic level. Biomass was derived from dry weight by drying samples at 60°C until constant weight.

In each survey, three replicates were sampled at each tidal flat for surface sediment temperature, salinity of the percolate water and depth of the apparent redox discontinuity layer, as the boundary from light color (oxygen-rich) to a darker layer of sediment (oxygen-poor). Sediment samples were also taken to determine total phosphorus (TP), total nitrogen (TN) and total organic carbon (TOC) contents, as well as granulometric parameters (mud content, grain size, sorting, $CaCO_3$ and organic matter). The concentrations of TN and TP were obtained according to Grasshoff et al. (1983), and TOC was determined with the oxidation method described by Strickland and Parsons (1972). Sediment samples were processed according to Suguio (1973), and granulometric parameters were determined on the R software (R

Development Core Team, 2013) using the package rysgran (Gilbert et al. 2012). Calcium carbonate ($CaCO_3$) and organic matter contents were determined using acid digestion and furnace combustion at 550°C for 1 hour, respectively.

Fecal sterol contents were used as chemical indicators of the level of sewage derived material. Fecal sterols are reliable stable organic markers of sewage input, and their concentration is significantly correlated to macrofaunal variation patterns (Barboza et al., 2013). In every survey, one sediment sample was taken from each tidal flat for fecal sterol analysis, with the method described by Kawakami and Montone (2002). Instrument specifications and calibration procedures are described by Montone et. al. (2010). The detection limits (DLs) were <0.01 µgg-1 for all analyzed compounds. Measured concentrations of target steroids in the IAEA-417 reference material were within 90–110% of the certified values provided by the International Atomic Energy Agency (IAEA).

Macrobenthic trophic guild assignments

Assignment of benthic fauna to trophic guilds of ITI index, hereafter called *broad method,* were based on Word (1979). Four trophic groups were stablished: TG1 corresponded to suspension feeders, TG2 was formed by a combination of suspension and surface-detritus feeders (0.5 cm upper layer of the sediment), TG3 was composed of surface deposit feeders (upper 5 cm), and TG4 by subsurface deposit feeders (upper 10 cm). TG1 and TG2 are a subdivision of detritus feeders that usually lack sediment grains in stomach contents, and both deposit feeders of TG3 and TG4 have either sediment and/or plant debris in stomach.

The second approach for trophic guild assignment, or the *narrow method*, was based on both feeding behavior and food type. Trophic groups included suspension and filter feeders (SUS), surface deposit feeders (SURDEP), and subsurface deposit feeders (SUBDEP). Taxa with facultative (split) feeding as either SUS or SURDEP depending on water-current intensity, like some spionids, were classified into the interface feeder group, INT (Dauer *et al.* 1981). Carnivores, omnivores, predators and scavengers formed the last group (COPSH). Herbivores were rare, representing less than 0.03% of the total abundance, and thus were grouped within the COPSH group. Trophic assignments were based on feeding morphology, feeding behavior and food preferences documented in the literature (Jumars et al., 2015; Gamito et al., 2012; Magalhães and Barros, 2011; Venturini et al., 2011; Abrahão et al., 2010; Jacobucci et al., 2009; Malaquias et al., 2009; Resgalla & Piovezan, 2009; David et al., 2008; Martin et al., 2008; Ramírez-Álvarez et al., 2007; Pardo and Amaral, 2006; Doi et al., 2005; Drumm, 2005; Pagliosa, 2005; Arruda et al., 2003; Dauer et al., 2003; Pardo and Dauer, 2003; Narchi and Domaneschi, 1993; Lana and Guiss, 1992).

Data analysis

Spatiotemporal variations in macrofaunal trophic guilds were assessed using the following linear mixed model ("µ" is the overall mean and "e" is the error term or residual, which corresponds to the variability among replicates):

$$X = \mu + CO_i + TF(CO)_{j(i)} + SE_k + EV(SE)_{l(k)} + FO(EV(SE))_{m(l(k))} + CO_i{*}SE_k + CO_i{*}EV(SE)_{l(k)} + CO_i{*}FO(EV(SE))_{m(l(k))} + TF(CO)_{j(i)}{*}SE_k + TF(CO)_{j(i)}{*}EV(SE)_{l(k)} + TF(CO)_{j(i)}{*}FO(EV(SE))_{m(l(k))} + e_{n(m(l))k(j(i))}$$

We first applied the model to a permutational multivariate analysis of variance (PERMANOVA) based on a Bray-Curtis dissimilarity matrix to simultaneously test responses of all trophic guilds, with both abundance and biomass data sets. Secondly, we performed a series of univariate analysis using the same model to assess the patterns of variability of individual trophic guilds. To this purpose, we used a permutational analysis of variance, sometimes referred to as PERANOVA (e.g., Fanelli et al., 2011, Ezgeta-Balić et al., 2011), based on the Euclidean distance (Anderson, 2001). The use of Euclidean distance as the measure of association makes this univariate test similar to a traditional ANOVA (Anderson et al.., 2008). However, since five of the eleven terms generated by the model could not be tested, they were approximated using the linear combination of effects procedure (Satterthwaite, 1946) implemented by Anderson et al. (2008). The approximation was performed in the PERMANOVA+ add-on of the PRIMER 6 software (Clarke and Gorley, 2006). The high number of sources of variation inherent in our linear model could raise the occurrence of type I error. To increase the robustness of the analysis, estimates of components of variation were also calculated to evaluate the proportion of variation attributable to each source (or term), specially to the residuals. Negative estimates of components of variation were set to zero, and the proportion estimates of the remaining factors were recalculated.

A Canonical Correspondence Analysis (CCA) was then performed to correlate faunal responses to abiotic variables representing the potentially structuring drivers operating in the sub-estuary, related to both natural and sewage-derived processes. In this analysis, we used the abundance of trophic guilds per tidal flat of all surveys (n = 92). We produced two CCAs using the TGs of the broad and the narrow methods. A stepwise selection model was used for the CCAs, in which the term choice is based on Akaike's Information Criterion (AIC) by permutation tests. The significance of the canonical axes and vectors was tested by individual ANOVAs.

Results

General structure of trophic guilds

The trophic structure resulted very similar between the two distinct methodologies (Fig. 3). All trophic guilds were present in both contaminated and non-contaminated tidal flats, but with varying abundance patterns. Contaminated sites were numerically dominated by subsurface deposit feeders (SUBDEP and TG4), commonly representing more than 50% of the total abundance of the tidal flats. The remaining trophic guilds were less abundant under sewage discharges than in sites further away from the pollution source. In Non-contaminated sites, the proportion of trophic guilds was more evenly distributed among sites despite the higher abundances of surface deposit feeders (SURDEP and TG3).

There is an opposite relationship between the abundance- and biomass-based trophic patters. For example, at contaminated sites, subsurface deposit feeders were numerically dominant but maximal biomass was represented by suspension feeders (SUS and TG1), which in turn were less abundant. Subsurface deposit feeders were represented largely by small-bodied oligochaetes (see Annex) with a small contribution to biomass, and suspensivores by large-bodied bivalves.

The trophic structure of macrobenthic assemblages did not seem to respond to seasonal changes. The only exception were the interface feeders (INT), which increased in abundance and biomass from summer to winter in both years.

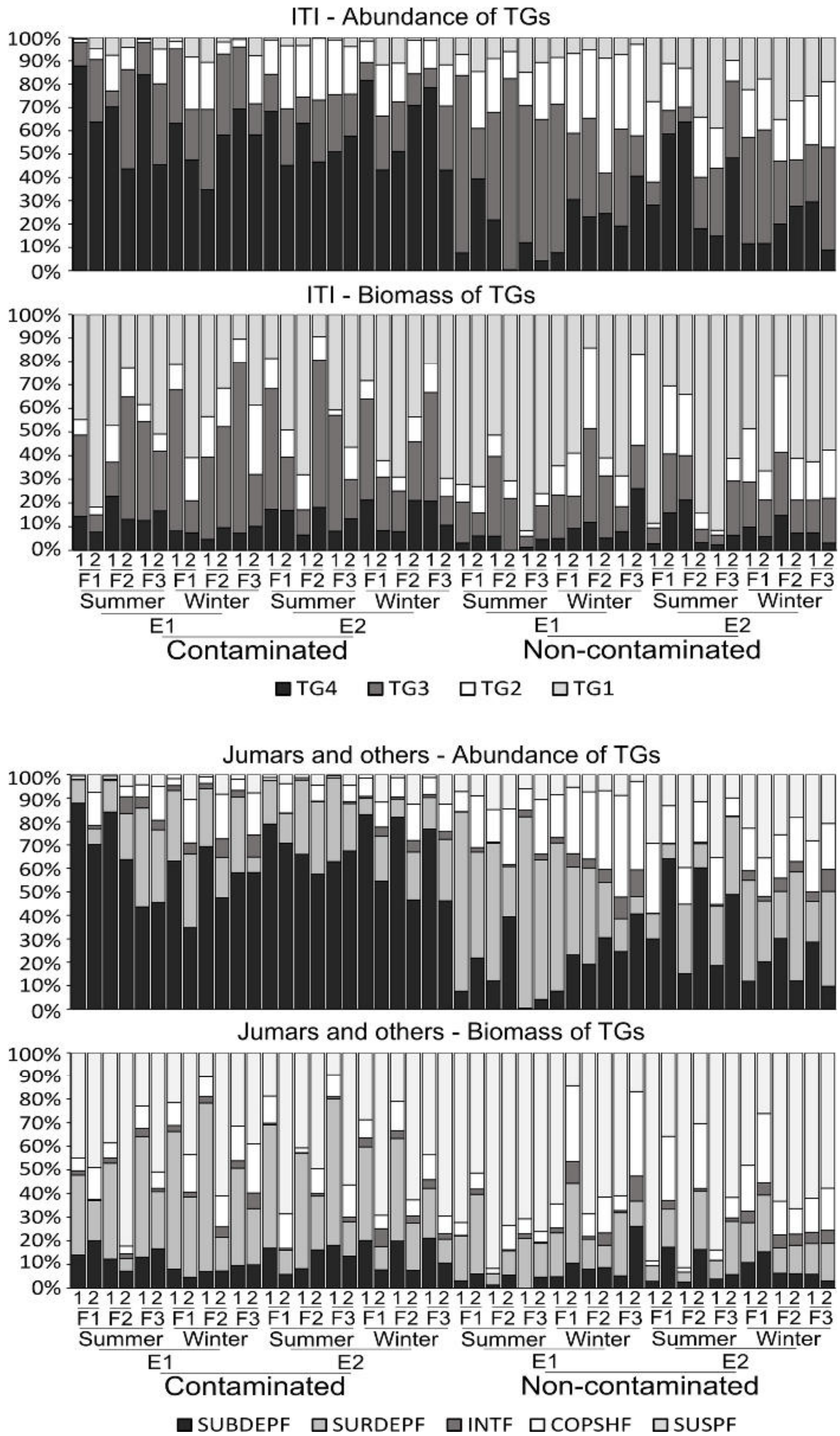

Figure 3: Variation in the relative abundance and biomass of trophic guilds per tidal flat (1 and 2) according to the groups of the ITI index (Word, 1978) and the groups based on the classification of Jumars et al. (2015) and other authors. TG1: suspension feeders; TG2: detritus feeders; TG3: surface deposit feeders; TG4: subsurface deposit feeders. SUSP: suspension feeders; SURDEP: surface deposit feeders; SUBDEP: subsurface deposit feeders; INT:

Interface or suspension/surface deposit feeders; COPSH: Carnivores, omnivores, predators, scavengers, and herbivores.

Consistency of patterns shown by PERMANOVAs and PERANOVAs

The general patterns of spatiotemporal variation between the two methods for TGs assignment resulted quite similar in terms of sensitivity to pollution (Tables 1 and 2). The trophic structure of assemblages and trophic guilds significantly varied between the distinct conditions of contamination through time, which was supported by high components of variation in several cases (Tables 1 and 2; terms COxEV, COxSE, COxFO). Thus, for most analysis the differences between contaminated and non-contaminated sites (shown in Fig. 3) were not of similar magnitudes for all temporal scales. Nevertheless, in the broad method using both abundance and biomass measures, *condition* (CO) only interacted with *events* (EV), while for the narrow method this interaction involved all temporal scales, including *seasons* (SE) and *fortnights* (FO).

The variability in the scale of *seasons* was only significant in the narrow methodology using abundance as a predictive variable. In this approach, both trophic guilds of COPSH and INT were largely significant at the SE scale, which was corroborated by the high percentages of components of variation (Table 2; term SE).

Differences at the remaining scales of *tidal flats* (10^2m), *fortnights* and *events* were also significant for abundance and biomass in both methodologies, interactively or not. However, these factors were not always a very large source of variation (note the low proportions of components of variation for these factors in analyses in Tables 1 and 2).

Table 1 – Results of the PERMANOVA for the trophic guilds of ITI and PERANOVAs for each of the TGs separately. TG1: suspension feeders; TG2: detritus feeders; TG3: surface deposit feeders; TG4: subsurface deposit feeders.

		Abundance														
		Permanova			TG4			TG3			TG2			TG1		
	df	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)
CO	1	207450	2,31	16,6	2692200	2,03	18,6	115630	0,86	0,0	55225	1,07	1,0	2630,8	0,68	0,0
SE	1	42489	1,36	1,9	15,016	0,42	0,0	12183	1,61	2,1	1144,7	0,30	0,0	6840,7	1,19	2,9
TF(CO)	2	65244	5,98	14,6	1265600	25,57**	24,6	103060	2,20	10,7	20392	0,83	0,0	1065,7	2,74	3,3
EV(SE)	2	31240	1,56	3,3	110540	1,45	4,7	28373	0,52	0,0	57533	2,09	12,2	4963,9	7,13**	21,4
COxSE	1	6006,3	0,45	0,0	1630,1	0,57	0,0	4618,3	0,54	0,0	36896	0,74	0,0	118,27	0,11	0,0
FO(EV(SE))	8	11294	3,44***	6,4	49159	1,49	4,9	17583	3,61*	7,2	4207,7	1,62	1,9	323,71	2,72*	3,0
COxEV(SE)	2	29089	1,99*	8,6	82240	1,29	5,1	86358	1,76	14,9	54106	2,01	22,3	3407,9	8,11**	30,3
TF(CO)xSE	2	8148,3	0,75	0,0	7578,1	0,15	0,0	8256,3	0,18	0,0	29086	1,18	3,5	1096,8	2,82	6,9
COxFO(EV(SE))	8	5365,5	1,63	3,4	39529	1,20	4,4	5126,3	1,05	0,3	3583,8	1,38	2,3	45,97	0,39	0,0
TF(CO)xEV(SE)	4	10906	3,32***	8,2	49497	1,50	5,7	46807	9,61***	31,9	24591	9,48***	34,4	388,87	3,26*	5,3
TF(CO)xFO(EV(SE))	16	3285,2	4,41***	8,2	32950	6,49***	12,9	4872,9	5,64***	9,1	2594,2	13,24***	11,3	119,21	3,89***	5,2
Res	528	744,48		28,8	5076,3		19,1	864,36		23,7	195,94		11,0	30,652		21,6
Total	575															
		Biomass														
		Permanova			TG4			TG3			TG2			TG1		
	df	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)
CO	1	58469	1,46	2,9	4012,2	8,80*	36,7	21136	1,55	4,3	638,4	0,33	0,0	4315,4	0,30	0,0
SE	1	27575	1,72	2,0	335,8	1,29	0,9	7,3803	0,36	0,0	4693,4	6,20	14,0	3795,6	0,43	14,0
TF(CO)	2	27410	4,53*	6,1	352,0	5,33	5,8	10508	5,39	9,0	113,3	1,71	0,3	65015	4,25	0,3
EV(SE)	2	7760,6	0,74	0,0	305,5	1,76	3,0	2029,6	0,64	0,0	633,9	1,84	2,3	9793,1	0,67	2,3
COxSE	1	5934,1	0,42	0,0	5,9	0,62	0,0	83,114	0,26	0,0	98,7	0,08	0,0	55842	2,00	0,0
FO(EV(SE))	8	8474,7	2,79***	4,7	125,9	3,83*	5,7	2155,8	3,65*	4,9	318,3	4,27**	5,1	6585,6	1,34	5,1
COxEV(SE)	2	16813	2,10*	6,0	111,5	0,83	0,0	4428,1	1,87	4,9	1993,3	15,28***	27,1	1011,9	0,34	27,1
TF(CO)xSE	2	11818	1,95	3,3	5,4	0,08	0,0	3455	1,77	3,2	133,4	2,01	0,9	34524	2,26	0,9
COxFO(EV(SE))	8	3400,2	1,12	0,6	108,6	3,30*	9,2	738,27	1,25	0,9	69,0	0,92	0,0	2031,5	0,41	0,0
TF(CO)xEV(SE)	4	6046,6	1,99*	3,5	66,1	2,01	2,7	1949,6	3,30*	5,7	66,4	0,89	0,0	15300	3,10*	0,0
TF(CO)xFO(EV(SE))	16	3036,9	1,91***	5,0	32,9	3,15***	5,5	590,48	1,37	2,0	74,6	1,57	2,3	4928,5	1,34	2,3
Res	528	1591,9		65,9	10,4		30,5	430,11		65,1	47,5		48,0	3676		48,0
Total	575															

Some trophic guilds derived from both methods did not vary at the scale of *contamination* (CO), such as the abundance of SUBDEP and TG4 (which were composed of the same taxa, see Annex), COPSH and TG2 (also composed of the same taxa), and the biomass of SUS and TG3. Nevertheless, a distinct pattern of variation was evidenced for SUBDEP and TG4 when using biomass as measure, in which the scale of CO was significant, non-interactively (also note the large components of variation for this term in Tables 1 and 2).

Table 2 - PERMANOVA results among the trophic guilds based on Jumars et al., 2015 (and other sources) and PERANOVA results for each separate trophic guild. SUSP: suspension feeders; SURDEP: surface deposit feeders; SUBDEP: subsurface deposit feeders; INT: Interface or suspension/surface deposit feeders; COPSH: Carnivores, omnivores, predators, scavengers and herbivores.

		Abundance																	
		Permanova			SUBDEP			SURDEP			INT			COPSH			SUS		
	df	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)
CO	1	208070	2,30	16,3	2685600	2,03	21,6	103600	0,79	0,0	377,0	0,84	0,0	42,8	0,52	0,0	2605,3	0,67	0,0
SE	1	59136	2,10**	4,8	2,1267	0,42	0,0	4241,3	1,27	1,0	2889,1	20,07*	32,2	22563	115,95***	52,4	6895,9	1,21	3,1
TF(CO)	2	69583	7,00	15,8	1262600	25,41**	37,6	104640	2,40	12,2	66,5	1,39	0,4	622,8	1,62	1,1	1070,0	2,75*	3,3
EV(SE)	2	25154	1,32	1,8	110390	1,45	1,4	29265	0,56	0,0	108,4	1,36	0,9	81,6	0,17	0,0	4916,4	7,11	21,2
COxSE	1	3338	0,40***	0,0	1732,6	0,57	0,0	5556,5	0,54*	0,0	42,3	0,19	0,0	137,1	1,63	0,9	121,9	0,11*	0,0
FO(EV(SE))	8	11481	3,72	6,7	49389	1,50	1,5	16710	3,72	7,3	62,0	1,51	1,5	798,2	6,92***	9,4	318,3	2,69**	2,9
COxEV(SE)	2	25337	1,87	7,0	82472	1,29	1,6	82130	1,79	15,4	440,4	4,50*	17,3	203,1	0,65	0,0	3425,2	8,10	30,4
TF(CO)xSE	2	7704,4	0,77*	0,0	7641,7	0,15	0,0	8451,5	0,19	0,0	37,9	0,79	0,0	116,3	0,30	0,0	1109,1	2,85	7,0
COxFO(EV(SE))	8	5245,6	1,7***	3,4	39472	1,20	1,2	4625,9	1,03	0,2	59,3	1,45	2,5	106,9	0,93	0,0	48,1	0,41*	0,0
TF(CO)xEV(SE)	4	9945,6	3,22***	7,3	49695	1,51	2,1	43651	9,72***	31,4	47,8	1,17	0,6	384,0	3,33*	5,0	389,7	3,29***	5,3
TF(CO)xFO(EV(SE))	16	3085,5	4,00	7,4	32943	6,5***	10,4	4492,3	5,46***	8,8	41,0	3,75***	8,3	115,4	2,83***	4,1	118,5	3,85	5,1
Res	528	772,3		29,5	5074,9		22,7	822,06		23,7	10,9		36,3	40,8		27,0	30,8		21,7
Total	575																		
		Biomass																	
		Permanova			SUBDEP			SURDEP			INT			COPSH			SUS		
	df	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)	MS	Pseudo-F	CV (%)
CO	1	57189	1,47	2,8	4017,4	8,82*	36,7	3011,7	1,23	0,4	674,7	1,11	1,3	21,9	0,07	0,0	47,0	0,21	0,0
SE	1	31809	1,87	2,5	334,3	1,29	0,9	1158,6	0,21	0,0	47,2	0,19	0,0	14,2	0,16	0,0	141,4	0,03	0,0
TF(CO)	2	27077	4,39*	5,9	351,9	5,31	5,8	248,7	1,83	0,1	112,0	1,37	1,1	248,3	2,45	1,4	1977,9	2,12	0,4
EV(SE)	2	7776	0,74	0,0	306,0	1,76	3,0	5780,8	9,23**	7,7	656,0	4,21*	17,8	616,7	1,27	1,3	39606,0	8,87***	14,5
COxSE	1	5780,1	0,42	0,0	6,1	0,62	0,0	839,3	0,36	0,0	389,1	0,80	0,0	1532,4	1,03	0,5	785,4	0,56	0,0
FO(EV(SE))	8	8383,2	2,74***	4,5	125,9	3,81*	5,7	622,9	0,51	0,0	77,8	5,33**	6,6	413,1	10,97***	10,5	3861,1	1,33	1,1
COxEV(SE)	2	16007	1,94*	5,2	111,3	0,82	0,0	2316,5	3,18*	6,0	567,7	5,93***	33,7	1495,3	4,85**	22,6	2634,4	2,20	2,3
TF(CO)xSE	2	12521	2,03	3,6	5,3	0,08	0,0	430,7	3,16	0,7	19,0	0,23	0,0	89,4	0,88	0,0	428,3	0,46	0,0
COxFO(EV(SE))	8	3675,6	1,20	1,0	108,8	3,2*	9,2	977,3	0,80	0,0	16,5	1,13	0,4	214,7	5,71**	9,9	1585,9	0,55	0,0
TF(CO)xEV(SE)	4	6170,7	2,02*	3,5	66,2	2,01	2,7	136,2	0,11	0,0	81,7	5,59**	9,3	101,4	2,69*	2,4	932,8	0,32	0,0
TF(CO)xFO(EV(SE))	16	3056,1	1,86***	4,8	33,0	3,16***	5,5	1221,8	2,99***	12,1	14,6	2,83***	3,9	37,6	0,98	0,0	2896,1	1,96*	0,0
Res	528	1639,9		66,3	10,4		30,5	407,8		72,9	5,2		25,9	38,5		51,5	1479,6		81,7
Total	575																		

The two CCAs performed on macrofaunal trophic guilds displayed clear spatial gradient trends related to contamination and organic enrichment (Figs. 4a and b). The CCA for the trophic guilds of the broad method (Fig. 4a) explained 53% of the total variance, of which 48.3% was accounted by the first canonical axis ($F = 45.19$; $p = 0.005$) and 4.9% by the second axis ($F = 4.39$, $p = 0.01$). The significant abiotic parameters that best fitted the CCA model were coprostanol / coprostanol + cholestanol ratio, total organic carbon and organic matter contents, all indicative of fecal and organic inputs. The ordination of samples along axis 1 showed a clear environmental gradient formed by samples from the Contaminated site in the right side of the bi-plot, while samples from the Non-contaminated site in the opposite side. Contaminated samples were associated to TG4, of subsurface deposit feeders, and with high coprostanol / coprostanol + cholestanol ratio. This same ratio was negatively correlated to samples from the Non-contaminated site as well as TG1 (suspension feeders), and is considered a stable organic marker for the presence of sewage derived material. To a lesser extent, TG2 (detritivores) and

TG3 (surface deposit feeders) were related to total organic carbon and organic matter contents, all associated to the second axis of weakest explanatory power in the model.

The CCA showed similar dispersion patterns for the trophic guilds of the narrow method, with 55.8% of explanation for the total variance (Fig. 4b). Nevertheless, these patterns along the contamination gradient were not as strong since samples were more densely grouped around the origin region of the model and thus poorly explained. The first canonical axis accounted for 46.5% ($F = 46.35$, $p = 0.005$) and the second axis for 6.3% ($F = 6.32$, $p = 0.005$) of the variation. A segregation between contaminated and non-contaminated samples was also observed, with an association between contaminated samples and the abundance of subsurface deposit feeders (SUBDEP). This segregation was mainly related to higher coprostanol / coprostanol + cholestanol ratio in the Contaminated site, an evidence of responses to sewage discharges. Conversely, the trophic group formed by carnivores, omnivores, predators, scavenges and herbivores (COPSH) and to a lesser extent the suspensivores (SUS) were associated to non-contaminated samples and negatively linked to the fecal sterols ratio. The weakest relationships along axis 2 involved interface (INT) and subsurface deposit feeders (SUBDEP) with total organic carbon and sediment temperature.

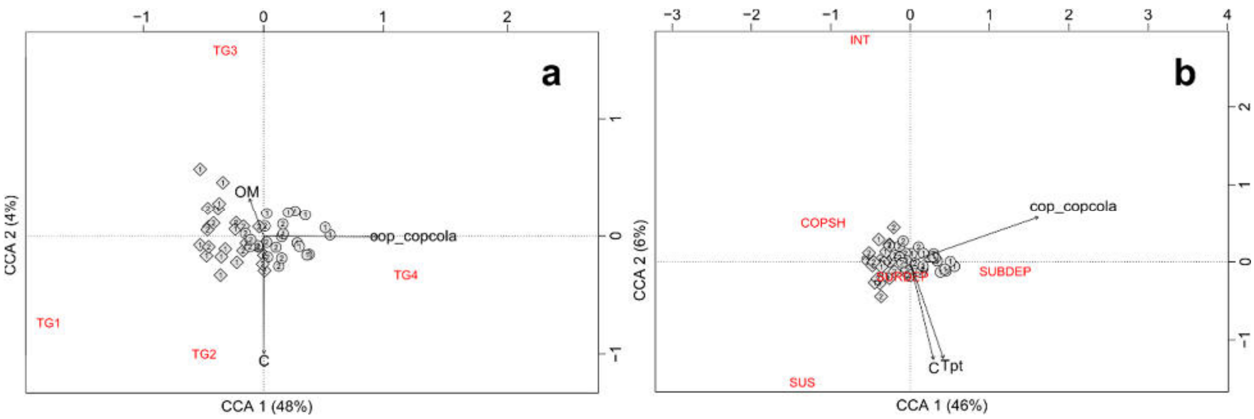

Figure 4: CCAs of trophic groups (according to the classification of: a- ITI index or *broad method* and b- Jumars and others or *narrow method*) with selected abiotic variables. Plots correspond to Tidal flats 1 and 2 of Contaminated (circles) and Non-contaminated (diamonds) sites. Vectors: cop_copcola: coprostanol / coprostanol + cholestanol ratio; C: total organic carbon; OM: organic matter; Tpt: temperature. Labels of trophic groups as in Fig. 3.

Discussion

The largest spatial scale or the scale of contamination (10^3m) was almost always the most significant source of variability in the trophic structure of macrofaunal assemblages and trophic guilds assigned with both methodologies. The recurrently significant scale of CO is a strong evidence of distinct trophic structures attributable to higher or lesser sewage input. In addition, trophic guilds were more closely related to sediment contaminant concentrations than to other abiotic variables (e.g. silt and clay content or grain size). The coprostanol / coprostanol + cholestanol ratio was mostly responsible for the spatial configuration of trophic guilds, irrespective of classification method, with higher concentrations in contaminated sites (as shown by CCA results). This reliably emphasizes the indicative value of macrofaunal trophic guilds to organic contamination as predicted in the classical work of Pearson and Rosenberg (1978) and established by many other studies (Brown et al., 2000, Afli et al., 2008, Culhane et al., 2014).

Our results also indicated that a more detailed TG assignment (the narrow method) does not improve the distinction between contaminated and non-contaminated sites. Broad or more inclusive macrofaunal groups may not be sensitive to moderate pollution (Maurer et al., 1999). In such conditions, mild or moderately tolerant organisms are likely to be numerically dominant, and still able to indicate pollution. The underestimation of these groups may decrease the effectiveness of environmental quality assessments. This is not necessarily true in terms of feeding modes.

Despite the moderate eutrophication state of our contaminated tidal flats (as shown in Souza et al., 2013), the inclusion of more trophic categories did not improve the indicative value of the trophic approach. Conversely, the newly added TGs of COPSH and INT were more responsive to seasonal variations than to the contamination levels. These trophic guilds include the carnivores *Sigambra* sp. and *Glycinde multidens,* and the interface feeders *Streblospio benedicti* and *Spiophanes duplex* (see Annex for species details). These species might be stimulated by excessive organic matter but still thrive under normal conditions (Borja et al., 2000). The interpretation of trophic patterns must be carefully done since the responses of feeding guilds to organic input might change according to the level of tolerance of the species included in each trophic guild. Tolerance levels are almost inherent to the life-strategy of organisms, whether closer to *k*- or *r*-strategy, which could be linked to their feeding mode. The interface feeders and carnivores might respond to natural processes such as the frequent deposition and resuspension of particulate matter during tidal cycles (Wieking and Kröncke, 2005), which are likely to change seasonally. The establishment of such causal relationships are yet to be properly explored using experimental approaches.

The patterns of composition and dominance shifts of trophic guilds to organic enrichment were in general similar to previous studies and indeed seem to fit typical responses to moderate organic inputs (e.g. Gaston et al., 1998, Brown et al., 2000, Grall and Chauvaud, 2002). The literature describes a progressive simplification of feeding guilds towards more eutrophicated conditions, with a tendency of dominance shifts from large-bodied suspension and surface deposit feeders to small-bodied subsurface deposit feeders. However, since the spatial scale of *condition* interacted with temporal scales in almost all analyses the resemblance between the trophic structure of contaminated and non-contaminated sites varied over time. This means that the trophic configuration of each condition might fit different parts of the models within the moderate range of responses depending on the sampling occasion. These models show theoretical linear responses according to the level of organic input, in fact likely to change at unpredictable temporal scales in real-world situations.

In the broad method for TG assignment, *event* was the only temporal scale to interact with *condition,* using both abundance and biomass. For the narrow method, on the other hand, all temporal scales (FO, SE and EV) interacted with *condition.* There were different patterns depending on whether abundance or biomass were used as predictors. If using biomass, the scales of variation become even more consistent between the methods (mainly between the Permanovas). Biomass seemed a more conservative ecological measure for pollution impact. Unlike abundance, biomass might be a most appropriate quantitative measure of species as it represents ingested food intake that was indeed turned into mass growth, or to resources provided by the organism to the ecosystem (Eleftheriou and McIntyre, 2005). Since smaller-sized organisms require less organic material to grow to adult sizes than larger organisms, it seems likely that more individuals of small size will be supported per unit of organic material than larger ones. This clearly evidences the success of small-bodied opportunists in organically enriched sites. Thus, the use of biomass would likely be a better, more powerful parameter of the community feeding relationships than their abundances (Word et al., 1980). The numerically dominant (and opportunists) subsurface deposit feeders (TG4 and SUBDEP) become largely significant at the scale of *condition* (no temporal interactions) only in terms of biomass. Interestingly, in our analyses the abundance of this important TG did not vary between conditions, mainly due to the largest variation at smaller scales (TF and TFxFO). In this sense, biomass was more efficient in showing differences between contaminated and non-contaminated sites.

The remaining scales of *tidal flat*, *fortnight* and *event* frequently interacted representing additional sources of variation in the data, though not as significant as the interactive scales involving *conditions*. They represent background signals from processes that operate at the scale of tidal flats through temporal scales different from seasons, with complex patterns of oscillation. This is likely to occur in estuaries, in which variations in tide, currents and winds, may further modulate the relationships between organic matter supply and consumer abundances (Grall and Chauvaud, 2002).

Both classifications of trophic guilds, either broader or narrower, were able to indicate the benthic trophic status at similar spatiotemporal scales of variation. For most analysis, the spatial scale of *condition* (10^3m) interacted with time, meaning that the differences between contaminated and non-contaminated sites were not of similar magnitudes for all temporal scales. The remaining scales of *tidal flat*, *fortnight* and *event* also varied, representing additional or secondary structuring forces in the system. Knowledge of spatial, temporal or interactive patterns of the macrofaunal trophic structure in contaminated estuarine areas is an important step towards distinguishing natural from man-induced processes that operate in the system, but does not, in itself, identify the many underlying ecological processes which cause these natural variations (Murphy et al., 2009). Manipulative experiments are now needed to unravel the complex interplay of ecological processes that cause macrofaunal variation and how these operate across a cascade of spatial and temporal scales. The use of biomass as predictive variable seems to improve the consistency between the patterns of variation for both methods of trophic guild assignment. Nevertheless, successful applications of trophic signatures to pollution detection will not only depend on the feeding mode of the involved species and the quality or quantity of organic enriched material, but also on their tolerance level to background or human disturbance.

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Annex - Taxa composition of each trophic guild in terms of percentage of numerically dominant taxa within each TG. The taxa accounted for at least 80% of the total abundance of the TG.

TGs according to Jumars and others			TGs according to ITI			% Total Dominance
TG	**Dominant taxa (feeding mode)**	**% Dominance within TG**	**TG**	**Taxa (feeding mode)**	**% Dominance within TG**	
SUS	*Anomalocardia flexuosa* (suspensivore)	66.2	TG1	*Anomalocardia flexuosa* (suspensivore)	66.2	4
	Tagelus divisus (suspensivore)	10.4		*Tagelus divisus* (suspensivore)	10.4	0,6
	Mytella sp. (suspensivore)	9.9		*Mytella* sp. (suspensivore)	9.9	0,6
COPSH	*Sigambra* sp. (carnivore)	61.8	TG2	*Sigambra* sp. (carnivore)	61.3	5,6
	Glycinde multidens (carnivore, omnivore, predator, scavenger)	10.5		*Glycinde multidens* (carnivore, omnivore, predator, scavenger)	10.4	0,9
	Bulla striata (omnivore)	7.3		*Bulla striata* (omnivore)	7.2	0,7
	Scoletoma tetraura (carnivore, omnivore)	6.0		*Scoletoma tetraura* (carnivore, omnivore)	6.0	0,5
SURDEP	*Laeonereis culveri* (surface deposit feeder)	55.9	TG3	*Laeonereis culveri* (surface deposit feeder)	52.6	14,4
	Heleobia australis (surface deposit feeder)	37.3		*Heleobia australis* (surface deposit feeder)	35.1	9,6
SUBDEP	Tubificinae sp1 (subsurface deposit feeder - deep)	77.6	TG4	Tubificinae sp1 (subsurface deposit feeder - deep)	77.6	44,7
	Tubificinae sp2 (subsurface deposit feeder - shallow)	10.8		Tubificinae sp2 (subsurface deposit feeder - shallow)	10.8	6,2
INT	*Streblospio benedicti* (interface feeder)	48.8				1,1
	Prionospio heterobranchia (interface feeder)	26.3				0,6
	Polydora websteri (interface feeder)	9.3				0,2

CONCLUSÕES GERAIS

Problemas relacionados à poluição por efluentes urbanos e anoxia são antigos, profundamente enraizados na sociedade e demandam urgente solução (Rockstrom et al., 2009). Os oceanos, de uma forma geral, ainda são vistos como imensos e profundos depositários com infinita capacidade de absorção de efluentes. De fato, uma fração não estimada da matéria orgânica proveniente de esgotos é consumida e assimilada por organismos bênticos, inevitavelmente entrando nas redes tróficas marinhas (Gonzalez-Silvera et al., 2015, Johnson et al., 2013). No entanto, esta capacidade de assimilação é reduzida com o desenvolvimento paralelo de condições de anoxia e hipoxia, até que o processo de eutrofização se complete e as perdas ecológicas, econômicas e os problemas à saúde humana sejam evidenciados. Protocolos científicos confiáveis e replicáveis nunca foram tão necessários para a tomada de decisões ambientais e para o desenvolvimento e melhoria das práticas de gerenciamento costeiro (Underwood & Chapman, 2013). Neste sentido, práticas lógicas e racionais podem contribuir grandemente não só para a melhoria de delineamentos amostrais, análises e interpretação de dados, mas também para a escolha de ferramentas confiáveis de detecção de impactos.

As práticas de proteção ambiental no Brasil ainda não se beneficiam plenamente da utilização do bentos marinho como ferramenta de detecção e monitoramento de áreas costeiras sujeitas ao impacto de esgotos. Índices de avaliação da qualidade ambiental baseados nestes organismos já são amplamente utilizados em países da Europa e nos Estados Unidos (Borja et al., 2014, Weisberg et al., 1997). Os diversos capítulos deste trabalho permitiram uma primeira aplicação mais integrada e uma primeira análise crítica da validade ou aplicabilidade destes índices em um estuário subtropical da costa brasileira. Esta análise crítica permitiu avaliar: (i) a confiabilidade dos índices (ou seja, até que ponto os diagnósticos de índices distintos refletem as condições do mundo real, expressa por marcadores orgânicos de contaminação), (ii) as influências da variabilidade espaço-temporal sobre o comportamento dos índices (a capacidade de identificar a contaminação mesmo com a atuação simultânea de outras forçantes naturais de fundo) e (iii) a congruência entre respostas de diferentes índices. Dos cinco índices bênticos utilizados, pelo menos três mostraram-se satisfatoriamente robustos para a aplicação em áreas costeiras brasileiras: AMBI (Borja et al., 2000), ITI (Word, 1978) e BO2A (Dauvin & Ruellet, 2007).

O valor da macrofauna bêntica como indicadora também foi evidenciado em termos de perdas ou ganhos funcionais ao ambiente utilizando uma abordagem trófica. Assim como alguns dos índices, diferenças na estrutura de guildas tróficas bênticas de locais contaminados e não-contaminados sinalizaram confiavelmente a qualidade ambiental. Este resultado não foi alcançado apenas com o uso dos grupos tróficos que compõem o índice ITI, previamente testado com sucesso, mas também com grupos tróficos menos abrangentes ou inclusivos. Neste sentido, abordagens tróficas são recomendáveis como uma complementação de outros tipos de indicadores da qualidade ambiental (Gamito et al., 2012). As respostas de guildas tróficas agregam uma perspectiva funcional das comunidades que nem sempre é contemplada pelos demais índices bióticos, embora não tenham uma linguagem tão amigável de comunicação ao público não-especialista quanto estes últimos.

Tanto os índices bióticos quanto a estrutura trófica do bentos, todavia, não devem ser indistintamente aplicados, sem problemas de pesquisa e hipóteses de trabalho claramente definidas, mesmo em rotinas de avaliação de impactos e monitoramento ambiental. As lições derivadas da aplicação de delineamentos robustos, caso dos hierarquizados extensivamente utilizados neste trabalho, são fundamentais para a melhoria e implementação de programas de conservação da qualidade ambiental costeira ou para sua eventual recuperação. Estes delineamentos revelaram a complexidade da dinâmica das populações bênticas, que podem aumentar ou diminuir entre locais mais próximos ou mais distantes em função de quinzenas, estações ou até anos. Vale ressaltar que no presente trabalho os modelos complexos não foram idealizados para que os processos ecológicos operantes sobre todas as escalas fossem revelados. Para o estabelecimento de relações de causalidade recomenda-se futuramente o uso de abordagens experimentais (Murphy et al., 2009). Na lógica dos delineamentos hierarquizados utilizados, as escalas menores foram aninhadas em escalas progressivamente maiores apenas para demonstrar que as diferenças mais significativas estão de fato nas escalas de topo quando se consideram gradientes espaciais ou temporais de contaminação (Underwood, 1996).

Na literatura, a visão da dinâmica dessas populações ainda é extremamente compartimentalizada. Quase todas as análises disponíveis na literatura contemplam poucas das escalas envolvidas nos processos sob avaliação, e componentes espaciais são geralmente desconectados de suas interações com o tempo (Underwood & Chapman, 2013). É evidente que a avaliação de impactos baseada em escalas apenas presumidamente importantes pode estar equivocada (como aconteceu com a sazonalidade). Como já apontado pelos nossos resultados e por diversos autores (Morrissey et al., 1992, Murphy et al., 2009, Underwood & Chapman, 2013), as diferenças observadas em uma certa escala podem na realidade ser mascaradas por diferenças em escalas menores. Entre as implicações práticas desta percepção estão a alocação mais racional das unidades amostrais e da replicação, de forma a aumentar a confiabilidade da avaliação de impactos e a eficiência de monitoramentos. Deve haver um balanço entre a quantidade e a distribuição de pontos amostrais e sua replicação para que o efeito que se deseja avaliar não seja confundido pela variabilidade natural de fundo.

Nossos resultados sugerem ainda que amostragens exaustivas com replicações pesadas não seriam necessárias para mostrar as diferenças entre áreas contaminadas e não-contaminadas no canal da Cotinga, já que esta escala foi importante mesmo face à variabilidade introduzida pelas demais escalas do modelo. Neste caso, o delineamento hierárquico apenas revelou que a magnitude dessas diferenças varia conforme as diferentes amostragens. Em outras palavras, houve interação espacial com as mais diversas escalas temporais. De forma geral, o impacto a ser avaliado deve ser minimamente replicado para que a variabilidade seja medida entre tratamentos e dentro deles (Underwood, 1996). O mesmo vale para monitoramentos, que incorporam a componente temporal em suas lógicas amostrais. Neles a otimização depende do balanço entre a periodicidade do estudo e a distribuição espacial ideal de amostras, como forma de se evidenciar adequadamente o impacto.

Seria conveniente adotar um instrumento normativo único que padronizasse os indicadores de qualidade ambiental e os delineamentos amostrais a serem utilizados na costa brasileira. Esta tarefa não é impossível, a exemplo do “Clean Water Act” norte-americano e do “Water Frame Directive” europeu (Llansó et al., 2009, Hering et al., 2010). O controle da origem, grau de tratamento ou até da frequência do despejo de esgotos que atingem as áreas costeiras é complexa e pouco transparente. Enquanto problemas de saneamento, fiscalização e conscientização ambiental persistirem, o monitoramento será a única estratégia que pode garantir a saúde de ambientes costeiros. Para tanto, será fundamental o uso de indicadores confiáveis a partir de delineamentos amostrais que tragam resultados mais realistas. A utilização de invertebrados bênticos como indicadores, seja compondo índices ou em uma abordagem funcional com guildas tróficas, certamente contribuirá para a preservação da integridade das águas costeiras e para que as sociedades continuem a usufruir de seus bens e serviços.

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Spatial variability of three benthic indices for marine quality assessment in a subtropical estuary of Southern Brazil

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ABSTRACT

Indices based on macrobenthic responses to disturbance remain to be adequately tested for the detection of spatial variability by robust sampling designs. We present herein a congruence test to real-world data of the widely used indices AMBI, M-AMBI and BENTIX in tidal flats of a subtropical estuary. We used a hierarchical sampling design to evaluate the spatial variability of the indices in response to distinct levels of sewage contamination. Indices were then tested for correlations with chemical proxies of contamination and for the similarity of responses. BENTIX and M-AMBI produced over- and underestimations of ecological status. We found a low degree of similarity among indices as an expression of the spatial variation of macrofaunal assemblages on their performances. Only AMBI varied at the contamination scale (10^3 m) and was congruent with physical–chemical proxies. Ambiguous responses indicated effects of natural inputs of organic matter rather than environmental quality associated to sewage.

1. Introduction

A large array of ecological indicators is available for application to environmental health assessment. The number of accessible tools and techniques, such as biotic indices, is rapidly increasing. Macrobenthic animals are considered effective indicators of pollution stress, as they show predictive responses to different levels of natural and anthropogenic impact (Pinto et al., 2009). Therein lies a challenge for the future: to select appropriate monitoring designs and ecological indicators that will provide convincing scientific underpinnings for management and policy decisions on real-world problems (Niemi and McDonald, 2004).

Variation or patchiness in the distribution of benthic assemblages occurs at different spatial scales (Fraschetti et al., 2005; Morrisey et al., 1992). Real changes of the environmental quality associated to biotic indices are frequently confounded with such variability in the distribution of the macrobenthic assemblages (Tataranni and Lardicci, 2010; Borja et al., 2008). Though patchiness patterns are evident at certain scales and absent at others, they are not adequately addressed in the literature due to a lack of appropriate spatial replication. There is evidence that biotic indices can likewise vary or respond to natural disturbances (Muniz et al., 2012). The efficiency of biotic indices or any inferences on their suitability requires some degree of congruence with criteria for degraded and undegraded sites based on nonbiological measures such as chemical proxies of contamination (Benyi et al., 2009).

Hierarchical sampling designs are considered an appropriate method to estimate the contribution of each spatial scale to the total variation among samples, and to discriminate between natural and human induced changes (Underwood and Chapman, 2013; Chapman et al., 2010; Murphy et al., 2009). The meaningful usage of biotic indices is strongly dependent on the quality and quantity of available data, to avoid erroneous classification of environmental health (Tataranni and Lardicci, 2010). As yet, only two studies have assessed the variability of biotic indices using hierarchical sampling approaches (Muniz et al., 2012; Tataranni and Lardicci, 2010), and no previous attempts have been conducted in tropical and subtropical coastal environments. The choice of appropriate biotic indices also involves understanding the association among physico-chemical and biological parameters. Despite the extensive amount of literature concerning the usage of biotic indices in subtidal areas, the actual application of such indices in intertidal areas have rarely been systematically examined using robust sampling designs. Desirable responses from indices involve the ability to detect quality trends across distinct environments found in both subtidal and intertidal systems (Borja et al., 2011).

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We present herein a congruence test to real-world data of three of the most widespread macrobenthic community indices to assess environmental health (Forde et al., 2013; Wu et al., 2013; Munari and Mistri, 2010; Ponti et al., 2008), namely AMBI (AZTI marine biotic index), its multivariate extension M-AMBI and BENTIX in response to distinct levels of sewage contamination. The aim of this study was to assess the effects of spatial variation on the performance of these indices in non-vegetated tidal flats of a subtropical estuary in southern Brazil. We used a hierarchical sampling design to evaluate the variability of the indices in response to the distinct levels of sewage contamination of the tidal flats, at the scales of 10^3 (Conditions – Contaminated and Non-contaminated), 10^2 (Tidal flats) and 10^1 m (Plots). Indices were then tested for correlations with chemical proxies of contamination levels, and for the percentage of similar responses. In this paper, the term congruence refers to the strength and significance within indices responses and their correlation with chemical proxies across a set of hierarchically distributed sites. In terms of strong congruence, we hypothesized that effective indices should preferably: (i) be highly correlated with chemical indicators of contamination; (ii) present a high percentage of similarity among responses and (iii) vary significantly at the largest spatial scale (10^3 m), or the Condition scale.

2. Materials and methods

2.1. Study area

The study was carried out at the Paranaguá Estuarine Complex (PEC) (25°03′S, 48°25′W), which covers an area of 612 km^2 and is one of the main estuaries on the southern coast of Brazil regarding port and tourist activities. The tidal regime is semi-diurnal with estimated average flushing times of three days in the wet season and of ten days in the dry season in average (Mantovanelli et al., 2004). The Cotinga sub-estuary extends for nearly 20 km and is located in the polyhaline sector, near the mouth of the estuary (Fig. 1). Mean neap and spring tidal heights are, respectively, 1.3 and 1.7 m, with a mean depth of 5.4 m (Lana et al., 2000, Marone and Jamiyanaa, 1997). About 34% of the surface area of the sub-estuary, strongly influenced by tidal currents, is covered by mangroves and marshes or remain non-vegetated (Noernberg et al., 2006).

The Cotinga sub-estuary is the main dilution path for anthropogenic input of sedimentary organic matter, represented by sewage-derived material from Paranaguá city (Souza et al., 2013; Lana et al., 2000). Only 50% of the sewage output undergoes treatment, while the rest is released *in natura* to the sub-estuary (CAB-Águas de Paranaguá, 2010). *Escherichia coli* activity and concentrations of fecal steroids, highly stable organic markers, indicate a sharp and compressed gradient of domestic sewage contamination from the inner sector to the outer part of the sub-estuary (Barboza et al., 2013; Martins et al., 2010). However, strong sewage contamination indicated by coprostanol levels is confined to sites close to Paranaguá city (Martins et al., 2010). The sites near Paranaguá city can be considered Contaminated by sewage inputs as average coprostanol concentrations above threshold limits (>0.5 $\mu g\, g^{-1}$) have been recently found, of up to 1.69 $\mu g\, g^{-1}$. As the distance from the sewage source increase these concentrations decrease, ranging from >DL (detection limit) up to only 0.14 $\mu g\, g^{-1}$ (Abreu-Mota et al., 2014). Based on these evidences, we determined two contamination conditions, namely Contaminated and Non-contaminated. Our samplings were carried out in four tidal flats within each

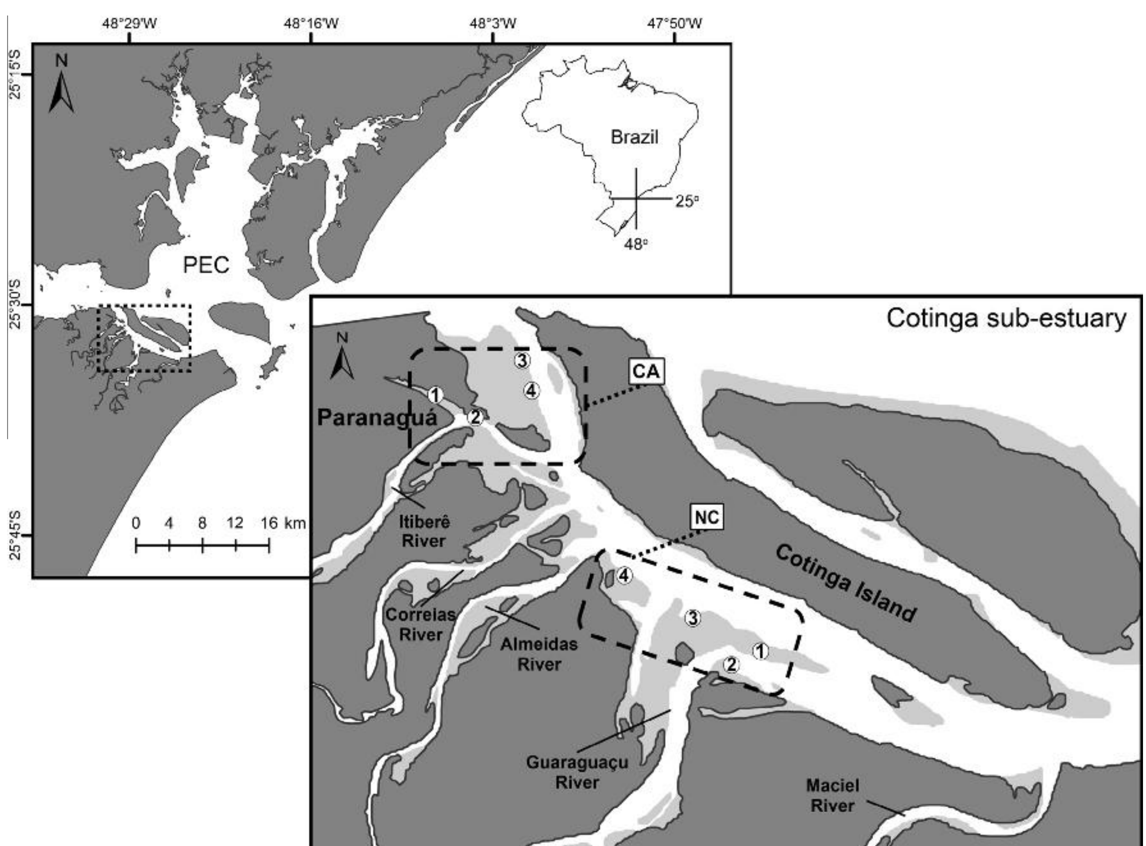

Fig. 1. Study area (modified from Souza et al., 2013). Paranaguá Estuarine Complex (PEC) and Cotinga Sub-estuary. Tidal flats 1, 2, 3 and 4 of the Contaminated area (CA) and the Non-Contaminated area (NC).

condition. All tidal flats corresponded to similar habitat types with no significant differences in salinity, granulometry, exposure to tides and slope (Souza et al., 2013; Noernberg et al., 2006).

We used a hierarchical sampling design to evaluate the variability of the indices in response to the distinct levels of sewage contamination of the tidal flats. The design incorporated three spatial scales, ranging from 10^0 m between replicate samples to 10^3 m between the two contamination conditions of Cotinga sub-estuary (Fig. 2). The factors of the mixed linear model were: Conditions – fixed, with two levels (10^3 m); Tidal flats – random, with four levels (10^2 m), nested in Conditions; and Plots – random, with three levels (10^1 m), nested in Tidal flats, with three replicates each (10^0 m).

Macrofauna was collected using plastic core tubes (10 cm diameter, 10 cm deep), and all plots were placed parallel to the water line, at similar tidal levels. All samples were sieved through a 0.5 mm mesh, fixed in 6% formaldehyde and preserved in 70% alcohol. In the laboratory, all organisms were counted and identified to the lowest possible taxonomic level.

Additional sediment samples were taken at each plot to determine total phosphorus (TP), total nitrogen (TN) and total organic carbon (TOC) contents. The concentrations of TN and TP were obtained according to the method described by Grasshoff et al. (1983), and the concentrations of TOC were measured with the oxidation method described by Strickland and Parsons (1972).

2.2. Biotic indices and data analysis

Three biotic indices were used to assess the ecological status of the Contaminated and Non-contaminated tidal flats (Table 1). AMBI and M-AMBI values were calculated using the software available at AZTI's web page (http://ambi.azti.es). The AMBI index is based on the percentage of abundance of five ecological groups according to their sensitivity to organic pollution, already listed in the software (Borja et al., 2003, 2000). However, some species or taxa present at Paranaguá bay are not as 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

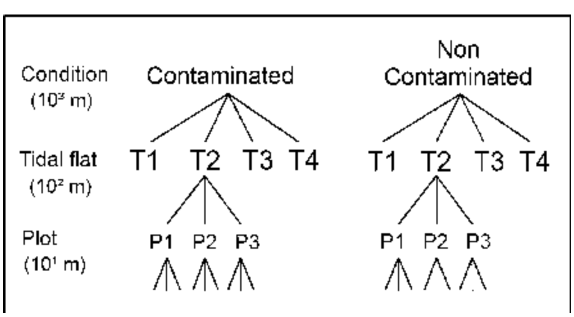

Fig. 2. Diagram of the experimental design (modified from Souza et al., 2013) and scales of spatial variability: Conditions (Contaminated and Non-contaminated); Tidal flats (T1, T2, T3 and T4); and Plots (P1, P2 and P3), with three replicate each.

(Ferrando and Méndez, 2011; Boehs et al., 2008; Gamito, 2008; Nalesso et al., 2005; Palacios et al., 2005; Barnett, 1983) 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, *Sigambra* sp. in GIII, Tubificinae sp1. and Tubificinae sp2. were in GV, while the polychaete *Dorvillea* sp. remained unassigned.

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; Bald et al., 2005; Borja et al., 2004). 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. We defined *a priori* reference conditions by adapting the default values that determine the 'high' and the 'bad' ecological status. We used a different dataset previously obtained in samplings from the same locations in the Cotinga channel (Unpublished data). Afterwards, the index was derived in relation to these values. We used as the highest AMBI value ('Bad' reference conditions) the number derived from the most polluted site of the dataset. Conversely, "High" reference conditions were calculated from the pristine site.

The BENTIX is based on the same proposal as AMBI, but the taxa are categorized in three ecological groups (Simboura and Zenetos, 2002). We adapted the classification of AMBI as following (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 indices values were calculated for each replicate and their ecological status was therefore attributed as *High*, *Good*, *Moderate*, *Poor* and *Bad* (Table 1). The spatial scales of variability were evaluated using a mixed nested ANOVA model for each index. The analyses were conducted in the R environment (R Development Core Team R, 2009) using the package GAD (Sandrini-Neto and Camargo, 2011). Estimates of components of variation were also calculated to evaluate the amount of variation attributed to each source, and were analyzed together with the analysis of variance. All analyses were performed using untransformed data to provide variance components comparable across all data (Fraschetti et al., 2005).

Redundancy analysis (RDA), a constrained linear ordination method, was carried out to explore the relationships among the biotic indices, the chemical proxies of nutrient enrichment (TOC, TN and TP), and the variation on the distribution of sampling plots along the gradient of sewage contamination. 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.

Table 1
Calculated indices and their ecological status threshold values.

Index		Classification				
		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

* Calculated by factorial analysis of AMBI, species richness and Shannon–Wienner diversity values.

3. Results

The three indices classified the majority of the sites as *poor* and *moderate* classes (Figs. 3 and 5).

Based on the AMBI classification, Cotinga sub-estuary exhibited some degree of disturbance. No values attained the *high* status, ranging from 1.8 (Non-contaminated site, T4, P3) to 5.7 (Contaminated, T1, P2) (Fig. 3). About 44% and 7% of the sites could be ranked as *poor* and *bad*, mostly located in the inner part of the channel near Paranguá city. *Good* (13%) and *moderate* status (36%) were found throughout the Non-contaminated tidal flats (Fig. 3). As expected, ecological groups V and I (opportunistic and sensitive species) dominated, respectively, Contaminated and Non-contaminated sites. However, high proportions of ecological group IV (represented mainly by the gastropod *Heleobia australis*) were found on Non-contaminated sites (Table 3). Differences in mean AMBI values were observed at the Tidal flat spatial scale (10^2 m) (Table 2). Components of variation also showed consistent variability at the contamination scale (10^3 m).

The situation was worse for BENTIX, with status values varying from *moderate* to *poor* (10% and 90% of the plots) (Fig. 3). Since no values attained the *high*, *good* or *bad* status, no gradient could be identified. Plots with *moderate* status were located at the Non-contaminated tidal flats. Ecological group III of BENTIX (equal to groups IV and V of AMBI) was made up by the so called second order opportunists *H. australis* and *Laeonereis culveri*, and by the first order opportunist Tubificinae sp1. (Table 3). The species that represented this ecological group were dominant at both Contaminated and Non-contaminated sites. Ecological groups I and II (sensitive and indifferent species) were also present, but with different proportions depending on the tidal flat. Significant spatial differences were only found at the Tidal flat scale (10^2 m), which was corroborated by the highest value of the component of variation (Table 2).

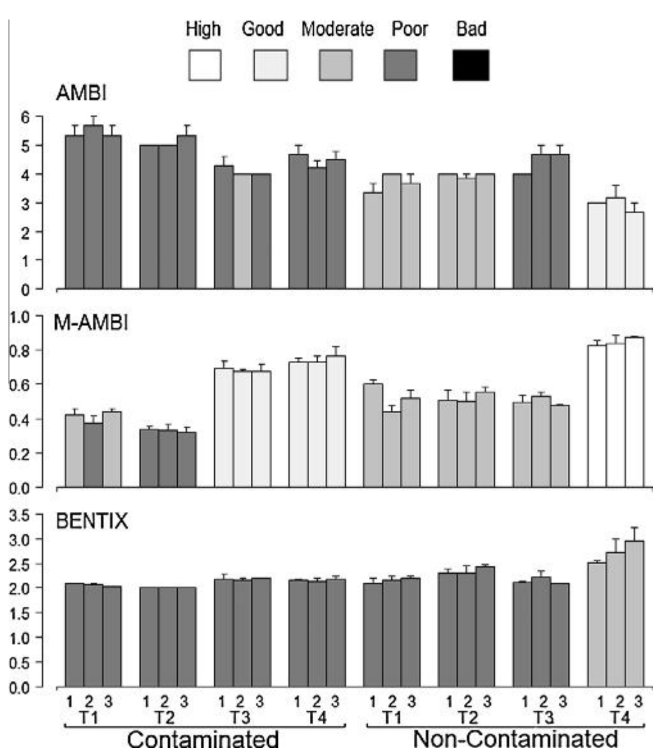

Fig. 3. Mean value (±SE) of the three biotic indices calculated for the Plots (1, 2 and 3) in each Tidal flat (T) of the Contaminated and Non-contaminated sites. Bar colors indicate the ecological status as defined by each index. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

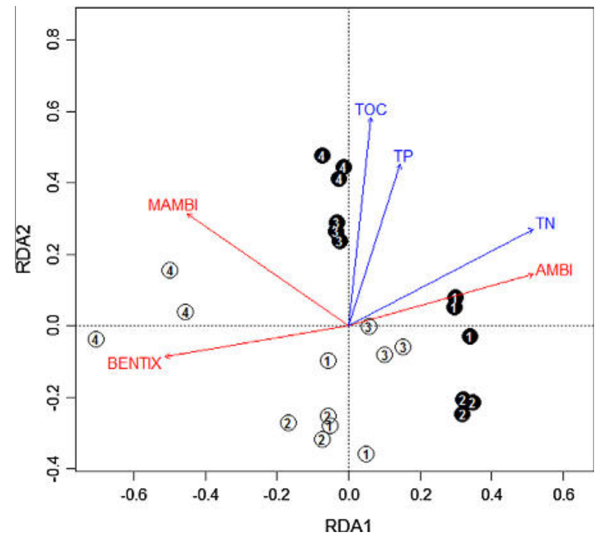

Fig. 4. Redundancy analysis (RDA) triplot of the relationships among biotic indices (red arrows), chemical indicators of contamination (blue arrows) and sampling plots distribution (circles). TOC – total organic carbon; TP – total phosphorus; TN – total nitrogen. Black circles – Contaminated tidal flats (T1 to T4); white circles – Non-contaminated tidal flats (T1 to T4). 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). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

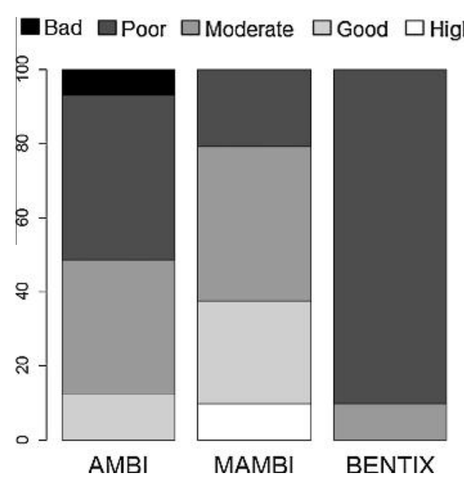

Fig. 5. Ecological status (%) derived from each index. Percentages were calculated for all tidal flats.

The classification of sites by the M-AMBI index was less severe, with values ranging from 0.25 (Contaminated site, T2, P2) to 0.92 (Non-contaminated site, T4, P2) (Fig. 3). M-AMBI was the only index to assess the *high* ecological status (10% of the plots) in Non-contaminated tidal flats. No site was considered as *bad*, whereas 28% of plots of the Cotinga sub-estuary were classified as *good*, 42% as *moderate* and 21% as *poor*. This index includes the species richness and the Shannon–Wiener diversity measures, which were either similar or higher in Contaminated tidal flats comparing to the Non-contaminated (see Souza et al., 2013 for details). M-AMBI was significantly variable at the scale of Tidal flats (10^2 m), a pattern equally important in terms of the percentage of the components of variation observed (Table 2).

Table 2
Hierarchical nested ANOVA results. Plot (P) nested in Tidal flat (T), Tidal flat nested in Condition (Cond). Freedom degrees, mean square (MS), statistical value (*F*), *p* value (*p*) and components of variation (CV%) are presented. Significant differences are given in bold ($p < 0.05$).

	Df	AMBI				M-AMBI				BENTIX			
		MS	*F*	*p*	CV%	MS	*F*	*p*	CV%	MS	*F*	*p*	CV%
Cond	1	19.107	5.6	0.0563	39	0.058	0.2	0.675681	0	1.038	2.9	0.1420	27
T(Cond)	6	3.434	18.8	**<0.001**	35	0.302	75.8	**<0.001**	74	0.363	15.0	**<0.001**	39
P(T(Cond))	16	0.182	0.9	0.53471	0	0.004	1.0	0.469207	1	0.024	0.8	0.6567	0
Residual	48	0.195			26	0.004			25	0.029			34

Table 3
Percentage of dominance of species within Non-contaminated sites, Contaminated sites and the Total. The respective Ecological Group following AMBI/M-AMBI (EGI, EGII, EGIII, EGIV and EGV) and BENTIX (EGI, EGII and EGIII) classifications are also shown.

Species	Ecological Group		Dominance (%)		
	AMBI/M-AMBI	BENTIX	Non-contaminated	Contaminated	Total
Tellina versicolor	I	I	2.59	0.48	0.92
Bulla striata	II	II	3.35	1.41	1.81
Sigambra sp.	III	II	8.16	3.65	4.59
Anomalocardia flexuosa	III	II	1.95	0.91	1.13
Streblospio benedicti	III	II	1.19	1.14	1.15
Heleobia australis	IV	III	47.5	7.19	15.61
Laeonereis culveri	IV	III	2.34	17.33	14.2
Tubificinae sp1.	V	III	14.86	46.21	39.66

The redundancy analysis considering the biotic indices and the chemical parameters of contamination displayed eigenvalues of 0.508 and 0.073 for axes 1 and 2, respectively (Fig. 4). The cumulative percentage of variance explained by the first two canonical axes accounted for 58.2% (50.82% and 7.4% respectively for the first and second axis) of indices data and 98.4% (86.0% and 12.4%) of index-environment relations.

The environmental parameters were significantly correlated with the first axis as evidenced by the Monte Carlo test ($p < 0.001$), and the test for all canonical axes was also significant ($p < 0.001$). Chemical parameters which measure the contamination level at the study sites (total nitrogen – TN, phosphorus – TP and organic carbon – TOC) played an important role in the dispersion of the samples along the first axis. The samples from Non-contaminated and from Contaminated sites were oppositely grouped along axis 1. However, T3 plots of the Non-contaminated site are closer to Contaminated plots. The mean values of the indices increased (AMBI) or decreased (M-AMBI and BENTIX) as expected from contaminated to Non-contaminated sites. AMBI was the only index positively correlated to all chemical variables, and the best correlation with the contamination proxies was for AMBI and total nitrogen (TN) in contaminated sites. M-AMBI was found among Non-contaminated and Contaminated samples, and was negatively correlated to TN. However, among the Non-contaminated sites T4 was the tidal flat with lower TN. BENTIX was inversely correlated to all chemical parameters and related to Non-contaminated sites.

The percentage of similarity or agreement among the three indices was low (Fig. 5). A same ecological status was assigned to only 12.5% of all studied plots. The highest agreement was between AMBI and BENTIX (48.6%), followed by AMBI and M-AMBI (29.2%) and M-AMBI and BENTIX (22.2%).

4. Discussion

A weak congruence was detected among the biotic indices, since they showed low correlations with the chemical proxies of contamination, their responses were of low similarity and significant spatial variability was not found at the *contamination* scale (10^3 m). The only exception was AMBI, which was congruent with the contamination proxies and varied significantly at the largest spatial scale, or the pollution scale.

The responses of biotic indices to disturbance need to be minimally congruent with chemical signals of anthropogenic stress, represented by biogeochemical markers of contamination. Total organic carbon, nitrogen and phosphorus may be considered as proxies of sewage input, although not necessarily unequivocal indicators. Souza et al. (2013) showed significantly higher values of these proxies on the Contaminated sites rather than Non-contaminated, which also sustain high background values of nutrients. Increased nutrient contents are a consequence of the massive sewage input to the contaminated sites near Paranaguá city. The local distribution of fecal steroids, much more conservative parameters than biological or other physico-chemical variables, support these assumptions (Martins et al., 2010).

However, our results and previous studies suggest a clear mismatch between the indices and the sewage impact (Souza et al., 2013), with the exception of AMBI. BENTIX overestimated the ecological status mainly in tidal flats classified by AMBI and M-AMBI as *high*, *good* and *moderate*. This index has been previously reported as less sensitive, overlapping two different intermediate responses into one quality status, since it classifies all species in only three ecological groups (Muniz et al., 2012; Dauvin et al., 2007). Conversely, the M-AMBI index produced an overestimation of the environmental status of the sites. The incorporation of diversity measures such as the Shannon diversity index and species richness, which are dependent on habitat type, sample size, seasonal variations and natural dominance of characteristic species, can lead to misinterpretations of M-AMBI (Simboura and Argyrou, 2010). The Pearson and Rosenberg (1978) paradigm predicts that benthic species richness or diversity should decrease with an increase in organic enrichment, above a certain threshold level. However, the highest records of diversity and richness at the contaminated sites of Cotinga channel are an unexpected pattern related to its moderate level of pollution (Souza et al., 2013). In these sites, the sewage load is constantly washed out by the tides but still provides enough organic matter to sustain a highly diverse community composed by tolerant and indifferent species, rather than leading to anoxia and habitat loss.

The three biotic indices varied at the hundreds of meters scale, or the tidal flat scale. Our findings are consistent with previous attempts to investigate the variability of indices using hierarchical sampling approaches, with evident patterns of variation at smaller spatial scales, from tens to hundreds of meters (Muniz et al., 2012; Tataranni and Lardicci, 2010). Patterns of distribution indicate how the ecological groups or key species defining the structure of the indices have responded to human pressure, directly influencing the performance of each index (Simboura and Argyrou, 2010). In intertidal systems species can be naturally more tolerant to a variety of stresses and the sewage effects could be minimalized during low tide levels, confounding indices assessments of the status of the benthic assemblages (Cowie et al., 2000; Dauer, 1984). Tidal flats may be exposed to low dissolved oxygen only at high tides, whether in low tides there is possible re-aeration of interstitial water from atmospheric diffusion. Nevertheless, the effects of contaminants can accumulate on the pore water and sediment, still selecting different patterns of occurrence and abundance of species according to the level of organic contamination. Efficient indices should respond to the contamination gradient, which is clearly reflected at our largest spatial scale (10^3 m). The only index to vary at the pollution scale was AMBI, which seems to be better suited for environmental quality assessment in the study area.

The responses of biotic indices at the scale of contamination may also be masked by natural organic inputs from mangroves near the Non-contaminated sites. Organic markers (low cholesterol/b-sitosterol ratios) have shown a greater contribution from organic matter of terrigenous origin in these sites (Barboza et al., 2013). This natural organic matter may represent an additional source of nutrients, somewhat simulating the sewage discharges at the Contaminated site. *H. australis* dominated the Non-contaminated sites (see Table 3), though being classified as a second order opportunist by the indices, a category favoured in slight to pronounced pollution situations (Borja et al., 2000). The unexpected high abundance of *H. australis* is probably related to the high inputs of natural organic matter in the Non-contaminated tidal flats. T3 of the Non-contaminated site was also grouped closer to Contaminated tidal flats according to the RDA results, as it shows high organic carbon content, however, probably derived from natural sources.

The sensitivity of marine species to certain stressors may change in different ecoregions, as their assignment into ecological groups (Borja et al., 2011). The shift in the numerically dominant *H. australis* sensitivity might influence the accuracy of the indices' responses. More effective indices would reflect the differences between Contaminated and Non-contaminated sites, consequently leading to significant variations at the spatial scale of contamination. The inconsistent assignment of several species into appropriate ecological groups due to the lack of information on their ecological sensitivity additionally contributed to the weak congruence. These indices accurately assessed the ecological status of other geographical regions, as has been documented in previous reports (Borja et al., 2008; Simboura and Reizopoulou, 2008). However, their application in the southern Atlantic coast remain to be carefully investigated and validated. AMBI has been applied in coastal areas of NE and S Brazil, as in the heavily polluted Todos os Santos (Bahia) and Guanabara (Rio de Janeiro) bays, near oil and sewage discharges (Omena et al., 2012; Muniz et al., 2005). The sites from Paranaguá bay, also subjected to urban effluents (Souza et al., 2013; Martins et al., 2010), clearly display a better ecological status.

The unexpected significance of the tidal flats spatial scale had similar effects on the low similarity among the responses of all biotic indices (AMBI, BENTIX and M-AMBI). Equivalent responses should vary at the contamination spatial scale, meaning that the macrofauna assemblages are structured by sewage effluents rather than other natural processes. The discrepancies among responses could also denote a low congruence in the numerical boundaries of disturbance categories of each index (Muniz et al., 2012). The verbal classes (e.g. *bad* or *poor*) are determined by numerical threshold values, and a low correspondence possibly indicates that adjustments on the threshold values could improve the level of agreement or discrepancies in indices responses. The highest agreement between AMBI and BENTIX was expected, since they are based on similar concepts (species level of sensitivity to organic enrichment). The opposite relationship was observed between BENTIX and M-AMBI, which showed the lowest agreement as a consequence of the overestimation of results by M-AMBI and underestimation by BENTIX.

Our results highlight some degree of ambiguity in less congruent indices. BENTIX and M-AMBI produced over- and underestimations of the ecological status of the studied sites. Only AMBI varied at the "pollution" scale (10^3 m) and was congruent with physical–chemical proxies of contamination. We found a low degree of similarity among AMBI, M-AMBI and BENTIX, which may be an expression of the spatial variation of macrofaunal assemblages on the performance of indices. We emphasize the importance of establishing unequivocal spatial configurations of macrobenthic assemblages directly driven by sewage contamination. Incongruences in biotic indices assessments of benthic condition mean that indices reflect different attributes of the environment, not the contamination itself. The fauna of our Non-contaminated sites was influenced by the natural massive input of nutrients from the marginal vegetation. Therefore, the application of indices in such context may be meaningless, as their ambiguous responses indicate the effects of natural inputs instead of environmental quality associated to sewage. Regardless of the employed index, generalities on spatial variation should incorporate nested sampling designs. Temporal scales might also represent an important source of variability, and need to be included for a robust assessment of scales and processes. Information about variability can be used to develop models to predict the environmental health of the entire bay, applied in effective monitoring programs (Underwood and Chapman, 2013; Norén and Lindegarth, 2005).

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