UNIVERSIDADE FEDERAL DO PARANÁ

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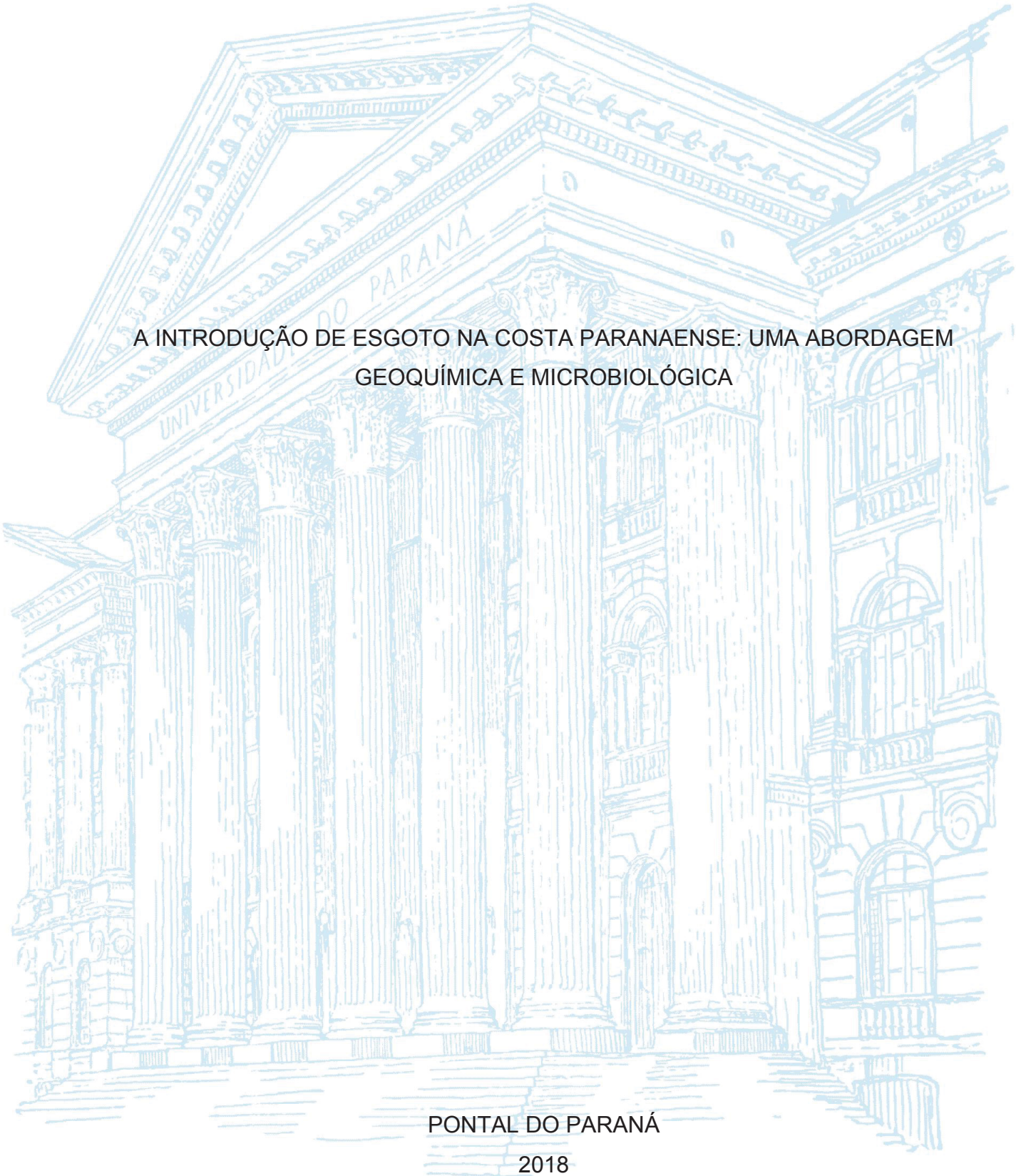

A INTRODUÇÃO DE ESGOTO NA COSTA PARANAENSE: UMA ABORDAGEM GEOQUÍMICA E MICROBIOLÓGICA

PONTAL DO PARANÁ

2018

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A INTRODUÇÃO DE ESGOTO NA COSTA PARANAENSE: UMA ABORDAGEM GEOQUÍMICA E MICROBIOLÓGICA

Tese apresentada ao curso de Pós-Graduação em Sistemas Costeiros e Oceânicos, setor de Ciências da Terra, Centro de Estudo do Mar, Universidade Federal do Paraná, como requisito parcial à obtenção do título de Doutora em Sistemas Costeiros e Oceânicos, linha de pesquisa Biogeoquímica e Poluição Marinha.
Orientador: Prof. Dr. César de Castro Martins.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em SISTEMAS COSTEIROS E OCEÂNICOS da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ANA CAROLINE CABRAL** intitulada: **A INTRODUÇÃO DE ESGOTO NA COSTA PARANAENSE: UMA ABORDAGEM GEOQUÍMICA E MICROBIOLÓGICA**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutor está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Pontal do Paraná, 29 de Agosto de 2018.

CÉSAR DE CASTRO MARTINS
Presidente da Banca Examinadora

ANA CECILIA RIZZATTI DE ALBERGARIA BARBOSA
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Avaliador Externo

CRISTINA ROSSI NAKAYAMA
Avaliador Externo

Às mães cientistas.

(https://www.youtube.com/watch?v=RukTR9VHcUg)

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“Tenho a impressão de ter sido uma criança brincando à beira-mar, divertindo-me em descobrir uma pedrinha mais lisa ou uma concha mais bonita que as outras, enquanto o imenso oceano da verdade continua misterioso diante de meus olhos.”

Isaac Newton

RESUMO

O esgoto é uma das principais fontes histórica e crônica de poluição em ambientes aquáticos no mundo e no Brasil, devido à precariedade (ou ausência) de serviços de coleta e tratamento de águas residuárias. Investimentos na ampliação destes serviços sanitários não acompanham o crescimento e a demanda da população, o que torna fundamental a detecção deste contaminante no ambiente a fim de monitorar áreas suscetíveis a eutrofização e prevenir o contato humano com águas contaminadas. O *status* atual e histórico da introdução de esgoto no litoral do Paraná, especificamente nas Baías de Guaratuba e Paranaguá, foi avaliado através da determinação de indicadores químicos e microbiológicos em sedimento superficial, material particulado em suspensão (MPS) e testemunhos sedimentares. Três artigos científicos são apresentados (capítulos 2, 3 e 4). No primeiro artigo foram avaliados valores limites de coprostanol e alquilbenzeno lineares (LABs) no MPS em relação aos valores estipulados para as bactérias indicadoras fecais (BIF) *Escherichia coli* e enterococci, estes últimos estabelecidos na legislação do Brasil e do mundo. A partir da análise de regressão logística, foi determinada uma faixa de valores limites de coprostanol (entre 1,00 e 2,23 μg g^{-1} MPS), que variou dependendo da BIF e da estação climática avaliada, sugerindo a temperatura como uma variável importante na definição de valores limites. Isso reforça a importância da calibração destes valores para diferentes condições climáticas. No segundo artigo, esteróis e LABs foram analisados no MPS e no sedimento para estabelecer a distribuição espacial da matéria orgânica biogênica e proveniente de esgoto em cada compartimento. Houve variação espacial homogênea desses marcadores no MPS, e mais restrita no sedimento, devido aos padrões hidrodinâmicos locais que podem promover homogeneização, dispersão e diluição das partículas provenientes do esgoto na coluna de água, mas com sedimentação preferencial em zonas específicas do estuário. Isto sugere que o MPS é uma boa matriz para avaliações de grande escala espacial e curta escala temporal, enquanto a avaliação sedimentar é mais indicada para definição de áreas prioritárias de introdução e deposição final de material oriundo do esgoto. As menores concentrações dos marcadores no sedimento em relação ao MPS, bem como a variação na sua composição entre ambas as matrizes foi relacionada principalmente à variação nas taxas de degradação, que são maiores na coluna da água. O objetivo do terceiro artigo foi descrever o histórico de contaminação por esgoto através da avaliação da variação vertical das concentrações de esteróis fecais e de isótopos estáveis ($\delta^{13}C$ e $\delta^{15}N$). Os *proxies* $\delta^{13}C$ e $\delta^{15}N$ não foram conclusivos na avaliação da contaminação por esgoto. Entretanto, as baixas concentrações de coprostanol, associado às análises de razões diagnósticas, indicaram que as regiões em que os testemunhos foram amostrados não estão contaminadas por esgoto. Dessa forma, valores de referência de esteróis fecais que podem representar um cenário ambiental não impactado, foram calculados. Com base nos resultados dos três produtos científicos, conclui-se que, no geral, as áreas estuarinas do litoral do Paraná não estão altamente contaminadas por esgoto, mas apresentam locais de maior contaminação ou suscetíveis a cenários de contaminação, especificamente nas zonas de mistura e fluvial, próximas às principais fontes de introdução de esgoto e sob condições sedimentares e hidrodinâmicas que favorecem a sedimentação dessas partículas.

Palavras-chave: Esgoto 1. Bactérias indicadoras fecais 2. Coprostanol 3. Alquilbenzeno linear 4. Sistema Estuarino de Paranaguá 5.

ABSTRACT

Sewage is one of the main historical and chronic sources of pollution in Brazilian and worldwide aquatic environments, due to precariousness (or absence) on sewage collection and treatment facilities. Investments in the expansion of sanitary services do not follow the population growth and demand. Thus, sewage detection in the environment is still necessary to monitor areas susceptible to eutrophication and to prevent human contact with contaminated water. The current and historical status of the sewage input in the Paraná coast, specifically in the Guaratuba and Paranaguá bays, was evaluated using chemical and microbiological indicators in surface sediment, suspended particulate matter (SPM) and sediment cores. Three scientific articles are presented (chapters 2, 3 and 4). In the first manuscript, thresholds values of the linear alkylbenzenes (LABs) and coprostanol in SPM were evaluated in relation to the indicator values of fecal indicator bacteria (FIB) *Escherichia coli* and enterococci, these last ones established in the Brazilian and worldwide legislation. A range of coprostanol values (between 1.00 and 2.23 μg g^{-1} SPM) was determined as thresholds values using logistic regression analysis. These values varied depending on the FIB and climatic season considered, suggesting that temperature is an important variable to establish these values. This reinforces the importance to calibrate threshold values for different climatic conditions. In the second paper, sterols and LABs were analyzed in the SPM and sediment to establish the spatial distribution of biogenic and sewage organic matter in each compartment. There was a homogeneous spatial variation of these markers in the SPM, and more restricted in the sediment, due the local hydrodynamic patterns that can promote homogenization, dispersion and dilution of the sewage particles in the water column, but with preferential sedimentation in specific areas of the estuary. It suggests that SPM is a good matrix for large-scale spatial and short-scale temporal evaluations, while sediment is more appropriate to define priority areas of input and final deposition of sewage residues. The lower concentrations of these markers in sediments, comparing to SPM, as well as the variation in their composition between both matrices was mainly related to the variation in the degradation rates, which are higher in the water column. The aim of the third paper was describe the historical sewage contamination analysing the vertical variation of the fecal sterols concentrations and the stable isotopes ($\delta^{13}C$ and $\delta^{15}N$). The proxies $\delta^{13}C$ and $\delta^{15}N$ were not conclusive to the evaluation of sewage contamination. However, the low concentrations of coprostanol, associated to diagnostic ratios analysis, indicated that the regions where the cores were collected are not under sewage contamination. Therefore, baseline values of fecal sterols that may represent a previous non-impacted environment scenario were calculated. Considering the three articles results, it is concluded that, in general, the estuarine areas of the Paraná coast are not highly contaminated by sewage, but they present sites of potential contamination or susceptible to chronic sewage impact, specifically in the mixing and fluvial zones, near the main sources of effluent input and under sedimentary and hydrodynamic conditions that favor the sedimentation of these particles.

Keywords: Sewage 1. Fecal indicator bacteria 2. Coprostanol 3. Linear alkylbenzene 4. Paranaguá Estuarine System 5.

SUMÁRIO

CAPÍTULO 1

1 INTRODUÇÃO GERAL

No Brasil, menos da metade das residências possuem sistema adequado de saneamento e a grande maioria dos municípios não possui redes eficientes de captação e tratamento de esgoto (IBGE, 2011; INSTITUTO TRATA BRASIL, 2015).

A introdução de esgoto no ambiente tem sido estudada através de marcadores orgânicos geoquímicos e/ou indicadores microbiológicos, mais comumente este último devido ao baixo custo e sua relativa confiabilidade, facilidade e rapidez de análise (GRIFFIN *et al.,* 2001; LIM *et al.*, 2017). Entretanto, indicadores microbiológicos (*Escherichia coli* e *Enterococcus* spp., por exemplo) apresentam ciclo de vida curto, são menos resistentes às variações de condições ambientais como temperatura, radiação solar, salinidade, nutrientes, agentes tóxicos entre outros, e nem sempre podem ser relacionados à contaminação fecal (SOLO-GRABRIELE *et al.*, 2000; BYAPPANAHALLI *et al.*, 2012; LIM *et al.*, 2017). Os marcadores orgânicos geoquímicos, como os esteróis e os alquilbenzeno lineares (LABs), aparecem como uma alternativa, pois são quantificados a baixos níveis e têm maior resistência à degradação ambiental que os microbiológicos (TAKADA & EGANHOUSE, 1998; ISOBE *et al.*, 2004).

Esteróis e LABs são compostos orgânicos de característica hidrofóbica, e por isso estão preferencialmente associados ao material particulado em suspensão (MPS) e aos sedimentos (LEBLANC *et al.*, 1992; KIM *et al.*, 2016). O MPS é a principal via de entrada e transporte desses marcadores no ambiente aquático, e sua análise permite traçar a fonte, caminho e destino desses marcadores e, consequentemente, da matéria orgânica proveniente do esgoto (ZENG *et al.*, 1997; DAUNER & MARTINS, 2015; CARREÓN-PALAU *et al.*, 2017). As taxas de degradação de esteróis e LABs na coluna de água (dissolvidos ou adsorvidos ao MPS) são mais altas que nos sedimentos, visto que neste último a atividade biológica (importante agente de degradação) tende a ser menor em comparação à coluna de água, devido à diferença de grau de oxigenação (geralmente maior na coluna de água que no sedimento) (JENG & HAN, 1996; WAKEHAM *et al.*, 1997; ZENG *et al.*, 1997). Portanto, os marcadores geoquímicos tendem a permanecer acumulados nos sedimentos durante anos, o que permite tanto a análise de

contaminação recente (sedimento superficial), como reconstruções históricas da introdução de esgoto através da análise de testemunhos sedimentares (JENG & HAN, 1996; MARTINS *et al.*, 2010a).

Sendo assim, tanto o MPS como sedimentos são utilizados como matrizes de determinação de marcadores de contaminação por esgoto. Porém, as diferentes condições físicas e químicas inerentes de cada compartimento (coluna de água e sedimentar), podem interferir na qualidade e quantidade dos marcadores orgânicos, dependendo da matriz analisada (WAKEHAM *et al.*, 1997; CARDOSO *et al.*, 2016).

Até o ano de 2017, cinco estudos geoquímicos sobre a distribuição de coprostanol e/ ou LABs foram realizados no litoral do Paraná. Quatro destes estudos abordaram a matriz sedimento superficial (MARTINS *et al.*, 2010b; 2012; ABREU-MOTA *et al.*, 2014; BET *et al.,* 2015) e um o MPS (DAUNER & MARTINS, 2015). A carência de estudos utilizando o MPS como matriz de determinação de marcadores geoquímicos no litoral paranaense pode ser reflexo do cenário mundial dos estudos geoquímicos, que são concentrados na matriz sedimento (DAUNER & MARTINS, 2015).

De forma geral, há uma imensa lacuna no que diz respeito a estudos relacionados à integração de marcadores orgânicos geoquímicos e indicadores microbiológicos, à determinação de marcadores orgânicos geoquímicos no MPS (bem como a comparação entre as matrizes MPS e sedimento) e, mais especificamente para litoral do Paraná, sobre a reconstrução histórica da introdução por esgotos utilizando esteróis fecais. Estes estudos são fundamentais para estabelecer níveis regionais de concentração de marcadores orgânicos geoquímicos capazes de indicar a contaminação por esgoto (tanto para MPS como para sedimento), e calibrar índices relacionados a estes compostos químicos para regiões subtropicais. Juntamente com a análise de testemunhos datados, estes estudos permitem avaliar o panorama atual e histórico da introdução de esgoto na área de estudo, bem como avaliar a eficiência dos esforços de regulação do saneamento e tratamento de esgoto na região que vem ocorrendo nos últimos anos. Conhecer estes panoramas permite a compreensão das tendências de poluição costeira e, consequentemente, o desenvolvimento de estratégias de manejo e conservação, que devem contribuir diretamente para embasar políticas públicas de monitoramento ambiental e qualidade da água, podendo afetar positivamente a saúde humana.

O presente trabalho teve como objetivo geral avaliar a Intercomparação dos marcadores geoquímicos LABs e esteróis fecais com os indicadores fecais microbiológicos *E. coli* e enterococci, mais usualmente utilizados no Brasil e no mundo, e avaliar o *status* histórico e contemporâneo da introdução de esgoto no litoral do Paraná, especificamente nas Baías de Guaratuba e Paranaguá, através da determinação destes marcadores nas duas principais matrizes de adsorção (sedimento e MPS).

A fim de atingir esses objetivos e, considerando que há a liberação de esgoto (sem, ou com precário tratamento) nas duas regiões estuarinas do Paraná (MARTINS *et al.*, 2010b, DAUNER & MARTINS, 2015; KOLM *et al*., 2018), as seguintes hipóteses foram avaliadas:

i) Se os marcadores moleculares fecais coprostanol e LABs, e as bactérias indicadoras fecais *E. coli* e Enterococci compartilham o esgoto, assim como suas fontes de introdução no ambiente, ambas as classes de indicadores (molecular e biológica) serão correlacionadas.

ii) Se a hipótese (i) é verdadeira, então é possível calcular valores limites de contaminação fecal para os marcadores moleculares com base nos valores limites já estipulados para os indicadores microbiológicos.

iii) Se o MPS é o principal meio de transporte dos marcadores orgânicos entre os compartimentos coluna de água e sedimento, interpretações sobre fontes de matéria orgânica e diagnóstico de contaminação, considerando MPS ou sedimento, serão semelhantes.

iv) Se as baías de Guaratuba e Paranaguá estão sujeitas historicamente à introdução de esgoto, será possível traçar este histórico de introdução de esgoto, através da variação de concentrações de marcadores moleculares ao longo de testemunhos sedimentares.

A tese está dividida em cinco capítulos. O primeiro capítulo corresponde à introdução geral e a revisão da literatura, com a apresentação dos principais conceitos relacionados ao tema central da tese. O segundo capítulo corresponde à comparação entre os marcadores geoquímicos (coprostanol e LABs) e os indicadores microbiológicos (*E. coli* e enterococci) de contaminação por esgoto, a fim de estabelecer valores limites (*threshold*) para os marcadores geoquímicos e seus respectivos índices de avaliação como indicativo de contaminação pelo descarte de efluentes em regiões subtropicais. No terceiro capítulo, foi avaliada a

variabilidade das concentrações de esteróis e LABs entre as duas principais matrizes ambientais (MPS e sedimentos superficiais), coletadas simultaneamente, a fim de avaliar as variações na matéria orgânica marinha, terrestre e de esgoto desde o momento mais próximo de sua introdução (MPS) até a deposição final (sedimentos) em um ambiente estuarino subtropical. No quarto capítulo, foi avaliada a variação vertical das concentrações de esteróis fecais e isótopos estáveis ($\delta^{13}C$ e $\delta^{15}N$) em testemunhos sedimentares a fim de descrever o *status* histórico da introdução de esgotos no litoral paranaense. Além disso, valores de referência indicativos de área estuarina sem contaminação por esgoto foram obtidos e apresentados. Estes valores podem ser utilizados como comparação a futuras avaliações de introdução e contaminação por esgoto em regiões costeiras, especialmente para a Baía de Paranaguá, considerando a instalação de novos empreendimentos portuários, planejados para a próxima década. Por fim, considerações gerais são apresentadas no quinto capítulo em relação ao *status* atual e histórico da contaminação por esgoto no litoral Paranaense.

2 REVISÃO DA LITERATURA

2.1 ESGOTO

O esgoto é uma mistura complexa de água e resíduos orgânicos e inorgânicos de origem doméstica, industrial e agrícola. Sua liberação no ambiente é considerada uma das principais causas do aumento da poluição de ambientes aquáticos de todo o mundo (UNEP, 2008; 2016). O descarte de resíduos em ambientes aquáticos é uma atividade antrópica histórica, realizada com a expectativa de que o ambiente fosse capaz de diluir, assimilar e degradar esses dejetos de forma ilimitada através de circulação local e processos de autodepuração (WEBER, 1992; MUNIZ *et al.*, 2013). No entanto, a introdução de efluentes ocorre geralmente em locais pontuais e rasos da zona costeira, os quais não apresentam circulação local que permita uma diluição e processamento eficiente do fluxo de entrada cada vez maior desses resíduos (WEBER, 1992; MUNIZ *et al.*, 2013; INSTITUTO TRATA BRASIL, 2015). Além de zonas rasas, regiões mais profundas e/ou afastadas da costa com maior circulação de correntes e, portanto, maior potencial de diluição, também são cenários de contaminação, onde sinais de esgoto são detectados em áreas

próximas da desembocadura de emissários submarinos, por exemplo (MARTINS *et al.,* 2008, LYONS *et al.*, 2015; MUNIZ *et al.*, 2015; SAEED *et al.*, 2015).

A introdução do esgoto no ambiente é fonte de problemas ambientais e de saúde pública, a citar enriquecimento orgânico e eutrofização, introdução de contaminantes emergentes (como fármacos e produtos de higiene), aumento da resistência bacteriana e *imposex*, bem como de proliferação de micro-organismos e uma variedade de patógenos que podem permanecer no ambiente aquático por longos períodos, sendo fontes de doenças de veiculação hídrica (MUNIZ *et al.*, 2013; ANDERSSON *et al.,* 2016; UNEP, 2016).

A coleta e tratamento de efluentes é um dos principais desafios para o manejo e conservação dos ambientes aquáticos, bem como da prevenção de doenças humanas (INSTITUTO TRATA BRASIL, 2015; UNEP, 2016). Ainda assim, o desenvolvimento de sistemas de coleta e tratamento de esgoto é lento (ou mesmo ausente), em comparação ao crescimento da demanda populacional humana, especialmente em países do Hemisfério Sul (BAUM *et al.*, 2013; ANDERSSON *et al.*, 2016; UNEP, 2016).

No Brasil, a coleta e o tratamento de esgoto são os serviços sanitários em situação de maior precariedade. As áreas privilegiadas com a ampliação e melhorias desse serviço são somente aquelas próximas às capitais estaduais e ao litoral do país. Mesmo nessas regiões, a ampliação dos serviços é defasada em relação ao aumento da densidade populacional, e a taxa de tratamento do esgoto coletado é ainda inferior (41% no Sudeste) (IBGE, 2011; INSTITUTO TRATA BRASIL, 2015). Desta forma, é comum que o esgoto chegue ao corpo receptor em sua forma bruta, sem nenhum tipo de tratamento.

O Conselho Nacional do Meio Ambiente (CONAMA) é o órgão brasileiro responsável pela legislação referente a análises de qualidade de água, incluindo valores de referência para avaliação de contaminação por esgoto. Em 1986, foi lançada a resolução nº 20 do CONAMA, a primeira a dispor sobre a classificação das águas doces, salobras e salinas do Brasil, segundo seus usos preponderantes e com tabelas de indicação de limites e condições de parâmetros de qualidade de água. Os critérios de balneabilidade foram revisados e alterados em 2000 pela resolução nº 274 (CONAMA, 2000). A resolução original de 1986 foi revogada no ano de 2005, pela resolução nº 357 (CONAMA, 2005), a qual incluiu condições e padrões de lançamento de efluentes em ambientes naturais. Esta última foi ainda

alterada e complementada em 2011 pela resolução nº 430 (CONAMA, 2011), que dispõe sobre as condições e padrões de lançamento de efluentes.

2.2 INDICADORES MICROBIOLÓGICOS DE CONTAMINAÇÃO POR ESGOTO

Membros dos grupos das bactérias coliformes e estreptococos fecais são amplamente utilizados como indicadores de qualidade de água e contaminação por esgoto em ambientes dulcícolos (ELHMMALI *et al*., 2000) e salinos (NICHOLS *et al*., 1993; GRIFFIN *et al*., 2001; COSTA & CARREIRA, 2005; LYONS *et al*., 2015). Estes micro-organismos geralmente não apresentam patogenicidade, mas indicam a presença de outros organismos patogênicos e agentes tóxicos presentes no esgoto, danosos ao meio ambiente e à saúde humana (CONAMA, 2000; GRIFFIN *et al*., 2001; WHO, 2003; HARWOOD, 2014).

Coliformes totais, particularmente os fecais, foram os primeiros grupos de bactérias avaliados como indicadores de qualidade de água. Este grupo é composto por um grande número de bactérias amplamente distribuídas na natureza, incluindo fezes humanas e de outros animais, solo, madeira, entre outras fontes (DAVIES *et al.,* 1995; HARWOOD, 2014). Assim, a utilização desse grupo como indicador de contaminação é restrita a alguns casos, como em análises de potabilidade, mas não para águas destinadas à recreação, por exemplo (NOBLE *et al*., 2003). Os coliformes fecais têm os intestinos de animais endotérmicos como principal origem e se distinguem dos coliformes totais pela habilidade de crescer em temperaturas elevadas (44,5 °C), sendo originalmente os principais indicadores de contaminação fecal (NOBLE *et al*., 2003). No entanto, este grupo contém espécies dos gêneros *Klebsiella* e *Enterobacter* que não são, necessariamente, de origem fecal, o que pode gerar interpretações errôneas quanto à qualidade da água (EDBERG *et al.*, 2000).

Atualmente, os órgãos de estudos e regulamentação de qualidade de águas recomendam a determinação dos coliformes fecais *E. coli* e *Enterococcus* spp. (tratados no presente trabalho como enterococci) como indicadores microbiológicos de contaminação fecal, pois seguem o conceito histórico de que esses micro-organismos são específicos de material fecal humano e outros animais endotérmicos (NOBLE *et al*., 2003; CONAMA, 2005; APHA, 2009). No entanto, estudos têm apontado que essas bactérias são amplamente distribuídas no ambiente, mesmo quando há pouca ou nenhuma introdução de fezes humanas ou animal (SOLO-

GRABRIELE *et al.*, 2000; BYAPPANAHALLI *et al.*, 2012), o que coloca em discussão a especificidade destes micro-organismos ao material fecal, bem como seu *status* como bom indicador de contaminação por esgoto.

2.3 ESTERÓIS E ALQUILBENZENO LINEARES (LABS) COMO MARCADORES DE CONTAMINAÇÃO POR ESGOTO

Marcadores orgânicos geoquímicos, como os esteróis e os alquilbenzeno lineares (LABs) apresentam especificidade de fonte e relativa resistência à degradação microbiológica durante a diagênese, sendo assim excelentes indicadores de fontes e eventos antropogênicos e naturais (TAKADA & EGANHOUSE, 1998; BULL *et al.,* 2002; DERRIEN *et al.*, 2017). Marcadores hidrofóbicos são preferencialmente adsorvidos a partículas do meio aquático, sendo possível o rastreamento da fonte, caminho e destino da matéria orgânica relacionada ao marcador analisado (TAKADA & EGANHOUSE, 1998; DACHS *et al.,* 1999; BIGUS *et al.,* 2014; MARTINS *et al.,* 2014). Devido à relativa estabilidade desses compostos, eles permanecem acumulados no sedimento, permitindo a realização de estudos de longa escala temporal, inclusive a reconstrução histórica de processos ecológicos, geológicos e de contaminação (TAKADA & EGANHOUSE, 1998; CARREIRA *et al.,* 2004; MARTINS *et al.,* 2011; BIGUS *et al.,* 2014).

Os esteróis são compostos orgânicos derivados do ciclopentano perhidrofenantreno, de ampla variedade estrutural, pertencentes ao grupo dos álcoois. Apresentam como principais características estruturais a presença de duplas ligações entre carbonos, um sistema de três anéis hexacíclicos e um anel pentacíclico, cadeia carbônica lateral e a presença de radicais metil nos carbonos C_4 e C_{14}, geralmente removidos na produção do colesterol (VOLKMAN, 2005).

A origem de matéria orgânica natural em estuários pode ser avaliada através de esteróis de fontes biogênicas, como colesterol (encontrado em material fecal de zooplâncton e algumas espécies de microalgas e cianobactérias), campesterol, sitosterol e estigmasterol (predominantes na constituição de plantas superiores) e dinosterol (associado a dinoflagelados e algumas espécies de diatomáceas) (VOLKMAN, 2005).

Dentre os esteróis, epicoprostanol e coprostanol (esteróis fecais) são utilizados como marcadores de contaminação fecal. O coprostanol é formado no trato intestinal de mamíferos superiores através da transformação bacteriana do colesterol, e

corresponde a cerca de 60% dos esteróis totais nas fezes humanas (TAKADA & EGANHOUSE, 1998). Embora o coprostanol seja encontrado também em fezes de outros vertebrados superiores, razões diagnósticas com outros esteróis podem ser aplicadas para confirmar a origem humana (DERRIEN *et al.*, 2017). O epicoprostanol é um isômero do coprostanol produzido principalmente durante os processos de digestão aeróbica em estações de tratamento de efluentes, e por isso é principalmente utilizado como indicador do nível de tratamento de efluentes ou idade do material fecal (TAKADA & EGANHOUSE, 1998; MUDGE & SEGUEL, 1999).

Coprostanol é o principal esterol utilizado como marcador de resíduos antropogênicos ao longo de áreas costeiras com adensamentos humanos em várias regiões do mundo (GONZÁLEZ-OREJA & SAIZ-SALINAS, 1998; LEEMING *et al.*, 1998; DACHS *et al.,* 1999; MARTINS *et al.,* 2005; CHOI *et al.,* 2009; MARTINS *et al.,* 2010b). No entanto, ainda não há um consenso de valores limites deste esterol referentes à contaminação por esgoto (por exemplo, foram estabelecidos os valores de 0,50 μg g^{-1} por GONZÁLEZ-OREJA & SAIZ-SALINAS, 1998 e 0,10 μg g^{-1} por GRIMALT *et al.* 1990 como valores limites para sedimentos). Isso ocorre porque as concentrações de coprostanol variam dependendo de características ambientais locais, como o tipo de sedimento e condições climáticas (TAKADA & EGANHOUSE, 1998). Razões entre esteróis fecais e demais esteróis têm sido utilizadas como uma forma de minimizar esses problemas, permitindo intercomparações entre resultados obtidos de ambientes com diferentes tipos de sedimentos, além de avaliações mais robustas na identificação de diferentes fontes e tipos de materiais fecais (GRIMALT *et al.,* 1990; BULL *et al.,* 2002; MARTINS *et al.,* 2014).

Grimalt *et al.* (1990) propuseram a razão [coprostanol]/([coprostanol+colestanol]) como uma ferramenta para a identificação de contaminação de sedimentos por esgoto, em que áreas que apresentassem valores próximos a 1,0 são influenciadas por esgoto. Faixas de resultados para essa razão foram posteriormente desenvolvidas em conjunto com as concentrações encontradas para coprostanol. Alta concentração de coprostanol associada à razão com valores entre 0,5 e 1,0 indica contaminação fecal (GONZÁLEZ-OREJA & SAIZ-SALINAS, 1998; LEEMING *et al.*, 1998), assim como baixa concentração de coprostanol e razão com valores entre 0,1 e 0,3 representam áreas prístinas com entrada biótica de colestanol ou redução *in situ* de colesterol em coprostanol

(LEEMING *et al.*, 1998). Valores entre 0,3 e 0,5 devem ser avaliados em conjunto com outros índices (MARTINS *et al.,* 2014). Com a razão [epicoprostanol/coprostanol] é possível estimar se o esgoto introduzido no ambiente recebeu tratamento, sendo que valores abaixo de 0,2 são relacionados à contaminação por esgoto sem tratamento prévio (MUDGE & DUCE, 2005).

Razões entre coprostanol e esteróis naturais, como colesterol e dinosterol são importantes para corroborar interpretações obtidas a partir das razões acima citadas (VENKATESAN & KAPLAN, 1990; MUDGE & SEGUEL, 1999; CARREIRA *et al.,* 2004; MARTINS *et al.,* 2014).

Os LABs são hidrocarbonetos aromáticos formados pela substituição de um átomo de hidrogênio do anel benzênico por uma cadeia alifática linear. Misturas de LABs comerciais, em geral contendo de 10 a 14 átomos de carbono na cadeia linear alifática, são utilizadas como matéria prima na produção do princípio ativo de detergentes, como o alquilbenzeno sulfonato linear (LAS), um surfactante aniônico de amplo uso doméstico e industrial (PENTEADO *et al.,* 2006). Durante o processo de sulfonação, cerca de 1 a 3 % dos LABs não reagem e permanecem como resíduo traço nestes produtos a serem comercializados. Este resíduo posteriormente é introduzido no ambiente aquático através de efluentes domésticos, podendo ser detectado e indicar a introdução e contaminação por efluentes (EGANHOUSE *et al.,* 1983; TAKADA & EGANHOUSE, 1998; PENTEADO *et al.,* 2006).

Os LABs apresentam uma variação isomérica dependendo da posição do grupo fenil na cadeia carbônica. A ligação do grupo fenil nas posições 2, 3 ou 4 da cadeia linear configura os isômeros externos (E) e nas posições 5 ou 6 os isômeros internos (I) (EGANHOUSE *et al.,* 1983; TAKADA & ISHIWATARI, 1990; TAKADA & EGANHOUSE, 1998). A biodegradação de isômeros externos tende a ocorrer antes que a dos internos e a razão entre a soma de suas concentrações (I/E, ou seja, [soma dos internos] / [soma dos externos]) permite estimar a extensão da degradação entre os isômeros e, consequentemente, se o esgoto detectado é de introdução recente ou antiga (EGANHOUSE *et al.,* 1983; TAKADA & ISHIWATARI, 1990).

Tanto os esteróis como os LABs são hidrofóbicos e, portanto, são preferencialmente adsorvidos a partículas em suspensão e / ou sedimentadas. Essa distribuição nos dois principais compartimentos aquáticos (coluna de água e sedimento), bem como sua maior resistência à degradação ambiental, permite o

rastreamento da fonte, rota e destino destes marcadores e da matéria orgânica relacionada a eles (TAKADA & EGANHOUSE, 1998; DAUNER & MARTINS, 2015; BIGUS *et al.,* 2014).

2.4 MATERIAL PARTICULADO EM SUSPENSÃO (MPS) E SEDIMENTOS COMO MATRIZES DE ESTUDO DE FONTE, TRANSPORTE E DESTINO DE MATÉRIA ORGÂNICA

O MPS é o conjunto de partículas orgânicas e inorgânicas não dissolvidas na coluna de água com diâmetro maior que 0,45 µm (MANZOLLI *et al.*, 2011). Essas partículas são capazes de adsorver grande variedade de compostos químicos, (TAKADA & EGANHOUSE, 1998; DAUNER & MARTINS, 2015; VOLKMAN & SMITTENBERG, 2017), e por isso age como um importante elo entre compostos presentes na coluna de água, no sedimento de fundo e a teia trófica (CLARK, 2001).

O MPS estuarino é composto de partículas de origem oceânica, fluvial, atmosférica e da produção biológica (SALOMONS & FORSTNER, 1984; CHESTER, 2000). Atividades decorrentes da densa ocupação humana na região costeira como desmatamento, processos de erosão, descarga de poluentes (principalmente esgoto) e dragagens também contribuem de forma significativa à quantidade e qualidade do MPS (SALOMONS & FORSTNER, 1984; CHESTER, 2000; ODRESKI *et al*., 2003). A intensidade de contribuição desses fatores varia de acordo com o tipo de estuário e com alterações ambientais de grandes (p. ex. sazonalidade) e pequenas (p. ex. ciclos de marés) escalas temporais, que alteram o padrão de circulação da água e processos de ressuspensão (SALOMONS & FORSTNER, 1984; JONES *et al*., 1998; CHESTER, 2000; PEREIRA *et al*., 2010). É comum, por exemplo, que a maior intensidade de transporte de MPS ocorra em períodos de sizígia, e este fator associado com outras condições ambientais, como períodos de maior pluviosidade, por exemplo, pode caracterizar o estuário como exportador ou importador de MPS, com significativas variações na concentração, composição e origem de suas partículas constituintes (JONES *et al*., 1998; PEREIRA *et al*., 2010).

Processos de intemperismo e introdução de material continental no ambiente aquático são contínuos. A maior parte deste material é transportada, estabilizada por meio de processos químicos e biológicos na coluna da água (adsorvidos ou não no MPS) e então depositada no leito de ambientes marinhos e estuarinos, podendo

permanecer acumulados durante longos períodos cronológicos (FÜTTERER, 2006; MARTINS & FIGUEIRA, 2008; VOLKMAN & SMITTENBERG, 2017).

Testemunhos sedimentares são amostras pontuais de perfis verticais de sedimento obtidas através de amostradores com a capacidade de penetração vertical no fundo marinho ou estuarino (GRIEP, 2011). Esta amostragem é aplicada quando se objetiva estudos estratigráficos e pode ser complementada com técnicas de datações, baseadas em métodos de determinação das taxas de sedimentação através dos radionuclídeos ^{210}Pb e ^{137}Cs, por exemplo (MARTINS & FIGUEIRA, 2008), em se tratando de períodos recentes (inferiores aos últimos 150 anos).

Através da distribuição de marcadores orgânicos geoquímicos ao longo de testemunhos datados é possível reconstruir padrões históricos de deposição sedimentar e contaminação em ambientes aquáticos ao longo de décadas ou mesmo séculos. Isso auxilia na interpretação de tendências de contaminação em diferentes escalas temporais e na avaliação de potenciais riscos ambientais (TAKADA & EGANHOUSE, 1998; CARREIRA *et al.,* 2004; MARTINS *et al.,* 2011).

A reconstrução histórica da contaminação através de estudos de colunas sedimentares só é possível devido às propriedades do sedimento e dos compostos orgânicos. Marcadores orgânicos geoquímicos de característica hidrofóbica são muito utilizados nesses estudos, já que sua característica apolar permite sua adsorção no MPS que eventualmente será depositado no fundo marinho, permanecendo como um registro da atividade antrópica em um determinado período de tempo (BOONYATUMANOND *et al*., 2007; BIGUS *et al.,* 2014). Isto torna o MPS e o sedimento, importantes matrizes de estudos relacionados a fontes de matéria orgânica, nutrientes e contaminação, bem como para acompanhar e prever processos ecológicos e geológicos (DACHS *et al.,* 1999; MANTOVANELLI *et al.,* 2004; CORDEIRO *et al.,* 2008).

2.5 LITORAL DO PARANÁ

O litoral do Paraná possui uma área total de 6.058 km² (IBGE, 2017) e 3.000 km² de ambiente marinho (incluídas as 12 milhas do mar territorial brasileiro) (PARANÁ, 2006). Duas regiões estuarinas interrompem a linha de costa: o Sistema Estuarino de Paranaguá (SEP; 25º30´S, 48º25´O) ao Norte, e a Baía de Guaratuba (25°52’S, 48°38'O) ao Sul. Remanescentes conservados do bioma Mata Atlântica, incluindo amplas faixas de manguezais, são característicos do litoral paranaense. As

regiões estuarinas de Paranaguá e Guaratuba contemplam o manguezal como principal tipo de vegetação, o qual se desenvolve por longas áreas (incluindo rios mais internos), e desaparece com a diminuição da salinidade e aumento da correnteza. Restinga é encontrada em algumas ilhas arenosas (BIGARELLA, 2001; PARANÁ, 2006). Essas características configuram a região litorânea como uma área de grande heterogeneidade biológica e de importância para conservação, de forma que um mosaico de unidades de conservação cobre mais de 80% da área (DENARDIN *et al.*, 2008).

O clima do litoral paranaense é influenciado pela atuação conjunta das massas de ar (tropicais: Atlântica e Continental, e; extratropicais: Massa Polar Atlântica e Frente Polar Atlântica), maresia, relevo do entorno e vegetação (LANA *et al.* 2001; VANHONI & MENDONÇA, 2008). Este conjunto de fatores gera na região o clima do tipo temperado chuvoso e moderadamente quente (CFa), com chuvas bem distribuídas ao longo do ano (média anual de 2.500 mm e máxima de 5.300 mm), de menor intensidade entre outono e inverno, mas permanentemente úmido (LANA *et al.* 2001; VANHONI & MENDONÇA, 2008). A pluviosidade aumenta em mais de três vezes no período chuvoso, que contempla o final da primavera e a maior parte do verão. As maiores temperaturas do ar são registradas nos meses de dezembro, janeiro e fevereiro (média das temperaturas máximas entre 29 e 30 °C) e as menores entre junho, julho e agosto (média das temperaturas mínimas entre 12 e 14°C) (LANA *et al.* 2001; VANHONI & MENDONÇA, 2008).

Sete municípios compõem o litoral do Paraná (Guaraqueçaba, Antonina, Morretes, Paranaguá, Pontal do Paraná, Matinhos e Guaratuba), os quais juntos totalizam uma população estimada para 2017 de 291.687 habitantes residentes, sendo Paranaguá o mais populoso (152.975 para o ano de 2017) (IBGE, 2017). O litoral paranaense foi a primeira região do Estado a ser colonizada, no entanto o desenvolvimento socioeconômico é restrito e o sistema sanitário é precário, sendo reconhecida como uma das regiões mais pobres do Paraná (ESTADES, 2003; DENARDIN *et al.*, 2008).

As principais atividades humanas na região são as portuárias, rurais e turísticas, concentradas respectivamente nas cidades de Paranaguá e Antonina; Morretes e Guaraqueçaba, e; Matinhos, Pontal do Paraná e Guaratuba (DENARDIN *et al.*, 2008). Dentre essas atividades, a portuária é a de maior destaque, inclusive nacional, com o porto de Paranaguá sendo o maior exportador de grãos da América

Latina (APPA, 2017).

Devido a estas atividades, o litoral do Paraná, especialmente as regiões estuarinas, está sujeito a variadas fontes de alteração antrópica como dragagens, ocupação irregular, introdução de poluentes derivados do petróleo, agrotóxicos, esgoto e atividades industriais entre outros (PARANÁ, 2006; MARTINS *et al.*, 2010b; COMBI *et al.*, 2013; DAUNER & MARTINS, 2015; CARDOSO *et al.,* 2016). Tanto para a Baía de Paranaguá como de Guaratuba, a introdução de esgoto é um dos principais problemas, considerando a precariedade de redes de esgoto e a presença de ligações clandestinas nas galerias pluviais. No geral, a Baía de Paranaguá apresenta pontos específicos e restritos de baixa qualidade de água, concentrados na borda sul da baía, especificamente em Antonina, foz do rio Sagrado (Paranaguá) e região de Encantadas (Ilha do Mel) (PARANÁ, 2006; MARTINS *et al.*, 2010b). Na baía de Guaratuba, há também alto risco de contaminação por agrotóxicos, principalmente em áreas da região mediana a interna da baía, e próximas ao centro urbano da cidade de Guaratuba (PARANÁ, 2006, DAUNER & MARTINS, 2015).

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CAPÍTULO 2

Uma avaliação integrada de algumas bactérias indicadoras fecais (BIF) e marcadores químicos como potenciais ferramentas de monitoramento de contaminação por esgoto em estuários subtropicais

An integrated evaluation of some faecal indicator bacteria (FIB) and chemical markers as potential tools for monitoring sewage contamination in subtropical estuaries

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Primeira página do artigo publicado no ANEXO 1.

Abstract

Sewage input and the relationship between chemical markers (linear alkylbenzenes and coprostanol) and fecal indicator bacteria (FIB, *Escherichia coli* and enterococci), were evaluated in order to establish thresholds values for chemical markers in suspended particulate matter (SPM) as indicators of sewage contamination in two subtropical estuaries in South Atlantic Brazil. Both chemical markers presented no linear relationship with FIB due to high spatial microbiological variability, however, microbiological water quality was related to coprostanol values when analyzed by logistic regression, indicating that linear models may not be the best representation of the relationship between both classes of indicators. Logistic regression was performed with all data and separately for two sampling seasons, using 800 and 100 MPN 100 mL^{-1} of *E. coli* and enterococci, respectively, as the microbiological limits of sewage contamination. Threshold values of coprostanol varied depending on the FIB and season, ranging between 1.00 and 2.23 $\mu g\ g^{-1}$ SPM. The range of threshold values of coprostanol for SPM are relatively higher and more variable than those suggested in literature for sediments (0.10 to 0.50 $\mu g\ g^{-1}$), probably due to higher concentration of coprostanol in SPM than in sediment. Temperature may affect the relationship between microbiological indicators and coprostanol, since the threshold value of coprostanol found here was similar to tropical areas, but lower than those found during winter in temperate areas, reinforcing the idea that threshold values should be calibrated for different climatic conditions.

Capsule: *Coprostanol levels present in estuarine suspended particulate matter can be used to predict microbiological water quality, when analyzed by logistic regression.*

Keywords: coprostanol, linear alkylbenzenes, *E. coli*, enterococci, logistic regression, South Atlantic.

Resumo

A introdução de esgoto e a relação entre marcadores químicos (alquilbenzeno lineares e coprostanol) e bactérias indicadoras fecais (BIF, *Escherichia coli* e enterococci) foram avaliados a fim de estabelecer valores limites para marcadores químicos no material particulado em suspensão (MPS) como indicadores de contaminação por esgoto em dois estuários subtropicais no Atlântico Sul, Brasil. Os dois marcadores químicos não apresentaram relação linear com BIF, devido à alta variabilidade espacial microbiológica. Entretanto, a qualidade microbiológica da água foi relacionada aos valores de coprostanol quando analisada através de regressão logística, indicando que modelos lineares podem não ser a melhor representação da relação entre ambas as classes de indicadores. A regressão logística foi realizada com todos os dados juntos e separadamente para as duas estações amostradas, usando 800 e 100 NMP 100 mL^{-1} de *E. coli* e enterococci, respectivamente, como os limites microbiológicos de contaminação por esgoto. Valores limites de coprostanol variaram dependendo do BIF e estação climática, variando entre 1,00 e 2,23 $\mu g\ g^{-1}$ MPS. A faixa de valores limites de coprostanol para MPS foram relativamente maiores e mais variáveis que aqueles sugeridos pela literatura para os sedimentos (0,10 a 0,50 $\mu g\ g^{-1}$), provavelmente devido à maior concentração de coprostanol no MPS do que no sedimento. A temperatura pode afetar a relação entre indicadores microbiológicos e coprostanol, já que os valores limites de coprostanol encontrados aqui foram similares ao de áreas tropicais, mas menores que aqueles encontrados durante o inverno em áreas temperadas, reforçando a ideia de que valores limites devem ser calibrados para diferentes condições climáticas.

Palavras chave: coprostanol, alquilbenzeno lineares, *E. coli*, enterococci, regressão logística, Atlântico Sul.

Highlights

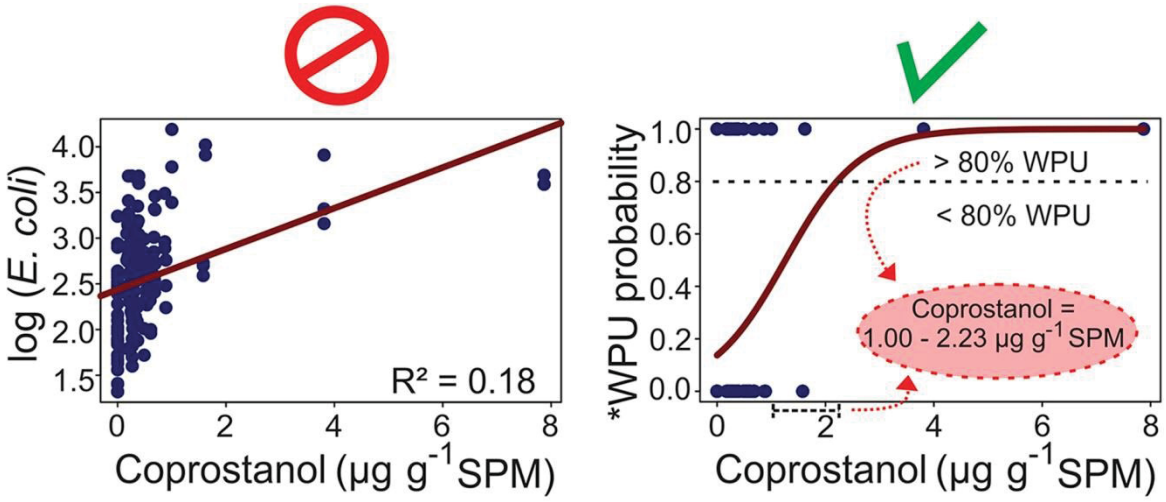
> LABs and coprostanol levels were determined on suspended particulate matter.

> Logistic regression was used to analyze relationships between sewage indicators.

> Coprostanol predicted microbiological water quality with 80% accuracy.

> Coprostanol threshold level of unsafe sewage input was 1.00 - 2.23 µg g^{-1} SPM.

Graphical abstract



* Waters potentially unusable

1. INTRODUCTION

Urban sewage is a source of contamination of major concern in estuarine systems and contributes to environmental and human health disturbances, decreasing the water quality for various human uses. Sewage input tends to become worse as populations grow due to the slower development of treatment infrastructure, and its detection is fundamental to provide warning of the need for improvements in treatment services (Who, 2003).

Sewage collection and treatment services in Brazil are poor and inefficient, with more than 70% of cities without an efficient network of these services (IBGE, 2011). Guaratuba and Paranaguá Bays, located in the South Atlantic, Brazil, are subtropical estuarine environments which present conflicting scenarios of diverse, pristine ecosystems with economic interest through permanent anthropogenic pressure resulting from port, agriculture and tourism activities (Lana et al., 2001; Dauner and Martins, 2015). Among the sources of pollution recognized in the region, sewage is still considered problematic due to poor collection and sewage treatment, and the unregulated discharges of sewage through to drains and channels (Lana et al., 2001; Kolm et al., 2002).

Escherichia coli and enterococci are fecal indicator bacteria (FIB) recommended by research agencies developing water quality regulations as sewage indicators (CONAMA, 2000; APHA, 2009). Nevertheless, the use of these indicators has been questioned due to variation in their reproduction and mortality rates under different environmental conditions (Anderson et al., 2005; Power et al., 2016).

Coprostanol and linear alkylbenzenes (LABs) have been also used to detect sources of domestic effluent contamination. Coprostanol is the main product of cholesterol degradation in the intestinal tract of higher mammals (about 60% of total sterols in human feces) and it is rarely detected in environments with no sewage input (Grimalt et al., 1990; Takada and Eganhouse, 1998) while LABs were used as raw material in detergent production, remaining as trace residue (about 3 – 5%) in the final product (Takada and Eganhouse, 1998). Both chemical markers are commonly studied in sediments rather than in suspended particulate matter (SPM). However SPM is highly relevant because it is included in environmental analyses for microbiological water quality and can indicate recent input of sewage, while sediments reflect historic deposition (Takada and Eganhouse, 1998). These chemical markers have been used for this purpose since the 1960s (coprostanol) and 1980s (LABs) (Murtaugh and Bunch, 1967; Vivian, 1986), however there is still no consensus regarding threshold indicator concentrations for sewage contamination (Isobe et al., 2004; Martins et al., 2014).

Some researchers have compared the abundance of chemical markers (even tried to find limit values of coprostanol) and FIBs with linear regression. Relationships between FIB and chemical markers in different matrices (as water, SPM and sediments) have been found to be positive and significant in temperate regions (Nichols et al., 1993; Leeming and Nichols, 1996), but showed some deviations when compared to studies in tropical or subtropical areas (Isobe et al., 2004; Costa and Carreira, 2005). This indicates that linear models may not be suitable for examining the relationship between these two effluent indicator categories (chemical and biological).

In this context, the present study aims to evaluate the integrated use of chemical markers (LABs and coprostanol) and FIB (*E. coli* and enterococci) in order to establish threshold values for LABs and coprostanol in SPM as indicative of environmental sewage impact to subtropical estuarine regions. For this purpose, we evaluated the relationship between these two effluent indicators categories (chemical and biological) with linear regression analysis, and compared this with logistic regression analysis.

2. STUDY AREA

Paranaguá (25º30´S, 48º25´W) and Guaratuba (25°52'S, 48°38'W) Bays are subtropical estuaries on the South Brazilian coast (Fig. 1a). The climate is wet and warm, with mean annual rainfall of 2500 mm, which is more intense during summer. The water surface area and the water residence time are 330 and 50 km² and three and nine days for Paranaguá and Guaratuba Bays, respectively (Lana et al., 2001; Marone et al., 2005). Hydrodynamics are mainly governed by river flow and tides, wherein Cachoeira and Nhundiaquara Rivers are the major contributors for Paranaguá Bay (Mantovanelli et al., 2004), and Cubatão and São João Rivers for Guaratuba Bay (Marone et al., 2006).

There is a resident population of about 217,066 and 66,915 around the Bays of Paranaguá and Guaratuba, respectively (IBGE, 2014), and this number can increase six-fold in summer as result of the intensive tourism activities in these estuaries (Dauner and Martins, 2015). Specific and restricted points of poor water quality and/or high contamination risk by pollutants occur at the southern margin of these bays, specifically in Encantadas inlet and around Antonina, Paranaguá (which have extensive urban development and harbors) and Guaratuba cities, (Kolm et al., 2002; Dauner and Martins, 2015). Three sewage treatment plants in Paranaguá and one in Guaratuba city (Fig. 1) were recently installed, however they

do not service all local inhabitants, especially during summer when the population density and rainfall increase, resulting in overflows of untreated sewage.

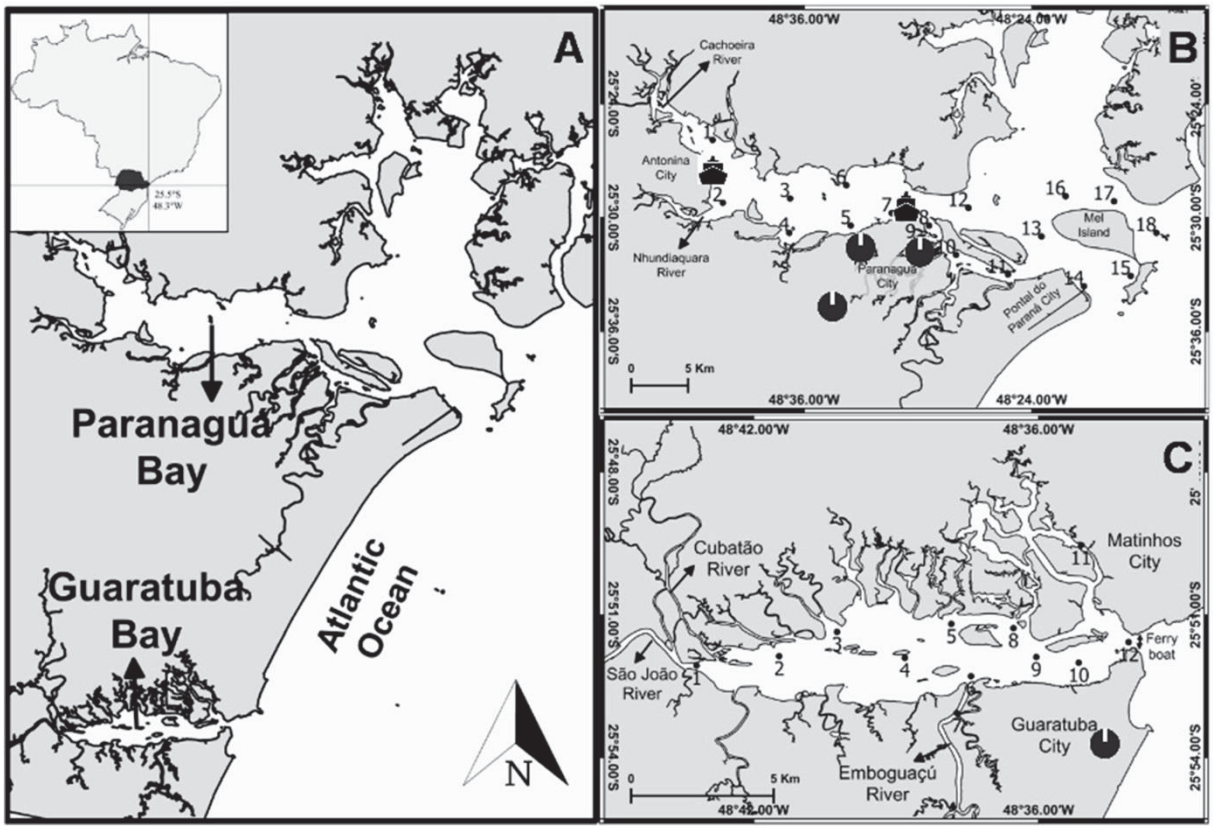


Fig. 1. (A) Map of study area indicating part of South Brazilian coast, and sampling sites in (B) Paranaguá Bay and (C) Guaratuba Bay. Ship symbol indicates harbor areas; cut circles indicate sewage treatment plants.

3. MATERIAL AND METHODS

3.1. Sample collection and physical - chemical water column parameters

One austral winter sampling campaign (August/2014), and one summer campaign (January and February/2015) were performed for both bays, during ebb spring tides. In each season, 12 sites were sampled in Guaratuba Bay and 18 in Paranaguá Bay (Fig. 1b and 1c), to obtain large spatial coverage, including sites where FIB and chemical markers have already been detected (Kolm et al., 2002; Martins et al., 2010). For FIBs, surface water was collected using previously autoclaved (121°C for 20 minutes) glass bottles (300 mL), in triplicate at each site. One sample of surface water was collected for each site for chemical analysis, using previously cleaned amber glass bottles (4 L).

Temperature, salinity and depth were measured at each site with CTD profiles (CastAway P/N 400313 SonTek). Water samples were also collected for determination of

dissolved oxygen (DO - determined by titration method using an automatic titrator – Metrohm 702SM Titrino), and pH (using a digital pH meter - PHTEK). Precipitation data were provided by the Meteorological Service of Paraná (SIMEPAR).

3.2. Analysis procedures for FIB

After collection, samples were stored in ice and taken to the laboratory to be processed within 24 h. *Escherichia coli* and enterococci were enumerated as most probable number (MPN) per 100 mL of water by the Quanti-Tray Method (Iddexx Laboratories, Maine, USA) (APHA, 2009). The Quanti-Tray Method details are described on supplementary information.

3.3. Analysis procedures for chemical markers

3.3.1. Sampling preparation

Water samples were vacuum filtered (approximately 3.5 L, within 12 h after collection) with GF/F Whatman® filters (0.7 μm), previously calcinated at 400 ºC for 4 h. Filters with SPM were frozen and freeze-dried before the extraction procedure. Seston data was obtained by gravimetric methods.

3.3.2. Sample extraction, clean up and instrumental analysis of chemical markers

The analytical procedure for sterols and LABs analysis was adapted from Wisnieski et al. (2016). SPM on filters were Soxhlet extracted for 8 h with 90 mL of *n*-hexane:dichloromethane (DCM) (1:1) and spiked with 500 ng of 5α-androstanol and 125 ng of 1-C_{12}LABs (including blanks). The extracts were concentrated to 1mL using a vacuum rotary evaporator, purified and fractionated by liquid chromatography on 5% deactivated silica and alumina columns with elution of 10 mL of *n*-hexane to obtain the LAB fraction and following this, 5 mL of ethanol/DCM (1:9, v/v) followed by 15 mL of ethanol to obtain the sterols fraction. LAB fractions were concentrated using a vacuum rotary evaporator and a slight stream of nitrogen, when necessary, and then were spiked with 125 ng of the internal standard 1-C_{19}LAB reaching a final volume of 250 mL. The sterol fractions were dried, derivatised (BSTFA/TMCS (99:1) for 90 min at 70 ºC), spiked with 500 ng of 5α-cholestane and had its volume adjusted to 250 mL with *n*-hexane before injection.

LABs were analyzed using an Agilent GC 7890A gas chromatograph coupled to an Agilent 5975C inert MSD with a Triple-Axis Detector Mass Spectrometer with helium (99.999% of purity) as the carrier gas. Sterols were analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC/FID), with hydrogen (99.999%

208 of purity) as the carrier gas. The details of instrumental analysis and analytical control are
209 described in supplementary information.
210
211 3.3.3. Data analysis
212 The microbiological water quality evaluation was based on *E. coli* and enterococci
213 limits described by Brazilian Council of Environment (CONAMA, 2000, Table 1) for marine
214 recreational waters of primary human contact. Enterococci results are also discussed in
215 relation to World Health Organization (WHO, 2003) and Environmental Protection Agency of
216 United States of America (USEPA, 2012) limits (Table 1), because they are used as the basis
217 for many countries guidelines. Narrower limits of each guideline were used for evaluation of
218 results, using the limit of "satisfactory" category as defined by CONAMA (2000) and class
219 "C" from WHO (2003). Sites with two or three of the triplicates with concentrations above
220 these limits are considered here as being in the "waters potentially unusable" category (WPU
221 - Table 1).
222

Table 1: Categories of marine recreational water quality according to regulations of Brazilian Council of Environment (CONAMA, 2000). Threshold values of World Health Organization (WHO, 2003) and Environmental Protection Agency of United States of America (USEPA, 2012) shown within CONAMA categories for comparison. ENT = enterococci; WPU = Waters potentially unusable.

	Excellent		Very good		Satisfactory		WPU[a]		Unusable	
Categories	*E. coli*	ENT	*E. coli*	ENT	*E. coli*	ENT	*E. coli*	ENT	*E. coli*	ENT
CONAMA	≤200	≤25	201-400	25-50	401-800	51-100	>800	>100	>2000	>400
WHO	-	-	-	≤40	-	41-200	-	201-500	-	>500
USEPA	-	-	-	-	-	-	-	>130[b]	-	-

[a] defined by the authors as CONAMA do not classify this range of values.

[b] According statistical threshold value (STV) described in USEPA (2012).

223
224 Principal Component Analysis (PCA) was performed using the water column
225 parameters (temperature, salinity, pH, DO and SPM) and precipitation (eight and two days
226 before sampling) to examine differences in climate and water column conditions under winter
227 and summer sampling. Comparisons of FIB concentrations between the two campaigns were
228 made using the Wilcoxon rank sum test. Two approaches were used to assess the relationship
229 between chemical markers and FIB. The first was Pearson correlation with raw data of
230 chemical markers and log transformed data of FIB. The second was logistic regression, where
231 FIB values were converted into binary data [1,0], where "1" (success) is the code for FIB

concentrations above satisfactory limits according CONAMA (2000) (or WPU) and "0" when FIB was below this limit (failure) and then, into probabilities of exceeding guideline limits, based on the binary distribution (Sheather, 2009). The logistic regressions tested whether FIB WPU water quality can be predicted by coprostanol. If there is a significant ($p < 0.05$) positive relationship, the threshold concentration limits of coprostanol are obtained by probability distribution that, in this study, was defined as an 80% of chance of achieving an acceptable FIB threshold limit. All analysis were performed in the R 3.3.1 software, using factor extra package (version 1.0.3) for PCA analysis.

4. RESULTS AND DISCUSSION

4.1. Water column parameters

Results for water column parameters at each site and each sampling campaign (winter and summer) are shown in Table S1. Accumulated precipitation (8 days before sampling), water temperature, salinity and pH were within expected ranges for spatial distribution and each season in both study areas (Lana et al., 2001; Dauner and Martins, 2015; Dauner et al., 2016). Lower DO levels recorded in summer (3.68 ± 0.77 mL L^{-1}) than winter (4.76 ± 0.72 mL L^{-1}) could be explained by higher summer water temperatures, which decreases oxygen dissolution. Moreover, the proximity to rivers and mangrove channels usually influence oxygen levels due to high rates of organic matter decomposition, and consequently higher oxygen consumption (Lana et al., 2001; Mizerkowski et al., 2012).

PCA confirmed distinct differences in climate and water column conditions between winter and summer sampling, with the principal components 1 and 2 explaining 49.9% and 17.5% of variability of the data set, respectively (Fig. S1). This analysis showed a clear pattern of seasonal differences, that was influenced mainly by higher temperature and precipitation in summer and higher salinity, pH and DO in winter. These parameters may influence the life cycle of FIBs, especially of reproduction and survival rates (Lipp et al., 2001; Byappanahalli et al., 2012). The amount of sewage released into the environment also tends to be greater in the high rainfall conditions and higher population density, as occurs in summer in this region.

4.2. Microbiological indicators of sewage input

4.2.1. Distribution of *E. coli* and enterococci in the estuarine systems analyzed

Concentrations of *E. coli* and enterococci are shown in Fig. 2 and Table 2. Concentrations of *E. coli* ranged from 21 to 15,531 MPN 100 mL^{-1} in winter, and from 26 to 10,462 MPN 100 mL^{-1} in summer, similar to levels previously recorded in these bays (Kolm et al., 2002; Forcelini et al., 2013). Concentrations of enterococci ranged from < 3 to 9,139 MPN 100 mL^{-1} in winter and from 4 to 855 MPN 100 mL^{-1} in summer. There are no previous enterococci records for waters of these bays according to the available literature.

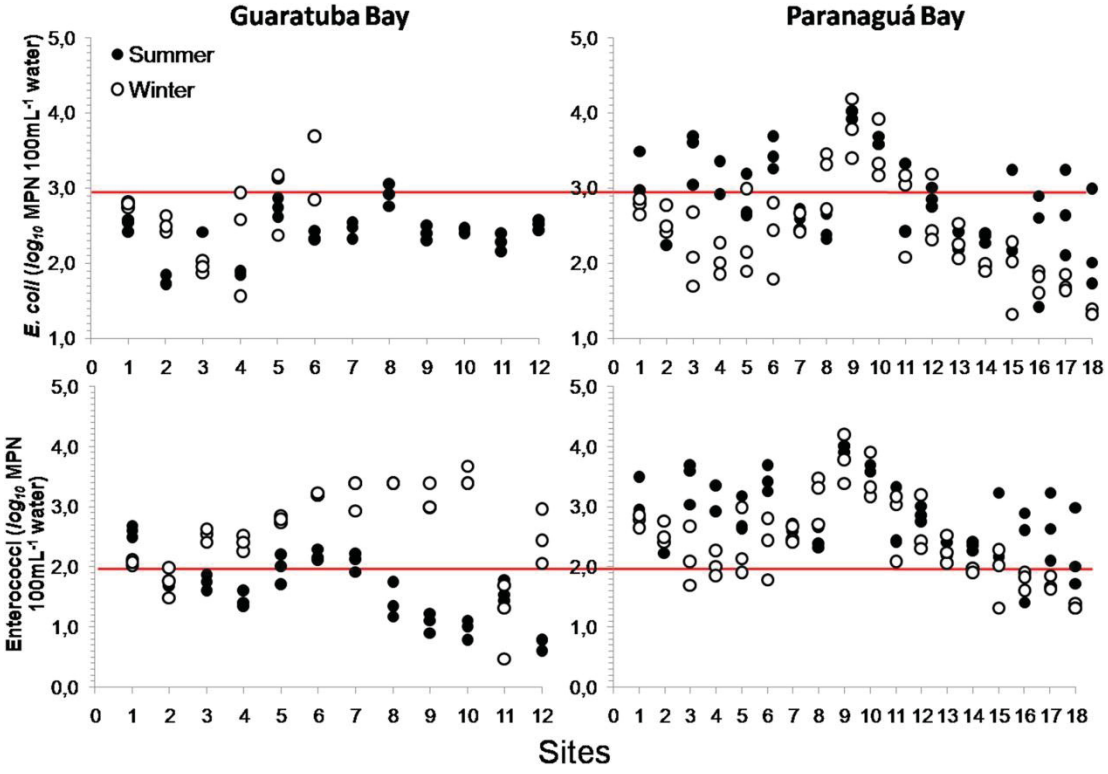


Fig. 2: Concentrations of *E. coli* and enterococci (log_{10} MPN 100 mL^{-1} water), distributed in bays, seasons (winter = open circle; summer = closed circle) and samplings sites. Limit line defined according to CONAMA (2000) concerning bathing conditions (= satisfactory limit or WPU as categorized here: 800 and 100 MPN 100 mL^{-1} water for *E. coli* and enterococci, respectively). Data of *E. coli* from sites 7 - 12 related to winter in Guaratuba bay are not available.

There was no significant difference in *E. coli* concentration between summer and winter ($p = 0.08$; Fig. S2a), while enterococci showed higher concentrations in winter (1191 ± 1729 MPN 100 mL^{-1}) than summer (121 ± 155 MPN 100 mL^{-1}) ($p < 0.01$; Fig. S2b). This is contrary to the expectations that values of FIB would be higher in summer due to the increase in population density and sewage release in this period (Dauner and Martins, 2015). However,

284 Forcelini et al. (2013) found higher values of *E. coli* in Guaratuba Bay in summer or no
285 significant difference between seasons, respectively, which also differ from our results.
286

Table 2: Concentrations of molecular markers (LABs and coprostanol) and microbiological indicators (*E. coli* and enterococci in minimum – maximum value) by site (G = Guaratuba Bay; P = Paranaguá Bay), and season (W: winter; S: summer). Samples with two or three of triplicates that fall into the WPU category are underlined. na = not available; < DL = lower than detection limits.

Samples	Total LABs ($\mu g\ g^{-1}$ SPM)		coprostanol ($\mu g\ g^{-1}$ SPM)		*E. coli* MPN 100 mL^{-1}		Enterococci MPN 100 mL^{-1}	
	W	S	W	S	W	S	W	S
G1	1.16	1.57	0.40	0.68	548 - 613	260 - 387	**104 - 134**	**308 - 488**
G2	0.76	1.02	< DL	< DL	260 - 435	52 - 69	31 - 96	48 - 59
G3	0.29	0.74	< DL	0.61	75 - 107	91 - 261	**261 - 435**	40 - 73
G4	0.34	0.62	< DL	< DL	36 - 866	70 - 78	**178 - 326**	22 - 40
G5	0.47	0.86	0.19	0.20	238 - **1462**	411 - 727	**549 - 687**	51 - **159**
G6	0.62	0.68	0.26	0.53	690 - **> 4839**	203 - 267	**1553 - 1733**	**129 - 199**
G7	0.53	0.53	< DL	0.59	na	208 - 345	**870 - >2420**	83 - **165**
G8	0.41	0.41	0.26	0.17	na	579 - **1120**	**>2420**	15 – 56
G9	0.56	0.42	0.20	0.22	na	198 - 313	**961 - > 2420**	8 – 17
G10	0.38	0.39	0.16	< DL	na	247 - 294	**>2420 - 4803**	6 – 13
G11	0.56	0.57	0.18	0.27	na	142 - 246	3 – 50	27 – 62
G12	0.58	0.45	< DL	< DL	na	271 - 370	**116 – 908**	4 – 6
P1	0.34	1.66	0.48	0.87	438 - 714	775 - **3106**	**548 - 1292**	41-75
P2	0.23	1.67	0.25	0.89	261 - 584	172 - 582	**219 - 1533**	48 – 72
P3	0.32	1.42	0.27	0.39	50 - 471	**1095 - > 4839**	**1130 - 2302**	64 - 147
P4	0.36	1.08	0.28	0.37	71 - 185	75 - **2240**	**194 - 3534**	76 - 106
P5	0.20	0.69	0.33	0.38	79 - 968	429 - 1540	**166 – 382**	73 – **2092**
P6	0.34	0.38	0.19	0.20	62 - 627	**1842 - 4839**	**833 - 2402**	99 – **177**
P7	0.23	0.92	0.56	1.58	259 - 464	393 – 538	**552 - 2978**	**189 – 228**
P8	0.18	1.44	0.69	0.60	520 - **2894**	211 – 455	98 – **2258**	52 – 85
P9	0.15	1.02	1.00	1.62	**2481 - 15531**	**8164 - 10462**	< 10 – 96	**624 – 855**
P10	0.17	0.79	3.81	7.87	**1459 - 8164**	**3873 - 4884**	**3762 - 9139**	**216 – 464**
P11	0.17	0.37	0.36	0.68	122 - **1459**	259 – 2092	< 10 - 627	85 - 119
P12	0.19	0.51	0.31	0.67	204 - 1555	562 – 1034	**2402 - > 6049**	**187 - 214**
P13	0.20	0.54	0.37	0.25	115 - 343	160 – 260	**833 - > 6049**	22 – 58
P14	0.24	0.86	< DL	0.54	94 - 137	187 – 256	10 - **220**	**156 - 216**
P15	0.32	0.53	< DL	< DL	21 - 191	146 – 1733	**969 - 2302**	34 – 63
P16	0.34	0.57	0.27	< DL	40 - 79	26 – 794	34 - **902**	27 – 46
P17	0.31	0.27	< DL	< DL	43 - 72	126 – 1724	< 3 - 369	15 – 19
P18	0.27	0.67	< DL	0.49	21 - 25	53 – 970	<3	8 – 24

287

288 Thus, seasonal human fluctuation seems not to be the main or unique factor that
289 influences the *E. coli* annual variation in these estuaries, and additional contributions may be
290 associated with possible biogenic sources of FIB found in livestock, domestic and wild
291 animals' feces and in association with soil, plants, zooplankton and algae (Solo-Gabriele et
292 al., 2000; Byappanahalli et al., 2012). These can contribute to elevate FIB densities during
293 periods where it may not be expected, as in winter. In fact, Santos et al. (2008) found high *E.*

294 *coli* concentrations in mangrove sediments of Guaratuba Bay far from sources of sewage
295 input.
296 Local environmental factors may also influence the distribution and survival of certain
297 microorganisms depending on the sampling period (Lipp et al., 2001). *Escherichia coli* and
298 enterococci have negative correlations with salinity and solar irradiation, wherein enterococci
299 have more resistance to high salinities, but higher decay rates from solar irradiation than *E.*
300 *coli* (Jin et al., 2004; Byappanahalli et al., 2012). In this study, lower rainfall and higher
301 salinity occurred in winter (Table S1), when there was also a lower average and peak of solar
302 irradiation in the 24 h before sampling (mean/peak: 83.5/522 W m^{-2} at winter; 288/1011W m^{-2}
303 at summer; Fig. S3), which could explain higher enterococci concentrations.
304
305 4.2.2. Water quality evaluation based on microorganism indicators and guidelines
306 The threshold values for microbiological water quality for recreational uses and the
307 percentage of samples classified as WPU for each season according CONAMA (2000), WHO
308 (2003) and USEPA (2012) are listed in Tables 1 and 3, respectively. WHO and USEPA limits
309 were in CFU 100 mL^{-1}, however comparisons among different methodologies found no
310 significant difference in the microbiological quantification for enterococci (Noble et al., 2003;
311 Cho et al., 2010).
312

Table 3: The percentage and number (in parenthesis) of Water Potentially Unusable (WPU)[a] samples for all data, separated by season, according CONAMA (2000), WHO (2003) and USEPA (2012) guidelines.

Guidelines		Winter	Summer	Both campaigns
CONAMA	*E. coli*	24 (17)	27 (24)	25 (41)
	Enterococci	79 (71)	36 (32)	57 (103)
WHO	Enterococci	68 (61)	14 (13)	41 (74)
USEPA	Enterococci	74 (67)	30 (27)	52 (94)
Mean (Enterococci)		73.7	26.7	50.3
% CV (Enterococci)		7.5	42.6	16.5

[a] defined by the authors as CONAMA do not classify this range of values. See table 1.

313
314 According to CONAMA (2000) and considering both campaigns, 25% and 57% of
315 samples were classified as WPU for *E. coli* and enterococci values, respectively. There are
316 higher percentages of WPU samples for enterococci than *E. coli* in both seasons. Enterococci
317 is more resistant in marine waters than *E. coli* which explains the higher percentages of WPU

318 samples for enterococci found here, while *E. coli* can also generate false negative results in
319 this environment (Jin et al., 2004; Byappanahalli et al., 2012).
320 Considering all enterococci values, the percentage of samples classified as WPU was
321 similar between CONAMA (2000), WHO (2003) and USEPA (2012) guidelines, with 57, 41
322 and 52% of total samples (% CV = 16.5%), respectively (Table 3). Analyzing by season, this
323 similarity between the guidelines remains in winter (% CV < 10%), while in summer there is
324 a greater variation, with a CV higher than 35% (Table 3). The similarity between the three
325 guidelines could be random, as there are no replicates of summer or winter campaigns.
326 However, we hypothesize that the similarity observed between guidelines for winter samples
327 may be due to similar climatic conditions in winter in these subtropical estuaries and in
328 temperate estuaries, where the key studies were made that the WHO and USEPA guidelines
329 are based on (WHO, 2003). This hypothesis reinforces the need to adapt the threshold values
330 of guidelines for different regions, as indicated by WHO (2003), but also for different seasons
331 of the year.
332 Guaratuba bay had sites 5 and 6 in winter and site 8 in summer classified as WPU
333 according to *E. coli* and almost all sites in winter (except sites 2 and 11), and sites 1, 5, 6 and
334 7 in summer according to enterococci (Table 2). Sites 6 and 7 are located on the dispersion
335 route of wastewater released by the sewage treatment plant of Guaratuba (STPG), which is
336 known to overflowor release untreated sewage in periods of high rainfall. Site 8 is close to
337 shellfish aquaculture where high concentrations of *E. coli* have been previously reported
338 (Forcelini et al., 2013). Sediments or biogenic sources of microorganisms may have
339 contributed to high values at sites 1 and 5 (Solo-Gabriele et al., 2000; Byappanahalli et al.,
340 2012).
341 Paranaguá Bay had sites 8, 9, 10 and 11 in winter and 1, 3, 4, 6, 9 and 10 in summer
342 classified as WPU according *E. coli* and almost all sites in winter (except 9, 11, 17 and 18),
343 and sites 5, 6, 7, 9, 10, 12 and 14 in summer for enterococci (Table 2). Sites 7 to 12 were
344 located around Paranaguá city and in Cotinga channel (Fig. 1b), where high concentrations of
345 sewage indicators were expected based on previous studies (e.g. Kolm et al., 2002; Abreu-
346 Mota et al., 2014), especially at sites 9 and 10, located on the mouth of Itiberê River which
347 runs through the Paranaguá city, and receives large amounts of wastewater. Evidence of
348 untreated domestic sewage release due the unreliability of sewer systems has also been
349 reported (Lana et al., 2001; Martins et al., 2010), such as around Antonina City (Kolm et al.,
350 2002), which would explain high concentrations at sites 1, 3 and 5.

Thus, there were clear patterns of sewage contamination concentrated near Guaratuba, Paranaguá and Antonina cities evidenced by high bacteria concentrations in both seasons and the proximity to known sewage sources.

4.3. Chemical markers of sewage input

4.3.1. Distribution of coprostanol in the estuarine systems

Coprostanol was detected in 70% and 77% of samples from winter and summer, respectively (Table 2). Coprostanol concentrations ranged from < DL to 3.81 μg g^{-1} SPM in winter and from < DL to 7.87 μg g^{-1} SPM in summer. The average and standard deviation were higher in summer (0.69 ± 1.42 μg g^{-1} SPM) than in winter (0.36 ± 0.69 μg g^{-1} SPM), but there was no significant difference between seasons (Wilcox test; $p = 0.07$; Fig. S2c). The highest coprostanol concentrations occurred in outflow areas of the major rivers (São João, Cubatão, Cachoeira and Nhundiaquara rivers) and areas with higher population density, such as Antonina, Guaratuba and Paranaguá cities (Fig. 1; sites 1, 3, 6, 7 and 8 of Guaratuba Bay and; sites 1, 2, 7, 9 and 10 of Paranaguá Bay), historically described as under sewage exposure (Martins et al., 2010; Abreu-Mota et al., 2014). In Paranaguá Bay this scenario seems to be more critical in summer, when the increase in coprostanol levels in nearby city areas (e.g. sites 2, 11 and 12; Table 2), reflected a spread of sewage contamination far from the source of sewage input.

The maximum concentration of coprostanol by volume of filtered water in the present study (0.31 μg L^{-1}) is within the same range as found in other coastal tropical and temperate regions under human influence, such as Guanabara Bay (Kalas et al., 2009) and Ebre River (Grimalt et al., 1990), which had concentrations below 1.00 μg L^{-1} (Fig. 3). Levels are in a much lower range than reported for other highly contaminated coastal regions, such as Western Malaysia and Mekong Delta (Isobe et al., 2002, 2004) and Morlaix and Seine Estuaries (Quemeur and Marty, 1992; Thoumelin et al., 1997), suggesting lower sewage contamination in Guaratuba and Paranaguá Bays. Nevertheless, it is important to highlight that sites with high coprostanol concentrations are included in the set of sites classified as WPU by FIB analysis in, at least one sampling season.

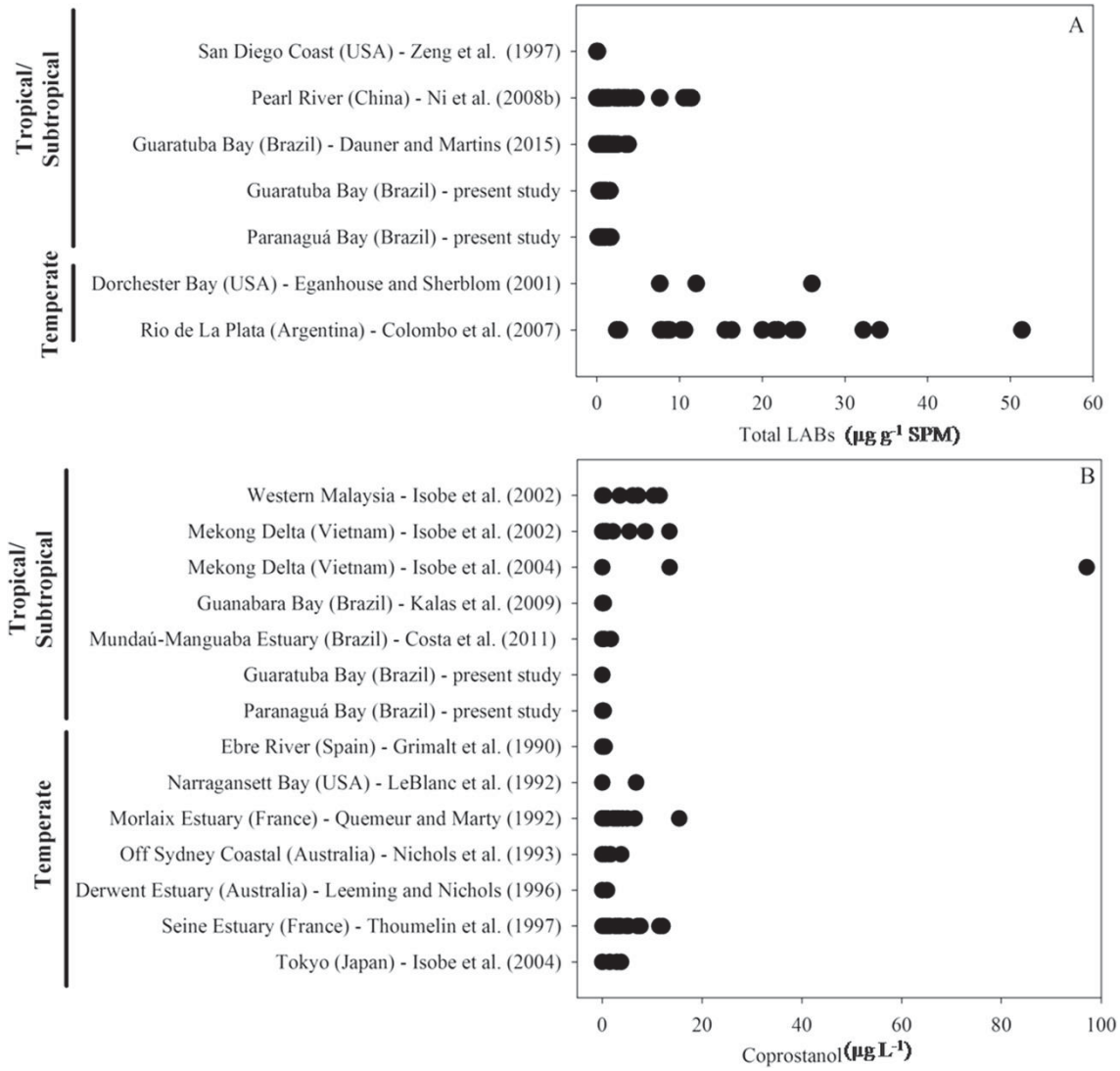


Fig. 3: Range of total LABs (in µg g^{-1} SPM) (A) and coprostanol (in µg L^{-1} for comparisons) (B) values obtained from SPM found in present and some previous studies, separated by climatic conditions (tropical/sub-supratropical and temperate) (Costa et al., 2011; LeBlanc et al., 1992; Zeng et al., 1997).

4.3.2. Distribution of total LABs in the estuarine systems

LABs were detected in all sites sampled in both bays and seasons (Table 2). Total LAB concentrations ranged from 0.15 to 1.16 in winter and between 0.27 and 1.67 µg g^{-1} SPM in summer, with significantly higher concentrations in summer (Wilcox test; $p < 0.01$; Fig. S2d). Similar to coprostanol, the highest LAB concentrations occurred in outflow areas of the major rivers (São João, Cubatão, Cachoeira and Nhundiaquara rivers) and areas near Antonina, Guaratuba and Paranaguá cities (Fig. 1), historically described as under sewage exposure (Martins et al., 2010; Abreu-Mota et al., 2014).

In general, total LAB concentrations detected here were lower than values found by Dauner and Martins (2015) in Guaratuba Bay in sampling carried out between April 2013 and

March 2014 and other places with high anthropogenic impact such as Pearl River (Ni et al., 2008b), Dorchester Bay (Eganhouse and Sherblom, 2001) and Río de La Plata (Colombo et al., 2007) (Fig. 3). This suggest that Guaratuba and Paranaguá Bays are generally only slightly affected by LAB input sources, except the inner regions that had the high values recorded in the present study (maximum of 1.57 μg g^{-1} SPM) and by Dauner and Martins (2015) (3.77 μg g^{-1} SPM).

LABs have been historically used as chemical markers of domestic sewage input (Eganhouse et al., 1983; Martins et al., 2014), however there were no significant correlations between total LABs and coprostanol (r between -0.25 and 0.14; Table 4). There was divergence between sites with high LAB levels and known sites of domestic effluent input, mainly in the inner region of the bays. Dauner and Martins (2015) also found this pattern and attributed it to possible organic matter accumulation due to natural chemical processes, such as intensified flocculation and coagulation processes in some sites of freshwater and salt mixing zones. In fact, our results show a negative correlation between total LABs and salinity (r =-0.72, $p < 0.01$). Thus, it is probable that these processes are intensified in summer due to higher rainfall (Table S1) that increases the river flows and reduces salinity, contributing to the increase of LABs in summer at sites with these physico-chemical conditions, even without known sewage input.

Table 4: Pearson correlation coefficients between total LABs, coprostanol, log (*E. coli*) and log (enterococci) in SPM collected in Guaratuba and Paranaguá Bays, Southern Atlantic, Brazil. Correlations were done with all data and also separately for season.

	coprostanol	log (*E. coli*)	log (enterococci)
Total data			
total LABs	0.13	0.16	-0.16
coprostanol	x	0.43	0.18
log (*E. coli*)		x	0.23
Winter data			
total LABs	-0.25	0.06	-0.08
coprostanol	x	0.49	0.25
log (*E. coli*)		x	0.28
Summer data			
total LABs	0.14	0.14	0.38
coprostanol	x	0.42	0.43
log (*E. coli*)		x	0.42

Therefore, LABs are not included in the integrated analysis with FIB because of the possibility of a stronger influence of natural chemical processes on the spatial distribution of LABs, not related to the sewage input.

4.4. Threshold values of coprostanol in SPM as an indicator of sewage contamination

Coprostanol has been proposed as a chemical marker of fecal contamination since the 1960s (e.g. Murtaugh and Bunch, 1967), however it has not been included in water quality legislation, probably because analysis is more expensive and harder to set up than the FIB analysis, but also to the lack of epidemiological studies relating coprostanol to waterborne illnesses, as done for FIBs (Leeming and Nichols, 1996; WHO, 2003).

A correlation between FIB and coprostanol was verified (Table 4), which led us to evaluate spatial trends in sewage contamination using chemical and bacteria data separately (see sections 4.2.2. and 4.3.1.).

A review of linear relationships between FIB and coprostanol ($R^2 > 0.50$), including (where possible) suggested threshold values of coprostanol, are listed in Table 5. Some studies have found that FIB parameters are in agreement with coprostanol data, particularly in areas near a sewage source or impacted by human activity (e.g. Mudge and Lintern, 1999; Jones et al., 2011, Table 5). In contrast, other studies (e.g. Dutka et al., 1974; Ottoson and Stenström, 2003; Shah et al., 2007, Table 5) have found no consistent relationship between FIB and fecal sterols. These contrasting findings cast doubt on the specificity and sensitivity of these previously proposed threshold values.

Tropical areas are more susceptible to these contrasting patterns than temperate or cold areas, due to high variation in the environmental conditions that may affect the survival and reproduction of microbiological organisms, as well as FIB contributions from natural sources (Isobe et al., 2002, 2004; Costa and Carreira, 2005). In regard to water temperature, the relationship between coprostanol and *E. coli* may vary between seasons (Churchland and Kan, 1982; Isobe et al., 2004) since lower temperatures during winter can inhibit the growth of *E. coli*, while coprostanol concentrations remain high. These results leads us to question whether linear regression is the best model to demonstrate relationships between these two different indicator classes in subtropical regions, and consequently, determine threshold values of coprostanol that predict water quality based on microbiological levels.

Smith et al. (2001) and Aranda et al. (2016) have found that logistic regression models may be more appropriate to identify parameters associated with FIB values above guideline

448 limits, as this approach decreases residuals arising from extreme variability inherent in
449 microbiological data that produces poor outcomes in linear regressions.
450

Table 5: Correlations between coprostanol and FIB found in previous studies. Threshold values of coprostanol (TVC) regarding FIB limits considered in each study (FIB lim), when calculated, with linear regression model; coefficient of determination (R^2); (95% confidence limits); nc = not calculated

Microbiological indicator	***R²***	**FIB lim (100 mL^{-1})**	**TVC (µg L^{-1})**	**Climate**	**Reference**
fecal coliforms	0.52	150	0.06	temperate	Leeming and Nichols (1996)
		1000	0.40	temperate	
Enterococcus	0.96	35	0.06	temperate	
		230	0.40	temperate	
fecal coliforms	x	1000	0.40 (winter)	temperate	Churchland and Kan (1982)
thermotolerant coliforms	0.89	x	nc	temperate	Nichols et al. (1993)[b]
Clostridium perfringens	0.98	x	nc	temperate	
coliforms	nc	200	0.5	temperate	Dutka et al. (1974)
fecal coliforms	0.94[a]	x	nc	temperate	Goodfellow et al. (1977)
total coliforms	0.79 [a]	x	nc	temperate	
E. coli	0.86	1000	0.03 (Mekong Estuary)	tropical	Isobe et al. (2002) [b]
E. coli	0.86	1000	0.10 (Malaysia)	tropical	
E. coli	0.86 - 0.91	1000	0.03 (wet season)	tropical	Isobe et al. (2004) [b]
			0.10 (dry season)	tropical	
E. coli	0.82 - 0.87	1000	0.03 (summer)	temperate	
E. coli		1000	0.40 (winter)	temperate	
Enterococci	0.27 - 0.86	x	nc	tropical	

[a] Calculated in present study with Goodfellow et al. (1977) data.

[b] Coprostanol from SPM.

451

452 Using logistic regression we found that coprostanol can predict the *E. coli* WPU
453 category, when analyzed with the full and winter data set. We found different threshold values
454 of coprostanol for each data set analyzed, which were 2.23 and 1.00 µg g^{-1} SPM, respectively
455 (Table 6). For enterococci, logistic regression showed that coprostanol can predict WPU
456 category when using all and the summer data sets. Based on these predictions, we found 1.71
457 and 1.16 µg g^{-1} SPM as threshold values of coprostanol for each data set analyzed,
458 respectively (Table 6).

Table 6: Threshold values of coprostanol (μg g^{-1} SPM and μg L^{-1}) for category "Waters Potentially Unusable"[a] for bathing, based on limits of microbiological indicators defined by Brazilian Council of Environment (CONAMA, 2000).

	E. coli		**Enterococci**	
	μg g^{-1} SPM	μg L^{-1}	μg g^{-1} SPM	μg L^{-1}
All data set	2.23	0.08	1.71	0.06
Winter	1.00	0.04	np	np
Summer	np	np	1.16	0.04

[a] defined by the authors as CONAMA do not classify this range of values. See table 1; np = not predicted.

In general, threshold values of coprostanol based on enterococci were lower than those obtained from *E. coli* considering all data (Table 6), probably due to the high enterococci concentrations found in winter, which resulted in a greater number of samples classified as WPU than when using *E. coli*. It should also be noted that *E. coli* is not the more indicated FIB for marine waters due its lower resistance in saline environments. Nevertheless, *E. coli* was also analyzed in this study because assessments of marine water quality for recreational use of primary contact at the Paraná coast (Brazil) have used *E. coli* as the main FIB (IAP, 2015).

Some previous studies reported coprostanol concentrations from SPM samples by water volume (Fig. 3 and Table 5). However, hydrophobic compounds, such as coprostanol, are strongly adsorbed in sediment and SPM (Takada and Eganhouse, 1998; Isobe et al., 2002). Thus, more precise results for concentration of coprostanol are obtained by normalization of coprostanol by the SPM mass, as done in present study, which can vary in a same water volume according to site studied (Ni et al., 2008a). The threshold values derived from this study were converted to μg L^{-1} for comparison with previous studies. This conversion was possible because there was strong linear relationship ($R^2 = 0.99$) between both measures of concentration of coprostanol.

Based on the equation obtained from a linear model ([cop] μg L^{-1} = 0.036 [cop] μg g^{-1} SPM), the threshold values of coprostanol by water volume corresponding to *E. coli* limits were 0.08 and 0.04 μg L^{-1} for the full and winter data set, respectively, and for enterococci were 0.06 and 0.04 μg L^{-1} for the full and summer data set, respectively (Table 6). These threshold values of coprostanol were similar to those found in tropical areas (0.03 - 0.10 μg L^{-1}; Isobe et al., 2002) or during summer in temperate areas (0.03 μg L^{-1}; Isobe et al., 2004), but

much lower than those found during winter in temperate areas (0.40 μg L^{-1}; Isobe et al., 2004). This reinforces the idea that threshold values of coprostanol based on FIBs may vary between temperate and tropical/subtropical regions, probably because the relationship between coprostanol and FIBs (especially *E. coli*) seems to be influenced by water temperature. Lower temperatures inhibit FIB growth and coprostanol degradation due to lower microbiological activity (Isobe et al., 2004), leading to higher threshold value of coprostanol in colder regions than in warmer ones. This variations between different seasons was expected, as observed by Isobe et al. (2004). However, threshold values of coprostanol based on FIBs were similar between winter and summer in the subtropical estuaries analyzed here (Table 6), probably due the lower seasonal variation of water temperature than that found in temperate regions.

The threshold value of coprostanol found for SPM samples (1.00 - 2.23 μg g^{-1} SPM) were higher and more variable than those suggested for sediments (0.10 - 0.50 μg g^{-1}; Grimalt et al., 1990; González-Oreja and Saiz-Salinas, 1998). Recent or chronic sewage input can hold higher coprostanol values in the water column that tends to decay from SPM to sediment due to high degradation in the water column (Wakeham and Ertel, 1988; Wakeham et al., 1997). In fact, the highest coprostanol values found in SPM were higher than those recorded in sediments of Paranaguá Bay (up to 2.22 μg g^{-1}; Martins et al., 2010; Abreu-Mota et al., 2014). Therefore, the higher concentrations of coprostanol in SPM justify the higher/more variable threshold values of coprostanol to SPM than sediments.

Using the proposed threshold values of coprostanol and FIB (WPU level), only sites 7, 9 and 10 of Paranaguá Bay were under the influence of high sewage input. Site 10 is more critical with coprostanol values > 2.23 μg g^{-1} SPM and classified as WPU considering both FIBs, followed by sites 9 and 7, with coprostanol between 1.00 and 2.23 μg g^{-1} SPM and at least, one FIB indicating WPU. These sites have been considered critically contaminated by sewage in previous studies done using sediment analysis (Martins et al., 2010; Abreu-Mota et al., 2014), corroborating the results found here.

It is interesting to note that if only FIB data were considered, the number of sites classified as WPU was much higher (see discussion in 4.2.2) than when considering coprostanol. Therefore, the currently applied FIB limit values may overestimate sewage contamination promoting “false positives” in these estuaries, probably due to increased FIBs from natural sources (Anderson et al., 2005; Santos et al., 2008; Byappanahalli et al., 2012). This overestimation can generate local economic losses, with respect to tourism, due to the closure of beaches (http://water.epa.gov) or decrease in visitors to areas characterized as

unusable for bathing, which, in reality, may not be contaminated. Therefore, the variability in interpretation of water quality and threshold values of coprostanol based on differing FIB demonstrates the importance of using more than one indicator to obtain a more reliable result.

The use of single threshold values to indicate poor water quality suffers from oversimplification of often complex environmental and ecological conditions. New approaches include the development of risk assessment matrices (e.g. Leeming et al., 2015), which combines threshold values (which can be from a range of markers) with other factors such as proximity to known sources and season to indicate the likely risk of problematic contamination, and coprostanol threshold values, for example, may be an important component of such approaches.

5. CONCLUSIONS

Relationships between coprostanol and LABs as chemical markers and *E. coli* and enterococci as FIBs were examined in order to perform an integrated environmental evaluation of water quality in two subtropical estuaries exposed to sewage input.

For these estuaries, the use of LABs in SPM as markers of sewage contamination is unreliable, since physical-chemical processes influenced by salinity may influence their spatial distribution. A different result was observed for coprostanol, which can accurately predict FIB categories of water quality by using logistic regression analysis. Logistic regression seems to be more appropriated to analyze these two fecal indicators categories (chemical and biological) reducing uncertainty due to extreme variability inherent in microbiological data that may produce poor outcomes in linear regressions.

Water temperature can affect the relationship between chemical markers and FIBs, which reinforces the idea of the need for thresholds of FIBs and chemical markers for specific climatic regions. The threshold values of coprostanol in SPM are higher than those suggested for sediments, and this was related to the possibility of higher coprostanol concentrations in the water column than in sediments in areas with recent or chronic sewage input.

This study does not propose replacing FIBs by coprostanol analysis, as FIB present some advantages in being cheap, quick, and easy analytical methods. However this study reinforces the need for further investigations into the currently used water quality indicators (FIBs), as well the need to develop new approaches to assess sewage contamination in coastal areas. One such approach is the use of a risk assessment matrix for environmental monitoring (e.g. Leeming et al., 2015), which incorporates multiple factors such as season, proximity to

551 human habitation or sewage inputs and concentrations other sewage derived contaminants.
552 These are used in combination with threshold values, such as for coprostanol, to assess the
553 relative likelihood of sewage contamination being of human origin and the level of health
554 risks it may represent. It is noted that although coprostanol analysis is more expensive than
555 FIBs, it is less variable, and therefore, can be analyzed with less sampling effort.
556 The evaluation of sewage chemical markers from SPM is a globally developing research
557 field, as most previous studies have been focused on sediments. Moreover, our results will be
558 useful in supporting the development of conservation policies and public health guidelines for
559 the study area.
560
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570
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752

753 **Appendix A. Supplementary information**

754 Supplementary information related to this article can be found at Supplement A.

CAPÍTULO 3

Fontes, distribuição e degradação de marcadores moleculares de esgoto e matéria orgânica natural em sedimentos superficiais e material particulado em suspensão de um estuário subtropical sob impacto humano

Insights about sources, distribution, and degradation of sewage and biogenic molecular markers in surficial sediments and suspended particulate matter from a human-impacted subtropical estuary

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Abstract

The molecular markers sterols and linear alkylbenzenes (LABs) were analyzed in the surficial sediments and suspended particulate matter (SPM) of a subtropical estuary in South Atlantic (Paranaguá Estuarine System). The purpose of this study was identifying the spatial distribution of sewage and the input of biogenic organic matter (OM) and to provide comparative insights about their behavior, compositions, and sources. The concentration of coprostanol ranged from < DL (detection limit) to 2.67 μg g^{-1} in SPM and from < DL to 0.94 μg g^{-1} in sediments. Total LABs ranged from 43.8 to 480.0 ng g^{-1} in SPM and from < DL to 21.0 ng g^{-1} in sediments. LABs homologs composition varied between the two matrices. The local hydrodynamic pattern may promote water column homogenization, dispersion, and dilution of sewage particles, and preferential sedimentation in fluvial and mixture zones. Results suggest that SPM is a good matrix for larger spatial and short time scale evaluation while sediments may help to define hot spot areas of input and final deposition of sewage particles. Marine sterols predominated in SPM while no dominance patterns of marine/terrestrial sterols occurred in surficial sediments. The higher degradation rates of sterols and LABs in the water column must be the main factor for the sharp drop in concentration towards the sediment and the variation of the preferential composition of these markers between compartments.

Capsule: *Sterols and LABs vary in concentration and composition between suspended particulate matter (higher concentrations) and surficial sediments (lower concentrations)*.

Keywords: coprostanol, linear alkylbenzenes, sewage, Paranaguá Estuarine System, organic matter.

Resumo

Os marcadores moleculares esteróis e alquilbenzeno lineares (LABs) foram analisados em sedimento superficial e material particulado em suspensão (MPS) de um estuário subtropical no Atlântico Sul (Sistema Estuarino de Paranaguá). A proposta deste estudo foi identificar a distribuição espacial de esgoto, a introdução de matéria orgânica (MO) biogênica e fornecer informações sobre o comportamento, composição e fontes destes compostos. A concentração de coprostanol variou de < LD (limite de detecção) a 2,67 μg g^{-1} no MPS, e de < LD a 0,94 μg g^{-1} nos sedimentos. LABs totais variaram de 43,8 a 480,0 ng g^{-1} no MPS e de < LD a 21,0 ng g^{-1} nos sedimentos. A composição dos homólogos de LABs variaram entre as duas matrizes. O padrão hidrodinâmico local pode promover a homogeneização da coluna de água, dispersão e diluição de partículas de esgoto, com preferencial sedimentação em zonas fluviais e de mistura. Os resultados sugerem que MPS é uma boa matriz de análise para avaliações de grandes escalas espaciais e pequenas escalas temporais, enquanto os sedimentos podem ajudar a definir áreas de risco de introdução e deposição final de materiais provenientes do esgoto. Esteróis de fontes marinhas predominaram no MPS, enquanto não foram observados padrões de dominância de esteróis marinho/terrestre nos sedimentos superficiais. A maior taxa de degradação de esteróis e LABs na coluna de água deve ser o principal fator para a brusca queda nas concentrações em direção ao sedimento e na variação da composição preferencial destes marcadores entre os compartimentos.

Palavras chave: coprostanol, alquilbenzeno lineares, esgoto, Sistema Estuarino de Paranaguá, matéria orgânica.

Highlights

> LABs and sterols were determined in suspended particulate matter and sediments.

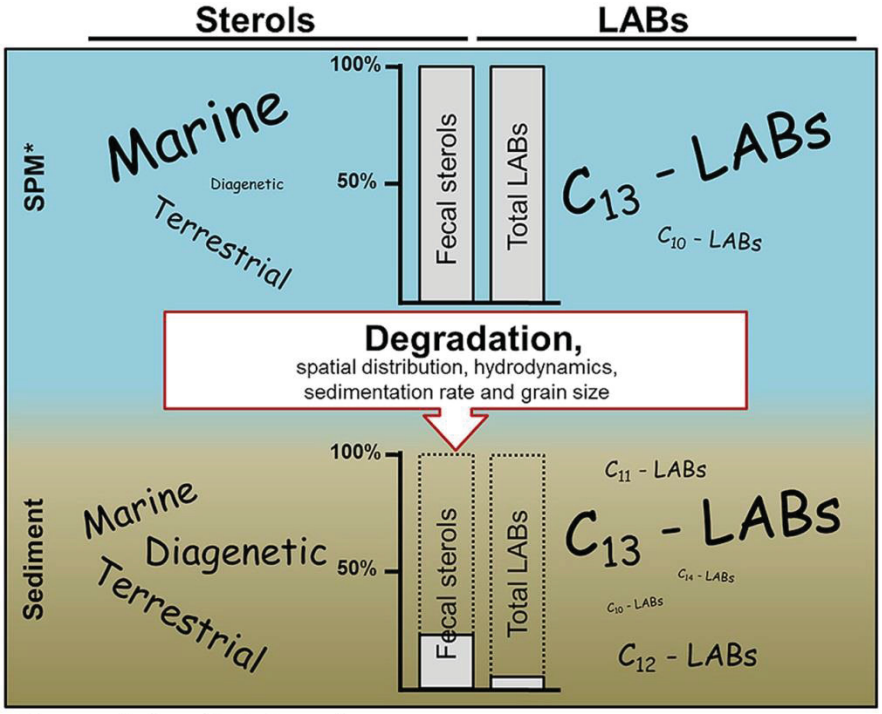
> Hydrodynamic factors (HF) allow water column homogenization of geochemical markers.

> HF and proximity of sources define local sedimentary deposition of sewage markers.

> Degradation is determinant to markers' values and composition in each compartment.

> SPM and sediments provide different spatial and temporal scales evaluation.

Graphical abstract



1. INTRODUCTION

Geochemical organic markers have been extensively used to characterize the input of organic matter (OM) in different environments and timescales and can be assigned to a specific source (Takada and Eganhouse, 1998; Derrien et al., 2017). Sterols are one of the most useful molecular markers for allochthonous (sewage and continental material) and autochthonous (zooplankton and phytoplankton) contributions (Volkman, 1986; Derrien et al., 2017). Linear alkylbenzenes (LABs) are aromatic hydrocarbons always related to anthropogenic (sewage and oil) sources (Takada and Eganhouse, 1998).

Sewage is one of the main stressor agents in coastal ecosystems, especially in estuaries where the water circulation is restricted, which hinders its dilution in the environment. The precariousness (or absence) of sanitary services in coastal regions allows the chronic input of sewage with a bulk of nutrients, pesticides, drugs, and pathogens which interferes with the natural ecosystem and public health (UNEP, 2008; Lim et al., 2017).

Because the development of sanitary systems does not keep up with accelerated increases in population density (UNEP, 2008; IBGE, 2011), the detection of environmental sewage is still the most used alternative to monitor areas susceptible to eutrophication and prevent human contact with contaminated water (e.g. WHO, 2003; CONAMA, 2000).

Because sewage is the main source of coprostanol and LABs in coastal environments, these molecular markers have been widely used in the detection of sewage input or contamination (Takada and Eganhouse, 1998; Bull et al., 2002). Coprostanol is the main product of cholesterol degradation inside the intestinal tract of homeothermic vertebrates and corresponds to about 60% of total sterols in human feces (Murtaugh and Bunch, 1967; Hatcher and McGillivary, 1979). LABs are used as raw matter in the manufacture of commercial detergents; the 3 - 5% that do not react remain as a tracing product in the final composition and can be detected in environmental samples (Eganhouse et al., 1983). Both groups of molecular markers are hydrophobic and are mainly adsorbed in the suspended particulate matter (SPM) and sediments in aquatic environments (Bull et al., 2002; Dauner and Martins, 2015).

The analysis of SPM provides information about recent input as well as origin and input pathways, while the analysis of sediments provides historical and chronic OM input information (LeBlanc et al., 1992) which is potentially useful in identifying sink areas of sediment deposition (with adsorbed OM) as probably contaminated hot spot areas (Roussiez et al., 2006; Cardoso et al., 2016). Moreover, the intense diagenetic transformation that occurs

in the water column can alter the concentration and composition of organic markers, therefore providing different results about OM sources and contamination in each compartment (Cardoso et al., 2016). Therefore, it is important to determine if hydrophobic markers such as sterols and LABs, also show different distributions in SPM and sediments.

This study investigated the spatial distribution of sterols and LABs in SPM and surficial sediments from a human-impacted subtropical estuary in order to determine sewage and biogenic OM inputs and provide comparative insights about their behavior, compositions, and sources in both compartments. The integrated approach has special relevance to estuarine environments, which have a restricted hydrodynamic circulation and are susceptible to contamination and eutrophication processes.

2. STUDY AREA

This study was performed in the E - W axis of the Paranaguá Estuarine System (PES), which includes Antonina (W side) and Paranaguá (E side) bays (25º30´S, 48º25´W) (Fig. 1). This area comprises approximately 46 km length, 10 km of maximum width and 330 km^2 of surface water area (Marone et al., 2005). The clime is rainy and moderately warm, with higher precipitation between austral spring and summer (three times more intense than in austral autumn and winter) (Lana et al., 2001). The hydrodynamic pattern is influenced by the rivers runoff and by the asymmetric variation of tides (Mantovanelli et al., 2004). The mean of the neap and spring tide are 1.3 and 1.7 m at the mouth of the bay and 2.0 and 2.7 m near to Antonina (Lana et al., 2001). Mangrove is the main vegetation at the margins of the bay (e.g. *Rhizophora mangle* and *Laguncularia racemosa*) (Bigarella, 2001); centric diatoms and phytoflagellates are the dominants algal groups on phytoplankton (Lana et al., 2001).

Previous analysis of surface water column parameters collected at the same sampling time (temperature, pH, dissolved oxygen, and salinity) indicated four sectors in Paranaguá Bay named as fluvial (sites 1 and 2), mixture 1 and 2 (sites 3 to 8), and marine (sites 9 to 15) (Cardoso et al., 2016). The two mixture zones were considered here as one because it coincides with the maximum turbidity zone (MTZ) recorded in previous studies (Mantovanelli et al., 2004; Mayerle et al., 2015).

The OM composition of this ecosystem is influenced by marine input seaward and continental supply through the Nhundiaquara and Cachoeira Rivers which flows at the inner estuary and adjacent margin runoff (Mantovanelli et al., 2004). Effluents are also introduced by these rivers, but mainly by the Anhaia, Itiberê, and Correias Rivers, which receive most of

the sewage produced in the city of Paranaguá (Martins et al., 2010a, 2011). According the last census about sanitary services on Brazil (IBGE, 2011), the city of Antonina has no service of sewage collection, and the city of Paranaguá has at least 65% of the sewage treated. Nevertheless, high concentrations of fecal sterols were recorded near the city of Paranaguá (Martins et al., 2010a).

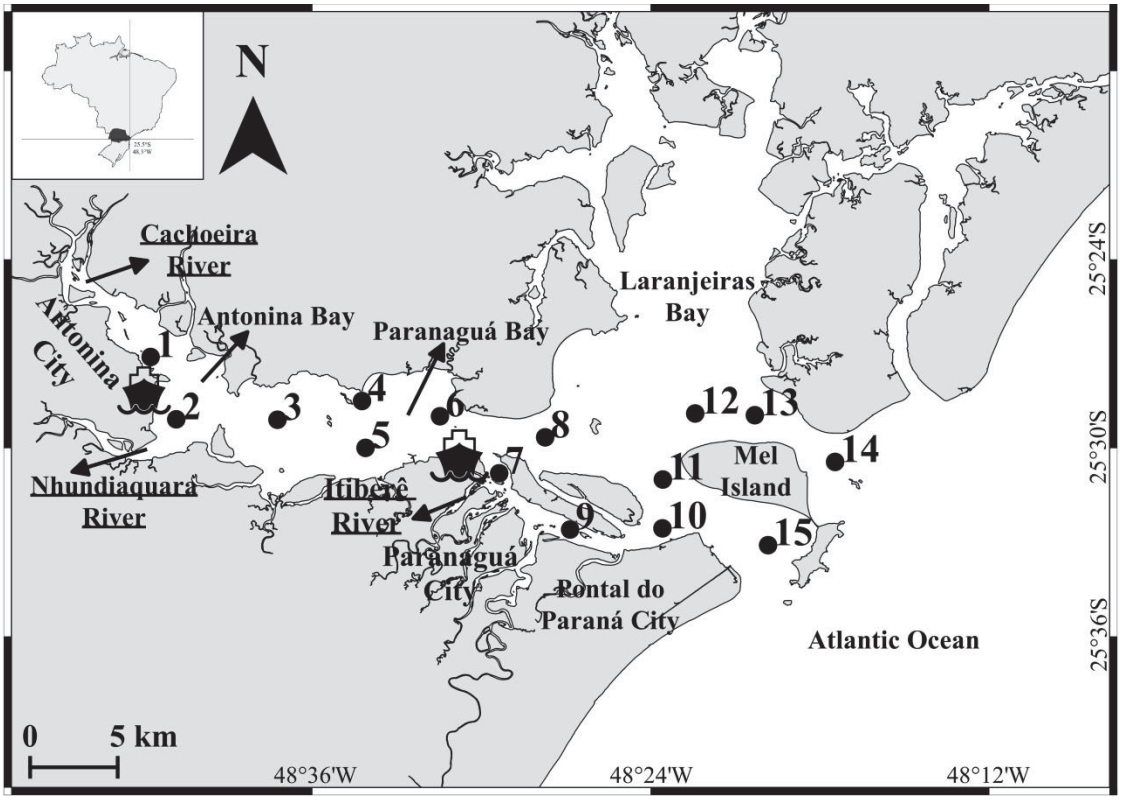


Fig. 1. Map of the study area indicating South Atlantic and sampling sites in the Paranaguá Estuarine System (PES). Ships indicate harbor areas.

The harbor activities at the Paranaguá, Antonina, and Ponta do Félix terminals, in addition to dredging, fishing, aquaculture, and effluent inputs are the main anthropogenic influence in the PES (Lana et al., 2001). Effluent inputs are enhanced with increasing local urbanization (188,935 residents estimated for 2017; IBGE, 2017). Previous geochemical and microbiological analyses recorded the highest concentrations of sewage indicators near Antonina and Paranaguá harbors and cities (Kolm et al., 2002; Martins et al., 2010a; Cabral et al., 2018), suggesting that these are the main source of effluents input to Paranaguá Bay.

3. MATERIAL AND METHODS

3.1. Sampling

Surface sediment and suspended particulate matter (SPM) were sampled in fifteen sites along the E-W axis of PES (Fig. 1) during one ebb spring tide in February of 2012. These sites were selected to obtain a wide spatial coverage according to a known salinity gradient in the area (Lana et al., 2001), and including sites where sewage markers have already been recorded (Martins et al., 2010a, 2011).

SPM samples (4 L of surface water) were collected in amber glass bottles at each site. Simultaneously, the surface layer of bottom sediment (0 - 2 cm) was collected at each site using a stainless-steel *Petite Ponar* bottom sampler with a 0.04 m^2 sampling area. The studied physicochemical parameters of the water column (sampling description in supplementary information) were previously discussed by Cardoso et al. (2016).

Total organic carbon (TOC), total nitrogen (TN) and total sulfur (TS) were determinate in homogenized surface sediments (0.8 - 1.0 g), treated by hydrochloric acid to remove inorganic carbon. Subsequently, samples were washed twice with deionized water to remove chloride, dried at 80 ºC overnight. TOC were determined using a Carlo Erba 1100 CHN Analyzer with a precision of < ± 8%. TN was determined directly on the same analyzer with precision < ± 10%. TS was determined on a Carlo-Erba NC 2500 elemental analyzer. All elemental analyses were performed in duplicate.

3.2. Analysis of geochemical markers

Three liters of water from each water column sample were vacuum filtered using GF/F Whatman® filters (ø = 0.7 µm) previously calcinated at 450 ºC for 12 h. Filters with SPM were frozen and freeze-dried before extraction. Seston data were obtained by the gravimetric method. Sediment samples were frozen, freeze-dried, macerated, sieved through a stainless steel mesh (1.0 mm), and stored in glass jars until extraction.

The analytical procedure for sterols and LABs analysis was adapted from Wisnieski et al. (2016). Sediments (about 20.00 g) and filters containing SPM were extracted in a Soxhlet apparatus over 8 h using 80 mL of a mixture of *n*-hexane:dichloromethane (DCM) (1:1, v/v) spiked with a surrogate mixture containing 1-C_{12}LABs and 5α-androstanol. Each extract was concentrated to a volume of 2 mL using a rotary vacuum evaporator (RVE) followed by clean up and fractionation using liquid adsorption chromatography on 5% deactivated silica and alumina columns; these columns were eluted with 10 mL *n*-hexane to obtain the LABs

fraction, followed by 15 mL of a mixture of *n*-hexane:DCM (7:3, v/v) to obtain the polycyclic aromatic hydrocarbons (PAHs) fraction (published in Cardoso et al., 2016), and 5 mL of an ethanol:DCM mixture (1:9, v/v) followed by 15 mL of ethanol to obtain the sterols fraction.

Extracts containing LABs were concentrated using RVE and a slight stream of nitrogen (when necessary) and spiked with the internal standard 1-C_{19}LAB, to a final volume of 250 and 500 µL for SPM and sediment samples, respectively. The sterols extracts were dried using nitrogen and derivatized with 40 µL of BSTFA/TMCS (99:1) over 90 min at 70 ºC. The derivatized extracts were subsequently dried in nitrogen, spiked with 5α-cholestane, and the final volume was adjusted with *n*-hexane, as described above for LABs.

LABs were analyzed using an Agilent GC 7890A gas chromatograph coupled to an Agilent 5975C inert MSD with a Triple-Axis Detector Mass Spectrometer on SIM mode (Selected Ion Monitoring - m/z 91 and 105). Sterols were analyzed using an Agilent 7890A gas chromatograph equipped with a flame ionization detector (GC/FID). The details of the instrumental analysis are described in the supplementary information.

3.3. Analytical control

Extracted blanks (every seven samples), certified sediment reference material, and recoveries of surrogate standards were used as quality controls. The detected molecular markers in blanks did not interfere in the analysis (< 3 times the detection limit - DL; values in supplementary information). The average recovery of 5α-androstanol was 82.7 (±16.9) and 102.2% (±17.3) in SPM and sediments, respectively.

The analysis of the certified sediment reference material, IAEA-408 (International Atomic Energy Agency) was satisfactory with recoveries between 90 and 110% (Martins et al., 2012). Reference material for the SPM was not available.

3.4. Statistical analysis

The Principal Component Analysis (PCA) was performed using the degradation ratio of LABs I/E of C_{13}-LABs and of 5α(H)-stanols/Δ^5-stenols (27Δ^0: 27Δ^5 and 29Δ^0: 29Δ^5) to examine the difference between LABs and sterols degradation in the two matrices (surficial sediment and SPM).

4. RESULTS AND DISCUSSION

4.1. Sedimentary bulk parameters

Sedimentary %TOC, %TS, and %TN ranged from 0.03 to 4.29% (average of 1.44 ± 1.46%), from 0.01 to 1.09% (average of 0.42 ± 0.37%), and up to 0.44% (average of 0.17 ± 0.13%), respectively (Table S2). The highest values of TOC, TS, and TN were recorded at fluvial and mixture zones (at site 3) following a decreasing gradient towards the east (Table S2). This pattern was expected due to grain size variation which tends to be larger towards the east (Lamour and Soares, 2007; Cattani and Lamour, 2016).

The TOC/TN ratios varied between 1.0 and 11.0 (average of 7.1 ± 3.8), suggesting a mixed origin of terrestrial and marine source for the sedimentary organic matter, except at sites 10, 11, 13, and 15 (TOC/TN up to 2.9) which reflected a predominantly marine input (Meyers, 1997). Finally, the TOC/TS ratio values ranged from 1.1 to 8.1 (average of 3.3 ± 2.0). Most of the sites showed TOC/TS values between 1.1 and 5.5, which is consistent with normal well oxygenated marine environments (~ 3.0; Borrego et al., 1998). Only site 15 showed a high TOC/TS value (8.1, Table S2).

4.2. Fecal sterols and LABs as markers of sewage contamination in the SPM and surficial sediment compartments

4.2.1. Fecal sterols

The concentrations of coprostanol ranged from < DL to 2.67 μg g^{-1} (average of 1.40 ± 0.92; Table S1; Fig. 2) (or < DL to 0.093 μg L^{-1}) in SPM, and from < DL to 0.94 μg g^{-1} (average of 0.30 ± 0.32) in the sediment (Table S2; Fig. 2). These concentration values are relatively lower than those registered in more populated regions such as Santa Monica Basin (USA), Mekong Delta (Vietnam), Western Malaysia, Tokyo (Japan) (up to 20.18 μg g^{-1} for sediments and 13.472 μg L^{-1} for SPM) (Isobe et al., 2002, 2004; Venkatesan et al., 2010), and in samples close to sewage outfalls (up to 45.26 μg g^{-1} for sediments; Venkatesan et al., 2010).

The sewage contamination can be evaluated by threshold values of coprostanol considered here as 0.50 μg g^{-1} of dry sediment (González - Oreja and Saiz - Salinas, 1998) and 1.71 μg g^{-1} SPM (Cabral et al., 2018), combined with diagnostic ratios between coprostanol and others biogenic sterols (Martins et al., 2014).

Sediment samples from sites 2, 3, 6, and 7 (concentrations ranging from 0.49 to 0.94 μg g^{-1}), and SPM samples from sites 1, 3, 5, 6, 7, 8, and 12 (concentrations ranging from 1.78 to

267 2.67 μg g^{-1} SPM) presented concentrations of coprostanol close to or higher than the threshold
268 values from sewage contamination mentioned above, indicating that fluvial and mixture zones
269 are subjected to sewage influence in both water column and sediments. The Antonina and
270 Paranaguá cities are located at opposite ends of these zones and are considered the main
271 sources of sewage input in this bay along with the Cachoeira, Nhundiaquara, and Itiberê rivers
272 (Fig. 1) (Martins et al., 2010a; Cabral et al., 2018). The hydrodynamics of the Paranaguá Bay
273 is governed mainly by the asymmetric variation of tides and is characterized by water and salt
274 export to the coastal region (Marone et al., 2005). However, this is not the pattern of the SPM
275 transport, which occurs toward the inside regions of the bay (Mayerle et al., 2015). The
276 presence of an MTZ in the mixture zone may act as a trap for SPM deposition (Dyer, 1995)
277 and the resulting salinity (between 5 and 10 PSU) of the mixing between riverine and marine
278 water in fluvial and mixture zones can intensify the coagulation and flocculation processes of
279 hydrophobic compounds such as sterols and LABs (Pietzsch et al., 2010). Moreover, high
280 %TOC in sediments of fluvial and mixture zones (Table S2) and a strong positive correlation
281 with sterols ($r > 0.80$, $p < 0.01$; Table 4) are observed. These factors along with low
282 granulometry would favor the accumulation of hydrophobic markers in these zones which
283 may become sinking areas for LABs and coprostanol. Therefore, the proximity to the main
284 sources of sewage input and the SPM and sediment transportation dynamics influence the
285 spatial distribution of these compounds in both compartments.
286 The diagnostic ratio levels which are usually applied (Table 1) were established for
287 sediments in temperate environments in the Northern Hemisphere and could underestimate
288 the sewage contamination in subtropical environments (Martins et al., 2014). When using
289 these levels for temperate environment sediments, only site 7 was near the threshold value of
290 sewage contamination of ratio I (0.49) (Table 1). However, using the levels calibrated for
291 subtropical regions (Martins et al., 2014), sites 2 and 3 were also under sewage
292 contamination, with at least one ratio above the subtropical threshold level.
293 The diagnostic ratio levels for SPM samples in this subtropical estuarine environment
294 (Fig. 3; Table 1) were calculated using equations originating from the linear regression ($R^2 >$
295 0.70) of diagnostic ratios involving sterols *vs.* coprostanol levels in the PES and the stricter
296 threshold limits of coprostanol for SPM reported by Cabral et al. (1.71 μg g^{-1} SPM; 2018).
297 Based on these values, sites 1, 3, 5, 6, 7, and 8 were under sewage contamination (Table 1).
298 These results highlight the need for the calibration of diagnostic ratio levels for different
299 environments and compartments to obtain more precise assessments of sewage contamination.

Additionally, this indicates that the mixture and fluvial zones are the most probable sewage contamination scenarios.

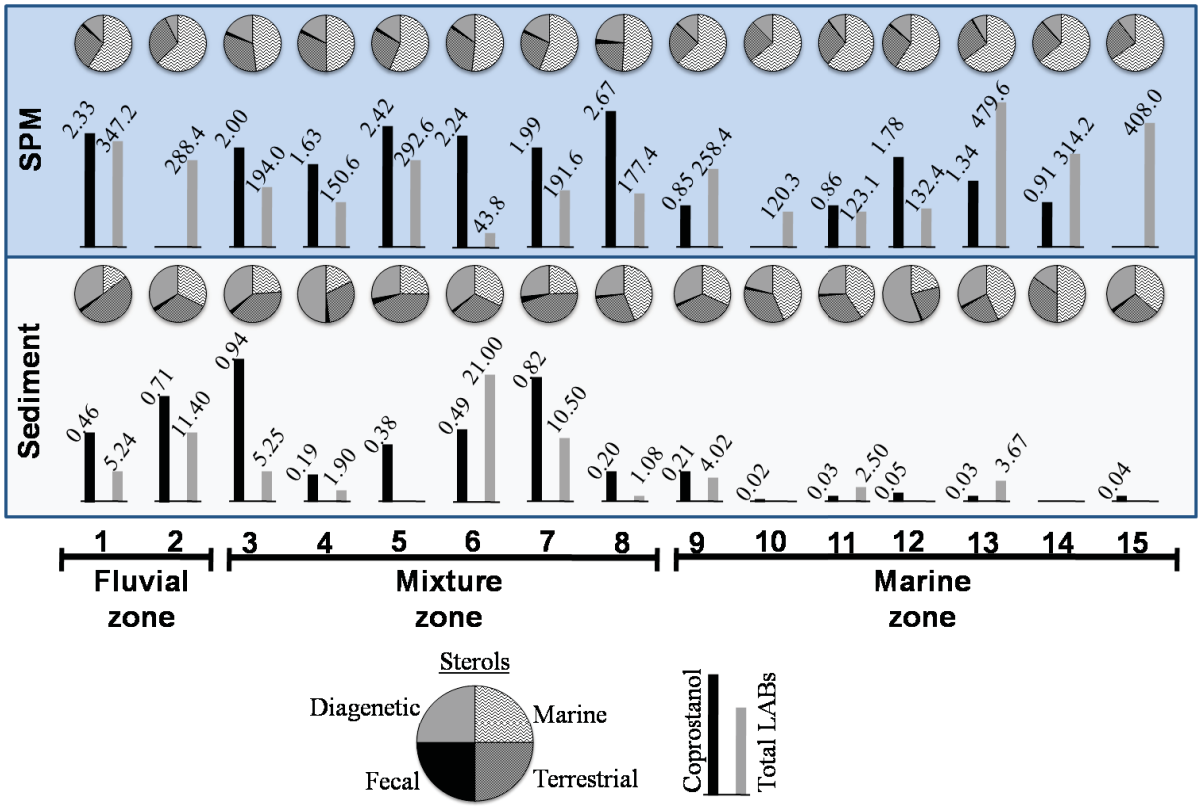


Fig. 2. Percentage of the contribution of marine, terrestrial, fecal, and diagenetic sterols (regarding total sterols) in SPM and sediments by sampling site (circle chart); distribution of the concentration of coprostanol (μg g^{-1}) and total LABs (ng g^{-1}) in suspended particulate matter (SPM) and sediments by sampling sites.

Evaluation of the fecal sterol epicoprostanol indicates whether or not the effluent was subjected to some treatment before its release into the environment (Mudge and Duce, 2005). Epicoprostanol was detected in only one sediment sample at a very low concentration (0.10 μg g^{-1}) and the ratio V of 0.14 (Table 1), confirms previous evidence that the sewage treatment before effluent released in the Paranaguá Bay is not effective (Martins et al., 2010a).

The concentration of coprostanol showed a considerable decrease between SPM and the sediment (about 4.7 times), which is probably related to its high rate of degradation in the water column (Wakeham et al., 1997). The spatial distribution, hydrodynamics, sedimentation rate, and granulometry also seem to influence this difference between matrices, such as the difference observed in the ratios between $SPM_{coprostanol}$ and $Sediment_{coprostanol}$ (Table 2). This ratio's average was 5.31 in fluvial and mixture zones and 18.83 in the marine zone (Table 2),

indicating an increased concentration decay toward the sediment in the marine zone. The fluvial and mixture zones have lower hydrodynamics energy, granulometry, and depth (between 5 and 22 m), and higher sedimentation rates than other zones (Lamour and Soares, 2007; Cattani and Lamour, 2016), which decreases the residence time of suspended particles and, consequently, the availability of sterols and LABs for degradation in the water column. The marine zone has opposite conditions, which allow longer particle floating time and, consequently, their spread to distant areas and increased time for degradation in the water column prior to sedimentation. Low sediment adsorption is also expected in this area due to the predominance of fine to medium sands (0.125 - 0.500 mm; Lamour and Soares, 2007).

Therefore, the proximity to main sources of sewage input and hydrodynamic processes also influence the spatial and compartmental distribution of coprostanol.

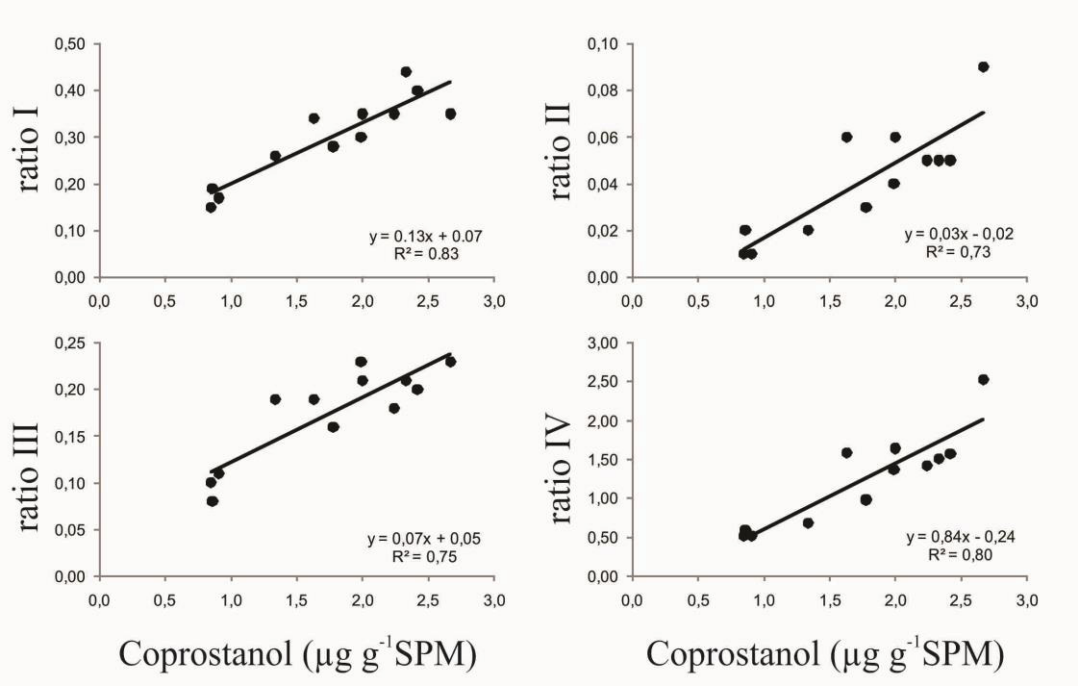


Fig. 3: Scatterplots of (ratio I) coprostanol/(coprostanol + cholestanol); (ratio II) coprostanol/(coprostanol + cholesterol); (ratio III) coprostanol/(coprostanol + dinosterol); and (ratio IV) % fecal sterols/total sterols *versus* coprostanol concentrations in suspended particulate matter (SPM) from Paranaguá Bay.

Table 1: Sterol diagnostic ratios, its values (in SPM and sediment matrix) by site from Paranaguá Bay and threshold levels found at literature for different climate conditions. Bold values: near or above the threshold value of sewage contamination and sites above thresholds values of coprostanol; nc = not calculated (one or more compounds < DL); sd = standard deviation.

Sites	**Matrix**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Threshold levels for sediments for temperate environments**	**Threshold levels for subtropical environments**
Diagnostic Ratios																		
I - coprostanol/ (coprostanol+cholestanol)	SPM	**0.44**	nc	**0.35**	0.34	**0.40**	**0.35**	**0.30**	**0.35**	0.15	nc	0.19	0.28	0.26	0.17	nc	< 0.30: pristine environments; > 0.50: sewage contamination (Grimalt et al., 1990; Leeming et al., 1998)	Contaminated Sediments > 0.28 (Martins et al., 2014); SPM > 0.29 (present study)
	Sediment	0.30	0.27	0.21	0.45	0.35	0.18	**0.49**	0.21	0.24	0.29	0.21	0.13	0.17	nc	0.17		
II - coprostanol/ (coprostanol+cholesterol)	SPM	**0.05**	nc	**0.06**	0.06	**0.05**	**0.05**	**0.04**	**0.09**	0.01	nc	0.02	**0.03**	0.02	0.01	nc	> 0.50: sewage contamination (Takada et al., 1994)	Contaminated sediments > 0.11 (Martins et al., 2014); SPM > 0.03 (present study)
	Sediment	0.14	**0.12**	**0.12**	0.20	0.22	0.08	**0.23**	0.04	0.08	0.09	0.09	0.05	0.06	nc	0.07		
III - coprostanol/ (coprostanol+dinosterol)	SPM	**0.21**	nc	**0.21**	0.19	**0.20**	**0.18**	**0.23**	**0.23**	0.10	nc	0.08	0.16	0.19	0.11	nc	> 0.50: sewage contamination (Venkatesan and Kaplan, 1990)	Contaminated sediments > 0.25 (Martins et al., 2014); SPM > 0.17 (present study)
	Sediment	0.31	0.17	**0.31**	0.26	0.33	0.13	**0.44**	0.24	0.17	0.20	0.27	0.33	0.18	nc	0.13		
IV - % fecal sterols/ total sterols	SPM	**1.51**	nc	**1.64**	1.58	**1.57**	**1.42**	**1.37**	**2.52**	0.52	nc	0.59	0.98	0.68	0.52	nc	> 50%: high sewage contamination (Hatcher and McGillivary, 1979)	Contaminated sediments: no calibration available; SPM > 1.20 (present study)
	Sediment	1.72	2.05	1.85	2.03	3.11	1.13	3.31	1.29	1.22	1.27	1.05	0.87	1.26	nc	1.69		
V - epicoprostanol/ coprostanol	SPM	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	< 0.20: untreated sewage input; > 0.80: treated sewage input (Mudge and Seguel, 1999)	not calibrated
	Sediment	nc	0.14	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc	nc		

337 4.2.2. LABs

338 The concentration of total LABs in SPM ranged from 43.8 to 480.0 ng g^{-1} SPM
339 (average of 234.8 ± 120.2 ng g^{-1} SPM) (Table S1); the highest concentrations were recorded
340 in marine zone sites, followed by fluvial sites (Fig. 2). In sediments, the total LABs ranged
341 from < DL to 21.0 ng g^{-1} (average of 4.44 ± 5.86 ng g^{-1}) (Table S2); the highest
342 concentrations were recorded in sites in the fluvial and mixture zones. LABs were absent in
343 most of the sites in the marine zone (Fig. 2).

344 SPM and sediments are considered the main way of transport and final reservoir of
345 hydrophobic compounds, respectively. Therefore, a similar spatial distribution between these
346 two matrices was expected, with the highest concentrations expected to be near sources of
347 LABs as near densely populated areas or river mouths, which are sources and routes of
348 effluent (Zeng et al., 1997; Luo et al., 2008). The spatial distribution in the studied sediments
349 followed this expectation (Fig. 2), with the highest concentrations of total LABs occurring in
350 sediments from the mixture and fluvial zones (near Paranaguá and Antonina cities,
351 respectively). A relatively different spatial distribution was observed in SPM, with the highest
352 concentrations recorded in the fluvial and marine zones. In the marine zone, site 15 is the only
353 one near tourist and residential areas (Mel Island; Fig. 1). Sites 13 and 14 are distant from
354 possible sources of sewage input. However, oil from boat and harbor activities is a possible
355 source of LABs in the region, and has been reported in other studies as influencing the
356 distribution and input of LABs (e.g., Martins et al., 2010b; Dauner et al., 2015).

357 The hydrodynamic factors of inner and middle estuarine regions, such as low wave
358 energy, presence of a MTZ that acts as barrier for the transport of fine particles towards the
359 coast, and high sedimentation rates (Lamour and Soares, 2007; Cattani and Lamour, 2016)
360 can also influence the high concentrations of total LABs in these regions of the bay (as
361 discussed for sterols). The spatial distribution of LABs in the sediment might be affected by
362 low OM %TOC (Table S2) in sediments and the more dynamic environment and coarse grain
363 size in the marine zone (Lamour and Soares, 2007), which can prevent the sedimentation and
364 adsorption of LABs on particles. This explains the low level of LABs in the sediment
365 compared to the high concentrations observed in the SPM of this zone.

366 Previous studies have already found a significant variation of total LABs concentration
367 between SPM and sediments, with higher concentrations always being found in SPM rather
368 than in sediments (Zeng et al., 1997; Isobe et al., 2004). Several factors influence the removal
369 of LABs from SPM prior to deposition on the sediment. Evaporation to the atmosphere and
370 bioaccumulation in organisms are still little studied. However, they are considered negligible

371 (detailed discussion in Ni et al., 2009). Their dilution by nonderived sewage particles was first
372 hypothesized by Zeng and Vista (1997) and recently observed by Dauner and Martins (2015)
373 who found a seasonal variation of LABs concentrations in SPM influenced by precipitation
374 variation and river runoff, being higher in the austral summer than in other seasons. The water
375 dissolution of LABs should not account for a significant decrease in total LABs toward the
376 sediment because only 10% of LABs in the water column are dissolved while the remaining is
377 adsorbed in SPM (Takada and Eganhouse, 1998; Isobe et al., 2004). Nevertheless,
378 degradation is considered the main factor in LABs removal from the water column (Zeng et
379 al., 1997), and should be the major cause for the difference in LABs between SPM and
380 sediment.
381 The degradation of LABs is commonly evaluated by the ratio between internal (I; the
382 sum of 6-C_m-LABs and 5-C_m-LABs) and external isomers (E; the sum of 4-C_m-LABs, 3-C_m-
383 LABs, and 2-C_m-LABs) of the dominant homolog (Takada and Ishiwatari, 1990; Martins et
384 al., 2014). This ratio is based on the preferential degradation of external isomers in relation to
385 internal isomers; an I/E greater than 1.00 indicates some degree of degradation (Takada and
386 Ishiwatari, 1990).
387 The I/E ratio of the predominant homolog C_{13}-LABs ranged from 0.42 to 0.96 (average
388 of 0.71 ± 0.17) in SPM and from 0.81 to 2.48 (average of 1.26 ± 0.55) in the sediment (Table
389 2). The observed values were higher in the sediment than in SPM, indicating additional LABs
390 degraded in the former matrix. This result confirms degradation as an important factor for the
391 LABs variation between the two compartments and is reinforced in the Principal Component
392 Analysis (PCA) based on biogenic sterols and the I/E-LABs ratio (section 4.2). It is also
393 important to highlight that resuspension processes, frequent in the fluvial and mixture zones
394 of the Paranaguá Bay, may favor the degradation of these markers since sewage particles are
395 fine and easily resuspended from the surficial sediment, which increases the availability of
396 these compounds to degradation processes that occur more intensely in the water column
397 (Zeng et al., 1997; Isobe et al., 2004).
398 The predominance of the C_{13}-LABs homologue, usually followed by C_{12}-LABs, C_{11}-
399 LABs, and C_{10}-LABs, is reported in most previous studies in both SPM (Zeng et al., 1997;
400 Colombo et al., 2007; Dauner and Martins, 2015) and sediments (Martins et al., 2014;
401 Venturini et al., 2015). This pattern is generally linked to the proportions found in the
402 composition of detergents manufactured in each studied area (Martins et al., 2014).

Table 2 - Ratios regarding sterols and LABs degradation in SPM and surface sediments from Paranaguá Bay, Southern Brazil. Cop = coprostanol; [1]fluvial and mixture sites; [2]marine sites; nc = not calculated (one or more compounds < DL); * ratio between internal (I) and external (E) isomers - Takada and Ishiwatari (1990).

Sites	Matrix	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Average ± sd
Sterols																	
$27\Delta^0/27\Delta^5$	SPM	0.07	0.10	0.12	0.13	0.08	0.10	0.10	0.18	0.08	0.10	0.08	0.08	0.06	0.07	0.08	0.10 ± 0.03
	Sediments	0.39	0.36	0.51	0.29	0.52	0.38	0.31	0.17	0.28	0.25	0.37	0.37	0.33	0.18	0.38	0.34 ± 0.10
$29\Delta^0/29\Delta^5$	SPM	0.25	0.00	0.42	0.36	0.44	0.32	0.43	0.50	0.26	0.25	0.25	0.27	0.16	0.30	0.22	0.30 ± 0.13
	Sediments	0.53	0.59	0.46	1.00	0.63	0.65	0.27	0.53	0.49	0.42	0.48	3.05	0.73	0.43	0.63	0.73 ± 0.66
$Cop_{SPM}/Cop^1_{sediment}$		5.07	nc	2.13	8.58	6.37	4.57	2.43	13.4								5.31 ± 4.20
$Cop_{SPM}/Cop^2_{sediment}$										4.05	nc	28.7	35.6	44.7	nc	nc	18.8 ± 19.9
LABs																	
I/E (*n*-C_{13}-LABs)*	SPM	0.57	0.67	0.74	0.83	0.96	nc	0.73	0.78	nc	nc	nc	nc	0.42	nc	nc	0.71 ± 0.17
	Sediments	1.25	1.16	1.75	0.84	nc	0.90	2.48	0.88	1.66	nc	0.89	nc	0.81	nc	nc	1.26 ± 0.55

Only C_{13}-LABs and C_{10}-LABs were recorded on SPM samples from the Paranaguá Bay with a predominance of C_{13}-LABs in most sites (Fig. 4). Ni et al. (2008) observed a similar pattern in SPM samples from Pearl River Delta (China), with a predominance of C_{10}-LABs, which is not in agreement with the homolog proportion found in detergents used in China (predominance of C_{12}-LABs). This variation can be associated with possible physical-chemical changes in LABs homologs after their environmental input, which may be related to homolog preferential degradation. Nevertheless, possible oil sources of LABs originating from harbor activities in the PES (Dauner et al., 2015) can also provide LABs of different composition pattern.

In the sediment, the four LABs homologs were recorded with a predominance of C_{13}-LABs, followed by C_{12}-LABs, C_{11}-LABs, C_{10}-LABs, and C_{14}-LABs (Fig. 4). Considering that SPM is the main way for hydrophobic compounds to reach the sediment and the hypothesis of a higher degradation rate in the water column, the larger variety of homologs found in the sediment rather than in SPM was not expected. The seasonal variations in dilution rates of these compounds and the difference in time scale between SPM and sediment may explain the vertical variation observed. Paranaguá Bay samples were collected in a season of high precipitation (austral summer); therefore, sewage particles in suspension may be more diluted in estuarine environments (Dauner and Martins, 2015), which could hamper the detection of some LABs homologs in SPM. Nevertheless, years of sedimentation contribute to the surface sediment layer, which may favor the detection of homologs not recently registered in the SPM samples. Moreover, considering the seasonal variation of precipitation and the short time scale that SPM represents, the seasonal variation of homologs diversity and total LABs concentrations in SPM may occur as previously observed by Dauner and Martins (2015) in Guaratuba Bay.

The maximum value of total LABs in SPM (up to 480.0 ng g^{-1} SPM) is below the range already registered in a previous study in Paranaguá and Guaratuba bays (up to 1670 and 3770 ng g^{-1} SPM, respectively, considering the rainy period), considered under low or moderated sewage contamination (Dauner and Martins, 2015; Cabral et al., 2018), and significantly below values recorded in areas with high sewage contamination or near outfalls such as Rio de La Plata (Argentina), Dorchester Bay (USA), and Pearl River (China) (up to 51,400, 26,000, and 11,400 ng g^{-1} MPS, respectively) (Eganhouse and Sherblom, 2001; Colombo et al., 2007; Ni et al., 2008). The same scenario is observed in the sediment samples where the concentrations recorded in this study (up to 21.0 ng g^{-1}) are similar to those in areas under low human influence such as Admiralty Bay in Antarctica (up to 23.0 ng g^{-1}) (Montone et al.,

2010), below values found in pristine areas from Rio de La Plata (up to 230 ng g^{-1}) (Venturini et al., 2015), and significantly lower than values recorded near submarine outfalls (up to 9342 ng g^{-1}; Venkatesan et al., 2010) or under high sewage contamination such as Montevideo Bay, in Uruguay (up to 7780 ng g^{-1}, Venturini et al., 2015).

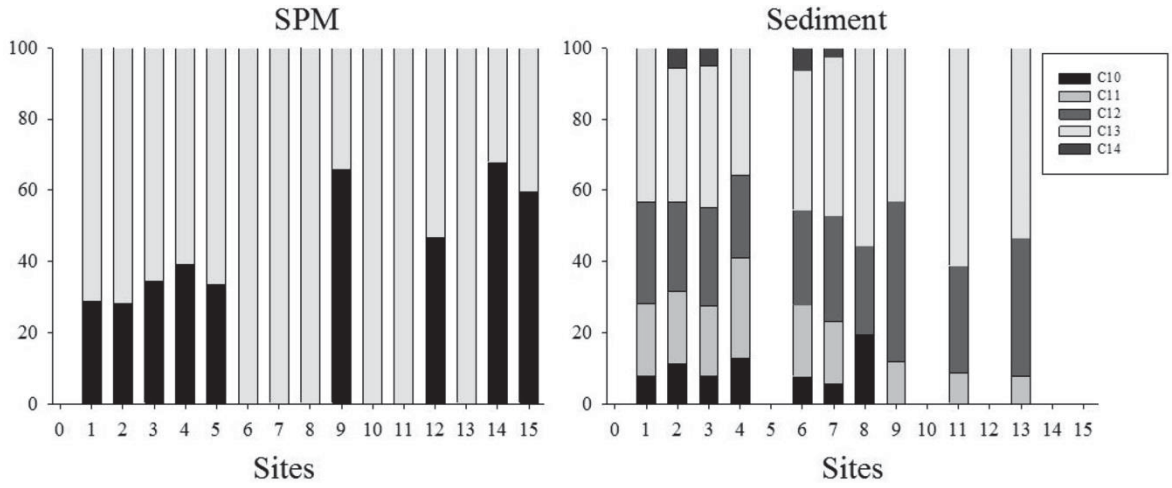


Fig. 4. Distribution of percentages of LABs homologues (n-C_{10}-LABs, n-C_{11}-LABs, n-C_{12}-LABs, n-C_{13}-LABs, and n-C_{14}-LABs) in suspended particulate matter (SPM) and sediments by sampling sites in the Paranaguá Estuarine System (PES).

These LABs results indicate that Paranaguá Bay is under low sewage impact considering both water column and surficial sediments. However, as discussed for coprostanol, fluvial and mixture zones are areas susceptible to showing scenarios of sewage contamination due to their proximity to LABs sources and the SPM and sediment transport dynamics, which may promote the accumulation of these markers in these zones. Moreover, the analysis of LABs degradation indicated recent sewage input in SPM (I/E ratio below 1.00), and chronical and historical sewage input in the surficial sediment (I/E ratio higher than 1.00 in sites 1, 2, 3, 7, and 9, near sources of sewage input). Therefore, it is important to highlight that even if the region is not under high sewage contamination, the sites in the fluvial and mixture zones should be the first impacted areas in a scenario of an increased sewage introduction.

4.2.3. Integration of the sewage molecular markers coprostanol and LABs

There is no correlation between total LABs and coprostanol in SPM (r = - 0.22) (Table 3). This result was also recently verified by Cabral et al. (2018) in SPM samples from Guaratuba and Paranaguá Bays (samples from 2014/2015) and suggests other sources or input

routes of LABs besides effluents, such as surfactants used in ship washing and surface runoff. There is no correlation analysis between both markers in SPM matrix in other estuaries for comparison.

Table 3: Pearson correlation coefficients between sewage markers total LABs and coprostanol from SPM and sediments samples of Paranaguá Bay, Southern Brazil. Bold values = significant Pearson correlation ($p < 0.05$).

	total $LABs_{sediment}$	$Coprostanol_{SPM}$	$Coprostanol_{sediment}$
total $LABs_{SPM}$	0.34	-0.22	
total $LABs_{sediment}$	x		**0.61**
$Coprostanol_{SPM}$		x	0.30
$Coprostanol_{sediment}$			x

Conversely, there are positive and significant correlations between coprostanol and LABs ($r = 0.61$; $p < 0.05$) and between both markers with %TOC (Table 4) in sediment samples which may indicate similar sources of contribution of detergents and fecal material for sediments, or, more probably, similar areas of final sewage particle deposition in Paranaguá Bay (as also observed by Venkatesan et al., 2010, Martins et al., 2014, Venturini et al., 2015 in sediments of other regions). These areas are preferably near a source of sewage input and fluvial and mixture zones as discussed above.

Table 4: Pearson correlation coefficients between total organic carbon (TOC), the molecular markers sterols and total LABs from sediments samples of Paranaguá Bay, Southern Brazil. All results had significant Pearson correlation ($p<0.05$).

	TOC
Coprostanol	0.83
Marine sterols	0.82
Terrestrial sterols	0.91
Diagenetic sterols	0.93
Total sterols	0.92
Total LABs	0.60

Therefore, the integrated analysis of the distribution of LABs and coprostanol in the two compartments indicates that the hydrodynamics conditions associated with the vicinity of sewage discharge areas may determine a common final sedimentary deposition of both markers, even with a more homogeneous distribution in the water column or a possible secondary source of LABs besides the sewage. This hypothesis is also reinforced by the

absence of a significant correlation between $LAB_{sediment}$ and LAB_{SPM} (r = 0.34, p = 0.22), and between $Coprostanol_{Sediment}$ and $Coprostanol_{SPM}$ (r = 0.30, p = 0.27) (Table 3).

4.3. Spatial and compartment distribution of natural sterols

A total of 15 sterols were identified including (1) C_{27}-sterols: cholest-5,22E-dien-3β-ol ($27\Delta^{5,22E}$), 5α-cholesta-22E-en-3β-ol ($27\Delta^{22E}$), cholest-5en-3β-ol ($27\Delta^{5}$), and 5α-cholestan-3β-ol ($27\Delta^{0}$); (2) C_{28}-sterols: 24-methylcholest-5,22E-dien-3β-ol ($28\Delta^{5,22E}$), 24-methyl-5α-cholestan-22E-en-3β-ol ($28\Delta^{22E}$), 24-methylcholest-5-en-3β-ol ($28\Delta^{5}$), and 24-methyl-5α-cholestan-3β-ol ($28\Delta^{0}$); (3) C_{29}-sterols: 24-ethylcholest-5,22E-dien-3β-ol ($29\Delta^{5,22E}$), 24-methyl-5α-cholestan-22E-en-3β-ol ($29\Delta^{22E}$), 24-ethylcholest-5-en-3β-ol ($29\Delta^{5}$), and 24-ethyl-5α-cholestan-3β-ol ($29\Delta^{0}$); (4) C_{30}-sterol: 4α,22,23-trimethylcholest-22E-en-3β-ol ($30\Delta^{22}$); and (5) the fecal sterols 5β-cholestan-3β-ol (coprostanol) and 5β-cholestan-3α-ol (epicoprostanol) (discussed in section sec4.2.1).

The concentration of total sterols in SPM ranges from 102.9 to 198.2 μg g^{-1} SPM (Table S1). The distribution of total sterols was practically homogeneous throughout Paranaguá Bay, except in sites 3, 4, 8, and 10 which showed lower values than those in other sites (Fig. 5a). Marine sterols ($27\Delta^{5,22E}$, $27\Delta^{5}$, $28\Delta^{5,22E}$, and $30\Delta^{22}$) predominated through the bay (average of 58.3 ± 5.9% of total sterols), followed by "terrestrial" ($28\Delta^{5}$, $29\Delta^{5,22E}$, and $29\Delta^{5}$; average of 27.1 ± 3.1% of total sterols) and diagenetic sterols ($27\Delta^{22E}$, $27\Delta^{0}$, $28\Delta^{22E}$, $28\Delta^{0}$, $29\Delta^{0}$, and $29\Delta^{22E}$; average of 13.6 ± 4.1% of total sterols) (Fig. 2). This predominance order of the contribution of sterol sources remained throughout the E-W axis in the PES.

The concentration of total sterols in sediments ranges from 0.5 to 50.8 μg g^{-1} dry sediment (Table S2) with the highest concentrations in sites of the fluvial and mixture zones (Fig. 5b) where % TOC was higher (Table S2). The contribution of marine, terrestrial, and diagenetic sterols was equivalent in average (32.1% ± 10.7, 34.1% ± 7.8, and 32.2 ± 10.2% of total sterols, respectively) with a tendency of an increasing contribution of marine sterols and decreasing contribution of terrestrial sterols toward the estuary's entrance (Fig. 2), as expected in estuarine environments and according to the TOC/TN variation with values < 4.0 in sites of marine zone (Table S2).

The spatial distribution of total sterol concentrations and the order of predominance between biogenic sterols were relatively uniform in SPM throughout the bay (Fig. 2 and 5a). It may be a consequence of the relatively short water residence time in Paranaguá Bay (3-4 days; Marone et al., 2005) and absence of a defined trend of OM exportation/importation to the ocean (Mantovanelli et al., 2004) that allows the homogenization of the OM in this

compartment. These two factors may be providing a constant mixture of marine and terrestrial OM sources in the water column throughout the estuarine extension. However, there was a high concentration of sterols in sediments from sites in the fluvial zone (sites 1 and 2), which receives a large amount of fluvial OM, and in the mixture zones (sites 3, 6, and 7), which has a tendency of sedimentation, especially of fine particles (Cattani and Lamour, 2016) (Fig. 5b). There is a predominance of fine to medium sands (0.125-0.500 mm; Lamour and Soares, 2007) in the entrance of the bay, which may explain the lower concentrations of sterols in the sediments of this region compared to that in the water column (Fig. 5b).

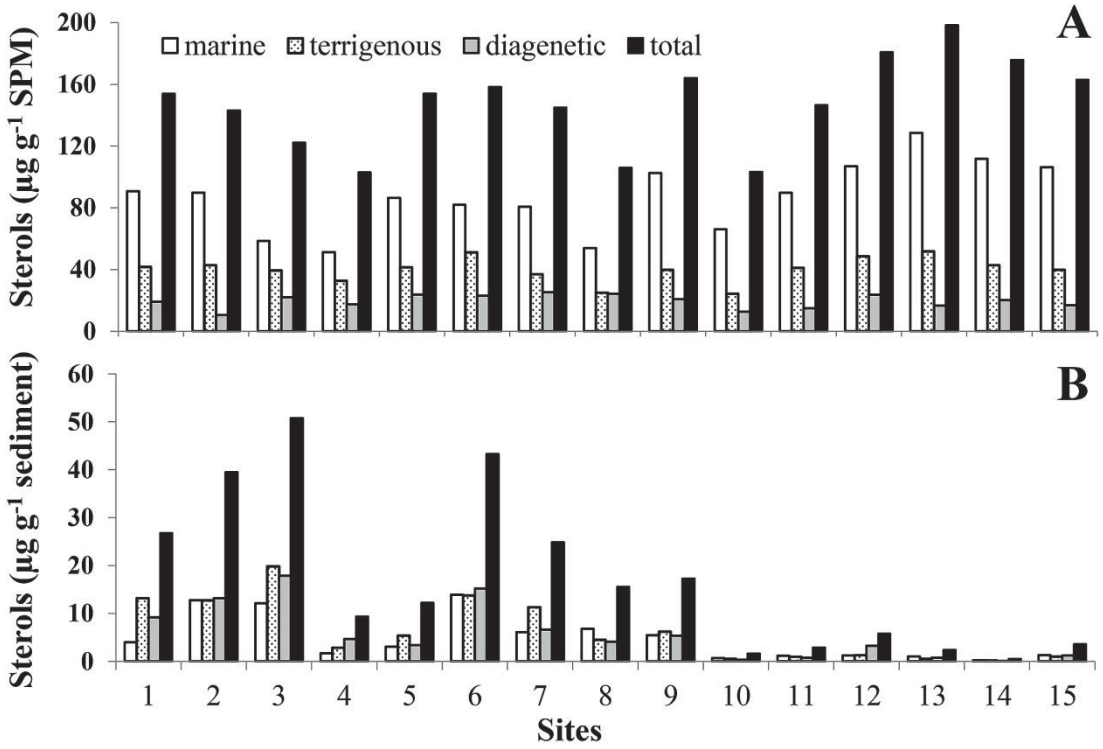


Fig. 5. Distribution of the concentrations of marine ($27\Delta^{5,22E}$, $27\Delta^{5}$, $28\Delta^{5,22E}$ and $30\Delta^{22}$), terrestrial ($28\Delta^{5}$, $29\Delta^{5,22E}$ and $29\Delta^{5}$), diagenetic ($27\Delta^{22E}$, $27\Delta^{0}$, $28\Delta^{22E}$, $28\Delta^{0}$ and $29\Delta^{0}$), and total sterols (µg g^{-1}) in suspended particulate matter (SPM) (A) and sediments (B) by samplings sites.

On average, the total concentration of sterols in the sediment was 90% lower than that in SPM (Fig. 5a and b). The decrease of the levels of these sterols toward the sediment is commonly recorded in comparative studies between these two compartments; lateral transport, seasonal variability, and preferential export from inner areas to offshore were some of the factors that influence this vertical variation in the concentrations of molecular markers (Wakeham et al., 1997; Jeng and Huh, 2004; Hernández-Sánchez et al., 2014). Moreover, degradation is still considered the main factor because most of the diagenetic reactions occur

538 in the water column through biological reactions (Wakeham et al., 1997; Hernández-Sánchez
539 et al., 2014).
540 The 5α(H)-stanols/Δ^5-stenols ratio may evidence degradation as an important factor in
541 the difference in sterols concentrations between SPM and sediment (Jeng and Huh, 2004)
542 because the conversion of stenols to stanols results from diagenetic transformation by bacteria
543 in sediments (Wakeham and Ertel, 1988). In the present study, these ratios increased towards
544 the sediment. The average of the $27\Delta^0$: $27\Delta^5$ and $29\Delta^0$: $29\Delta^5$ ratios in SPM were 0.10 ± 0.03
545 and 0.30 ± 0.13, respectively (Table 2) and 0.34 ± 0.10 and 0.73 ± 0.66 in the sediments,
546 respectively. These results confirm the predominance of more degraded OM in the sediment
547 than in SPM or the presence of "fresher" OM in SPM than in the sediments. Wakeham et al.
548 (1997) and Jeng and Huh (2004) report similar results in sites from the Equatorial Pacific and
549 East China Sea Shelf, respectively. The PCA with these degradation ratios (plus I/E ratio of
550 LABs) as variables (Fig. S1) confirms this pattern of degradation (toward sediments) with
551 PC1 explaining 57.3% and PC2 explaining 35.2% of the variation showing the highest values
552 of all ratios in the sediments.
553 Previous studies have observed a predominance of marine sterols in SPM in coastal
554 marine ecosystems (Wakeham and Ertel, 1988; Hudson et al., 2001) related to the constant
555 input of marine OM such as phytoplankton and zooplankton. In fact, Paranaguá Bay has
556 historical records of high phytoplankton stock in the central sector (Brandini et al., 1988;
557 Machado et al., 1997), which is influenced by the occurrence of a MTZ (Mantovanelli et al.,
558 2004) and which acts as a barrier against advective processes by slowing down the movement
559 of particles to the coast (Dyer, 1995), furthering planktonic organisms in that region.
560 Additionally, the samples in this study were collected during the summer when increased
561 precipitation occurs and continental drainage increases by up to four times (Marone et al.,
562 2005) allowing organic and nutrient enrichment and stimulating primary production that
563 consequently enhances the food supply for zooplankton. This fact is supported by the higher
564 $27\Delta^5$ and 28 $\Delta^{5,22E}$ contributions in relation to total sterols in SPM which are mainly related to
565 zooplankton and phytoplankton, respectively (Volkman, 1986). However, it should be noted
566 that SPM represents the state of the environment at the time of sampling and, therefore,
567 seasonal variations, or even a smaller time scale of the predominance of terrestrial or marine
568 OM contribution, are not discarded.
569 The differential degradation between sterols may also explain the decrease in the
570 contribution of marine sterols and the increase in the contribution of diagenetic sterols from
571 SPM to sediment because C_{27} and C_{28}-sterols are predominant in SPM and are preferably

572 degraded over C_{29} and C_{30}-sterols (Wakeham and Ertel, 1988). The results of the analysis of
573 the contribution percentage showed a higher decrease of marine C_{27} ($27\Delta^{5,22E}$ and $27\Delta^{5}$) and
574 C_{28}-sterols ($28\Delta^{5,22E}$) sterols than those in terrestrial C_{28} ($28\Delta^{5}$) and C_{29}-sterols ($29\Delta^{5,22E}$ and
575 $29D^{5}$) (Fig. 2). Considering that SPM is the main source of sterols to the sediment, the
576 degradation difference between marine and terrestrial can be evaluated through the ratio
577 between sterols of the same origin in both compartments: higher ratio values indicate higher
578 degradation rates (Jeng and Huh, 2004). Thus, the ratio of the average concentration of marine
579 and terrestrial sterols between SPM and sediments was calculated as presented below:
580
581

$$\frac{average\ (marine\ sterols)_{SPM}}{average\ (marine\ sterols)_{sediment}}$$

582
583 and

$$\frac{average\ (terrestrial\ sterols)_{SPM}}{average\ (terrestrial\ sterols)_{sediment}}$$

584
585
586 The ratio was 20.7 for marine sterols and 6.4 for terrestrial sterols, which reinforces the
587 hypothesis that differentiated degradation between sterols is one of the main factors in the
588 variation of the predominance of sterols from different sources in each compartment.
589
590 **5. CONCLUSIONS**
591
592 The local hydrodynamic factors seem to provide relevant water column homogenization
593 in this estuarine ecosystem. Consequently, the terrestrial and marine OM dispersion occurs
594 and explains the uniform distribution and composition predominance of total sterols in SPM.
595 These factors can also explain the relatively more homogeneous spatial distribution of LABs
596 and coprostanol in SPM than sediments because they tend to promote the dispersion and
597 dilution of sewage particles through the water column while there is a preferential final
598 deposition in fluvial and mixtures zones in sediments. Therefore, the SPM evaluation
599 indicates a contamination status on a larger spatial scale, more generalist to the environment,
600 while the sedimentary evaluation indicates more specific areas related to main sites of input
601 and final deposition of sewage particles that could be considered hot spot areas and should be
602 monitored.

603 The evaluation of 5α(H)-stanols/Δ^5-stenols and LABs degradation ratios in SPM and
604 sediments indicate that the pronounced degradation of these molecular markers in the water
605 column must be the main cause for the sharp drop in its concentration towards the sediment as
606 well as the variation of the preferential composition of sterols and LABs homologues between
607 these two compartments.
608 Lastly, the similarity of the coprostanol spatial distribution in both matrices showed that
609 SPM and surficial sediments could be used to evaluate sewage input, however, with different
610 purposes related to temporal and spatial scales. This would indicate that simultaneous
611 approaches of the two matrices should provide a complete history related to the source,
612 pathway, degradation, and final deposition of OM, nutrients, and contaminants, which will
613 serve to drive decisions in environmental and public health management. It is important to
614 highlight that the threshold values of sewage markers will be different for each matrix
615 because SPM has concentrations of geochemical markers higher than those in sediments; this
616 also applies to the threshold values of diagnostic ratios that must be calibrated to different
617 climatic regions and matrices.
618 This study is the first report on sources, sink, and deposition of marine, terrestrial, and
619 sewage OM using sterols and LABs in integrated evaluations of SPM and sediments in South
620 Atlantic. Moreover, it is of special relevance to estuarine environments, which have restricted
621 hydrodynamic circulation and are the first environments to receive continental OM from
622 natural and anthropogenic sources and, therefore, susceptible to contamination and
623 eutrophication processes.
624
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635

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829 **Appendix A. Supplementary information**

830 Supplementary information related to this article can be found at Supplement B.

CAPÍTULO 4

Histórico do aporte de esgoto em sistemas estuarinos subtropicais da América do Sul com base em esteróis fecais e isótopos estáveis ($\delta^{13}C$ e $\delta^{15}N$)

Tracking the historical sewage input in South American subtropical estuarine systems based on faecal sterols and bulk organic matter stable isotopes ($\delta^{13}C$ and $\delta^{15}N$)

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Primeira página do artigo publicado no ANEXO 3.

Abstract

Faecal sterols and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) from bulk organic matter (OM) were analysed in three sedimentary cores collected in two subtropical bays located in the South Atlantic to evaluate historical trends in the sewage input and to track possible changes in the bulk isotopic composition of OM in recent decades. The values of $\delta^{13}C$ and $\delta^{15}N$ ranged from -27.4 to -25.0 ‰ and from 0.5 to 3.9 ‰, respectively, without a clear trend in the variation over the whole period covered by sediment cores and with no conclusive interpretation of a specific range value typically related to the sewage input for these areas. The maximum coprostanol concentration was 0.19 μg g^{-1} in the upper 4 cm of one core, which was not considered contaminated by evaluation of the sterols diagnostic ratios. Even at low levels, the coprostanol concentrations followed variations in urban and economical regional development. Baseline values for faecal sterols (in average between 0.03 and 0.05 μg g^{-1}), which may represent a previous non-impacted environment scenarios, were calculated for use in comparative perspectives for future evaluations of the sewage input and contamination.

Keywords: coprostanol, Guaratuba Bay, Paranaguá Estuarine System, contamination, sediment cores.

Resumo

Esteróis fecais e isotópos estáveis ($\delta^{13}C$ e $\delta^{15}N$) de matéria orgânica foram analisados em três testemunhos sedimentares coletados em duas baías subtropicais localizadas no Atlântico Sul, a fim de avaliar tendências históricas da introdução de esgoto e traçar possíveis mudanças na composição isotópica da matéria orgânica (MO) nas décadas recentes. Os valores de $\delta^{13}C$ e $\delta^{15}N$ variaram de -27,4 a -25,0‰ e de 0,5 a 3,9‰, respectivamente. Não houve uma clara tendência de variação em todo o período analisado, e esses *proxies* não forneceram uma interpretação conclusiva quanto a uma possível faixa específica de valores relacionada à introdução de esgoto nessas áreas. A concentração máxima de coprostanol foi 0,19 μg g^{-1}, nos 4 cm superiores de um testemunho, que foi considerado não contaminado através da avaliação de razões diagnósticas envolvendo esteróis. Mesmo em baixos níveis, as concentrações de coprostanol acompanharam as variações relacionadas ao desenvolvimeto urbano e econômico regional. Valores de referência de esteróis fecais (em média entre 0,03 e 0,05 μg g^{-1}), que podem representar um cenário ambiental não impactado, foram calculados para serem usados em comparações a futuras avaliações de introdução e contaminação de esgoto na região.

Palavras chave: coprostanol, Baía de Guaratuba, Sistema Estuarino de Paranaguá, contaminação, testemunhos sedimentares.

Highlights

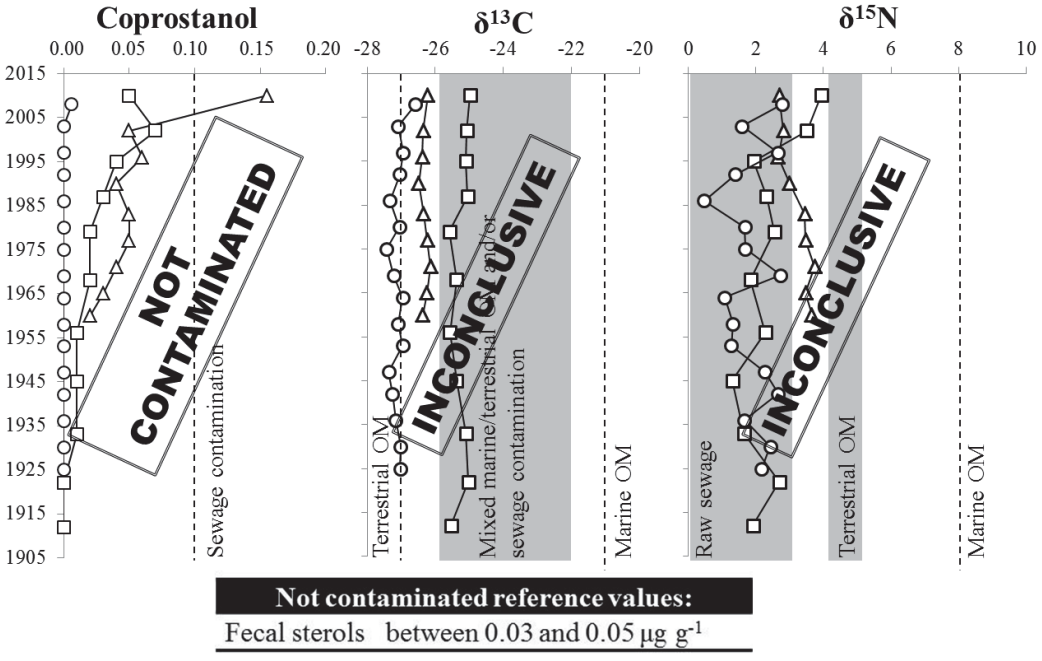
> $\delta^{13}C$ and $\delta^{15}N$ provided inconclusive indications of sewage contamination.

> The sedimentary cores were classified as uncontaminated by faecal sterols analysis.

> Coprostanol values were low but can follow the urban and economic development.

> Faecal sterols reference values were established in uncontaminated sediments.

Graphical Abstract



1. INTRODUCTION

Aquatic environment disposal of raw sewage is a historical and routine human practice that has continued due to a lack of investment in wastewater collection and treatment systems together with rapid urbanization and increases in population (Sato et al., 2013; WWAP, 2017). In South American countries, for instance, approximately 20% of collected wastewater is treated, and less than half of the population is connected to wastewater collection systems (Sato et al., 2013).

Coastal aquatic environments, mainly estuaries, are more susceptible to sewage contamination because they have more restricted hydrodynamics conditions (as poor water circulation and low depth, for instance) than offshore areas, in addition to high human density at the margins, which makes it difficult to dilute and degrade the large volume of waste material released into the environment (Muniz et al., 2013). The estuaries of the South American coast, in particular the Brazilian coast, are important examples of the historical sewage input and recent degradation, as recorded in several studies (e.g., Carreira et al., 2004; 2015; Muniz et al., 2015, Cabral et al., 2018; Costa et al., 2018; Kolm et al., 2018).

The Paraná coast has a great biological heterogeneity and importance for environmental conservation, with more than 80% of its area covered by areas of environmental conservation (Denardin et al., 2008). Nevertheless, anthropogenic influence related to harbour activities, overfishing, illegal human settlement and pesticide input (Lana et al., 2001; Denardin et al., 2008; Combi et al., 2013; Cardoso et al., 2016) has altered and currently threatens this ecosystem. In general, these estuaries are not contaminated by sewage, but recent studies have detected critical regions of faecal contamination, especially in the neighbouring areas of Antonina, Paranaguá and Guaratuba cities (e.g., Cabral et al., 2018; Cabral and Martins, 2018; Kolm et al., 2018).

Until 2010, on average, 80% of residences neighbouring the estuaries of Paraná had adequate sanitary services related to sewage, with a high percentage of coverage of sewage treatment services that reached 100% of the collected sewage from Guaratuba City (IBGE, 2018). However, the sewage collection network does not reach all residences, especially irregular occupations in the mangrove and preservation areas (Polidoro and Deschamps, 2013; Silva et al., 2015), which, for instance, are occupied by about 50% of the urban population of Paranaguá, the most populated city of the Paraná Coast (PDDIP, 2007).

Molecular and isotopic signatures of organic matter (OM) preserved in sedimentary cores are used to reconstruct histories of OM deposition from various sources in marine and

estuarine regions (e.g., Meyers, 1997; Barros et al., 2010; Zhao et al., 2015). Faecal sterols (coprostanol and epicoprostanol) are molecular markers used in the detection of domestic effluent input (and contamination) in the environment because are associated with human faeces (Takada and Eganhouse, 1998; Bujagić et al., 2016; Costa et al., 2018). Coprostanol is generated by microbial activity on cholesterol in the intestinal tract of higher vertebrates, and epicoprostanol is mainly produced by microbial degradation of cholesterol in sewage treatment plants (McCalley et al., 1981; Bull et al., 2002).

Carbon and nitrogen stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) are typically used to distinguish between marine and terrestrial OM inputs (Meyers, 1997). However, the values of $\delta^{13}C$ and $\delta^{15}N$ in sedimentary OM from faecal material may vary compared to the regular patterns observed in biogenic OM (Carreira et al., 2002; Barros et al., 2010; Savage et al., 2010). Sediment with sewage input has $\delta^{13}C$ ranging from -26 to -22 ‰, while terrestrial and marine OM has $\delta^{13}C$ around -27 ‰ and between -20 and -22 ‰, respectively (Bachtiar et al., 1996; Meyers, 1997; Barros et al., 2010). In general, $\delta^{15}N$ is lower in raw sewage (< 2.0‰) and in terrigenous OM (~4.0 ‰) (Bachtiar et al., 1996; Bueno et al., 2018). The OM from marine sources is ^{15}N-enriched ($\delta^{15}N$ = ~ 8.0‰), and sewage effluent that receives tertiary treatment is even more enriched ($\delta^{15}N$ > 15.0 ‰) (Vaalgamaa et al., 2013; Savage et al., 2010).

The aim of this study was to evaluate the vertical variation in the faecal sterol concentrations and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) in three sedimentary cores from various subtropical bays located in the South Atlantic to describe the historical sewage contamination in these regions and track possible changes in the bulk isotopic composition of OM found in these environments under anthropogenic pressure. In addition, values of faecal sterols from bottom sediment core sections that may represent a previously un-impacted environment scenario are presented, which could be used in a comparative perspective for future evaluations of the sewage input and contamination.

2. STUDY AREA

The Paraná Coast has a total area of 6,058 km² (IBGE, 2018). Preserved remnants of Atlantic Forest, particularly the mangrove, characterize the coastal vegetation, which develops over large areas (Bigarella, 2001; Denardin et al., 2008). The harbour, rural and tourism are the main human activities on the coast of Paraná State, particularly the first, since the Harbour of Paranaguá is the principal Brazilian Harbour for grains exportation (APPA, 2018). The Paranaguá Estuarine System (25°30´S, 48°25´W) (PES - including the Bays of Laranjeiras,

Antonina and Paranaguá) and Guaratuba Bay (25°52’S, 48°38'W) are the two estuarine environments of the Paraná state (Fig. 1).

Paranaguá Bay represents the major portion of the East–West axis of PES and is the most economically important estuarine area of the Paraná coast due to intense harbour activity. Urban areas are concentrated at the south margin of this bay and the Antonina Bay, including Paranaguá and Antonina cities (152,975 and 19,420 inhabitants estimated to 2017, respectively) (IBGE, 2018). Paranaguá Bay (including Antonina Bay) is 46 km long and 10 km wide (maximum). The water residence time is approximately three days, influenced by semi-diurnal tides with diurnal irregularities (Bigarella, 2001; Lana et al., 2001; Marone et al., 2005). The average of the neap and spring tide height are, respectively, 1.3 and 1.7 m in the entrance of the bay, and 2.0 and 2.7 m near to Antonina City (Lana et al., 2001). Due to the seasonal variation in rainfall (higher precipitation during summer season), the hydrodynamics of the Paranaguá Bay is influenced mainly by tide currents and variation of the fresh water input from rivers such as Cachoeira and Nhundiaquara, that is more intense during the rainy season (Lana et al., 2001; Mantovanelli et al., 2004). According surface water column parameters (temperature, pH, dissolved oxygen, and salinity), Paranaguá Bay may be sectored in fluvial (near Antonina), mixture 1 and 2 (middle of the bay), and marine (entrance of the bay) zones (Cardoso et al., 2016). Bottom sediments present a predominance of silt and clay in Antonina Bay with very fine sand in the intermediary region of Paranaguá Bay and fine to medium sands at the entrance of the PES (Cattani and Lamour, 2016). In general, the sedimentary compartment receives an equivalent mixture of terrestrial and marine organic matter (Cabral and Martins, 2018).

Agriculture, fishery and tourism are the main economic activities in the Guaratuba Bay and adjacent areas (Pietzsch et al., 2010; Dauner and Martins, 2015). Guaratuba Bay is 15 km long and 5 km wide (maximum) (Bigarella, 2001). The water residence time is approximately nine days with semi-diurnal tides with diurnal irregularities (Marone et al., 2006). The tidal range is, in average, 1.50 and 0.65 m in spring and neap tides, respectively (Marone et al., 2006). Continental drainage and rainfall are the main drivers of water quality dynamics during the rainy season, and tide fluctuations are the dominant hydrodynamic processes during the lower rainfall season (Mizerkowski et al., 2012). Urban areas are concentrated at the south margin (Guaratuba city - 35,986 inhabitants estimated to 2017), and there is high risk of pesticide contamination, mainly in the middle and inner areas, near Guaratuba city (Dauner and Martins, 2015).

3. MATERIAL AND METHODS

3.1. Sampling

Three sediment cores were collected, one in each studied bay: Antonina, Paranaguá and Guaratuba cores (AC, PC and GC, respectively. Fig. 1). The AC (25°27'14''S; 48°40'47''W) and PC (25°27'55''S; 48°30'49''W) cores were collected in November, 2013 (10 cm inner diameter and 27 cm long each). AC is from an area near the discharge mouth of the Nhundiaquara River in the harbour and urban areas of Antonina city, and the PC from the north margin of the Paranaguá Bay, opposite the harbour and urban areas of Paranaguá city. The GC (25°52'27''S; 48°39'39''W) was collected in November, 2010 (7 cm inner diameter and 32 cm long). The three cores were sliced into 2 cm sections each. Subsamples were stored individually in pre-cleaned aluminium foil at -20 ºC for the faecal sterol and stable isotope analyses, and in plastic bags for grain size and radionuclides analyses. In the laboratory, the samples were freeze-dried, carefully homogenized and stored in pre-cleaned glass vessels.

3.2. Dating of sediment cores

The sedimentation rate and respective dating of studied cores were previously presented by Combi et al. (2013) and Martins et al. (2015). The sedimentation rate was based on unsupported ^{210}Pb activity measurements and were made using the concentration initial constant (CIC) model described by Appleby and Oldfield (1978). The ^{210}Pb was determined by gamma-ray spectrometry using a low-background EG&G ORTEC spectrometer and a hyperpure Ge detector model GXM25190P (ORTEC, Oak Ridge, TN, USA). The average of sedimentation rates were 0.49 ± 0.05 cm yr^{-1} for AC, 0.26 ± 0.03 cm yr^{-1} for PC and 0.36 ± 0.02 cm yr^{-1} for GC, which correspond to periods of 52 years (1960 to 2012), 98 years (1912 to 2010) and 83 years (1925 to 2008), respectively, as detailed in Combi et al. (2013) and Martins et al. (2015).

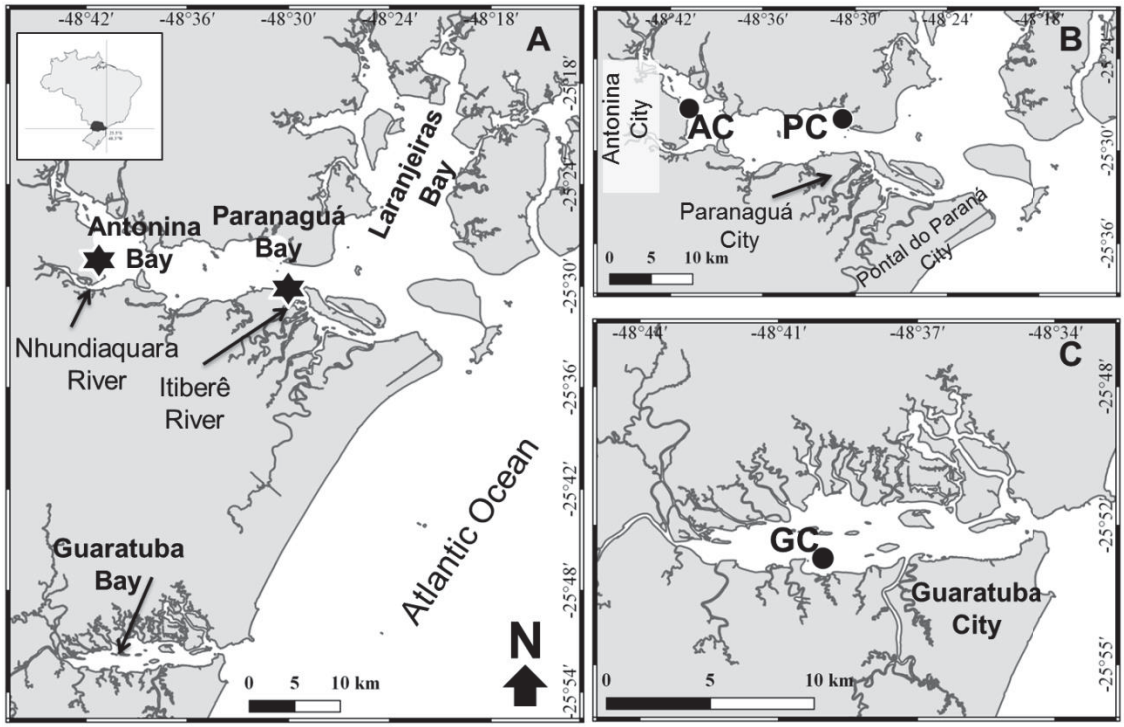


Fig. 1: Map of study area indicating (A) Paraná Coast with two estuarine environments; (B) sampling sites (black circles) in Antonina Bay and Paranaguá Bay and; (C) sampling site in Guaratuba Bay. AC = Antonina core; PC = Paranaguá core; GC = Guaratuba core; Stars indicate harbour areas.

3.3. Bulk organic marker parameters

Total organic carbon (TOC), $\delta^{13}C$ and $\delta^{15}N$ were determined using a Costech Elemental Analyser coupled with a Thermo-Finnigan Delta V Plus mass spectrometer. For $\delta^{15}N$ analysis, dry sediment aliquots were directly packaged into tin capsules. For TOC and $\delta^{13}C$, samples were first decarbonated by acidification using HCl 1 mol L^{-1}, dried and then, packaged into tin capsules. The $\delta^{13}C$ and $\delta^{15}N$ results were recorded relative to vPDB (Vienna Pee Dee Belemnite) and atmospheric air, respectively.

USGS-40 (L-glutamic acid, United States Geological Survey, USGS) and IAEA-600 (caffeine, International Atomic Energy Agency) were used as standards reference material before and after sets of 40 samples. Analytical errors are 0.01‰ for both isotopic ratios using USGS-40, and, 0.03‰ and 0.09‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively, using IAEA-600. The reference sediment material used for TOC was Soil LECO (LECO Corporation USA).

3.4. Sample extraction and instrumental analysis of faecal sterols

The sterols were determined according the method described by Wisnieski et al. (2016). Approximately 15 g of sediments of each core sub-sample was used. The extraction was performed in a Soxhlet apparatus for 8 hours using 80 mL of an *n*-hexane/dichloromethane

(DCM) mixture (1:1, v/v). A volume of 100 μL of a surrogate standard mixture, containing 5α-androstanol (20 ng μL^{-1}), was added before each blank or sample extraction. The resulting extracts were concentrated using a vacuum rotary evaporator, purified and fractionated via glass column chromatography filled with 5% deactivated silica and alumina. The elution sequence was 10 mL of *n*-hexane followed by 15 mL of a DCM/*n*-hexane mixture (3:7, v/v) (aliphatic and aromatic hydrocarbons fractions, see Martins et al. 2015) and finally 5 mL of ethanol/DCM mixture (1:9, v/v) followed by 15 mL of ethanol to obtain the sterols. The last eluted fraction was dried with a gentle stream of nitrogen, derivatised using BSTFA/TMCS (99:1) for 90 min at 65 °C and spiked with 500 ng of 5α-cholestane. The final volume was adjusted to 500 μL with *n*-hexane before injection.

Instrumental analyses of sterols were performed using a gas chromatograph (Agilent GC; model 7890A) equipped with a flame ionization detector (FID). The injection was performed in splitless mode using an Agilent 19091J-015 chromatographic column (50 m long, 0.32 mm inner diameter and 0.17 μm thickness). Hydrogen was used as the carrier gas (99.999% of purity). The oven temperature initiates at 40 °C and increased to 240 °C at 5 °C min^{-1}, then to 250 °C at 0.25 °C min^{-1} (holding for 5 min), to 280 °C at 5 °C min^{-1} and finally to 300 °C to 20 °C min^{-1} (holding for 8 min). The quantification of sterols was performed by integrating the chromatographic peaks of the compounds using the HP Chemstation program (G2070BA). Calibration was based on external standard mixtures of selected sterols (coprostanol, epicoprostanol, cholesterol, cholestanol, and sitosterol) at nine different concentrations (0.25 - 15.0 ng μL^{-1}; $R^2 > 0.995$).

Extracted blanks (every seven samples), certified sediment reference material, and recoveries of surrogate standards were used as quality controls Procedural blanks were performed with each series of eleven extractions, and no peaks interfered with the analyses of target compounds. The analysis of the sediment reference material, IAEA-408 (International Atomic Energy Agency) was satisfactory within 85% to 110 % of referenced values (Martins et al., 2012). The recovery of surrogates ranged from 66% to 125% (mean = 88 ± 25%), considering samples of the three cores. The detection limit (DL) for sterols was 0.002 ng g^{-1} based on the lowest sensitive concentration (0.05 ng μL^{-1}, respectively) multiplied by the final extracted volume (500 μL) and divided by the sediment weight (15 g) before extraction.

3.5. Data analysis

Principal Component Analysis (PCA) was performed using TOC, grain size (% of silt + clay), faecal sterols, $\delta^{13}C$ and $\delta^{15}N$. The analysis considered the data from all cores together,

to examine differences between the sampled areas, and separately, to examine possible variations of the parameters along each core.

4. RESULTS AND DISCUSSION

4.1. Bulk parameters

The Principal Component Analysis using the bulk parameters (and faecal sterols) showed a clear separation between the three cores (Fig. 2a). TOC, $\delta^{15}N$ and faecal sterols were the main variables that separated AC from the other two cores, while PC and GC were separated from each other mainly by the fine-grained and, $\delta^{13}C$ variation.

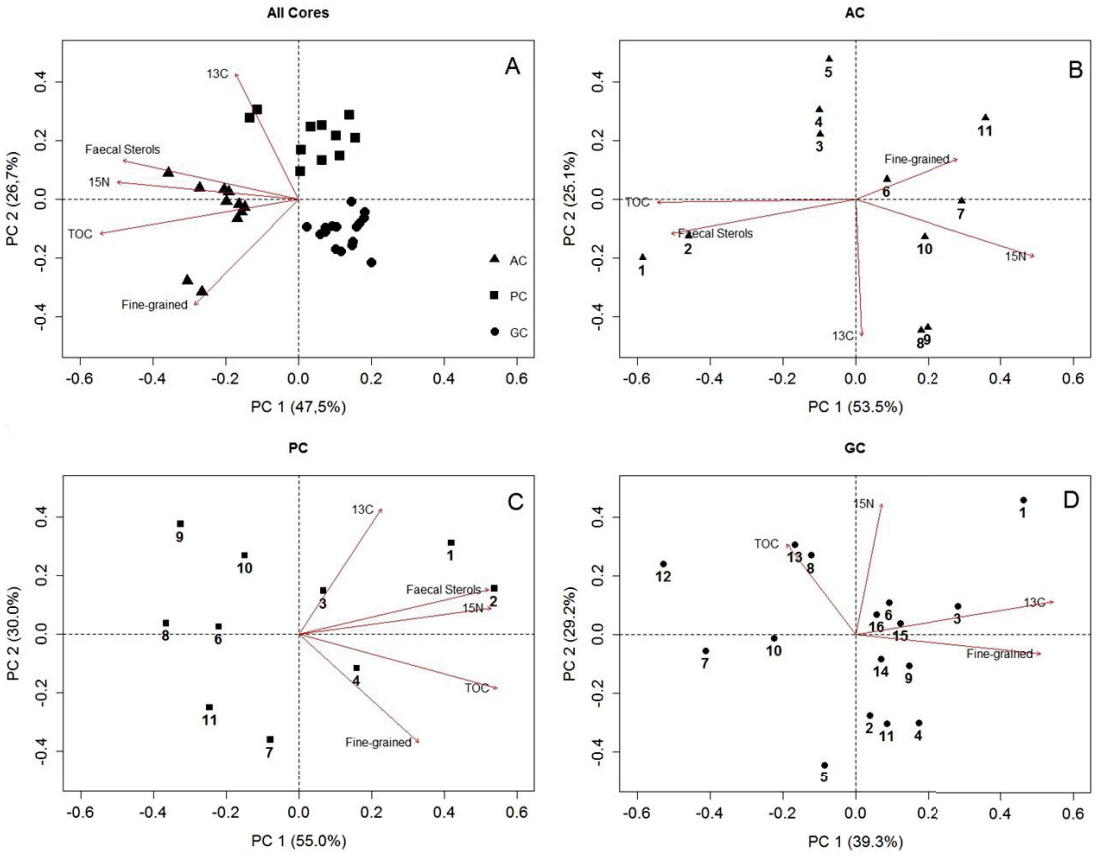


Fig. 2: Principal Component Analysis (PCA) based on % of fine-grained, total organic carbon (TOC), faecal sterols (except for Guaratuba core), δ13C and δ15N using all cores data together (A) and separated for Antonina core (B), Paranaguá core (C) and Guaratuba core (D). The numbers represent the subsamples of each core in ascending order considering top-to-bottom direction (see the number corresponding to each depth in table 1).

274 Grain size analysis was previously presented in Combi et al. (2013) for GC and Martins
275 et al. (2015) for the AC and PC. Briefly, the percentage of fine-grained (% silt + clay)
276 fractions varied according to the sampling site with a slightly higher average of fine sediments
277 in the AC (36.8% ± 1.92, not considering the 1950s and 1970s peaks) than in the PC and GC
278 (20.2% ± 7.1 and 29.4% ± 9.5, respectively) (Table 1). The AC also showed less grain size
279 variation through the core profile than did the PC and GC (Fig. 3). It seems that, in these
280 cores, grain size does not influence the concentrations of the other parameters, because, in
281 general, % of fine-grained did not follow its variations (only a weak tendency to follow TOC
282 in PC and $\delta^{13}C$ in GC) (Fig. 2b; c and d).
283

Table 1: Values of fine grained (silt + clay), total organic carbon (TOC), $\delta^{13}C$, $\delta^{15}N$ and concentrations of coprostanol (Cop) and epicoprostanol (Epicop) in sediment cores (AC: Antonina core; PC: Paranaguá core; GC: Guaratuba core). < DL = Detection limit.[1]Previously published in Combi et al. (2013) for GC and Martins et al. (2015) for AC and PC.[2]Faecal sterols (FS) = coprostanol + epicoprostanol. nc = not calculated. *not consideredin the calculation of average and standard deviation.

Depth (cm)	Subsamples	Age	fine-grained (%)[1]	TOC (%)	Cop (μg g^{-1})	Epicop (μg g^{-1})	FS[2] (μg g^{-1})	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
AC									
0-2	1	2011	31.5	3.97	0.19	0.02	0.21*	-26.22	2.69
2-4	2	2007			0.12	0.02	0.14*		
4-6	3	2003	32.6	3.71	0.05	< DL	0.05	-26.35	2.82
6-8	4	1999	30.4	3.62	0.06	< DL	0.06	-26.38	2.65
8-10	5	1995	33.8	3.73	0.04	0.01	0.05	-26.49	3.00
11-13	6	1989	30.0	3.6	0.05	0.01	0.06	-26.35	3.45
14-16	7	1982	77.9*	3.51	0.05	0.01	0.06	-26.23	3.47
17-19	8	1976	27.8	3.58	0.04	0.01	0.05	-26.13	3.74
20-22	9	1970			0.04	< DL	0.04		
23-25	10	1964	30.7	3.54	0.03	< DL	0.03	-26.24	3.47
26-27	11	1959	76.6*	3.57	0.02	< DL	0.02	-26.37	3.66
Mean±sd			**36.8±1.92**	**3.65±0.14**	**0.06±0.05**	**0.01±0.01**	**0.05±0.01**	**-26.32±0.11**	**3.28±0.40**
PC									
0-2	1	2009	18.2	1.09	0.05	0.01	0.06	-24.98	3.93
2-4	2	2001	22.5	1.19	0.07	0.02	0.09	-25.08	3.50
4-6	3	1994	19.4	0.96	0.04	0.01	0.05	-25.09	1.94
6-8	4	1986	31.7	0.97	0.03	< DL	0.03	-25.06	2.31
8-10	5	1978	31.0	1.23	0.02	< DL	0.02	-25.58	2.55
11-13	6	1967	15.1	0.79	0.02	< DL	0.02	-25.38	1.84
14-16	7	1955	24.2	0.94	0.01	< DL	0.01	-25.57	2.28
17-19	8	1944	14.2	0.71	0.01	< DL	0.01	-25.37	1.31
20-22	9	1932	7.81	0.72	0.01	< DL	0.01	-25.10	1.65
23-25	10	1921	16.8	0.71	< DL	< DL	< DL	-25.03	2.70
26-27	11	1911	20.9	0.81	< DL	< DL	< DL	-25.53	1.91
Mean±sd			**20.2±7.1**	**0.92±0.19**	**0.02±0.02**	**< DL±0.01**	**0.03±0.03**	**-25.25±0.24**	**2.36±0.79**

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Table 1: Continuation

Depth (cm)	Subsamples	Age	fine-grained (%)[1]	TOC (%)	Cop (μg g^{-1})	Epicop (μg g^{-1})	FS² (μg g^{-1})	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
GC									
0-2	1	2011	39.2	1.19	0.006	< DL	< DL	-26.59	2.77
2-4	2	2005	26.8	0.81	< DL	< DL	< DL	-27.09	1.58
4-6	3	2000	37.0	0.90	< DL	< DL	< DL	-26.94	2.65
6-8	4	1994	35.6	0.86	< DL	< DL	< DL	-27.04	1.38
8-10	5	1989	38.1	1.06	< DL	< DL	< DL	-27.33	0.46
10-12	6	1983	39.1	1.25	< DL	< DL	< DL	-27.05	1.67
12-14	7	1977	17.0	1.08	< DL	< DL	< DL	-27.42	1.69
14-16	8	1972	26.7	1.11	< DL	< DL	< DL	-27.22	2.73
16-18	9	1966	37.6	1.16	< DL	< DL	< DL	-26.96	1.07
18-20	10	1960	16.7	1.16	< DL	< DL	< DL	-27.09	1.31
20-22	11	1955	24.1	0.83	< DL	< DL	< DL	-26.95	1.27
22-24	12	1949	7.2	1.19	< DL	< DL	< DL	-27.36	2.27
24-26	13	1944	27.5	1.19	< DL	< DL	< DL	-27.26	2.65
26-28	14	1938	38.4	1.06	< DL	< DL	< DL	-27.16	1.66
28-30	15	1932	29.9	0.90	< DL	< DL	< DL	-27.02	2.42
30-32	16	1927	28.8	1.01	< DL	< DL	< DL	-27.03	2.17
Mean±sd			**29.4±9.5**	**1.05±0.15**	**nc**	**nc**	**nc**	**-27.09±0.20**	**1.86±0.69**

Sedimentary TOC contents ranged from 3.51 to 3.97%, 0.71 to 1.23% and between 0.81 and 1.25% for the AC, PC and GC, respectively (Table 1; Fig. 3). The higher % TOC values in the AC suggest a more favoured depositional environment than in the PC and GC. The lower energy environment in the AC region should explain these features (van Rijn, 1993), and consequently become easy the accumulation of sedimentary OM. There is no significant correlation between % TOC and % silt + clay in the AC (r = 0.48; p = 0.27; not considering the 1950s and 1970s peaks), which means that fine sediments are not accompanied by a % TOC increase, that is also observed in PCA (Table 1; Fig. 2b). The PC1 of PCA from AC (Fig. 2b) presents a gradient of grain size (fine-grained decreases towards the top) and of faecal sterols and TOC (increase towards the top). However, in the AC profile it is observed low variation in % TOC and fine sediments in the AC, which indicates that the depositional pattern of this region was maintained throughout the analysed period (with the exception of the 1950s and 1970s peaks). In PC, there are a positive correlation between these two parameters (r = 0.69; p < 0.05), also observed in PCA analysis (Fig. 2c), suggesting a grain size influence on the sedimentary OM concentrations in this area of Paranaguá Bay, which is not observed in AC (as mentioned above) and GC (r = -0.07; p = 0.79; Fig. 2b and 2d). In addition, there was a slight but distinct increasing tendency for % TOC from the 1980s to the top core of AC, while there was no trend in PC and GC (Fig. 2b and 2c; Fig. 3).

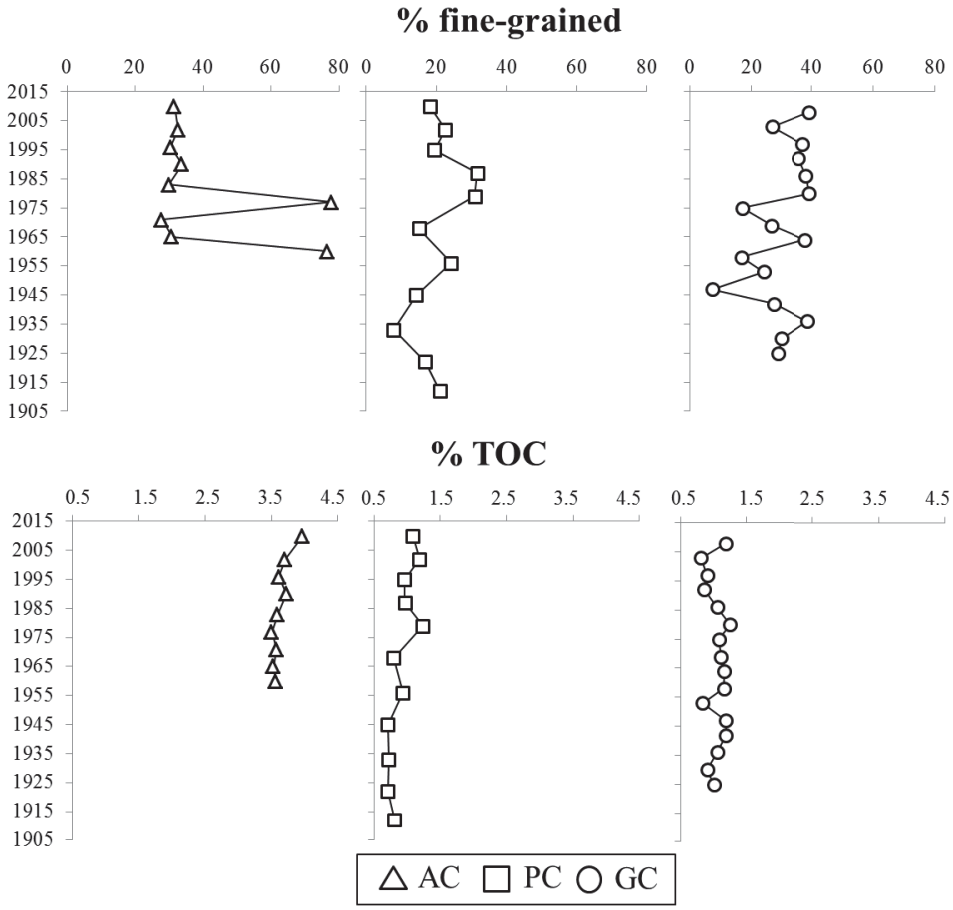


Fig. 3: Profiles of % fine-grained (silt + clay – previously published in Combi et al. (2013) and Martins et al. (2015)) and % total organic carbon (TOC) in cores of Antonina (AC - triangles), Paranaguá (PC - squares) and Guaratuba (GC - circles).

Considering the three cores, the values of $\delta^{13}C$ ranged from -27.4 ‰ and -25.0 ‰ (Table 1), similar to that found in tidal-dominated estuaries, which have intermediate values of $\delta^{13}C$ (between -26 and -24 ‰), compared to the values related to marine and riverine/terrestrial endmembers (Middelburg and Herman, 2007). The $\delta^{13}C$ values vary between the groups of the primary producers due to different photosynthetic pathways (Meyers, 1997; Bouillon et al., 2008). The most typical discrimination is between terrestrial plants (Calvin pathways - C_3) and phytoplankton, which have $\delta^{13}C$ around of approximately -27 ‰ and between -20 and -22 ‰, respectively (Meyers, 1997). Accordingly, the three cores from estuarine areas of the Paraná coast appeared to be influenced predominantly by terrigenous OM ($\delta^{13}C$ between -27.0 and -25.3 ‰). This may be related mainly to the significant mangrove cover near these estuaries. In fact, preserved remnants of Atlantic Forest are characteristic of these estuarine areas, and mangrove (represented mainly by *Rhizophora mangle, Laguncularia racemosa* and *Avicennia tomentosa*) is the main vegetation found on

the margins of these bays, especially at the northern margins (Bigarella, 2001; Lana et al., 2001).

Sewage OM input may also influence the $\delta^{13}C$ variation, ranging from -26 to -22 ‰, but this range of values overlaps the typical range of $\delta^{13}C$ found in estuarine environments (Bachtiar et al., 1996; Carreira et al., 2002; Barros et al., 2010), as the range found in the bays studied here (Table 1). Thus, these values should be interpreted in association with other proxies, as well as data or the historical facts of urban development (consequently sanitary services) near the estuaries (e.g., Bachtiar et al., 1996; Andrews et al., 1998; Barros et al., 2010; Bueno et al., 2018).

Analysing the cores separately, the average of $\delta^{13}C$ was -26.3 ‰ (± 0.1) for AC, -25.3 ‰ (± 0.2) for PC and -27.1 ‰ (± 0.1) for the GC (Table 1). This variation in isotopic signature between the cores may reflect location in the studied bays, considering the natural gradient from OM with less C_{13} produced by terrigenous vegetation to OM enriched in C_{13} produced by marine primary producers (Meyers, 1997). The AC and GC were sampled in inner and intermediate areas of the considered bays and receive substantial loading from large drainage basins, while the PC is near the mouth of Paranaguá Bay (Fig. 1). Moreover, the GC is a retainer of terrestrial OM from land runoff and mangrove (Brandini, 2008), which may reinforce the greater depletion of ^{13}C than in the AC and PC.

The three cores showed relatively stable $\delta^{13}C$ values downcore (Fig. 4) with coefficients of variation ranging from 0.41 to 0.94% and no gradient of $\delta^{13}C$ observed on the PCAs (Fig. 2), which suggests the same sources of OM deposited in the last few years. Nevertheless, weak oscillations are observed in the core profiles. The AC showed increasing $\delta^{13}C$ from the 1960s to early 1970s, followed by a decrease until the end of the 1980s and another increase until the top core. The PC showed a constant oscillation from the bottom core until the period between the 1970s and 1980s, when the $\delta^{13}C$ values increased markedly and remained constant until 2009. The $\delta^{13}C$ distribution in the GC is similar to that in the PC, varying constantly and without a clear tendency until 1985, when it slightly increased to the top core (Fig. 4). Between the 1970s and 1980s, an important access route to the coast and to the harbour was completed, making the region more attractive to tourism and to economic activities with urban expansion to provide human resources to the harbour sector (Estades, 2003; Castro et al., 2015). The access route construction promotes deforestation, which increases the terrigenous material input on the estuary, which can alter the signs of $\delta^{13}C$. These events tend also to promote accelerated population growth (Castro et al., 2015), which could increase the sewage input. However, the $\delta^{13}C$ variation in the periods before and after

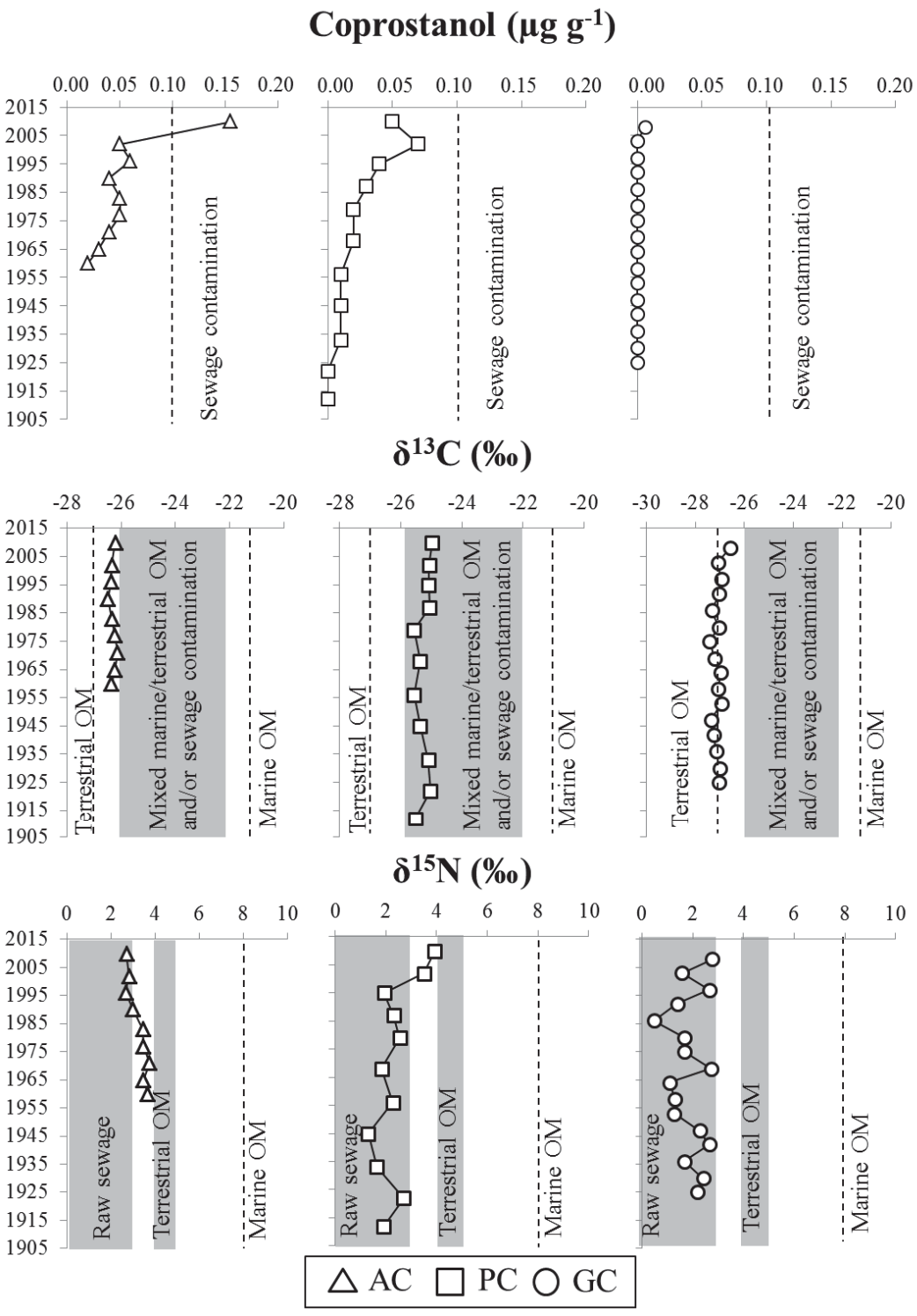
355 the 1980s in the AC and PC is discrete (a difference approximately 0.02 ‰ to the AC and
356 0.32 ‰ to the PC, in average; Table 1). Moreover, the values remain in the common range
357 related to estuarine sediments (between -24 and -26 ‰; Middelburg e Herman, 2007),
358 requiring further indicators to generate a better assessment of the possible influence of sewage
359 in the sedimentary OM of the studied area, which can also are overlapped by the greater
360 introduction of terrigenous material in that period. Therefore, at first, these oscillations must
361 be related to variations in local productivity, terrestrial material input or other natural
362 interferences.

363 The $\delta^{15}N$ composition is usually used combined with $\delta^{13}C$ to confirm changes in the
364 sedimentary OM composition due to the sewage input (e.g., Barros et al., 2010; Bueno et al.,
365 2018). The AC was the core more enriched in ^{15}N with $\delta^{15}N$ average of 3.2 ‰ (2.7 to 3.7),
366 this being one of the main variables that separated AC from the other cores (Fig. 2a). The
367 averages of $\delta^{15}N$ for the PC and GC were 2.4 ‰ (1.3 to 3.9) and 1.9 ‰ (0.5 to 2.8 ‰),
368 respectively (Table 1). The higher $\delta^{15}N$ values for the AC were recorded from the bottom of
369 the core until the 1980s (3.5 to 3.7 ‰), when there was a decrease until the end of the 1990s
370 (minimum of 2.6 ‰), that remained constant until 2009. In the PC and GC, the $\delta^{15}N$ values
371 oscillate continuously and without a clear trend until the 1980s, when there is an increase until
372 the top (Fig. 4).

373 The signs of $\delta^{15}N$ are highly variable due to several factors inherent to the complexity of
374 the global nitrogen cycle and diagenesis (Thornton and McManus 1994; Carreira et al., 2002;
375 Bueno et al., 2018), especially in transitional environments such as estuaries and the shallow
376 continental shelf, in which the $\delta^{15}N$ values may vary between 0 to ~10 ‰, even without
377 sewage input (e.g., Thornton and McManus 1994; Tucker et al., 1999).

378 Given the values found in other estuaries of the Brazilian coast (Carreira et al., 2002;
379 Barros et al., 2010) and other harbour regions (Bachtiar et al., 1996; Tucker et al., 1999), the
380 range of values between 0.4 and ~3.5; from 4.0 to 5.1 and >8.0 ‰ may be related to sewage,
381 terrestrial and marine OM endmembers, respectively. Based on the above ranges, the values
382 recorded in the AC, PC and GC (0.46 to 3.93 ‰) could indicate sewage input for the entire
383 period covered in the three estuarine areas with a decrease in sewage OM influence on the
384 upper layers (reflected by the slight trend of $\delta^{15}N$ increase, Fig. 4). However, the $\delta^{15}N$ profiles
385 were quite variable for the entire period covered, even in periods prior to the main urban
386 development and increase in population densities in the region (between the 1970s and 1980s)
387 without a clear trend. Therefore, it is presumable that the range of $\delta^{15}N$ found here (0.46 to

388 3.93 ‰) is a consequence of several factors, where sewage input is one factor together with a
389 strong terrigenous OM contribution.
390



391
392 Fig. 4: Profiles of coprostanol (µg g^{-1}), $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) in cores of Antonina (AC - triangles), Paranaguá
393 (PC - squares) and Guaratuba (GC - circles). Limit values of marine and terrestrial OM endmembers (for $\delta^{13}C$
394 and $\delta^{15}N$) and for sewage contamination (for coprostanol) are in dashed lines and shaded areas.

395 **4.2. Faecal sterols**

396 Stable isotope analyses did not generate a conclusive interpretation or indicate a specific
397 range value typically related to the sewage input in all cores studied. The mixed isotope
398 signatures in estuarine sediments reflect multiple sources of carbon and nitrogen that
399 comprise the bulk material and therefore represent the entire mixture of the OM components
400 (Meyers, 1997), which may generate an overlap of stable isotope sign ranges. Therefore, the
401 analysis together with specific molecular markers of the OM source, such as sterols, can
402 support and strengthen the interpretation of the origin of OM found in sediments (e.g.,
403 Bachtiar et al., 1996; Zhao et al., 2015).

404 The concentrations of coprostanol ranged from 0.02 to 0.19 μg g^{-1} in the AC and from <
405 DL to 0.07 μg g^{-1} in the PC. In the GC, coprostanol was detected only in the upper layer with
406 0.006 μg g^{-1}(Table 1). Epicoprostanol, the other faecal sterol, was detected in the three cores.
407 In the AC and PC, epicoprostanol ranged from < DL to 0.02 μg g^{-1} and was detected in more
408 layers in the first core (from the top to 4 cm and between 8 and 19 cm). In the GC,
409 epicoprostanol was not detected in any layers (Table 1).

410 Sewage contamination analysis by faecal sterols was performed using threshold values
411 of coprostanol and diagnostic ratios between coprostanol and other biogenic sterols (e.g.,
412 Grimalt et al., 1990; Martins et al. 2014a). The recommended threshold values of coprostanol
413 that indicate sewage contamination in sediments varied between 0.10 (Writer et al., 1995) and
414 0.50 μg g^{-1} (Gonzalez-Oreja and Saiz Salinas, 1998). For the cores analysed here, even given
415 the more restrictive threshold value of coprostanol (0.10 μg g^{-1}), it was only possible to
416 identify sewage output in the AC, specifically on the upper 4 cm of this core, ranging from
417 0.12 to 0.19 μg g^{-1} (2 – 4 and 0 – 2 cm, respectively) (Fig. 4). When the coprostanol values
418 are higher than the threshold value considered (here 0.10 μg g^{-1}; Writer et al., 1995),
419 diagnostic ratios such as (i) coprostanol/coprostanol+cholestanol (Grimalt et al, 1990) and (ii)
420 coprostanol/coprostanol + cholesterol (Takada et al., 1994; Mudge and Seguel, 1999) are used
421 to confirm the status of faecal contamination. It is possible to calibrate the threshold values of
422 these diagnostic ratios for sediments in subtropical estuarine environments considering the
423 regression models proposed by Martins et al. (2014a). Applying 0.10 μg g^{-1} as a threshold
424 value for coprostanol in the regression models (0.07.[coprostanol]+0.23 for ratio (i) and
425 0.06.[coprostanol] +0.08 for ratio (ii); Martins et al., 2014a), the threshold values for ratios (i)
426 and (ii) are 0.24 and 0.09. The results of the ratios of the upper layers of the AC (with
427 coprostanol > 0.10 μg g^{-1}) were 0.13 and 0.15 to ratio (i) and 0.07 and 0.05 to ratio (ii) for the
428 layers 0 – 2 and 2 – 4, respectively, suggesting no/low sewage input. Nevertheless, high and

429 moderate coprostanol values were previously recorded in areas close to the cities of
430 Paranaguá (up to 2.22 μg g^{-1}; Martins et al., 2010) and Antonina (up to 0.71 μg g^{-1}; Cabral and
431 Martins, 2018), considered sinking areas for coprostanol and hot spot areas for sewage
432 contamination monitoring.

433 In general, the range of coprostanol values recorded in AC and PC are similar to that
434 recorded in environments with low or without sewage contamination, such as Admiralty Bay
435 (Antartica; up to 0.15 μg g^{-1}) and Camamu Bay (Brazil; 0.01 μg g^{-1}) (Martins et al., 2014b;
436 Carreira et al., 2016), and much lower than areas of severe sewage contamination, such as the
437 sites of Guanabara Bay, Brazil (high as 40 μg g^{-1}; Carreira et al., 2004) and near urban
438 outfalls, for instance in Cienfuegos Bay, Cuba (up to 5,400 μg g^{-1}; Tolosa et al., 2014).

439 This evaluation of faecal contamination using threshold values of coprostanol,
440 diagnostic ratios and comparison of coprostanol values recorded in other regions (with or
441 without sewage contamination) reinforces that the faecal sterols values found in these cores
442 may be representative of a previous non-impacted environment scenarios by sewage. By using
443 the mean concentration of these values in each core (Table 1), the reference values of faecal
444 sterols which may represent a previous non-impacted environment scenarios were 0.05 ± 0.01
445 and 0.03 ± 0.03 μg g^{-1} for AC and PC, respectively, and < DL for GC core. The two upper
446 layers from the AC were not considered because the concentrations of faecal sterols were
447 higher than 0.10 μg g^{-1} and could be associated with recent sewage input.
448
449 **4.3. Sedimentary record of coprostanol and human development history of studied**
450 **estuaries**

451 Even when low, coprostanol concentrations tend to follow variations in urban and
452 economical regional development, as verified by the slight increase in values in the upper
453 layers of the AC and PC cores (Fig. 4) and the gradient observed in the PCAs (Fig. 2). In the
454 AC, coprostanol values slightly increased from the 1950s until the mid-1970s. Then, the
455 values are constant until the early 2000s, when they increase sharply until 2011. Between the
456 1940s and 1950s, Antonina city experienced economic development reflected by the human
457 migration due to the establishment of the main regional industrial zone of this period
458 (Matarazzo Industrial Complex) and by intensification of harbour activities (APPA, 2018). In
459 the 1970s, the Matarazzo Industrial Complex was closed and harbour activities declined,
460 resulting in the stagnation of economic activities of Antonina and consequent population
461 evasion. The marked increase of coprostanol values in 2011 may be related to strong storms

462 that caused high urban outflow to the estuarine region (Pinto et al., 2012; Martins et al.,
463 2015).
464 In the PC, faecal sterols were detected since the 1930s with a constant level (up to 0.02
465 μg g^{-1}) until the end of the 1970s. The values for coprostanol tended to increase from the
466 1980s with a peak (0.09 μg g^{-1}) in the early 2000s. Similar to AC, the increase in coprostanol
467 values in the upper layers of the PC follow the urban development of Paranaguá city. Its
468 economy and urban development were stimulated by harbour activities, which in turn were
469 enhanced by the completion of important access routes (by road and rail) to the Paraná coast
470 and by Paranaguá harbour infrastructure that was enhanced after the 1970s (Pierri et al., 2006,
471 Castro et al., 2015). The increase in harbour activities generated another important migration
472 to the region around Paranaguá city, leading to a fast population increase between 1950 and
473 2010 (24,638 inhabitants in 1950 to 140,469 in 2010) (Martins et al., 2015; IBGE, 2018).
474 Future projects related to a new harbour area and access road (AMB, 2007) tend to stimulate
475 population growth in that region, occupying mangrove and environmental protection areas, as
476 currently recorded (Silva et al., 2015).
477
478 **5. CONCLUSION**
479
480 The determination of faecal sterols (coprostanol and epicoprostanol) and the diagnostic
481 correlated ratios provided an unambiguous interpretation of the evidence of the historical
482 sewage input on the analysed cores. The molecular markers clarified the interpretation of
483 stable isotopes, showing that biogeochemical processes and/or sources other than sewage
484 should be the dominant factors in the variation in the $\delta^{13}C$ and $\delta^{15}N$ distributions. In addition,
485 molecular markers also showed that the AC, PC and GC are in areas outside of the influence
486 of the effluent plume and can provide reference values for the studied subtropical estuarine
487 areas.
488 It is a challenge to use carbon and nitrogen stable isotopes as proxies of the sewage
489 input in transition environments such as estuaries since ranges for sewage OM overlap the
490 terrestrial and marine OM endmembers (or terrestrial OM only, in the case of $\delta^{15}N$). This
491 overlap reinforces the need for an evaluation of sewage contamination integrating indicators
492 using different approaches, especially molecular markers, as faecal sterols, in sediment cores,
493 as well together microbiological indicators in recent sediments and / or in particles from water
494 column.
495

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CAPÍTULO 5

CONSIDERAÇÕES FINAIS

A liberação do esgoto em ambientes aquáticos é uma atividade histórica, podendo ser considerada uma das formas mais primitivas de contaminação dos oceanos. Ambientes aquáticos continentais e/ou costeiros geralmente não apresentam condições físico–químicas e biológicas capazes de assimilar, diluir e/ou degradar a grande quantidade de matéria orgânica, patógenos, contaminantes e outros constituintes do esgoto. Dessa forma, o tratamento de águas residuárias previamente à sua liberação no ambiente seria a melhor solução para a prevenção de deterioração do ambiente e da disseminação de doenças humanas de veiculação hídrica.

No Brasil (e muitos outros países do mundo), entretanto, só nas décadas recentes estão havendo maiores investimentos nos serviços de saneamento que, porém, não acompanha a demanda gerada pelo acelerado crescimento populacional (o que ocasiona maior produção de esgoto). Sendo assim, a detecção do esgoto no ambiente tem papel importante para identificar áreas de risco de contaminação (ou contaminadas) a fim de se evitar o contato humano com este contaminante e de monitorar áreas suscetíveis a este tipo de poluição.

Uma série de parâmetros indicadores de contaminação por esgoto já está estabelecida por órgãos reguladores de qualidade de água (nacionais e internacionais). No entanto, os parâmetros microbiológicos usualmente utilizados (*Escherichia coli* e enterococci) possuem algumas desvantagens que podem gerar interpretações errôneas referentes à qualidade de água, especialmente de ambientes estuarinos subtropicais. Desta forma, a detecção do esgoto no ambiente através da investigação de outros indicadores, como os marcadores moleculares e / ou isótopos estáveis investigados no presente trabalho (em conjunto ou não com os já usualmente aplicados) deve trazer interpretações mais realistas com a situação atual e/ ou histórica da introdução e contaminação por esgoto no ambiente.

Os resultados obtidos neste trabalho responderam os objetivos propostos em cada artigo científico, bem como fornecem um panorama geral da contaminação por esgoto nos ambientes estuarinos do litoral paranaense.

A hipótese (i) foi refutada, já que não houve correlação significativa entre os marcadores moleculares e os indicadores microbiológicos analisados. No entanto, foi possível calcular valores limites de contaminação fecal para os marcadores moleculares com base nos valores limites já estipulados para os indicadores microbiológicos (hipótese (ii)), isso porque, embora as concentrações de coprostanol e de bactérias indicadoras fecais possam não apresentar relações lineares, categorias de qualidade de água baseada nos parâmetros microbiológicos indicados na legislação podem ser preditas por concentrações de coprostanol no MPS através da análise de regressão logística, inclusive gerando valores limites de coprostanol relacionados a essas categorias. É importante ressaltar, no entanto, que diferentes temperaturas (resultantes de estações ou zonas climáticas), bem como diferentes matrizes ambientais analisadas (MPS e sedimento, como analisados nesta tese) influenciam no estabelecimento de valores limites de contaminação por esgoto, e devem ser considerados em análises de qualidade ambiental.

Considerando que a maioria dos estudos de marcadores químicos de matéria orgânica foca em análises sedimentares, a presente tese trouxe importantes contribuições em escala regional e mundial referentes às análises de marcadores químicos no MPS. Essas contribuições estão relacionadas principalmente à considerável variação de composição e concentração dos marcadores entre MPS e sedimento, o que resultou em diferentes valores limites de concentração de coprostanol indicativo de contaminação por esgoto e variação na predominância de fonte de matéria orgânica em cada matriz (refutando a hipótese (iii)). Esses resultados reforçam a importância da seleção da matriz de acordo com a escala espacial e temporal objetivada, sugerindo que análises de MPS indica um *status* de contaminação atual (curta escala temporal) e mais generalista para o ambiente (grande escala espacial), enquanto análises no sedimento indicam áreas preferenciais de deposição de matéria orgânica e de maior escala temporal.

Em relação ao problema do esgoto no litoral do Paraná, as análises de indicadores de esgoto no MPS, sedimento superficial e testemunho permitiram estabelecer o panorama atual e histórico da introdução de esgoto na área de estudo. Os resultados indicaram que em geral as regiões estuarinas do Paraná não apresentam um cenário crítico de contaminação por esgoto, mas que áreas específicas de introdução e acúmulo de matéria orgânica proveniente do esgoto merecem atenção especial para monitoramento, a citar áreas das zonas fluvial e de

mistura, bem como áreas próximas das cidades de Antonina, Guaratuba e Paranaguá.

Os testemunhos sedimentares disponíveis não apresentaram concentrações significativas de marcadores geoquímicos que indicassem contaminação por esgoto. Por isso, não foi possível avaliar historicamente a eficiência dos esforços de regulação do saneamento e tratamento de esgoto na região que vem ocorrendo nos últimos anos (hipótese (iv)). Porém, estes testemunhos “não contaminados” geraram valores de referência de área não contaminada, e são de grande importância para a comparação em futuras avaliações de introdução e contaminação por esgoto em áreas estuarinas, especialmente para a Baía de Paranaguá, considerando a instalação de novos empreendimentos portuários, planejados para a próxima década.

Por fim, é importante ressaltar que tanto os marcadores moleculares como os microbiológicos possuem vantagens e desvantagens em sua utilização como indicadores de contaminação fecal, relacionadas à exatidão de suas indicações (como discutido no capítulo 2), bem como em relação aos custos e tempo de análises (sendo inferiores para os microbiológicos, em relação aos moleculares analisados no presente trabalho). Desta forma, os resultados aqui encontrados não sustentam e não sugerem a utilização de somente uma classe de indicadores, mas sim um conjunto destes, com diferentes características complementares entre si, de forma a se obter uma avaliação cada vez mais próxima da exatidão e precisão indispensáveis à detecção de áreas de risco ao contato humano.

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APÊNDICE 1 - MATERIAL SUPLEMENTAR DO CAPÍTULO 2

Uma avaliação integrada de algumas bactérias indicadoras fecais (BIF) e marcadores químicos como potenciais ferramentas de monitoramento de contaminação por esgoto em estuários subtropicais

An integrated evaluation of some faecal indicator bacteria (FIB) and chemical markers as potential tools for monitoring sewage contamination in subtropical estuaries

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Supplementary Information

1. Quanti-Tray Method

Quanti-Tray Method uses chromogenic substrates with the culture medium Colilert® and Enterolert® for enumeration of *E. coli* and enterococci, respectively (APHA, 1995; 2009). After the sealing and incubation (at 36°C for 18 hours for *E. coli* and at 41ºC for 24 hours for enterococci), the quantification trays were analyzed under ultraviolet light (365 nm). The number of positive cells from each dilution series were recorded and converted to MPN using a standard reference table with 95% of confidence intervals (APHA, 1995).

2. Instrumental analysis of molecular markers

For LABs, it was used an Agilent 19091J-433 capillary fused silica column coated with 5% diphenyldimethylsiloxane (30 m, 0.25 mm ID, 0.25 μm film thickness). The oven temperature was programmed from 40 to 60 °C at 20 °C min^{-1}, then to 290 °C at 5 °C min^{-1}, and to 300 °C at 5 °C min^{-1}. SIM (System Ion Monitoring) mode and the HP Enhanced Chemstation G1701CA were used for data acquisition and quantification, respectively. Calibration was based on an external standard solution containing 1-C_m-LABs (m = 10 - 13) at six different concentrations (0.10, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50 and 2.00 ng μL^{-1}; R^2 > 0.995). Compounds were identified by ion mass fragments (m/z 91, 92 and 105) and by matching the retention times with a mixture of all of the n-C_m-LABs (m = 10 – 13) provided

by Deten Química S.A. (LABs Mix Lot LPS 0025/08). The n-C_{14}-LABs were identified by ion mass fragments and estimated retention index based on general literature. Quantitation LABs ions used were m/z 91 and 105, considering the area of each main fragment of the analyzed compound.

For sterols, it was used an Agilent 19091J-015 capillary fused silica column coated with 5% phenylmethylsiloxane (50 m, 0.32 mm ID and 0.17 μm film thickness). The oven temperature was programmed from 40 to 240 °C at 5 °C min^{-1}, then to 250 °C at 0.25 °C min^{-1} (holding for 5 min), then to 280 °C at 5 °C min^{-1} and to 300 °C at 20 °C min^{-1} (holding for 10 min). The HP Enhanced Chemstation G2070BA program was used to perform the measurements. Calibration was based on external standard mixtures of sterols (coprostanol, epicoprostanol, cholesterol, cholestanol, campesterol, stigmasterol, sitosterol and dinosterol) at nine different concentrations (0.25, 0.50, 0.75, 1.00, 2.50, 5.00, 7.50, 10.0 and 15.0 ng μL^{-1}; $R^2 > 0.995$). Compounds were identified by matching retention times with results from standard mixtures of sterols above. For the sample quantification, it was considered the peak area of each compound multiplied by its own response factor, present in calibration curve, in relation to the mass/area ratio of surrogate standards added before extraction.

3. Analytical control of molecular markers analysis

The analytical control was based on extraction blanks (for each 11 samples) and the recoveries of the surrogate standards in all samples. The molecular markers detected in the blanks were low (<3 times the detection limit - DL) do not interfere on the target compounds. The DL for LABs and sterols were 1.43 ng L^{-1} and 3.57 ng L^{-1}, respectively, based on the lowest sensitive LAB or sterols concentration (0.02 ng μL^{-1} and 0.05 ng μL^{-1}, respectively) multiplied by the final extracted volume (250 μL) and divided by the filtered water volume (3.5 L). The surrogate recoveries were considered satisfactory, with mean values of 62 ± 16% for 1-C_{12}-LAB and 84 ± 34% for 5α-androstanol for at least 80% of the samples analyzed.

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Table S1: Water column parameters and precipitation data at each site (G = Guaratuba Bay; P= Paranaguá Bay), and two seasons (W: winter; S: summer). Temp.: temperature; DO: dissolved oxygen; na = not available

Samples	rainfall (mm) Σ8 days[a]		rainfall. (mm) Σ2 days[a]		Surface water Temp. (°C)		Salinity (UPS)		pH		DO (mL L^{-1})		DO (% saturation)		Depth (m)		Seston (mg L^{-1})	
	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S	W	S
G1	0.0	86.5	0.0	86.5	23	30	12	7	6.7	6.7	5.10	1.64	91.2	32.3	5.2	5.2	19.9	13.3
G2	0.0	86.5	0.0	86.5	23	30	17	9	6.7	6.7	5.48	4.40	100.9	87.7	2.2	2.2	19.2	15.8
G3	0.0	86.5	0.0	86.5	23	31	19	14	7.0	6.9	5.35	3.50	99.6	72.9	3.1	2.2	25.8	28.0
G4	0.0	86.5	0.0	86.5	22	31	26	17	7.6	7.5	5.51	3.14	105.0	66.3	4.6	4.7	21.8	25.1
G5	0.1	87.3	0.0	39.5	23	31	23	18	7.8	7.7	5.50	3.19	105.0	67.5	1.5	1.8	41.2	54.8
G6	0.1	87.3	0.0	39.5	21	30	30	22	7.8	7.7	4.81	4.06	92.1	86.8	4.5	7.7	30.8	35.7
G7	0.1	87.3	0.0	39.5	21	30	30	25	7.9	7.9	5.10	5.32	97.7	115.7	4.9	5.6	30.2	42.1
G8	0.1	87.3	0.0	39.5	23	31	23	21	8.0	7.9	5.93	3.64	113.2	78.7	5.0	5.0	30.6	47.3
G9	0.1	87.3	0.0	39.5	21	30	33	31	8.0	7.9	5.03	4.38	98.0	98.0	6.3	6.4	34.2	46.4
G10	0.1	87.3	0.0	39.5	21	30	33	32	8.0	7.9	4.92	4.44	95.9	100.3	3.6	2.8	44.3	35.9
G11	0.1	87.3	0.0	39.5	23	31	26	21	7.8	7.8	5.41	3.37	105.0	72.9	2.4	2.2	37.9	29.4
G12	0.1	87.3	0.0	39.5	21	30	34	32	8.1	8.0	5.01	4.54	98.3	102.4	13.7	23.0	45.9	40.3
P1	0.4	171.3	0.0	19.7	21	27	20	10	6.2	6.6	4.14	3.06	74.8	58.2	4.4	4.2	26.9	26.5
P2	0.4	171.3	0.0	19.7	21	27	25	13	6.7	6.5	4.15	3.05	77.1	58.9	3.5	3.8	31.6	32.6
P3	0.4	171.3	0.0	19.7	21	27	25	14	7.0	6.5	4.22	3.18	78.4	61.9	2.4	6.1	33.8	35.8
P4	1.2	209.0	0.0	10.8	21	28	26	17	7.2	6.5	4.16	3.52	77.7	70.7	8.2	8.7	31.6	37.6
P5	1.2	209.0	0.0	10.8	21	28	26	19	7.3	6.6	4.82	3.51	90.2	71.6	3.9	1.3	35.9	52.0
P6	1.2	209.0	0.0	10.8	21	28	27	20	7.4	6.7	4.42	3.52	83.1	72.0	5.1	4.6	36.0	172.1
P7	1.2	209.0	0.0	10.8	21	28	27	21	7.4	6.7	2.58	3.32	48.4	68.4	3.2	0.9	35.6	61.3
P8	1.2	209.0	0.0	10.8	21	28	28	20	7.6	6.7	4.42	3.75	83.8	76.8	13.0	11.6	39.2	43.3
P9	1.0	182.8	0.0	11.2	21	28	29	20	7.7	6.6	4.15	3.01	79.0	61.5	0.9	0.5	31.0	77.7
P10	1.0	182.8	0.0	11.2	21	28	29	12	7.6	6.3	4.44	2.31	84.5	45.2	3.4	2.7	36.5	39.1
P11	1.0	182.8	0.0	11.2	21	28	29	16	7.8	6.5	3.77	3.09	71.8	61.8	6.4	6.0	27.7	30.7
P12	1.0	182.8	0.0	11.2	21	28	30	20	7.9	6.9	3.30	3.45	63.3	70.6	5.4	5.5	39.0	35.8
P13	1.0	182.8	0.0	11.2	21	28	29	22	7.7	6.9	4.95	3.86	94.3	79.8	15.8	14.7	26.7	48.9
P14	1.0	182.8	0.0	11.2	21	28	30	23	7.8	7.0	5.13	3.70	98.4	77.0	11.3	10.4	33.9	40.8
P15	2.2	297.6	0.0	17.9	21	28	30	25	8.0	7.3	5.18	4.40	99.2	92.7	19.7	19.0	24.1	33.2
P16	2.2	297.6	0.0	17.9	21	28	31	27	7.9	7.3	5.30	4.60	102.1	98.0	11.3	11.7	36.6	43.5
P17	2.2	297.6	0.0	17.9	20	28	32	27	8.0	7.8	5.19	4.72	98.6	100.4	1.4	na	39.2	37.9
P18	2.2	297.6	0.0	17.9	20	28	32	27	8.1	7.1	5.25	4.61	99.8	98.1	1.4	1.6	43.1	34.5

[a] before sampling

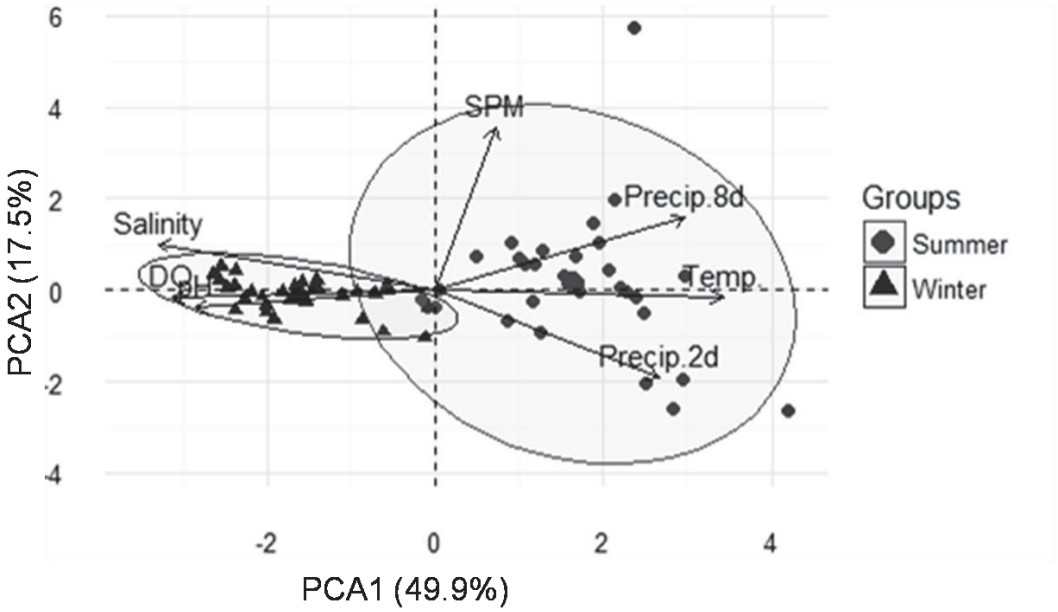

Fig. S1: Principal Component Analysis (PCA) based on water column parameters and precipitation data of two sampling campaigns (winter and summer). DO = dissolved oxygen; SPM = suspended particulate matter; Precip.8d = total precipitation in 8 days before sampling; Precip.2d = total precipitation in 2 days before sampling; Temp. = temperature.

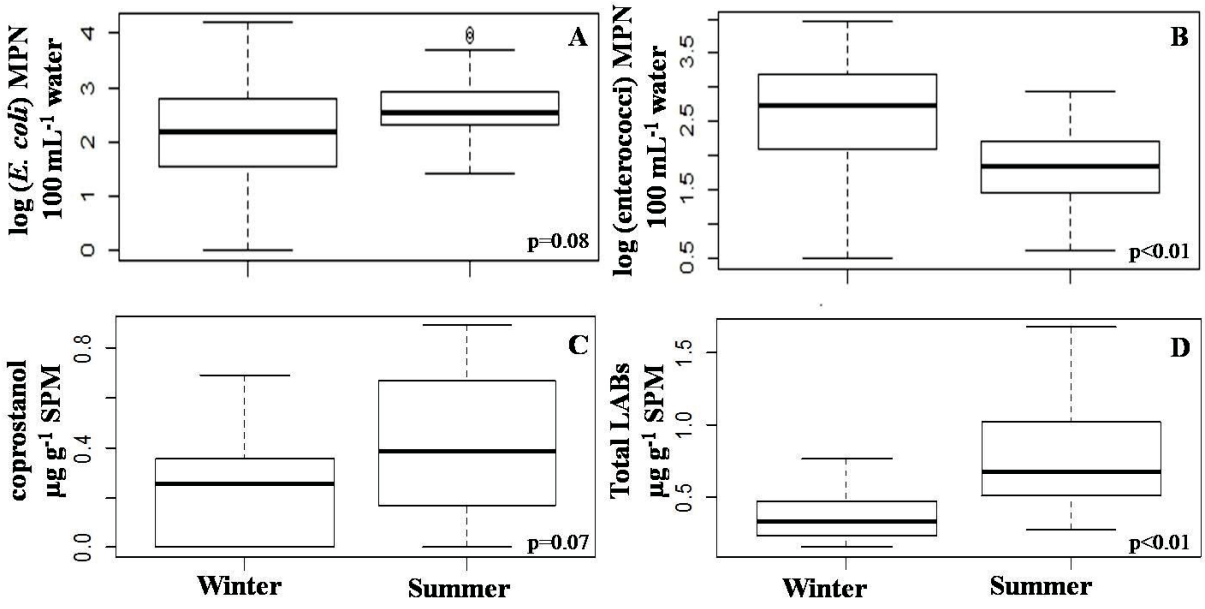

Fig. S2: Boxplot of log (*E. coli*) and log (enterococci) MPN 100 mL^{-1} water (A and B, respectively) and of coprostanol and total LABs (µg g^{-1} SPM; C and D, respectively) of two sampling campaigns (winter and summer).

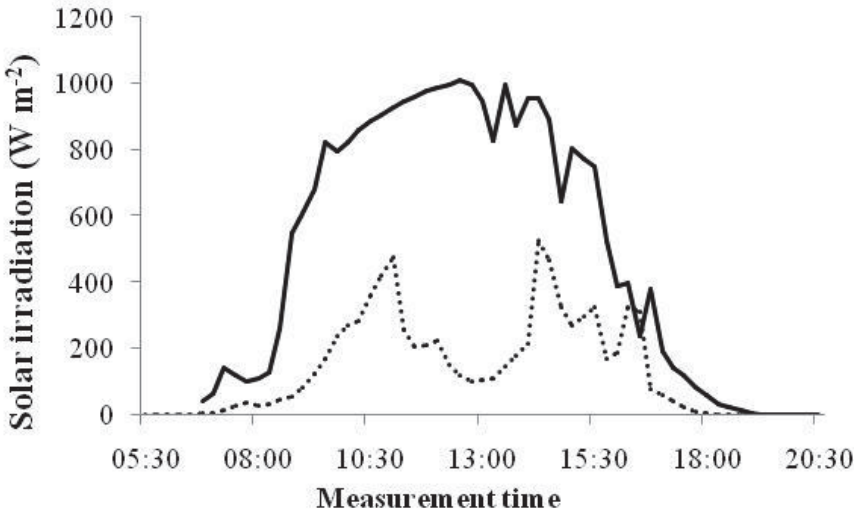

Fig. S3: Solar irradiation profiles for Brazilian South coast (Paraná State) over a period of 24 hours before winter (dashed line) and summer (continuous line) sampling.

APÊNDICE 2 - MATERIAL SUPLEMENTAR DO CAPÍTULO 3

Fontes, distribuição e degradação de marcadores moleculares de esgoto e matéria orgânica natural em sedimentos superficiais e material particulado em suspensão de um estuário subtropical sob impacto humano

Insights about sources, distribution, and degradation of sewage and biogenic molecular markers in surficial sediments and suspended particulate matter from a human-impacted subtropical estuary

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Supplementary Information

1. Sampling of physicochemical parameters of the water column

Simultaneously to geochemical samples, *in situ* data of depth, water temperature and salinity were determinate at each site using CTD profile (CastAway P/N 400313 SonTek). Water samples were also collected for in lab determination of pH (Denver UP-25) and dissolved oxygen (DO - titration method using an automatic titrator - Metrohm 702SM Titrino).

2. Instrumental analysis of chemical markers

The procedures were based on described in Cabral et al. (submitted), as below.

For LABs, it was injected 1.0 µL and 2.0 µL of SPM and sediments extracts, respectively. It was used an Agilent 19091J-433 capillary fused silica column coated with 5% diphenyldimethylsiloxane (30 m, 0.25 mm ID, 0.25 µm film thickness). The oven temperature was programmed from 40 to 60 °C at 20 °C min^{-1}, then to 290 °C at 5 °C min^{-1}, and to 300 °C at 5 °C min^{-1}. The injection temperature was adjusted to 280 ºC and splitless was the injection mode. Temperature of detector and ions source were adjusted to 290 °C and 180 °C, respectively. SIM (System Ion Monitoring) mode and the HP Enhanced Chemstation G1701CA were used for data acquisition and quantification, respectively. Compounds were identified by ion mass fragments (m/z 91, 92 and 105) and by matching the retention times

with a mixture of all of the n-C_m-LABs (m = 10 – 13) provided by Deten Química S.A. (LABs Mix Lot LPS 0025/08). The n-C_{14}-LABs were identified by ion mass fragments and estimated retention index based on general literature. For quantification, the area of the main fragment of each isomer was multiplicated by its response factor, at the calibration curve, regarding to ratio mass/area of the surrogate pattern added in each sample. Calibration was based on an external standard solution containing 1-C_m-LABs (m = 10 - 13) at six different concentrations (0,125; 0,25; 0,50; 0,75; 1,00; 1,25 e 1,50 ng μL^{-1}; $r^2 > 0.995$).

For sterols, it was injected 1.0 µL and 2.0 µL of SPM and sediments extracts, respectively. It was used an Agilent 19091J-015 capillary fused silica column coated with 5% phenylmethylsiloxane (50 m, 0.32 mm ID and 0.17 µm film thickness). The oven temperature was programmed from 40 to 240 °C at 5 °C min^{-1}, then to 250 °C at 0.25 °C min^{-1} (holding for 5 min), then to 280 °C at 5 °C min^{-1} and to 300 °C at 20 °C min^{-1} (holding for 8 min). Splitless was the injection mode. The HP Enhanced Chemstation G2070BA program was used to perform the measurements by the integration of the compounds peaks. Calibration was based on external standard mixtures of sterols (coprostanol, epicoprostanol, cholesterol, cholestanol, campesterol, stigmasterol, sitosterol and dinosterol) at nine different concentrations (0.25, 0.50, 0.75, 1.00, 2.50, 5.00, 7.50, 10.0 and 15.0 ng μL^{-1}; $r^2 > 0.995$). Compounds were identified by matching retention times with results from standard mixtures of sterols above. For the sample quantification, it was considered the peak area of each compound multiplied by its own response factor, present in calibration curve, in relation to the mass/area ratio of surrogate standards added before extraction.

3. Detection limits of geochemical markers

Detection limit (DL) of SPM samples ranges from 40.0 to 80.0 ng g^{-1} SPM for LABs and from 0.20 to 0.40 µg g^{-1} SPM for sterols (regarding to the lowest concentration of LABs – 0.02 ng μL^{-1} – and sterols – 0.10 ng μL^{-1} –multiplied by the final volume extracted – 250 µL – and divided by the SPM mass (g) of each sample). DL of sediment samples ranges from 0.002 to 0.009 µg g^{-1} for sterols and the average of LABs DL was 0.21 ng g^{-1}. The average of 1-C_{12}-LABs recover was 66.7 (± 12.6) and 56.7% (± 8.4) for SPM and sediment, respectively.

References

Cabral, A.C.; Stark, J.S.; Kolm, H.E.; Martins, C.C., 2018. An integrated evaluation of some faecal indicator bacteria (FIB) and chemical markers as potential tools for monitoring sewage contamination in subtropical estuaries. Environmental Pollution 235, 739–749.

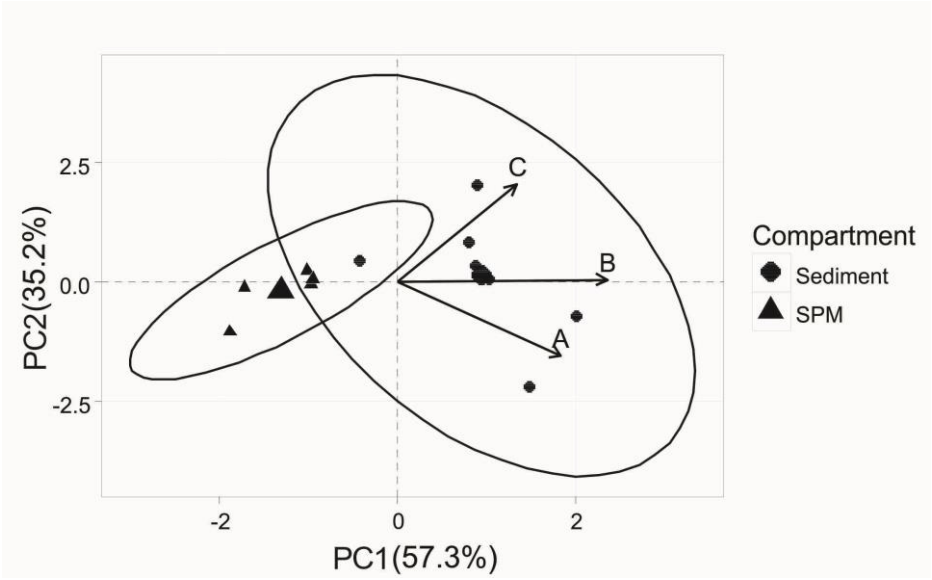


Fig. S1: Principal Component Analysis (PCA) based on the degradation ratio of LABs (A) I/E of C_{13}-LABs and 5α(H)-stanols/Δ^5-stenols, (B) 27 Δ^0: 27 Δ^5, and (C) 29 Δ^0: 29 Δ^5.

Table S1 - Concentrations of the molecular markers sterols (µg g^{-1} SPM) and LABs (ng g^{-1} SPM) in suspended particulate matter (SPM) from Paranaguá Bay, Southern Brazil. < DL = lower than detection limits; sd = standard deviation; % source sterols regarding total sterols; nc = not calculated; [1]fecal sterols; [2]marine sterols; [3]terrestrial sterols; [4]diagenetic sterols.

Sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Average ± sd
Sterols																
DL (µg g^{-1} SPM)	0.36	0.32	0.26	0.21	0.33	0.20	0.40	0.28	0.36	0.35	0.35	0.26	0.32	0.33	0.39	
Coprostanol[1]	2.33	< DL	2.00	1.63	2.42	2.24	1.99	2.67	0.85	< DL	0.86	1.78	1.34	0.91	< DL	1.40 ± 0.92
Epicoprostanol[1]	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	nc
$27\Delta^{5,22E}$ [2]	13.2	9.38	7.31	6.68	12.5	10.7	12.0	7.98	18.7	12.1	14.6	20.3	31.5	22.1	21.3	14.7 ± 6.85
$27\Delta^{22E}$ [4]	2.02	6.10	1.72	1.52	1.66	1.96	2.26	2.16	2.31	1.84	2.41	2.48	1.56	2.21	2.55	2.32 ± 1.10
$27\Delta^{5}$ [2]	43.9	43.8	29.9	25.0	43.2	41.6	47.7	27.2	56.7	34.4	46.5	56.4	65.8	60.7	49.0	44.8 ± 12.1
$27\Delta^{0}$ [4]	2.94	4.44	3.65	3.13	3.60	4.17	4.67	4.92	4.69	3.50	3.76	4.65	3.88	4.47	4.05	4.03 ± 0.60
$28\Delta^{5,22E}$ [2]	24.9	26.0	13.7	12.8	21.1	19.3	14.4	9.79	19.7	13.5	18.9	20.6	25.4	21.2	24.5	19.1 ± 5.14
$28\Delta^{22E}$ [4]	3.25	< DL	2.30	3.05	2.99	3.14	3.68	3.88	3.04	2.25	3.64	4.89	3.64	3.11	5.80	3.24 ± 1.27
$28\Delta^{5}$ [3]	16.3	17.6	14.2	12.9	16.2	20.7	13.0	9.89	12.2	8.93	13.1	20.4	24.1	16.7	10.7	15.1 ± 4.30
$28\Delta^{0}$ [4]	3.62	< DL	3.65	2.52	4.01	3.18	5.05	4.93	3.06	2.74	< DL	2.86	2.56	2.89	< DL	2.74 ± 1.61
$29\Delta^{5,22E}$ [3]	5.23	8.36	6.22	3.51	5.55	4.02	8.86	3.16	8.93	5.93	7.59	5.74	6.27	7.94	8.64	6.40 ± 1.93
$29\Delta^{22E}$ [4]	2.42	< DL	2.89	1.34	2.95	1.97	3.21	2.45	2.96	< DL	< DL	2.63	1.61	2.09	< DL	1.77 ± 1.21
$29\Delta^{5}$ [3]	20.1	16.8	19.0	16.2	19.6	26.4	15.1	11.9	18.6	9.49	20.3	22.4	21.3	18.2	20.4	18.4 ± 4.15
$29\Delta^{0}$ [4]	4.94	< DL	7.94	5.88	8.63	8.55	6.45	5.96	4.84	2.40	5.08	6.14	3.37	5.48	4.50	5.34 ± 2.28
$30\Delta^{22}$ [2]	8.70	10.6	7.63	6.74	9.54	10.3	6.60	8.96	7.45	6.10	9.70	9.53	5.87	7.66	11.4	8.45 ± 1.73
Fecal sterols	2.33	< DL	2.00	1.63	2.42	2.24	1.99	2.67	0.85	< DL	0.86	1.78	1.34	0.91	< DL	1.40 ± 0.92
Marine sterols	90.7	89.8	58.5	51.2	86.3	81.9	80.7	53.9	102.6	66.1	89.7	106.8	128.6	111.7	106.2	87.0 ± 22.4
Terrestrial sterols	41.6	42.8	39.4	32.6	41.4	51.1	37.0	25.0	39.7	24.4	41.0	48.5	51.7	42.8	39.7	39.9 ± 7.97
Diagenetic sterols	19.2	10.5	22.2	17.4	23.8	23.0	25.3	24.3	20.9	12.7	14.9	23.7	16.6	20.3	16.9	19.5 ± 4.48
Total sterols	153.9	143.1	122.1	102.9	154.0	158.2	145.0	105.9	164.0	103.2	146.4	180.8	198.2	175.7	162.8	147.8 ± 28.7
% Fecal sterols	1.51	0.00	1.64	1.58	1.57	1.42	1.37	2.52	0.52	< DL	0.59	0.98	0.68	0.52	0.00	0.99 ± 0.74
% Marine sterols	59.0	62.8	47.9	49.8	56.1	51.8	55.7	51.0	62.5	64.1	61.3	59.1	64.9	63.6	65.2	58.3 ± 5.90
% Terrestrial sterols	27.1	29.9	32.3	31.7	26.9	32.3	25.5	23.6	24.2	23.6	28.0	26.9	26.1	24.4	24.4	27.1 ± 3.11
% Diagenetic sterols	12.5	7.4	18.1	17.0	15.5	14.5	17.5	23.0	12.7	12.3	10.2	13.1	8.39	11.5	10.4	13.6 ± 4.10

Table S1 – Continuation.

Sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Average ± sd
LABs																
DL (ng g^{-1} SPM)	71.0	64.0	53.0	42.0	65.0	40.0	80.0	57.0	71.0	70.0	71.0	52.0	65.0	66.0	78.0	
Σn-C_{10}-LABs	99.7	81.0	66.5	59.1	97.7	< DL	< DL	< DL	170.0	< DL	< DL	61.8	< DL	213.0	243.0	72.8 ± 80.9
Σn-C_{11}-LABs	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	nc
Σn-C_{12}-LABs	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	nc
Σn-C_{13}-LABs	248.0	207.0	128.0	91.5	195.0	43.8	192.0	177.0	88.1	120.0	123.0	70.6	480.0	101.0	165.0	162.0 ± 104.7
Σn-C_{14}-LABs	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	nc
Total LABs	347.0	288.0	194.0	151.0	293.0	43.8	192.0	177.0	258.0	120.0	123.0	132.0	480.0	314.0	408.0	234.8 ± 120.2

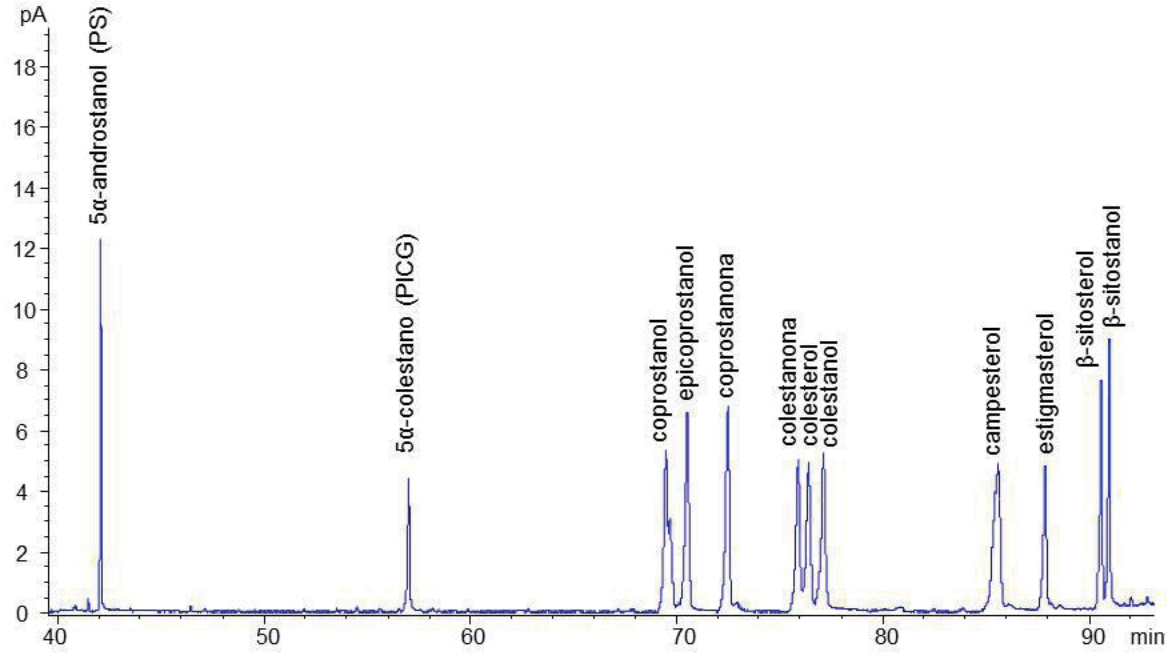
Table S2 - Concentrations of the molecular markers sterols (μg g^{-1}) and LABs (ng g^{-1}) dry sediment and the bulk parameters total nitrogen (TN; %), total organic carbon (TOC; %), and total sulphur (TS; %) in sediments from Paranaguá Bay, Southern Brazil. < DL = lower than detection limits; sd = standard deviation; % source sterols regarding total sterols; nc = not calculated; [1]fecal sterols; [2]marine sterols; [3]terrestrial sterols; [4]diagenetic sterols.

Sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Average ± sd
Sterols																
Coprostanol[1]	0.46	0.71	0.94	0.19	0.38	0.49	0.82	0.20	0.21	0.02	0.03	0.05	0.03	< DL	0.04	0.30 ± 0.32
Epicoprostanol[1]	< DL	0.10	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	0.01 ± 0.03
$27\Delta^{5,22E}$ [2]	0.16	0.66	0.35	0.06	0.10	0.97	0.34	0.50	0.41	0.09	0.25	0.08	0.14	0.03	0.16	0.29 ± 0.26
$27\Delta^{22E}$ [4]	0.43	0.43	< DL	0.08	< DL	0.53	0.21	0.17	0.20	0.03	0.06	0.12	0.07	0.02	0.17	0.17 ± 0.17
$27\Delta^{5}$ [2]	2.76	5.31	6.93	0.78	1.35	5.76	2.79	4.32	2.46	0.20	0.30	0.90	0.46	0.11	0.50	2.33 ± 2.27
$27\Delta^{0}$ [4]	1.09	1.92	3.50	0.23	0.70	2.20	0.87	0.74	0.68	0.05	0.11	0.33	0.15	0.02	0.19	0.85 ± 0.98
$28\Delta^{5,22E}$ [2]	< DL	3.37	2.72	0.28	0.84	3.93	1.94	1.34	1.52	0.32	0.53	0.14	0.29	0.05	0.35	1.17 ± 1.28
$28\Delta^{22E}$ [4]	< DL	1.92	2.55	0.37	< DL	2.40	1.04	0.70	0.82	0.05	0.23	0.38	0.15	< DL	0.31	0.73 ± 0.87
$28\Delta^{5}$ [3]	1.99	2.85	4.91	0.51	1.25	3.60	2.02	1.35	1.38	0.13	0.26	0.34	0.12	0.06	0.22	1.40 ± 1.46
$28\Delta^{0}$ [4]	2.97	2.52	4.10	0.25	< DL	2.66	1.56	0.77	0.79	0.06	0.10	0.43	0.08	< DL	0.07	1.09 ± 1.34
$29\Delta^{5,22E}$ [3]	4.58	2.07	3.81	0.70	1.18	2.41	3.27	0.67	1.42	0.15	0.37	0.37	0.10	0.03	0.32	1.43 ± 1.47
$29\Delta^{22E}$ [4]	1.16	1.78	2.59	2.13	0.82	2.37	1.29	0.41	1.21	0.03	0.07	0.18	0.08	< DL	0.21	0.96 ± 0.92
$29\Delta^{5}$ [3]	6.61	7.80	11.1	1.61	2.95	7.71	5.99	2.43	3.41	0.26	0.31	0.58	0.33	0.07	0.43	3.44 ± 3.54
$29\Delta^{0}$ [4]	3.51	4.63	5.14	1.61	1.87	5.05	1.64	1.28	1.66	0.11	0.15	1.77	0.24	0.03	0.27	1.93 ± 1.82
$30\Delta^{22}$ [2]	1.03	3.40	2.12	0.55	0.78	3.22	1.04	0.64	1.06	0.08	0.08	0.10	0.14	0.04	0.28	0.97 ± 1.10
Fecal sterols	0.46	0.81	0.94	0.19	0.38	0.49	0.82	0.20	0.21	0.02	0.03	0.05	0.03	< DL	0.06	0.31 ± 0.32
Marine sterols	3.95	12.7	12.1	1.67	3.07	13.9	6.11	6.80	5.45	0.69	1.16	1.22	1.03	0.23	1.29	4.76 ± 4.70
Terrestrial sterols	13.2	12.7	19.8	2.82	5.38	13.7	11.3	4.45	6.21	0.54	0.94	1.29	0.55	0.16	0.97	6.27 ± 6.29
Diagenetic sterols	9.16	13.2	17.9	4.67	3.39	15.2	6.61	4.07	5.36	0.33	0.72	3.21	0.77	0.07	1.22	5.72 ± 5.69
Total sterols	26.8	39.5	50.8	9.35	12.2	43.3	24.8	15.5	17.2	1.58	2.85	5.77	2.38	0.46	3.54	17.1 ± 16.5
% Fecal sterols	1.72	2.05	1.85	2.03	3.11	1.13	3.30	1.29	1.22	1.27	1.05	0.87	1.26	0.00	1.69	1.59 ± 0.83
% Marine sterols	14.8	32.3	23.9	17.9	25.1	32.1	24.6	43.8	31.6	43.7	40.7	21.1	43.3	50.0	36.4	32.1 ± 10.71
% Terrestrial sterols	49.3	32.2	39.1	30.2	44.0	31.7	45.5	28.7	36.0	34.2	33.0	22.4	23.1	34.8	27.4	34.1 ± 7.77
% Diagenetic sterols	34.2	33.4	35.2	50.0	27.7	35.1	26.6	26.2	31.1	20.9	25.3	55.6	32.4	15.2	34.5	32.2 ± 10.2

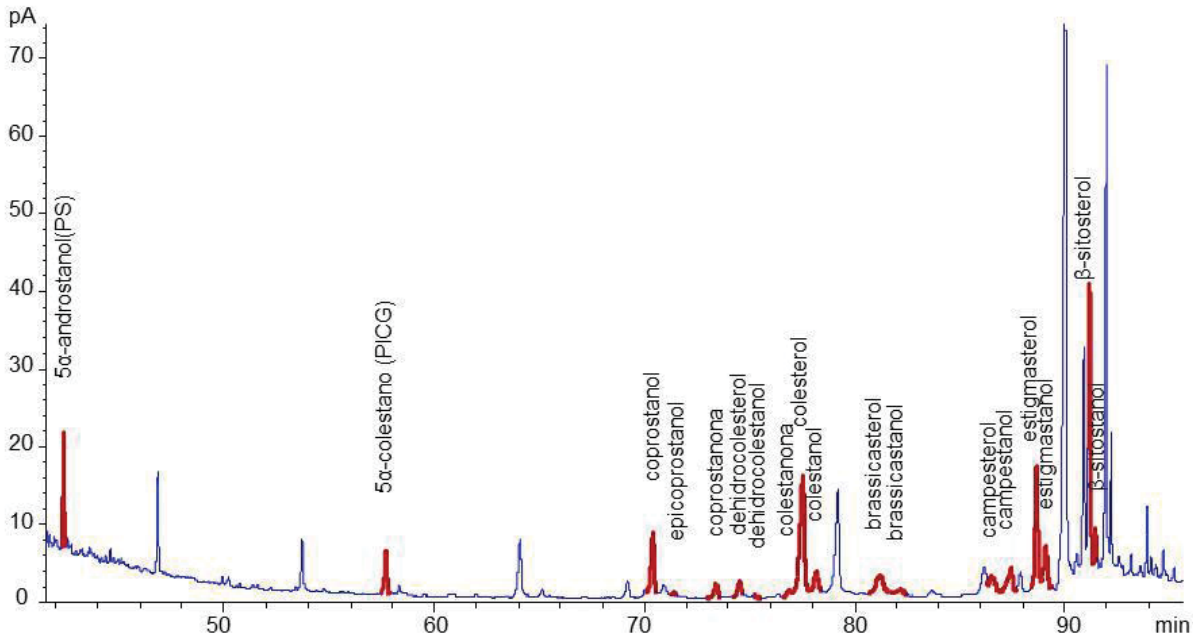
Table S2 – Continuation.

Sites	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Average ± sd
LABs																
Σn-C_{10}-LABs	0.41	1.29	0.40	0.24	< DL	1.60	0.59	0.21	0.00	< DL	< DL	< DL	< DL	< DL	< DL	0.32 ± 0.50
Σn-C_{11}-LABs	1.06	2.34	1.05	0.54	< DL	4.20	1.85	< DL	0.48	< DL	0.22	< DL	0.29	< DL	< DL	0.80 ± 1.19
Σn-C_{12}-LABs	1.50	2.84	1.45	0.44	< DL	5.61	3.12	0.27	1.81	< DL	0.75	< DL	1.42	< DL	< DL	1.28 ± 1.58
Σn-C_{13}-LABs	2.27	4.32	2.09	0.68	< DL	8.21	4.70	0.60	1.73	< DL	1.53	< DL	1.96	< DL	< DL	1.87 ± 2.32
Σn-C_{14}-LABs	< DL	0.65	0.26	< DL	< DL	1.35	0.27	< DL	< DL	< DL	< DL	< DL	< DL	< DL	< DL	0.17 ± 0.37
Total LABs	5.24	11.4	5.25	1.90	< DL	21.0	10.5	1.08	4.02	< DL	2.50	< DL	3.67	< DL	< DL	4.44 ± 5.86
Bulk parameters																
TN	0.28	0.32	0.44	0.16	0.21	0.34	0.16	0.12	0.07	0.03	0.04	0.07	0.06	0.00	0.27	0.17 ± 0.13
TOC	3.04	3.33	4.29	1.57	2.30	3.41	1.13	0.89	0.69	0.03	0.07	0.45	0.16	0.03	0.26	1.44 ± 1.46
TS	0.89	0.61	1.09	0.74	0.98	0.53	0.31	0.29	0.22	0.03	0.03	0.40	0.14	0.01	0.03	0.42 ± 0.37
TOC/TN	10.9	10.4	9.8	9.9	11.0	10.1	7.3	7.5	9.3	1.1	1.6	6.1	2.9	nc	1.0	7.1 ± 3.8
TOC/TS	3.4	5.5	3.9	2.1	2.3	6.4	3.7	3.1	3.1	1.1	2.6	1.1	1.2	1.8	8.1	3.3 ± 2.0

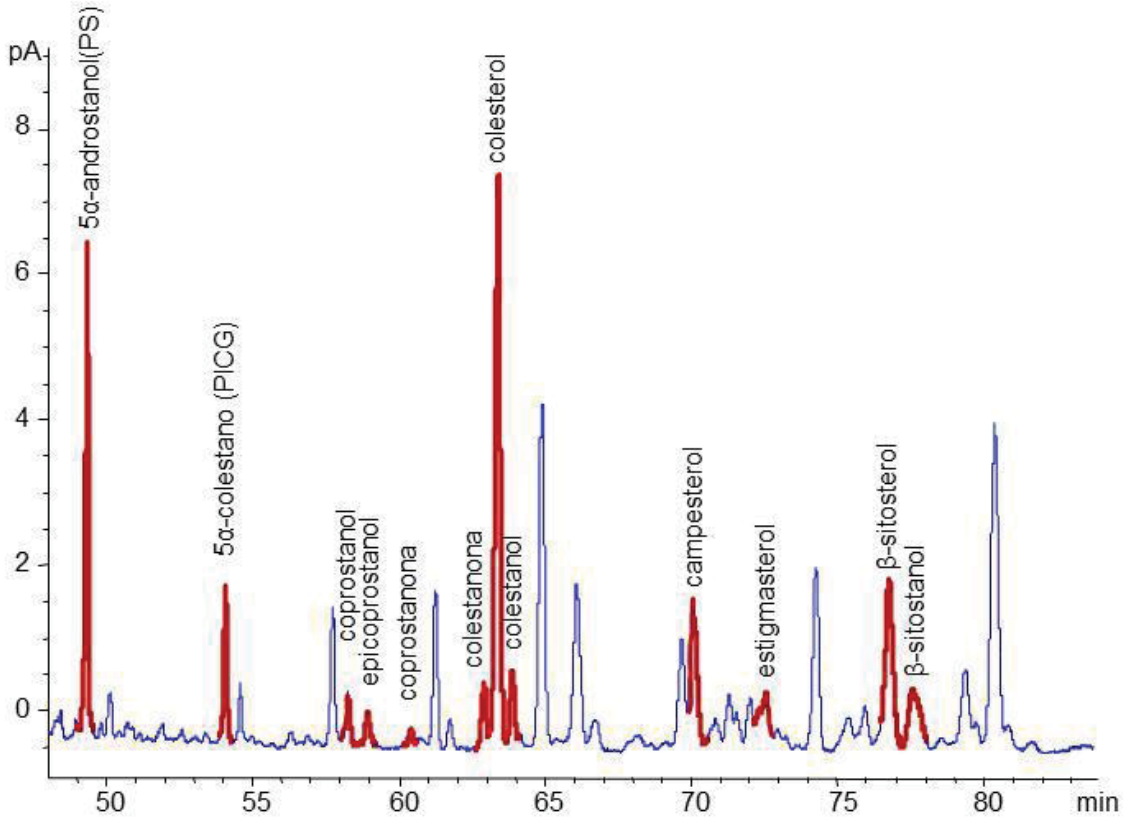
APÊNDICE 3 - CROMATOGRAMA DA CURVA DE CALIBRAÇÃO PARA ESTERÓIS (PONTO DE CONCENTRAÇÃO 5,0 NG ML^{-1}).



APÊNDICE 4 - CROMATOGRAMA DE ESTERÓIS DE UMA AMOSTRA DE MATERIAL PARTICULADO EM SUSPENSÃO DA BAÍA DE PARANAGUÁ (P10 VERÃO – CAPÍTULO 2).



APÊNDICE 5 - CROMATOGRAMA DE ESTERÓIS DE UMA AMOSTRA DE SEDIMENTO SUPERFICIAL DA BAÍA DE PARANAGUÁ (P3 – CAPÍTULO 3). EM DESTAQUE OS PRINCIPAIS ESTERÓIS DETECTADOS.



ANEXO 1 – CAPA DO ARTIGO 1 PUBLICADO

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An integrated evaluation of some faecal indicator bacteria (FIB) and chemical markers as potential tools for monitoring sewage contamination in subtropical estuaries☆

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ABSTRACT

Sewage input and the relationship between chemical markers (linear alkylbenzenes and coprostanol) and fecal indicator bacteria (FIB, *Escherichia coli* and enterococci), were evaluated in order to establish thresholds values for chemical markers in suspended particulate matter (SPM) as indicators of sewage contamination in two subtropical estuaries in South Atlantic Brazil. Both chemical markers presented no linear relationship with FIB due to high spatial microbiological variability, however, microbiological water quality was related to coprostanol values when analyzed by logistic regression, indicating that linear models may not be the best representation of the relationship between both classes of indicators. Logistic regression was performed with all data and separately for two sampling seasons, using 800 and 100 MPN 100 mL^{-1} of *E. coli* and enterococci, respectively, as the microbiological limits of sewage contamination. Threshold values of coprostanol varied depending on the FIB and season, ranging between 1.00 and 2.23 $\mu g\, g^{-1}$ SPM. The range of threshold values of coprostanol for SPM are relatively higher and more variable than those suggested in literature for sediments (0.10–0.50 $\mu g\, g^{-1}$), probably due to higher concentration of coprostanol in SPM than in sediment. Temperature may affect the relationship between microbiological indicators and coprostanol, since the threshold value of coprostanol found here was similar to tropical areas, but lower than those found during winter in temperate areas, reinforcing the idea that threshold values should be calibrated for different climatic conditions.

1. Introduction

Urban sewage is a source of contamination of major concern in estuarine systems and contributes to environmental and human health disturbances, decreasing the water quality for various human uses. Sewage input tends to become worse as populations grow due to the slower development of treatment infrastructure, and its detection is fundamental to provide warning of the need for improvements in treatment services (WHO, 2003).

Sewage collection and treatment services in Brazil are poor and inefficient, with more than 70% of cities without an efficient network of these services (IBGE, 2011). Guaratuba and Paranaguá Bays, located in the South Atlantic, Brazil, are subtropical estuarine environments which present conflicting scenarios of diverse, pristine ecosystems with economic interest through permanent anthropogenic pressure resulting from port, agriculture and tourism activities (Lana et al., 2001; Dauner and Martins, 2015). Among the sources of pollution recognized in the region, sewage is still considered problematic due to poor collection and sewage treatment, and the unregulated discharges of sewage through to drains and channels (Lana et al., 2001; Kolm et al., 2002).

Escherichia coli and enterococci are fecal indicator bacteria (FIB) recommended by research agencies developing water quality regulations as sewage indicators (CONAMA, 2000; APHA, 2009).

☆ This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

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ANEXO 2 – CAPA DO ARTIGO 2 PUBLICADO

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Insights about sources, distribution, and degradation of sewage and biogenic molecular markers in surficial sediments and suspended particulate matter from a human-impacted subtropical estuary☆

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Organic matter

ABSTRACT

The molecular markers sterols and linear alkylbenzenes (LABs) were analyzed in the surficial sediments and suspended particulate matter (SPM) of a subtropical estuary in South Atlantic (Paranaguá Estuarine System). The purpose of this study was identify the spatial distribution of sewage and the input of biogenic organic matter (OM) and to provide comparative insights about their behavior, compositions, and sources. The concentration of coprostanol ranged from < DL (detection limit) to 2.67 $\mu g\ g^{-1}$ in SPM and from < DL to 0.94 $\mu g\ g^{-1}$ in sediments. Total LABs ranged from 43.8 to 480.0 $ng\ g^{-1}$ in SPM and from < DL to 21.0 $ng\ g^{-1}$ in sediments. LABs homologs composition varied between the two matrices. The local hydrodynamic pattern may promote water column homogenization, dispersion, and dilution of sewage particles, and preferential sedimentation in fluvial and mixture zones. Results suggest that SPM is a good matrix for larger spatial and short time scale evaluation while sediments may help to define *hot spot* areas of input and final deposition of sewage particles. Marine sterols predominated in SPM while no dominance patterns of marine/terrestrial sterols occurred in surficial sediments. The higher degradation rates of sterols and LABs in the water column must be the main factor for the sharp drop in concentration towards the sediment and the variation of the preferential composition of these markers between compartments.

1. Introduction

Geochemical organic markers have been extensively used to characterize the input of organic matter (OM) in different environments and timescales and can be assigned to a specific source (Takada and Eganhouse, 1998; Derrien et al., 2017). Sterols are one of the most useful molecular markers for allochthonous (sewage and continental material) and autochthonous (zooplankton and phytoplankton) contributions (Volkman, 1986; Derrien et al., 2017). Linear alkylbenzenes (LABs) are aromatic hydrocarbons always related to anthropogenic (sewage and oil) sources (Takada and Eganhouse, 1998).

Sewage is one of the main stressor agent in coastal ecosystems, especially in estuaries where the water circulation is restricted, which hinders its dilution in the environment. The precariousness (or absence) of sanitary services in coastal regions allows the chronic input of sewage with a bulk of nutrients, pesticides, drugs, and pathogens which interferes with the natural ecosystem and public health (UNEP, 2008; Lim et al., 2017).

Because the development of sanitary systems does not keep up with accelerated increases in population density (UNEP, 2008; IBGE, 2011), the detection of environmental sewage is still the most used alternative to monitor areas susceptible to eutrophication and prevent human contact with contaminated water (e.g. WHO, 2003; CONAMA, 2000).

Because sewage is the main source of coprostanol and LABs in coastal environments, these molecular markers have been widely used in the detection of sewage input or contamination (Takada and Eganhouse, 1998; Bull et al., 2002). Coprostanol is the main product of cholesterol degradation inside the intestinal tract of homeothermic vertebrates and corresponds to about 60% of total sterols in human feces (Murtaugh and Bunch, 1967; Hatcher and McGillivary,

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ANEXO 3 – CAPA DO ARTIGO 3 PUBLICADO

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Tracking the historical sewage input in South American subtropical estuarine systems based on faecal sterols and bulk organic matter stable isotopes ($\delta^{13}C$ and $\delta^{15}N$)

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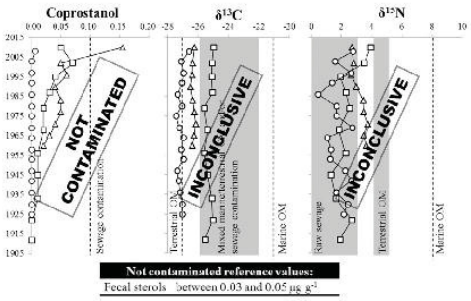
HIGHLIGHTS

- $\delta^{13}C$ and $\delta^{15}N$ provided inconclusive indications of sewage contamination.
- The sedimentary cores were classified as uncontaminated by faecal sterols analysis.
- Coprostanol values were low but can follow the urban and economic development.
- Faecal sterols reference values were established in uncontaminated sediments.

GRAPHICAL ABSTRACT



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ABSTRACT

Faecal sterols and stable isotopes ($\delta^{13}C$ and $\delta^{15}N$) from bulk organic matter (OM) were analysed in three sedimentary cores collected in two subtropical bays located in the South Atlantic to evaluate historical trends in the sewage input and to track possible changes in the bulk isotopic composition of OM in recent decades. The values of $\delta^{13}C$ and $\delta^{15}N$ ranged from −27.4 to −25.0‰ and from 0.5 to 3.9‰, respectively, without a clear trend in the variation over the whole period covered by sediment cores and with no conclusive interpretation of a specific range value typically related to the sewage input for these areas. The maximum coprostanol concentration was 0.19 μg g^{-1} in the upper 4 cm of one core, which was not considered contaminated by evaluation of the sterols diagnostic ratios. Even at low levels, the coprostanol concentrations followed variations in urban and economical regional development. Baseline values for faecal sterols (in average between 0.03 and 0.05 μg g^{-1}), which may represent a previous non-impacted environment scenarios, were calculated for use in comparative perspectives for future evaluations of the sewage input and contamination.

1. Introduction

Aquatic environment disposal of raw sewage is a historical and routine human practice that has continued due to a lack of investment in wastewater collection and treatment systems together with rapid urbanization and increases in population (Sato et al., 2013; WWAP,

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