




# Microscale Toxicity Identification Evaluation (TIE) for interstitial water of estuarine sediments affected by multiple sources of pollution

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## Abstract

Estuaries in the world are affected by different contamination sources related to urbanisation and port/industrial activities. Identifying the substances responsible for the environmental toxicity in estuaries is challenging due to the multitude of stressors, both natural and anthropogenic. The Toxicity Identification and Evaluation (TIE) is a suitable way of determining causes of toxicity of sediments, but it poses difficulties since its application is labour intensive and time consuming. The aim of this study is to evaluate the diagnosis provided by a TIE based on microscale embryotoxicity tests with interstitial water (IW) to identify toxicants in estuarine sediments affected by multiple stressors. TIE showed toxicity due to different combinations of metals, apolar organic compounds, ammonia and sulphides, depending on the contamination source closest to the sampling station. The microscale TIE was able to discern different toxicants on sites subject to different contamination sources. There is good agreement between the results indicated in the TIE and the chemical analyses in whole sediment, although there are some disagreements, either due to the sensitivity of the test used, or due to the particularities of the use of interstitial water to assess the sediment toxicity. The improvement of TIE methods focused on identifying toxicants in multiple-stressed estuarine areas are crucial to discern contamination sources and subsidise management strategies.

**Keywords** Toxicity identification and evaluation · Effect-directed analysis · Embryo-larval development · Porewater · Toxicity test · Pollution · Contamination · Ecotoxicology

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## Introduction

Estuaries worldwide are affected by multiple stressors as a consequence of population growth and anthropogenic activities, such as urbanisation, food harvesting, tourism, recreation, industrial and port settlements (Kennish 2005; Halpern et al. 2012). Physical and chemical characteristics of these environments, such as high quantities of suspended particulate matter, high concentrations of dissolved organic matter, predominantly fine bed sediments and increased salinity compared to freshwaters, favor deposition and accumulation of contaminants in the sediments (Chapman and Wang 2001; Eggleton and Thomas 2004), which often lead to negative effects to the biota.

Apart from being an environmental issue, sediment quality is also economically relevant especially for the industrial and port sector. This is because of, for example, the legal imposition of monitoring the environmental quality of water bodies, and the need for dredging material characterisation for environmental remediation or the installation and maintenance of ports and waterways. Although assessing environmental quality in estuaries is not an easy task due to natural confounding factors, contaminants synergies (Beyer et al. 2014) and interactions between contaminants and environmental conditions (Choueri et al. 2009; Gusso-Choueri et al. 2015), even more complex is to establish causality between exposure and effects.

This challenge, however, is important to be addressed by scientists and environmental managers. It not only provides accuracy in the development of Sediment Quality Guidelines (by detecting false-positives), it also supports environmental forensics for the identification of sources of pollution, assists in the establishment of priority pollutants for remediation efforts and allows the identification of confounding factors (i.e. when naturally occurring substances such as non-ionised ammonia and sulphides lead to a false-positive decision error about sediment toxicity) (Phillips et al. 1997; Anderson et al. 2001; Pusceddu et al. 2007; USEPA 2007a; Ho and Burgess 2013).

The Toxicity Identification and Evaluation (TIE) approach is a kind of effect-directed analysis, i.e. a sequence of analysis targeted to identify substances causing toxicity in complex sample matrices. By means of a combination of toxicity tests and physical and chemical manipulations of the samples, the TIE allows a progressive narrowing of research focus on the substance suspected to cause toxicity. TIE techniques were designed to, as much as possible, preserve the linkages between the original test sample and the observed toxicity (Ankley et al. 2011). Therefore, although initially developed for application in industrial effluents, the TIE approach has evolved to be applied in the identification of substances responsible for toxicity in environmental samples worldwide, including marine sediments (Ho and Burgess 2013; Montero

et al. 2013; Camargo et al. 2014; Poleza et al. 2014; Greenstein et al. 2014; Ferraz et al. 2017; Moreira et al. 2019; Campos et al. 2019; Cruz et al. 2019). The development or adaptation of TIE frameworks for routine monitoring in estuaries and other coastal environments involves the utilisation of rapid, cost-effective ecotoxicological tests and sample manipulations aimed to assess the toxicity of the main substances potentially causing toxicity in sediments from multi-stressed estuaries, including common confounding factors for sediment toxicity in these environments.

The choice of the matrix to be used in the TIE (e.g. whole sediment, interstitial water, water column) must reflect the scientific questions to be addressed, including the sediment: water partitioning of potential pollutants and ways of exposure of the environmental receptors of interest. Furthermore, they have different characteristics regarding the logistics involved in conducting the tests and the type of information collected (Di Toro et al. 1991). Interstitial water (IW) is the main route of exposure to contaminants for many organisms, and toxicity tests focused on IW are able to eliminate or distinguish the effects caused by factors unrelated to contamination (e.g. sediment particle size), providing information regarding the bioavailability of chemical contamination in the site (Chapman et al. 2002). In addition, the use of IW in toxicity tests is advantageous since it is relatively simple to conduct, and may be based on water column toxicity tests (Mehler et al. 2010). On the other hand, IW may not be the main way of exposure to contaminants for many organisms, such as sediment-ingesting organisms (Hoss et al. 2001; Hoss et al. 2011; Ferraz et al. 2020). The use of this environmental matrix alone in toxicity tests may lack correspondence with the toxicity in the exposure scenario (field conditions) due to, for example, loss of volatile and semivolatile contaminants (solid phases can buffer this process), sorption to the test chamber walls of organic chemicals with moderate to high octanol:water partition coefficient ( $K_{ow}$ ) and changes in the concentration or bioavailability of potentially toxic substances due to IW oxidation after sampling, extraction and testing (Chapman et al. 2002; Ho and Burgess 2008). Thus, IW tests and analysis can be effective tools, as long as their limitations are well understood by researchers and managers (Chapman et al. 2002).

TIE frameworks are notably time-consuming and labour-intensive due to the large number of sample manipulations and ecotoxicological trials. One of the greatest difficulties related to TIE focused on IW is the demand for large quantities of interstitial water (IW) (USEPA 2007b; Macken et al. 2009; Ferraz et al. 2017). Therefore, the development and application of interstitial water TIE at small scales (hereafter referred to as microscale TIE) is important to provide feasibility for the routine use for management purposes.

The sea urchin embryolarval development test is a reliable, sensible and inexpensive tool to assess toxicity of chemicals

and environmental samples (Marroni et al. 2016; Bonaventura et al. 2021). The test is well established by standard protocols (ASTM 1995; USEPA 1995) and is commonly used to test IW toxicity (e.g. Carr et al. 1996; Nipper et al. 2002; Losso et al. 2009). However, its use as part of a microscale TIE was tested only for sediments affected by domestic sewage (Ferraz et al. 2017). For it to be a viable alternative, the assessment of the effectiveness of the microscale TIE depends on its experimental application on sites subject to multiple sources of contamination, where contamination profiles may differ, in order to understand the limitations and potentialities of the technique.

The current study aimed to assess the suitability of a microscale TIE approach in identifying individual substances or classes of substances responsible for toxicity in IW of sediments from an estuary subjected to multiple stressors, such as urban sewage, dredging, port and a variety of industrial activities. The tested hypothesis is that this approach is able to separate the load of toxicity of the main groups of substances present in estuarine sediments under the influence of different sources of contaminants, including ammonia and sulphides, which can be natural confounding factors or a result of anthropogenic sources, depending on the environmental context. The TIE was methodologically divided into steps (e.g. manipulation of toxicity and chemical analyses), which were compared to each other for congruences and inconsistencies in order to assess the effectiveness of the microscale TIE. The current study contributes to the improvement of techniques for monitoring and assessing of environmental quality in coastal waters (especially estuaries), which are commonly subject to multiple sources of pollution.

## Materials and methods

### Study area and sediment sampling

The study area is the Santos Estuarine System (SES), Southeastern Brazil, located at 23°30'5" and 46°05' at 46°30". It occupies the central portion of the coast of the State of São Paulo and stands out due to its economic and environmental importance. This coastal environment is subject to multiple sources of contamination of different natures, such as steel, chemical, petrochemical and fertiliser industries; navigation and port activities; and domestic/sanitary effluents (Cesar et al. 2006; Sousa et al. 2014; Abessa et al. 2019). Contaminants at concentrations above water and sediment environmental quality standards (e.g. CCME 2001; Choueri et al. 2009; Brasil 2012) or related to toxicity effects have been reported in previous works for different contaminants, such as metals, organic compounds, sulphides, ammonia, surfactants, pharmaceutical compounds and even illicit drugs (Cesar et al. 2007; Choueri et al. 2009; Cortez et al. 2012; CETESB 2013; Krull et al. 2014; Torres et al. 2015; Pereira et al. 2016; Ferraz

et al. 2017; Maranhão et al. 2017; Abessa et al. 2018; Perina et al. 2018; Fontes et al. 2019). Since this is an estuarine system, naturally occurring substances (such as non-ionised ammonia and sulphides) can also cause sediment toxicity, although the natural origin of these substances in anthropogenically disturbed environments is arguable (Pusceddu et al. 2007; Ferraz et al. 2017).

Sediment samples were collected at four different sampling sites of the Santos Estuarine System (Fig. 1). Site 1 (S1), located at the Bertioga Channel (23°51'28" S–46°09'13" W), is less influenced by sources of anthropogenic contamination. Site 2 (S2), located in the Santos Bay, is at the vicinities of the submarine sewage outfall of Santos city (24°00'041" S–46°21'048" W) through which pre-conditioned (screened and chlorinated) urban sewage is discharged. Site 3 (S3) is located at an area inner the estuary with intense port activities related to handling of oil and by-products, chemical liquid bulk and containers (23°55'132" S–46°22'151" W). Site 4 (S4) is at the innermost portion of the estuary, comprising the final portion of the Santos port channel and being strongly affected by the industrial complex of Cubatão City (23°55'132" S–46°22'389" W).

In the field, sediment samples were collected with the aid of a Van Veen grab sampler, placed in identified plastic bags and kept cooled with ice in thermal boxes. In the laboratory, the sediments were immediately homogenised and kept at 4 °C in the dark. Aliquots of sediment were separated into plastic bags for analysis of particle size, organic matter content and concentrations of metals. The samples for the analysis of organic compounds were kept in aluminium vessels. All sediment samples for chemical analyses were kept at –20 °C and in the dark.

Before the TIE manipulations and toxicity tests, the IW was extracted by centrifugation at 2500 rpm (769×g) at 4 °C for 20 min and stored in acid-washed amber glass bottles and kept refrigerated at 4 °C for a maximum of 24 h until the beginning of the assays. TIE tests started immediately after IW collection and sediment samples were stored for a period no longer than 8 weeks (USEPA/ACOE 1998).

### TIE overview

In the current study, the TIE approach was conceptually divided into two phases (phases I and II), being the second one sub-divided in two (phase IIa and IIb). In phase I, physical and chemical manipulations of the samples were used to build an initial profile of the substances responsible for the toxicity in the sample. The manipulations were selected according to the sources of contamination in the study area, as well as based on USEPA guidelines for sediment TIE (USEPA 2007c) and previous studies on interstitial water TIE (Carr et al. 2001; Kwok et al. 2005; Picone et al. 2009). It was performed the EDTA addition test, to assess metal toxicity; the C18 test, to



**Fig. 1** Location of the sediment sampling sites in the Santos Estuarine System

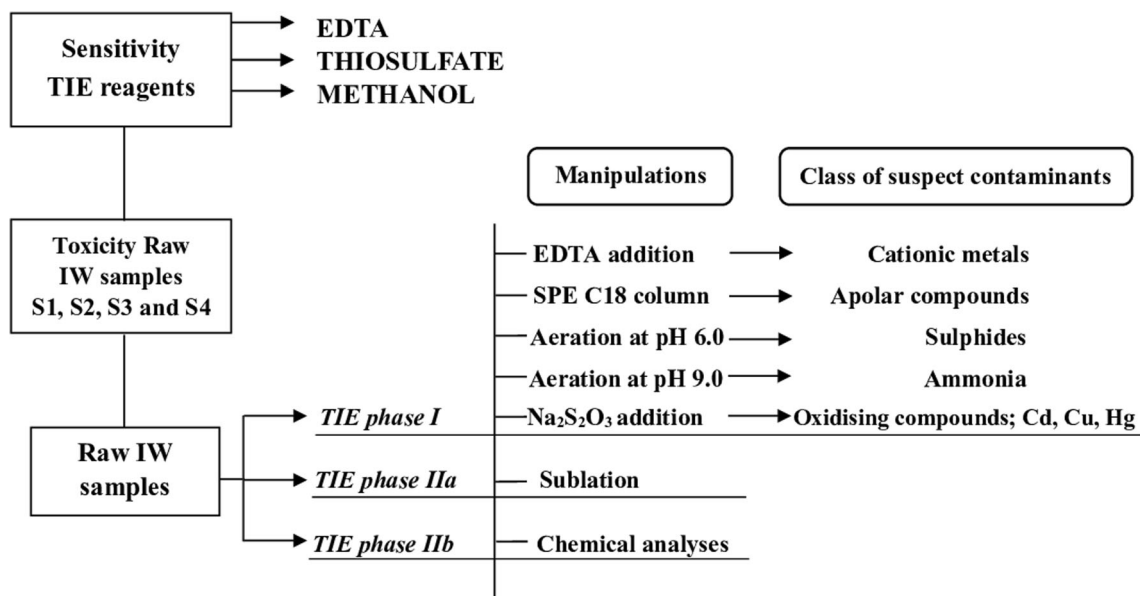
assess toxicity of apolar compounds (PAHs, PCBs, and some pesticides, for example); manipulations involving pH graduations followed by aeration, in order to remove substances whose volatility strongly varies with pH (e.g. ammonia and sulphides); and the sodium thiosulfate addition test, aiming to reduce the toxicity of oxidising compounds as well as some cationic metals. Phases I and II of the TIE are shown in Fig. 2.

In phase IIa and IIb, the analysis is narrowed on a specific substance (or class of substances) based on the outcomes of the phase I, in order to detail the identification of substances responsible for the observed toxicity. In phase IIa, the sublation test was used, aimed at the toxicity of surfactants. Phase IIb (identification) relied on chemical analyses of the IW or whole sediment samples and comparison with sediment quality guidelines (SQGs) (both international SQGs and locally derived SQGs), with the aim of assessing the congruence between the toxicity manipulations measured by the microscale toxicity tests and concentrations of potentially toxic

substances to provide an integrated diagnosis of culprit substances for IW toxicity.

It is important to consider that some manipulations aimed at manipulating the toxicity of a certain class of substances can also interfere in the toxicity of another class of chemicals. For example, column C18 can also remove metals from the sample (USEPA 1992). In these cases, it is important to compare the results of the C18 test with tests based on metal chelation, in addition to the joint analysis of the chemical concentrations of metals and apolar organic substances.

In phases I and IIa, the identification of the substances causing toxicity is based on the difference of toxicities between manipulated and non-manipulated samples (hereafter referred as “baseline toxicity”) (phase I) or blank reagents (phase IIa). The treatments for the toxicity tests (both baseline and after-manipulation tests) were a dilution series of IW samples (ranging from 100 to 1.56% of IW) with dilution water (reconstituted water prepared in the laboratory using Pro Coral



**Fig. 2** Toxicity identification evaluation (TIE) approach with phase I and II manipulations for each class of suspect contaminants. *IW* interstitial water

RedSea® sea salt added to distilled water to salinity 30). For all TIE toxicity tests, a control of the dilution water and a TIE-manipulation control (dilution water submitted to the same manipulations and dilution series as for IW samples) were done concomitantly with the toxicity tests. Before the application of the TIE, the toxicity of the reagents used to manipulate the samples were previously tested to determine the concentrations to be used in the TIE manipulations.

### Toxicity assays

Toxicity assays with sea urchin embryos *Lytechinus variegatus* or *Echinometra lucunter* were used in the TIE with sediment IW following the ABNT protocol (NBR 15350/2012) (ABNT 2012) with adaptations (Ferraz et al. 2017) for the use of a small sample volume (2.5 mL) in 24-well microplates.

The organisms were collected in the field by free diving and acclimatized in the laboratory until the tests were carried out. Sea urchins were stimulated by the application of 35 V electrical pulses for the release of the gametes. Sperm was collected directly from the gonopores with the aid of a Pasteur pipette and accommodated in a small glass beaker kept surrounded by ice. Unfertilised eggs were directly released in a 250 mL glass beaker containing dilution water. For the fertilisation, 1 to 2 mL of the sperm suspension (1 mL of sperm and 24 mL of dilution water) were added to the container with unfertilised eggs, maintaining gentle but constant agitation to promote fertilisation.

Fertilisation ratio was checked under microscope (400×) (less than 90% of fertilisation is unsatisfactory) and the density of zygotes (units per volume) was estimated to allow the addition of 300 units into each microplate well containing 2.5

mL of the test solution (adapted from Nilin et al. 2008, which successfully tested with 500 zygotes/2.5 mL). During the exposure period, the microplates were kept in an incubator chamber, with a temperature of  $25 \pm 2$  °C and controlled photoperiod (16 h/8 h-light/dark).

At the end of the exposure time (24 h to 28 h for *L. variegatus* and 36 h to 42 h for *E. lucunter*), the organisms were fixed with 0.5 mL of buffered formaldehyde (40%) to pH 7.0 with 10% borax. With the aid of a Sedgewick-Rafter counting chamber, the ratio of abnormal development (individuals with delayed development or morphological anomalies) was estimated for the first 100 organisms observed under optical microscope (400×). The test was deemed valid only in cases when more than 80% of normal development was observed in the negative control treatment (dilution water) after the exposure time. The sensitivity of each batch of organisms was tested with a toxic reference substance ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  at concentrations ranging from 0.02 to 0.22 mg L<sup>-1</sup>) and the results were compared to a control chart kept by the laboratory to assess the reliability of the ecotoxicological data produced. For all tests, respective controls (dilution water and/or TIE-manipulation controls) were performed. Four replicates were done for each dilution treatment and controls.

Physico-chemical variables (salinity, pH, temperature, and dissolved oxygen) were analysed at the beginning and at the end of each test to verify that the test conditions remained within acceptable levels for the organism. Salinity was measured with the aid of an induction refractometer (Shibuya, model I45), pH was analysed by direct measurement using a pH-specific glass electrode (Micronal, model B474), dissolved oxygen was measured through an electronic oximeter (WTW, model OXI 315i). The equipment used for each analysis was calibrated by the *Rede Brasileira de Calibração*



(Brazilian Calibration Network) in accordance with the requirements of the *Instituto Nacional de Metrologia* (National Institute of Metrology).

### Sensitivity to TIE reagents

In the current study, ecotoxicological assays to assess the sensitivity of the organisms to test reagents were performed in 3 trials for each reagent. The results were used to set the concentrations for the TIE manipulations. The tested concentration ranges were 12.5 to 200 mg L<sup>-1</sup> for EDTA, 3.1 to 50 mL L<sup>-1</sup> for methanol and 0.2 to 3.2 g L<sup>-1</sup> of sodium thiosulfate.

### TIE phase I—initial toxicity identification profile

In phase I, a reduction in toxicity is expected after a given manipulation if the respective targeted class of substances plays a significant role in the toxicity of the sample. For the removal of the toxicity of cationic metals, 40 mg L<sup>-1</sup> of EDTA was added to the sample (EDTA addition test). Interaction between the chelator and metals was allowed to occur for 3 h in the dark (to avoid photochemical degradation of EDTA). The C18 test, in turn, is designed to remove from the samples substances with high affinity for the octadecyl resin, such as apolar organic compounds (PAHs, PCBs, and some pesticides). The Octadecyl I solid phase extraction (SPE) C18 6 mL-column (Bakerbond®) was used in the current study. Initially, the column was activated with 10 mL of methanol and then cleaned with 10 mL of distilled water (USEPA 1991). Then, 15 mL of filtered dilution water was passed through the column and kept to be used as the manipulation control in the toxicity assays (for safety, the first 5 mL were discarded to avoid the presence of any methanol still remaining in the column). Then, 20 mL of the IW sample were passed through the SPE C18 column and collected for toxicity testing.

The manipulations of pH gradations followed by aeration were used considering that potentially toxic substances in the IW samples have variable volatility with changes in pH. Regarding IW of estuarine sediments, the most probable substances to be removed by these manipulations are ammonia (higher volatility at high pH) and sulphides (higher volatility at low pH). The pH of the samples were modified by the addition of HCl (final pH: 6.0) or NaOH (final pH: 9.0) and then samples were aerated for 30 min. After aeration, the pH was adjusted to the initial value (with NaOH or HCl) and the sample was subsequently tested for its toxicity.

The sodium thiosulfate (Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>) addition test is intended to reduce the toxicity of oxidising compounds and some metals (especially Cd, Cu, and Hg) (Hockett and Mount 1996; USEPA 2007d). The equilibrium time kept for the reduction/chelation reactions to occur was 1 h.

### TIE phase IIa—sublation test

This phase aims for the identification of surfactants toxicity by means of the sublation test. Interstitial water sample (30 mL) was aerated for 30 min in a glass vessel and discarded thereafter. The vessel was then washed with methanol (20 mL) in order to recover the toxic agents that have adhered to the walls. Toxicity of the solvent was tested and if samples contained surfactants at toxic levels, the methanol used to rinse container walls should be more toxic compared to the methanol “blank” (pure methanol).

### TIE phase IIb—chemical analyses and comparison with sediment quality guidelines

Sediment grain size was characterised by wet sieving (CETESB 1995) and grain size distribution was classified according to the Wentworth scale (Wentworth 1992). The organic matter content was estimated using the loss on ignition method (Luczak et al. 1997) and carbonates content was quantified with the addition of hydrochloric acid (HCl) in the sediment sample (Hirota and Szyper 1975).

Concentrations of ammonia, sulphides, nitrate and nitrite were quantified in IW samples. Total ammonia were analysed immediately after extraction by measuring the absorbance in a UV/VIS Spectrophotometer Kasuaki with a wavelength of 640 nm (Grasshoff et al. 1983) and then the concentration of non-ionised ammonia was estimated taking into account the values of pH and temperature. Sulphides, nitrate and nitrite were quantified according to the colorimetric method described in Standard Methods for the Examination of Water and Wastewater (APHA 2005) (absorbance measured at 650 nm wavelength). Concentrations of metals, As, PAHs, PCBs, sulphides and surfactants were quantified in whole sediment samples.

Before metal determination, dry samples were acid digested in a microwave system model MARS 6 (CEM Corporation). The extraction solution was a mixture of 9 mL of HNO<sub>3</sub> and 3 mL of HCl (3:1) (3051A method) (USEPA 2007c). This mixture was added to 0.5 g of samples in Teflon flasks, which were properly locked and placed in the microwave system. After cooling, the extracts were transferred to 50 mL Falcon flasks and the volume was made up with ultrapure water (Milli-Q, 18 MΩ.cm at 25 °C). The elements Cd and Pb were determined by a graphite furnace atomic absorption spectrometer (GF AAS- AAnalyst 800 Perkin Elmer). As, Cr, Mn, Co, Ni, Cu e Zn were determined by an optical emission spectrometer coupled with argon plasma (ICP-OES). Particularly, the Hg concentration was measured by a cold vapor generation (FIMS, Perkin Elmer). For the results of ICP-MS and FIMS, the method validation was performed by analysing the percentage of each metal recovery of the certified reference material (San Joaquin NIST2709a e SS-2).

Concentrations of PAHs (benzo[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[ghi]perylene, benzo[k]fluoranthene, chrysene, dibenzo[a, h]anthracene, acenaphthene, acenaphthylene, anthracene, phenanthrene, fluoranthene, 2-methylnaphthalene, naphthalene, pyrene and indeno [1,2,3-cd] pyrene) and PCBs (PCB-28, PCB-101, PCB-153) were quantified using the USEPA method 8270D (USEPA 2014) and 8082a, respectively. The samples were prepared according to USEPA 3550C (USEPA 2007a). The accuracy of the analyses was validated using certified reference material, and the mean percentage of recovery was  $109.0 \pm 4.3\%$ . Concentrations of surfactants were determined following the Standard Methods for the Examination of Water and Wastewater (SMWW-5540C). For sulphide analysis, sediment samples were prepared according to USEPA 9034 (USEPA 1996) and concentrations determined following USEPA 9034 (USEPA 1996) recommendations. The results are expressed in terms of  $\text{mg kg}^{-1}$  dry weight for metals and As, and  $\mu\text{g kg}^{-1}$  dry weight for PAHs and PCBs.

The sediment quality guidelines (SQGs) used for comparison in the present study were derived mainly from data from field studies in which sediment chemistry and biological effects data were obtained for the same location (co-occurrence approach). Biological data for Interim Sediment Quality Guidelines (ISQGs) and probable effects level (PEL) were mainly apical endpoints (i.e. observable outcomes in a whole organism) measured through laboratory toxicity tests (CCME 1999) whilst Choueri et al. (2009) also included macrobenthic community structure parameters in the derivation of SQGs.

## Data analysis

The dispersion of variances was tested through the Permutational Dispersions method (PERMIDISP) based on centroids (Anderson et al. 2008). The lowest observed effect concentration (LOEC) and the no observed effect concentration (NOEC) of TIE reagents were estimated by univariate permutational analysis of variance (PERMANOVA) (Anderson et al. 2008) with two fixed factors: (i) reagent concentration, with six levels; and (ii) trial, with three levels. Univariate PERMANOVA was also used to assess the effect of the TIE manipulation, with two fixed factors: (i) IW concentration with five levels, and (ii) TIE manipulation (two levels: baseline and

after manipulation tests). The advantage of doing PERMANOVA instead of traditional ANOVA is that, in the first case,  $p$  values are obtained by permutation, thus avoiding the assumption of normality (Anderson 2017). All PERMANOVA tests were performed on Euclidean distance similarity matrices and pairwise a posteriori multiple comparisons tests, which were conducted when significant differences were detected ( $p < 0.05$ ). When identified the number of permutations lower than 50, Monte Carlo  $p$  values were used (for more information about the program to be performed in the context of 999 permutations, see Anderson et al. 2008). The residuals were permuted using unrestricted permutation of raw data. For all statistical tests, a significance level of 5% ( $\alpha = 0.05$ ) was used. All data were analysed using the statistical software PRIMER-E (version 6.1.16) (Anderson et al. 2008).

## Results

### Physical-chemical parameters and sensitivity to TIE reagents

Dissolved oxygen, salinity, pH and temperature at the beginning and at the end of the tests did not change significantly during the experiments and were within acceptable limits for the organism as established in the ABNT 15350/2012 (Table 1). Sensitivity to  $\text{ZnSO}_4$  of organisms of all batches ranged within the acceptable limits of the control chart maintained by the laboratory.

NOEC and LOEC values obtained in the sensitivity tests for the reagents used in the TIE are shown in the Table 2. NOEC was  $25.0 \text{ mg L}^{-1}$  for EDTA, and ranged from  $0.8$  to  $1.6 \text{ g L}^{-1}$  for sodium thiosulfate (PERMANOVA,  $p \leq 0.05$ ). Methanol showed significant toxicity at the lowest concentration tested ( $3.1 \text{ mL L}^{-1}$ ) (PERMANOVA,  $p \leq 0.05$ ) in the first two trials, therefore NOEC estimation was possible only to the third trial ( $3.1 \text{ mL L}^{-1}$ ). Methanol showed significant toxicity at the lowest concentration tested ( $3.1 \text{ mL L}^{-1}$ ) (PERMANOVA,  $p \leq 0.05$ ) in the first two trials; therefore, NOEC estimation was possible only to the third trial ( $3.1 \text{ mL L}^{-1}$ ). From these results, the concentrations of test reagents in the TIE were defined. EDTA was added at  $40 \text{ mg L}^{-1}$ , and sodium thiosulfate at  $1.6 \text{ g L}^{-1}$ . In both cases, although the concentration used was

**Table 1** Results of the initial (after manipulations) and final (end of the test) physico-chemical analysis of the interstitial water samples from sediments from the sampling sites. (i) = initial; (f) = final

	T (i) °C	T (f) °C	Sal (i)	Sal (f)	DO (i) ( $\text{mg L}^{-1}$ )	DO (f) ( $\text{mg L}^{-1}$ )	pH (i)	pH (f)
<b>Bertioga channel (S1)</b>	25	25	26	30	6.7	5.5	7.80	7.70
<b>Santos submarine sewage outfall (S2)</b>	25	25	34	30	6.6	6.1	7.92	8.18
<b>Port Terminal (S3)</b>	25	25	30	30	6.0	5.5	7.53	8.17
<b>Piaçaguera channel (S4)</b>	25	25	26	30	6.5	6.1	8.00	8.15

**Table 2** NOEC and LOEC results (means±standard deviations) of TIE reagents obtained from tree trials

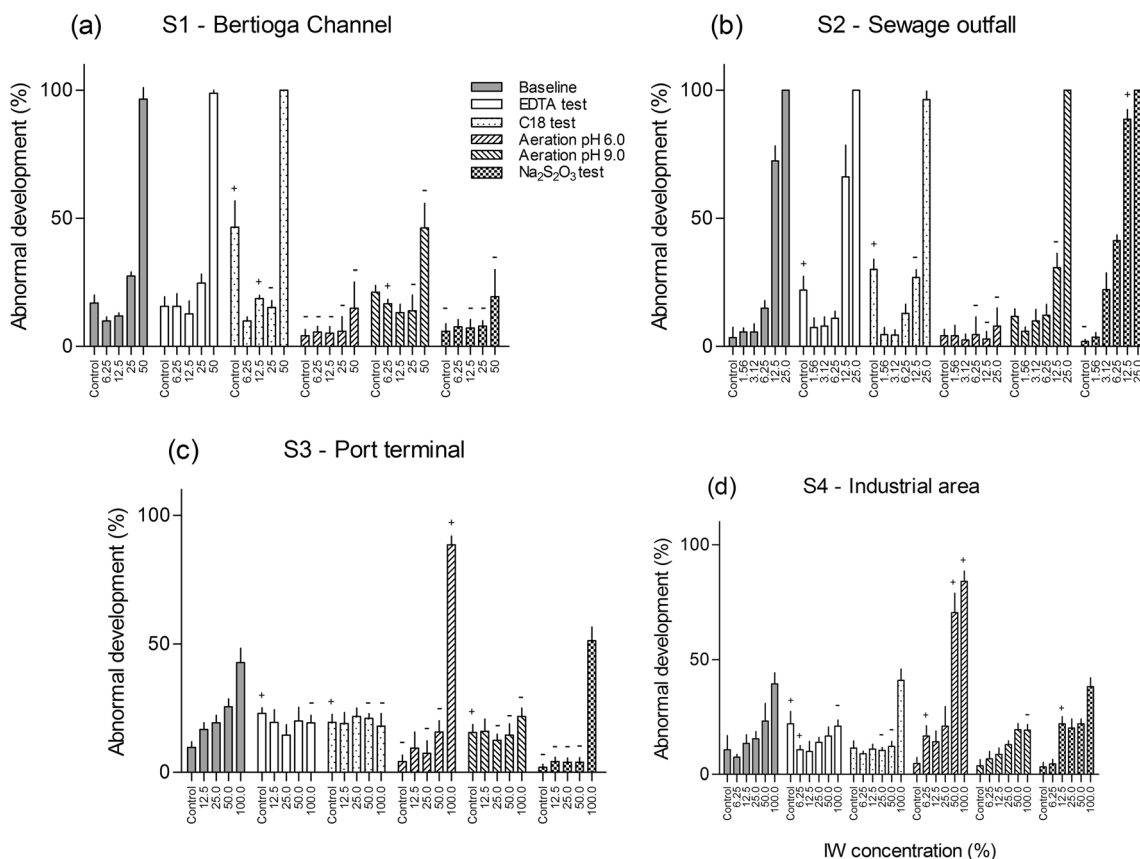
Trial		EDTA (mg L <sup>-1</sup> )	Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> (g L <sup>-1</sup> )	Methanol (mL L <sup>-1</sup> )
1	NOEC	25.0 ± 1.41	0.8 ± 3.20	–
	LOEC	50.0 ± 0.58	1.6 ± 1.63	3.1 ± 0.50
2	NOEC	25.0 ± 0.58	0.8 ± 33.13	–
	LOEC	50.0 ± 0.58	1.6 ± 17.33	3.1 ± 0.50
3	NOEC	25.0 ± 0.96	1.6 ± 3.00	3.1 ± 0.70
	LOEC	50.0 ± 0.96	3.2 ± 10	6.2 ± 0.43

slightly above the estimated NOEC, low or none toxicity was observed in the TIE manipulation control, as expected from the results obtained in the sensitivity tests. For the manipulations involving methanol, a concentration ranging from 3.1 to 50 mL L<sup>-1</sup> was tested, so the toxicity of the methanol sample used to rinse vessels in the sublation manipulation could be compared to the toxicity methanol blank.

**TIE phases I and IIa**

Mean (±standard deviation) results of sea urchin embryolarval development for TIE phases I (Fig. 3) and IIa (Fig. 4)

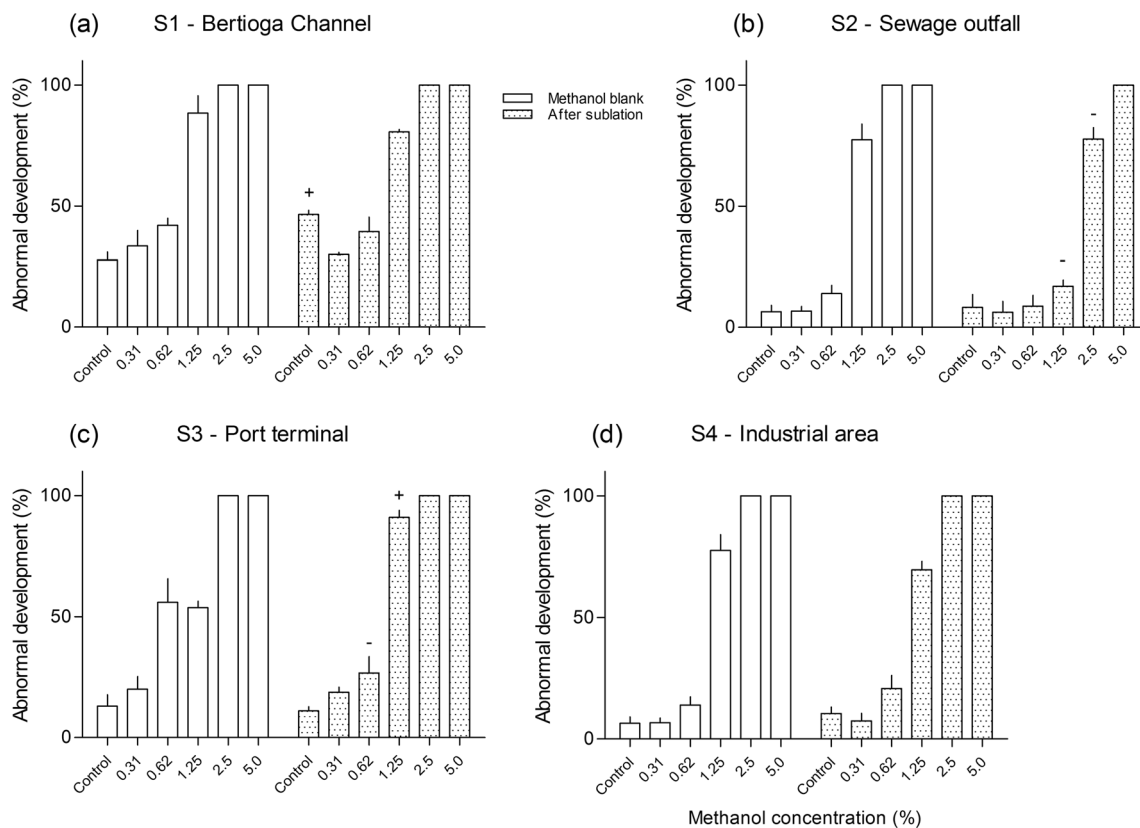
performed on the IW of sediments from each sampling site (S1 to S4) are presented in the Figs. 3 and 4. Site 1 presented significant and consistent toxicity reduction after the aeration test both at basic (9.0) and acidic pH (6.0) conditions, as well as in the Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> addition test (PERMANOVA, *p* ≤ 0.05) (Fig. 3a). The results of the C18 were significantly different from the baseline, but the results were not consistent, i.e. showed both a reduction and an increase in toxicity, depending on the IW concentration (Fig. 3a). Toxicity of IW samples from S2 (submarine sewage outfall) was significantly reduced in the aeration test both at basic (9.0) and acidic pH (6.0) test, and in the C18 SPE column test (Fig. 3b). For S3 (port terminal), all the phase I TIE manipulations consistently abated the baseline toxicity of IW (PERMANOVA, *p* ≤ 0.05) (Fig. 3c). In addition, IW from S3 showed significantly increased toxicity when compared to the methanol blank in the sublation test (PERMANOVA, *p* ≤ 0.05) (Fig. 4c). The IW samples from S4 (Cubatão industrial complex) showed clear toxicity reduction in the EDTA addition test, and aeration at pH 9.0 compared to the baseline toxicity (PERMANOVA, *p* ≤ 0.05) (Fig. 3d). The C18 SPE column test showed a tenuous reduction in toxicity, statistically significant only in some intermediate dilutions of IW (Fig. 3d).



**Fig. 3 a–d** Mean (± standard deviation) results of sea urchin embryolarval development for TIE phase I manipulations performed on the IW of sediments from S1 (a), S2 (b), S3 (c), and S4 (d). The error bars

represent the standard deviation. Signals above the bars represent a statistically significant increase (+) or decrease (–) in toxicity relative to the corresponding baseline IW concentration





**Fig. 4 a–d** Mean ( $\pm$  standard deviation) results of sea urchin embryolarval development for TIE phase IIa manipulations performed on the IW of sediments from S1 (a), S2 (b), S3 (c) and S4 (d). The

error bars represent the standard deviation. Signals above the bars represent a statistically significant increase (+) or decrease (–) in toxicity relative to the corresponding methanol blank concentration

## TIE phase IIb

Results for TIE phase IIb are presented in the Table 3. Levels of non-ionised ammonia in the interstitial water were higher than the toxicity threshold for sea urchin embryolarval development (Prósperi 2002) in S1, S2 and S4, and levels of sulphides were above the detection limit only in S1 and S2. All sampling sites showed more than one exceedance of SQGs for metals or As in whole sediment samples. Hg and Ni showed values above the threshold for high pollution (L2) according to locally derived SQGs (Choueri et al. 2009) for all four sampling sites. Concentrations of Hg are even higher than PEL in all sites, but S1. Sites S3 and S4 showed Pb concentrations above L2 (Choueri et al. 2009), As above TEL and Zn above both L2 and TEL. Zinc was also higher than L1 in the vicinity of the submarine sewage outfall (S2).

Sites S3 (port terminal) and S4 (Cubatão industrial complex) were the only sampling sites that presented values of PAHs higher than the guideline values. Site 3 showed exceedance for acenaphthylene (slightly above TEL) and the sum of the 13 PAHs (above L1 of locally derived SQGs). Site S4 showed several exceedances for individual PAHs, i.e. chrysene, acenaphthene, acenaphthylene, anthracene, fluoranthene

and pyrene (above TEL), and benzo(a)anthracene, benzo(a)pyrene and dibenzo(a,h)anthracene (above PEL).

PCBs were not found at concentrations above the detection limit of the analytical method used. Total Kjeldahl nitrogen was higher at S2 and concentrations of surfactants (LAS) in whole sediment did not vary much between sampling sites (from 0.9 to 1.7 mg kg<sup>-1</sup>). Neither Choueri et al. (2009) nor CCME (2001) provide SQGs for these substances.

## Discussion

Interstitial water of sediments from all sampling sites showed significant toxic effects in the present study. For all sites, the results of TIE phase I suggested a strong contribution of both ammonia and substances volatile in acidic conditions (probably sulphides for most sites) for the toxicity in all sites. Although the sea urchin embryo-larval development test is standardised and widely used for assessing sediment toxicity (whole sediment, IW, sediment elutriates, sediment-water interface) (ASTM 1995; USEPA 1995; Marroni et al. 2016; Bonaventura et al. 2021), the sensitivity of sea urchin embryos/larvae to typical natural characteristics of sediments

**Table 3** Results for sediment characterisation and concentrations of the analysed substances in the whole sediment or interstitial water samples (S1 to S4) and comparison with SQGs. LD = limit of detection; ISQG = interim sediment quality guideline; PEL = probable effect level; L1 = threshold below which sediments are not polluted (Choueri et al. 2009); L2 = threshold above which sediments are highly polluted (Choueri et al. 2009); values in bold represent exceedances to the SQGs

Parameters	Sites				Sediment quality values			
	S1	S2	S3	S4	ISQG	PEL	L1	L2
<b>Sediment characterisation (%)</b>								
Organic matter	9.90	7.00	28.61	17.80	–	–	–	–
Carbonates	12.80	17.70	28.90	17.90	–	–	–	–
Fine sediments	41.38	64.73	92.98	74.78	–	–	–	–
<b>Interstitial water (mg L<sup>-1</sup>)</b>								
Nonionised ammonia	0.12	0.23	0.04	0.05	–	–	–	–
Sulphides	0.06	0.09	< LD	< LD	–	–	–	–
<b>Whole sediment</b>								
<b>Metals/As (mg kg<sup>-1</sup>)</b>								
As	6.57	6.27	<b>11.55</b>	<b>9.29</b>	7.24	41.60	–	–
Cd	< LD	< LD	0.27	0.19	0.70	4.20	< 0.75	≥ 0.75
Cr	21.90	25.50	40.00	39.40	52.30	160.00	≤ 65.80	≥ 65.80
Cu	15.00	12.70	28.60	27.20	18.70	108.00	< .69.00	≥ .69.00
Co	<b>10.00</b>	<b>9.10</b>	<b>11.60</b>	<b>14.70</b>	–	–	≤ 4.10	≥ 10.30
Mn	96.60	213.70	168.00	96.70	–	–	–	–
Pb	9.38	<b>14.55</b>	<b>22.22</b>	<b>24.41</b>	30.20	112.00	≤ 10.30	≥ 22.10
Ni	<b>16.00</b>	<b>14.70</b>	<b>22.10</b>	<b>27.00</b>	–	–	≤ 3.89	≥ 21.20
Zn	< LD	<b>90.0</b>	<b>138.60</b>	<b>134.20</b>	124.00	271.00	≤ 37.90	≥ 110.40
Hg	<b>0.40</b>	<b>1.00</b>	<b>0.98</b>	<b>0.97</b>	0.13	0.70	≤ 0.08	≥ 0.32
<b>Organics (µg kg<sup>-1</sup>)</b>								
Benzo(a)anthracene	< LQ	1.88	25.60	<b>740.00</b>	74.80	693.00	–	–
Benzo(a)pyrene	< LQ	2.33	33.20	<b>1140.00</b>	88.80	763.00	–	–
Chrysene	< LQ	1.65	23.70	<b>763.00</b>	108.00	846.00	–	–
Dibenzo(a,h)anthracene	< LQ	< LQ	4.64	<b>246.00</b>	6.22	135.00	–	–
Acenaphthene	< LQ	< LQ	3.31	<b>12.60</b>	6.71	88.90	–	–
Acenaphthylene	< LQ	< LQ	<b>6.24</b>	<b>121.00</b>	5.87	128.00	–	–
Anthracene	< LQ	0.58	8.84	<b>108.00</b>	46.90	245.00	–	–
Phenanthrene	< LQ	2.14	15.10	59.00	86.70	544.00	–	–
Fluoranthene	0.87	4.14	38.10	<b>1060.00</b>	113.00	1494.00	–	–
Fluorene	< LQ	< LQ	5.50	16.80	21.20	144.00	–	–
2-Methylnaphthalene	< LQ	0.61	2.63	15.00	20.20	201.00	–	–
Naphthalene	< LQ	0.49	3.12	4.65	34.60	391.00	–	–
Pyrene	0.61	2.83	42.70	<b>973.00</b>	153.00	1398.00	–	–
Total PAHs	1.48	<b>25.15</b>	<b>328.38</b>	<b>9016.05</b>	374.00	6982.00	≤ 15.0	≥ 1660.0
Total PCBs	< LD	< LD	< LD	< LD	21.50	189000	≤ 0.94	–
<b>Others (mg kg<sup>-1</sup>)</b>								
Surfactants	1.70	0.90	0.90	1.50	–	–	–	–
Chlorine	3720	4070	1290	1600.00	–	–	–	–
Kjeldahl total nitrogen	545	988	623	623	–	–	–	–

(e.g. high levels of unionised ammonia, sulphides, dissolved organic carbon including humic acids, fine sediment grain size) is extensively discussed (Novelli et al. 2003; Carr et al. 2006; Picone et al. 2009). A review study on the application

of TIE in sediments showed that ammonia is often a cause of toxicity, especially in IW samples (Ho and Burgess 2013). Since the study area in the current study is an estuarine system, naturally occurring substances can also

be the cause of sediment toxicity, apart from anthropogenic inputs.

The identification of the same toxic substances for all study sites may initially suggest that the microscale TIE is ineffective in discerning the anthropogenic substances responsible for toxicity in estuarine areas subject to multiple stressors, especially in cases where the toxicity of confounding factors (i.e. natural factors causing toxicity) is suspected to overshadow the toxicity of contaminants. However, it is important to consider that at least part of the ammonia and sulphides causing toxicity in the current study is a result of anthropogenic pressures, e.g. introduction of sanitary effluents. There is evidence of the widespread introduction of sanitary effluents from houses not connected to the sewage network in the SES (Pusceddu et al. 2019). Therefore, since ammonia and sulphides cannot be considered as natural confounding factors in IW toxicity in the SES, the microscale TIE fulfils its role of detecting the toxicity of substances whose presence is originated from anthropogenic sources.

Perhaps more importantly to test the efficacy of the technique, other TIE manipulations were able to discern different toxic substances for sites with different sources of contamination. Besides the toxicity reduction in the manipulations aimed for ammonia and sulphides, the toxicity of the IW samples was significantly reduced in the sodium thiosulfate addition test in S1 (Bertioga Channel) and S3 (port terminal), in the EDTA test in S3 and S4 (industrial complex), and in the C18 test in S2 (submarine sewage outfall), S3 and S4 (although not as clear as in the previous ones), which indicates toxicity by oxidant agents (e.g. some cationic metals), cationic metals and apolar organic substances, respectively. The sublation test showed toxicity recovery due to the presence of surfactants only in S3. Discerning differences in the toxicity profile of sites with different pollution sources is good evidence that the microscale TIE is a useful tool for its purpose of identifying substances responsible for toxicity in complex samples.

### Correspondence between toxicity manipulations and chemical analyses

Another evidence of the effectiveness of microscale TIE is the agreement between the toxicity profile (phase I and phase IIa) and chemical analyses (phase IIb). In the present study, chemical analyses in IW or whole sediment generally corroborate the results obtained in the toxicity manipulations. Non-ionised ammonia was detected at all sites at concentrations very close to or above the threshold of toxicity for embryo-larval development of the test species ( $0.05 \text{ mg L}^{-1}$ ) (Prósperi 2002). Sulphides, where analytically detected, were pointed out as one of the substances responsible for toxicity. Only the S3 sample showed a decrease in toxicity after the aeration test at low pH, but the chemical analysis did not detect sulphides. In this case, either sulphide toxicity occurred at concentrations

below the detection limit of the method ( $0.025 \text{ mg L}^{-1}$ ), or substances not measured in this study are responsible for the observed toxicity.

The role of unmeasured substances in this study was also suspected in the sample from the vicinity of the sewage submarine outfall of the city of Santos (S2). S2 showed toxicity abatement after the C18 manipulation, although PCBs were not detected and concentrations of PAHs were well below SQGs. It is reasonable to suggest a possible contribution of other compounds with chemical characteristics similar to those of apolar organic pollutants, such as several pharmaceuticals and personal care products. Many recent studies have shown the presence of such compounds at concentrations able to cause toxicity in the area under the influence of the submarine outfall of Santos Bay (Cortez et al. 2012; Pereira et al. 2016; Maranhão et al. 2017; Fontes et al. 2019). Although other types of chromatography columns can be used for the specific separation of drugs, pharmaceutical compounds, and personal care products the SPE C18 is widely used as well for the purposes of chemical analyses (Petrovic et al. 2005).

Another incongruity between the toxicity manipulation and quantification of contaminants phases was observed in the S2 sample. Whilst the EDTA test did not show metals as causing toxicity in IW, whole sediment chemical analyses showed As, Co, Pb, Ni and Zn in concentrations above the toxicity threshold, and Hg concentration above probable toxic effect concentration when compared to SQGs. The explanation may be related to other substances producing toxicity, such as non-ionised ammonia and sulphides, because these substances are in concentrations so high (due to the input of sanitary effluents) that they required TIE to be performed with high dilutions of IW (maximum concentration was 25% in S2). Thus, Hg and other cationic metals found in S2 at concentrations able to cause toxicity did not do so due to the need of initially diluting the IW to perform the TIE.

The C18 test also showed some incongruity in the case of S4. On this site, whilst the C18 test shows only a slight reduction in toxicity, the chemical analyses show high concentrations of PAHs in sediments. The unclear role of PAHs in the IW toxicity at this site, despite the high concentrations measured in the whole sediment, suggests that these apolar substances may be sorbed by the sediment particles, i.e. not bio-available in IW. Thus, chemical exposure to sediment-sorbed contaminants can be overestimated by bulk sediment analyses (Chapman et al. 2002), at least for organisms whose main exposure route is IW.

It is important to note that the apparent inconsistencies discussed here were the exceptions. Much more broadly, the TIE microscale phase I results were corroborated by phase II chemical analyses. More interestingly, in some cases, the joint interpretation of the different TIE manipulations and phases of the microscale TIE bring more precise information about the identification and evaluation of the toxicity. This is the case of

S1 (Bertioga Channel), in which the addition of sodium thiosulfate decreased the toxicity of the IW sample. Sodium thiosulfate strongly diminishes the toxicities of  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$  and  $\text{Cd}^{2+}$ , whilst toxicities of  $\text{Pb}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Ni}^{2+}$  are weakly affected (Hockett and Mount 1996). Since in the current study Pb was found at a low concentration in sediments from Bertioga, and Cu, Cd and Zn concentrations were below the LQ, the abatement of toxicity was probably mainly due to chelation of Hg (found at concentrations above ISQG and L2). Nickel, found at a concentration above L1 of local SQG, may have not contributed significantly to toxicity; otherwise, an important effect of EDTA (a strong Ni chelator) (Hockett and Mount 1996) would be observed.

### Correspondence of the microscale TIE with pollution sources in the SES

The classes of substances pointed out as responsible for the toxicity in each of the sites are in accordance with the known sources of pollution and are corroborated by previous studies. In Bertioga Channel (S1), it is likely that natural sources have some contribution in the amount of ammonia and sulphides in the sediments due to the presence of dense mangrove forests in their surroundings. However, the proximity to sources of pollution in the city of Bertioga, such as untreated sanitary effluents, public garbage dumps (Duarte et al. 2016, 2017) and intense nautical activities due to operations of many recreational marinas (Oliveira and Vidal-Torrado 2007; CETESB 2008; Gonçalves et al. 2013; Sousa et al. 2013 al. 2014; Salaroli et al. 2018) may have contributed to the ammonia and sulphides toxicity, as well as corroborates the evidence that metals are causing IW toxicity in S1.

Ammonia and sulphides toxicity has also been observed or discussed in past studies on the sewage outfall of the city of Santos (S2). TIE studies at this location indicated toxicity due to these substances (Rachid 2002; Ferraz et al. 2017) and Abessa et al. (2005) discussed the contribution of ammonia and sulphides (besides other contaminants) to the observed acute toxicity to amphipods exposed to whole sediments from sites around the submarine sewage outfall. Clearly, ammonia and sulphides cannot be considered as natural confounding factors on this site, since there is a continuous introduction of huge volumes of sanitary sewage in Santos Bay through the outfall.

Metal contamination or pollution at the inner portions of the SES is extensively reported (Lamparelli et al. 2001; Abessa et al. 2008; Choueri et al. 2009; Buruaem et al. 2013; Torres et al. 2015; Kim et al. 2016; Perina et al. 2018; Abessa et al. 2019). Siderurgical, petrochemical and chemical industries (including chloralkali industry) are part of the Cubatão industrial complex. Along with industries, port activities have also been held responsible for impairing environmental quality in this area. Previous studies have reported sediment toxicity and concentrations of Ni, Hg, Pb, Zn and

less frequently, Cd, exceeding national and/or international SQGs (in some instances, concentrations above PEL) (Hortellani et al. 2008; Choueri et al. 2009; Buruaem et al. 2013; Torres et al. 2015; Perina et al. 2018; Abessa et al. 2019). PAHs in high concentrations in the sediments of the innermost portions of the SES were also previously reported and the contamination was attributed to industrial and port activities, besides domestic sewage from subnormal agglomerates (Lamparelli et al. 2001; Choueri et al. 2009; Buruaem et al. 2013; Torres et al. 2014; Perina et al. 2018).

In general, the microscale TIE using the sea urchin embryolarval development test was able to discern different toxicants on sites subject to different contamination sources. There was good agreement between the results indicated in the toxicity manipulation tests (phase I), chemical analyses in whole sediment (phase IIb) and the literature on contamination and pollution in SES. However, due to the high sensitivity of the sea urchin embryo/larva to ammonia and sulphides, these substances when in high concentrations can overshadow the toxicity of other substances. Another limitation is the underestimation of sediment toxicity due to unavailability of sediment-sorbed contaminants in the IW, since these contaminants may be available through other ways of exposure, i.e. to organisms that are in direct contact with sediment and/or feed through sediment ingestion (Chapman et al. 2002; Ferraz et al. 2020).

### Conclusion

The current results showed the suitability of a microscale TIE approach in identifying individual substances or classes of substances responsible for toxicity in IW of sediments from different sites subjected to different kinds of stressors. Despite the increased efforts for improving environmental quality monitoring in the area since the 1980s, the Environmental Agency of the State of São Paulo still rely on techniques which do not allow the identification of substances or classes of substances causing toxicity. The TIE approach employed in the present study used the microscale sea urchin embryolarval development with reduced volumes of the experimental medium (2.5 mL), which was a rapid, cost-effective, and reliable test for identifying culprit substances in complex environmental samples of sediment interstitial water.

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execution. ICB, DITF, MEBC: study execution, manuscript production. RBC: study conceptualization, execution, data analysis, manuscript production, supervision, project administration.

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**Data availability** Data will be available as requested.

## Declarations

**Ethics approval and consent to participate** This study complies with all Brazilian institutional guidelines on the use of invertebrate animals in scientific research.

**Consent for publication** We declare neither the article nor portions of it have been previously published elsewhere. The manuscript is not under consideration for publication in another journal, and will not be submitted elsewhere until the editorial process is completed. All authors consent to the publication of the manuscript.

**Competing interests** The authors declare no competing interests.

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