Article

When the Detail of Organism Makes the Difference in the Seascape: Different Tissues of *Phallusia nigra* Have Distinct Hg Concentrations and Show Differences Resolution in Spatial Pollution

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This study indicates the use of *Phallusia nigra* as a potential biomonitor of mercury contamination. In this way, Hg levels were measured in seawater and different parts of ascidians (tunic, hepatopancreas, and branchial basket) from eight different sites in the Todos os Santos Bay, Salvador-Bahia. The ascidians were lyophilized, weighed, and taken to the DMA-80 (direct mercury analyzer); the method accuracy was confirmed by analyzing the certified material DORM-4 muscle tissue and MEES-3 marine sediment with a confidence level of 95%. The results were evaluated through the Tukey's test and it was possible to observe a higher concentration of Hg (82.00-312.7 ng g^{-1}) in the branchial basket, followed by the hepatopancreas (69.67-130.7 ng g^{-1}) and tunic (21.63-33.27 ng g^{-1}). Thus, the branchial basket was the only tissue capable of identifying spatial differences in pollution between the points.

Keywords: ascidian, trace metals, biomonitor

Introduction

Due to the great impact of human activities, different trace metals are released into the marine environment and are a threat to organisms and human life. Biological monitoring of trace metals is necessary to identify potential sources of contamination and assess the current state of an area under anthropogenic pressure. This information is sometimes difficult to obtain when evaluating water samples, because metal concentrations may be close to or below the limit of detection of analytical techniques.[1,2] To circumvent this problem, some organisms are used to biomonitoring the environment. Biomonitoring species can accumulate trace metals for a long period, thus providing a robust evaluation of the state of contamination of the environment.[1] Several marine species accumulate these trace metals, either from the aquatic environment or from food, depending on the bioavailability of the metal in the water or the diet and bioaccumulation rates.[3,4]

Hg has presented high levels in the environment and toxic effects on living organisms, mainly through its bioaccumulation and biomagnification processes. In contact with the human organism, it affects the nervous, gastrointestinal, respiratory, and immune systems, and even at low concentrations, the risks of Hg pollution to human health are worrying.[5-7] There are three main sources of Hg in the environment: natural deposits in soil; anthropogenic

release and wet and dry atmospheric decomposition from both sources. However, human actions are the main causes of increasing Hg levels in the atmosphere, soil, and aquatic environments.[8]

Some invertebrates have relatively simple physiological functions and are sensitive to detecting trace metals, making them important monitors of contamination and pollution.[9] Examples of these organisms are ascidians, which feed through water filtration and manage to accumulate trace metals in their mantle and tunic at different stages of individual development.[10-13] Ascidians are sessile marine organisms that are represented by both solitary and colonial forms; they have to inhale and exhale siphons that connect to the branchial basket for breathing and feeding functions. These animals inhabit coastal and estuarine environments and their distribution is determined by temperature, salinity, and hydrodynamics.[14,15]

Phallusia nigra ascidian is distributed worldwide, although it is considered cryptogenic in many regions.[16] This marine invertebrate has been investigated as a bioindicator of pollution by heavy metals and organic compounds.[17-20] Report on Hg in *P. nigra* is rare, it appears only in works related to bioremediation, monitoring program of metals in edible tunicates, and the influence of metals on reproduction mechanisms.[18,21,22]

By its feeding characteristics and distribution, *P. nigra* presents a potential biomonitor to chemical contaminants.[3] However, studies indicate that different parts of the animal contain different amounts of metals. *In vitro* experiments indicate that the accumulation of metals in sea squirts occurs preferentially in the branchial baskets, while the available data from *in situ* experiments published so far are insufficient to compare the accumulation of Hg in different tissues (tunica, hepatopancreas, and branchial basket).[14,23]

In this context, due to the numerous and worrying problems caused by Hg contamination and its accumulation in organisms, developing methodologies that use a small amount of sample to detect metal is important to monitor Hg contamination in the environment, even at low concentrations. For that purpose, we evaluated the potential bioindicator of *Phallusia nigra* to Hg levels and analyzed spatial differences of this contamination in Todos os Santos Bay by performing direct Hg quantification without sample pre-treatment.

Experimental

Sampling location and methods

This experiment was performed in Todos os Santos Bay, Brazil (BTS), which has gradients of environmental pollution, including Hg.[8] *Phallusia nigra* is classified as a cryptogenic species in Todos os Santos Bay.[24] BTS is the second-largest bay in Brazil (ca. 1200 km^2), located in the vicinity of Salvador, the third-largest metropolitan area in Brazil.[25] This bay harbors many different and important ecosystems such as mangroves, coral reefs, and seagrass meadows, among others, thus more than three million habitants living surround it.[26] However, this area receives impacts of diverse human activities, which come from sugar cane cultivation, dating back to colonization times (ca. 1500s), and a complex range of industrial activities that started around the 1950s with the exploration of oil and the installation of a refinery, followed by the development of several chemicals, metallurgical and petrochemical industrial complexes.[8,25]

The samples were collected at eight sites of BTS in December 2020, selected by the representative abundance of *Phallusia nigra*, the easy access for diving and different levels of anthropization. Besides the ideal number of individuals for the sampling, *P. nigra* is easy to recognize by its black tunic and effortless to get sampled during the fieldwork. Sampling was performed by scuba diving around 4-9 m depth at eight BTS sites (three specimens *per* site): (P1) Ilha dos Frades Sul, −12°48'37,43240'' −38°37'37,85345''; (P2) Poste 4, −12°48'52,48724'' −38°34'18,42605''; (P3) Poste 1, −12°49'21,57579'' −38°33'36,87196''; (P4) Pedra Cardinal, −12°50'14,90532'' −38°32'58,25449''; (P5) Pedra Cardinal Norte, −12°49'47,00113'' −38°32'51,26297''; (P6) Pedra do Português, −12°49'39,78300'' −38°32'04,75055''; (P7) Pedras Alvas, −12°52'22,02352'' −38°31'46,99174''; and (P8) Pedra do Dentão, −12°50'02,03454'' −38°31'32,52260'' (datum WGS 84).

The ascidians were stored in previously decontaminated Ziploc bags in 10% aqueous nitric acid solution and transported to the laboratory on the same day in a cool box with dry ice. In the laboratory, individuals were dissected in a tunic (T), branchial basket (BB), and hepatopancreas (H), without anesthesia to avoid any new sources of contamination. The different tissues were freeze-dried in a SL-404 Solab lyophilizer (Piracicaba, Brazil) for 42 h, approximately, then dried tunics were macerated in the ball mill, while hepatopancreas and branchial baskets were manually crushed in a mortar and pestle for subsequent analysis.

Seawater samples were collected in acid-cleaned glass tubes by SCUBA from each study site. The tubes were pre-cleaned with 10% HNO_3 followed by a rinse with water (Milli-Q system, 18.2 MΩ cm). In the laboratory, samples were brought to pH < 2 with concentrated HNO_3 (14.4 M) (Merck, Darmstadt, Germany), filtered using a membrane

(0.45 μm pore diameter), and then kept at −20.0 °C until the time of analysis.

Total Hg determination

Measurements of Hg concentrations were performed on ascidians samples (previously divided into a tunic, hepatopancreas, and branchial basket), and seawater using a direct mercury analyzer DMA-80 Tri Cell spectrometer (Milestone, Sorisole (BG), Italy). As the DMA-80 does not require pre-treatment of the samples, the organisms were weighed: tunic (100 mg), hepatopancreas (40 mg), and branchial basket (20 mg), in nickel balls, which were inserted into the equipment. For each analysis of seawater samples, 300 μL was taken. In the analysis step, the samples were submitted to a heating program: (*i*) drying (200 °C for 80 s); (*ii*) thermal decomposition (650 °C for 180 s); (*iii*) detection and cooling (100 s). The analyzes were carried out under a continuous flow of oxygen that is responsible for carrying the decomposition products through a heated path to the catalyst, where the gaseous Hg is trapped. All kinds of Hg are reduced to Hg^0 and are loaded into a gold amalgamator where the Hg is selectively trapped. After being trapped, the amalgamator is heated, resulting in the release of Hg, and taken to the detection cells.

The accuracy of the results was evaluated and confirmed by the analysis of two certified materials (CRM), MESS-3 marine sediment from the National Research Council of Canada (NRC-CNRC) and DORM-4 muscle tissue from the National Research Council of Canada (NRC-CNRC). Recoveries of 101 and 96%, respectively, were observed. The limits of detection and quantification were set at 0.004 and 0.012 ng, respectively.

Statistical analysis

A two-ways analysis of variance (ANOVA) was tested (software StatSoft Statistica (version 8))[27] to indicate the Hg accumulation in the tissues and the difference in contamination levels among sites with certain gradients of pollution. In the cases that presented significant differences, Tukey as a post hoc test was used.[28]

Results

The Hg concentrations differ among the different tissues ($F_{2,46} = 35.0890$, $p < 0.001$), and from each other (Tukey $p < 0.001$) (Table S5, Supplementary Information (SI) section). The lower concentration was recorded in the tunic, followed by hepatopancreas, and the higher

concentration was in the branchial basket. Total Hg concentrations in the different tissues analyzed are shown in Table 1.

The Hg concentration in seawater ranged from < limit of quantification (LOQ) to 0.5554 ± 0.2205 ng mL^{-1}. The concentration of Hg in the tunic of *P. nigra* ranged from 21.63 ± 2.620 to 33.27 ± 12.15 ng g^{-1}. The minimum level was found at the P8 site and the maximum level at the P7 site. In the hepatopancreas of the ascidians, the concentration of total Hg ranged from 69.67 ± 14.37 to 130.7 ± 12.10 ng g^{-1} corresponds to the P4 and P7 sites the minimum and maximum levels, respectively. The minimum level of Hg in the branchial basket of *P. nigra* was found in P2 82.00 ± 19.99 ng g^{-1}, whereas the maximum level was found at P7 312.7 ± 54.27 ng g^{-1}.

There is a difference among sites ($F_{7,48} = 358.178$, $p < 0.001$), the site 7 have a significantly higher concentration than sites P1, P2, P3, P4, P5, and P6 (Tukey $p < 0.001$) and site P8 with a higher level than sites P2, P3 and P5, beyond P7 (Tukey $p = 0.001$, $p = 0.002$, and $p = 0.007$, respectively) (Figure 1). The spatial result among the different tissues ($F_{14,46} = 18.6360$, $p < 0.001$) and branchial basket show a difference between sites, P7 and P8 and all others, including between them, (Tukey $p < 0.001$) (Tables S5-S8, SI section). The P7 and P8 points drew attention to the higher Hg concentration values in the branchial baskets (312.7 and 192.7 ng g^{-1}), respectively (Table 1).
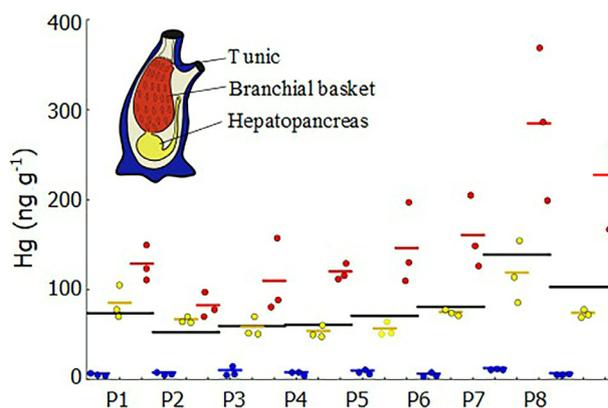

Figure 1. Mercury concentration in different ascidian tissue at each site. Blue dots represent each tunic measured, yellow ones represent each hepatopancreas measured, and red ones represent the branchial basket measured. The color lines represent each site's mean mercury concentration in respective colors dots tissue in each site and the black line represent the mercury concentration mean in all tissue samples in the respective site.

Low concentrations of Hg found in seawater indicate that ascidians can accumulate this element in tissues at several orders of magnitude. The significant statistical differences found in the determination of Hg in different

Table 1. Amount of mercury in *Phallusia nigra* tissues and seawater

Point	Tunic / (ng g^{-1})	Hep / (ng g^{-1})	Bb / (ng g^{-1})	Seawater / (ng mL^{-1})
P1	26.23 ± 15.94	92.00 ± 10.06	128.9 ± 22.10	0.5129 ± 0.1203
P2	22.27 ± 12.47	82.33 ± 16.68	82.00 ± 19.99	0.5554 ± 0.2205
P3	27.70 ± 12.19	81.00 ± 11.14	89.00 ± 41.16	0.0304 ± 0.0272
P4	30.23 ± 1.880	69.67 ± 14.37	141.7 ± 12.46	< LOQ
P5	26.43 ± 0.730	71.00 ± 22.09	119.5 ± 10.78	< LOQ
P6	23.33 ± 5.82	90.33 ± 19.41	129.0 ± 7.84	< LOQ
P7	33.27 ± 12.15	130.7 ± 12.10	312.7 ± 54.27	< LOQ
P8	21.63 ± 2.620	73.67 ± 3.640	192.7 ± 5.10	< LOQ

Hep: hepatopancreas; Bb: branchial basket; LOQ: limit of quantification.

tissues (tunic, hepatopancreas, and branchial basket) suggest its preferential accumulation in the branchial basket. A plausible explanation is that the main vital functions occur predominantly in the branchial basket, so it is expected that this tissue would accumulate greater concentrations of metal.[14]

Discussion

Testing three parts of the ascidian, it was found a significant difference in Hg concentration. The tunic contains less Hg than the branchial basket, which has the highest concentration (Figure 1). The greater level of Hg in the branchial baskets is due to their filtering function, which in fact, also regulates some vital functions such as nutrient absorption, circulation, and storage.[14] Thus, these results are in congruence with some observations in the literature that show that the branchial basket of ascidians is a suitable tissue for biomonitoring metals in the environment.

Although the study of Abdul Jaffar Ali *et al.*[18] suggests that *P. nigra* could effectively be used as a biomonitor to metals contamination, including Hg, the ascidians were only divided into two parts, tunic, and mantle body (a soft body inside tunic, including branchial basket and hepatopancreas). According to the authors, the metal content in the tunic was lower than in the mantle body, but there is a lack of information about the different concentrations in the inner parts of the body.[18] Leatherland and Burton[29] analyzed concentrations of metals in ascidian *Styela clava*, among other marine invertebrates. The concentration of Hg in the soft tissue of the whole organism was 130 ng g^{-1} but details about different concentrations in ascidian tissues were not presented. To avoid loss of information about the difference in Hg concentration, this current work (Table 2, entry 1) divided the ascidians into three body parts (tunic, branchial basket, and hepatopancreas).

Philp *et al.*[30] (Table 2, entry 4), who studied metal contamination in sediment, water, and in two ascidians

species (other benthic organisms as well), affirm that the organisms accumulate metals even when the environmental levels are low. However, the low concentrations of Hg found in ascidians (200 ng g^{-1} for *Styela partita* and 130 ng g^{-1} for *Molgula occidentalis*) can be explained by the analysis of the ascidians as a whole, once the authors did not dissect the ascidians in different tissues. Our results show that branchial baskets accumulate more Hg than other tissues, and allowed the identification of different concentrations among sites.[30]

Bellante *et al.*[1] (Table 2, entry 7) reported higher concentrations of Hg (and other metals) in the branchial basket when compared to the tunic and hepatopancreas in the solitary ascidian *Styela plicata.* They found 3.0 ng g^{-1} of Hg in branchial baskets but by the limit of detection, did not measure Hg in tunic and hepatopancreas. Despite that, Bellante *et al.*[1] suggested that the tunic would be more useful for the analysis as it has a sufficient amount of tissue to provide individual analysis. Testing the capacity of these different tissues to reveal an environmental gradient of Hg pollution, we did not differ among sites using tunic and hepatopancreas Hg concentrations. Indeed, we found that only the branchial baskets showed statistical differences amongst sites.[1]

In our study, the highest concentrations of Hg were found at sites 7 and 8, both close to Salvador city. Although site 8 is close to Itapagipe Bay, where a chlor-alkali industry was localized in the early 1970s, the highest values were found at site 7, which could influence the Hg pollution recorded in Mataripe, north of this bay.[9] The difference between the sites was supported only by the concentrations of Hg in the branchial baskets, which may indicate that this tissue is more sensitive to detecting this contamination. Besides, this study is in line with some literature remarks which show the branchial basket of ascidians as a suitable tissue for metal biomonitoring in the environment. Parrinello *et al.*[14] and Cheney *et al.*[23] confirmed the accumulation of metals in branchial baskets of ascidians

Table 2. Mercury studies in solitary ascidian species

entry	Experiment	Ascidian specie	Analyzed tissue	Hg / (ng g^{-1})	Method	Sample pre-treatment	Sufficient weight	Reference
1	*in situ*	*Phallusia nigra*	tunic	21.63 to 33.27	direct mercury analyzer	not-require	individual analysis	this study
			hepatopancreas	69.67 to 130.67				
			branchial basket	82.00 to 312.7				
2	*in situ*	*Phallusia nigra*	tunic	13.1 to 20.4	atomic absorption	digestion	–	18
			body	10.7 to 34.7				
3	*in situ*	*Styela clava*	whole organism soft tissue	130	neutron activation	digestion	–	29
4	*in situ*	*Styela partita*	whole organism	200	ICP-MS	digestion	–	30
		Molgula occidentalis		130				
5	*in vitro*	*Styela plicata*	hepatopancreas	1.0 to 8.0	ICP-OES	digestion	pool of 5 organs	14
			branchial basket	1.0 to 53.0			pool of 5 organs	
6	*in vitro*	*Ascidia ceratodes*	branchial basket	–	–	–	–	23
		Ciona intestinalis						
		Styela montereyensis						
7	*in situ*	*Styela plicata*	tunic	< limit of detection	ICP-OES	digestion	pool of 5 organs	1
			hepatopancreas	< limit of detection				
			branchial basket	3.0				

ICP-MS: inductively coupled plasma mass spectrometry; ICP-OES: inductively coupled plasma-optical emission spectrometry.

in vitro experiments. Parrinello *et al.*[14] (Table 2, entry 5) used hepatopancreas and branchial baskets of *Styela plicata*. In turn, Cheney *et al.*[23] (Table 2, entry 6) used only the branchial basket of *Ascidia ceratodes*, *Ciona intestinalis*, and *Styela montereyensis*. As the authors stated, the cuts in the branchial baskets may expose the blood cells and increased the chance to accumulate metals.[14,23]

Conclusions

It was shown that the branchial basket is the most suitable tissue to monitor Hg in *P. nigra*. Also, this species adequately meets the criteria of a bioindicator, for being sedentary, reasonably abundant in places of interest, easy to identify and sample, and large enough for analysis. In addition, we use an accessible and easy-to-operate method without pre-treatment of the sample, with a sensitive capacity to quantify individual tissues without performing a pool of samples, which allows us to perform a greater number of analyzes *per* site with fewer individuals. Furthermore, this work includes results of metal accumulation in *P. nigra* in natural contexts. Future studies should focus on the geographical distribution of Hg bioavailability since it is affected by trophic state, quite variable in most coastal areas. In addition, studies on the physio-morphological effects of Hg in animals are also necessary.

Supplementary Information

Supplementary data (Tables S1-S8 and Figure S1) are available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

Author Contributions

Sabrina T. Martinez was responsible for conceptualization, formal analysis, investigation, visualization, and writing original draft; Caio S. Félix was responsible for formal analysis, investigation, visualization, and writing original draft; Rayane Sorrentino was responsible for investigation, visualization, writing original draft; Igor C. S. Cruz was responsible for investigation, validation, visualization, writing original draft, and Jailson B. de Andrade for conceptualization, formal analysis, visualization, and supervision.

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