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Feeding Habit and Lifestyle Influence the Baseline Micronuclei Frequency of Crab Species in Pristine Mangroves

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Abstract

Metals are harmful inorganic pollutants in aquatic environments when their concentrations are higher than expected (or tolerated) and, in low concentrations, they can lead sublethal genetic injuries. Baseline frequencies of micronucleated cells (MN‰) of three mangrove crab species were established in a pristine mangrove (Juréia-Itatins Ecological Station, JIES). *Aratus pisonii*, *Ucides cordatus* and *Goniopsis cruentata* belong to different functional groups, regarding the diet and lifestyle. Overall, the baseline MN‰ of *G. cruentata* (1.7 ± 1.2; mean ± sd) was higher than that of *A. pisonii* (0.9 ± 1.1) and *U. cordatus* (1.3 ± 0.9). These differences can be explained by the diet (gl, green leaves; sl, senescent leaves; a, animal items; or their combination) and lifestyle of these species, as their degree of contact with abiotic compartments (w, water; s, sediment). *Aratus pisonii* is an arboreal crab and specialist herbivore, associated with few compartments (w + gl); *Ucides cordatus* is a digger crab, generalist herbivore, using three compartments (w + s + sl); and *Goniopsis cruentata* is a cursorial crab, omnivorous, exploring more compartments (w + s + sl + a). Thus, using a broader range of compartments and a more diverse diet were correlated with a higher genotoxicity. Metals in JIES were registered in environmentally safe concentrations but seem to influence the baseline MN‰ in crab species. Higher genotoxicity was registered in species that interact with more compartments (especially the sediment), a fact that should be considered in monitoring processes.

Keywords Conservation · Crustacean · Estuary · Genotoxicity · Metal · Monitoring

Introduction

Mangroves are one of the most productive and relevant coastal environments worldwide (Kathiresan and Bingham 2001; Schaeffer-Novelli et al. 2016; Souza et al. 2018). In Brazil, they are considered as Permanent Protection Areas (PPAs), according to the Brazilian Forest Code (see Brasil 2012; Federal Law 12.651/2012). Despite their well-known ecological, economic and social role (Robertson and Duke 1987), 35% of the world's mangroves have been lost for several reasons (Valiela et al. 2001), resulting in diversity losses (Burnside 2018). Thus, mangrove management and preservation are urgent and crucial issues. Moreover, studies in pristine mangroves are very scarce and required to better understand the natural patterns of biological processes.

Metal concentrations have become an important criterion in the diagnosis of environmental quality due to their toxicity to biota, persistence in abiotic compartments (water and sediment), and role as indicator of human local disturbance (Luoma and Rainbow 2008; Abraham and Susan 2017; Duarte et al. 2017, 2019). These contaminants are difficult to break down, accumulate in the biota, and can be magnified through the trophic chains (Rainbow 2007; Pinheiro et al. 2013). Essential metals perform an important role in biological systems whereas non-essential are toxic even in trace amounts (Rainbow 2007).

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Due to the threatening environmental pollution, Brazilian Conservation Units (UCs) have become even more relevant to the protection of biodiversity (Bruner et al. 2001; Pinheiro et al. 2012). Considering the intense human population growth and loss of natural habitats, these UCs have become refuges for most of the biota that are intolerant to human activities, and thus have attracted a global interest (Kramer et al. 1997; Marques et al. 2017). Studies in such areas are essential as they help to understand how an ecologically healthy environment functions (Fraser and Bernatchez 2001; Duarte et al. 2019).

Since new monitoring techniques have been developed, the use of biomarkers has gained prominence in the assessment of environmental risk in natural populations (Moore et al. 1986; Depledge and Galloway 2005; Amiard-Triquet et al. 2013; Araújo et al. 2018). According to these authors, these biological assays can detect sublethal effects on organisms exposed to metal contamination. Biomarkers have been widely applied in studies with invertebrates, such as mollusks (Klobucar et al. 2003; Pereira et al. 2014; Reguera et al. 2018), annelids (Gomes et al. 2015; Barrick et al. 2016) and crustaceans, such as copepods (Raisuddin et al. 2007) and decapods (Boudet et al. 2013; Duarte et al. 2016, 2017, 2019). Crustaceans, in particular, have been considered an expressive biological model by several studies with toxic chemicals (as metals) that can be absorbed with the food and/or by contact with contaminated water and sediment (Marsden and Rainbow 2004; Luoma and Rainbow 2008; Abraham and Susan 2017; Harris et al. 2019).

Brachyuran crustaceans stand out due to their abundance in mangroves, where some species are endemics (Ellison 2008). According to Melo (1996), three crabs species are common in western Atlantic mangroves (from Florida-USA to the southern region of Brazil): *Aratus pisonii* (H. Milne Edwards, 1837), a sesarmid crab known as 'aratu-marinheiro'; *Goniopsis cruentata* (Latreille, 1803), a grapsid known as 'maria-mulata' or 'aratu-vermelho'; and *Ucides cordatus* (Linnaeus, 1763), the ocypodid 'uçá-crab'. These species are characterized by reduced vagility, distinct interactions with abiotic and biotic compartments, and diverse diets. In Brazil, *U. cordatus* has been used as a mangrove sentinel species (Pinheiro et al., 2013; Duarte et al. 2016, 2017) and the other two endemic species possibly have a high potential to assess the environmental quality of the mangroves (Pinheiro et al. 2017), a fact that has not been confirmed.

In crustaceans the absorption of pollutants occurs directly through the articulation membranes, gills, and by feeding (Rainbow 2007). Long term exposure to pollutants promotes negative effects on crustaceans, as some compounds bioaccumulate in the tissues and may cause malformations (Pinheiro and Toledo 2010; Lezcano et al. 2015).

In aquatic organisms, pollution may lead to genetic alterations that can be detected through a simple genotoxicity test such as the micronucleus assay (Carrano and Heddle 1973). Micronuclei are abnormal cytoplasmic structures formed by fragmented and/or lost chromosomes after cellular division. Since they are easily perceptible at any stage of the cell cycle (Countryman and Heddle 1976), they constitute a quick and accurate method to detect chromosomic aberrations (Heddle et al. 1983). This technique has been broadly used in studies with vertebrates (e. g., fishes: Ahmed et al. 2011; and mammalians: Narumi et al. 2012), and also successfully applied to invertebrates, such as bivalves (Scarpato et al. 1990; Pereira et al. 2014) and crabs (Pinheiro et al. 2013; Duarte et al. 2016, 2017, 2019). According to Bolognesi and Cirillo (2014) and Pereira et al. (2014), the micronucleus assay has a great ecological relevance, since it detects genetic damages that can harm the populations. Thus, studies aiming to characterize the biological variation of this biomarker in aquatic organisms are essential, especially in pristine areas, where a better distinction between normality (baseline) and abnormalities (Cenov et al. 2018) can be made. The micronuclei assay method quantifies the micronuclei frequency (Pinheiro et al. 2013). Duarte et al. (2016) indicated that an area considered as 'Probably Not Impacted' (well-preserved) has specimens of *U. cordatus* showing less than three micronucleated cells for one thousand analyzed. Therefore, the description of a species' baseline micronuclei frequency allows further comparisons between conditions and areas. This technique can be used as an environmental monitoring tool, to classify areas according to the species' responses to metal contamination by anthropic or natural ('background') sources.

Mangrove crab species occupy distinct ecological niches and have different lifestyles, regarding the diet, feeding frequency, and variety of abiotic and biotic compartments they explore (e. g., water, sediment, and/or vegetation). Thus, the combined study of species from different functional groups can portray a more complete picture of an ecosystem's quality (Jha 2008). Some native species of western Atlantic mangroves (e. g., *Goniopsis cruentata* by Davanso et al. 2013; and *Ucides cordatus* by Pinheiro et al. 2017 and Duarte et al. 2016, 2017, 2019) have been used to monitor the quality of mangroves.

This study aimed to establish the baseline frequency of micronuclei cells (MN‰) of three crab species (*Aratus pisonii*, *Goniopsis cruentata* and *Ucides cordatus*), and to evaluate its seasonal variation (summer/rainy vs. winter/dry). Knowing the genetic baseline of these species is particularly important in order to establish what would be expected in a pristine mangrove. These species belong to distinct functional groups and coexist in the pristine mangroves of Juréia-Itatins Ecological Station (JIES) in the state of São Paulo, Brazil. Since they interact with abiotic (water and sediment) and biotic (distinct food items) compartments in different ways, a secondary aim was to quantify the metals in these compartments in order to link the micronuclei baseline with diet and lifestyle of the crab's species. Finally, in order to validate the assumption that JIES mangroves are pristine, the metal contamination was also quantified in a well-known polluted mangrove from the same Brazilian region, and used for comparison. The data obtained here can be used as reference values and/or compared to other species of the same functional groups living in mangroves (Depledge and Fossi 1994).

Materials and Methods

Mangrove Study Area and Sampling

Two samplings, covering two seasons (summer/rainy season: February 2014; and winter/dry season: August 2014), were carried out in the state of São Paulo, southeastern region of Brazil. The mangrove area belongs to the Juréia-Itatins Ecological Station (JIES) (Fig. 1), a conservation unity integrated to the Conservation Mosaic (São Paulo 2013). The JIES mangroves occupy 40 km of coastline and harbor several well-preserved rivers, due to the reduced human contingent and anthropogenic impacts (Pinheiro et al. 2013). According to Por et al. (1984), Duleba and Debenay (2003) and Marques and Duleba (2004), this region has semidiurnal tides (0.1–1.5 m) and narrow temperature variation. Salinity and pH are higher in the dry season than in the rainy one. According to the climatic classification of Köppen-Geiger (Alvares et al. 2014), JIES mangroves are within the tropical humid region, where the highest average temperature and rainfall occur from January to March, and the lowest from May to August.

Specimens were captured in three mangrove subareas in the JIES estuary of river 'Una do Prelado', as follow: **JUR1** (24°26′14.4"S; 47°04′34.2"W), **JUR2** (24°26′3.8"S; 47°04′32.2"W) and **JUR3** (24°25′48.3"S; 47°04′52.7"W). For standardization purposes, five individuals of each crab species (*A. pisonii*, *G. cruentata* and *Ucides cordatus* – Fig. 2) were manually captured in each subarea and season ($n = 15$/species/season), all of them males in intermolt stage (see Pinheiro and Fiscarelli 2001). This approach avoids the confounding effects of sex and molting stage, previously reported for other decapod crustaceans (see Pinheiro et al. 2012). A precision caliper (0.05 mm) was used to record the body size (CW, carapace width), and the individuals were considered as replicates ($n = 30$ specimens/species). Only animals with body size >2/3 of the maximum size (CW) of each species were included in the study, as follow: CW > 17 mm in *A. pisonii*, according Leme et al. (2014); CW > 37 mm in *G. cruentata*, as informed by Moura and Coelho (2004); and CW > 64 mm in *Ucides cordatus*, based on the review by Pinheiro et al. (2005). The animals were kept individually in plastic bags (with leaves and twigs of mangrove trees), to avoid agonistic behaviors during transport. They were transported to the laboratory in plastic boxes with 1–3 cm of brackish water (salinity 15), depending of the individual size.

Fig. 1 Juréia-Itatins Ecological Station (JIES), state of São Paulo, Brazil. Location of the three mangroves subareas (JUR1, JUR2 and JUR3), in the estuary of river 'Una do Prelado'. Source: Google Earth Image © 2020 DigitalGlobe

In the laboratory, after the biometry, each crab specimen was submitted to hemolymph puncture to the confection of slides used in the analysis of macrolesions evaluation (see details at 'Micronucleus assay' item), following by extraction

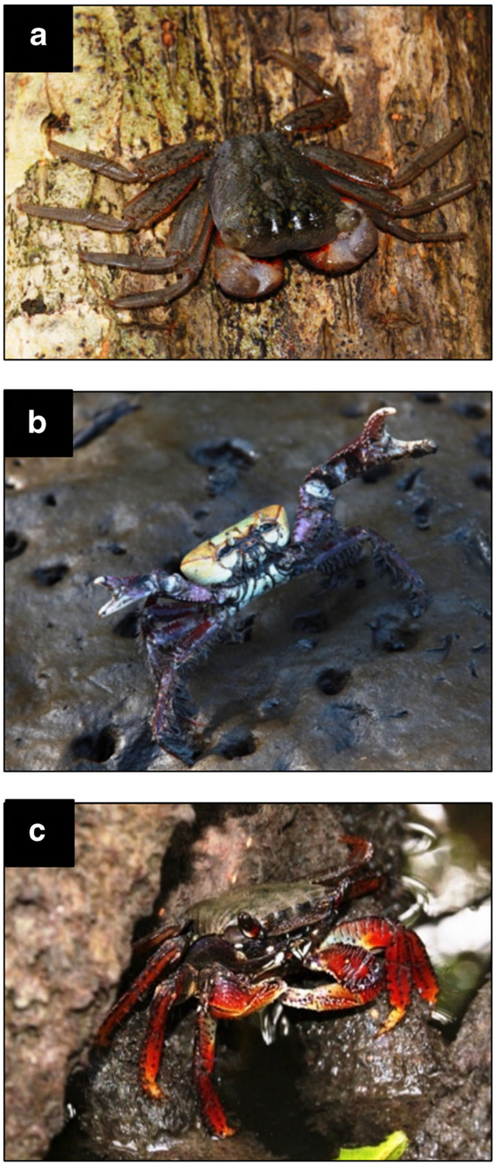

Fig. 2 Mangrove crab species and their lifestyle and feeding habit. A) *Aratus pisonii* (H. Milne Edwards, 1877), an arboreal sesarmid crab that feeds on green leaves; B) *Ucides cordatus* (Linnaeus, 1763), a digger ocypodid crab that feeds on senescent leaves; and C) *Goniopsis cruentata* (Latreille, 1803), an omnivore cursorial grapsid crab that feeds on vegetal and animal items, under decomposition (or not)

of tissues of each one to metal quantification (see the following item). Before the latter procedure (dissection), each animal was cryo-anesthetized (−20 °C), by submersion in crushed ice for 15 min, followed by euthanasia (insertion of a forceps or scissors inside the crab mouth and rotating to disrupt the cerebral ganglia). This is a recommended procedure but not mandatory because according to Brazilian Law for Good Practices and Animal Welfare, scientific experimentations or projects don't need authorization emitted by a specific committee when non-chordate animals (invertebrates) are sacrificed.

Sampling, Sources of Contamination and Metal Quantification

In order to assert the environmental quality of JIES mangroves, the metal concentrations were compared to those of Cubatão (**CUB1:** 23°53′02.9"S - 46°21'54.6"W; **CUB2:** 23°54′02.4"S - 46°22'56.9"W; and **CUB3:** 23°55′08.0"S - 46°23'04.8"W). Cubatão is located 95 km away from JIES and is characterized by an intense history of metal contamination (Pinheiro et al. 2013; Duarte et al. 2016, 2017). In both mangroves, samples of water and sediment were obtained to quantify the total concentrations of six metals (Cd, Cu, Pb, Cr, Mn and Hg), using a specific atomic spectrophotometer (GBC - 932 AA) after the acid digestion of the samples, according to Athanasopoulos (1994) and the protocol previously described by Pinheiro et al. (2013). In each mangrove subarea, three water samples (100 mL) were collected by suction with a silicone hose and stored in labeled polyethylene bottles (n = 18: 6 subareas vs. 3 samples). Surface sediments samples (< 10 cm) were collected with nitrile gloves in each studied area by a composite sampling method, which provide a more adequate resolution to characterize the contamination (Garner et al. 1988). These samples (500 g) were equally obtained in the same subareas (n = 18) and kept in labeled polyethylene bottles, after removal of roots by sieving.

In JIES mangroves the bioaccumulation was studied through the quantification of metal concentrations in green and senescent leaves (used by specialist and generalist herbivores, respectively, and by omnivores), crustacean tissues (used by carnivores and omnivores), thus representing all sources of contamination. Leaves of the red-mangrove tree (*Rhizophora mangle*), in two maturation stages (gl, green mature; and sl, pre-abscission senescent), were collected following Pinheiro et al. (2012). Twenty leaves of each stage were removed with pruning shears, placed in labeled plastic bags, transported to the laboratory, and washed to avoid contamination by atmospheric pollution (1st running water: water +5% neutral detergent; 2nd running water: distilled water + HCl saturated; and finally with distilled water). To evaluate the contamination of an animal prey available to omnivore crabs we used muscle and hepatopancreas samples from *Ucides cordatus* (n = 3 ind./subarea).

The total concentrations of Cd, Cu, Pb, Cr, Mn and Hg were determined in each sample (water, sediment, leaves of *R. mangle*, and tissues of *U. cordatus*) using the mineralization method with HNO_3 at 65%, according to Basset et al. (1981). Analyses were optimized by hollow cathode lamps (LCO), according to the metallic element, and samples were read using a GBC-932 AA atomic absorption spectrophotometer (Athanasopoulos 1994). The equipment was calibrated with metal stock solutions (1000 ppm). Metal concentration was expressed in a dry weight basis. The units of measurement and detection limits were as follows: Cd < 0.01 μg/g; Cu and Mn < 0.02 μg/g; Pb and Cr < 0.05 μg/g; and Hg <1.10^{-6} μg/g.

Micronucleus Assay

A hemolymph sample (0.2 mL) was taken from each crab using a hypodermic syringe (1 mL) coupled to a 21-gauge needle to avoid damage to the hemocytes, as recommended by Nudi et al. (2010). The method described in Scarpato et al. (1990) and adapted to brachyurans by Pinheiro et al. (2013) was used to prepare slides of hemolymph ($n = 5$/specimen). The slides were air-dried (20 min.), immersed in Carnoy solution (3:1, methanol: acetic acid) for cell fixation (20 min.), and air-dried again. Each slide was stained (15 min.) with Giemsa solution (2%) ($Na_2HPO_4 + KH_2PO_4$, pH 6.8), washed with deionized water and air-dried. In order to identify the number of micronucleated cells per 1000 analyses (MN‰), the slides were observed under a Zeiss® optical microscope (1000 x). Notes about the nuclear formations were taken according to the characteristics proposed originally by Countryman and Heddle (1976).

Statistical Analyses: Crab Lifestyle and Genetic Damage

The homogeneity and normality of the variances were inspected using Levene's (L) and Shapiro-Wilk's (SW) tests, respectively. The confirmation of normality ($P > 0.05$) allowed the use of parametric tests. Thus, the means were compared by ANOVA (subarea factor) and t-tests (species and seasons) (Zar 1999; Faraway 2002).

Micronuclei frequencies (MN‰) were compared by a three-way factorial ANOVA to test the significance of the effects of three sources of variation (factors) – two seasons (SE): summer/rainy and winter/dry; three species (SP): *G. cruentata*, *A. pisonii* and *U. cordatus*; and three subareas (LO): JUR1, JUR2 and JUR3. We also included in the model the respective first- and second-order interactions. Data were analyzed in R Version 3.3.2 (Ihaka and Gentleman 1996), considering a statistical significance level of 5%. In all cases, the multiple comparison of means was followed by a Tukey's honestly significant difference test (Zar 1999).

We investigated whether the baseline micronuclei frequency was influenced by metals (even if present in safe concentrations), or natural and intrinsic to each crab species. For this purpose, the concentration of the six metals was measured in each environmental/food compartment used by the crabs in JIES, which was assumed based on their lifestyles: **1)** ***A. pisonii*** (water + green leaves), are primarily herbivorous sesarmid species that feed mainly on green leaves (Erickson et al. 2003) that can be easily picked from the tree canopy. Being an arboreal sesarmid species, they contact with the sediment is very limited, but they are exposed to the water that is used to renew the oxygen in the gill chambers (Erickson et al. 2008; Riley et al. 2014); **2)** ***U. cordatus*** (water + sediment + senescent leaves), is a digger ocypodid species that has a close contact with the sediment and water during the mangrove flooding; they are not adapted to climb trees and access green leaves. As detritivores (Christofoletti et al. 2013) they feed on senescent leaves available on the sediment during lower tides (Duarte et al. 2017); and **3)** ***G. cruentata*** (water + sediment + senescent leaves + animals), is a typical omnivorous cursorial grapsid species and a generalist feeder which exploits most of the items available on the mangrove sediment (Gomes et al. 2011; Ferreira et al. 2013). As their diet includes live and dead animals, we considered *U. cordatus* as a prey of *G. cruentata*. The contamination of this compartment was taken as the sum of metal concentration in two tissues: hepatopancreas (main detoxification organ of decapod crustaceans – see Rainbow 2007 and Eisler 2010) and muscles (accumulation already reported by Pinheiro et al. 2012).

Based on the known distinct toxicity potential of metals and potential risk to the aquatic environment (i. e., Hg > Cu > Cd > Pb > Mn > Cr; adapted according to studies performed by Wong and Bradshaw 1981, Sinha et al. 1993, Luoma and Rainbow 2008 and Eisler 2010), we propose the application of a weighted toxicity potential, represented by the hierarchical sequence 3 > 2.5 > 2 > 1.5 > 1 > 0.5 (relative to the previous sequence of metals). These results were then multiplied by a correction factor, according to the type: 1x for essential metals (Cu, Mn and Cr) and 2x for non-essential metals (Hg, Cd and Pb). Taking into account that concentrations of metals have different orders of magnitude, the data were standardized by the equation,

$$MC_i = log[(M_i{*}T_i{*}C_i) + 1]$$

where: MC_i, log-transformed concentration of each 'ith'-metal in each compartment; M_i, concentration of the 'ith'-metal (in μg/mL or μg/g) in each compartment; T_i, weighed toxicity potential of the 'ith'-metal; and Ci, correction factor of the 'ith'-metal. A total standardized concentration value (MCS) was calculated, comprising the sum of MC_i by compartment, posteriorly summed per species, according to the compartments used by them. A figure was made to better illustrate the relationship between the exposure of crab species to the metals (MCS) and their species-specific genetic damage (MN‰).

In the analysis of the total concentrations of six metals in the water and sediments of JIES and Cubatão, the normality of the data was evaluated with the Shapiro-Wilk test (SW). Then, the concentrations were compared using non-parametric (Mann-Whitney, Z) or parametric (*t*-test) tests (Zar 1999). The results were compared with the reference values established by CONAMA (resolution n° 357/2005 – Brasil 2005), and North American (EPA 2017) and European Council (DIRECTIVE 2008/105/EC 2008) legislation, for estuarine waters, and by Environmental Canada (1999), for sediments (see PEL, Probable Effect Level; and TEL, Threshold Effect Level).

Results

The metal concentrations in the water, sediment and green and senescent leaves of both mangroves (JIES and Cubatão) were not normally distributed (SW≤0.95, $p > 0.05$), requiring a comparison by non-parametric tests (KW, Kruskal-Wallis).

The metal concentrations in the water samples of JIES mangroves were below the detection limits, in contrast with Cubatão (Table 1). Sediment samples from Cubatão mangroves were contaminated by Cd and Pb (non-essential metals), which were only found in this area. The concentration of Hg was 1.8x higher than in JIES. On the other hand, the concentrations of two essential metals (see Cr and Mn, Table 1) were higher in the sediments of JIES. The inverse occurred with Copper, which was 2.3x higher in Cubatão. Copper concentrations in the green and senescent leaves were similar, and 1.7 to 2.9x higher in Cubatão, respectivelly. In contrast Mn concentrations were 56.5 to 69.5x higher in JIES. In Cubatão, the tissues of *U. cordatus* were significantly contaminated by Cu (2.0x higher than in JIES), while the concentration of Mn and Hg was similar in the studied mangroves.

Table 1 shows the MC_i per metal and per compartment (environment: water and sediment; food item: green leaves, senescent leaves and animal tissues) as well as the MCS of each compartment in JIES. These data corroborated our initial assumption that JIES mangroves are pristine, given the lower

Table 1 Concentration of metals (in µg/mL or µg/g) per compartment (environment: water and sediment; diet item: green leaves, senescent leaves and animal), recorded in the mangroves of Juréia-Itatins Ecological Station (JIES) and Cubatão, in the state of São Paulo, Brazil. Abbreviations: x, mean; se, standard error; Z, Mann-Whitney test; p, statistical significance; MCi, log-transformed concentration of the 'ith'-metal, based on the equation $MC_i = log[(M_i * T_i * C_i) + 1]$; Mi, 'ith'-metal concentration (in µg/mL or µg/g); T_i, toxicity potential factor of the 'ith'-metal; C_i, correction factor to 'ith'-metal type; MCS, MC_i sum of each compartment; and nd, not detectable

Compartment (Environmetal or Food)	Metals	JIES x±se	Cubatão x±se	Z	p	JIES's Metal Concentration	
						MC_i	MCS
Water	Cu	nd	0.013±0.003	–	–	0	0.00
	Cr	nd	nd	–	–	0	
	Mn	nd	0.0027±0.0002	–	–	0	
	Cd	nd	nd	–	–	0	
	Pb	nd	0.18±0.01	–	–	0	
	Hg	nd	nd	–	–	0	
Sediment	Cu	2.47±0.10	**5.76±0.44** *	3.576	0.0004	0.86	6.52
	Cr	**18.24±0.41**	8.00±0.40	−3.576	0.0004	1.01	
	Mn	**77.81±21.18**	0.72±0.12	−3.576	0.0004	1.90	
	Cd	nd	0.88±0.03	–	–	0	
	Pb	nd	9.74±0.64	–	–	0	
	Hg	92.53±12.19	**166.10±9.64**	3.488	0.0005	2.75	
Green Leaves	Cu	0.72±0.10	**2.13±0.22**	3.576	0.00035	0.45	3.02
	Cr	3.69±0.14	nd	–	–	0.45	
	Mn	**130.64±12.26**	1.88±0.17	−3.576	0.0004	2.12	
	Cd	nd	0.22±0.01	–	–	0	
	Pb	nd	1.86±0.15	–	–	0	
	Hg	nd	nd	–	–	0	
Senescent Leaves	Cu	0.58±0.09	**0.99±0.05**	2.428	0.0152	0.39	2.91
	Cr	3.75±0.47	nd	–	–	0.46	
	Mn	**114.05±13.55**	2.02±0.09	−3.576	0.0004	2.06	
	Cd	nd	0.23±0.02	–	–	0	
	Pb	nd	1.95±0.15	–	–	0	
	Hg	nd	nd	–	–	0	
Animal	Cu	6.74±0.70	**13.08±0.48**	3.576	0.0004	1.25	3.84
	Cr	nd	nd	–	–	0	
	Mn	1.79±0.20	1.88±0.24	0.353	0.7239	0.45	
	Cd	nd	nd	–	–	0	
	Pb	nd	nd	–	–	0	
	Hg	22.63±9.04	10.28±1.76	−0.397	0.6911	2.14	

[*] bold numbers represent contrasting and higher values with statistical difference

metal concentrations in almost all compartments. Also, between Cubatão and JIES there were the significant differences in Cu and Hg in the sediment, Cu in green/senescent leaves and in animal tissues ($P < 0.05$). Water contamination by metals in JIES was below the detection limits (MCS = 0), while the sediment had the highest weighted toxicity potential (MCS = 6.52), about twice the value of each food compartment (MCS ranging from 2.91, in senescent leaves to 3.84, in the animal tissues).

The metal concentrations (%) of specific compartments were evaluated in order to understand their potential toxicity (Fig. 3). These compartments were either environmental (w, water; s, sediment) or part of the crab's diet (gl, green leaves; sl, senescent leaves; and a, animal tissues). The results were as follows: Copper, a > s > (gl ~ sl); Chromium, s > (gl ~ sl); Mercury, s > a; and Manganese, (gl ~ sl) > s > a. Overall, the most contaminated sources were sediment (Hg, 56%; and Cr, 53%) and animal tissues (Cu, 43%; and Hg, 44%). Green and senescent leaves (vegetal compartments) were responsible mainly by Mn and Cr contamination, represented by 64% and 47%, respectively.

Considering the metal concentrations of each compartment studied, we calculated the relative contribution of each metal as a contamination source, depending on the number of compartments used by each crab species (Fig. 4 – left). The results were Mn > Hg > (Cu ~ Cr). Figure 4 (right) shows the expected higher contamination effect of the sediment, followed by animal tissue, while the two types of leaves were similar and less contaminated, i. e., s > a > (gl ~ sl).

The body size of *A. pisonii* ranged from 18.3 to 28.3 mm (mean ± s.d.: 22.1 ± 2.5 mm CW), while the size of *U. cordatus* and *G. cruentata* ranged from 63.6 to 94.2 mm CW (78.0 ± 7.6 mm CW) and 33.2 to 53.1 mm CW (44.5 ± 4.7 mm CW), respectively.

The frequency of micronucleated cells varied significantly among species ($F = 4.59$; $P = 0.01$; Fig. 5). *Aratus pisonii* had the lowest mean value (mean ± se: 0.9 ± 0.15 MN‰), *G. cruentata* the highest one (1.7 ± 0.13 MN‰) ($p = 0.01$), and *Ucides cordatus* ($P = 0.19$) had intermediate values (1.3 ± 0.12 MN‰). The MN‰ of each species was slightly higher in winter than in summer, but these differences were not statistically significant ($P > 0.05$).

A three-way factorial ANOVA (Table 2) confirmed a significant variation of frequency of micronucleated cells (MN ‰) between 'Species' (code 'SP': $F = 4.59$, $p = 0.01$) and between 'Seasons' (code 'SE': $F = 8.16$, $P = 0.01$), but not between 'Localities' (code 'LO': $F = 0.74$, $P = 0.47$). First order interactions between treatments were not significant ($F > 0.28$, $p > 0.67$), but there was one significant second order interaction: SP x LO x SE. This can be explained by the fact that the highest frequency of micronucleated cells was recorded in winter, in the case of *G. cruentata*, and in summer, in the case of *A. pisonii* and *U. cordatus* it ($P < 0.05$).

The micronuclei frequency of *G. cruentata*, *A. pisonii* and *U. cordatus* was independent of the broad climatic variation between summer/rainy and winter/dry season. However, it was related to the metal toxicity effect in the compartments that are explored by each species. Corroborating our assumptions about the degree of exposure to metals, the results indicate that *G. cruentata* is relatively more exposed than

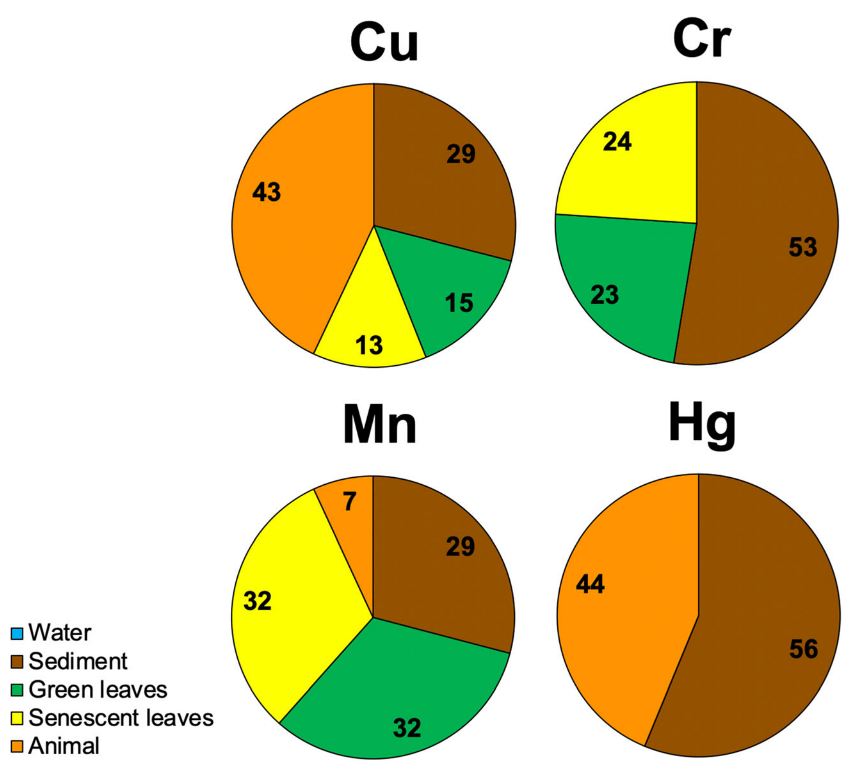

Fig. 3 Potential toxicity (%) of each metal (Cu, Cr, Mn and Hg) per compartment (environmental: water and sediment; and biotic / food item: green leaves, senescent leaves and animal), recorded at the Juréia-Itatins Ecological Station (JIES)

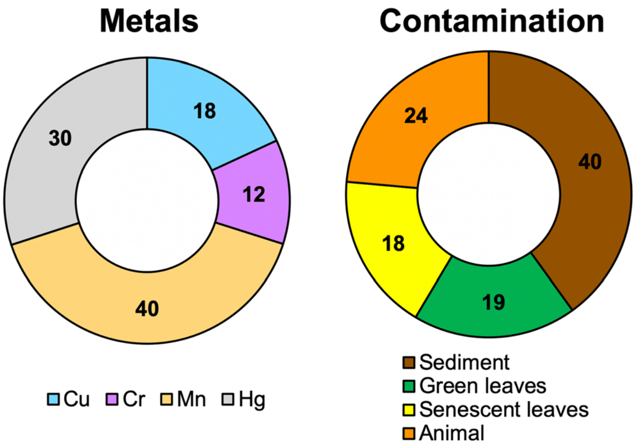

Fig. 4 Total potential toxicity (%) of each metal (Cu, Cr, Mn and Hg), considering all compartments studied (left), as well as the contamination potential of each compartment (right) in the Juréia-Itatins Ecological Station (JIES)

U. cordatus and *A. pisonii*, since their weighted toxicity potential (MCS%) were 81.5, 57.9 and 18.5%, respectively (Fig. 6). These values are correlated with the baseline micronuclei frequency of these species (1.7, 1.3 and 0.9 MN%, respectively), and with the number of compartments explored by them (3, 2 and 1, respectively).

Discussion

According to the studies of Souza and Barrela (2001) and Pinheiro et al. (2013) the Juréia-Itatins Ecological Station (JIES) has a high conservation status, certainly due to the low human density (only artisanal fishermen and local farmers live there) and absence of activities like industrial and urban areas, large ports, etc.). Our assumptions about its pristine condition was confirmed, especially when we compared our results with other mangroves from the central coast of São Paulo, where anthropic effects were recorded (see Pinheiro et al. 2013; Duarte et al. 2016, 2017). In JIES, five out of the six metals studied were not detected in the water, when compared with CONAMA (# 357/2005), North American (EPA 2017) and European Council (DIRECTIVE 2008/105/EC 2008) legislation. Metal concentrations in the sediment were also below the reference values established by Environmental Canada (1999). In contrast, in the mangroves of Cubatão, also characterized in this study, higher concentrations of Cu and Pb were detected in the water, indicating that this mangrove is near the contamination sources and there is a continuous discharge of soluble pollutants (Rainbow 2007). Also, the concentrations of Cd and Hg in the sediment were above the threshold considered environmentally safe.

The frequency of micronucleated cells (MN‰) of each crab species was below three, similar to the results of Pinheiro et al. (2013) and Duarte et al. (2016, 2017, 2019). A narrower interval (0–1 MN‰) was reported in a review of twenty-six fish species in pristine areas by Bolognesi and Hayashi (2011). It seems that aquatic animals, even from distinct taxonomic groups, have similar basal values in well-preserved environments. Nonetheless, the increase in stressors in estuarine environments and the contamination of biotic and abiotic compartments could affect the biochemical and physiological responses of organisms and result in expressive genetic damages (Monserrat et al. 2007).

Aratus pisonii, *U. cordatus* and *G. cruentata* belong to three families of the Infraorder Brachyura (Sesarmidae, Ocypodidae and Grapsidae, respectively), which stand out due to the diversity of morphological, physiological and behavioral adaptations used to maintain homeostasis (Mantel and Farmer 1983).

Aratus pisonii is an arboreal crab that lives in tree trunks and branches but often descends from the treetops during high tides to hydrate and replace the water in the gill chambers. Through this behavior it avoids the osmotic stress by desiccation in warmer days (Wolcott and Wolcott 2001), controls the body temperature (Young 1972), and optimizes respiration and ammonia excretion (Weihrauch et al. 1999, 2004; Henry et al. 2012). Despite the high concentration of tannins and polyphenolic substances found in mangrove leaves, they make up to 42% of the stomach content of *Aratus pisonii* (Lacerda et al. 1991), which is thus considered as an herbivore. Green leaves, however, have a higher concentration of nutrients (Faraco and Lana 2004; Christofoletti et al. 2013).

Goniopsis cruentata is a very active grapsid crab that has an expressive cursorial behavior on mangrove sediments. It does not build galleries but occupies crevices among roots and burrows of other crab species, when it needs protection from predators. Due to its intense foraging activity, *G. cruentata*

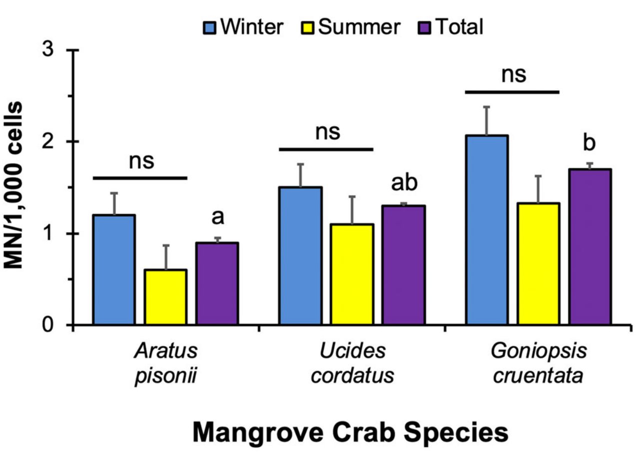

Fig. 5 Number of micronucleated cells per thousand (MN‰) in mangrove crab species of the Juréia-Itatins Ecological Station (JIES), per season (winter, dry season; summer, wet season; $n = 15$ crabs/species/season) and in total ($n = 30$ crabs/species). The bars indicate the average and the vertical lines, the standard error; ns = non-significant ($P > 0.05$); different letters indicate significant differences ($P < 0.05$)

Table 2 Three-way factorial ANOVA used to compare the frequency of micronucleated cells (MN‰) in relation to three sources of variation (SE, Seasons: summer/rainy and winter/dry; SP, Species: *Goniopsis cruentata*, *Aratus pisonii* and *Ucides cordatus*; and LO, Localities: three subareas sampled), in mangroves of the Juréia-Itatins Ecological Station, state of São Paulo, Brazil. Abbreviations: F, F-test; df, degrees of freedom; *P*, level of statistical significance; MQ, mean square; SQ, sum of squares

Sources of Variation	*df*	*SQ*	*MQ*	*F*	*P*
Species (SP)	**2**	**9.60**	**4.800**	**4.593**	**0.01**
Localities (LO)	2	1.55	0.775	0.742	0.47
Seasons (SE)	**1**	**8.53**	**8.533**	**8.165**	**0.01**
SP x LO	4	1.35	0.337	0.323	0.86
SP x SE	2	0.60	0.300	0.287	0.75
LO x SE	2	0.82	0.408	0.391	0.67
SP x LO x SE	**4**	**10.15**	**2.538**	**2.428**	**0.05**

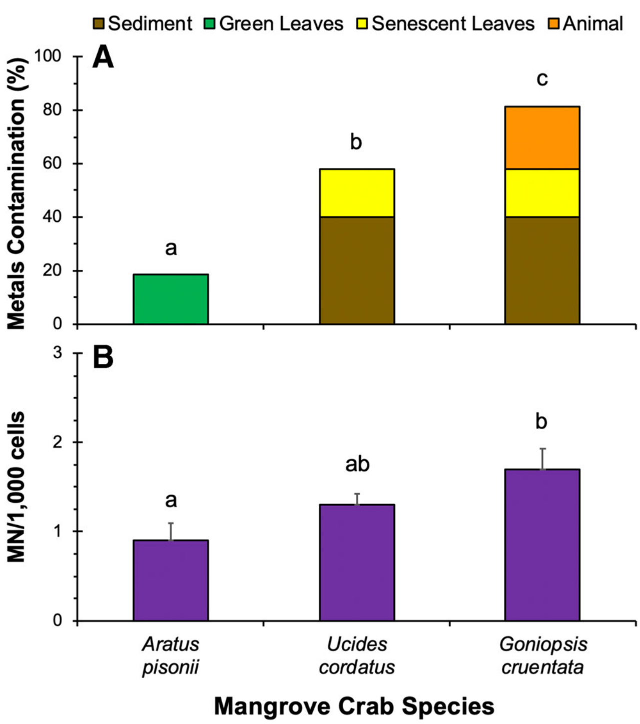

Fig. 6 Metal contamination and crab genotoxicity. (**a**) Percent of metal contamination by compartment (environmental: w, water; s, sediment; and food items: gl, green leaf; sl, senescent leaves; a, animals). The compartments are used by the mangrove crab species, as follow: *Aratus pisonii* (w + gl), an arboreal, herbivore crab that feeds on green leaves; *Ucides cordatus* (w + s + sl), a digger, herbivore crab that feeds on senescent leaves; and *Goniopsis cruentata* (w + s + sl + a), a cursorial, omnivore crab. The bars indicate the metal concentration (%) after log-transformation and correction by toxicity and metal type, to each resource used (see text for details). (**b**) Baseline frequency of micronucleated cells in mangrove crab species in the Juréia-Itatins Ecological Station (JIES), Brazil. The vertical lines indicate the mean and standard error. In both (**a**) and (**b**), different letters indicate significant differences ($P < 0.05$)

can find and consume diverse food resources within its preference, i. e., decaying vegetal (leaves and propagules) and animal tissues (Lima-Gomes et al. 2011). This species is an opportunistic predator, which is corroborated by strong mouthparts adapted for food maceration. Its animal preys are captured by active predation or necrophagy (Lima-Gomes et al. 2011). During the flooding of mangrove areas at high tides, it climbs on the arboreal vegetation (up to ~3 m) and descents to replace the water of its gill chambers, which, in the case of JIES, is not contaminated by metals. According to Ferreira et al. (2013) this species is in contact with a broad range of compartments and is an active predator. In addition, *G. cruentata* is a great osmoregulator (Zanders 1978; Zanders and Hammer 1984) due to the good performance of their antennal glands (Zanders 1978). It avoids stressful of osmotic and heat conditions by climbing trees or sheltering among the roots (Zanders and Hammer 1984).

Although metals are within safe concentrations in all compartments of JIES mangroves, we investigated whether their merely presence could be genotoxic to crabs and bioaccumulated through physical contact and/or feeding. In fact, the differences in micronuclei frequency between the three species can be explained by this hypothesis, since they occupy different trophic positions in the mangrove, and the concentration of contaminants is higher in the highest trophic levels (Baumard et al. 1998; Evans et al. 2000; Bodin et al. 2008). This fact can explain the higher frequency of MN‰ in *G. cruentata* (an omnivorous/generalist species) than in *A. pisonii* and *U. cordatus*. These two last species are primarily herbivores; *U. cordatus* forages on senescent leaves and propagules that compose the mangrove litter (Lacerda et al. 1991; Christofoletti et al. 2013). *Ucides cordatus* and *G. cruentata* interact more often with the mangrove sediment, where they forage for leaves and seedlings, promoting the recycling of nutrients (Wellens et al. 2015). The 'uçá'-crab is a well-known agent of sediment bioturbation, which promotes the oxygenation of sediments and influences the dynamics and bioavailability of metals (Vilhena et al. 2013; Araújo-Junior et al. 2016; Silva et al. 2018). Although *G. cruentata* does not dig burrows in the sediment, it explores the substrate at low tides, foraging on algae, animal carcasses, and parts of higher plants (Lima-Gomes et al. 2011). It also acts as a mangrove predator, eventually using the burrows of 'uçá'-crab and tree roots as a refuge during the high tides (Wellens et al. 2015). On the contrary, *A. pisonii* feeds mainly on green leaves (herbivore) and has a reduced contact with the sediment due to its arboreal habits (Díaz and Conde 1989). Thus, it is safe to assume that *G. cruentata* and *Ucides cordatus* are in contact with a wider range of environmental compartments than *A. pisonii*. In turn, the use of more contamination sources, even at extremely low levels, explains the higher MN‰.

Seasonal differences in biological responses, indicated by biomarkers, are known in several aquatic organisms (Bodin et al. 2004; Hagger et al. 2010; Cenov et al. 2018). However, most of these studies were done in temperate regions, where seasonal variation is more pronounced than in the tropical region of Brazil. The higher frequency of MN‰ in *G. cruentata* and *Ucides cordatus* in winter (compared to *A. pisonii*) indicates a greater sensitivity to stress caused by natural physical and biochemical variability in their habitats. According to studies on fishes (see Hughes and Hebert 1991; and Amado et al. 2006), the activity of DNA repair enzymes may decrease during winter, leading to an increased chromosomal damage. Also, the reproductive cycle can influence the antioxidative defense mechanisms, leading to more sublethal effects during reproduction. Similar results have been found in mussels (Bocchetti et al. 2008), where higher MN‰ values were recorded in late autumn. The absence of species-specific seasonal differences in MN‰ indicates a reduced seasonal effect in the tropical region.

The study of sentinel species and biomarkers in pristine environments brings relevant information that helps to understand their conservation status. These studies are especially important in the case of widely impacted coastal ecosystems such as mangroves. Therefore, the study of three endemic and abundant species of Brazilian mangroves, with reduced vagility and different lifestyles, contributes to the better understanding of the conservation of western Atlantic mangroves. We propose that mangroves can be considered as well-preserved (or pristine) when the mean MN‰ of two very different species — i. e., one that uses few compartments (like *A. pisonii*), and another who uses many (like *G. cruentata*) — is <1 and < 2 MN ‰, respectively. Similar values (MN‰ < 3) were recorded by Duarte et al. (2016) in *U. cordatus* in six mangrove areas ranging from pristine to impacted. In their study, a MN‰ < 3 indicated that an area is 'Probably Not Impacted' (PNI), that is, well-preserved. We propose that the assessment and monitoring of mangrove environmental quality can be minimally based on two sentinel species. This approach allows the comparison of different areas, considering specific morpho-physiological adaptations (Pinheiro and Fiscarelli 2001), and pollutant pathways and their kinetics in these organisms (Luoma and Rainbow 2008; Duarte et al. 2017, 2019; Ortega et al. 2016, 2017). These procedures optimize the performance (Bruner et al. 2001) and effectiveness of monitoring (Chape et al. 2005).

Long-term sublethal damage caused by pollutants may be irreversible in species with higher levels of exposure. The genotoxicity can be influenced by body size (individual age vs. exposition time), contamination levels in the environmental/food compartments used, habitat preference, behavior, and morpho-physiological characteristics of species. Thus, our assessment can be used as a guide in the environmental diagnosis and development of management strategies to be applied to mangroves. It is a useful tool to categorize the conservation status of mangroves along the western Atlantic coast, where these three crab species occur.

We presented evidence that metals, even at concentrations considered environmentally safe, may influence the baseline frequency of micronucleated cells in crabs of well-preserved mangroves. Our results can be used to build a protocol that, after a short training by different organizations (governmental or non-governmental), can be employed in the monitoring and conservation of western Atlantic mangroves. Our approach represents a new protocol, which can be used to better understand the potential toxicity by metals in mangrove areas.

Conclusions

The assumed pristine condition of Juréia-Itatins Ecological Station was confirmed, based on the quantification of metal concentrations in environmental compartments. The mean number of micronucleated cells per thousand (MN‰) of three mangrove crab species was <3 MN‰, which is expected in pristine environments, according to the literature. The overall mean MN‰ was higher in winter than in summer, indicating a higher genotoxicity in this period. However, there were no differences when seasonality was compared within each species, probably due to lack of striking differences between these two seasons in the tropical region. Metals, even at safe concentrations in environmental compartments, can influence the baseline micronuclei frequency of crab species in pristine mangroves. The longer the species have contact with a broad range of environmental compartments and diets, the higher the frequency of micronucleated cells. Thus, crab species that interact more with the sediment (where contaminants usually are proportionally more concentrated), such *Goniopsis cruentata* and *Ucides cordatus*, are suitable species to be used as sentinels of mangrove environmental quality. Our results provide new insights on the baseline micronuclei frequency of crab species from pristine mangroves, which may support the management of mangrove ecosystems.

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Authors' Contributions MAAP had the concept and sampling design of this study, obtain financial resources to be executed, participated in all fieldwork, executed the statistical analysis, build graphics/photos, took principal responsibility for writing the manuscript and addressed all revisions. NK participated in all fieldwork, assisted in data analysis, and contributed to writing the manuscript. CAS participated in all fieldwork, contributed to writing the manuscript, and aid in all revisions. LFAD participated sometimes fieldwork, assisted in data analysis and statistics, contributed to writing the manuscript and to responding the reviewers.

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Availability of Data and Material Not applicable.

Code Availability Not applicable.

Declarations

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

Conflicts of Interest/Competing Interests Not applicable.

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