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Polycarpa aurata as biomonitor to assess potential toxic elements across the Coral Triangle

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Abstract Coral reefs are increasingly threatened by multiple stressors, including pollution from potentially toxic trace elements (PTEs) released in the environment by many human activities, like widespread industrial and artisanal/small-scale gold mines (ASGMs), especially in the Coral Triangle. In the present study, concentration of 12 PTEs (Al, As, Ba, Cr, Cu, Hg, Fe, Mn, Mo, Sb, V, and Zn) in suspended particulate matter and their bioaccumulation in tissues (tunic and body without tunic) of the ascidian *Polycarpa aurata* were investigated by inductively coupled plasma mass spectrometry at four putative impact sites, downstream of four mining areas with different industrial and ASGM activities, and at four control sites in the North Sulawesi, Indonesia. Compared to control sites, *P. aurata* significantly accumulates Hg, Cu, and V in the tunic and, with higher values, Hg,

Cu, Mo, and Cr (respectively, up to 3.35 ± 0.71 , 12.63 ± 1.52 , 9.58 ± 1.48 , and $1.60 \pm 0.47 \mu\text{g g}^{-1}$) in the rest of the body, especially in the presence of less regulated mining activities (i.e., ASGMs). Mercury showed a bioaccumulation factor for suspended particulate matter greater than 1 at all sites. As a combination of easy-to-recognize and sample, widespread, and very abundant throughout the Coral Triangle, an efficient filter feeder resistant to pollution, very effective in accumulating potentially toxic trace elements, especially mercury, copper, and chromium, *P. aurata* may be considered as an ideal biomonitor organism. To measure PTEs contamination, this species should be included in monitoring programs throughout the Coral Triangle by developing a proper standard protocol.

Keywords Monitoring · Heavy metals · Pollution · Golden sea squirt · Artisanal mines · Indo-Pacific ocean

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Introduction

Coral reef ecosystems are threatened by climate-change-driven stressors at a global scale and by human disturbance at local and regional scales (Hughes et al. 2017; Ponti et al. 2021). These stressors and disturbances result in degraded coral reef conditions, impacting ecosystem functions and, consequently, local economies, and community livelihoods (Pandolfi et al. 2003; Hernández-Delgado 2015). The Coral Triangle area (sensu Veron et al. 2009) is recognized as a major marine biodiversity hotspot (Roberts et al. 2002), accounting for 76% of all known reef-building coral species and 53% of world's coral reefs (De Vantier and Turak 2017), and a global priority for coral reef conservation (Bryant et al. 1998; Burke et al. 2011; Selig et al. 2014; Asaad et al. 2018). During the past century, this area has been exposed to intense human pressure resulting from rapid industrialization, economic development, and urbanization in coastal regions, which contributed to an increase of potentially toxic elements in seas, above all in estuarine and coastal habitats, including coral reefs (Edinger et al. 1998; Todd et al. 2010; Analuddin et al. 2017). Potentially toxic trace elements (PTEs; sensu Nieder and Benbi 2024) include heavy metals with a density higher than 5 g cm^{-3} , and non-metals according to their physiological functions and toxicity. They can accumulate in marine and coastal environments, originating from both natural processes, including rock weathering during soil formation, volcanic emissions, and aeolian transport, and human activities (e.g., run-off from agricultural activities, sewage, combustion of fossil fuels, and mining residues) (Hader et al. 2020). PTEs, introduced onto coastal marine environments via wastewaters, watersheds, groundwater infiltration, and atmospheric inputs, represent a potential threat to aquatic species, natural habitats, and human health (Long and Chapman 1985). Due to their toxicity, persistence, and bioaccumulation, they can lead to a decrease in species diversity and abundance, degrading coral reefs at the local and regional scales (Elumalai et al. 2024). Although some of these elements are naturally occurring, anthropogenic sources, particularly gold mining activities that may release over 99% of the extracted ore as waste into the environment, have contributed substantially to their increase, causing long-term adverse effects on the environment and public health (Fashola et al. 2016).

The Coral Triangle is abundant in metal ores, including gold deposits especially in Indonesia, Papua New Guinea, and the Philippines (Chung 2022). Mining activities, including the extraction and refining stages, can cause the mobilization and release of PTEs into the surrounding environment, leading to air, water, and soil pollution (Marmolejo-Rodríguez et al. 2011). Industrial and artisanal gold mining activities are particularly widespread in Sulawesi Island, Indonesia (Ido et al. 2015). Industrial gold mines

generally use the cyanidation method to extract gold from ores, while artisanal and small-scale gold mines (hereinafter ASGMs) still use mercury amalgamation (Bose-O'Reilly et al. 2010; Edinger 2012). At present, ASGMs are considered a major source of Hg emission and release worldwide (Cordy et al. 2011; Soe et al. 2022), with 410–1040 tons of employed per year corresponding to ca. 37% of total anthropogenic emissions (Futsaeter and Wilson 2013). Moreover, Hg (as cinnabar, a form of mercury sulfide) and As can naturally occur in many gold deposits (Edinger 2012). Mine tailings, even when stored in artificial closed ponds, as should be in properly managed industrial mines, may be dispersed in the catchment basin due to heavy rain and flooding events, two extreme events that occur frequently in tropical areas (González-Valoys et al. 2022). Nowadays, PTE contamination, especially Hg, from anthropogenic activities is a major concern in the Coral Triangle.

Given their bioaccumulation and toxic effects on aquatic organisms, many PTEs are usually included in international convention and marine environmental monitoring programs (e.g., Badr et al. 2009; Tornero and Hanke 2017; Greggio et al. 2021). However, their detection in seawater and suspended particulate matter (SPM) is difficult due to the low concentrations (Rainbow 1995; Cebrian et al. 2007).

Suspended particulate matter in seawater generally includes organic and inorganic fractions from both natural and biogenic origins (e.g., sediments, living and dead organisms, and shell debris) and anthropogenic sources (e.g., from industrial activities), which are brought to the sea mainly via river run-off and atmospheric deposition (Iwamoto and Uematsu 2014). These particles are critical in controlling substance' reactivity, transport, and biological impacts in marine environments and provide an essential link for chemical constituents between the water column, bed sediments, and the food chain (Turner and Millward 2002). When PTEs are associated with particles, they can be taken with the diet by suspension feeder organisms. Otherwise, they can settle to the bottom and become available to the deposit feeders or be re-released into the water column via resuspension, adsorption/desorption, reduction/oxidation reactions, or degradation of organisms. Therefore, the chemical properties of SPM play a crucial part in controlling the composition of PTEs in marine coastal environments by reflecting the characteristics of the water column (Balls 1990; Fernández-Severini et al. 2013; Iwamoto and Uematsu 2014).

Living organisms can be used as biomonitors (sensu Rainbow and Phillips 1993) to yield a relative measure of the amount of PTEs accumulated in the environment over time, as they are selectively absorbed and accumulated into their tissues whether or not these elements are essential for their metabolism (Rainbow 2002; Stankovic et al. 2014). Among benthic marine invertebrates, filter feeders, processing relatively large amounts of water during feeding

activities, maximize their exposure to PTEs in the water column (Wang and Rainbow 2000; Bellante et al. 2016). Organisms particularly suitable as biomonitors are those that are abundant, widely distributed, sessile, tolerant to salinity variations, and capable of accumulating a broad range of contaminants over a long period (Rainbow 1995; Chiarelli and Roccheri 2014).

The bioaccumulation of PTEs and their effects have been extensively studied on edible bivalves and holothurians (e.g., Tunca et al. 2016; Cammilleri et al. 2020); more recently sessile suspension feeders organisms, such as sponges, corals, bryozoans, polychaetes, barnacles, and ascidians have also been suggested as suitable biomonitors due to their physiological and ecological features (Roveta et al. 2021). Choosing an appropriate biomonitor species is crucial to any successful biomonitoring program (Chiarelli and Roccheri 2014).

Ascidians are highly efficient filter-feeding organisms that can accumulate and concentrate PTEs, especially from suspended particulate matter (SPM), even in low concentrations (Monniot et al. 1994). They have been used in the monitoring of different marine pollutants, including aluminum, cadmium, cesium, cobalt, copper, iron, lead, manganese, mercury, nickel, vanadium, and zinc (e.g., Monniot et al. 1994; Jiang et al. 2009; Chiarelli et al. 2014; Abdul Jaffar Ali et al. 2015; Aydin-Onen 2016; Tzafriri-Milo et al. 2019). *Polycarpa aurata* (Quoy and Gaimard 1834), commonly known as the golden (or gold-mouth, or ink-spot) sea squirt, or ox heart ascidian, is an iconic solitary ascidian belonging to the family Styelidae that is easily identifiable, abundant, and widely distributed in the Coral Triangle (Fig. 1), and thus a good candidate as biomonitor organism.

The aim of this study is to assess the suitability of *Polycarpa aurata* as biomonitor species for PTEs accumulating in the Coral Triangle coral reefs. To this end, levels of selected PTEs (As, Cr, Cu, Hg, Fe, Mn, Mo, Sb, and Zn) and other selected elements (Al, Ba, and V) were measured in SPM and *P. aurata* tissues (tunic and body without tunic) collected in coastal areas downstream of four mines, including both ASGMs and industrial ones, and at four control sites in the North Sulawesi province, Indonesia. To our knowledge, no previous studies were conducted on SPM and ascidian bioaccumulation in this area.

Materials and methods

Study area and watershed analysis

The present study was carried out in the North Sulawesi province and included four sites downstream gold mines and four control sites, randomly interspersed among them (Fig. 2). North Sulawesi presents a long and widespread

history of unregulated ASGMs and, more recently, regulated industrial mining activities. Contamination of some PTEs (i.e., As, Cd, Cr, Cu, Ni, Pb, Zn, and Hg) associated with gold mining has been an emerging environmental concern in the Indonesian province of North Sulawesi since the early 2000s (Lasut and Yasuda 2008; Lasut et al. 2010; Palapa and Maramis 2015; 2018).

As in Tamburini et al. (2022), putative impact sites were chosen close to the mouth of streams or rivers flowing through mining areas, identified by a watershed analysis based on the digital elevation model from the Shuttle Radar Topography Mission (SRTM 4.1; horizontal resolution of 90 m at the equator) and performed with the GRASS' "r.watershed" algorithm in QGIS 3.16 (QGIS Development Team 2021). These sites correspond to different types and ages of gold mining activities. Totok Bay site (labeled as TB; Fig. 2) corresponds to a still active ASGM dated back to the mid-nineteenth century (Bentley and Soebandrio 2017). Totok Bay and the nearby Buyat Bay (Buyat-Ratatotok district) are already known for gold mining-related pollution (Turner et al. 1994). From 1996 to 2004, Buyat Bay was also affected by an underwater tailing disposal of an industrial gold mine (Blackwood and Edinger 2007; Lasut and Yasuda 2008; Edinger 2012). However, separated by a discontinuous peninsula, Totok Bay is primarily affected by the sewage and surface leaching from the ASGM (Blackwood and Edinger 2007). Total mercury and methylmercury (MeHg) were detected in sediments from the beach, river estuary, and shallow marine bottom, and some specimens of soft corals, seaweeds, seagrasses, mollusks, crabs, and fishes from both bays, as well as the scalp hair of residents in the area, with the highest concentrations at Totok Bay and intermediate concentrations at Buyat Bay compared to the control area, respectively, 2 and 1 order of magnitude higher (Lasut et al. 2010). In Totok Bay, the present study site was established on the outermost part of a fringing reef near Hogouw Islet at the end of the bay.

The second putative impact site was chosen on the outermost part of a fringing coral reef located south of Talawaan river mouth (TW; Fig. 2) downstream of the Talawaan-Tatelu ASGMs, established in 1997. This area experienced a gold rush with an increasing number of miners working in ASGM, assessed to be ~ 10 thousand in 2003 (Castilhos et al. 2006). In 2010, the Toka Tindung Project was started here under the supervision of PT Archi Indonesia. The Toka Tindung Project covers an area of about 40 thousand ha, located 35 km north-east of the regional capital of Manado. Until December 2016, the Toka Tindung Project produced about 25,200 kg of gold. Kambey et al. (2001) found that fish from the affected area had a mercury content 30 times higher than the reference sites.

The third impact site was chosen at patches of coral reef located in the middle of the bay of Pantai Surabaya (PS;

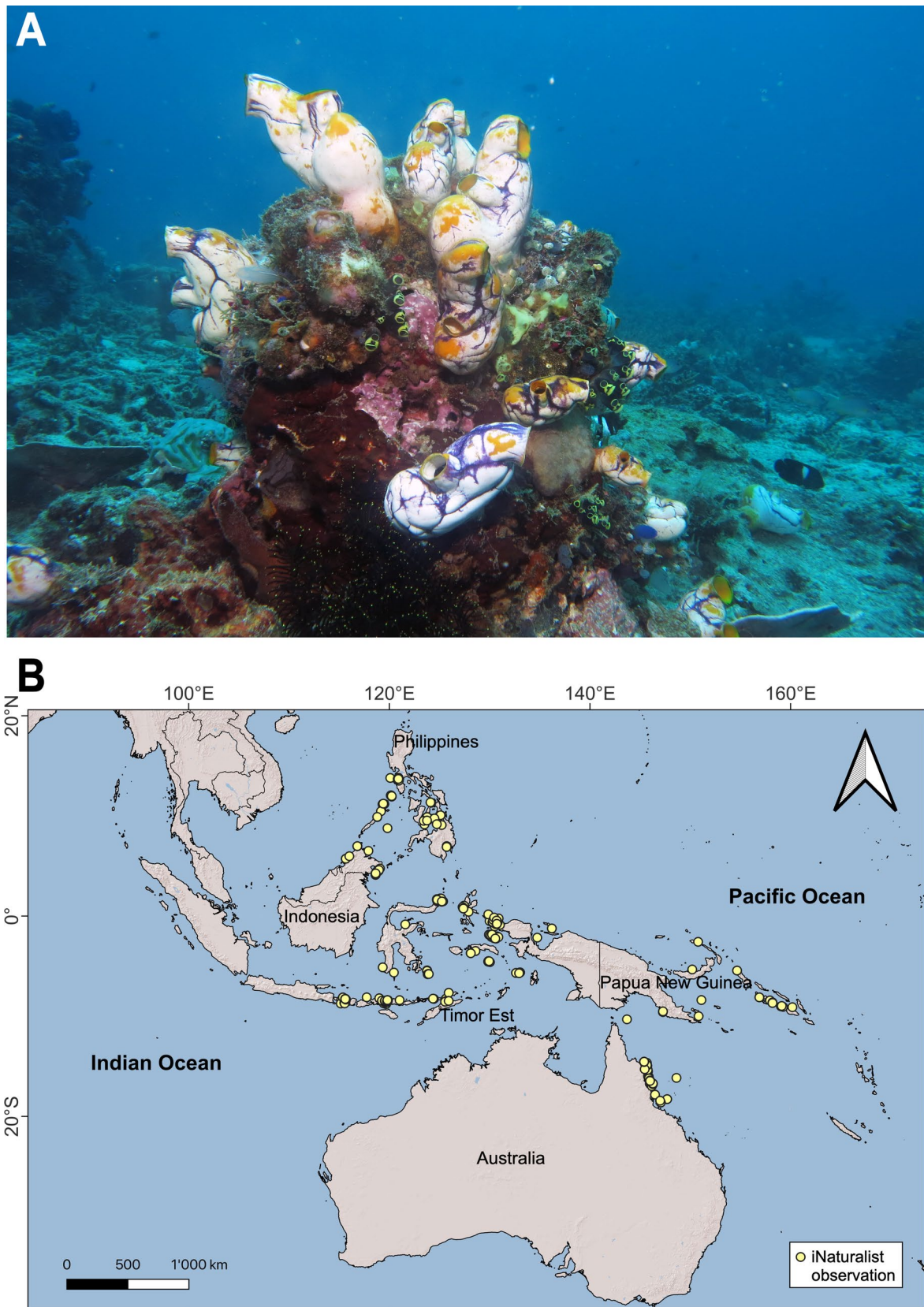


Fig. 1 A Dense group of *Polycarpa aurata* specimens at a study site; and B photographically documented occurrences of *P. aurata* retrieved from the iNaturalist community (www.inaturalist.org; last access August 25th, 2023)

Fig. 2 Map of the study area and sampling sites (Datum WGS84, Projection UTM zone 51). Included active gold mines: Totok Bay (TB); Talawaan River (TW); Pantai Surabaya (PS); Mining site in preparation: Coral Eye (CE); Control sites: Batu Belah (BB); Batu Nona (BN); Talisei Island (TA); Tasik Ria Resort (TR)

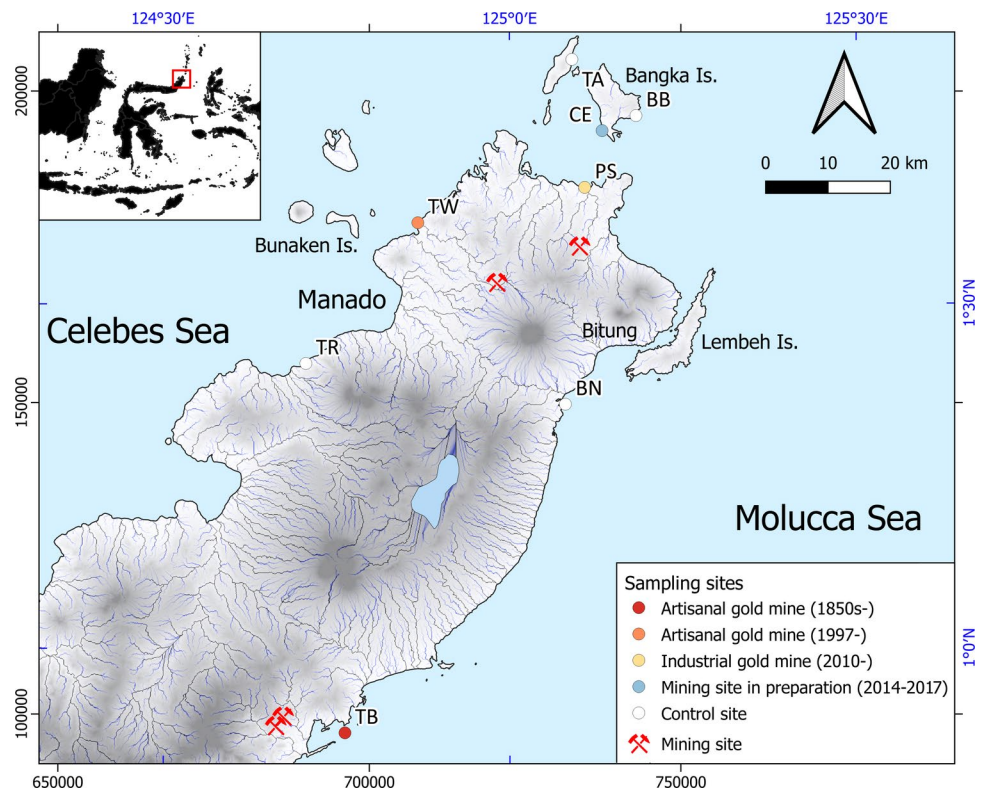


Fig. 2), a few hundred meters from the river mouth downstream to the Toka Tindung industrial gold and silver mine, which has been operating since 2010. In this area, significant sediment contamination of several PTEs (i.e., As, Sb, Co, Cr, Cu, Ni, Zn and Hg) and their bioaccumulation in the sea cucumber *Holothuria (Halodeima) atra* was found (Tamburini et al. 2022).

The fourth impact site was at Bangka Island on the fringing reef in front of the Coral Eye resort (CE; Fig. 2), near a mining site under preparation. Earthmoving work began here in 2014, immediately raising concerns about the health of local coral reefs (Ponti et al. 2016). The mine was officially closed in March 2017 after the Jakarta High Court accepted a lawsuit filed by residents and tour operators.

Control sites were randomly located at Batu Belah on Bangka Island (BB), Talisei Island (TA), Batu Nona (BN), and near Tasik Ria Resort (TR), the latter two on the North Sulawesi Peninsula (Fig. 2; see also Supplementary Information, Table S1).

Sampling and sample processing

From June to August 2017, three replicate samples for suspended particulate matter (SPM) were collected at each site. A known volume of surface seawater (from 12.5 to 38 L) was filtered onsite, using a manual vacuum pump, through pre-combusted (450 °C, 5 h) Whatman GF/C filters (47 mm diameter, 1.2 µm pore size). All containers for seawater

sample collection and processing were acid cleaned and rinsed three times with the sample before collection. Filters were oven-dried at 50 °C for 24 h in a clean chemical laboratory, weighed (± 0.01 mg), and stored in low-density polyethylene (LDPE) grip seal bags. The filters were preserved in sealed polypropylene boxes in the dark until further analysis (protocol adapted from Duinker et al. 1979).

At all sites, the population density of *P. aurata* was estimated by counting individuals along three belt transects (50 × 2 m, i.e., 100 m²), carried out at least 10 m apart by scuba diving (Hill and Wilkinson 2004). Then, eight specimens of *P. aurata* were randomly manually collected by scuba divers at each study site to measure potentially toxic trace element concentrations. To avoid differences in bioconcentration among individuals due to different ages and, therefore, time exposures, the individuals collected were of similar size (~8–10 cm in length; Kosyanenko et al. 2021). Specimens were transported alive in plastic grip seal bags with seawater until they were placed in 20-L aquariums with aerators within 2–3 h of collection. Menthol (0.1 g L⁻¹) was added to relax the animal's muscles before dissection (Tzafriri-Milo et al. 2019). The eight specimens for each study site were dissected, separating the tunic (TUN) from the rest of the body (BWT, which stands for “body without tunic”) using ceramic scissors and tweezers to avoid metal contamination (Tzafriri-Milo et al. 2019). Tissue samples were washed in a stream of distilled water to remove the salt; then, they were dried at no more than 50 °C for 24 h

and individually stored in new LDPE grip seal bags until chemical analysis.

Chemical analysis

After additional drying for 24 h at no more than 50 °C, both SPM samples on filters and ascidian tissues were grinded in the laboratory. Approximately 1 g of each sample was powdered in a ceramic mortar and put in a plastic screw-top vial. SPM subsamples (~0.075 g each) were subjected to acid digestion with 0.35 g of HNO₃ (69%) and 0.75 g of HCl (37%) in 15 ml closed polypropylene vials at 100 °C for 2 h, then added 3 ml of aqua regia solution diluted to 1% by weight and heated again at 100 °C for 4 h; and finally diluted to 33% by weight. Tissue subsamples (~0.075 g) were also subjected to acid digestion with 0.35 g of HNO₃ (69%) and 0.75 g of HCl (37%) heated in closed polypropylene vials for 3 h at 100 °C and then diluted with Milli-Q water to obtain 5% by weight (EPA Method 3050B:1996—Acid Digestion of Sediments, Sludges, and Soils; adapted for biological tissues). All tools, vials, and jars were cleaned before every use with 20% nitric acid and deionized water to avoid cross-contamination among samples (Badocco et al. 2015a; 2015b).

Twelve elements (Al, As, Ba, Cr, Cu, Hg, Fe, Mn, Mo, Sb, V, and Zn) were quantified both in SPM and ascidian tissues by using inductively coupled plasma mass spectrometry (ICP-MS, Agilent Technologies 7700x, Agilent Technologies International Japan, Ltd., Tokyo, Japan; following the EPA Method 6020B:2014—Inductively Coupled Plasma–Mass Spectrometry—and the ISO method 17,294–2:2016—Water quality—Application of ICP-MS—Part 2: Determination of selected elements). The instrument was equipped with an octupole collision cell operating in kinetic energy discrimination mode, which was used to remove polyatomic and argon-based interferences. The operating conditions and data acquisition parameters were the same as reported in Badocco et al. (2015c). Multi-element standard solutions for calibration were prepared in aqua regia (5%) by gravimetric serial dilution at 12 different concentrations—from a minimum of 1 ng L⁻¹ to a maximum of 100 mg L⁻¹. All solutions were prepared in Milli-Q ultrapure water using a Millipore Plus System (Milan, Italy, resistivity 18.2 MΩ cm⁻¹). All regressions were linear with a determination coefficient (R²) larger than 0.9999. The ICP-MS was tuned daily using a tuning solution containing 1 μg L⁻¹ of ¹⁴⁰Ce, ⁷Li, ²⁰⁵Tl, and ⁸⁹Y (Agilent Technologies, UK). The internal standard mixture (Agilent, 5183–4681) containing Bi, Ge, In, Sc, Tb, Y, and Li at 10 μg mL⁻¹, each in 5% HNO₃, was used. The internal standard was added to the sample solution via a T-junction. The Critical Limit (CL), Limit of Detection (LOD), and Limit of Quantification (LOQ) of each element were computed based on the methods described in Lavagnini et al. (2011) and Badocco

et al. (2015a, 2015b; 2014; 2015c). Instrumental operating conditions are reported in the Supplementary Information, Table S2.

Due to the lack of certified reference materials for ascidian tissues, method accuracy was assessed through spike recovery tests at known concentrations, directly applied to the real biological matrix. Recovery values for all analyzed elements (Al, As, Ba, Cr, Cu, Hg, Fe, Mn, Mo, Sb, V, and Zn) ranged between 80 and 95%, which falls within internationally accepted limits (EPA method 6020B:2014; AOAC Guidelines for Single Laboratory Validation, 2019; ISO 17025:2018—General requirements for the competence of testing and calibration laboratories). This approach is considered suitable for trace element determination in non-standard biological matrices when certified materials are unavailable (e.g., Choi et al. 2014).

Data analysis

Trace element concentration values below the Critical Limit (CL) were replaced by one-half of the minimum Limit of Detection (LOD_{min}) only for statistical purposes (Paschal et al. 1998). Since there were no reasons to hypothesize that the concentrations of trace elements in SPM and ascidian tissues were affected in the same way in the different basins subject to gold mines, each putative impact mining site was analyzed separately against the four control sites (as in Guerra et al. 2009; Ponti et al. 2009; Tamburini et al. 2022). Differences in mean concentrations in SPM and tissues (tunic, TUN, and body without tunic, BWT) between Impact and Control, and among sites nested in the interaction Impact × Control, where Control has four sites, were assessed by a two-way asymmetrical permutational analysis of variance (PERMANOVA; α=0.05) based on Euclidean distances (Anderson and Robinson 2008). When less than 100 unique permutations were available, the asymptotic Monte Carlo p-value was used instead of the permutational one. When no significant differences in mean trace element concentrations were found among control sites (p > 0.25), site data were pooled to improve the power of the test for differences between Impact and Control (Anderson et al. 2008).

Possible correlations between the concentrations of trace elements in the tunic and the rest of the body of individuals of *P. aurata* (n=64) and between the mean concentrations in the SPM of the different sites and those in the tunic and body without tunic of *P. aurata* (n=64) were estimated with Pearson's moment-product correlation coefficient (r), whose difference from zero was assessed by t-test (α=0.05).

Bioaccumulation is the concentration in the organism of a chemical resulting from the dynamic equilibrium between the uptake rate (by all possible means, including contact, respiration, and ingestion) and the elimination rate (Chojnacka and Mikulewicz 2014). Since *P. aurata* mainly

feeds on suspended particulate matter, for each element, the bioaccumulation in the field can be assessed by the bioaccumulation factor (BAF), calculated as the ratio between the mean element concentrations in tissues (i.e., tunic and body without tunic) and its concentration in the suspended particulate matter at a given site (Arnot and Gobas 2006). Separate BAFs were calculated for the tunic and the rest of the body of *P. aurata*. Possible bioaccumulation from SPM in the study area was confirmed when the mean BAF across sites was significantly greater than 1 according to a *t*-test ($\alpha=0.05$, $n=8$).

PERMANOVA analyses were done using PRIMER 7 with PERMANOVA + addon package (Anderson et al. 2008), while linear correlations and *t*-tests were conducted with the computational language R (R Core Team 2024). Mean values were reported with their standard errors, unless otherwise indicated.

Results

Trace elements contamination in suspended particulate matter

Bar plots presented in Fig. 3 illustrate the mean concentrations of trace elements in suspended particulate matter (SPM) at the putative impact and control sites. Statistical analysis assessing the significance of differences between mean concentrations at each individual putative impact mining site and the control ones is reported in the Supplementary Information (Tables S3.1–4).

The mean mercury (Hg) concentration in SPM ranged from below the CL, as occurred at all control sites and two mining sites (i.e., PS and CE), to $0.14 \pm 0.01 \mu\text{g g}^{-1}$ at TB (Fig. 3A). A statistically significant enrichment of Hg was detected at both TB and TW relative to the control sites.

The mean antimony (Sb) concentration ranged from $0.50 \pm 0.06 \mu\text{g g}^{-1}$ at CE to $0.84 \pm 0.02 \mu\text{g g}^{-1}$ at TB, the latter being the only mining site where Sb concentration was significantly higher than at the control sites (Fig. 3J).

The mean copper (Cu) concentration ranged from $14.68 \pm 1.30 \mu\text{g g}^{-1}$ at BB to $31.58 \pm 0.43 \mu\text{g g}^{-1}$ at PS. Significantly higher Cu concentrations were observed at TW and PS mining sites compared the control sites (Fig. 3F). Similarly, the mean concentration of manganese (Mn) ranged from $58.73 \pm 6.30 \mu\text{g g}^{-1}$ at BB to $1'015 \pm 260 \mu\text{g g}^{-1}$ at TW, with both TW and PS exhibited significantly higher Mn concentrations than control sites (Fig. 3H).

The mean iron (Fe) concentration ranged from $2'334 \pm 46 \mu\text{g g}^{-1}$ at BB to $16'114 \pm 3'341 \mu\text{g g}^{-1}$ at TW. Among the mining sites, only TW exhibited a statistically significant increase in Fe concentration relative to the control sites (Fig. 3G). Likewise, mean zinc (Zn)

concentration ranged from $43'060 \pm 1'684 \mu\text{g g}^{-1}$ at BB to $61'377 \pm 4'339 \mu\text{g g}^{-1}$ at TW, which also exhibited significantly higher concentrations than the control sites (Fig. 3L).

No statistically significant differences were found between mean concentrations at any of the mining sites compared to the control sites for either arsenic (As), which varied from $7.19 \pm 0.64 \mu\text{g g}^{-1}$ at TA to $11.08 \pm 1.37 \mu\text{g g}^{-1}$ at TW (Fig. 3C), or chromium (Cr), ranging from $16.31 \pm 0.49 \mu\text{g g}^{-1}$ at BB to $21.22 \pm 0.72 \mu\text{g g}^{-1}$ at BN (Fig. 3E). Mean molybdenum (Mo) concentration ranged from $1.53 \pm 0.15 \mu\text{g g}^{-1}$ at PS to $5.18 \pm 0.91 \mu\text{g g}^{-1}$ at BB (Fig. 3I). A significant variability was observed among control sites ($p < 0.05$); however, Mo concentration at the impact sites did not differ significantly from those of the controls.

The mean aluminum (Al) concentration ranged from $38'227 \pm 1'319 \mu\text{g g}^{-1}$ at BB to $63'857 \pm 5'440 \mu\text{g g}^{-1}$ at TW. Among all mining sites, only TW exhibited a statistically significant enrichment of Al concentration in SPM compared to control sites (Fig. 3B). A similar pattern was observed for vanadium (V), with mean concentration ranged from $7.52 \pm 0.50 \mu\text{g g}^{-1}$ at BB to $34.93 \pm 6.19 \mu\text{g g}^{-1}$ at TW, where it was significantly higher than those recorded at control sites (Fig. 3K). Likewise, TW was the sole mining site to display a significant enrichment of barium (Ba) concentration compared to the control sites, with mean concentrations ranging from $12'902 \pm 956 \mu\text{g g}^{-1}$ at PS to $43'554 \pm 8'602 \mu\text{g g}^{-1}$ at TW (Fig. 3D).

Trace elements concentration in ascidian tissues

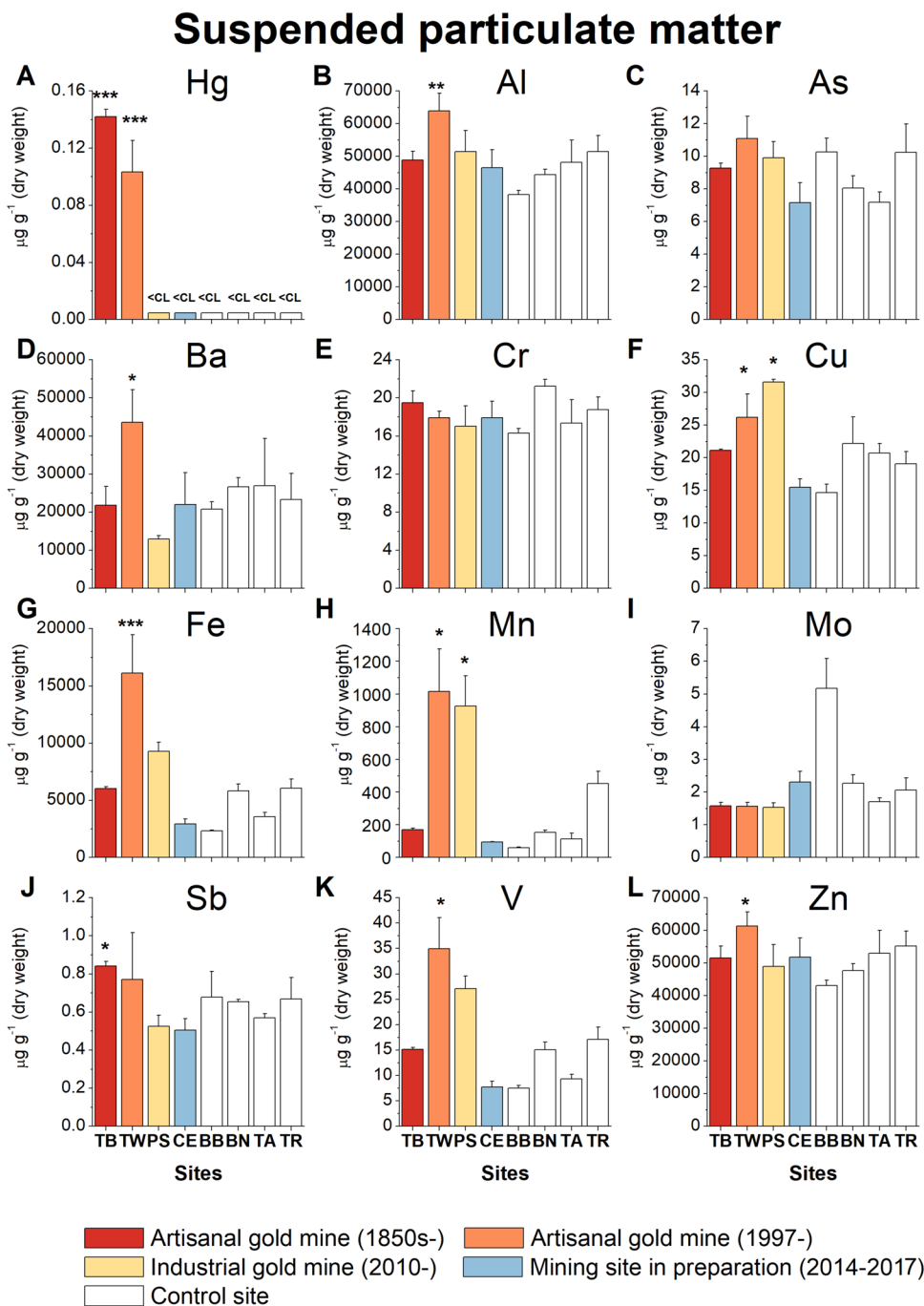
Across the different study sites, the mean densities of *P. aurata* populations ranged from 0.053 ± 0.003 at BB to $0.963 \pm 0.111 \text{ ind. m}^{-2}$ at PS, with no relation to the presence of mines in the area (Fig. 4).

Mean concentrations of trace elements in tunic and body without tunic of *P. aurata* at putative impact and control sites are presented as bar plots in Figs. 5 and 6, respectively. Statistical tests evaluating the significance of differences in mean concentrations at each single putative impacted mining site to the control ones are provided in Supplementary Information (Tables S4.1–4 and S5.1–4).

Overall, higher concentrations in the bodies without tunic than in the tunics are a common finding for many of the elements analyzed, except for Cr and V.

The mean Hg concentration in tunic of *P. aurata* specimens ranged from $0.07 \pm 0.01 \mu\text{g g}^{-1}$ at BB to $0.53 \pm 0.06 \mu\text{g g}^{-1}$ at TW, and they were significantly higher at the TW and TB artisanal mining sites than at the control ones (Fig. 5A). A similar result was found for the mean concentrations in the body without tunic, although the values in these tissues were much higher than in the tunic, up to $3.35 \pm 0.71 \mu\text{g g}^{-1}$ at TB (Fig. 6A). While, the other two

Fig. 3 Mean concentrations (\pm s.e.) of Hg (A), Al (B), As (C), Ba (D), Cr (E), Cu (F), Fe (G), Mn (H), Mo (I), Sb (J), V (K), and Zn (L) in suspended particulate matter at each study site (TB: Totok Bay; TW: Talawaan River; PS: Pantai Surabaya; CE: Coral Eye; BB: Batu Belah; BN: Batu Nona; TA: Talisei Island; TR: Tasik Ria Resort). CL: critical limit. Impact sites that differ significantly from controls are marked with asterisks: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$



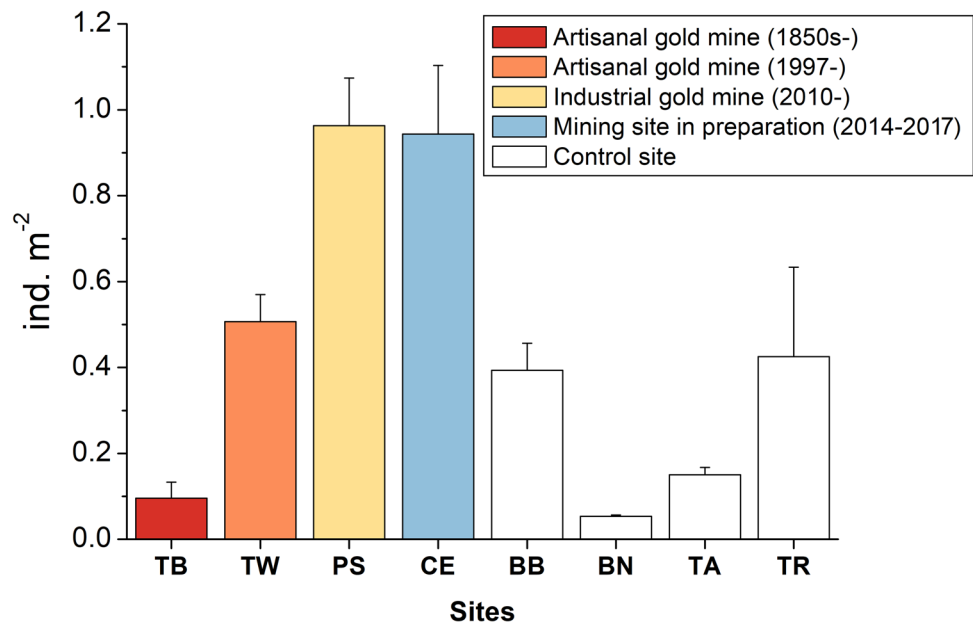
mining sites, namely the industrial and in preparation ones, did not show higher tissue concentrations than the controls.

Similarly to Hg, the mean Cu concentration in both tissues resulted significantly higher close to an artisanal mining, namely TB, the oldest one, where on average it was $12.63 \pm 1.52 \mu\text{g g}^{-1}$ in body without tunic (Figs. 5F, 6F). A quite similar pattern was observed for Mo at TB, where the mean concentration in body without tunic ($9.58 \pm 1.48 \mu\text{g g}^{-1}$) was significantly higher than at control

sites (Fig. 6I), although no such difference was detected in the tunic (Fig. 5I).

Cr also followed this trend of site-specific enrichment at TB, with a significantly higher concentration in body without tunic ($1.60 \pm 0.47 \mu\text{g g}^{-1}$; Fig. 6E), but not in tunic (Fig. 5E). Unlike the elements described above, at the industrial mining site, for what concerns the body without tunic, and at the site in preparation, in the tunic, significantly lower values were found than at the control sites.

Fig. 4 Mean density (\pm s.e.) of *P. aurata* at each study site: TB: Totok Bay; TW: Talawaan River; PS: Pantai Surabaya; CE: Coral Eye; BB: Batu Belah; BN: Batu Nona; TA: Talisei Island; TR: Tasik Ria Resort



Arsenic was the only element that showed mean values lower than those of controls at TB, but only in the tunic (Figs. 5C and 6C).

The mean V concentrations in tunic showed higher values at the TW and PS mining sites than those recorded at the control. At PS, the mean concentration in the tunic reached $1.98 \pm 0.43 \mu\text{g g}^{-1}$ (Fig. 5K). However, this trend was not reflected in the body without tunic (Fig. 6K).

All other elements considered (i.e., Al, Ba, Fe, Mn, Sb, and Zn) showed similar mean concentrations in both tissues between mining and control sites.

Trace elements bioaccumulation

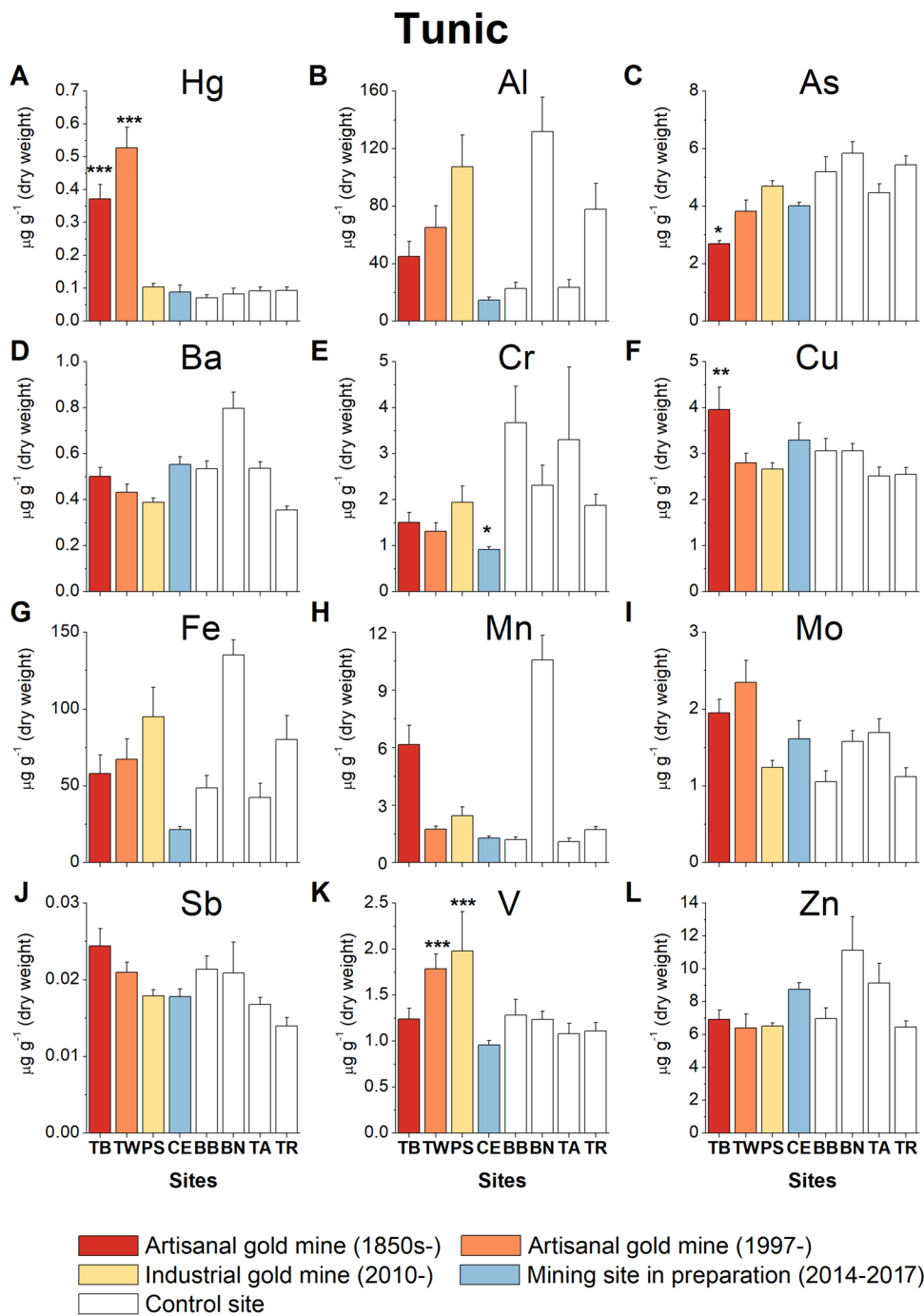
The relationships between the mean concentrations of trace elements in suspended particulate matter and ascidian tissues (tunic and body without tunic) are shown in Fig. 7, while Pearson's product-moment correlation coefficients (r) and corresponding t-tests for difference from zero are reported in Table 1. Significant positive correlations were found for the concentrations in the SPM and in *P. aurata* tissues, TUN and BWT, both for Hg and Sb, with very high correlation coefficients for Hg. V showed a significant positive correlation between SPM and TUN, but not for BWT; while for Mo a significant negative correlation was found between SPM and TUN. As exhibited a significant, albeit weak, positive correlation between SPM and BWT, but not TUN. Comparing the concentrations of trace elements in TUN and BWT of single individuals, strong positive correlations were found for Hg, Al, As, Ba, Fe, Mn, Mo; moreover, weak but significant positive correlations were also detected for Cu and Sb.

Bioaccumulation factors for SPM concentrations (BAF) largely varied among elements, sites, and tissues from $9.93\text{E-}06$ to 99.43 (Supplementary Information Table S6). In general, higher values were obtained for BWT compared to TUN. The most bioaccumulated element was Hg, whose BAF resulted in greater than 1 at all sites, where at TR was found the highest value in BTW (i.e., 99.43). By averaging the BAF values of each element across all study sites in the region, Hg was significantly bioaccumulated both in TUN (14.97 ± 2.53 ; $p < 0.001$) and BWT (57.39 ± 10.53 ; $p < 0.001$) (Fig. 8). The second most bioaccumulated element was Mo in BWT, with a BAF greater than one at all sites except BB, with a mean value of 2.48 ± 0.59 , significantly higher than 1 ($p < 0.05$). Arsenic showed a mean BAF in BWT of approximately one (1.08 ± 0.14), as well as no other elements exhibited mean BAS across the region significantly higher than 1.

Discussion

This study provides the first data on the concentration of potentially toxic trace elements in suspended particulate matter (SPM) and their bioaccumulation in the ascidian *Polycarpa aurata* in relation to different gold mining activities in North Sulawesi, Indonesia. The investigation spanned several putative impact and control sites strategically selected along the coastline, encompassing drainage basins with and without gold mining activities. The study included areas influenced by both long-standing artisanal and small-scale gold mines (ASGMs; e.g., Totok Bay, operational since the 1850s) and more recent activities, including active artisanal operations at the Talawaan River mouth,

Fig. 5 Mean concentrations (\pm s.e.) in *P. aurata* tunics of Hg (A), Al (B), As (C), Ba (D), Cr (E), Cu (F), Fe (G), Mn (H), Mo (I), Sb (J), V (K), and Zn (L) at each study site (TB: Totok Bay; TW: Talawaan River; PS: Pantai Surabaya; CE: Coral Eye; BB: Batu Belah; BN: Batu Nona; TA: Talisei Island; TR: Tasik Ria Resort). Impact sites that differ significantly from controls are marked with asterisks: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$



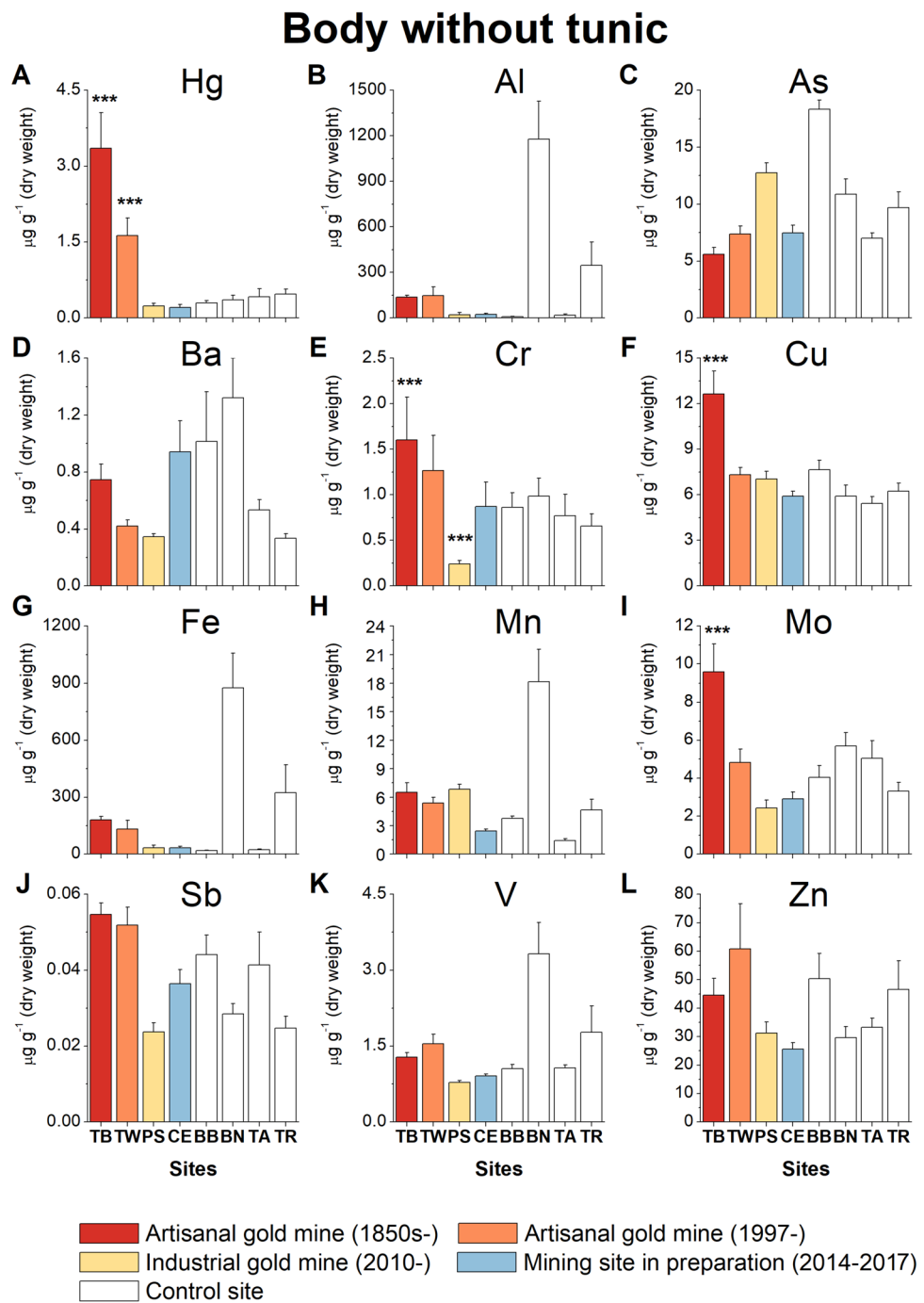
industrial mining at Pantai Surabaya, and a mine in preparation at Coral Eye, which had ceased activity before production began.

In the study area, the distribution patterns of many PTEs in the SPM were closely aligned with the presence and intensity of mining operations. Compared to control sites, SPM from areas proximate to ASGMs exhibited significant enrichment in Hg, Al, Ba, Cu, Fe, Mn, Sb, V, and Zn. Elevated concentrations of Cu and Mn were also founded downstream of the industrial gold mine. Meanwhile, no evidence

of PTE enrichment was observed at Coral Eye, where preparing mining activities ceased a few months before sampling and production never started.

The PTE that deserves more attention and is of greatest environmental concern in the particulate matter is certainly mercury. It has been reported that SPM is the primary carrier of mercury in river systems, with spatial and temporal variability in concentration, affecting the ecosystem and human health in downstream regions (e.g., Appleton et al. 1999; Moreno-Brush et al. 2016, 2020). While mercury was not

Fig. 6 Mean concentrations (\pm s.e.) in *P. aurata* bodies without tunic of Hg (A), Al (B), As (C), Ba (D), Cr (E), Cu (F), Fe (G), Mn (H), Mo (I), Sb (J), V (K), and Zn (L) at each study site (TB: Totok Bay; TW: Talawaan River; PS: Pantai Surabaya; CE: Coral Eye; BB: Batu Belah; BN: Batu Nona; TA: Talisei Island; TR: Tasik Ria Resort). Impact sites that differ significantly from controls are marked with asterisks: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$



detectable in the SPM at the control sites and downstream of the industrial mine and the mine preparation site, its concentration was particularly high close to the active ASGMs, which still use mercury amalgamation, especially the oldest one at Totok Bay. These results mirror the high sedimentary Hg levels reported in earlier studies in the same areas (Lasut et al. 2010; Palapa and Maramis 2015; Kaunang et al. 2018; Tamburini et al. 2022).

It has long been known that ascidians can actively accumulate PTEs, including manganese, iron, molybdenum,

chromium, and most commonly vanadium (e.g., Carlisle 1968). In the present study, *P. aurata* demonstrated the ability to accumulate a wide suite of PTEs in its tissues, particularly Hg, Cu, and V in the tunic, as well as Hg, Cu, Cr, and Mo in the rest of the body, especially in the presence of unregulated mining activities. PTEs content was generally higher in the body without tunic than in the tunic, as observed in other ascidian species (e.g., Abdul Jaffar Ali et al. 2015).

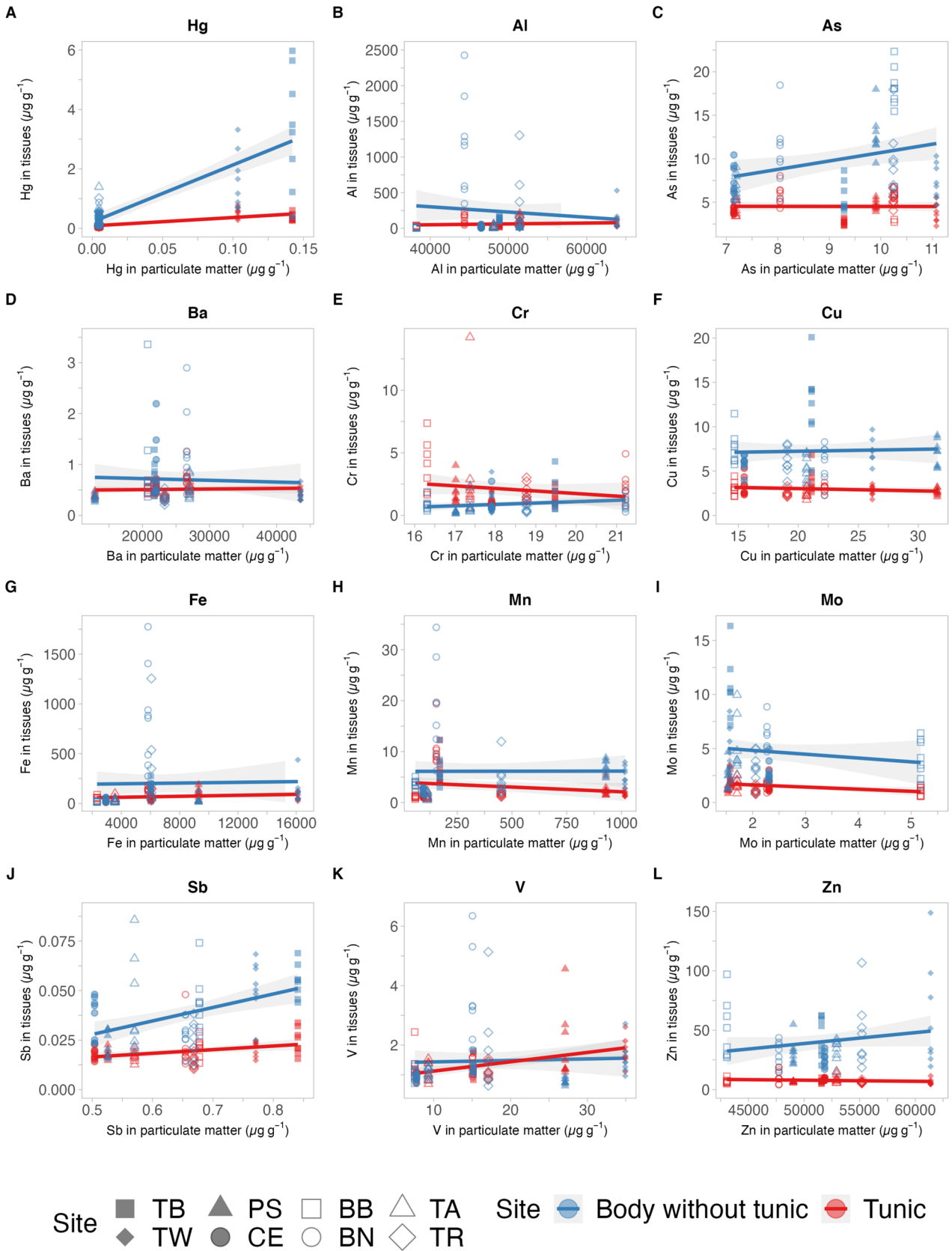


Fig. 7 Relations between the mean concentrations of Hg (A), Al (B), As (C), Ba (D), Cr (E), Cu (F), Fe (G), Mn (H), Mo (I), Sb (J), V (K), and Zn (L) in suspended particulate matter and their accumulation in ascidian tissues (blue: body without tunic, red: tunic). Impact sites are represented by solid symbols (TB: Totok Bay; TW: Talawaan River; PS: Pantai Surabaya; CE: Coral Eye), while control sites by open symbols (BB: Batu Belah; BN: Batu Nona; TA: Talisei Island; TR: Tasik Ria Resort). Lines indicate the linear tendencies, and the gray shadow areas the corresponding 95% confidence intervals

Mercury was always found in the ascidian tissues, both tunic and body without tunic, at all study sites, including the control sites, even where it was not detectable in the suspended particulate matter. This suggests a high bioaccumulation capacity by *P. aurata*, even at very low environmental concentrations. The differences in tissue concentration between the contaminated and control sites were highly significant. Indeed, mercury concentration in *P. aurata* was extremely high and easily detectable at the two sites affected by artisanal mining, especially in the body without tunic resulting in about an order of magnitude higher than in the tunic. Although this species is not of interest for human consumption, it is important to note that the concentrations of mercury found in its tissues, especially in the body without tunic (up to $3.35 \mu\text{g g}^{-1}$), are remarkably higher than the maximum level set by the European Union in wild fish ($0.02 \mu\text{g g}^{-1}$, wet weight; European Directive on environmental quality standards in the field of water policy, 2008/105/EC) and in fishery products ($0.3\text{--}1.0 \mu\text{g g}^{-1}$, wet weight; Commission Regulation (EU) 2023/915).

Mercury concentrations found in the present study were much higher than the ones observed in other solitary ascidian from sites with different sources of pollutants (Supplementary Information, Table S7). Particularly, according to a study conducted in Brazil, *Phallusia nigra* has been indicated as a potential biomonitor of mercury contamination (Martinez et al. 2023); however, the highest values found there ($0.31 \pm 0.05 \mu\text{g g}^{-1}$, \pm Std. Dev., in the branchial basket) was an order of magnitude lower than those found in the body without tunic of *P. aurata* at Totok Bay ($3.35 \pm 2.00 \mu\text{g g}^{-1}$, \pm Std. Dev.).

High mercury contamination levels may adversely affect many marine species, including ascidians. For instance, high Hg concentration had sublethal effects on the reproduction of *Ciona intestinalis* (Bellas et al. 2001, 2004; Gallo et al. 2011) and resulted cytotoxic for *Styela plicata* hemocytes (Cammarata et al. 2007). However, the density of *P. aurata* populations at the study sites did not appear to be affected by mercury levels found in SPM or tissues.

The second PTE of environmental concern in the particulate matter here was copper. Along with antimony, cobalt, chromium, nickel, and zinc, copper is consistently associated with gold deposits in North Sulawesi (Turner et al.

1994; Edinger et al. 2007). Copper is an essential trace metal required for various processes, including its role as a cofactor of multiple enzymes, but, besides that, at a high level, it can be considered one of the most toxic elements after mercury for marine life, albeit well tolerated by some ascidians (Osborne et al. 2018). Its release into the sea in this area appeared related to both artisanal and industrial mines, as indicated by the higher concentrations in the particulate matter at Talawaan River and Pantai Surabaya mining site, as well as in the sediments at Totok Bay and Pantai Surabaya (Tamburini et al. 2022). Although *P. aurata* does not appear to significantly bioaccumulate this metal, its concentrations were significantly higher in both tissues analyzed at Totok Bay, downstream of the older artisanal mine. Although its effects on this species have never been studied, some sublethal effects of copper have been demonstrated in the reproduction of *Ciona intestinalis* (Bellas et al. 2001, 2004).

Copper concentration was investigated in several solitary ascidians (Table S7), finding values even higher than those found in the present study, such as in *Phallusia nigra* and *Microcosmus exasperatus* (respectively, 41.15 ± 22.47 and $12.31 \pm 5.96 \mu\text{g g}^{-1}$, \pm Std. Dev., in the Red Sea; Tzafiriri-Milo et al. 2019), and *Halocynthia aurantium* ($25.70 \pm 1.30 \mu\text{g g}^{-1}$, \pm Std. Dev., in the Sea of Japan; Kosyanenko et al. 2021).

Ascidians can accumulate high levels of vanadium mainly in blood cells (i.e., vanadocytes; Michibata et al. 2003) or store it at the tunic surface (Hirose et al. 2001). Although vanadium is present in almost all ascidian species, those belonging to the suborder Phlebobranchia appeared to contain higher levels than those belonging to the Stolidobranchia, like *P. aurata* (Michibata et al. 2003). The role of vanadium in ascidians is still controversial. It has not always been shown to have an anti-fouling and -predatory defense, as initially hypothesized (Odate and Pawlik 2007 and references therein). Significant enrichment of vanadium in SPM was found at the Talawaan River mouth downstream of the ASGM, initiated in 1997. Its concentration in tunic tissue resulted significantly higher at both the Talawaan River and Pantai Surabaya mining sites. A positive correlation was also found between the accumulation in SPM and the tunic concentration.

Although molybdenum is not a main target element of mining activities in North Sulawesi, it is known as a conservative-type element in open ocean settings despite its involvement in bio-cycling processes (Collier 1985). The concentration of molybdenum in SPM was generally low at all sites. However, *P. aurata* has demonstrated a capacity for Mo bioaccumulation in BWT at all active mining sites, especially at Totok Bay (BAF up to 6), whose concentration was significantly higher than controls.

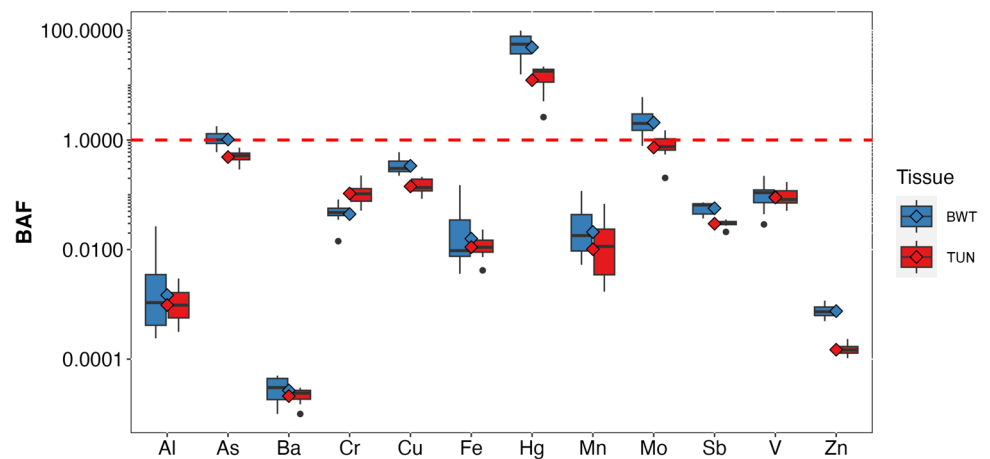
Chromium is an essential nutrient element, which appears not to be associated with gold deposits in North

Table 1 Pearson's product-moment correlation coefficients (r) and corresponding t -tests for difference from zero of trace element concentrations between suspended particulate matter (SPM) and tunic (TUN) or body without tunic (BWT), and between tunic (TUN) and body without tunic (BWT) ($n=64$)

	SPM vs TUN		SPM vs BWT		TUN vs BWT	
	r	t -test	r	t -test	r	t -test
Hg	0.82	***	0.78	***	0.74	***
Al	0.15	ns	-0.11	ns	0.49	***
As	0.00	ns	0.30	*	0.51	***
Ba	0.06	ns	-0.05	ns	0.60	***
Cr	-0.15	ns	0.20	ns	0.01	ns
Cu	-0.15	ns	0.04	ns	0.31	*
Fe	0.22	ns	0.02	ns	0.51	***
Mn	-0.19	ns	0.00	ns	0.77	***
Mo	-0.35	**	-0.14	ns	0.50	***
Sb	0.34	**	0.45	***	0.32	**
V	0.48	***	0.04	ns	-0.06	ns
Zn	-0.15	ns	0.19	ns	0.02	ns

Significant levels were indicated by the following symbols: ns=not significant; $*$ = $p<0.05$; $**$ = $p<0.01$; $***$ = $p<0.001$

Fig. 8 Boxplots (median, interquartile, 1.5 interquartile and outliers) of the biota-suspended particulate matter accumulation factors (BAFs) for each element in tunic (TUN, in red) and body without tunic (BWT, in blue) among sites. Mean values are also shown (diamond symbol)



Sulawesi. Besides little information on the bioaccumulation of Cr by marine organisms, filter feeder organisms, like barnacles and mussels, seem to bioaccumulate this metal directly from the seawater and retain it in soft tissues (e.g., van Weerelt et al. 1984; Cheung and Wong 1992). Although to a lesser extent than Hg and Cu, chromium has also been found to have sublethal effects in the early developmental stages of *Ciona intestinalis* (Bellas et al. 2001, 2004). Although particulate matter had similar concentrations at all sites, *P. aurata* showed significantly higher values at Totok Bay. Present study found higher values in the tunic than in the rest of the body, contrary to all the other elements. This result is also observed in *S. plicata*, another solitary ascidian species with broad geographic distribution and considered of interest for bio-monitoring, especially in ports (Aydin-Onen 2016; Bellante et al. 2016). However, the maximum values found in

S. plicata are up to about 40 times lower than those found in *P. aurata* (Table S7).

Although many of the elements here found at significantly higher concentrations in tissues of ascidians exposed to mining contamination than controls have been shown to have sublethal effects in several ascidian species, populations of *P. aurata* in the study area do not appear to be affected by possible negative effects, as demonstrated by high densities even in contaminated sites. This, together with the wide abundance and distribution throughout the Coral Triangle, seems to indicate an excellent resistance of the species to pollution.

Potentially toxic trace element contamination from anthropogenic activities poses a threat to marine ecosystems and human populations. National regulations generally provide concentration limits for different environmental matrices (e.g., sediments and marine waters), eventually depending on their use. For example, in Table 2, the marine water

Table 2 National marine water quality standard for selected heavy metals in Indonesia (Government of Indonesia 2021) and in the United States of America (U.S. Environmental Protection Agency2024; CMC: Criterion Maximum Concentration and CCC: Criterion Continuous Concentration (concentration of elements dissolved in water in mg L⁻¹)

Element	Indonesian regulation			US EPA	
	Port/harbor	Marine recreational area	Marine biota (*)	Saltwater CMC	Saltwater CCC
Hg	0.003	0.002	0.001	0.0018	0.00094
As	–	0.025	0.012	0.0690	0.03600
Cr	–	0.002	0.005	1.1000	0.05000
Cu	0.050	0.050	0.008	0.0048	0.00310
Zn	0.010	0.095	0.050	0.0900	0.08100

(*) refers to natural marine habitats, including mangrove ecosystems, seagrass beds, and coral reefs

quality standard provided by Indonesian and US legislation for the considered elements are compared. If it were possible to dissolve in the seawater the elements found in the particulate matter, considering the concentration of particulate matter (range 0.88–3.37 mg L⁻¹), we would generally obtain values much lower than these limits, nevertheless ascidians have bioaccumulated several elements making them easily measurable in their tissues. However, despite the recognized importance and effectiveness of environmental biomonitoring, it is still little applied in marine environments and often limited to species of commercial interest, especially in the Indo-Pacific (e.g., Fry et al. 2016; Médieu et al. 2022). Therefore, there is an urgent need to integrate traditional monitoring methods (i.e., sediment and water chemical analysis) by employing reliable and cost/time-effective biomonitors and adopting a unique and shared methodology for assessing environmental health in the whole area. *P. aurata* appears to have all the characteristics of an ideal biomonitor species in the Coral Triangle (Rainbow and Phillips 1993; Rainbow 1995; Chiarelli and Roccheri 2014). It is an efficient, pollution-resistant filter feeder that has been shown to be very effective in accumulating potentially toxic trace elements, especially mercury, copper, and chromium, without suffering effects that compromise the abundance of local populations. It is easy-to-recognize, widespread, and very abundant throughout the Coral Triangle. It is also easy to sample and to handle, dissect, and preserve by drying onsite, even in very remote places. Finally, it is of no economic interest and presumably not threatened by fishing. These findings and considerations should encourage more in-depth bioaccumulation tests, the study of sublethal effects, and the development of standard protocols to include this iconic species in monitoring programs throughout the entire Coral Triangle region.

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Author contributions Conceptualization: MP, ET; formal analysis: RE, DB, ET, FG, LB, RG, PP, MP; funding acquisition: MP, DB, PP, ML; resources: MP, DB, PP, ML, FG, LB, RG, DM, JM, JS, RT; visualization: ET, MP; writing—original draft: ET, MP, RE; Writing—review and editing: all authors. All authors contributed to the article and approved the submitted version.

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Data availability The data that support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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