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Bioconcentration and cellular effects of emerging contaminants in sponges from Maldivian coral reefs: A managing tool for sustainable tourism

Cristiana Rizzi [a], Davide Seveso [a,b,*], Chiara De Grandis [a], Enrico Montalbetti [a,b], Stefania Lancini [a,b], Paolo Galli [a,b,c], Sara Villa [a]

[a] Department of Earth and Environmental Sciences, University of Milano - Bicocca, Piazza della Scienza, 1, Milano 20126, Italy
[b] MaRHE Center (Marine Research and High Education Centre), Magoodhoo Island, Faafu Atoll, Maldives
[c] University of Dubai, PO Box: 14143, Dubai Academic City, United Arab Emirates

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ABSTRACT

Tourism is the main income source for the Maldives, but concurrently, it represents a growing threat to its marine ecosystem. Here, we monitored the bioaccumulation of 15 emerging contaminants (ECs) in the Maldivian reef sponges *Spheciospongia vagabunda* collected in two resort islands (Athuruga and Thudufushi, Ari Atoll) and an inhabited island (Magoodhoo, Faafu Atoll), and we analysed their impact on different sponge cellular stress biomarkers. Caffeine and the insect repellent DEET were detected in sponges of all the islands, whereas the antibiotic erythromycin and the UV filter 4-methylbenzylidene camphor were found in resort islands only. Although concentrations were approximately a few ng/g d.w., we quantified various induced cellular effects, in particular an increase of the levels of the enzyme glutathione S-transferase involved in cell detoxification. Our results highlight the importance to increase awareness on ECs pollution, promoting the use of more environmental friendly products to achieving the sustainable development goals.

1. Introduction

Emerging contaminants (ECs) represent a growing problem for ecosystems and human health because most of these chemicals are not currently regulated or continuously monitored but are extensively used worldwide (Da Silva et al., 2011). ECs include pharmaceuticals, personal care products, disinfection products, UV filters, persistent organic chemicals, preservatives, and their degradation products (Daughton and Ternes, 1999). Although ECs are usually polar and non-persistent molecules, they are continuously released and ever-present in the environment; therefore, they are defined as "pseudo-persistent" (Daughton and Ternes, 1999). Most ECs are discharged from wastewater following human consumption or use. However, wastewater treatment plants (WWTPs) are often inefficient in removing ECs, as many of them are stable against common water treatment techniques, such as UV light irradiation and ozonation (Gartiser et al., 2012; Ahmed et al., 2021). This led municipal WWTPs to be the greatest sources of ECs for receiving water bodies. Surface water or water from sewage treatment plants is the most investigated compartment; however, recently, attention has been given to the marine coastal environment and seawater contamination (Richardson et al., 2005; Langford and Thomas, 2008; Birch et al., 2015; Ojemaye and Petrik, 2019; Madikizela et al., 2020). EC contamination in seawater mostly occurs near estuaries and marine outfalls (Arpin-Pont et al., 2016). Despite low concentrations in the aquatic compartment, usually of the order of a few ng/mL, ECs can accumulate in biological tissues and cause adverse effects (Daughton and Ternes, 1999; Arpin-pont et al., 2016; Birch et al., 2015). Bioaccumulation of ECs in marine organisms is related to a small portion of unionised species, with a high affinity for lipophilic matter in the aqueous phase, or the uptake of pollutants present in the dissolved phase or adsorbed onto colloids through the digestive tract (Fabbri and Franzellitti, 2015). Although scientific research on the occurrence of ECs in oceans is still ongoing, with monitoring programs designed to detect the quantity and distribution of ECs, little is known about the bioaccumulation of ECs in specific marine taxa.

Sponges are important functional components of marine ecosystems (Bell, 2008; Beepat et al., 2013, 2021), are an important link between the benthos and the water column (De Goeij et al., 2013; Rix et al., 2018), play a role in nutrient cycling (Maldonado et al., 2012; De Goeij et al., 2017), act as substrate stabilisers (Wulff, 2016), and provide

* Corresponding author at: Department of Earth and Environmental Sciences, University of Milano - Bicocca, Piazza della Scienza, 1, Milano 20126, Italy.
E-mail address: davide.seveso@unimib.it (D. Seveso).

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habitats for a suite of invertebrates (Beepat et al., 2014; Ávila and Ortega-Bastida, 2015). Sponges represent a valid organism for determining the presence of ECs in a habitat because they possess many characteristics that make them suitable as effective biomonitors, such as being sessile filter-feeding organisms, having a wide distribution in coastal environments, and their tolerance to different pollutants (Berthet et al., 2005; De Mestre et al., 2012). Several studies have reported the use of sponges as biomonitors to detect metals (Cebrian et al., 2007; Pan et al., 2011; De Mestre et al., 2012; Orani et al., 2020), polychlorobiphenyl (Perez et al., 2003), and polycyclic aromatic hydrocarbons (Batista et al., 2013). However, to date, few studies have analysed the occurrence of ECs in sponges (Rizzi et al., 2020) and no information on the effects of ECs on the physiology and cellular homeostasis of sponges is available.

Organisms attempt to metabolise pollutants through defence mechanisms that minimise cellular damage. Such cytoprotective processes include alterations in enzyme activities and cellular protein expression, which can be considered molecular biomarkers of exposure or effects (Livingstone, 1993; Cheung et al., 2001; Downs et al., 2005). Among these, glutathione-dependent detoxifying enzymes are crucial contributors to cell defence against both xenobiotics and endogenous compounds derived from oxidative stress (Liska, 1998; Hayes and McLellan, 1999; Srikanth et al., 2013). Glutathione S-transferase (GST) and glutathione reductase (GR) are two major glutathione-dependent enzymes involved in cell detoxification pathways. GST catalyses the conjugation of glutathione with xenobiotics and endobiotics, making them more hydrophilic and non-reactive, and consequently, excretable from the cell (Edwards et al., 2000). GR catalyses the NADPH-dependent reduction of glutathione disulphide to the sulfhydryl form of glutathione, which, in addition to representing a fundamental substrate for the activity of GST, is a critical molecule for counteracting oxidative stress and maintaining the reducing environment of the cell (Deponte, 2013). The expression of molecular chaperones involved in the maintenance of protein homeostasis, such as heat shock proteins (Hsps), has also been frequently adopted as cellular stress biomarkers in organisms subjected to different environmental stressors, including pollutants and xenobiotics (Venn et al., 2009; Gupta et al., 2010; Tomanek, 2011; Downs et al., 2012). Hsps usually increase their expression when organisms face conditions that may affect their cellular protein structure by facilitating proper protein folding and translocation, reducing the aggregation of other proteins, and assisting in degrading stress-damaged proteins (Balchin et al., 2016). Among the different Hsp families, 60-kDa Hsp (Hsp60) is a mitochondrial chaperonin that plays a crucial role in the synthesis and transportation of mitochondrial proteins, and in the folding of newly imported or stress-denatured proteins (Arya et al., 2007).

The Republic of Maldives is an archipelago that builds its foundation on coral reefs that are essential for the country's economy (Statistical Yearbook of Maldives, 2020). Sponges are one of the most abundant associated organisms that contribute to the functionality and services provided by the ecosystem (Rizzi et al., 2020). Tourism provides 30 % of the gross domestic product (GDP) as >1,500,000 people visit this example of one of the greatest reef ecosystems in the world each year (NBSAP, 2015; Maldives bureau of statistics, 2019; Di Fiore et al., 2020). Although the Maldives represent an excellent example of sustainable tourism (Domroes, 2001), several negative impacts and damage to coral reefs caused by touristic activity have been reported (Allison, 1996; Scheyvens, 2011; Zubair et al., 2011; Cowburn et al., 2018). Additionally, tourist presence has been found to increase waste production and the presence of xenobiotics released in the marine environment (World Bank, 2017). These impacts are exacerbated by the lack of adequate systems for wastewater treatment and solid waste disposal on many local islands (Kundur and Murthy, 2013; Rizzi et al., 2020). In 2016, only 48 % of the total population had access to sewerage networks in the Maldives (National Water and Sewerage Policy, 2017). Alternatively, WWTPs are present in the resort islands and capital island, where wastewater is pumped and discharged directly into the sea, causing potential damage to marine organisms (Zubair et al., 2011; Fallati et al., 2020). In this context, the aim of this study was to use the marine sponge *Spheciospongia* cf. *vagabunda* as a biomonitor to assess and quantify the presence and distribution of 15 ECs in the seawater surrounding the Maldivian resort islands and to compare their abundance with that found around a local non-tourist island. In all the islands, different reef sites characterised by different levels of human impact and different distances from the sewage pipe were analysed to track the contaminant extension. In addition, the effect of each EC on sponge cellular homeostasis was evaluated by investigating the enzymatic activity of GST and GR, and the expression of Hsp60. For this purpose, the correlation between the levels of each contaminant found in sponges and those of each biomarker was explored.

2. Materials and methods

2.1. Study area and sampling design

Samplings of the marine sponge *Spheciospongia* cf. *vagabunda* (Clionaidae; Ridley, 1884) were performed in the water surrounding three Maldivian islands, namely Athuruga (South Ari Atoll, 3°88′70″N; 72°87′64″E), Thudufushi (South Ari Atoll, 3°78′62″ N, 72°73′10″ E), and Magoodhoo (Faafu Atoll, 3°4′49″ N, 72°5757″ E), (Fig. 1A-B). Athuruga and Thudufushi are luxury resort islands hosting 46 and 47 beach bungalows, respectively, and 25 water villas each. Both islands have an oval/circular shape, surrounded by their own reef system, and a land area of approximately 0.045 km^2. Magoodhoo is an inhabited island (~900 inhabitants) measuring 900 m × 450 m, located in the southeastern part of the atoll rim, and its reef is approximately 2.9 km long and 1.55 km wide (Montano et al., 2012). The reefs of all the islands exhibit the features of typical low-energy reefs, with luxuriant growth of coral and gentle slopes on all sides (Montano et al., 2013; Montalbetti et al., 2019, 2022). In these reefs, the bioeroding sponge *S.* cf. *vagabunda* is a very common species being an important and abundant component of lagoon benthic communities of Maldives, and through all the Indo-Pacific reefs, where mainly occurs on soft-bottom substrate, although it can also occurs on hard-bottom, dead corals, coral rubble and on steep reef walls (Sutcliffe et al., 2010; Lim et al., 2012; Beepat et al., 2013, 2020; Becking et al., 2013; Calcinai et al., 2017; Marlow et al., 2018, 2021). Specimens from Maldives showed a massive morphology with surface generally smooth to moderately lumpy and dark coloration, with short conical oscular chimneys-like projections of few cm of diameter, protruding from the sponge surface as fistules (Fig. 1C).

On each island, sponge specimens were sampled from two different sites, characterised by different distances from the potential source of contaminants (e.g. sewage effluent outfall or solid waste landfill). In particular, in both Athuruga and Thudufushi, the first site was located close to the sewage pipe outfall on the eastern sides of the islands and at a depth ranging between 30 and 35 m, directly facing the deep sea (pipe site). The second site was situated along the same reef system approximately 200 m from the pipe and at the same depth (control site) (Fig. 1). In Magoodhoo, sewage effluent outfalls were absent. Therefore, one site was selected because it was characterised by a degraded and almost totally dead coral reef near the island's solid waste landfill (~30 m from the landfill, landfill site). Another site was characterised by a healthy coral reef located on the opposite side of the landfill, approximately 250 m from the island (control site), as previously described (Rizzi et al., 2020), (Fig. 1). At all these sites, fragments from different sponges ($n = 3$ to 6, from 5 to 10 g each) were collected by snorkelling or SCUBA diving, depending on the depth. All the fragments were squeezed to remove as much water as possible, were wrapped in aluminium foil, and frozen at −20 °C until the subsequent chemical analysis of contaminants. The sponges collected from Athuruga and Thudufushi were divided into two sub-samples. The first was preserved as described above to perform the chemical analysis, while the second sub-sample used for the enzyme and

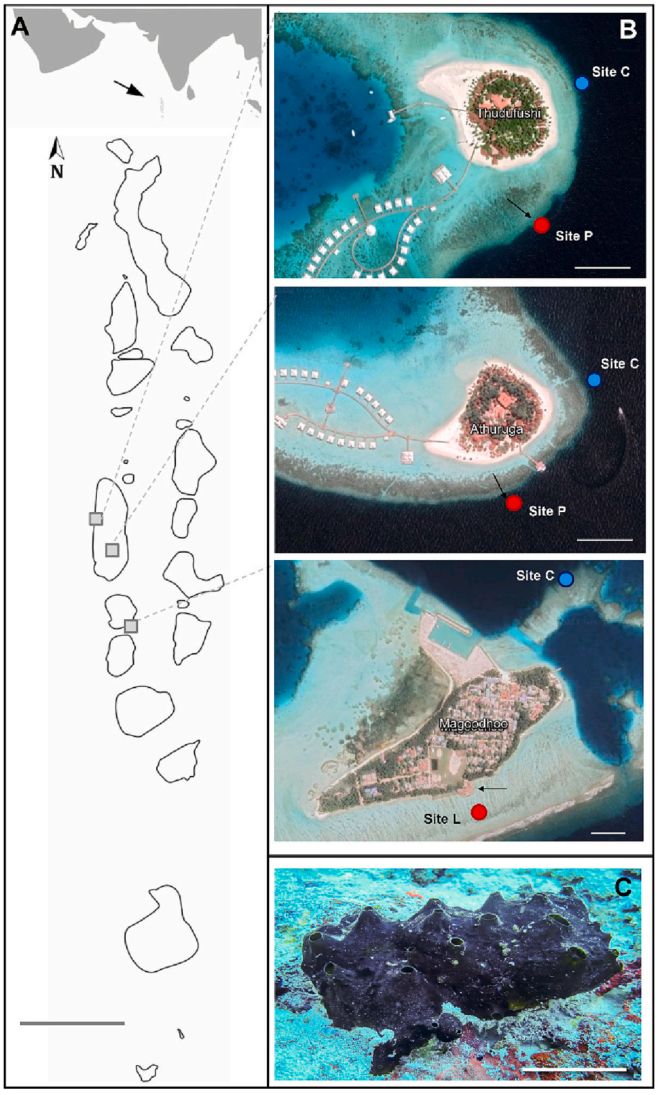

Fig. 1. A. Map of the 26 atolls composing the Republic of Maldives archipelago. The location of the three study islands is also represented by the three rectangles (Scale bar: ~ 100 km). B. Details of the three islands selected for the sampling activities: starting from the bottom, the inhabited island of Magoodhoo located in Faafu Atoll, and the resort islands of Athuruga and Thudufushi (Ari Atoll). The sampling sites of sponges in each island are also indicated (see Materials and methods for additional information; C: control sites; P: pipe sites; L: landfill site). In Athuruga and Thudufushi, black arrows indicate the position of sewage pipes. In Magoodhoo, the arrow indicates the solid waste landfill (Scale bars: ~ 100 m.) C. Specimen of the sponge *Spheciospongia* cf. *vagabunda* collected in the reefs of the three islands (Photo: Inga Denhert; Scale bar: ~ 5 cm).

protein analysis was immediately frozen at −80 °C, using an immersion cooler, and kept at that temperature until the protein extraction.

2.2. Chemical analysis and quality control

Fifteen ECs, belonging to 6 different compound categories, were selected as possibly related to tourism and released into the wastewater network system, and analysed in sponge samples: (I) caffeine; (II) pharmaceuticals (the antidepressant fluoxetine and its metabolite norfluoxetine, the non-steroidal anti-inflammatory drug diclofenac, the antibiotic erythromycin, the antipyretic and analgesic paracetamol, the anticonvulsants carbamazepine, and the two phosphodiesterase type 5 inhibitors such as sildenafil and tadalafil); (III) insect repellents (DEET); (IV) artificial sweetener (sucralose); (V) preservatives (methylparaben); and (VI) UV filters (3-(4-methylbenzylidene) camphor, benzophenone-1, benzophenone-3). All analytical standards were purchased from Merck Life Sciences (Milan, Italy). Freeze-dried samples (200 mg each) were placed in a Teflon vessel containing 8 mL of acetonitrile (HiPerSolv CHROMANORM, VWR Chemicals, Milan, Italy). Four grams of Al2O3 (Merck Life Science (Milan, Italy) were added to inhibit co-extraction of lipids and other hydrophobic matrix constituents (McEneff et al., 2013). Microwave assisted extraction (MAE) was performed using a Multiwave 5000 microwave reaction platform (Anton Paar GmbH, Graz, Austria). The extraction temperature was programmed as follows: ramp to 25 °C in 1 min, 5 °C/min until reaching 55 °C, holding at 55 °C for 5 min. The sample was left to cool before opening the vessel. The extract was evaporated to 0.5 mL under gentle nitrogen flow and loaded into an Oasis Prime HLB cartridge (200 mg, Waters, Milford, MA, USA). The eluates were evaporated to dryness and reconstituted with 0.1 mL of water (Milli-Q, CHROMASOLV Plus, Honeywell-Riedel-de Haën)/acetonitrile (98v/2v). Atrazine d-5 PESTANAL® (Merck Life Science, Milan, Italy) was used as the internal standard. Extracts were then analysed using ultra-performance liquid chromatography (Acquity UPLC H-class, Waters, USA) coupled with a QDa detector (Waters, USA). Three different analytical methods were developed and optimised for the determination of all studied contaminants (SI- 1). Further details on ULPC methods and MS setup are provided in the supplementary information.

Procedural blanks were used in all the steps of extraction and clean-up. The limits of detection (LOD) and limits of quantification (LOQ) were estimated as three and ten times the standard deviation of the procedural blanks, respectively LOD and LOQ for each substance are reported in Table S1. To calculate the means, values below the LOD and LOQ were substituted with ½ LOD and ½ LOQ, respectively.

The overall process efficiency, which combines recovery of analytes from the matrix and matrix effect on analytes ionization (Matuszewsky et al., 2003), was tested prior to sample analysis and the results are reported in Table S1.

2.3. Analysis of the enzymatic activities

2.3.1. Protein extraction

Sponge fragments were mashed with a pre-chilled mortar and pestle, transferred into tubes, and homogenised in 750 µL lysis buffer (50 mM Tris-HCl, pH 7.4, NaCl 150 mM, glycerol 10 %, NP40 detergent 1 %, EDTA 5 mM) containing 1 mM phenylmethylsulfonylfluoride (Sigma-Aldrich, Sigma-Aldrich, St. Louis, MO, USA). After the first centrifugation step (5 min, 3000 rpm) to remove calcium carbonate components, the cells were broken by sonication (6 × 10 s pulse on ice, amplitude 10 µm, Soniprep 150, Sanyo). Samples were then subjected to a second centrifugation step (15 min, 14,000 rpm, 4 °C), and the supernatant was collected and frozen immediately (−80 °C) until subsequent assays. The total protein content of each sample was determined using the Bradford method with bovine serum albumin (BSA) to design a calibration curve.

2.3.2. GR activity assay

The enzymatic assay for GR was performed as described by Wang et al. (2001). The activity of GR was evaluated by spectrophotometric detection of the absorbance at 340 nm (Varian Cary 50 Scan spectrophotometer, Agilent Technologies) of NADPH oxidation to NADP+ reaction, which occurs in conjunction with glutathione reduction and is proportional to the decrease in absorbance over time. In particular, the NADPH reaction was initially detected in the reaction mix (containing 0.1 M potassium phosphate buffer pH 7.6, 0.16 mM NADPH, 1 mg ml^{-1} BSA, and 4.6 mM oxidized glutathione), and then by adding different volumes of samples. The GR activity was determined from the difference between the two absorbance values. One unit of GR activity was defined as the oxidation of 1 nmol NADPH/min at 25 °C. The results were expressed as units (U) of enzyme per mg of protein.

2.3.3. GST activity assay

GST activity was assessed by considering the reaction of the enzyme with the 1-Chloro-2,4-dinitrobenzene (CDNB) substrate, according to Hayes and Strange (2000). The reaction solution (containing 200 mM potassium phosphate buffer pH 6.5, 20 mM CDNB dissolved in 95 % ethanol, and 20 mM reduced glutathione) was mixed in a 1 mL cuvette with different volumes of samples, and the formation of CDNB-oxidized glutathione conjugate was followed spectrophotometrically at 340 nm (Varian Cary 50 Scan spectrophotometer, Agilent Technologies). GST activity is expressed as units (U) of enzyme per mg of protein and is proportional to the increase in absorbance caused by conjugated product formation.

2.4. Analysis of the expression of Hsp60

To extract total proteins, frozen sponge tissue portions were mashed with a pre-chilled mortar and pestle and homogenised in SDS buffer (0.0625 M Tris-HCl, pH 6.8, 10 % glycerol, 2.3 % SDS, 5 % 2-mercaptoethanol) containing 1 mM phenylmethylsulfonylfluoride (Sigma-Aldrich) and complete EDTA-free protease inhibitor cocktail (Roche Diagnostic). Extracts were stored at −80 °C until further processing. Aliquots were used to determine total protein concentrations with the Bradford method using BSA as reference. Equal amounts of proteins from each sample were separated by SDS-PAGE on 8 % polyacrylamide gels (Vai et al., 1986), then run in duplicates using a Mini-Protean Tetra Cell (Bio-Rad Laboratories). After electrophoresis, the gel was stained with Coomassie Brilliant Blue to visualise total proteins, while the other was electroblotted onto a nitrocellulose membrane (Amersham Protran 0.45 mm) for western blot analysis, as previously described (Seveso et al., 2012, 2013). Filters were stained with Ponceau S Red (Sigma-Aldrich) to confirm the correct protein transfer. The following primary antibodies were used: anti-Hsp60 monoclonal antibody (IgGI1 mouse clone LK-2, SPA-807, Enzo Life Sciences) and anti-β-actin monoclonal antibody (IgG1k mouse clone C4, MAB1501, Millipore). Hsp60 antibody was diluted 1:1000 in TBS-0.1 % Tween 20 and 5 % skimmed milk, whereas β-actin antibody was diluted 1:3000 in the same solution. After washing three times with fresh changes of TBS-0.1 % Tween 20 (15 min each), filters were incubated with anti-mouse IgG polyclonal secondary antibodies conjugated with horseradish peroxidase (ADI-SAB-100, Enzo Life Sciences) diluted 1:10000 for Hsp60 and 1:15000 for β-Actin in TBS-0.3 % Tween 20 and 5 % skimmed milk. Western blots were developed using Pierce ECL Western Blotting substrate, followed by exposure of filters to Amersham Hyperfilm ECL.

Densitometric analysis was performed as previously described (Seveso et al., 2013). Films were scanned on a Bio-Rad GS-800 calibrated imaging densitometer, and the pixel density of the scanned bands was quantified using ImageJ free software (http://rsb.info.nih.gov/ij/) of the NIH Image software package (National Institutes of Health, Bethesda, Md., USA). For each blot, the scanned intensity of the bands of Hsp60 was normalized to the total protein content. Densitometric data are expressed as relative levels (arbitrary units).

2.5. Statistical analysis

Data normality was verified using the Shapiro-Wilk test, and where assumptions were violated, the data were corrected by transformations. For each EC, differences in their concentrations among islands and sites (control vs. pipe/landfill sites) and a combination of both factors were analysed with two-way ANOVA followed by Tukey's HSD post hoc tests. For each biomarker analysed, a one-way ANOVA was performed to detect significant differences between the control and pipe sites, considering the sponges sampled only in Athuruga and Thudufushi. Values were considered statistically significant at $p < 0.05$, and all data are presented as arithmetic mean ± SE. Spearman's rank correlation order test was used to examine whether the abundance of each EC was correlated with the level of each biomarker analysed (GST, GR, and Hsp60), considering all sponges sampled only in Athuruga and Thudufushi. All statistical analyses were performed using the SPSS ver. 26 (IBM). Similarities among sponges sampled in the different islands based on the concentration of ECs found were illustrated using a non-metric multidimensional scaling plot (nMDS) performed using Primer v7 (Primer-E). nMDS was performed using Bray-Curtis similarity without transformation.

3. Results and discussion

Among the studied compounds, fluoxetine, norfluoxetine, diclofenac, paracetamol, carbamazepine, sildenafil, tadalafil, sucralose, methylparaben, benzophenone-1, and benzophenone-3 were not detected in any sample. On the other hand, caffeine and DEET were found in sponges collected in reefs of all three investigated islands, whereas erythromycin and 3-(4-methylbenzylidene) camphor (4-MBC) were detected only in sponges living in reefs of the tourist islands Athuruga and Thudufushi (Table S2).

In the tourist islands, sponges collected near the sewage pipe showed significantly higher levels of GST and Hsp60 than those collected from the control sites, far from the pipe (ANOVA, $p = 0.001$ and $p = 0.002$, respectively), while no difference was reported in the enzymatic activity of GR (Fig. 2).

3.1. Caffeine

Caffeine residues were uniformly present among the islands, but significant differences were detected between the control and pipe/landfill sites, regardless of the island (Table 1, Fig. S1A). In Athuruga, caffeine showed a significantly higher concentration in sponges collected near the pipe than at the control sites (Table S2; Fig. 3A), indicating that sewage pipes strongly contribute to caffeine emissions. In Thudufushi, caffeine was detected, but no significant differences were observed between sites, even though a lower amount was recorded in the control site. However, the presence of caffeine in the control site of this island could be attributed to marine currents, which could transport wastewater along the coast, especially after the construction of submerged breakwaters (Jameel, 2010). Finally, in Magoodhoo, caffeine was only found near the landfill site (Table S2; Fig. 3A). The detection of caffeine confirms its potential bioconcentration despite its low LogKow, equal to −0.07 (Hansch et al., 1995), suggesting a link with lipid-independent mechanisms (Ali et al., 2018). This process could explain the variability (even if less than one order of magnitude) in the bioconcentration of caffeine observed in previous studies (Bayen et al., 2015, 2016; Rizzi et al., 2020). The concentrations detected in this study were in the range of those observed by other authors in marine organisms. In previous studies, caffeine concentrations were found to vary from a few ng/g d.w. to 73.6 ng/g d.w. in fish (Ojemaye and Petrik, 2019; Ali et al., 2018) and up to a maximum of about 140 ng/g d.w. in bivalves (Maruya et al., 2014; Bayen et al., 2015, 2016). Caffeine is a stimulant that is used worldwide as an effective marker of anthropogenic presence and activity (Nehlig et al., 1992; Dafouz et al., 2018). It is usually associated with wastewater pollution, as it is mainly excreted by the human body through urine (Seiler et al., 1999).

Recently, several studies have demonstrated that environmentally realistic concentrations of caffeine have significant adverse effects on several marine coastal species, such as algae, bivalves, polychaetes, and sea urchins (reviewed in Vieira et al., 2022). These effects include growth inhibition, impairment of reproduction and development, alteration of energy reserves and metabolic activity, neurotoxic effects, oxidative stress, and cellular damage (Vieira et al., 2022). However, to date, no studies have analysed the effects of caffeine on sponges. A significant positive correlation was detected between the level of caffeine and the levels of all three biomarkers analysed (Table 2). This indicated that sponges containing higher concentrations of caffeine showed up-regulation of the activity and expression of GST, GR, and

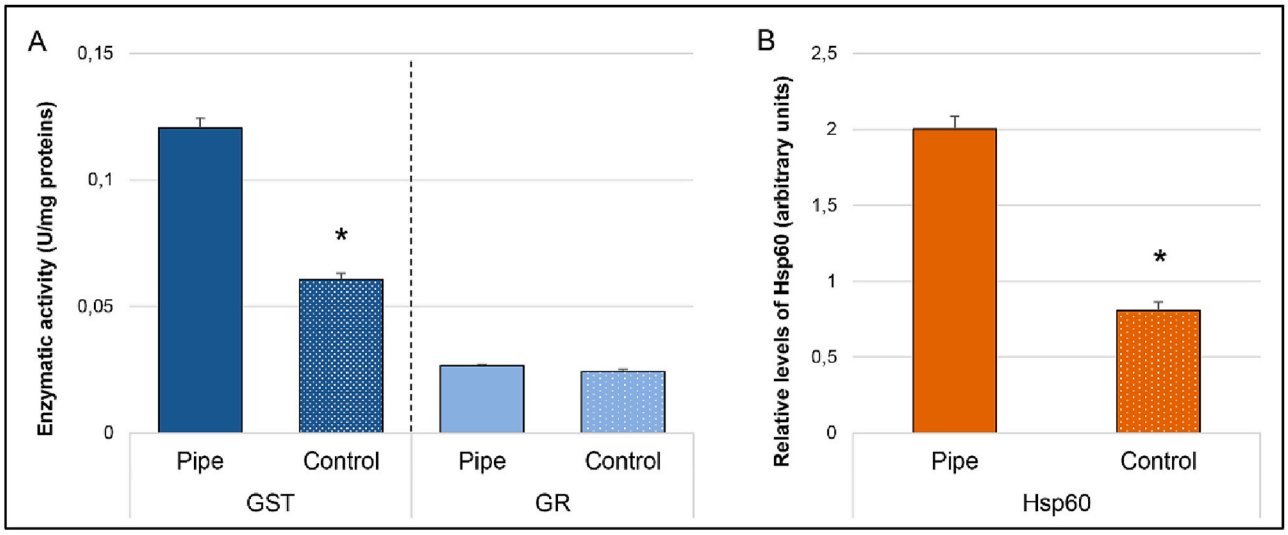

Fig. 2. Enzymatic activity of GST and GR (A) and levels of Hsp60 (B) detected in sponges sampled in control and pipe sites of Athuruga and Thudufushi islands. Data are expressed as units U of enzyme per mg of proteins (A) and as arbitrary units (B). In both graphs, data are calculated considering the sponges sampled only in Athuruga and Thudufushi together and are reported as mean ± SEM (n = 12). For each biomarker, asterisks indicate significant differences between sites.

Table 1
Summary of the two-way analysis of variance (ANOVA) of the concentrations of caffeine, erythromycin, DEET, and 4-MBC among islands and sites (control vs. pipe/landfill sites) and a combination of both factors. Significant values (p < 0.05) are in bold.

EC	Factors	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Caffeine	Island	2	21.310	10.655	3.174	0.065
	Site	1	60.473	60.473	18.014	**0.0001**
	Site*Island	2	9.318	4.659	1.388	0.268
	Residuals	25	83.926	3.357		
Erythromycin	Island	2	108.343	54.171	6.925	**0.004**
	Site	1	5.716	5.716	0.731	0.401
	Site*Island	2	22.549	11.274	1.441	0.256
	Residuals	25	195.570	7.823		
DEET	Island	2	11.016	5.508	2.494	0.103
	Site	1	0.031	0.031	0.014	0.907
	Site*Island	2	12.574	6.287	2.847	0.077
	Residuals	25	55.207	2.208		
4-MBC	Island	2	1857.537	928.768	4.328	**0.020**
	Site	1	15.534	15.534	0.072	0.790
	Site*Island	2	26.632	13.316	0.062	0.940
	Residuals	25	5364.861	214.594		

Hsp60, suggesting that these enzymes/proteins may be useful biomarkers of caffeine contamination. Therefore, the caffeine levels found in the tourist island reefs may be toxic to the sponge cells, producing oxidative stress and affecting cellular protein homeostasis, thus resulting in the activation of cell detoxification pathways and defence mechanisms. In other coral reef organisms, such as scleractinian corals and sea anemones, caffeine has been shown to increase the expression of Hsp70 and to have affected protein phosphorylation, causing bleaching (Sawyer and Muscatine, 2001; Pollack et al., 2009). Moreover, recent evidence has demonstrated that, in several marine organisms, caffeine may enhance the production of reactive oxygen species (ROS), which represent early warning indicators of toxic effects in aquatic organisms (Aguirre-Martínez et al., 2015, 2016), inducing a wide spectrum of antioxidant responses, including GST and GR (Aguirre-Martínez et al., 2013, 2016; Maranho et al., 2015; Pires et al., 2016; Cruz et al., 2016; Li et al., 2020).

3.2. Erythromycin

Erythromycin concentration was significantly different in sponges sampled from different islands but was similar between the pipe and control sites (Table 1, Fig. S1A). In Magoodhoo, erythromycin was not detected in any sample (Table S2; Fig. 3B), confirming previous evidence (Rizzi et al., 2020). In contrast, erythromycin was detected in both touristic islands (Fig. 3B), suggesting the role of tourism in increasing contamination levels. However, in both Athuruga and Thudufushi, no significant differences in its concentration were recorded between sites, even though the Athuruga values of erythromycin found in sponges collected close to the sewage pipe were higher than those in sponges inhabiting the control site (Fig. 3B). This homogeneous distribution in coastal waters may be due to its half-life in seawater (DT50), estimated to be approximately 40 days (Kwon, 2016). Erythromycin persistence in the marine environment may favour its distribution away from the emission sites. Because of its lipophilic nature (LogKow = 3.06, McFarland et al., 1997), bioaccumulation is expected and has been observed in aquatic organisms (Gaw et al., 2014; Liu et al., 2014; Cheng et al., 2018). Notwithstanding this, to the best of our knowledge, only a few studies have reported the presence of erythromycin in wild marine animals, and in all these, the reported concentrations were always lower than those found in Maldivian sponges (Li et al., 2012; Wu et al., 2021).

Erythromycin poses high ecological risks to aquatic organisms and has been included on the watch list by the European Union (EU) decision 2015/495. As a pharmaceutical, it is biologically active, thus leading to a greater possibility of affecting non-target organisms. In previous studies, both acute and chronic effects have been observed, including an increase in mortality, DNA damage, ROS production, and bacterial resistance to antibiotics in aquatic organisms, such as *Daphnia magna* and fish (Meinertz et al., 2010; El-Nahhal and El-Dahdouh, 2015). Nevertheless, adverse effects are usually observed when organisms are exposed to higher concentrations than those reported in the environment. Here, a significant positive correlation was observed between erythromycin and GST only (Table 2), indicating that the levels of this EC detected in Athuruga and Thudufushi sponges could be toxic to these organisms and sufficient to trigger cellular detoxification. In contrast, the level of erythromycin observed in this study did not appear to affect protein homeostasis.

3.3. Insect repellent (DEET)

The DEET concentrations were similar among islands, sites, and within sites on each island (Table 1, Fig. S1A). In all islands, DEET levels were lower in the control sites than in the pipe/landfill sites, although

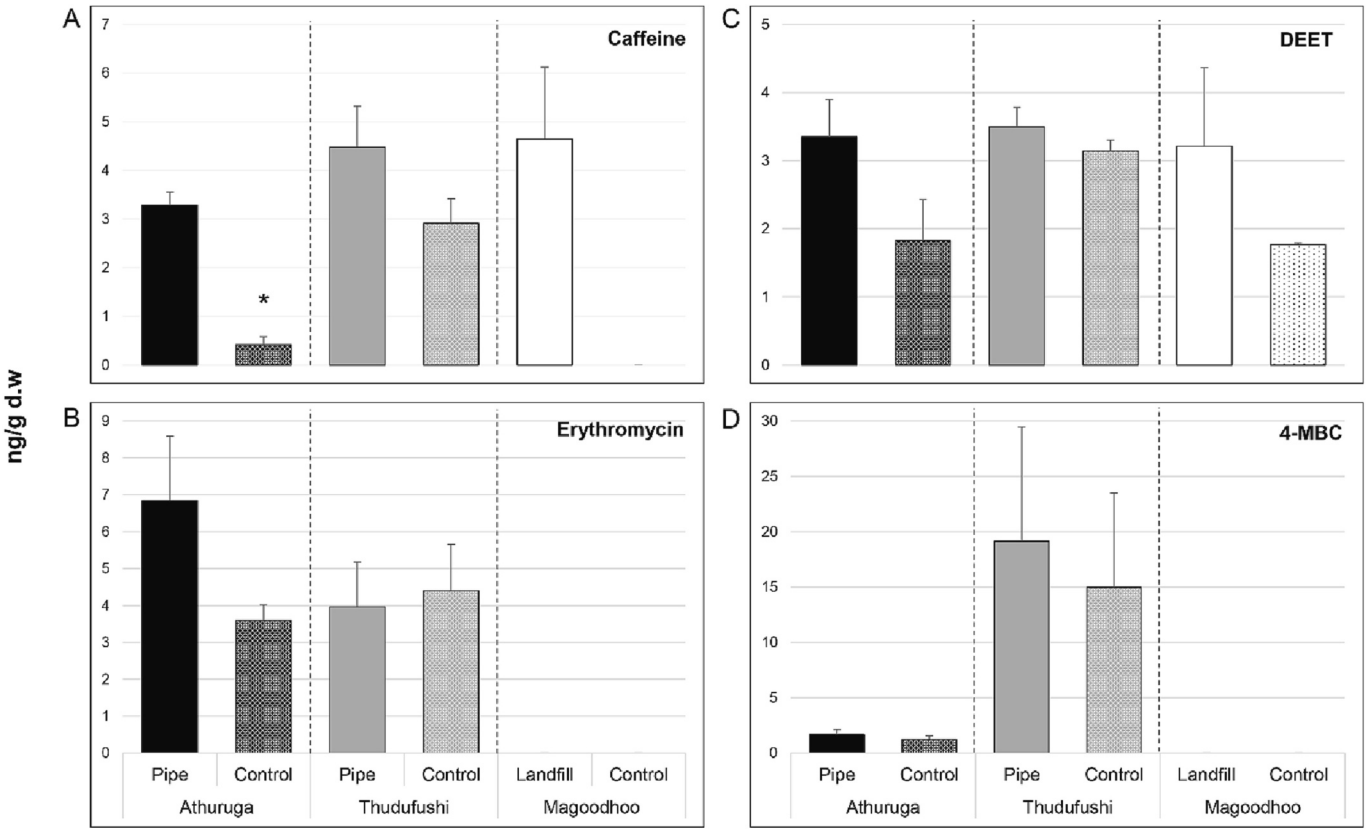

Fig. 3. Levels of caffeine (A), erythromycin (B), DEET (C), and 4-MBC (D) detected in sponges collected in the different sites (Control, Pipe, Landfill) of the three investigated islands (Athuruga, Thudufushi, and Magoodhoo). Data are expressed and mean ± SEM. For each island, asterisks indicate significant differences in the level of each compound between sites.

Table 2
Spearman's rank order correlation values between the level of GST, GR, and Hsp60 and each EC detected in sponges from Athuruga and Thudufushi (caffeine, erythtomycin, DEET, and 4-MBC). Statistical significance was defined as $p < 0.05$ and N is 24 for each biomarker analysed in correlation with each EC.

		Caffeine	Erythromycin	DEET	4-MBC
GST	Spearman coefficient	0.821	0.427	0.042	0.566
	Sign. (two-tailed)	**0.001**	**0.037**	0.846	**0.022**
GR	Spearman coefficient	0.568	0.243	0.116	0.265
	Sign. (two-tailed)	**0.004**	0.253	0.59	0.211
Hsp60	Spearman coefficient	0.89	0.329	0.145	0.521
	Sign. (two-tailed)	**0.001**	0.116	0.499	**0.04**

these differences were not significant (Fig. 3C). No significant differences were highlighted when comparing the tourist and local islands (ANOVA, $p = 0.247$). The presence of DEET in control sites may be due to its atmospheric transportation after spray application (Weeks et al., 2012), or by volatilisation from wastewaters (Ferrey et al., 2018). DEET can also enter the aquatic environment while swimming, showering, or bathing. Moreover, in areas characterised by the presence of diseases transmitted by mosquitoes (i.e. Zika and dengue fever), such as the Maldives, the use of insect repellents is recommended for public health (Merel and Snyder, 2016). Consequently, DEET is frequently detected in various water matrices worldwide (Merel and Snyder, 2016; Montes-Grajales et al., 2017). For this reason, there is growing concern regarding the potential relevance of DEET in the environment. In the Maldives, DEET pollution is expected to be continuous because of the year-round presence of tourists and the constant presence of insects (Dudouet et al., 2020).

EU assessments conducted prior to 2010 classified DEET as an R52/53 chemical, which required the labelling of the active ingredient (but not products containing <25 % DEET) as "harmful to aquatic organisms, may cause long-term adverse effects in the aquatic environment" (EC, 2008). The EU classification is expected to change to R52 because recent assessments have concluded that DEET is readily biodegradable (KemI, 2010).

Acute toxicity tests have shown that environmental concentrations should not affect biota (Costanzo et al., 2007; Weeks et al., 2012). However, exposure to DEET, even at high concentrations, has been demonstrated to lead to adverse effects such as a decrease in catalase and GST activities, inhibition of acetylcholinesterase, and a decrease in adult body size in the aquatic midge (Campos et al., 2016). Very limited data are available regarding chronic effects. Aronson et al. (2012) showed that chronic aquatic toxicity would not be expected based on environmental fate and concentration. In this study, no correlations were detected between DEET and GST, GR, or Hsp60 (Table 2), indicating that at the concentrations reported here, DEET did not cause cellular toxicity in the sponges.

3.4. UV filters

Among the studied UV filters, only 4-MBC showed quantifiable concentrations in the analysed samples. A significant difference in its concentration was detected only among the islands and not among the sites (Table 1, Fig. S1A). In all islands, no significant difference in 4-MBC levels was observed between the control and pipe sites (Fig. 3D). The mean concentration of 4-MBC was markedly higher in Thudufushi than in Athuruga, whereas it was never found in sponges from Magoodhoo (Table S2, Fig. 3D). Therefore, a UV filter was detected in tourist islands only, showing that tourist presence may play a role in intensifying its introduction into the marine environment. Moreover, the presence of 4-MBC at the control sites of both tourist islands may be due to the same

factors influencing DEET distribution in the environment. In fact, as a main ingredient in solar creams and sprays, it is applied to the skin and transferred to the environment indirectly after showering, through wastewater discharge, or directly from recreational activities such as swimming (Falfushynska et al., 2021). Therefore, the aquatic compartment is the main receiver of this compound (Gago-Ferrero et al., 2015).

4-MBC is characterised by high lipophilicity (LogKow = 5.14; EC, 2006) and poor degradability; therefore, it is expected to be found in biota (Gago-Ferrero et al., 2015). Vidal-Liñán et al. (2018) investigated its bioaccumulation potential in mussel tissues. A growing interest in such a contaminant is also linked to its toxicity. Recently, several studies analysed the deleterious effects of UV filters, and in particular 4-MBC, on different aquatic animals (Kunz et al., 2006; Fent et al., 2010; Paredes et al., 2014; Sang and Leung, 2016; Santonocito et al., 2020; Araújo et al., 2021; Hong et al., 2021). Although UV filters have been detected and analysed in coral reefs and related organisms (Danovaro et al., 2008; Downs et al., 2016; Tsui et al., 2017; Corinaldesi et al., 2018; Kung et al., 2018; Horricks et al., 2019), the potential effects of 4-MBC on sponges have not been investigated. Our results showed that an increase in the level of 4-MBC in the sponges led to a significant increase in the levels of both GST and Hsp60, thus indicating a possible toxic effect of this compound at the cellular level. In previous studies, *Chironomus riparius* insect larvae exposed to the UV filter benzophenone-3 altered their HSPs gene expression but not GST activity (Muñiz-González and Martínez-Guitarte, 2020). In the loggerhead turtle *Caretta* a significant positive correlation between oxidative stress biomarkers, such as GST and Hsp60, and total sunscreen agent concentrations in their blood was observed (Cocci et al., 2020).

The European Chemical Agency identified 4-MBC as a substance of very high concern because of its endocrine disrupting properties relevant to human health (ECHA, 2021) and included this substance in the Candidate List for eventual inclusion in Annex XIV (lists all the substances which uses are subject to authorisation in the EU). In addition, the potential environmental impacts originating from the use of UV filters have led several nations to take action to protect marine ecosystems. Hawaii, Key West (Florida, USA), the U.S. Virgin Islands, and the Republic of Palau (Levine, 2020; Miller et al., 2021; Carve et al., 2021) have already banned some sunscreen ingredients owing to their toxic effects on marine ecosystems, particularly coral reefs (Suh et al., 2020; Raffa et al., 2018; Mitchelmore et al., 2021; Moeller et al., 2021). To achieve sustainable tourism development, the Maldivian Republic should develop a monitoring protocol to assess the environmental level of these emerging but harmful reef compounds. At the same time, it is essential to raise awareness among citizens on the adverse effects of chemicals on the coral ecosystems and to encourage tourists to use "reef friendly" products.

3.5. Multivariate analysis results

The nMDS analysis identified two main clusters composed of tourist (Athuruga and Thudufushi) and local (Magoodhoo) islands, highlighting that tourism could play an important role in the release of the investigated ECs in the coral reef ecosystem (Fig. 4). Moreover, the graph showed that caffeine, erythromycin, and 4-MBC were the most important compounds in determining the distribution of the sample groupings.

4. Conclusions

Tourism has had several negative impacts on the environment of the Maldivian archipelago. This study confirms that the presence of tourists can introduce hazardous substances into the marine environment. Indeed, the wastewaters of tourist islands bring to the coral reef a different charge of EC contaminants, particularly antibiotics and UV filters. Moreover, exposure of marine sponges to ECs leads to a significant increase in the levels of enzymes involved in cell detoxification pathways. In particular, GST was the most sensitive biomarker for ECs, as it is the most directly involved in the detoxification processes (phase II) and is less generic than Hsp and GR. As coral reefs worldwide are already suffering from disease and climate change consequences, it is important to reduce the effects of other sources of danger. Nevertheless, political issues and a lack of coordination of risk-mitigation measures may slow this process. For this reason, it is fundamental to invest in increasing tourists' awareness of the stress on coral reef communities due to the presence of ECs, for example, by promoting the use of more environmentally sustainable and less hazardous types of UV filters.

Preventing and contrasting such evidence is fundamental to

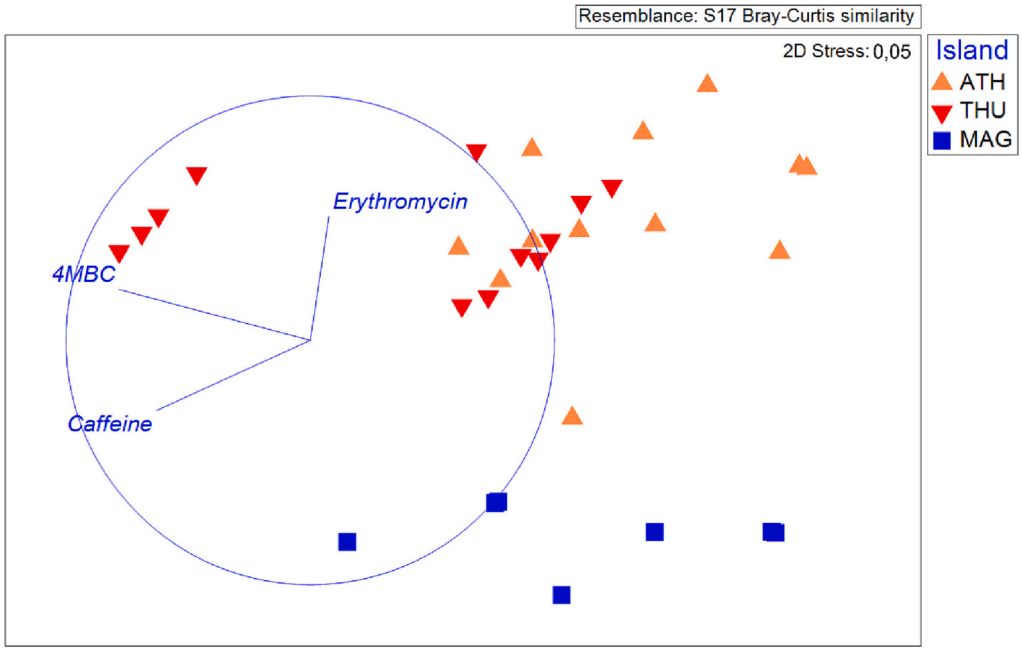

Fig. 4. Non-metric multidimensional scaling plot (nMDS) showing the similarities among sponges sampled in the different islands, regardless of site, based on the concentration of ECs, with vectors (Pearson's correlations ≥0.7) representing the ECs that drive significant similarities among islands.

achieving the sustainable development goals adopted by the United Nations Member States, in particular Goal 14, which aims to conserve and sustainably use oceans, seas, and marine resources for sustainable development.

CRediT authorship contribution statement

Cristiana Rizzi: Methodology, Writing - original draft. Davide Seveso: Conceptualization, Writing - review & editing, Formal analysis. Chiara De Grandis: Investigation. Enrico Montalbetti: Methodology, review & editing. Stefania Lancini: Investigation and Laboratory experiments. Paolo Galli: Funding acquisition. Sara Villa: Conceptualization, Resources, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2023.115084.

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