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Application of marine sponges for biomonitoring active pharmaceutical ingredients (APIs) in coral reefs. Optimization of an SPME and ESI-LC-MS/MS method

Francesco Saliu [a,*], Alessandro Becchi [a], Enrico Montalbetti [a,b], Valerio Isa [a,b,c], Tommaso Gatti [a], Davide Riseri [a], Marina Lasagni [a], Paolo Galli [d], Davide Seveso [a,b]

[a] Department of Earth and Environmental Science, University of Milano Bicocca, Piazza della Scienza 1, 20126 Milano, Italy
[b] MaRHE Center (Marine Research and High Education Center), Magoodhoo Island, Faafu Atoll, Maldives
[c] Costa Edutainment SpA - Acquario di Genova, Area Porto Antico, Ponte Spinola, 16128 Genoa, Italy
[d] University of Dubai, Dubai, P.O. Box 14143, United Arab Emirates

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ABSTRACT

Chemical pollution is a threat to coral reefs. To preserve them, it is crucial to monitor novel contaminants and assess the related risks. The occurrence of active pharmaceutical ingredients (APIs) in coral reefs has been poorly investigated until now. Under this light, we tested the use of the marine sponge Cf. *Hyrtios* as bio-monitors and conducted a pilot study in the Faafu Atoll (Maldives). Analyses were carried out by in vivo solid-phase microextraction (SPME) and liquid chromatography (LC) electrospray ionization (ESI) tandem mass spectrometry (MS/MS). Twelve APIs were selected for method optimization. Limits of quantitation (LOQs) were in the 0.6 and 2.5 ng/g range, accuracy between 86.5 % and 104.7 %, and precision between 3.0 % and 14.9 %. All the sponges located in the inner reefs resulted contaminated with at least one API. Gabapentin and Carbamazepine displayed the highest detection rates, while Ketoprofen had the highest concentration (up to 15.7 ng/g).

1. Introduction

Chemical pollution is nowadays considered one of the most significant threats to the stability of crucial Earth system processes and subsystems (Steffen et al., 2015). According to Persson and colleagues (Persson et al., 2022), the increasing release of new chemicals is surpassing our capacity to evaluate the connected risks and monitor their spread in the environment. This has already lead to a situation where the world is exceeding the safe limits of the related "planetary boundary". Particularly concerning is the lack of data regarding the accumulation of chemical pollutants in the marine environment (Fabbri and Franzellitti, 2016; Branchet et al., 2021). The issue has been substantially underestimated until now, under the assumption that "the infinite dilution" occurring in seawater may represent a safety factor. Only in the last decades, the observation of massive tarball strandings in coastal environments and of garbage patches in oceanic gyres (Saliu et al., 2023; Suaria et al., 2020), and the discovery of the accumulation of persistent organic pollutants (POPs) in seawater, sediments, and marine organisms (Gavrilescu et al., 2015; Isa et al., 2022; Saliu et al., 2022), has substantially changed the overall perspective: monitoring marine pollution has become a priority recognized in the goal 14 of the 2030 Agenda for sustainable development. Although there is growing data on the toxicity of certain POPs to different marine organisms, new compounds of emerging concern have been released into the marine environment and we lack of sufficient scientific information for accurately assessing the associated risk. Among the emerging contaminants, active pharmaceutical ingredients (APIs) have been largely overlooked despite they may exhibit a high recalcitrance and a potent biological activity (Mezzelani and Regoli, 2022). APIs can enter water systems through various pathways, including patient excretion and improper disposal of medications (Mezzelani et al., 2018). Moreover, conventional wastewater treatment plants (WWTPs) might be ineffective in removing APIs from wastewater (Jones et al., 2005; Afonso-Olivares et al., 2017; Rogowska et al., 2020). In the aquatic environment, APIs may display unexpected toxicological effects since they have finely tailored chemical structures that were purposely designed to interact with specific enzymatic targets and elicit potent physiological responses (in humans) at relatively low doses (Miller et al., 2018). In this respect, the dispersion of

* Corresponding author.
E-mail address: francesco.saliu@unimib.it (F. Saliu).

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antimicrobial agents is considered extremely concerning since it may lead to the development of antibiotic-resistance genes (Munita and Arias, 2016).

Currently, regulatory bodies do not require a comprehensive assessment of the eco-toxicological effects of APIs (Vambol et al., 2021). Only in the last years, pharmaceutical companies have started to take in consideration the entire lifecycle of their products and to discuss plans for the mitigation of the related environmental risks. However, these initiatives are still not integrated into a regulatory framework.

Overall, >3000 APIs are available on the market (Richardson and Ternes, 2022). To the best of our knowledge, only 27 APIs are included by international legislation on a Watch list that requires or suggests their monitoring in the marine environment, e.g. the 'OSPAR Convention' (OSPAR Commission, 1992) and the Water Framework Directive (WFD) 2000/60/EC (European Commission Directive, 2013; Tornero and Hanke, 2016; Ojemaye and Petrik, 2019; Branchet et al., 2021).

In the coming years, the loads of APIs into the marine environment are expected to increase mostly due to the growing global demographic trends and the increasing number of people living along coastlines (Ortúzar et al., 2022). Therefore, it is urgent to evaluate the incoming exposure scenario and asses the environmental impacts. By the adoption of new analytical technologies the collection of data may be improved. For example, field operations may be substantially speed up and the areas covered by surveys enlarged. In this context, the use of marine invertebrates to monitor the presence of chemical pollutants represents an efficient solution that is gaining more and more popularity. Mussels have been the first marine invertebrates exploited for biomonitoring, due to their high capacity of accumulating inorganic contaminants, and their use is now included in official methods (O'Connor, 1996). In recent years, also marine sponges have received some attention, both for monitoring organic (Batista et al., 2013; Rizzi et al., 2020; Saliu et al., 2020a) and inorganic pollutants (Venkateswara Rao et al., 2009; de Mestre et al., 2012). Marine sponges are important functional components of marine ecosystems (Bell, 2008) very abundant and adapted to a variety of ecological niches (Pawlik et al., 2018). They act as a link between the benthos and the water column (de Goeij et al., 2013) and play a crucial role in nutrient cycling, especially in carbon fluxes (Maldonado et al., 2017). Because they are sessile, long-living, and very efficient filter feeders, they may process large volumes of water and concentrate pollutants in their tissues (Bell et al., 2018). For all these reasons marine sponges may be used to trace the occurrence of new contaminants, and at the same time, to collect indications of the health status of the marine environment.

Starting from this basis, we aimed to research the use of marine sponges as biomonitors of the occurrence of APIs in coral reefs. Specifically, we optimized a non-lethal method based on the use of solid phase microextraction (SPME) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Twelve APIs were selected among those most commonly found in sewage and known to not be completely removed by conventional wastewater treatment: the antibiotics Ofloxacin and Clarithromycin, the anti-inflammatory compounds Diclofenac, Propyphenazone, Ketoprofen, and Amisulpride, the neuroactive compounds Gabapentin-lactam, the beta-blocker Metoprolol and the anti-epileptic Carbamazepine, the antidepressant Escilatopram, and the antihypertensive Ibertesan.

To test the applicability of the proposed approach in a real case scenario, we carried out a preliminary survey in the coral reefs of the Republic of Maldives, where sponges are among the most prominent and abundant reef-associated fauna, contributing to the functionality and integrity of the related ecosystems. More specifically, we examined the occurrence of APIs in specimens of the sponge Cf. *Hyrtios* sp. (Demospongiae), by surveying two different reef sites characterized by a different distance from an inhabited island and different hydrodynamic conditions.

2. Material and methods

2.1. Chemicals

Acetone and dichloromethane were purchased from Promochem (Promochem, Milano, Italy) and used for glassware cleaning. Ultra-grade methanol was purchased from Merck (Merck KGaA, Darmstadt, Germany) and used for chromatographic elution. Ultrapure Water (resistivity, 18.2 MΩ-cm) was produced on a Milli-Q Plus apparatus (Millipore, Milan, Italy). Cylindrical SPME fibers with 0.08 cm^2 surface area were purchased from Sigma Aldrich (Sigma Aldrich Part No 57281-U). Certified analytical standard (purity ≥98 %) of Ofloxacin, Clarithromycin, Diclofenac Propyphenazone, Ketoprofen, Amisulpride, Gabapentin-lactam, Metoprolol, Sulfamethoxazole Carbamazepine, Ibertesan, Escilatopram and their analytical surrogates Fluoxetine-d6, Ofloxacin-d3, Carbamazepine-d10 were purchased from Merck (Merck KGaA, Darmstadt, Germany) and used to prepare the stock calibration solutions at 10 μg/mL in methanol. This solution was then diluted as specified further for method calibration and validation.

2.2. Study area and collection of sponges

Samples were collected in the Republic of Maldives and, more specifically in the Faafu Atoll (3°04′N, 72°57′E), located in the south-east region (Fig. 1). Faafu Atoll is about 140 km away from the capital, Male. The atoll has a circular shape with a diameter of approximately 30 km, and is composed of 23 islands of which only 5 are inhabited and a tourist resort occupies one. The population of the atoll is about 5000 people. Following several pre-surveys of the area, in May 2022, two coral reef sites with different morphological, physical, and ecological features were selected. The first site, Beyrufushi Reef, was on the external ocean-facing side of the atoll rim. This site is usually subjected to intense hydrodynamic conditions and characterized by steep walls, and thus classified as an "outer" reef. In addition, this site is far from any inhabited island of the atoll. As an open ocean-facing reef, this site was characterized by massive coral growth morphologies, sea fans, and marine sponges. The second site, Magoodhoo Reef, is the house reef of Magoodhoo Island. 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., 2020). The Magoodhoo reef exhibits the features of typical low-energy reefs, with luxuriant growth of coral and gentle slopes located inside the atoll rim, inside the calm atoll lagoon, and thus classified as an "inner" reef. As most of the Maldivian inner lagoon reef, the site is characterized by Acroporidae, Pocilloporidae, and Poritidae corals (Montano et al., 2020). Both sites were heavily impacted by the coral bleaching event of 2016, corallivore outbreaks, and coral diseases, but by the time of our sampling, a natural recovery process could be observed (Cowburn et al., 2018; Raguso et al., 2022). In both sites, we selected individuals belonging to the genus: Cf. *Hyrtios* sp. (Demospongiae), that is a common sponge genus, abundant in the lagoon benthic communities of Maldives and through all the Indo-Pacific reefs, where mainly occurs on hard-bottom substrate, dead corals, coral rubble and on steep reef walls (Rizzi et al., 2020; Cleary et al., 2021). In total, 11 individuals of *Hyrtios* sp. were selected at the same depth (5-15 m) and sampled in Beyrufushi Reef (7 samples) and Magoodhoo Reef (4 samples). Three samples were collected for method validation, as specified in the following section.

2.3. Solid phase micro-extraction (SPME)

The scheme of the analytical procedure employed for the analysis of APIs in marine sponges is reported in Fig. 2. Briefly, the procedure include three steps: the first step is the insertion of the SPME fiber into sponge tissue. The fiber is left in contact with the tissue for the selected time that is designated to guarantee an efficient extraction of the

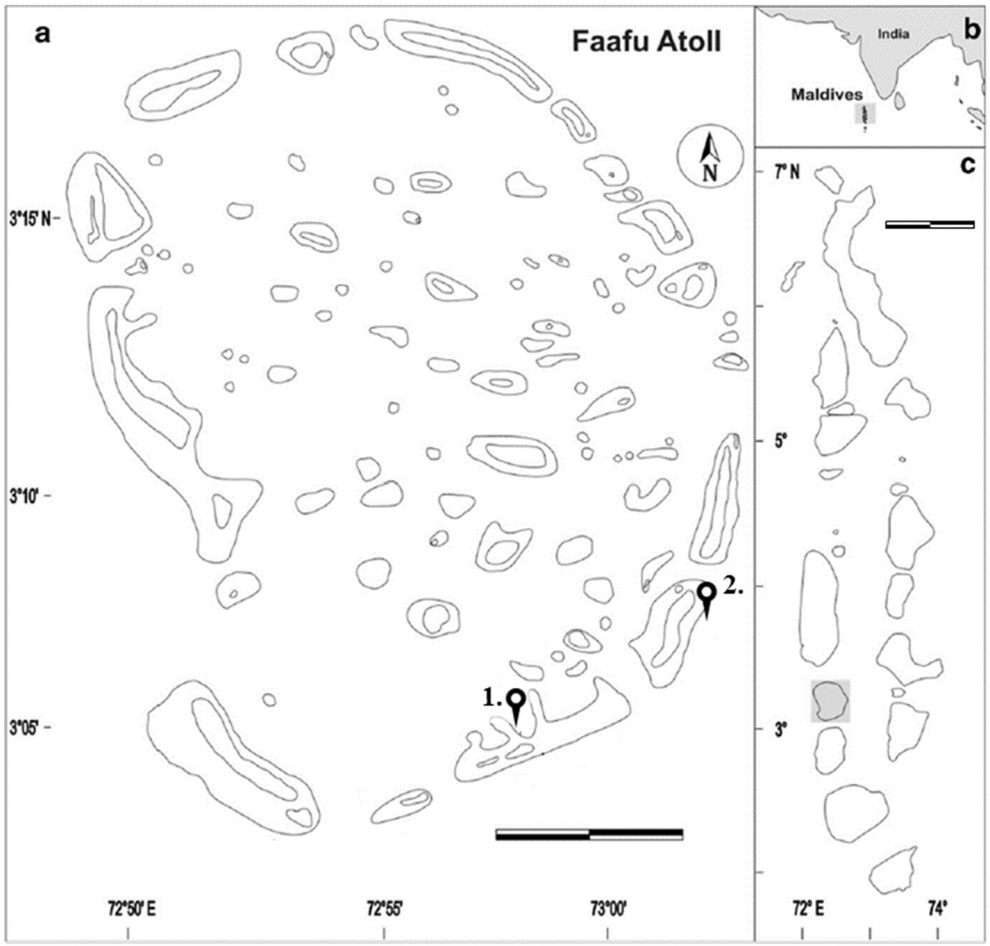

Fig. 1. (A) Detailed map of Faafu Atoll, with an indication of the selected sampling points: 1 = House Reef (H.R.), 2 = Beyrufushi Reef (B.R.). Scale bar: 10 km. (B) Map of the North-Central Indian Ocean. The Maldives are indicated within the grey square. (C) Detailed map of the Maldives' atolls. Faafu Atoll is indicated within the grey square. Scale bar: 100 km.

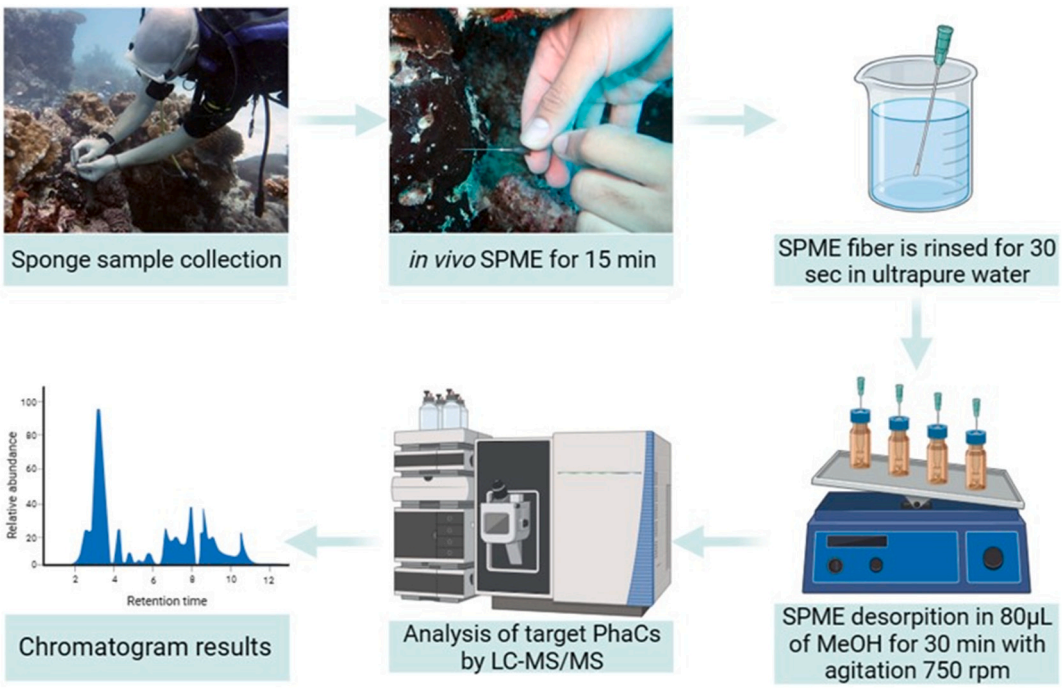

Fig. 2. Scheme of the procedure adopted for the analysis of APIs in marine sponges.

targeted compounds. After that, the analytes that are sorbed onto the fiber polymeric phase must be desorbed in a solvent mixture to be then injected in a chromatographic system. The desorption is achieved by placing the fiber into a vial insert containing 80 μL of the selected desorption solution and left for the required time in agitation. At the end of the desorption process, the fiber is removed from the vial, and the vial is placed in the LC-MS/MS autosampler for performing the analytical determination by tandem mass spectrometry. The extraction performance is influenced by several parameters e.g. desorption time, temperature, chemical composition of the fiber, and desorption solvent mixture (Souza Silva et al., 2013) that ultimately impact on method selectivity and sensitivity. The optimization of this condition for APIs in sponge tissue is detailed in the following sections.

2.4. ESI-LC-MS/MS

ESI-LC-MS/MS analyses were carried out by employing a TSQ Quantum Access Max instrument (Thermo Scientific) equipped with a liquid chromatograph (UHPLC/HPLC), an ESI interface, and a triple quadrupole mass analyzer. The chromatographic separation was performed using a Thermo Scientific Accucore C-18 aQ column (100 mm × 2.1 mm I.D., 2.6 μm). Gradient elution was obtained with a binary mobile phase mixture. Specifically, flow rate was set up at 0.5 mL/min, bottle A was filled with water and 0.1 % of formic acid, bottle B was filled with methanol 98 % and 2 % water, and the gradient elution was set up as follow: during the first minute of elution the pump B percentage was kept at 20 %, then it was raised from 30 to 70 % for the successive 4 min, followed by a 5-minute linear gradient to 96 %, and then maintained at 96 % for 5 min. At the end of the gradient elution, 10 min were used to restore the initial condition (pump B at 20 %) and the equilibration of the system before the successive chromatographic run. Sample introduction was performed by using an autosampler, and the injector was operated in the partial loop injection mode. The injection volume was set up at 10 μL. For mass spectra acquisition, the ESI-MS interface was operated in the positive ion mode. The spray voltage was set up at 3500 V. The vaporizer temperature at 350 °C and the capillary temperature at 270 °C. Sheath gas pressure was set up at 50 arbitrary units and auxiliary gas pressure at 15 arbitrary units ion sweep gas pressure 2 arbitrary units. The triple quad mass spectrometer was operated in selected reaction monitoring mode (SRM). For each target analyte, one qualifier and one quantifier were researched (details are reported in Table 1). Collision gas pressure was set up at 1.0 m Torr and the cycle time at 0.6 s. Data acquisition and data processing were performed using the software Xcalibur (Thermo Scientific).

2.5. Method optimization and validation

SPME conditions were optimized by employing a portion of sponge tissue of approximately 10 g. This portion was artificially contaminated at 100 ng/g concentration level with the API's stock solution. Because references of sponge tissues contaminated with API's are no commercially available, the same matrix build-up for method optimization was used also as a reference standard for method validation. The method optimization was primarily carried out to determine the conditions that would result in the highest extraction efficiency and reproducibility. Specifically, our tests aimed to evaluate how extraction time, extraction temperature, solvent polarity, and elution time affected these two parameters. At first, we assessed the recoveries at three different extraction temperatures (25, 30 and 35 °C) and by using four different solvent extraction mixtures (methanol:water 25:75 methanol: water 50:50 methanol:water 80:20 methanol:water 95:5). We also assessed the extraction kinetic from 5 to 90 min, by considering five points of the extraction time curves (more details of the experiments and the related results are reported in the following result section).

Method validation was carried out according to the guidelines of the European Union Commission Decision 2002/657EC (Publication Office of the European Community, 2022). Specifically, matrix-matched calibration curves were drawn by spiking the SPME extracts of the sponge tissue with the standard calibration mixture and by measuring the relative area versus the internal standard. Calibration points were prepared at 0.5, 2, 5, 10, 30, 60, and 150 ng/g. Accuracy was evaluated by comparing the expected concentration in the reference matrix with the mean concentration obtained from 12 replicated analyses. The concentration was measured by following the back-calculation approach described by Martins et al. (2011). Precision was assessed from the relative chromatographic peak areas standard deviations (RSDs). The matrix effect was determined by comparing the peak areas obtained from each API when spiked in 1 g of methanol: water 80:20 ($n = 7$), and in 1 g of SPME extract of sponge tissue (in both cases we spiked 25 ng of each API as the absolute amount for the peak areas comparison).

2.6. Statistical analysis

All the collected data were expressed as mean ± standard error (SE). One-way analysis of variance (ANOVA) was used to investigate differences in the distribution of the APIs in the different sponges. Data normality was assessed by the Shapiro-Wilk tests. Non-parametric Kruskal-Wallis test was applied when the normality assumption was not met and in this case, the differences between groups were visualized with Tukey's H test. Values were considered statistically significant at $p < 0.05$. Non-parametric Spearman rank correlation test was performed to examine the occurrence of correlation in the abundance of the most frequently detected APIs. All these analyses were performed using XLSTAT (XLSTAT, Lumivero, Denver USA).

3. Results

3.1. Method optimization

The first parameter optimized was the extraction temperature. Tests

Table 1
List of the twelve APIs researched in the present study with the related mass spectrometry targeted ions and collision energies (C.E.) applied.

Compound[a]	Formula	CAS n°	Molar Mass (g/mol)	Log Kp	Parent	Quantifier (C.E.)	Qualifier (C.E.)	Tube lens
Ofloxacin	$C_{18}H_{20}FN_3O_4$	82419-36-1	361.37	−0.39	362.0	318.0 (19)	260.9 (25)	100
Clarithromycin	$C_{38}H_{69}NO_{13}$	81103-11-9	747.95	3.16	748.2	157.9 (26)	590.2 (18)	107
Diclofenac	$C_{14}H_{11}Cl_2NO_2$	15307-86-5	296.15	4.51	295.8	213.9 (36)	249.8 (16)	56
Propyphenazone	$C_{14}H_{18}N_2O$	479-92-5	230.31	1.94	231.0	56.2 (32)	189.0 (21)	82
Ketoprofen	$C_{16}H_{14}O_3$	22071-15-4	254.28	3.12	254.9	77.1 (39)	208.9 (13)	83
Amisulpride	$C_{17}H_{27}N_3O_4S$	53583-79-2	369.48	1.10	208.9	208.9 (42)	242.0 (27)	85
Metoprolol	$C_{15}H_{25}NO_3$	37350-58-6	267.36	1.88	268.1	74.1 (21)	116.0 (17)	82
Gabapentin-lactam	$C_9H_{15}NO$	64744-50-9	171.24	−1.10	154.1	67.1 (29)	67.1 (16)	100
Carbamazepine	$C_{15}H_{12}N_2O$	298-46-4	236.27	2.45	236.9	192.0 (24)	193.9 (19)	87
Escitalopram	$C_{20}H_{21}FN_2O$	128196-01-0	324.39	1.34	325.1	262.0 (22)	109.1 (27)	67
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	723-46-6	253.28	0.89	254.0	156.0 (13)	108.1 (23)	76
Irbesartan	$C_{25}H_{28}N_6O$	138402-11-6	428.53	4.50	429.1	206.9 (22)	180.0 (35)	92

[a] Data from U.S. National Library of Medicine ChemIDPlus Advanced (https://chem.nlm.nih.gov/chemidplus/

were conducted within the temperature range of 25–35 °C and the effect on extraction yields registered. This limited range of temperature conditions was selected because it is consistent with the temperature range typically observed in Maldivian reefs (Saliu et al., 2018). As depicted in Fig. 2, the maximum absolute recoveries were obtained by operating at 35 °C. At this temperature, the relative increments in extraction recovery in respect to the lowest extraction temperature was +45.6 %. However, we decided to validate the method at a temperature of 30 °C, considering that a temperature of 35 °C is not usual and may induce significant stress in the organism. At this temperature, the recoveries resulted from 0.3 to 27.2 % lower than the recoveries obtained at the highest temperature, which we consider acceptable for further method development (Fig. 3).

The eluotropic strength of solvent mixtures applied during the fiber desorption step was the second parameter scrutinized to enhance the recoveries of the analytes. Results showed a correlation between the extraction yields and the polarity of the solvent mixture (Fig. 4), with most of the APIs scoring the highest recoveries by using 100 % methanol as fiber desorption solution ($\varepsilon = 0.73$ on Snyder's scale). However, we also observed that 80:20 methanol: water provided the highest precision in replicated extractions (S·D = 3.8 %). Therefore, we retained this condition for the method validation. Finally, extraction time curves were drawn by considering the absolute amounts extracted at 5, 10, 20, 30, 45, and 90 min (supplementary, S1). The curves showed that in any case, equilibrium conditions were reached, and that in all the cases, 5 min fiber insertion was a sufficient time to extract absolute amounts of APIs that were detectable at the mass spectrometer. Overall, we found at 15 min a good compromise in terms of the amount of analytes extracted, matches with the mass spectrometer sensitivity and application of pre-equilibrium conditions. This short exposure time also helps limit stress on the sponges caused by contact with the fiber. The analytical benefits of operating the solid-phase microextraction (SPME) in pre-equilibrium have been extensively described by Pawliszyn (1997). This is due to the extraction process being controlled by diffusion, which in turn provides comparable response factors for different classes of analytes.

3.2. Method validation

Table 2 reports the figure of merits of the method obtained by drawing matrix-matched calibration curves and by performing replicated analysis of the quality control samples. Overall, all the compounds tested showed a good linear response within the 0.2–150 ng/g concentration range, with R^2 values comprised between 0.990 and 0.998. The limit of detection calculated from matrix-matched calibration curves considering a value equal to 3.3 times the residual standard deviation divided by the slope in the linear regression, resulted in varying from 0.2 to 0.8 ng/g, while quantification limits (LOQs) between 0.6 and 2.5 ng/g. Method accuracy, evaluated through back-calculated concentration level onto a quality control spiked at 100 ng/g, resulted comprised between 86 % to 104 %. Precision calculated from RSDs levels of the inter-day assays of quality control samples spiked at 25 ng/g was comprised between 2.8 % and 15.3 %. No significant matrix effect was highlighted (Table 3) for any of the tested APIs under the applied conditions. Finally, the recoveries obtained after 15 min insertion in the 100 ng/g spiked reference matrix are reported in Table 4. Values, expressed as absolute amounts of API, varied between 0.2 and 1.8 ng (in all the cases the value corresponds to a relative recovery lower than 1 % of extraction of the total amount of target compounds spiked in the matrix volume, which is in line with the concept of the analytical micro-extraction).

3.3. Analysis of sponges

The results of the survey carried out in the Faafu Atoll are reported in Table 5. All the sponge collected in the lagoon (samples H.R.) resulted to be contaminated with a least one of the target compounds. Outside the atoll rim (samples B.R.), only one sample displayed a certain level of contamination (in trace amounts). Among the researched APIs, Carbamezapine and Gabapentin displayed the highest detection rates with 5/8 and 4/8 detections, respectively, while Ofloxacin, Amilsulpride, Chlaritromicyn, Ecilatopram, Ibertesan, Sulfamethazole were not found in any of the examined samples. The maximum concentration for a single API was 15.7 ng/g caused by Ketoprofene in a sample collected from the inner reef (sample H.R. 1). The same sample resulted to be the most contaminated also for the sum of targeted APIs (26.6 ng/g). Also, the samples H.R.4 and H.R.3 displayed notable levels of APIs (21.8 and 9.6 ng/g) respectively. From the concentration levels, we calculated bio-concentration factors, considering the average concentration found in the water of the lagoon during a previous survey (Becchi et al., 2024). Average values resulted in 2700 L/Kg for Carbamazepine, 2282 L/Kg for Ketoprofen and 137 L/Kg for diclofenac with a maximum value of 3670 L/Kg for the specimens H.R.1 (Supplementary, S3).

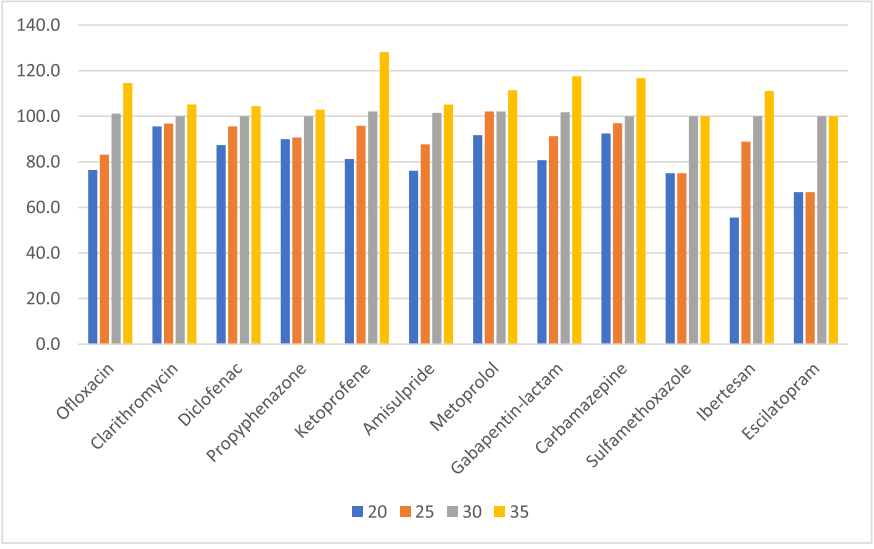

Fig. 3. Effect of extraction temperature on API's recovery. Values are expressed as relative responses (response observed at the tested condition vs the response obtained in the validated method).

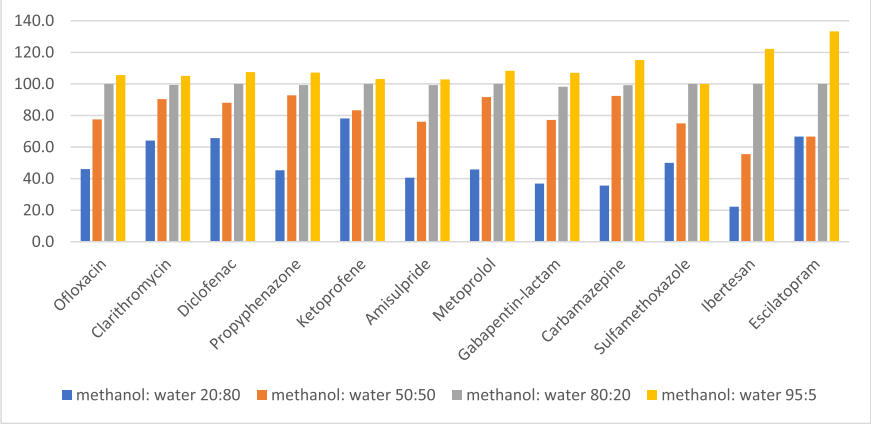

Fig. 4. Effect of solvent mixture used during the desorption step on the recoveries. Values are expressed as relative responses (response observed at the tested condition vs the response obtained in the validated method).

Table 2
Figure of merits obtained for the bioSPME and ESI-LC-MS/MS analysis of APIs in marine sponges.

Name	RT	Linear range (ng/g)	R^2	LOD (ng/g)	LOQ (ng/g)	Accuracy	intra-day RSD (%)	Inter day RSD (%)
Ofloxacin	4.52	0.8–150	0.992	0.8	2.5	95.3 %	2.3	6.8 %
Metoprolol	5.45	0.3–150	0.989	0.3	0.8	89.6 %	2.1	14.9 %
Sulfamethoxazole	1.49	0.4–150	0.997	0.4	1.3	99.2 %	3.7	10.9 %
Escilatopram	6.83	0.4–150	0.987	0.4	1.2	86.5 %	5.2	14.4 %
Amisulpride	1.92	0.4–150	0.998	0.4	1.3	101.4 %	1.2	3.0 %
Gabapentin-lactam	7.74	0.3–150	0.994	0.3	0.8	97.9 %	3.6	8.9 %
Propyphenazone	8.17	0.3–150	0.996	0.3	1.0	97.2 %	0.9	3.1 %
Carbamazepine	8.29	0.2–150	0.995	0.2	0.6	97.6 %	0.8	2.5 %
Clarithromycin	8.46	0.3–150	0.992	0.3	0.9	98.0 %	2.5	5.9 %
Ibertesan	9.07	0.7–150	0.988	0.7	2.2	91.4 %	4.6	12.5 %
Ketoprofene	9.51	0.4–150	0.993	0.4	1.2	104.7 %	3.9	7.5 %
Diclofenac	11.75	0.2–150	0.989	0.2	0.7	101.8 %	1.2	3.9 %

Table 3
Matrix effect obtained by applying bioSPME and ESI-LC-MS/MS to the reference matrix obtained by spiking the sponge tissue with the selected APIs at 100 ng/g. Results are expressed as mean values from six replicates with standard deviation. Values higher than 120 % represent a significant effect of ionization enhancement, whereas values smaller than 80 % represent a significant ion suppression effect.

Compound	Matrix effect	RSD %
Ofloxacin	86.2 %	9.8
Clarithromycin	87.5 %	11.4
Diclofenac	89.6 %	7.5
Propyphenazone	90.3 %	10.8
Ketoprofene	86.7 %	14.2
Amisulpride	96.1 %	10.5
Metoprolol	102.0 %	11.9
Gabapentin-lactam	87.7 %	8.1
Carbamazepine	101.0 %	14.1
Sulfamethoxazole	95.9 %	8.2
Escilatopram	88.0 %	9.7
Ibertesan	92.7 %	13.1

Table 4
Extraction efficiency of the twelve APIs by bioSPME. The recovery tests were carried out by using an in-house prepared quality control sample, obtained by spiking at 100 ng/g concentration level of the targeted analytes into a pre-homogenized *Hyrtios* sp. sample.

Compound	Amount extracted (ng)	RSD %
Ofloxacin	0.89	7.2
Clarithromycin	1.56	13.9
Diclofenac	1.34	11.4
Propyphenazone	1.39	12.0
Ketoprofene	0.96	7.3
Amisulpride	1.38	7.5
Metoprolol	0.96	6.9
Gabapentin-lactam	0.57	5.0
Carbamazepine	1.32	7.1
Sulfamethoxazole	0.04	12.9
Ibertesan	0.09	8.8
Escilatopram	0.03	5.5

4. Discussion

4.1. Potential of marine sponges for biomonitoring by SPME

In the recent literature, there are examples of the application of marine sponges as bio-monitors for tracing contaminants in the marine environment (Orani et al., 2018). This is not surprising, since sponges have a high capacity for contaminant accumulation (Padovan et al., 2012) by filtering large quantities of seawater (100–1200 $mLh^{-1}g^{-1}$), more than any other marine invertebrates (Olesen and Weeks, 1994). In addition, the effectiveness of sponges as model organisms for the bioremediation of polluted marine sites has already been demonstrated (Illuminati et al., 2016). Solid-phase microextraction (SPME) is a well-established sample-preparation technique for environmental studies. In recent years, thanks to the development of direct immersion probes coated with biocompatible polymers, the application of SPME has extended from the headspace extraction of volatile compounds (VOCs) to the capture of active components in living organisms (Zhou et al., 2008; Togunde et al., 2012; Bojko et al., 2012; Souza Silva et al., 2013; Panio et al., 2020; Saliu et al., 2020b; Yu et al., 2022). For bio-monitoring, SPME represents a significant improvement to traditional extraction techniques such as solvent extraction (LE) or solid phase

Table 5
Concentrations of APIs in the sponge of the Faafu Atoll. n.d. = not detect (concentration under the detection limit). HR = House Reef (located inside the atoll rim near the inhabited island) BR = Beyrufushi Reef (located outside the atoll rim). For values above the detection limit and under the quantitation limit, concentration was set up as half of the quantitation limit.

	H.R. 1	H.R. 2	H.R. 3	H.R. 4	B.R. 1	B.R. 2	B.R. 3	B.R. 4	Frequency	Mean	SE
Gabapentin	1.2	0.4	0.4	0.4	n.d.	n.d.	n.d.	n.d.	4/8	0,5	0,1
Propylphenazone	0,9	0.5	0,9	n.d.	n.d.	n.d.	n.d.	n.d.	3/8	0,3	0,1
Carbamazepine	6.4	6.8	1.7	6.9	0.3	n.d.	n.d.	n.d.	5/8	3,1	0,9
Ketoprofene	15,7	n.d.	6.1	14.1	n.d.	n.d.	n.d.	n.d.	3/8	4.7	1.8
Metoprocol	n.d.	0.8	n.d.	0.4	n.d	n.d.	n.d.	n.d.	2/8	0,2	0,1
Diclofenac	2.4	n.d.	0.5	n.d.	n.d.	n.d.	n.d.	n.d.	2/8	0,4	0,2
Ofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/8	0.0	0.0
Amisulpride	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/8	0.0	0.0
Clarithromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/8	0.0	0.0
Sulfamethazole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/8	0.0	0.0
Escitalopram	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/8	0.0	0.0
Ibertesan	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0/8	0.0	0.0
SUM	26.6	8.5	9.6	21,8	2.1	0.0	0.0	0.0	5/8	8.5	2.7

extraction (SPE) because it helps to avoid the suppression of collected specimens. The compact setup of the coated fiber allows extraction from a very small tissue portion without significantly disturbing the tested living system. During the survey, we monitored the health of tested *Hyrtios* sp. before and after the in vivo sampling procedure. We did not observe any signs of stress or tissue damage. Moreover, the validated method displayed good analytical performances, both in terms of the limit of quantification and reproducibility, enabling the detection of APIs at ppb concentration levels. This may be related to the biomolecular composition of the sponge tissue, which allows a relatively fast diffusion of the analytes from the matrix to the bioSPME fiber. The differences observed in the recovery yields of the APIs might be mainly ascribed to their different chemo-physical properties. According to the boundary layer model (Pawliszyn, 2000), the diffusion of the analytes is mainly influenced by their molecular weights, steric hindrance, and tissue-fiber partition constants. Differences in these parameters lead to the different diffusion kinetics that ultimately leads to the observed differences in the extraction yields, which are also partially corrected by the different instrumental responses of the mass spectrometer.

4.2. APIs in coral reefs: associated risks for marine life

The presence of APIs in the Maldivian archipelago can be attributed to the rise in the population and the greater access to modern healthcare services that the Republic of Maldives has faced in the last decades, even in the more remote villages. A significant contribution to the environmental contamination may be related also to the increase in international tourism, sustained by the new form of hospitality such as the diffusion of guesthouses in the inhabited that has been added to the traditional form of international resorts. The lack of proper systems for wastewater treatment and solid waste disposal represents a significant increase in the potential hazard of these compounds in these fragile environments. Because no data regarding the occurrence in the sewage of any API is available from the Maldivian institution; we used as reference the data available in Western countries (Zuccato et al., 2000). The APIs surveyed in this study were therefore selected and monitored as they are the most diffused in Western countries and regularly found as contaminants of the aquatic environment, and also because included in the list of approved drugs by the Maldivian Health Authority e (National Bureau of Statistics Ministry of National Planning, 2020).

Considering the possible impacts of these contaminants on aquatic life, it must be highlighted that the available literature is mostly focused on freshwater ecosystems, while there is a still lack of information for the marine environment. Numerous evidence indicates that APIs may trigger toxic effects in different aquatic organism. For instance, test with Ofloxacin and the microalgae *M. aeruginosa*, *L. minor*, and *P. subcapitata* showed EC50s of 21, 126, and 12,100 μg/L, respectively (Robinson et al., 2005). Test with *Daphnia magna* showed low mortality (<10 %) at a concentration of 10 mg/L indicating that ofloxacin may not be a treat considering the environmental concentrations (Robinson et al., 2005). This was also confirmed by Nguyen et al. (2021) that studied the long-term effect (42 days) on *D. magna* exposed to different concentrations of ofloxacin (50, 500, and 5000 μg/L). Mortality never happened but a reduction in fertility was observed as the production of dead eggs was significant at the higher exposure. Diclofenac was tested to determine acute and chronic toxicity on *D. magna* showing an EC50 of 123 mg/L, which is considerably higher than environmental concentrations (De Oliveira et al., 2016). Even if *D. magna* seems quite resistant to Diclofenac, Quinn et al. (2011) highlighted that this is not true for zebra mussel (*Dreissena polymorpha*). Different biomarkers were monitored after 24 h and 96 h of exposure to two different concentrations (1 and 1000 μg/L nominal concentrations). Both exposures triggered an increase in lipid peroxidation. Oxidative stress was also found in the freshwater fish *Cyprinus carpio* exposed to 0.31 μg/L of diclofenac, causing cell death by apoptosis and DNA damage was observed at 72 h in liver cells (Quiroga-Santos et al., 2021). Chronic toxicity tests of Ofloxacin, Sulfamethoxazole, and Clarithromycin were performed by Isidori et al. (2005) on the green algae *Pseudokirchneriella subcapitata* showing strong results especially for the last compound (EC50 of 2 μg/L after 3 days of exposure), while EC50 was higher for the other two (1.44 and 0.5 mg/L). Ofloxacin was also the only compound among the studied ones to show genotoxic properties. Sulfamethoxazole is known to be toxic for green microalgae like *Chlorella vulgaris* (Xu et al., 2022). Inhibition of cell growth, a decrease of chlorophyll content, damage in cell membrane, and abnormal production of reactive oxygen species were also observed on the green algae *Scenedesmus obliquus* exposed to different levels of this micropollutant (0, 5, 10, 20, 40, 80, and 160 μM). The EC50 8.42 μM after 96 h of exposure while permeability of the cell membrane increased when sulfamethoxazole was higher than 10 μM (Xu et al., 2022). Clarithromycin did not show acute effects on *D. magna* as no immobilization was observed after 48 h of exposure to different concentrations (0.01, 0.1, 1, 10 mg/L), (Yamashita et al., 2006). However, the reproduction ratio dropped from 100 % in the control to 40 % with a concentration of 0.1 mg/L. Metoprolol toxicity was low for *D. magna* (EC50 = 438 mg/L) while other trophic levels seems more sensible as an EC50 of 7.9 mg/L was found for the microalgae *Desmodesmus* (Cleuvers, 2005). Toxicity of Metoprolol was also studied on fish embryos (*Danio rerio*), where growth retardation was detected for concentration above 12.6 mg/l (van den Brandhof and Montforts, 2010). The toxicity of Gabapentin and Irbesartan was studied by Minguez et al. (2016), showing low effect for the two compounds (EC50 > 100 mg/L) both on *D. magna* (after 48 h) and *Pseudokirchneriella subcapitata* (after 72 h). Carbamazepine showed toxicity in L. *minor* (EC50 = 50.17 mg/L) but relevant effects were not found on Vibrio fisheri and *D. magna* (Grabarczyk et al., 2020). Fish embryos (*Danio rerio*) was affected by growth retardation when exposed to concentrations above 30.6 mg/L

(van den Brandhof and Montforts, 2010). Acute toxicity test on *Daphnia similis* was performed studying Ofloxacin by De Castro et al. (2014) highlighting an EC50 of 4.41 mg/L. No effect was shown on D.magna after 21 days (NOEC = 8.9 μg/L) while the lowest effect (LOEC) was shown with an exposure of 31 μg/L. NOEC <0.6 μg/L after 48 h, EC50 of 24 μg/L, and LC50 of 2 mg/L are reported for green microalgae (Fent et al., 2006; Oakes et al., 2010). In summary, according to the above-mentioned data, APIs can determine toxic effects on different levels of the water trophic chain. For the best of our knowledge no data were collected till now regarding the possible impacts onto marine sponge.

4.3. Need of developing monitoring program in the Maldives for emerging contaminants

The Maldivian archipelago is characterized by 1192 coralline islands grouped in 20 atoll systems for administrative purposes and located on top of two parallel submerged ridges (Hilmi et al., 2023a). The Maldivian reefs cover an area of~ 4500 km^2, comprised of 2041 distinct coral reefs, corresponding to nearly 5 % of the world's reefs and representing the 7th largest coral reef system in the world in terms of area covered (Spalding et al., 2001). From an economic standpoint, the Maldivian Republic builds its foundation on coral reefs, whose health and integrity strongly affect the livelihood of the Maldivian population (Di Fiore et al., 2020). Reefs are essential for the country's economy since they support an intense fishery activity and tourism that provides 30 % of the gross domestic product as >1,500,000 people visit the country each year (Di Fiore et al., 2020; Rizzi et al., 2020). The Maldivian reefs host incredible biodiversity, with about 300 species of corals, >500 species of other invertebrates, and 1100 species of fishes (Hilmi et al., 2023a) that represent a touristic attraction recognized worldwide (Hilmi et al., 2023b). Moreover, reefs act as natural protections for the islands against wave action, having the islands an average altitude of 1.5 m above mean sea level and no point higher than 2.3 m above mean sea level (Di Fiore et al., 2020). Since the beginning of the '90s, the Republic of Maldives has experienced a long-term demographic increase, with a recorded population of 213.215 inhabitants in 1990, 270.102 inhabitants in 2000, and 402.071 inhabitants in 2017 (Duvat, 2020). This sudden demographic increase was accompanied by equally rapid technological development and access to essential services, among which the availability of drugs and medical resources experienced a fast improvement. On the other hand, wastewater management in the Republic of Maldives still needs substantial development since the sewerage networks do not serve most of the islands, and the wastewater treatment (where available) consists mainly of septic tanks to allow settlement and biological digestion before the final discharge (Malatesta et al., 2015). In addition to the pollution produced in the local islands, the hospitality and tourism industry contributes to the release into the water of waste substances to an unknown extent. With >1,500,000 people visiting the Maldivian coral reefs each year, the lack of environmental monitoring and reporting represents a serious environmental issue to be addressed and implement sustainability in the economic development of the country. According to our study results, it will be therefore crucial to monitor contamination levels, particularly in the enclosed lagoons near the inhabited island where water mixing is limited. At present, due to the lack of pharmaceutical prescription data in the entire republic, it is impossible to predict the loads of APIs in the waterways and create a model for the contamination pathways. Moreover, no direct measurements of flows and concentrations were carried out until now by local authorities to establish inputs from sewage. This substantial lack of information limits the adoption of proper coral reef management plans from a conservational and legislative point of view. Considering that the preliminary results of this study, demonstrate the occurrence of APIs in the Maldivian archipelago, we would like to strongly emphasize how urgent the collection of toxicological data is. This information and the knowledge regarding environmental concentration are the basis for assessing the risks posed to the reefs by these emerging contaminants. At the same time, our findings should also encourage local authorities to adopt new standards and regulatory testing of these compounds in seawater.

5. Conclusion

In conclusion, in this study, we developed an analytical method based on the use of bioSPME and ESI-LC-MS/MS for determining in vivo the occurrence of twelve APIs in marine sponges, and we successfully tested the use of these marine invertebrates for biomonitoring the occurrence of these emerging contaminants in coral reefs. Our preliminary survey carried out in the Faafu atoll displayed the occurrence of five of the targeted APIs, with detection rates up to 100 % in the lagoon near the inhabited island of Magodhooo. The occurrence of these emerging contaminants in such remote tropical islands indicates that toxicological data are urgently required to assess the possible impact on the related reef ecosystems.

CRediT authorship contribution statement

Francesco Saliu: Writing – original draft, Supervision, Methodology, Conceptualization. **Alessandro Becchi:** Investigation, Formal analysis, Data curation. **Enrico Montalbetti:** Investigation. **Valerio Isa:** Investigation. **Tommaso Gatti:** Investigation. **Marina Lasagni:** Funding acquisition. **Paolo Galli:** Funding acquisition. **Davide Seveso:** Investigation.

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

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