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A non-lethal SPME-LC/MS method for the analysis of plastic-associated contaminants in coral reef invertebrates†

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Current approaches to evaluate microplastic contamination of the marine environment include the use of marine organisms as bioindicators and the detection of plastic-associated contaminants in their tissues. Liquid/liquid extraction and/or solid-phase extraction are the usual methods of choice for sample preparation. However, these methods suffer from background contamination, due to the large volume of solvents used and the ubiquity of plasticizers in laboratory environments. Moreover, organisms used in the study may belong to species that are rare or endangered and these should preferably not be sacrificed as a consequence of the relatively sizeable biological material required for exhaustive sample extractions and the destructive nature of mass spectrometry analysis. In this study, we evaluated, as a non-lethal alternative, a procedure involving solid phase microextraction (SPME) coupled with liquid chromatography mass spectrometry (LC/MS) analysis. Two coral reef invertebrates that may be sensitive to microplastic contamination were used for the tests, *i.e.*, the scleractinian *Danafungia scruposa* and the bivalve mollusk *Tridacna maxima*. The results showed that the method was effective in quantifying phthalate esters within ten minutes of exposure, offering at the same time an improved control of the background contamination compared to the classical extraction procedure.

1. Introduction

In the last few decades, there has been a growing scientific awareness regarding microplastics (plastic fragments smaller than 5 mm, MPs) in marine environments.[1] Several efforts were devoted to the development of reliable methods to evaluate their concentrations.[2] In this context, associated plastic contaminants such as phthalate esters (PAEs) were researched in the tissues of several marine organisms, and some of them were proposed as bio-indicators of microplastic contamination, *i.e.*, sea-squirts,[3] cetaceans,[4] and basking sharks.[5] PAEs are, in fact, common plastic "ingredients" blended with plastic polymers to enhance the flexibility of the plastic materials[6] that may easily leach into the environments.[7] Moreover, PAEs are associated with several adverse effects and therefore capable of enhancing the toxic effect of microplastics: the US EPA categorizes them as priority pollutants,[8] and they are categorized as substances suspected of producing endocrine alterations by the European Union (EU).[9]

Usually, the determination of PAEs in marine organisms is carried out after exhaustive extraction techniques such as liquid–liquid extraction and/or solid-phase extraction (SPE).[10] This approach has some drawbacks such as low automatization, the use of large amounts of solvents, and frequent changes in handling equipment, that may easily lead to secondary contamination.[11] PAEs are, in fact, commonly present in laboratory environments, especially in the air, organic solvents, and adsorbed on glassware and other devices.[12]

Besides background contamination, when the organisms selected for bio-monitoring are small, they must be suppressed to recover utilizable amounts of biological material. This point should be carefully taken into account for conducting research programs in vulnerable marine environments involving endangered species.

To overcome these issues in this work, we, therefore, evaluated the use of solid-phase micro-extraction (SPME), a technique based on the equilibration of analytes between a relatively "large" sample matrix and a "small" organic polymeric phase.[13] Thanks to the recent introduction of biocompatible coating, SPME may be used in direct immersion mode[14] with a biological matrix and even *in vivo*.[15] For the tests, we selected the bivalve mollusk *Tridacna maxima* (Röding, 1798) and the scleractinian

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coral *Danafungia scruposa* (Klunzinger, 1879). *Tridacna maxima* is a giant clam that has been recently reported to be a sink of microplastics by a combination of ingestion and adhesion processes.[16] Thanks to its large dimensions, extreme mantle thickness, and high water filter capacity, *Tridacna maxima* may ingest large amounts of microplastics and accumulate the associated plastic contaminants in its tissues.[17] *Danafungia scruposa* is a solitary, free-living coral that may be considered a representative of monostomatous large-sized coral reef invertebrates that could be sensitive to plastic pollution. Like many other "mushroom coral" species, this species displays a single big mouth that allows it to ingest big prey, such as jelly fish,[18] salps and sea slugs.[19] Owing to their predatory activity,[20] growth form and rugosity, corals are able to impound (both actively or passively) microplastics.[21] At present, evidence that corals may ingest microplastics comes mostly from controlled aquaria system experiments and they underline the fact that diversified interactions are possible between corals and plastics. In line with this, contaminants related to microplastic pollution have already been detected in scleractinian corals,[22] suggesting that microplastics may be a potential threat to their health. The present work aimed to introduce a new and simple analytical tool to determine the uptake of plastic-associated contaminants in coral reef environments, where euthanization of marine organisms that are already depauperated due to several environmental stresses, is undesirable.

2. Experimental

2.1 Chemicals and materials

Solvents used for the SPME procedure were of high purity grade (pico-grade) from Promochem (Promochem, Milano, Italy). LC/MS analyses were carried out by using ultra-grade methanol (MeOH) from Sigma Aldrich (Sigma Aldrich, Darmstadt, Germany). Ultrapure Water (resistivity, 18.2 MΩ cm) was produced on a Milli-Q Plus apparatus (Millipore, Milan, Italy). A phthalate ester standard mix was purchased from Sigma Aldrich (EPA 506 phthalate ester mix) containing dimethyl phthalate (DMP), diethyl phthalate (DEP), dibutyl phthalate (DBP), benzyl butyl phthalate (BBP), bis(2-ethylhexyl)phthalate (DEHP), bis(2-ethylhexyl)adipate (DEHA), each component added at 500 μg ml^{-1} in methanol. Bis(2-ethylhexyl)phthalate-3,4,5,6-d_4 (DEHP d_4, 98%) and monoethyl phthalate (MEHP, 98%) were also provided by Sigma Aldrich and were used to prepare individual stock solutions in methanol at a concentration of 500 μg ml^{-1} in an amber flask. All the standards were stored at 4 °C and used to spike the control samples for method optimization and to prepare the native solutions for calibration after appropriate methanol dilution. A SPME fibre in the needle probe format, with a C18 cylindrical fiber core of 200 μm diameter, a length of 40 mm, an extraction phase surface area of 8.1 mm^2 was purchased from Sigma Aldrich (part no. 57234-U).

2.2 Marine invertebrates

One individual of *D. scruposa* of approximately 15 cm in diameter and one individual of *T. maxima* of 20 cm in maximum length were used for the tests. The specimens were sampled in the coral reefs near the Faafu Atoll and then carried to the marine laboratory facility at the Marine Research and High Education Center (MaRHE), located in the Magoodhoo Island (Maldives) for the tests. More detailed indications regarding the sampling area and related microplastic contamination can be found in a previous paper.[23] Before setting up the experiments, the coral and the bivalve were acclimated for 72 hours in an aquaria system consisting of four 60 l seawater tanks, connected to a 330 l sump containing a gravel-bed filter, a protein skimmer and a 500 W Titanium Heater (Aqua Medic) connected to a temperature controller. In all tanks, light was provided by 400 W metal halide lamps (Powerstar HQI-T, Osram), which were turned on at 09:00 for the day cycle and turned off at 21:00 (12 : 12 light : dark cycle). After the experiments, both specimens were brought back to their original sampling sites.

2.3 *In vivo* sampling

In vivo sampling was performed by inserting the SPME fiber in the tissue close to the mouth of the mushroom coral and in the mantle of the bivalve mollusk for 10 min. After that, the fibers were removed from the tissue and rinsed with distilled water before immersion in the desorption solvent mixture (100 μl of 6 0 : 40 methanol : water) for 50 min at 45 °C, maintaining 500 rpm of agitation; 20 μl aliquot of the desorption solution was then injected into a LC/MS instrument for analysis. The health condition of both organisms was monitored during exposure, 15 minutes and two hours after the exposure, and then each day for the following three days. A change in health condition as an indicator of stress in reaction to stimuli was evaluated by the excretion of mucus in the mushroom coral, and by the ability to open and close the shell of the giant clam. For both organisms we used the "bleaching" status, in terms of the loss of the zooxanthellae symbionts, as an additional indicator of stress.

2.4 LC/MS analysis

LC/MS analysis was performed with a TSQ Quantum Access Max Liquid instrument (ThermoScientific) equipped with a liquid chromatograph (UHPLC/HPLC), an ESI interface, and a triple quadrupole mass analyzer. Chromatographic separation was performed using a Thermo Scientific Accucore C-18 aQ column (100 mm × 2.1 mm I.D., 2.6 μm). Chromatographic elution was carried out with a binary system comprising water with 0.1% of acetic acid for pump A and methanol for pump B. A gradient was applied from 80 to 96% of B at 0.6 ml min^{-1} of the total flow in order to enable the separation of the six PAEs in less than 15 min and to minimize the co-elution of analytes with the residual interfering components in the matrix. To prevent contamination of the ion source by undesired non-volatile components and to avoid the drop of MS sensitivity, during the first minute and the last four minutes the column effluent was directed to waste through a post-column switching valve. ESI-MS was operated in the positive ion mode. The spray voltage was maintained at 3500 V. The vaporizer temperature was fixed at 350 °C and capillary temperature at 270 °C. The sheath gas

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pressure was set up at 50 arbitrary units and the auxiliary gas pressure at 15 arbitrary units, whereas the ion sweep gas pressure was set at two arbitrary units. The collision gas pressure was fixed at 1.0 mTorr and with a cycle time of 0.6 s. The injection was performed by using an autosampler in partial loop mode by injecting 20 μl. A time segmented selected reaction monitoring mode was used to enhance the selectivity of the mass spectrometry detection. Table 1 shows the transition and the applied collision energy used for the detection of each analyte. For quantitation we adopted the kinetic calibration approach.[13] This approach uses the symmetric relationship that is established between the extraction kinetics of the target analytes and the desorption kinetics of the preloaded surrogate standards in order to define the rate of adsorption of the target analytes from the tissues to the extraction phase, according to the reference equation

$$C_s = nKV_f(1 - Q/q_0)$$

Here C_s is the concentration of target analytes in the sample, n is the amount of the extracted analyte under pre-equilibrium sampling time, K is the distribution coefficient of the analyte between the fiber coating and the sample matrix, V_f is the volume of the fiber coating, Q is the standard remaining in the SPME fiber coating after sampling, and q_0 is the preloaded standard on the fiber. The SPME fiber was therefore pre-loaded before use with a deuterated standard by immersion in 50 ng ml^{-1} spiked phosphate buffer solution at 500 rpm for 1 h.

2.5 Evaluation of the extraction kinetic

Due to the limited availability of marine invertebrates for determining extraction kinetics and performing the optimization of extraction efficiency of the method, tests were carried out in spiked 1% (w/v) agarose gel samples. Previous studies already detailed the possibility of using agarose gel for mimicking *in vitro* the free diffusion of analytes in living tissues, due to the similarity of this gel in terms of porosity and tortuosity. Agarose was therefore weighed and dissolved in hot phosphate buffer (pH 7.4) and allowed to solidify at room temperature for 3 h. Just before solidification, a known volume of agarose gel was spiked (100 ng ml^{-1}, $n = 3$) with a mixture of the selected PAEs and vortexed for 10 min in a 4 ml vial to homogenize the drug distribution in the gel. Pre-equilibrium extraction time profiles were then determined at six points (0, 5, 20, 30, 60, and 340 min) using the C18 fibers (each time point corresponded to the immersion of one fiber in a vial). After each interval, fibers were removed from their respective spiked gels and PAE analyses were carried out as described for the *in vivo* procedure.

Table 1 LC-MS selected reaction monitoring parameters: RT = retention time and CE = collision energy

Abbreviation	RT	Precursor ion (polarity)	Product ion (CE)	Product ion (CE)
MEHP	1.19	277.3 (−)	135.2 (20)	121.1 (29)
DMP	1.60	194.8 (+)	163.1 (11)	77.3 (33)
DEP	1.86	223.1 (+)	149.1 (18)	121.1 (28)
DBP	4.50	279.1 (+)	149.1 (19)	121.1 (30)
BBzP	4.73	313.1 (+)	149.1 (21)	91.3 (29)
DEHP	7.10	391.2 (+)	149.1 (25)	121.0 (27)

2.6 Validation tests

Since certified reference materials are not commercially available for the selected marine macroinvertebrates, extraction efficiency and accuracy were evaluated with estimated values for back-calculation[24] by using homogenized tissue (4.0 ± 0.2 g) of *T. maxima* spiked with 100 μl of 4 mg l^{-1} native standard mixture (PAE mix) solution. Extraction was performed for 10 min using C18 fibers in the direct immersion mode. Desorption and analysis were carried out as described for the *in vivo* procedure. Linearity and limit of quantification were evaluated by using matrix-matched calibration curves obtained from each different matrix based on the relative area *versus* the internal standard. Calibration points were prepared at 0.5, 1, 5, 10, 25, 50, 100 and 250 ng g^{-1}. Linearity was assumed when the correlation coefficient (R^2) was >0.990 with randomly distributed residuals (<20%). LODs and LOQs were calculated following the IUPAC recommendations, from five blank extractions as the signal of the blank plus three and ten times the standard deviation, respectively. In case no peak was found at the retention time of the analyte, the LODs and LOQs were estimated as three and ten times the signal-to-noise (S/N) ratio, respectively. Repeatability was evaluated on the relative chromatographic peak areas of the 50 ng g^{-1} sample (with respect to internal standards) standard deviations (RSDs) over six replicates, determined by using an intra-day assay. A total of 15 procedural blanks were analyzed on five different days to determine possible issues with background contamination.

3. Results and discussion

3.1 Choice of the SPME fibers

The marine invertebrates considered in this study display a simple biochemical composition if compared, for instance, to fish tissues: the soft tissue of cnidaria (such as *D. scruposa*) is mostly composed of water (98%) with collagen and a number of lipids ranging from 9% to 47% of their dry weight.[25] Mollusks such as *T. maxima* have a water content of approximately 70%, and carbohydrates, lipids, and proteins of 8–12%, 4–5%, and 70% in their soft body tissue, respectively.[26] The application of SPME looks particularly suitable for these organisms. SPME has already proven itself, in fact, as an alternative analytical method that is particularly relevant for non-lethal extraction.[15] The special coating applied onto the fibers excludes water and prevents fouling by other high molecular weight components present in biological matrices (*i.e.*, polysaccharide, proteins, and particulates). This sample clean-up promotes the enhancement of the signal-to-noise ratio and counteracts the low recovery related to the non-exhaustive nature of SPME. Moreover, thanks to the minimization of sample manipulation

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the chance of external contamination is lowered, and this is considered the critical point of PAE analysis.

3.2 Study of the extraction kinetic

Fig. 1 shows the kinetic extraction curve obtained in agarose gel for the selected PAEs. The recoveries obtained after 20 min from the most hydrophilic PAEs (MEP and DEP which display log K_{OW} values of 1.61 and 2.54, respectively) are significantly lower than the recoveries obtained from the most hydrophobic PAEs (DBP and DEHP) which display log K_{OW} values of 4.70 and 7.73, respectively. A time of 10 min is sufficient to extract detectable amounts of phthalates. Moreover, for this short exposure time, according to the pre-equilibrium SPME theory, the rate of extraction is determined by the diffusion coefficients that display small differences among the PAEs. This feature enables better control of the linear dynamic range.

3.3 Method evaluation

Table 2 shows the most relevant analytical parameters obtained by performing matrix-matched calibrations and analysis of pre-spiked homogenized tissue samples, used as quality control. Satisfactory linearity was achieved within the tested concentrations. R^2 values ranged between 0.992 and 0.997 and MDLs from 0.3 to 0.7 ng g^{-1}.

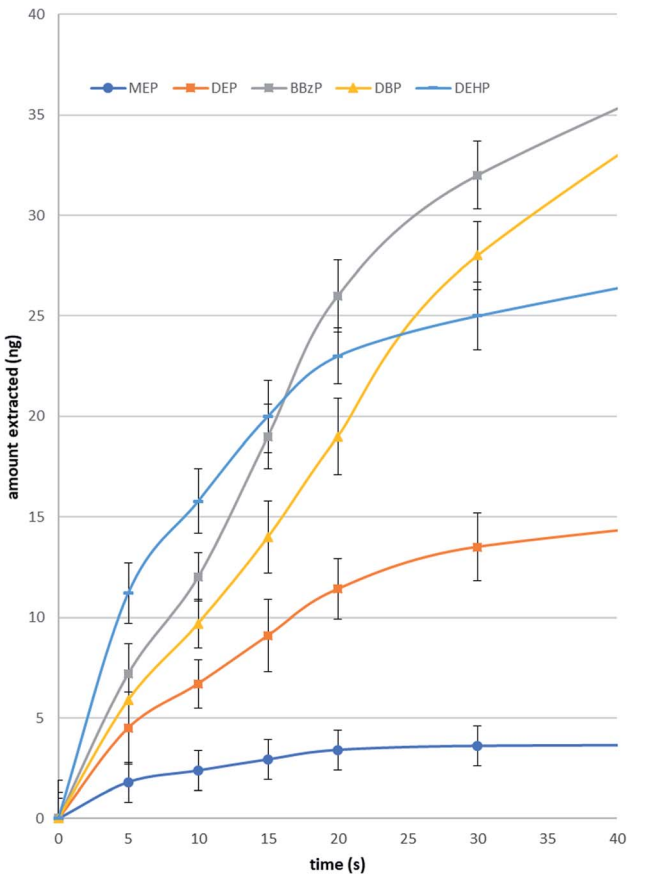

Fig. 1 SPME extraction kinetic obtained from 5 g of agarose gel spiked with MEP, DEP, BBzP, DBP and DEHP at 100 ng g^{-1}.

Table 2 Analytical data for the determination of PAEs in spiked homogenized tissue

Analyte	LOD (ng g^{-1})	R^2	Accuracy%	% intra-day RSD ($n = 6$)
MEHP	0.7	0.995	94	5
DMP	0.4	0.993	99	6
DEP	0.5	0.994	97	5
DBP	0.5	0.998	99	5
BBzP	0.6	0.994	94	7
DEHP	0.3	0.993	101	5

Method accuracy was back-calculated through estimated concentration values, as indicated by Martins *et al.*[30] This procedure is typical for SPME that is characterized by low but consistent absolute recoveries. The obtained mean values ranged from 94 to 101% (Table 2). Precision for inter-day assays displays RSDs under 11%.

Analysis of blanks showed that the method provided precise control of background contamination: the residual levels of PAEs in the procedural blanks were always found to be under the detection limit. This represents a remarkable upgrade compared to other conventional extraction methods. Our previous experience with liquid–liquid extraction from scleractinian corals displayed average blank values up to 18.2 ng g^{-1} for the sum of six PAEs;[21] Shenkar's group reported that applying accelerated solvent extraction to ascidians,[3] results in blank values up to 31.0 ng g^{-1} for DEHP. This better control of contamination offered by SPME may be explained by the ease of sample manipulation, the limited use of the solvent, and the single/distinct use of fibers and vials for each sample, which limits the chances of cross-contamination.

3.4 *In vivo* analyses

To perform non-lethal analyses on living organisms in their natural environment, SPME is commonly applied to relatively large and uniform tissue samples such as venous blood, fish muscle, and porpoise blubber.[27] Recently[28] it was demonstrated by using a smaller SPME format that it is possible to probe tiny and discrete tissues (*i.e.* for fish from the adipose fin and the dorsal-epaxial muscle), to underline differences in the analyte partition (which may be influenced by lipids and protein binders), and to track tissue burdens over time. In our tests, we preferred to reduce the invasiveness of the procedure and to improve the chances of recovery of the invertebrates. Therefore, the extractions were performed on a single point on the surface: near the mouth in *D. scruposa* and in the mantle for *T. maxima*. This represented the most suitable solution for the introduction of the fiber and sampling of a representative tissue section. Fig. 2 shows the pictures taken before (Fig. 2a), during (Fig. 2b and c) and after (Fig. 2d–f) the *in vivo* extraction procedure. A thorough observation indicated that the 10 min exposure time did not affect the test animals significantly. In *D. scruposa*, the exposure caused only a slight displacement of mesentery filaments, suggesting a potentially stressful situation for the coral.

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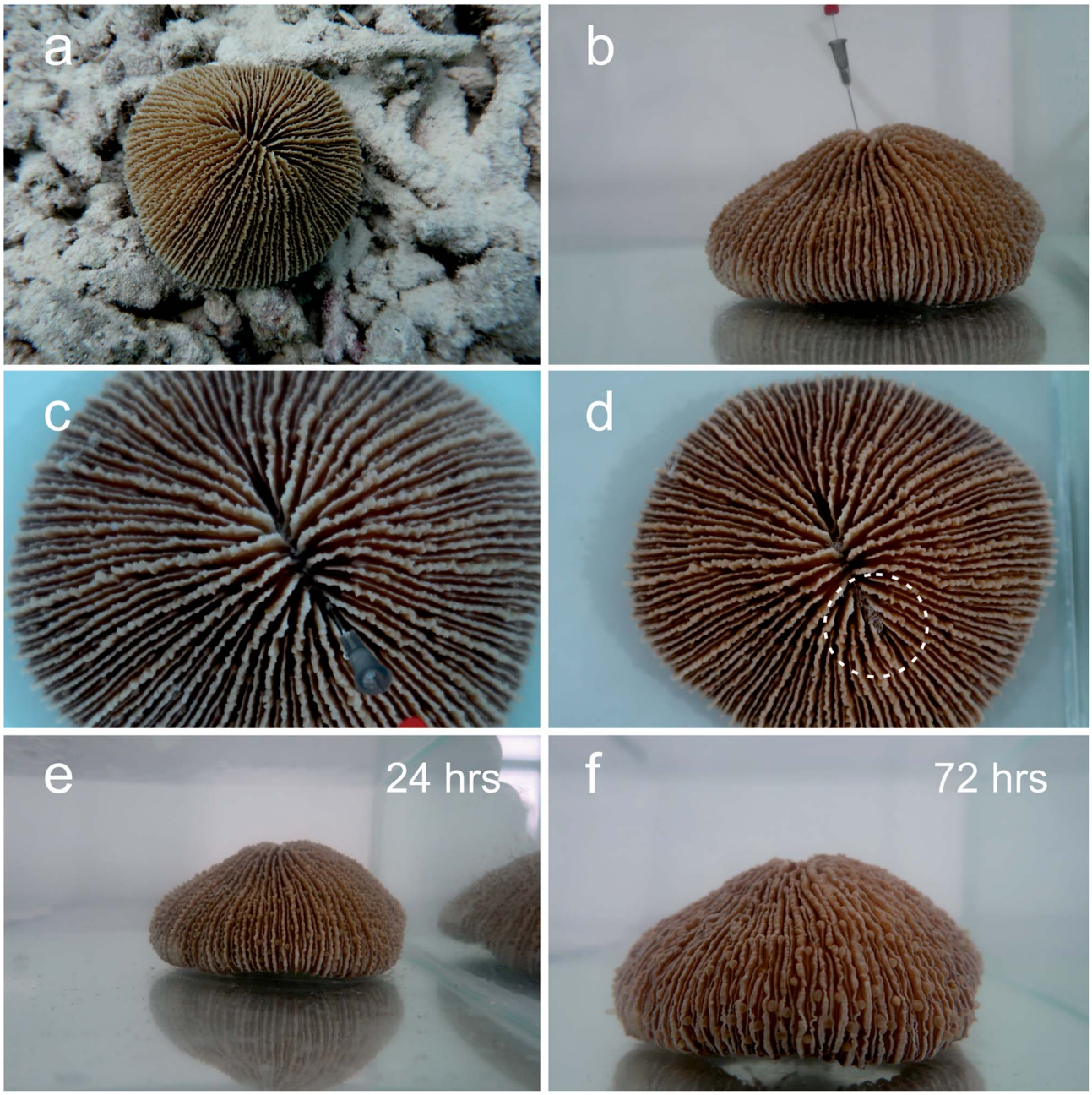

Fig. 2 Representative picture of solitary scleractinian *Danafungia scruposa* (a) *D. scruposa* at 5 m depth in its environment; (b and c) microfiber inserted in the mouth tissue (d) mesenterial filaments close to the point where the microfiber was inserted, 2 h after the exposition (e) *D. scruposa* after 1 day (f) *D. scruposa* after 3 days.

Although it was temporary, and corals recovered towards a normal homeostasis within the first two hours after exposure, it is reasonable to presume that the needle used to insert the microfiber may potentially damage the soft and thin coral tissue. In this context, it is advisable that this standard procedure should be applied only to big monostomatous mushroom corals and other colonial scleractinians with a polyp diameter over 2 cm. On the other hand, the development of a new SPME format that may be less invasive for tiny corals is currently underway in our lab. Fig. 3a shows the *in vivo* extraction procedure followed for *T. maxima*. After 10 min of exposure (Fig. 3b and c) no signs of tissue damage had been observed, and the area where the microfiber was inserted (Fig. 3d) did not show any significant difference before and after contact with the microfiber. Similar morphological observations have already been reported for previous non-lethal mantle biopsy experiments involving bivalve mollusks.[29] This lack of damage may be due to the larger quantity of marine invertebrate tissue in relation to the small size of the needle. On the other hand, the mollusk remained responsive to stimuli until the 3rd day of the experiment (Fig. 3f).

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Fig. 3 Representative picture of *Tridacna maxima*. (a) *T. maxima* at 7 m depth in its environment (b) microfibers inserted in the mantle of the bivalve mollusk (c) close-up of the microfiber (d) area of microfiber application 2 h after the exposure (e) *T. maxima* after 1 day (f) *T. maxima* after 3 day.

After the extraction, the criteria applied to selectively identify and quantify PAEs in the samples were: (a) detection of the representative SRM transitions (one qualifier and quantifier as confirmation ions) of the specific phthalate at the exact m/z at unit resolution (b) detection of the chromatographic peak of the analyte within a 15 s interval at the mean retention time

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Table 3 Amounts of PAEs (ng g^{-1}) determined in *Danafungia scruposa* and *Tridacna maxima* by SPME-LC-MS (BDL = under the detection limit)

Analyte	*Danafungia scruposa*	*Tridacna maxima*
MEHP	BDL	BDL
DMP	BDL	BDL
DEP	0.6	BDL
DBP	2.7	1.8
BBzP	BDL	0.9
DEHP	0.8	4.6
∑PAEs	4.1	7.3

obtained during analysis of the reference compounds in the calibration standards; and (c) the measure of the signal-to-noise ratio for the representative ions ≥3. The analyses of the two specimens showed a trace amount of PAEs (Table 3). Specifically, DEHP was found in the highest relative concentrations in *T. maxima* (4.6 ng g^{-1}), whereas DBP in *D. scruposa* was less (2.7 ng g^{-1}). The total PAE concentration in *T. maxima* was 7.3 ng g^{-1} whereas in *D. scruposa* it was 4.1 ng g^{-1}. For comparison, we contrasted aliquots of samples previously analyzed by a solvent extraction method[30] with the presently described direct immersion procedure. The results (ESI S1†) did not show any significant differences between the two approaches, confirming that the use of SPME as a non-lethal alternative method may also result in reliable determination. Since the direct immersion mode selectively extracts free analytes, whereas solvent extraction (due to the swelling of the matrix) extracts bounded and unbounded analytes together, it is reasonable that the phthalates accumulate in the lipid fraction and/or by transdermal absorption. It should be pointed out that, because *ex vivo* determination methods are also affected by the metabolite stability, it is possible that the use of *in vivo* SPME in monitoring campaigns may highlight differences in the observed profiles and capture metabolites that are not observed by *ex vivo* methods.

4. Conclusion

In this study, *in vivo* SPME was applied to quantitate phthalates in coral reef macroinvertebrates. Extraction kinetic studies carried out in agarose gel showed that detectable recoveries were attainable within 10 min of exposure time. Tests with homogenized tissues showed satisfactory performance in terms of sensitivity and repeatability, and enhanced control of background contamination compared to the classic solvent extraction methods. The insertion of the C18 fibers into the tissue of a mushroom coral (*D. scruposa*) and into the mantle of a giant clam (*T. maxima*) did not cause any temporary macroscopic change in the health status of both organisms. In conclusion, this study demonstrates the feasibility of SPME-LC/MS for the analysis *in vivo* of plastic-associated contaminants in marine macroinvertebrates and offers a method to improve the control of background contamination with respect to classical and lethal approaches based on solvent extraction.

Conflicts of interest

There are no conflicts to declare.

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