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Factor #1: Two size classes of *Tridacna maxima*

Factor #2: Four concentrations of microplastic

Retention

Adhesion

Microplastic removal by Red Sea giant clam (*Tridacna maxima*)

Silvia Arossa*, Cecilia Martin, Susann Roszbach, Carlos M. Duarte

King Abdullah University of Science and Technology (KAUST), Red Sea Research Centre (RSRC) and
Computational Bioscience Research Center (CBRC), Thuwal, 23955, Saudi Arabia

*Corresponding author

Declarations of interest: none

Abstract

This study assesses for the first time the ingestion of microplastics by giant clams and evaluates their importance as a sink for this pollutant. A total of 24 individuals of two size classes were collected from the Red Sea and then exposed for 12 days to 4 concentrations of polyethylene microbeads ranging from 53 to 500 μm . Experiments revealed that clams actively take up microplastic from the water column and the average of beads retained inside the animal was $\sim 7.55 \pm 1.89$ beads individual⁻¹ day⁻¹ (5.76 ± 1.16 MPs / g dw). However, the digestive tract itself cannot be considered the only sink of microbeads in Tridacnids. Indeed, shells play a key role as well. The abundance of microplastic adhering to the shells, which was estimated directly, was positively correlated to the concentration of beads found in the surrounding seawater. Therefore, clams' shells contribute to the removal of 66.03 ± 2.50 % of the microplastic present in the water column. Furthermore, stress responses to the exposure to polyethylene were investigated. Gross Primary Production:Respiration (GPP:R) ratio decreased throughout of the experiment, but no significant difference was found between treatments and controls.

Keywords: Microplastics, *Tridacna maxima*, plastic pollution, giant clam, ingestion

Capsule: Giant clams remove almost 70% of plastic from the water column mainly through a passive removal process of plastic adhesion to the shell.

1. Introduction

The continuous rise of global plastic production (up to 322 million metric tons per year in 2015; Worm et al., 2017) and the lack of proper disposal and recycling provisions has led to an increase in plastic loads marine environment, where it tends to be persistent. Large plastic debris undergo natural degradation due to UV radiation, oxidative reactions and hydrolytic properties of seawater, which act synergistically together with the mechanical activity of waves to yield microplastics (<5 mm diameter; Ashton et al., 2010). Microplastics also enter directly the marine environment, as plastic microbeads used in cosmetics, facial cleanser and synthetic fibers from clothing (Browne et al., 2008; Cole et al., 2011). Both these types of microplastics, called

respectively secondary and primary, have become ubiquitous in the marine environment.

Global concern on the presence of plastic in the oceans is due to sublethal and lethal effects on marine organisms ingesting the plastics and those connected to them in the food web (Von Moos et al., 2012; Gregory, 2009). Whereas both macro- and microplastics can be ingested by marine biota, microplastics are more likely to enter the food chain, bioaccumulate and represent a hazard across a wide range of marine organisms (Ashton et al., 2010), such as seabirds, crustaceans (Murray and Cowie, 2011; Watts et al., 2014; Setälä et al., 2016), lugworms (Thompson et al., 2004), polychaetes (Setälä et al., 2016), sea cucumbers (Graham and Thompson, 2009), scleractinian corals (Hall et al., 2015), zooplankton (Cole et al., 2013), barnacles and bivalves (Thompson et al., 2004; Mathalon and Hill, 2014; Setälä et al., 2016; Sussarellu et al., 2016). Available evidence suggest that filter feeders ingest large amounts of plastics (Setälä et al., 2016).

Ingestion of microplastics may cause obstruction and injury to the digestive system (Cannon et al., 2016; Stamper et al., 2006), starvation or malnutrition due to the reduction of food intake (Gregory, 2009) and/or potential translocation of microplastics into the circulatory system (Browne et al., 2008). The plastic persistence in the body of marine biota could lead to the release of hazardous chemicals, known as “plasticizers”, incorporated in the plastic itself during the manufacture process (Cole et al., 2013). Then, they can be absorbed by the surrounding tissues causing toxic effects (Rochman et al., 2015). Furthermore, microplastics can absorb and transport other hydrophobic contaminants (e.g. heavy metals, polybrominated diphenyl ethers (PBDE) and organic pollutants) from the surrounding seawater (Mato et al., 2001; Endo et al., 2005; Ashton et al., 2010).

Biological removal of microplastics has been proposed to be a major sink for microplastics in the marine environment, resulting in loads in the open ocean corresponding to a few percent of that expected from inputs (Cózar et al., 2014). Semi-enclosed basins, which are characterized by a limited capacity to export floating debris are expected to act as high plastic accumulation zones (Cózar et al., 2014). Despite this, the Saudi Arabian waters of the Red Sea has been reported to support one of the lowest floating microplastic concentrations found worldwide (Marti et al., 2017). This may either be explained by a low input of plastic waste from land and/or by the presence of a removal processes acting as plastics sinks. Indeed, it has been

hypothesized that fast removal rates of plastics debris from the surface by the marine biota could actively contribute to the shortage of anthropogenic debris. The latter could be also related to different processes such as shore deposition, nano-fragmentation, sinking due to epiphytic growth or trapping by mangrove and coral reef ecosystems (Marti et al., 2017; Martin et al., 2018).

Here we experimentally assess, for the first time, passive (i.e. attachment to shells) and active (i.e. retention inside the animal) removal of microplastics by the giant clam *Tridacna maxima*, and explore possible effects of microplastics retention on the clam metabolism, filtration activity and survival. Since there were no available studies reporting the size range of particles filtered by *Tridacna* species, we investigated a size range from 53 to 500 μm , since microplastics below 0.5 mm have been reported missing in the Red Sea (Marti et al., 2017) and represents, more broadly, the size range where losses of microplastic from the floating pool are inferred (Cózar et al., 2014). We focused on the Red Sea giant clam, *T. maxima*, because this species is highly abundant in the Red Sea and plays a key role in Red Sea coral reefs, where they contribute significantly to total system particle removal, carbonate formation, and, through the activity of their photosynthetic symbionts, primary production (Neo et al., 2015). Finally, the shells of giant clams contribute to reef building and provide substrate for colonization by epibionts, thus improving the complexity of coral reefs. Despite their important role in Indo-Pacific coral reefs, plastic removal by *Tridacna* species has not yet been examined.

2. Materials and methods

2.1. Clams' collection and husbandry

A total number of 24 individuals of *T. maxima* were collected in the Central Red Sea using Scuba Diving (22° 18' 35" N, 38° 53' 09" E) in June and August 2018. As this species is often embedded in the substrate, specimens were removed with a hammer and chisel, and the byssus cut with a knife, according to (Ishikura et al., 1997). All the individuals were collected from the same depth ranging from 3 to 7 m.

Clams were kept in ambient seawater, transported back to aquarium facilities at KAUST University (Coastal and Marine Resources Core Lab, CMOR), scrubbed in order to remove biofouling organisms from the shells and allowed to acclimate for 1 week. Temperature ($27.15 \pm$

0.20 °C), salinity (40.7 ± 0.2 psu) and light intensity ($1113.25 \text{ watts m}^{-2}$ during day mode) were maintained constant for the whole duration of the acclimation and experiment. Aquaria were illuminated using Radion LED Lights (EcoTech Marine, USA). Water temperature and salinity were measured daily using a handheld YSI Professional Plus multiparameter meter (Xylem Water Solutions and Water Technology, USA), while light intensity was evaluated with HOBO Pendant Temperature/Light 64K Data Logger (Onset Computer Corporation, USA).

2.2 Microplastic characterization

To ascertain whether clams ingest microplastics, an exposure using fluorescent green polyethylene microspheres (Cospheric LLC, USA) was conducted. Polyethylene (density of $\sim 1 \text{ g/cc}$) was chosen because it represents the most common type of plastic found in the Red Sea ($\sim 69\%$ out of the total) (Marti et al., 2017). Five different size classes of microplastics ranging from 53 to 500 μm (53-63 μm , 125-150 μm , 212-250 μm , 300-350 μm , and 425-500 μm) were selected according to (Marti et al., 2017; Kolandhasamy et al., 2018). Indeed, the study by Marti and colleagues (2017) showed that microplastic $< 0.5 \text{ mm}$ was missing from the surface. Furthermore, since there was no specific literature about the range of particle's size filtered by this clam species, we based our choice upon the papers by (Ezgeta-Balic et al., 2012), which focuses on the size range of zooplankton ingested by different species of bivalves, and by Kolandhasamy and colleagues (2018).

Then, fourteen mixes of microbeads, containing $\sim 5975.9 \pm 67.7$ beads per size class and $\sim 29879.7 \pm 156.8$ beads in total were prepared in 50 ml of filtered seawater (Reichert et al., 2018) and then poured into 10 l of seawater in the feeding chambers. Thus, obtaining a final concentration equal to $2.99 \pm 0.02 \text{ beads ml}^{-1}$. In the absence of previous information that quantifies how *T. maxima* microplastics ingestion depends on plastic concentration in the water column, we used a high concentration of microbeads (Davidson and Dudas, 2016). This concentration was used to assess if *T. maxima* can actively and/or passively take up microplastics and if ingestion affects its metabolism.

Using the same method, we prepared additional twelve plastic mixes in 50 ml falcon tubes, in order to obtain the following final concentrations: $0.33 \pm 0.01 \text{ beads ml}^{-1}$, 0.78 ± 0.03

beads ml^{-1} and 1.61 ± 0.03 beads ml^{-1} (approximately $\frac{1}{8}$, $\frac{1}{4}$ and $\frac{1}{2}$ of the original concentration used in the first experiment) to be used to assess whether there is an influence of the concentration on active and passive removal. These concentrations are comparable to those used in other studies such as Xu et al. (2017) and those reported in Ribeiro et al. (2017).

Plastic mixes were kept under the direct sunlight and exposed to natural temperature and environmental conditions for 2 weeks in 50 ml falcon tubes, in order to allow the bacterial biofilm to form and to mimic the natural conditions (Lobelle and Cunliffe, 2011).

In order to maintain the microbeads in suspension during the experiment, feeding chambers were provided with a pump, which was directed towards the surface. As a result, the surface tension was broken and the beads were forced into the water column as suggested by (Allen et al., 2017).

2.3. Experimental set-up

After the acclimation period, two main experiments were conducted. The first one was performed in order to understand if the exposure to microplastic beads causes stress responses in clams (i.e. measuring clams' metabolism), to assess whether clams ingest microplastics and to understand if ingestion depends on the clam size. The second experiment allowed us to evaluate if the abundance of microplastics ingested depends on the initial concentration of plastic beads in the water.

Moreover, we experimentally assessed the total plastic removal by *Tridacna maxima* and partitioning these between the contributions of active removal, by retention with filter feeding, and passive removal, through the possible attachment of plastic material to their shells, both estimated directly through the dissection of the animal and exploration for plastic beads and screening the entire shell surface to detect attached plastic beads, respectively. We also looked for possible egestion with fecal pellets, but none were produced during the experiments.

2.3.1. Effects of clams' size

Microplastic retention in 10 individuals of *T. maxima* belonging to two different size classes (5 juvenile-adults $\sim 13.8 \pm 1.7$ cm and 5 adults $\sim 19.8 \pm 2.2$ cm) was investigated. These

two sizes were selected because it is known that juvenile-adults usually obtain most of the nutrients they require from their endosymbiotic algae (e.g. zooxanthellae), on the contrary adults have a higher rate of filter feeding activity useful to extract food from seawater (Klumpp et al., 1992). Therefore, we expected to see a difference between the two size classes.

The 10 individuals of *T. maxima* were placed individually in 10 l feeding chambers and exposed to microplastics for 12 days (288 h). In addition, three different types of control were run together with the treatments. The first control included two empty feeding chambers with microplastics to evaluate the number of microbeads dispersed in the environment or attaching to the walls of the feeding chamber. The second was constituted by two feeding chambers including two “dummies” exposed to microplastics. Dummies were clams’ shells, obtained from dead clams and bleached for conservation purposes, used to mimic the presence of a three-dimensional object in the system and to account for microplastics lost for adhesion to the object surface. A 13 cm and a 23 cm dummies were selected to simulate the two size classes juvenile-adult and adult. The last control included two feeding chambers with 1 clam each not exposed to microplastics, used as metabolic controls, as explained in the next paragraph.

The 16 feeding chambers were then randomly disposed in seven open flow system water baths. The feeding chambers were provided with a frontal 25 μm mesh, in order to allow water exchange and to avoid microplastics loss during the experiment. Water current in the feeding chambers was generated by a pump, which allowed us to reduce the surface tension and as a consequence, floating microplastic was forced into the water column (Allen et al., 2017).

The first fourteen microplastic mixes (giving a concentration of equal to 2.99 ± 0.02 beads ml^{-1}), mentioned in the previous paragraph, were poured into the 10 feeding chambers containing 5 juvenile-adults and 5 adults, into the two feeding chambers with dummies and into the two empty boxes, but not into the controls for metabolism.

Since a depletion of the microbeads concentration was expected to occur in the water column, seawater aliquots in 3 replicates were collected from each chamber straight after pouring microplastics into the tanks and after 24, 48, 72, 144, 216, 288 hours (12 days). Aliquots were not collected from metabolic controls without microplastics. Aliquots were then vacuum-filtered onto 25 μm filters, dried and subsequently microplastics were quantified with a fluorescent microscope (Axioscope, Carl Zeiss AG, Germany) in combination with computer assisted image

analysis software (Axiovision, Carl ZEISS AG, Germany) used to measure the beads size.

In order to directly examine plastic attachment to the shell, clams and dummies were collected at the end of the experiment from the feeding chambers and rinsed thoroughly with seawater, in order to collect the plastic attached to their shells during the exposure. In particular, shells, of both clams and dummies, were inspected under dissecting microscope for the presence of plastic beads, which were counted for each individual shell. Furthermore, in order to estimate the abundance of beads left in the box and adhered to its surfaces, the feeding chambers were rinsed as well. The plastic collected in these latter steps was concentrated up to 200 ml and subsequently, a 5 ml sub-sample was observed under a fluorescent microscope and the number of beads was recorded.

2.3.2. Metabolic rate measurements

Metabolic rates were assessed before and after the plastic retention experiment as a proxy of stress in the clams. Although water aliquots were collected for 12 days (288 h), plastic exposure lasted 305 h in order to allow metabolism measurements to be performed exactly at the same hours before and after the experiment to obtain consistent results. This physiological study was conducted on all 10 experimental clams, on 2 clams used as metabolic controls (1 from each size class) and 2 empty feeding chambers to evaluate the background oxygen production due to other sources. Clams were moved in 10 l incubation chambers which were hermetically closed and filled with 0.2 micron filtered natural seawater, provided with a pump to maintain a constant water movement. Net oxygen production and dark respiration ($\mu\text{mol O h}^{-1}$) were assessed for 1.5 h respectively and logged in 15 min intervals using optodes (miniDOTs; Precision Measurement Engineering Inc., USA). Standardization of metabolic rates in giant clams is conceptually difficult, because photosynthetic algae mostly occur within the upper 5 mm of the mantle (Ishikura et al., 1997) and photosynthesis may vary in proportion to the mantle area. However, respiration applies to the entire holobiont (clam and the algal symbionts) and varies therefore rather with mass rather than area. As the main purpose of the assessment of metabolic rates was to check for potential stress responses in *Tridacna maxima* and as especially respiration is typical proxy, not only in bivalves, but all animals (Koehn and Bayne, 1992), we normalized obtained metabolic rates for tissue dry mass (g). The latter was estimated using a regression line obtained

from previous experiments (not shown here), because during our study it was not possible to measure the dry weight of the clams, which were immediately digested for microplastics measurements. Therefore, for data normalization, a linear regression ($R^2 = 0.70$), describing the shell length (SL) to - dry mass (DM) relationship in *Tridacna maxima* ($n = 27$) was established (Fig. S4 in Supplementary Materials). Shell length (cm) and tissue dry mass (g) were correlated as follows: $DM = 1.15 SL - 10.46$. Dry mass of experimental clams was estimated between 7.09 g.

2.3.2 Effect of the concentration of microplastics

Then, 12 clams randomly selected were exposed for 12 days to 3 different concentrations of fluorescent polyethylene microbeads, equal to 0.33 ± 0.01 beads ml^{-1} , 0.78 ± 0.03 beads ml^{-1} and 1.61 ± 0.04 beads ml^{-1} . Four replicates for each concentration were used and water aliquots were collected after 0, 72 and 288 hours from the start of the experiment. Metabolic measurements were not conducted during this experiment. The four concentrations used in both the experiments will be named respectively low (0.33 ± 0.01 beads ml^{-1}), mid-low (0.78 ± 0.03 beads ml^{-1}), mid-high (1.61 ± 0.04 beads ml^{-1}) e high (2.99 ± 0.02 beads ml^{-1}). Data was normalized using the duration of the exposure to microbeads. Similarly, to the first experiment, plastic adhered to the shell and remained in the feeding chamber at the end of the experiment was quantified and measured in size. The same approach already described for the first experiment was used to count microplastics in the aliquots, to dissect and digest the clams.

2.4. Dissection of clams and acid digestion

Before to process the actual samples deriving from the experiments, we tested different digestion methods on only stomach tissue according to the review done by (Lusher et al., 2017). Two different acid digestions were first tested: chloridric acid (HCl) 38% at room temperature for 3 hours (Desforges et al., 2015) and nitric acid (HNO_3) at 90 °C for 3 hours (Davidson and Dudas, 2016). At the end of this first test, we observed that HNO_3 was showing us the best performance. However, the short digestion period (3 h) was not strong enough to digest clams' tissue. The digestion was therefore extended and checked every hour. We found that the best digestion duration for the thick stomach tissue of *Tridacna maxima* was about 12 h, when the solution appeared almost completely clear.

Moreover, we decided to test whether this strong acid digestion could damage the polyethylene microbeads. During this test, clam's stomach and a known concentration of beads were subjected to HNO_3 overnight digestion at 90 °C. Despite our efforts, a lack of fluorescence in the beads was observed, but we managed to count the microbeads in the samples because they maintained their green color. Indeed, at the end of the process we were able to retrieve 85.5% of the beads added at the beginning (results shown in Fig. S1 of Supplementary Materials). As a consequence, we adopted this method for the actual samples. Therefore, immediately after the end of the last metabolic rate measurement, clams were transported into the laboratory and dissected. Gills and digestive system were collected, conserved at -20 °C and then digested to isolate microbeads. The extraction of microplastics from clams' tissues was performed using a modified acidic digestion based on (Claessens et al., 2013; Desforges et al., 2015; Davidson and Dudas, 2016). According to this modified procedure, samples of the digestive system were soaked into 20 ml of 70% nitric acid (HNO_3), while gill samples were soaked into 15 ml of the same solution. They were digested overnight in a water bath set up at ~90 °C. The digestion was considered completed when there was no visible tissue. The resulting clear solution was then diluted 1:10 with warm deionized water (~90 °C) and then vacuum filtered over a 25 μm filter. All the equipment used during this step was rinsed thoroughly with deionized water, in order to reduce the amount of microbeads and sample lost during the different steps. Microplastics on the filters were counted according to the method used for the aliquots.

The dry weight of the clams was calculated using the linear regression already mentioned in the previous paragraph (dry weight vs. length in cm), which was obtained during previous experiments with *Tridacna maxima* (shown in Fig. S4 in Supplementary Materials).

2.5. Surface and volume measurements

The quantification of morphometric features of clams was performed through the protocol described by Lavy (2015). For all the clams used in this experiment a shooting covering 360 ° of the object was conducted. At least 20 images were uploaded to Autodesk ReCAP Version 5.0.5.58 software. Then the model was used to obtain surface area (cm^2) and volume (cm^3).

2.6. Data Analysis

Statistical analysis was performed using R (Foundation for Statistical Computing, Vienna, Austria, Version 1.0.143). Data was tested for normality and homogeneity of variance with manual examination of QQ-plots. In order to meet these assumptions, we used the natural logarithmic transformation on our data. The R function for testing linear models was used to verify the linear regression of depletion of plastic in time and the distribution of plastic's size classes. The Analysis of Covariance (ANCOVA) was used to compare linear models between clam size classes and among the different concentrations of beads. Since the data concerning ingested and attached plastic were not meeting the normality assumptions, non-parametric tests were performed. Mann-Whitney was used to compare two populations, such as juvenile-adults and adults' classes, while Kruskal-Wallis followed by Tukey Kramer post hoc test was used to compare more than 2 populations of data, for instance different concentrations of microplastic or sizes of beads. Finally, in order to correlate two variables, such as clam size and ingested beads, Spearman's correlation was performed. Spearman's correlation, two-way ANOVA and t-test were used to test the effects of ingestion of microplastics on clam's GPP:R ratio. ANOVA was used to test the differences of CR among concentrations. Data was presented as the mean $\pm$ standard error, unless otherwise indicated. We reported the specific p-values as they appear in our statistical analysis according to (Amrhein et al., 2019).

3. Results

The decrease in the concentration of microplastics in the feeding chambers over time was highly statistically significant in all 10 chambers with treated individuals (p-value = 1.37, Fig. 1), referred hereafter as live *Tridacna*, involving a daily loss of 13.1% of the beads, with 81.5 % of the beads added lost over after 12 days (Fig. 1). However, plastic abundance also decreased significantly in the controls without dummy (linear model, p-value = 4.28, F=62.41, N=15), although with a much lower loss rate of $\sim 4.23\% \text{ day}^{-1}$ and 40.4 % of beads lost over 12 days (Fig. 1). The loss in the controls with dummies was, however, comparable (ANCOVA, p-value = 0.28), to those in the treatment with living *Tridacna* ($\sim 22\% \text{ day}^{-1}$, with 95 % of beads lost after 12 days, Fig. 1). Finally, no difference in bead depletion rate between juvenile and adult clams was found (ANCOVA, p = 0.5).

The abundance of beads remained into the feeding chambers at the end of the experiment accounted for $\sim 18.4 \pm 2.99$ % of the initial concentration.

Figure 1. Depletion of microplastic beads through time in the feeding chambers: (a) controls without dummy (linear model, p-value = 4.28, F-value = 62.41, N = 2), (b) controls with dummy (linear model, p-value = 0.01, F-value = 17.67, N = 2) and c. live *Tridacna* (linear model, big clams: p = 0.01, F-value = 6.5, N = 5 and small clams: linear model, p-value < 0.001, F-value = 19.3, N = 5). The lines in the graph represent linear models obtained with natural log-transformed data of the number of beads (y-axis).

The plastic bead loss rate (beads lost individual⁻¹ day⁻¹) increased linearly with the initial concentration of beads (beads individual⁻¹, Fig. 2 and further data are shown in Fig. S2 in Supplementary Materials).

Figure 2. Depletion of microplastic beads per day during the experiment: plot showing that the plastic lost individual⁻¹ day⁻¹ increased linearly according to the initial concentration of beads (beads ml⁻¹: linear model, p-value = 2.95, F=599.4, N=12).

3.1. Active removal of microplastics

Microplastics were found inside all the treated clams used in the experiment with dummies (Fig. 3), averaging ($\pm$ SE) 100.5 ± 35.9 beads individual⁻¹ (5.76 ± 1.16 MPs / g dw) in the digestive system, with a minor retention of plastic beads in the gills (1.3 ± 0.5 beads individual⁻¹). The 80% of clams retained all the size classes used for this experiment. The uptake process by clams accounted for $\sim 0.34 \pm 0.12$ % of beads out of the initial number of beads added into the feeding chambers.

The number of microbeads retained in the two clams' size classes (adult and juvenile-adult) were then compared. Our results showed that the difference in plastic retained between the two sizes was not significant (Mann-Whitney, p-value = 0.29, Fig. 4a). Moreover, there was a significant correlation between the plastic retained and the clam size (Spearman's correlation, p-value = 0.01, R = 0.53) and the volume of the clams (Spearman's correlation, p-value = 0.03, R=0.46).

Figure 3: Microplastics retained in the first experiment by *T. maxima*. (a) A scheme showing clams' internal anatomy. (b) (d) Polyethylene microbeads retained by two adult clams, clearly visible within the digestive system. (e) Fluorescent microbeads observed under a fluorescent microscope. Image acquired using the Axiovision software (Carl ZEISS AG, Germany). (c) (f) fluorescent microbeads attached to the clams' surface.

Distribution of retained beads in size classes was different between the two clams' sizes (ANCOVA, p -value = 0.02, Fig. 4b): big clams tend to retain bigger beads (linear model, p -value = 0.03, $F=5.52$, $N=24$, Fig. 4b), while no preference arises for small clams.

At the end of the experiment, 0.65 ± 0.33 % of total beads lost from suspension was retained by the clams (i.e. active removal). We have also estimated the same value without taking into account missing beads and obtaining a value 1.59 ± 0.52 %.

Figure 4: Microplastics retained in adult and juvenile-adult clams. (a) Number of retained microplastics in adult (in dark grey) and juvenile-adult (in light grey) clams. The dark horizontal line in the center of boxes represents the median value of data, the edges of the boxes are quartile and error bars are the extreme values. **(b)** Distribution of retained microplastics in beads size classes for adult (in dark grey; linear model, p-value = 0.03, F = 5.52, N = 5) and juvenile-adult (in light grey; linear model, p-value = 0.5, F = 0.48, N = 5) clams. The x-axis shows the mean value of each beads size class. The two lines represent the linear model obtained using untransformed data and the equation is referred to the linear regression in adult clams.

Polyethylene microbeads were also observed in the digestive system of all the 12 clams used in this experiment without dummies. No significant difference was found among the different concentrations (Kruskal-Wallis, $p = 0.08$, Fig. 5).

The percent of the beads lost from suspension that was retained by the clams did not differ significantly with initial bead concentration. The number of beads retained by clams treated with low, mid-low and mid-high concentrations accounted respectively for: (1) 0.28 ± 0.02 %, (2) 1.82 ± 0.90 % and (3) 0.61 ± 0.30 % and it was equal to: (1) $\sim 9.5 \pm 0.65$, (2) $\sim 138.75 \pm 78.85$, (3) $\sim 99 \pm 49.73$ beads clam⁻¹.

The retention of microbeads was also estimated excluding the number of missing beads and beads attached to the boxes in low, mid-low and mid-high treatments. The values were respectively equal to: (1) 0.49 ± 0.06 %, 3.39 ± 1.87 % and 1.17 ± 0.60 %.

Figure 5: Results of the experiment without dummies and comparison with the experiment with dummies. Number of beads retained by clams exposed to the four different concentrations: low (dark grey), mid-low (white), mid-high (grey) and high (light grey). No significant difference was found among them (Kruskal-Wallis, $p = 0.08$).

3.2. Passive removal of microplastics

Our calculations showed that the passive removal process accounted for $35.95 \pm 2.10 \%$, therefore about 1/3 of the microbeads added were retrieved attached to the shells at the end of the experiment with dummies, resulting in an average density of beads attached to the shells of 50.36 ± 5.22 beads cm^{-2} . We also obtained the same value excluding the number of missing beads and beads attached to the boxes and it was equal to $66.03 \pm 2.50 \%$. Larger clams supported a larger number of attached beads than smaller ones (46.49 ± 13.14 beads cm^{-2} and 54.22 ± 8.99 beads cm^{-2} Mann-Whitney, p -value < 0.0001 , Figure 6a), although no significant simple correlation was found between attached plastic microbeads and clam volume or surface area (Spearman's correlation, p -value $= 0.46$, $R = 0.1667$). Likewise, we found no significance difference in distribution of plastic beads in size classes between juvenile-adult and adult clams (ANCOVA, p -value $= 0.91$, Fig. 6b). Finally, microbeads loss showed a significant difference between living clams and dummies (linear model, p -value $= 0.02$, $F=5.38$, $N=60$) and the number of beads attached to the clams was different between living clams and dummies (linear model, p -value $= 0.02$, $F=71.93$, $N=60$). The contribution to the removal process of adhesion to dummies

accounted for 48.06 ± 1.12 %.

In the experiment without dummies, the number of beads attached to the shells increased with increasing initial concentration of beads (ANOVA, p -value = 9.11, $R = 0.90$, Fig. 7a), from 6.89 ± 1.78 beads cm^{-2} at low bead density, to 13.72 ± 2.36 beads cm^{-2} at intermediate bead density and 30.38 ± 7.5 beads cm^{-2} at high bead density (linear model, $p = 4.67$, $F = 71.93$, Fig. 7b). As a result of this linear relationship, beads passive removal accounted for 67.50 ± 0.28 % (63.38 ± 6.21 %, 65.30 ± 4.06 %, and 73.82 ± 3.37 % for low, mid and high bead density, respectively). About 38 % of the beads lost from the suspension were found attached to the clam shell independently of initial concentration (38.44 ± 7.73 %, 38.36 ± 3.22 %, and 37.34 ± 4.54 % for low, mid and high bead density, respectively).

Figure 6: Microplastic adhered to clam's surface in the experiment involving the use of dummies. (a) Box plots summarizing the distribution of the number of polyethylene microbeads attached to the shells in adult (in dark grey) and juvenile-adult (in light grey). Larger clams supported a larger number of attached beads than smaller ones (Mann-Whitney, p -value < 0.0001). The dark horizontal line in the center of boxes represents the median value of data, the edges of the boxes are quartile and error bars are the extreme. **(b)** Distribution of microplastics of different size attached to the shells of adult (in black; linear model, p -value = 0.46, $F = 0.57$, $N = 5$) and juvenile-adult (in light grey; linear model, p -value = 0.28, $F = 1.22$, $N = 5$) clams. No significant difference between the two distributions was observed (ANCOVA, p -value = 0.91, $R = 0.30$). The two lines represent the linear model obtained using untransformed data.

Figure 7. Passive removal of microplastic from the water column in the different treatments. (a) Box plots showing the distribution of the average number of beads attached to the clams' shells during the experiment in the different treatments: low (dark grey), mid-low (white), mid- high (grey) and high (light grey). The dark horizontal line in the center of boxes represents the median value of data, the edges of the boxes are quartile and error bars are the extreme values (ANOVA, p -value = 9.11, R = 0.90). **(b)** Boxplots indicating the bead density found for beads attached to the shells for each concentration of microplastic used in this study low (dark grey), mid-low (white), mid- high (grey) and high (light grey) (linear model, p =4.67, F = 71.93).

3.3 Mass balance of microplastic beads

A total of 88.2% of microplastic beads was lost from the suspension at the end of both the experiments. Moreover, the number of microplastic adhered to the shells excluding missing microbeads was 35.95 ± 2.10 , and the retained microplastic accounted for $0.65 \pm 0.20\%$ of the initial number of beads. $45.64 \pm 2.19\%$ of plastics was not retrieved at the end of the experiment. The controls, without dummies or living *Tridacna*, allowed quantification of the beads lost to the surfaces of the experimental aquaria at 69.81 %. The percent of the beads lost from suspension that was retained or attached to the shell surface was independent of the initial bead concentration, averaging about 1% (see above) and 66 % ($63.39 \pm 6.21\%$, $65.30 \pm 4.06\%$, $73.82 \pm 3.37\%$ and $64.27 \pm 4.48\%$ for low, mid, high and very high bead density, respectively).

3.4. *Tridacna* performance

Exposure to microplastic beads was not observed to cause any change in behavior or mortality events, and no anatomical anomalies were observed during the dissection. The

experiment had an influence on the metabolism of the clams, indeed a significant difference between the initial and final GPP/R ratio was found (Two-way ANOVA, p -value = 0.02, data shown in Fig. S3 in Supplementary Materials). However, no difference was observed among the controls (Two-way ANOVA, p -value = 0.33) or between adult and juvenile-adult clams (Two-way ANOVA, p -value = 0.645, data shown in Fig. S3 in Supplementary Materials).

4. Discussion

This study confirms that the Red Sea giant clam *T. maxima* can remove microplastics from the surrounding water, and reveals that this occurs through two independent processes: an active retention of the beads and a, dominant, passive process involving the attachment of plastic beads to the shell. Dissection of the animals showed that retention leads to accumulation of the microplastics beads inside their body, mainly into the digestive system. However, the digestive tract itself represents a minor sink for plastic microbeads in Tridacnids, as passive attachment to *Tridacna* shells accounted for $66.03 \pm 2.50\%$ (or $35.95\% \pm 2.1\%$ excluding missing microbeads), on average of the microplastics beads removed. We also demonstrate that the passive removal rate and the density of microplastic beads found in the shells increase linearly with microplastic concentration in the environment, as expected from the fact that a higher microplastic density should result in an increased likelihood of encounter, and subsequent attachment with the shell.

Most studies published to-date focused mainly on plastic ingestion, but few (Cole et al., 2013) considered the microplastic attached to the surface of marine organism as an important factor. For the first time, we demonstrated that passive attachment to shells represents the dominant component of microplastic removal in giant clams. It was also observed that, even though clams were exposed to different concentrations, the percentage of plastic attached to the shells remained constant. The attachment process to shells is potentially related both to the complexity of the clams' shell surface and the presence of epibionts. Indeed, even though clam shells were thoroughly scraped off epibionts at the beginning and at the end of the experiment, they may have trapped a high number of beads. In this regard, we have noticed that the shells of living animals removed more beads than bleached shells/dummies, ($66.03 \pm 2.50\%$ and $48. \pm 1.12\%$, respectively), thus confirming that the presence of both microbial film and epiphytes on the shells of living organisms increase the attachment of plastic. Nevertheless, if we compare the

depletion of microbeads in the feeding chambers, dummies seem to remove more plastic than living animals (Fig. 1b and 1c). The explanation for this discrepancy is due to the fact that the beads attached also to the internal part of the dummies, leading to a higher removal rate (Fig. 1c). However, the passive removal contribution in percentage was obtained using only the number of beads directly attached to the external part of the dummies.

It has been widely documented that microplastics can be detected in an extensive range of bivalves worldwide. Mussels have been widely studied, because of their ubiquity and commercial importance, while other ecologically relevant species, such as *Tridacna*, had not been investigated as yet. Indeed, plastic ingestion may differ greatly among bivalve species, due to different feeding strategies and filtration rates (Cusson et al., 2005). For instance, the blue mussel, *Mytilus edulis*, has been used for both experimental and environmental studies and it is considered the quintessential model organism, because of its intense filter-feeding activity. Indeed, thanks to this feeding strategy it is exposed directly to microplastics in the surrounding seawater (Cusson et al., 2005). Recently, it has been found that if exposed to high level of microplastics (approximately 411 million beads in 400 ml), the blue mussel takes up microbeads into the stomach, hepatopancreas, gills and ovaries in quantity ranging from 65 (hepatopancreas) to 1025 (stomach) beads (Farrell and Nelson, 2013). This means that *M. edulis* ingests ~ 0.00024% out of the total stock of microplastic beads, whereas our results show that *T. maxima* can take up about 1.59 %. This difference could be related to the dissimilarities between the sizes of these two bivalves.

If ingested by bivalves, microplastics are likely to impede the digestion of natural food, as plastic items take the place of food (Watts et al., 2014; Wright et al., 2013; Cole et al., 2015), resulting in a false sense of satiation (Trench et al., 1981; Murray and Cowie, 2011). If the amount of plastic ingested is high, this may potentially lead to the obstruction of the digestive tract (Stamper et al., 2006). Moreover, it has been shown that ingested microplastics could potentially translocate from the digestive system cavity to the circulatory system of mussels (Browne et al., 2008). Hence, further investigations will be necessary to understand whether this process could happen in giant clams as well. However, it must be taken into account that ingested beads could be egested, leading to a temporary accumulation of plastic inside marine biota and then to its release into the water column as pseudofeces (Jørgensen, 1975; Levinton et

al., 1996; Cole et al., 2013; Frydkjær et al., 2017; Ory et al., 2018). Indeed, it has been observed that clams used in our experiments can expel water and microplastics along with it, if subjected to stress leading to the closure of the shells, however this process was not common during our experiment. Metabolism measurements highlighted that the exposure and retention to microplastics decreases the GPP:R ratio of the holobiont throughout the experiment, but no difference between controls and treatments was found. This result does not conform to the finding of stress responses related to reproduction and nutrition upon plastic exposure for many organisms, in different organisms.

Besides, recent studies have pointed out that in most of the above-mentioned papers, researchers exposed organisms to unrealistic conditions, including concentrations of microplastics higher than those observed in the aquatic environment and exposure to very small virgin microspheres, which do not reflect the size, nature and status of microplastics present in the oceans. As a consequence, these studies cannot be directly extrapolated to nature (Lenz et al., 2016; Paul-Pont et al., 2018). Therefore, experimental conditions in the present study were chosen according to (Xu et al., 2017; Ribeiro et al., 2017; Kolandhasamy et al., 2018; Marti et al., 2017; Ezgeta-Balic et al., 2012). However, the main finding of this research is that passive removal was 35-fold higher than active removal independently from the initial concentration used.

Tridacnids show a complex feeding strategy, which includes several processes. First of all, the transfer of the excess of carbon from the symbiotic zooxanthellae to the host's tissues, which seems to satisfy the main nutritional requirements (Trench et al., 1981; Fisher et al., 1985). Furthermore, they possess a digestive system typical of heterotrophic filter-feeding bivalves (Morton, 1978; Reid et al., 1984). Thanks to these autotrophic and heterotrophic nutritional sources, which act synergistically, giant clams can act as trophic opportunists in oligotrophic environments, such as tropical coral reefs (Yonge, 1982; Heslinga and Fitt, 1987). They can also obtain nutritional benefits from the digestion of zooxanthellae (Morton, 1978; Reid et al., 1984) and from the direct ingestion of dissolved organic carbon (Frankboner, 1971; Goreau et al., 1973). It has been shown that filter feeding represents the major organic carbon source (65%) for both respiration and growth in small (100 mg dry tissue) clams, while these sources provide only 34% of the carbon used by large clams (10 g dry tissue) (Klumpp et al.,

1992). Nevertheless, we did not observe a significant difference between the amount of retained microbeads in small and large clams, and there was no correlation between retention and specific size of the individuals. The most plausible explanation for the lack of correlation between size and quantity of plastic retained by clams is the high variability found in the big clams. Furthermore, the size range of *Tridacna* tested was limited (i.e. small clams 13.8 ± 1.7 cm in length vs. 19.8 ± 2.2 cm in length for large clams), and greater differences may occur if yet smaller and larger individuals are compared. Even though pumping rate was not measured in this study, it has been shown that the effect of organism's size on microplastic load reported in mussels could be explained by the size-dependence of pumping rate/g (Catarino et al., 2018; Bråte et al., 2018). Moreover, no difference in retention (as % of beads removed) was found when clams were exposed to different microplastic concentrations. A plausible reason for this is related to the egestion process which can flatten possible differences, but which was not measured in this study.

The Saudi Arabian coast of the Red Sea supports unexpectedly low density of floating microplastics, on average 1.08 g Km^{-2} (Marti et al., 2017), two orders of magnitude below that found in the adjacent Mediterranean Sea ($423\text{-}672 \text{ g Km}^{-2}$; Cózar et al., 2014). This contrast suggests the existence of effective removal process in the Red Sea, which may include some of the filter-feeding organisms in the extensive reef systems of the Red Sea. Our study highlighted that indeed *T. maxima* can remove microplastics from the water column, largely through adhesion to the shells. Given the high abundance of *T. maxima* in Red Sea coral reefs (about $0.22 \text{ individuals m}^{-2}$; Bodoy, 1984), microplastic removal by *T. maxima* may represent an important sink process. However, provided that most of the surface of clams in the natural environment is embedded in the substrate, and, therefore, it is not entirely exposed to the water, and that the concentration of microplastics in the Red Sea is very low ($0.00005 \text{ items L}^{-1}$), the *in situ* removal rates are expected to be significantly below those reported here. Hence, further studies are needed to clarify the actual role of passive removal in the natural environment. Furthermore, high calcification rates by *Tridacna* clams suggest the possibility that the microplastics adhered to the shells may become eventually embedded into the calcium carbonate shells.

The finding that the shell of *Tridacna* acts as a sink for microplastics, supporting removal rates 35-fold higher than ingestion, points at microplastic removal by the large and complex

structures developed by coral reefs, including carbonate structures developed by corals, as a potentially intense sink for microplastics, a possibility that need be examined. Our results do, therefore, support the suggestion that the massive coral reefs of the Red Sea may act as effective filters, possibly dominated by passive removal processes, accounting for the low microplastic concentration in the Red Sea (Marti et al., 2017).

5. Conclusions

Although microplastic-related research has been mainly focused on ingestion and retention activities by marine organisms, a new perspective has been highlighted in this study. Surface of marine biota and its structural complexity can contribute to the removal process of microplastics from the water column as well. Moreover, in the case of *Tridacna maxima*, the contribution of passive removal seems to be significantly more intense than active retention of microbeads. These findings lay the basis for further studies to be conducted using other species, in order to better understand the interactions between 3D structure of organisms and microplastics. However, studying single species is not sufficient, in the future studies there will be the need to consider the interactions of microplastics and coral reefs as a whole.

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Microplastic removal by Red Sea giant clam (*Tridacna maxima*)

Silvia Arossa*, Cecilia Martin, Susann Rossbach, Carlos M. Duarte

Highlights

- Removal of plastic from the water by giant clams is assessed for the first time
- We identified two removal processes: active (ingestion) and passive (adhesion)
- Passive removal is 35-fold higher than active removal
- Adhesion contributes to remove > 60% of plastic from the water column
- Removal rate depends on the concentration of plastic

Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: