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Sea cucumbers reduce nitrogen, bacteria and transparent exopolymer particles in *Anemonia sulcata* aquaculture tanks

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Abstract

Traditional aquaculture produces wastewater with high nutrient and organic matter concentrations. Poly-culture can improve this problem including "extractive species" such as sea cucumbers along with the primary species. The influence of sea cucumbers on transparent exopolymer particles (TEP) (i.e. biofilm precursors) has not been previously explored. Here, we monitored during 1 year the concentration of nutrients, total organic carbon (TOC), particulate organic matter (POM), TEP, chlorophyll-*a* and bacteria in two tanks of 50,000 L. One tank only contained *Anemonia sulcata*, whereas the other tank also included holothurians. To complement these time-series, we performed three short-term experiments in smaller (300 L) tanks. Three tanks contained *A. sulcata* plus *Holothuria tubulosa* (+H treatment) and other four tanks contained only *A. sulcata* (−H treatment). In the time-series, we found that the concentration of ammonium, nitrate, TOC, POM, TEP and bacteria in the effluent of the tank with holothurians was lower than in the effluent of the tank without holothurians. The three experiments confirmed that the holothurians reduced significantly nitrates, bacterial abundance and TEP concentration. Therefore, these invertebrates can control bacterial proliferation and prevent biofilm formation minimizing likely the risk of outbreak of pathogenic bacteria and improving the hygiene of the tanks.

KEYWORDS

bacteria, extractive species, nitrates, poly-culture, sea cucumbers, transparent exopolymer particles

1 | INTRODUCTION

During last decades, human activities such as overfishing, addition of pollutants and climate change are substantially affecting species stocks and diversity in marine ecosystems (Halpern et al., 2008; Purcell et al., 2013). Moreover, the exponential growth of human population has boosted the global demand of fish and seafood, however extractive fisheries are more and more limited. In fact, aquaculture now accounts for approximately 50% of human consumption of fish and seafood (Bostock et al., 2010; FAO, 2018). Therefore, a responsible aquaculture is a global challenge for both marine biologists and food producers (Diana et al., 2013). These last authors proposed poly-culture and -integrated multitrophic aquaculture (IMTA) as alternative procedures to alleviate, to some extent, the environmental problems derived from traditional aquaculture.

Traditional aquaculture produces wastewater that usually contains high loads of organic and inorganic nutrients, antibiotic and uneaten food pellets (Black, 2001; Klinger & Naylor, 2012; Read & Fernandes, 2003). The influence of this wastewater on the marine environment depends on the production system (extensive vs. intensive/semi-intensive), aquaculture system type (tank, pond and cage), the cultured species, as well as the carrying capacity of the recipient waters. During wastewater discharges from aquaculture facilities or under the cages in offshore installations, processes such as eutrophication and anoxia in sediments can happen (Crab, Avnimelech, Defoirdt, Bossier, & Verstraete, 2007). Aquaculture wastewater can be a relevant source of nitrogen stimulating primary producers and increasing the risk of algal blooms or red tides (Ajin, Silvester, Alexander, Nashad, & Abdulla, 2016). Similarly, oxygen depletion in sediments can cause the release of ammonia and sulfide changing the physicochemical properties of water affecting fishes and corals (Kalantzi & Karakassis, 2006). Faecal waste and uneaten foods constitute a fraction of particulate organic matter that is deposited in the bottom of the tanks or below the cages in offshore installations affecting bacterial activity and sediment properties. These changes also modify the biomass and diversity of macrobenthos (Yokoyama, 2002). In the particular case of inshore installations, wastewater from aquaculture tanks usually has to be treated before being returned to the aquatic ecosystems.

Several procedures to treat aquaculture wastewaters are in practice. To decide which treatment is more appropriate several factors should be considered such as land and water availability, wastewater local regulation and operational expenses. Wastewater treatments such as Fenton's oxidation (Lee & Shoda, 2008), sequencing batch reactor (Fontenot, Bonvillain, Kilgen, & Boopathy, 2007), up-flow anaerobic sludge bed or integrated anaerobic/aerobic biological treatments (Bortone, 2009) are used, although they imply high economical costs and eventually can generate toxic by-products and membrane fouling in comparison with alternative biological treatments. In general, biological treatments are more acceptable by fish producers and policy makers. For instance, Da, Phuoc, Duc, Troell, and Berg (2015) proposed the reuse of wastewater from Striped Catfish farms in rice crops. Other authors proposed the recovering of phosphorous from wastewaters using the gastropod shell (Oladoja, Adelagun, Ahmad, & Ololade, 2015) or aquatic plants (Buhmann & Papenbrock, 2013,and Zhang, Achal, Xu, & Xiang, 2014). Diana et al. (2013) recommended the integrated multitrophic aquaculture (IMTA) and poly-culture as responsible procedures that can decrease inorganic and organic nutrient loads in the effluents using “extractive” species that can reduce the costs of wastewater treatments.

IMTA and poly-culture procedures, unlike mono-specific aquaculture, use complementary species where the excretion, faecal and food wastes from the primary species are nutritional resources of the “extractive” species (Chopin, Cooper, Reid, Cross, & Moore, 2012). Therefore, these aquaculture procedures include primary species (e.g., finfish), extractive species that filter or ingest the suspended or deposited organic matter (e.g., mussels, oysters, holothurians), and extractive species that assimilate inorganic nutrients (e.g., seaweeds) reducing the loads of mineral nutrients and organic matter in the effluents. Therefore, it is desirable that the upcoming expansion of aquaculture develops these co-culture procedures to remove or, at least, reduce these organic and inorganic loads in the effluents and, simultaneously, provide extra species with an additional economical or ecological value.

Sea cucumbers are species with high extractive capacity for organic matter in sediments (Nelson, MacDonald, & Robinson, 2012a, b; Slater & Carton, 2009; Yokoyama, 2013, 2015). However, to the best of our knowledge, the specific effects of sea cucumbers on biofilm precursors such as transparent exopolymer particles (TEP) have not been previously explored. In addition, these marine invertebrates are very demanded for human consumption in some countries of Asia; where overfishing have declined their stocks (Purcell et al., 2013). Therefore, sea cucumber cultures could, on the one hand, mitigate the overfishing problem in some regions and, on the other, improve water quality in poly-culture installations. Another asset of sea cucumbers is their biotechnological potential. Their microbiomes appear to be an important source of new antimicrobial substances (Chludil, Muniain, Seldes, & Maier, 2002; Gowda, Goswami, & Khan, 2008; Haug, AK, Styrvold OB, Sandsdalen E, Olsen ØM, & Stensvag K., 2002; Kumar, Chaturvedi, Shukla, & Lakshmi, 2007; León-Palmero et al., 2018). Therefore, the use of sea cucumbers in poly-culture has also a pharmaceutical interest (Bhatnagar & Kim, 2010; Valliappan, Sun, & Li, 2014).

In this study, we assess the effects of holothurians as extractive species of nutrients and organic matter in aquaculture tanks with *Anemonia sulcata* as primary species. During 1 year, we monitored the changes in the concentration of total organic carbon, particulate organic matter, bacteria, chlorophyll-*a*, transparent exopolymer particles and major nutrients (ammonium, nitrate, nitrite and total phosphorous) in two big tanks of 50,000 L that only differed in the presence of holothurians. Afterwards, to corroborate the observations obtained in the big tanks, we performed three short-term experiments manipulating the presence of holothurians in smaller tanks (300 L). We observed that the presence of holothurians reduced significantly the concentration of nitrate, transparent exopolymer particles and bacterial abundance both in the big tanks and in the short-term experiments. TEP reduction by sea cucumbers was very remarkable. Therefore, TEP and associated bacteria appear to be a food resource for holothurians and its ingestion might have implications for the keeping of tank hygiene as well as for the gut microbiota of these invertebrates.

2 | MATERIAL AND METHODS

2.1 | Time-series in the big-volume tanks

We monitored for 1 year two aquaculture tanks at iMareNatural S.L. facilities (https://www.imarenatural.com) in Southern Spain (36°44′38″ N, 3°35′59″ W). Each tank (radius = 3 m, high = 1.8 m with ca. 50,000 liters of capacity) was connected directly with the coastal water by one inlet pipe (inlet waters) and the water from each tank was released by one outlet pipe located in the bottom of the tank (effluent). The seawater was pumped into the tanks at a continuous flow of 1,200 L/hr. Therefore, water residence time in the tanks was about 42 hr. In one of the tanks, 811 ± 125 individuals of the primary species, the sea anemone *Anemonia sulcata*, and 93 ± 3 adults of sea cucumbers with an average weight of 293 g and length of

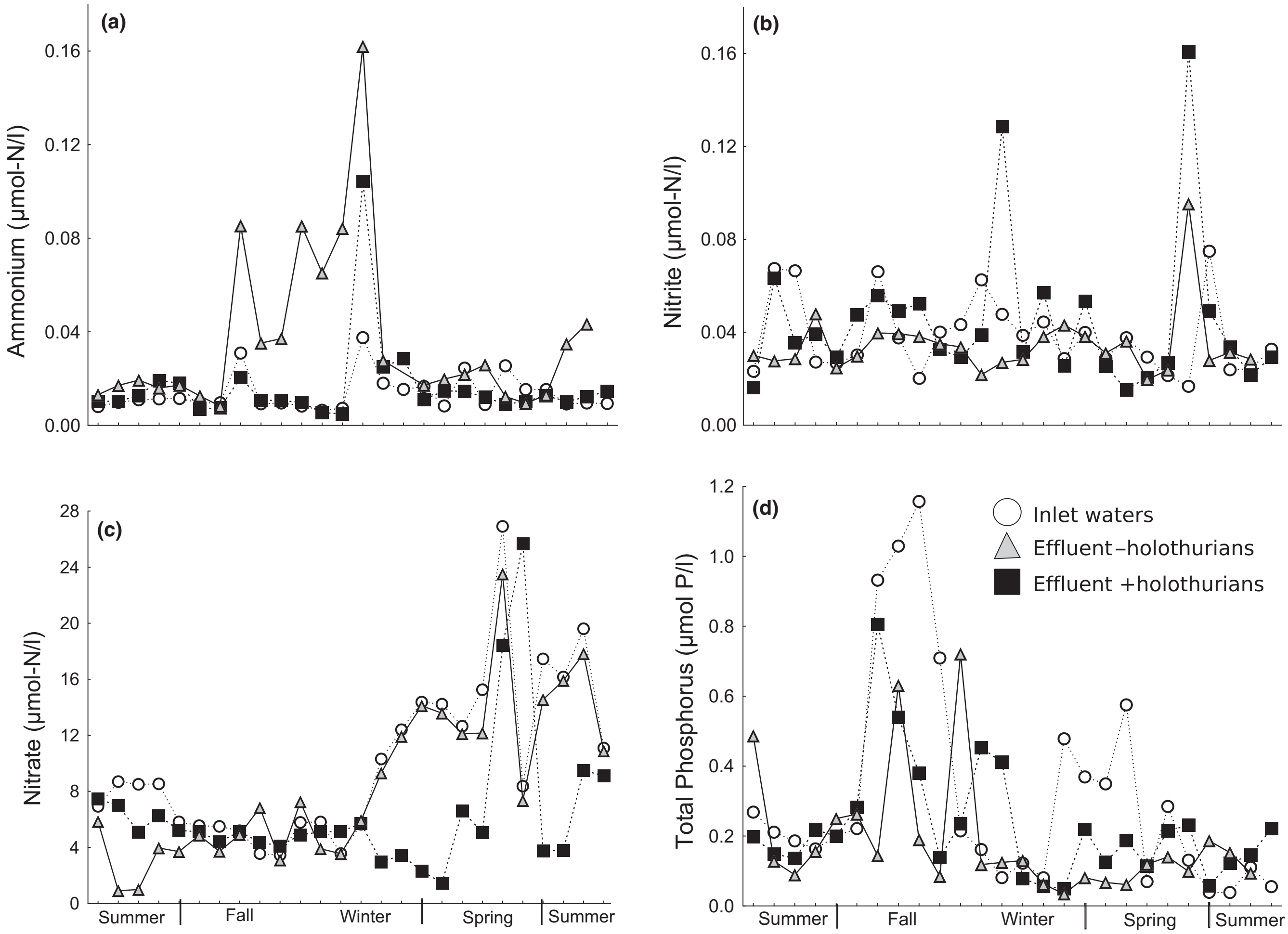

FIGURE 1 Time series of the concentration of (a) ammonium, (b) nitrite, (c) nitrate, and (d) total phosphorus in the inlet waters (white circles), in the effluent waters of the *-holothurian* tank (grey triangles) and in the effluent waters of the *+holothurian* tank (black squares) in the big volume tanks

24 cm (approximately 80% *Holothuria tubulosa* and 20% *H. forskali*) were included (hereafter designated as *+holothurian* tank). This tank had a stocking density of ca. 3 holothurians m^{-2} and a ratio of anemone to holothurian between 8 and 9. These two species of holothurians are the most common in the nearby coastal area. In the other tank only 690 ± 87 individuals of *A. sulcata* were included (hereafter designated as—*holothurian* tank). Sea anemones were placed on floating plastic boxes in the surface of the tanks and holothurians were free in the bottom and walls of the tanks. Sea anemones were fed with about 900–1,800 g of fresh chopped fish, mainly *Scomber scombrus* (Chintiroglou & Koukouras, 1992; Van-Praët, 1985) twice per week. *Anemonia sulcata* was selected as the primary species because is a very palatable species, highly demanded for gourmet catering in Southern Spain (U. Granada, 2013). Moreover, this species has also a great biotechnological potential (León-Palmero et al., 2018; Silva, Andrade, Paiva-Martins, Valentão, & Pereira, 2017). In particular, the company iMareNatural S.L., where this study was performed, is involved in the study of this species because this pharmacological potential (https://www.tascmar.eu) and has also started its commercialization for gourmet catering. Species diversification in aquaculture is another main goal for a sustainable aquaculture as well as for environmental conservation and restoration (Diana et al., 2013; Froehlich, Gentry, & Halpern, 2017).

Water samples from each tank were collected biweekly from July 2013 to August 2014. We took the samples from the centre of the tanks, transferred to sampling bottles and immediately placed on ice for their transportation to the laboratory. Sampling bottles were previously cleaned with acid, rinsed with bi-distillated water and several times with seawater. Before each sampling, basic parameters such as temperature (°C), pH, salinity (psu), total dissolved solids and conductivity (mS cm^{-1}) were measured in the tanks using a multi-parameter HANNA probe (HI9828 model). For the total organic carbon (TOC) samples, we used 40 ml amber EPA vials previously combusted at 500°C. Once in the laboratory (about 1 hour from the tanks), the samples for dissolved nutrients were filtered through Whatman GF/F filters and the filtrates were stored at −20°C until analysis. Samples for bacterial abundance were fixed with 1% paraformaldehyde and 0.05% glutaraldehyde and then immediately stored at −80°C.

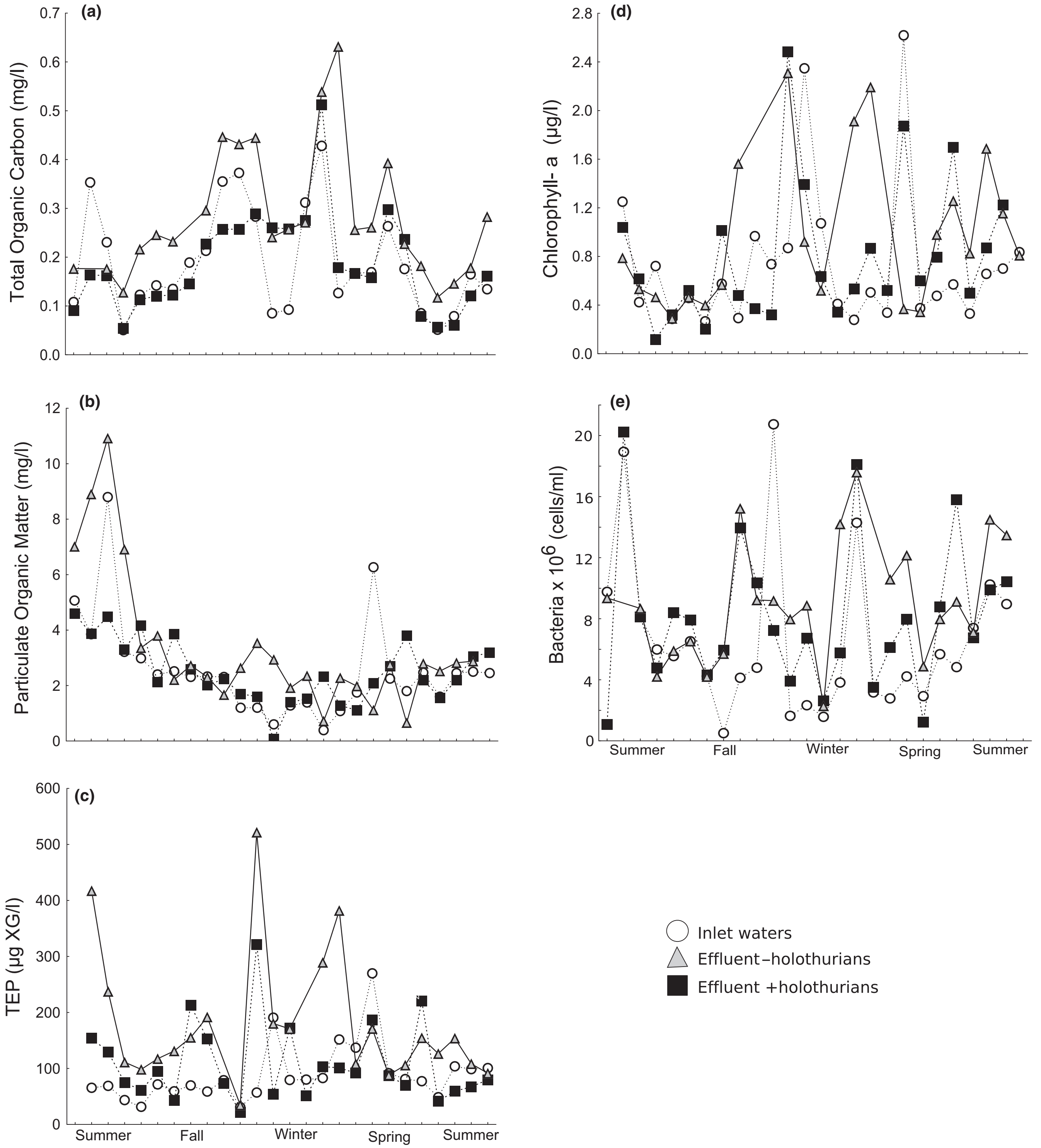

FIGURE 2 Time series of the concentration (a) total organic carbon, (b) particulate organic matter, (c) transparent exopolymer particles (TEP), (d) chlorophyll-*a*, and (e) bacterial abundance in the inlet waters (white circles), in the effluent waters of the *-holothurian* tank (grey triangles) and in the effluent waters of the *+holothurian* tank (black squares) in the big volume tanks

2.2 | Nutrient analysis

To determine the concentrations of ammonium, nitrite, nitrate and total phosphorous (TP) we used the standard methods (APHA, 1992). The dissolved fraction of the nutrients was previously filtered using Whatman GF/F filters and total nutrients were analyzed from unfiltered samples. All samples were analyzed by triplicate. To determine ammonium concentration we used the phenate method (APHA,

TABLE 1 Results of paired *t*-tests (for normally distributed variables) and Wilcoxon matched pairs test (for not normally distributed variables) between the inlet waters and the effluents from the +*holothurian* tank and −*holothurian* tank

	Statistical Analysis	*t* or *z*	*p*-value
Inlet waters versus +*holothurian* effluent			
Ammonium	Wilcoxon matched pairs test	0.74	0.4537
Nitrite	Paired *t* test	0.58	0.5639
Nitrate	Paired *t* test	2.19	0.037
Total phosphorus	Paired *t* test	0.64	0.5253
Total organic carbon	Paired *t* test	0.03	0.9691
Particulate organic matter	Paired *t* test	0.19	0.8443
Transparent exopolymer particles	Paired *t* test	1.51	0.1439
Chlorophyll-*a*	Paired *t* test	0.61	0.5455
Bacteria abundance	Paired *t* test	1.49	0.1468
Inlet waters versus −*holothurian* effluent			
Ammonium	Wilcoxon matched pairs test	3.37	0.0008
Nitrite	Wilcoxon matched pairs test	1.00	0.3130
Nitrate	Paired *t* test	0.60	0.0058
Total phosphorus	Paired *t* test	1.93	0.0644
Total organic carbon	Paired *t* test	6.17	0.0000
Particulate organic matter	Paired *t* test	2.05	0.0493
Transparent exopolymer particles	Paired *t* test	5.43	0.0000
Chlorophyll-*a*	Paired *t* test	1.41	0.1737
Bacteria abundance	Paired *t* test	2.97	0.0070

1992) with the Spectroquant® Test Kit (Merck Millipore). Nitrate concentrations were measured following the ultraviolet spectrophotometric method. Briefly, 25 ml-samples were acidified with 0.5 ml of hydrochloric acid (1 M), shaked and the absorbance at 220 nm and 275 nm were measured using 10 cm cuvettes in an UV-VIS Perkin-Elmer spectrophotometer connected to a computer equipped with UV Winlab software. The nitrite concentration was determined spectrophotometrically through the formation of a reddish purple azo dye produced at pH 2.0 – 2.5 by coupling diazotized sulfanilamide with N-(1-naftile)-ethylendiamine dihydrochloride. The concentration of TP was determined spectrophotometrically using the ascorbic acid technique (Murphy & Riley, 1962).

2.3 | Organic components

We measured total organic carbon (TOC) concentration by a high-temperature catalytic oxidation as non-purgeable organic carbon using a Shimadzu TOC-V CSN analyzer. Samples by triplicate were acidified with hydrochloric acid and purged for 20 min to eliminate dissolved inorganic carbon. Three to five injections were analyzed for each sample and the blanks (Milli-Q water). Standardization of the instrument was done with potassium hydrogen phthalate (4-point calibration curve).

To determine the concentration of particulate organic matter (POM) we filtered between 1.5 and 2.0 L of water from the tanks through pre-weighed and precombusted (500°C for 4 hr) Whatman GF/F glass fibre filters (0.7 μm nominal pore size). The filters containing all the solids were dried at 60°C for >24 hr and reweighed to determine the total mass (mineral +organic matter). Then, the filters were combusted at 500°C for 6 hr to burn the organic fraction. Finally, the filters were reweighed again to determine the mineral residue. POM was obtained after the subtraction of the mineral residue to the total mass.

We determined the concentration of chlorophyll-*a* spectrophotometrically after pigment extraction with methanol (APHA, 1992). In the laboratory, a volume of 2 L of water from the tanks was filtered through Whatman GF/F filters. The filters were covered with aluminum foil and frozen at −20°C until analysis. Pigments were extracted with methanol during 24 hr at 2–4°C. To obtain the concentration of chlorophyll-*a*, absorbance at 665 nm was measured using a spectrophotometer UV/VIS Perkin Elmer and, if needed, corrected for turbidity using the absorption at 750 nm.

We determined bacterial abundance by triplicate using flow cytometry (Gasol & del Giorgio, 2000) with a FACScalibur Becton Dickinson cytometer equipped with a laser emitting at 488 nm. Samples were stained for 10 min in the dark with a DMSO diluted Syber Green I (Molecular Probes) at 10 μM final concentration. A volume of 10–20 μl of a solution of yellow-green 0.92 μm Polysciences latex beads was added as an internal standard. Bacterial abundance was detected by their signature in bivariate plots of Side scatter (SSC) versus FL1 (green fluorescence). Samples were acquired in log mode and run at low speed (for 2 min at 12 μl/min) for bacterial abundance until ca. 10^5 events. Dilution of the samples was performed for events higher than 800 cells s^{-1}. Data were processed using Cell quest software.

Transparent exopolymer particles (TEP), on the one hand, are biofilm precursors (Bar-Zeev, Berman-Frank, Girshevitz, & Berman, 2012); which can affect recirculation systems and tank hygiene (Joyce & Utting, 2015). On the other, TEP can be considered a food source for marine invertebrates as well a potential source of gut microbiota (Joyce & Utting, 2015; Passow, 2002a). We determined TEP concentration using the alcian blue method (Passow & Alldredge, 1995) with minor modifications after Mazuecos, Ortega-Retuerta, and Reche (2012). Briefly, water samples (100–250 ml), previously fixed with formaldehyde (1% final concentration), were filtered through 0.4 μm polycarbonate filters. Then, the filters were dyed with 0.5 ml of alcian blue (0.02%) and after 30 s filtered again. The filters were soaked in 80% sulphuric acid (5 ml) for 3 hr and the solution was measured at 787 nm in a UV/VIS Perkin Elmer spectrophotometer. Stained filters without sample were used as blanks. Alcian blue absorption was calibrated using a solution of xanthan gum (XG) that was homogenized using a tissue grinder and measured

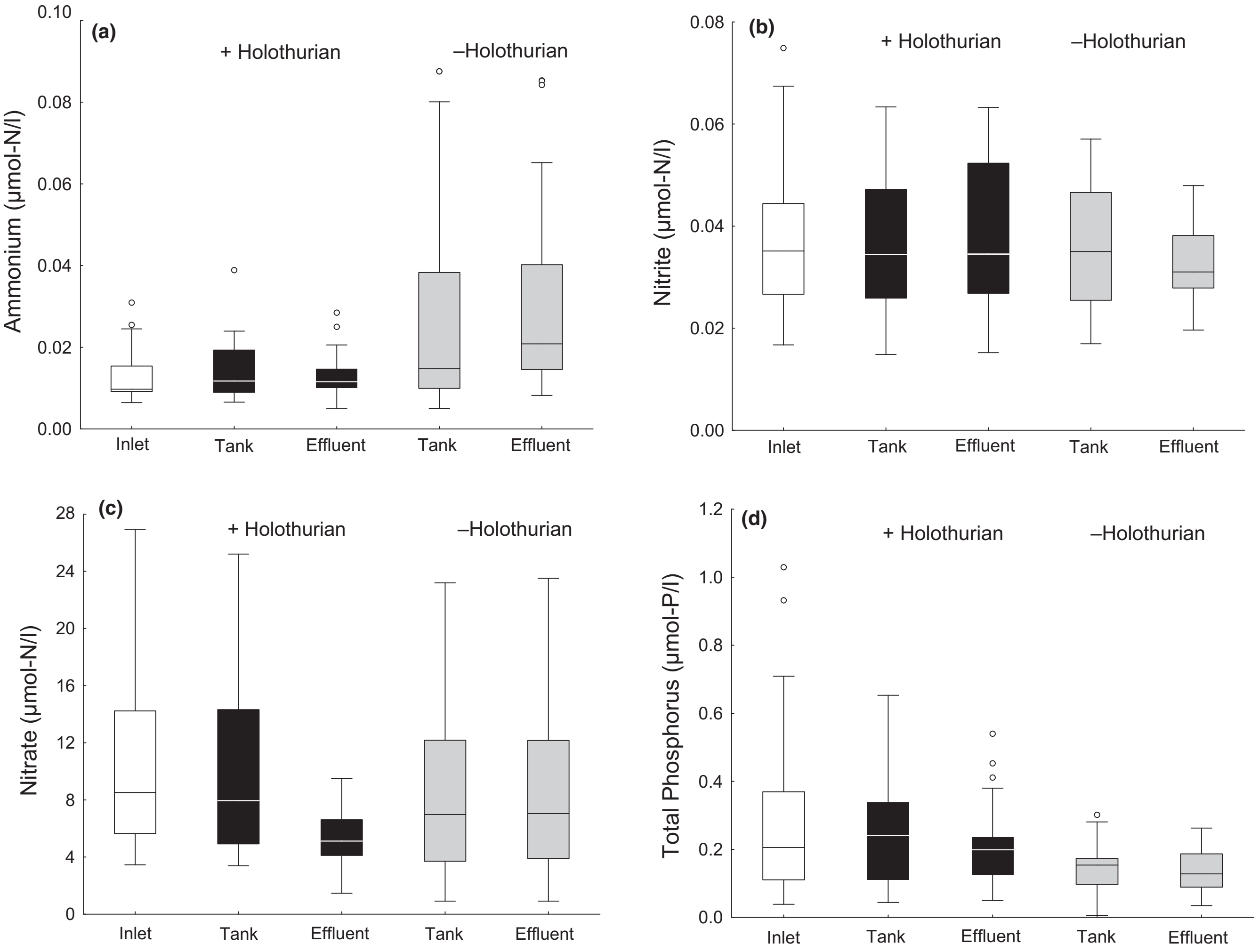

FIGURE 3 Median (line), the 25-75% percentile (box), the non-outliers range (whisker), and outliers (dots) of (a) ammonium, (b) nitrite, (c) nitrate, and (d) total phosphorous in the inlet waters (white box), in the *+holothurian* tank and effluent (black boxes), and in the *–holothurian* tank and effluent (grey boxes)

by weight. Therefore, TEP concentration was expressed in μg XG eq L^{-1}.

2.4 | Short-term experiments

To corroborate the results obtained in the time-series, we performed three short-term (3 days) experiments manipulating the presence of holothurians under similar conditions to the big tanks but considering only the variables that changed in the time-series. Each experiment was carried out in seven tanks of 300 litres that contained a floating plastic box with 80 individuals of *A. sulcata* per tank and consisted of two treatments: *+holothurians* (+H) and *–holothurians* (–H). At the initial time, in three of the tanks we included 10 individuals of *H. tubulosa* in each tank (i.e. a ratio anemone to holothurian of eight). These three tanks are the replicates of the *+holothurians* treatment. The other four tanks only contained the 80 individuals of *A. sulcata* and represent the replicates of the *–holothurians* treatment. The experiment one was carried out from 6th to 9th October 2017, the experiment two from 27th to 30th October 2017, and the experiment three from 3rd to 6th November 2017. During the duration of each experiment the anemones were not fed to control the net effect of holothurian activity and avoid interactions with the food supply. At the initial and final time we took samples for nitrate, total phosphorus, bacteria and transparent exopolymer particles. To analyze the samples we followed the same procedures used in the time-series.

2.5 | Statistical analyses

To compare the tank with holothurians versus the tank without holothurians over time we performed paired *t* tests for normally-distributed variables and Wilcoxon matched pairs tests for not normally-distributed variables using the software Statistica (V8) and R 3.2.2. These statistical analyses ameliorate the problem of temporal pseudoreplication in this type of studies (Millar & Anderson, 2004). These paired tests have been commonly used in marine sciences to compare changes over time among different sites (e.g. Ault & Johnson, 1998; Greenstreet & Hall, 1996; Rodney & Paynter, 2006). In

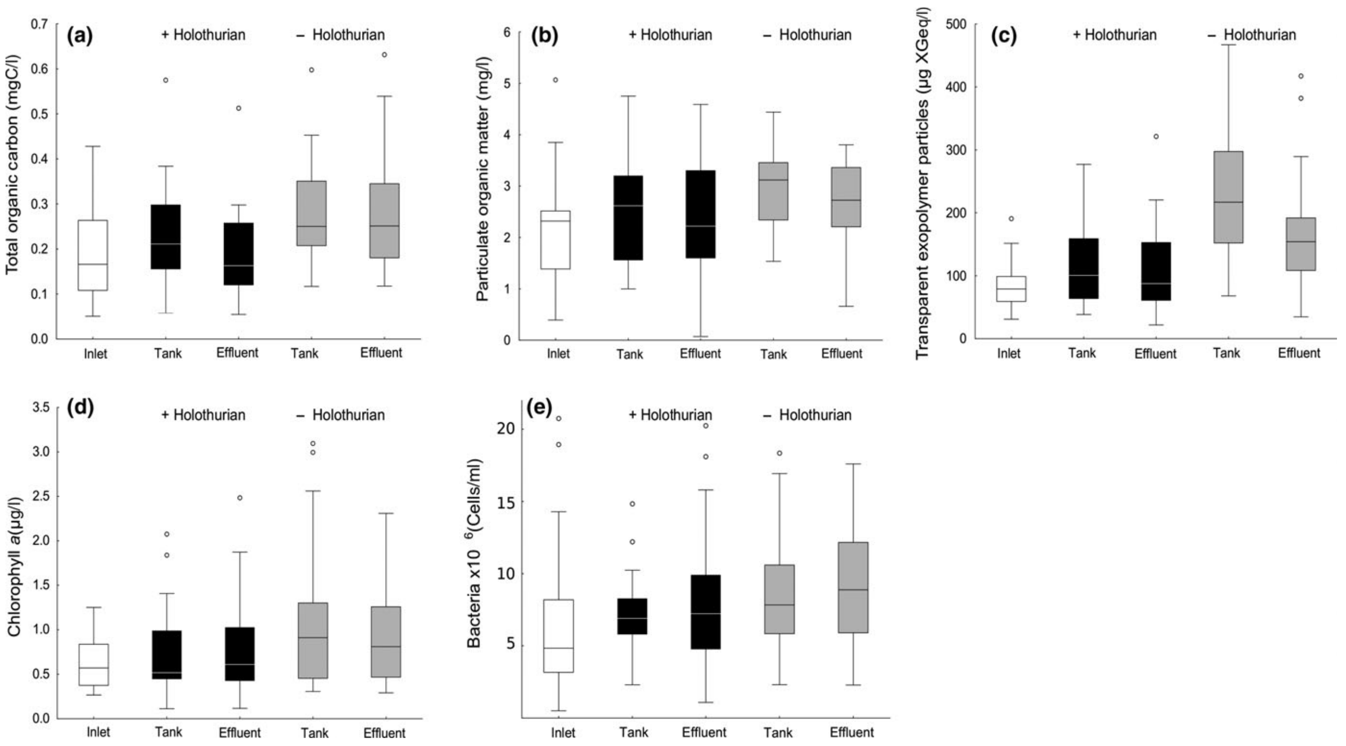

FIGURE 4 Median (line), the 25-75% percentile (box), the non-outliers range (whisker), and outliers (dots) of the concentration of (a) total organic carbon, (b) particle organic matter, (c) transparent exopolymer particles, (d) chlorophyll-*a* (a), and (e) bacterial abundance in the inlet waters (white box), in the *+holothurian* tank and effluent (black boxes), and in the *–holothurian* tank and effluent (grey boxes)

TABLE 2 Results of the analysis of variance (ANOVA) in the three experiments performed to compare the nitrate, bacterial abundance, transparent exopolymer particles, in the treatments with holothurians (+H) versus the treatments without holothurians (–H) at the initial and the final times

	Experiment # 1		Experiment # 2		Experiment # 3	
	F	*p-value*	*F*	*p-value*	*F*	*p-value*
Initial time						
Nitrate	2.71	0.116	3	0.125	384.2	0.0831
Bacterial abundance	3.04	0.097	2.958	0.101	0.550	0.467
TEP	17.84	0.118	8.4	0.134	0.45	0.516
Final time						
Nitrate	605.93	<0.001	354.4	<0.001	174.81	<0.001
Bacterial abundance	7.06	0.015	89.366	<0.001	83.221	<0.001
TEP	1,162.64	<0.001	5,158.59	0.00	6,916.2	0.00

the short-term experiments, to determine the statistical significance of the presence of holothurians we performed analysis of variance (ANOVA) to compare the tanks with holothurians (+H treatment) with the tanks without holothurians (–H treatment) using Statistica software (V8).

3 | RESULTS

3.1 | Time-series in the big-volume tanks

During the study period, in the inlet waters, we found pH values that ranged from 7.71 to 8.31, temperature values from 13.58 to 25.58 °C, salinity from 35.8 to 41.6 psu, conductivity between 52.28 and 61.96 mS cm^{-1}and total dissolved solids from 18.26 to 30.84 ppt.

Ammonium concentration in the inlet waters ranged from 0.006 to 0.038 µmol-N L^{-1} with values usually below 0.025 µmol-N L^{-1} (Figure 1a, white circles). However, in the effluent of the *–holothurian* tank we detected punctual higher values during fall 2013 and winter 2014 reaching concentrations up to 0.162 µmol-N L^{-1} (Figure 1a, grey triangles). The ammonium concentration in the effluent of the *+holothurian* tank (Figure 1a, black squares) was consistently lower than in effluent of the *–holothurian* tank (Figure 1a, grey triangles). No relevant changes in the nitrite concentration between the

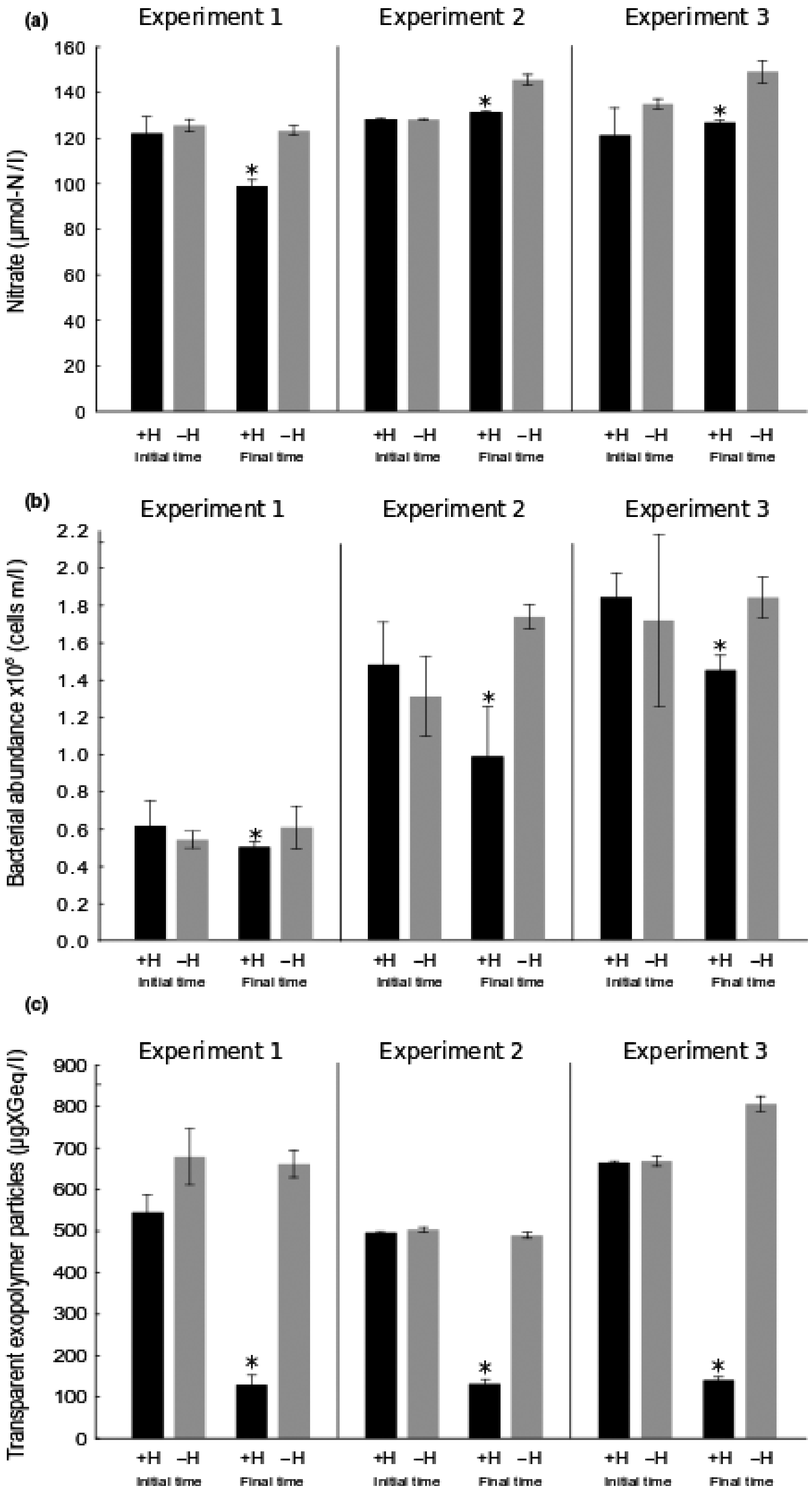

FIGURE 5 Changes in the concentration of nitrates, bacterial abundance and transparent exopolymer particles in the short-term experiments. Mean (bars) and the standard deviations (whiskers) of the replicates of (a) nitrates, (b) bacterial abundance and (c) transparent exopolymer particles in the treatments with holothurians (+H) and without holothurians (–H) at the initial and final times. Black bars represent the treatment with holothurians and grey bars represent the treatment without holothurians. Asterisks show the statistically significant differences at the final time of the experiments (more details in Table 2)

inlet waters (white circles) and the effluents of both tanks (grey triangles and black squares) were observed (Figure 1b). The nitrate concentration was usually lower in the effluent of +*holothurian* tank (Figure 1c, black squares) than in the effluent of –*holothurian* tank (Figure 1c, grey triangles) and in the inlet waters (Figure 1c withe circles). The nitrate concentration in the inlet waters ranged from 3.45 to 26.91 µmol-N L^{-1}. The maximum value of nitrate in the inlet waters was observed in the spring of 2014. Total phosphorus (TP) ranged from 0.039 to 1.157 μmol-P L^{-1} in the inlet waters. In fall and spring, total phosphorus (TP) was higher in the inlet waters (Figure 1d, white circles) than in the effluents of both tanks (Figure 1d, black squares and grey triangles).

Total organic carbon (TOC) ranged from 0.05 to 0.43 mg C L^{-1} in the inlet waters (Figure 2a, white circles) similar to the values measured in the effluent of *+holothurian* tank from 0.05 to 0.51 mg C L^{-1} (Figure 2a, black squares). However, the values in the effluent of *-holothurian* tank were higher ranging from 0.12 to 0.63 mg C L^{-1} (Figure 2a, grey triangles). The maximum values were reached in all cases during spring of 2014, particularly in the *−holothurian* effluent (Figure 2a). In general, the concentration of particulate organic matter (POM) in the effluent of the *−holothurian* tank (Figure 2b, grey triangles) was higher than in the inlet waters (Figure 2b, white circles) and in the effluent of *+holothurian* tank (Figure 2b, black squares). The concentration of transparent exopolymer particles (TEP) ranged from 32 to 270 μg XG eq. L^{-1} in the inlet waters (Figure 2c, white circles) and from 22 to 321 μg XG eq. L^{-1} in the effluent of *+holothurian* tank (Figure 2c, black squares) and from 35 to 522 μg XG eq. L^{-1} in the effluent of *-holothurian* tank (Figure 2c, grey triangles). Chlorophyll-*a* ranged one order of magnitude in the inlet waters from 0.27 to 2.62 μg/l (Figure 2d, white circles) and from 0.20 to 2.48 μg/l in the effluent of *+holothurian* tank (Figure 2d, black squares) and from 0.29 to 2.31 μg/L in the effluent of *−holothurian* tank (Figure 2d, grey triangles). Bacteria abundance ranged from 0.5 to 20.7 × 10^6 cell ml^{-1} in the inlet waters, (Figure 2e, white circles) and from 1.1 to 20.2 × 10^6 cell ml^{-1} in the effluent of +holothurian tank (Figure 2e, black squares) and from 2.3 to 17.6 × 10^6 cell ml^{-1} in the effluent of *-holothurian* tank (Figure 2e, grey triangles).

To determine if the holothurians produce significant changes in the water quality of the tank and its effluent we compared the tanks using paired *t* tests or Wilcoxon matched-pairs tests over time (Table 1). We did not obtain significant differences between the inlet (white box) and the *+holothurian* tank waters and its corresponding effluent (black boxes) neither for ammonium (Figure 3a), nitrite (Figure 3b), nitrate (Figure 3c) nor TP (Figure 3d) (Table 1). By contrast, we obtained significant differences between the inlet waters and *−holothurian* effluent waters (grey boxes) for ammonium (Figure 3a) and nitrate (Figure 3c) (Table 1). It is particularly remarkable the increase of ammonium in the *−holothurian* tank and in its effluent in comparison with the inlet waters and the *+holothurian* tank and its effluent (Figure 3a, grey boxes).

We did not find statistically significant differences between the inlet waters and the *+holothurian* effluent waters for TOC, POM, TEP, chlorophyll-*a* and bacterial abundance (Table 1). By contrast, the *-holothurian* effluent waters showed significantly higher concentrations than the inlet waters of TOC (Figure 4a, grey boxes), POM (Figure 4b, grey boxes) and TEP (Figure 4c, grey boxes) (Table 1). It is particularly remarkable the higher values of TEP in the *−holothurian* tank and in its effluent waters (Figure 4c, grey boxes) than the values in the inlet waters (Figure 4c, white box) and in the *+holothurian* tank and its effluent waters (Figure 4c, black boxes). We also found significantly higher abundance of bacteria in the *−holothurian* effluent waters (Figure 4e, grey boxes) than in the inlet waters (Figure 4e, white box) and in the*+holothurian* tank and its effluent waters (Figure 4e, black boxes) (Table 1).

3.2 | Short-term experiments

In the experiments, at the initial time, we did not observe significant differences in the concentration of nitrate, TEP nor bacterial abundance between both treatments (+H and −H) indicating that the experiments started with identical conditions (Table 2, Figure 5 initial times). By contrast, after three days of the introduction of holothurians (i.e. at the final times) we found a statistically significant reduction in the concentration of nitrates (Figure 5a), bacterial abundance (Figure 5b) and transparent exopolymer particles (Figure 5c) in treatment with holothurians (+H) in comparison with the treatment without holothurians (−H) (Table 2). This reduction was particularly elevated for the case of TEP concentration (Figure 5c).

4 | DISCUSSION

Sea cucumbers are considered highly marketable species both as food and a pharmaceutical resource (Farouk, 2007; Gowda et al., 2008; León-Palmero et al., 2018; Nelson et al., 2012a; Nelson, MacDonald, & Robinson, 2012b; Silchenko et al., 2005). Since holothurians are detritus-feeders, they are also used as an extractive species in integrated multitrophic aquaculture (Yokoyama, 2013; Zamora, Yuan, Carton, & Slater, 2016). In fact, here we showed that the introduction of holothurians in tanks with sea anemones reduced significantly the concentration of dissolved nitrogen, transparent exopolymer particles and bacterial abundance. Therefore, this environmental potential of holothurians, besides their marketable value, should be also considered for the development of sustainable aquaculture installations.

The reduction in the concentration of ammonium and nitrates by holothurians was likely indirect through the interaction bacteria-detritus. We did not observe significant changes of dissolved nitrogen in the tank with holothurians (Table 1, Figure 3a,b black bars), whereas the concentration of ammonium and nitrate was significantly higher in the tank without holothurians in comparison with the tank with holothurians consistently over time (Table 1, Figure 3a, b grey bars). Considering that the concentration of TOC, POM, TEP and the abundance of bacteria were significantly higher (Table 1) in the *−holothurian* tank (Figure 4 grey bars) than in the *+holothurian* tank (Figure 4 black bars), we can assume that bacterial mineralization of detritus and organic matter was likely higher in the *−holothurian* tank than in the *+holothurian* tank. This additional microbial mineralization of detritus in the *–holothurian* tank might eventually have increased the concentration of mineral nutrients such as ammonium and nitrate in the tank. In the short-term experiments, we also observed at the final times statistically significant higher values of the concentration of nitrates in the −H treatment (Figure 5a grey bars) than in the +H treatment (Figure 5a black bars)

(Table 2) suggesting an increase in the mineralization process in the −H treatment during the experimental time (3 days) that was similar to the water renovation time in the big tanks. Another alternative (non-exclusive) explanation for nitrogen reduction is the direct assimilation of dissolved nitrogen by symbiotic bacteria living in the sea cucumbers tissues. Recently, Brothers, Lee, and Nestler (2015) demonstrated, using stable isotopes, the uptake of free amino acids in several tissues of the sea cucumber *Parastichopus californicus*. The assimilation of amino acids and other nutrients have been associated to the presence of subcuticular symbiotic bacteria in sea cucumber species such as *Stichopus mollis* (Lawrence, O'Toole, Taylor, & Davy, 2010) and deep-sea holothurians (Robert, Billett, McCartney, & Hayes, 1991). More detailed studies on the role of these symbiotic bacteria on dissolved nutrients assimilation by holothurian are needed to determine its relevance. Therefore, detritus consumption is not the unique way to obtain energy in the sea cucumbers. They can also absorb dissolved organic matter (Brothers et al., 2015; Sadeghi-Nassaj, Catalá, Álvarez, & Reche, 2018), ingest mucus aggregates and microorganisms (Navarro, García-Sanz, Barrio, & Tuya, 2013; Tamura & Tsuchia, 2011).

Several authors (e.g. Amon & Herndl, 1991; Coulon & Jangoux, 1993) have reported that *H. tubulosa* is a selective deposit feeder containing higher concentrations of particulate organic matter, total particulate carbohydrates and bacteria biomass in its foregut in comparison with their concentrations in the surrounding sediment. Most holothurians have a digestive tract specialized for the assimilation of organic matter from sediments (Roberts, Gebruk, Levin, & Manship, 2000). Indeed, they consume sediments particularly enriched in organic matter selecting bacteria, pigments, chlorophyll, and fungi (Navarro et al., 2013; Paltzat, Pearce, Barnes, & McKinley, 2008; Yokoyama, 2013, 2015), although there are also species with a lesser selective capacity (Slater, Jeffs, & Sewell, 2011). The non-selective consumption usually appears in species living in benthic zones on surface sediments with low nutritional value (Slater et al., 2011; Zamora & Jeffs, 2011). In our study, we observed a significant decrease in the concentration of TOC, POM and TEP in the tank that included holothurians in comparison with the tank without holothurians over time (Table 1). These results suggest a net consumption of most particulate organic compounds by the study holothurians. This consumption was particularly remarkable for the case of transparent exopolymer particles (Table 1, Figure 4c).

Transparent exopolymer particles (TEP) have strong adhesive and polymerization forces being biofilm precursors and key substrates for microbial colonization (Bar-Zeev et al., 2012). Both phytoplankton and bacteria release TEP precursors and form aggregates (Iuculano, Mazuecos, Reche, & Agustí, 2017; Ortega-Retuerta, Duarte, & Reche, 2010; Passow, 2002b). Recently, Joyce and Utting (2015) have emphasized the complex role of TEP in hatcheries. On the one hand, TEP can be considered as food for invertebrates and fish larvae and, in the other, they can be colonized by commensal bacteria and sequester micronutrients and toxins, which can be essential in the hygiene of hatcheries. In our study, we observed a very relevant reduction in the TEP concentration in the tanks containing holothurians both in the time-series and in the short-term experiments (Tables 1 and 2, Figure 4c and Figure 5c), suggesting that TEP were ingested as a food source by the study holothurians. In fact, Wotton (2005, 2011) observed that the reduction of TEP in the water column is mostly due to marine invertebrate. Since bacteria are part of the biofilms and TEP aggregates (Bar-Zeev et al., 2012), TEP ingestion by holothurian could have also implications for the formation and keeping of their gut microbiome (Joyce & Utting, 2015). Hence, holothurians could have an excellent role regulating biofilms in aquaculture tanks.

The importance of chlorophyll-*a*, as a surrogate of rich organic food, has been highlighted in previous studies (Hudson, Wigham, Solan, & Rosenberg, 2005; Uthicke, 2001; Uthicke & Karez, 1999). However, we did not find significant differences in the concentration of chlorophyll-*a* between the tank containing holothurians and the tank without holothurians over time (Table 1, Figure 4d). Bacteria are also considered a food source for holothurians (Amon & Herndl, 1991; Moriarty, 1982; Moriarty, Pollard, Hunt, Moriarty, & Wassenberg, 1985). Unlike chlorophyll-*a*, we found significant differences in the abundance of bacteria between the *−holothurian* effluent and the inlet waters (Table 1, Figure 4e). The bacterial abundance in the *+holothurian* tank was lower than in the *−holothurian* tank suggesting a net consumption of bacteria by holothurians. In fact, in the short-term experiments, we found statistically significant lower values in the abundance of bacteria in the +H treatment with respect the −H treatment (Table 2, Figure 5b). Therefore, holothurians appear to control bacterial populations in term of days. This bacterial consumption by holothurians could have also beneficial consequences to avoid pathogen bacterial outbreaks such as, for instance, *Vibrio* sp. that is a serious problem in aquaculture (Roux et al., 2015).

5 | CONCLUSIONS

Sea cucumbers in aquaculture tanks reduce the load of nitrogen, total organic carbon, particulate organic matter, transparent exopolymer particles and bacteria in tanks and effluents of aquaculture. Therefore, they are a relevant extractive species both for mineral nutrients and particulate organic components. Nitrate reduction in aquaculture effluents can be beneficial to reduce eutrophication problems associated with aquaculture installations. It is also particularly remarkable the TEP and bacteria consumption by the study holothurians. This consumption can be relevant in the maintenance of the tank hygiene and in the control of pathogenic bacterial outbreaks. This extractive facet of the holothurians confers them a high environmental value in multitrophic or polyculture aquaculture beyond their economical and pharmaceutical intrinsic value.

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