Microplastics Ingestion in the Ephyra Stage of *Aurelia* sp. Triggers Acute and Behavioral Responses

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Abstract

For the first time, we report a ***correspondence*** between microplastics (MP) ingestion and ecotoxicological effects in gelatinous zooplankton (Cnidarian jellyfish). The ephyra stage of the jellyfish *Aurelia* sp. was exposed to both environmental and high concentrations of fluorescent 1-4 µm polyethylene MP (0.01-10 mg/L). After 24 and 48 hours, MP accumulation, acute (Immobility) and behavioral (Frequency pulsation) endpoints were investigated. MP were detected by confocal and tomographic investigations on gelatinous body and mouth, either attached on the surface or ingested. This interaction was responsible for impairing ephyrae survival and behavior at all tested concentrations ***after 24 h***. Acute and behavioral effects were also related to mechanical disturbance, caused by MP, triggering a loss of radial symmetry. Contaminated ephyrae exposed to clean seawater showed full recovery after 72 h ***highlighting the organisms without the microspheres, attached on body jellyfish surface around the mouth and lappets***. In conclusion, short-term exposure to MP affects ephyrae jellyfish health, impairing both their survival and behavior. Polyethylene MP temporarily affect both Immobility and Frequency of pulsation of *Aurelia* sp. jellyfish. This study provides a first step towards understanding and clarifying the potential impacts of MP contamination in gelatinous zooplankton.

Key-words: gelatinous zooplankton, ecotoxicology, microplastic, 3D-holotomographic microscope, polyethylene, toxicity

1. Introduction

Plastic items account for around 75% of marine litter recorded on shorelines worldwide in terms of numerical abundance (Williamson et al. 2016). Plastic is the result of scientific progress and economic development, which ***has*** undoubtedly significantly improved man's quality of life. Globally, around 300 million tons of plastics are produced each year and about 4.8-12.7 million tons accumulate in the marine environment annually (Jambeck et al. 2015). The combination of thermo-oxidative breakage of polymeric chains, UV degradation and leaching of plasticizing additives make plastics susceptible to mechanical abrasion (Andrady, 2011; Beiras et al. 2018), promoting their fragmentation into small plastic particles (0.1 µm-5mm), known as microplastics (MP, Gewert et al. 2015). MP have been found in the sea surface, in the water column, and on the seabed (including deep sea), as well as in marine biota (Barnes et al. 2009; Batel et al. 2016; Thompson et al. 2004). There are concerns that MP ingestion by marine organisms could lead to toxicological harm, either as a consequence of the transfer of persistent contaminants from sea water (Rochman et al. 2013; Teuten et al. 2009), or of the release of chemicals incorporated during manufacture, such as plasticizers, flame retardants, and anti-microbials (Browne, 2013). Consequently, their presence is considered as an emerging threat for the marine ecosystem, more than larger plastic items (GESAMP, 2015). Indeed, as MP occupy the same size range as sand grains and planktonic organisms (Fendall and Sewell, 2009), they happen to be available to a wide range of marine organisms. Thus, MP ingestion has been shown in several taxa, including marine zooplankton (Bergami et al. 2016; Cole et al. 2013, 2015; Gambardella et al. 2017; Lee et al. 2013). Zooplankton are an important food source for many secondary consumers, thus playing a crucial role in nutrient cycling. They mainly feed in surface waters, where MP abundance is high, with increasing chances of encounter and ingestion (Cozar et al. 2014). Once ingested, MP can affect zooplankton feeding capacity, energy reserves, reproduction, and growth, as well as trigger detrimental alterations to their intestinal function (Nelms et al. 2018). This evidence has been found in several zooplankton species (crustaceans, rotifers, mussels, and sea urchin larvae; Beiras et al. 2018; Cole et al. 2013; Della Torre et al. 2014; Desforges et al. 2015; Gambardella et al. 2017; Jeong et al. 2016); however, little research is available to date on gelatinous ***organisms*** (Sun et al. 2017). Gelatinous zooplankton include approximately 2,000 species (Daly et al. 2007) widely heralded as a key member of ocean ecosystems, playing a central role in the trophic organization of marine food webs (Boero et al. 2008; Epstein et al. 2016; Richardson et al. 2009). Different animal phyla include taxa of gelatinous zooplankters, such as Cnidaria (e.g. cnidarian jellyfish, hydromedusae, hydroids, siphonophores), Ctenophora (e.g. ctenophores, comb jellies), Chordata (e.g. pelagic tunicates such as salps, doliolids, and pyrosomes), ***and*** Mollusca (e.g. pteropods, heteropods). Among cnidarian jellyfish, *Aurelia* sp. (misidentified as *Aurelia aurita;* recently revised by Scorrano et al. 2016) is a promising model organism for ecotoxicology to predict the effects of chemicals and other stressors in the marine environment (Almeda et al. 2013; Costa et al. 2015; Faimali et al. 2014, 2017; Gadreaud et al. 2016; Gambardella et al. 2015). Although jellyfish blooms have been observed in regions of plastic accumulation (Ziveri et al. 2013) and MP ingestion has been recently documented in natural samples of jellyfish (adults) (Macali et al. 2018; Sun et al. 2017), no ***correspondence*** between MP exposure/ingestion and ecotoxicological effects has ***yet*** been reported. The overall objective of this study was to ***test*** this assumption in the ephyra stage of the jellyfish *Aurelia* sp. exposed to fluorescently labelled polyethylene MP. Firstly, the ingestion of polyethylene MP in the ephyra stage was investigated by means of epi-fluorescent, confocal and three-dimensional (3-D) optical microscopes. Secondly, acute and behavioral endpoints (Immobility, Frequency pulsation) were investigated in ephyrae exposed to environmentally relevant ***(Koelmans et al. 2015)*** and high concentrations of MP. Thirdly, it was assessed whether ecotoxicological responses were temporary or permanent.

2. Materials and methods

2.1. Recruitment of ephyrae

Colonies of polyps attached on PVC tubes were obtained from the laboratories of the "Acquario di Genova, Costa Edutainment S.p.A.", and transported to CNR-IAS. They were placed in a thermostatic room at 20 °C in 1.5 L dark plastic tanks, covered with a lid in order to keep polyps in dark conditions. Tanks were filled with filtered natural seawater (FNSW, 37‰ salinity) and gently aerated. Polyps were fed daily with nauplii of *Artemia salina* (about 40 nauplii/mL); seawater was changed every two days. ***Strobilation was induced by thermic shock and food starvation: PVC tubes with polyps were moved to 10 °C into 1.5 L dark plastic tanks filled with FNSW , the polyps were not fed and sweater was not change for one month.*** Once released by strobilation, ephyrae (0 days old) were immediately collected, poured into a beaker and used for the toxicity tests. Ephyrae were exposed to environmental (0.01-0.1 mg/L) and high concentrations (1-10 mg/L) of MP in both static and semi-dynamic conditions, according to literature data (Batel et al. 2016). Semi-dynamic conditions were selected to simulate ***more closely*** environmental conditions found in marine ***ecosystems***.

2.2 Microplastics

1-4 µm ***diameter*** polyethylene (PE) MP ***spheres*** (CPMS-0.96, 0.99 g/cm^3 density) were purchased from Cospheric (CA, USA) and Sigma-Aldrich (Germany). In addition, 1-5 µm ***diameter*** fluorescent green polyethylene MP (1.3 g/cm^3 density, 414 nm excitation/515 nm emission) purchased from Cospheric were used for documenting particle ingestion in jellyfish ephyrae. Stock solutions of MP (10 mg/L) were prepared in 0.22 µm of FNSW. ***Ephyrae were exposed to environmental (0.01-0.1 mg/L, corresponding to 1,8x10^3 and 1,8x10^4 particles/ml) and high concentrations (1-10 mg/L corresponding to 1,8 x10^5 and 1,8 x 10^6).***

2.3. MP Ingestion

To detect MP ingestion, tests were performed by exposing ephyrae to fluorescent green MP in semi-dynamic conditions, according to Batel et al. (2016). In detail, 10 ephyrae that had been collected immediately after strobilation were incubated in a glass beaker filled with 100 mL of FNSW with serial MP dilutions (0-0.01-0.1-1-10 mg/L). Aeration was supplied during exposure, to keep the ephyrae up and off the bottom of the beaker without bouncing them off the wall (Widmer, 2008), as well as to have a constant mixing of MP solutions (Batel et al. 2016). Three replicates were prepared for each dilution. ***A total of 150 ephyrae were used for the test.*** Beakers were then sealed and kept in a thermostatic room at 20 °C in dark conditions for 24 and 48 hours. After exposure, the ephyrae were removed and washed three times with fresh FNSW to remove any MP bound to the exoskeleton (Nasser and Lynch, 2015). ***The*** organisms were ***then*** anesthetized with menthol crystals according to Williams and Van Syoc, (2007), fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS, pH 7.4), and mounted in glycerol-PBS (1:1) to be observed in by epi-fluorescence microscope (Olympus) and Confocal Laser Scanner Microscope (CLSM Leica SP2). Fluorescent MP were illuminated using ***an*** ArKr laser in the blue channel (wavelength 458 nm). The corresponding transmitted and green fluorescence channels were simultaneously acquired using integrated photomultipliers. To further address MP internalization by ephyrae, samples were also observed using a 3D holotomographic microscope (Tomocube Inc. model HT-2). With this recent technology implementing optical diffraction tomography, the fluorescence signal and a three-dimensional map of the sample refractive index can be acquired at the same time (Soto et al. 2017). A 3D holotomography map is thus rendered showing the different structures (different refractive index ranges) with different colours, together with the fluorescence signal associated to MP.

2.4 Toxicity tests

Toxicity tests were performed in both static and semi-dynamic conditions (Table 1), according to previous studies (Batel et al. 2016; Beiras et al. 2018). For static exposure, ephyrae collected immediately after strobilation were individually placed into a multiwell plate containing 2 ml of MP (0-0.01-0.1-1-10 mg/L) dilutions (one individual for each well). For each dilution, 3 multi-well replicate plates were prepared, each containing 8 ephyrae individually placed in each well to avoid interactions among organisms (Faimali et al. 2014).

Bioassays in semi-dynamic conditions were performed as described in paragraph 2.3, exposing ephyrae to MP at the same dilutions reported for static exposure. ***A total of 300 ephyrae (150 for both static and semi-dynamic exposition) were used for toxicity tests.***

In addition, bioassays were also performed against a reference toxicant – Cadmium nitrate – according to Faimali et al. (2014).

After 24 and 48 hours, acute and sub-lethal endpoints were assessed in ephyrae jellyfish (Costa et al. 2015). The acute endpoint – namely % of Immobility – measures an ***organism's*** ability to perform any kind of movement. In detail, completely motionless ephyrae were counted as immobile organisms, and the percentage of Immobility (% I) was calculated for each dilution compared to controls. The term 'motionless' means organisms that do not change their own barycentre position and fail to move any appendages in 5 seconds, as described in Garaventa et al. (2010). The sub-lethal endpoint, namely Alteration of Frequency pulsation (AFp %) of the ephyrae was calculated recording the number of pulsations (Frequency pulsation, Fp) made by each ephyra in 1 minute. For each dilution and controls, the average Fp was calculated, and the AFp % was derived for each dilution against controls according to the following formulae:

$$\%AFp= [(Fp\ treated-Fp\ control)/Fp\ control]/100;$$

Both endpoints were assessed using an automatic recording system coupled with a specifically designed video graphics analyzer (Swimming Behavioral Recorder, SBR; Faimali et al. 2014). The SBR developed at CNR-IAS is a video camera-based system, coupled with image analysis software, specifically designed to track and analyze linear swimming behavior of aquatic invertebrates (Faimali et al. 2006; Garaventa et al. 2010, Morgana et al. 2016). For this purpose, in the semi-dynamic exposure, each ephyra was transferred into a single Petri dish after 24 and 48 h and analyzed under the SBR system in order to evaluate their Immobility and Frequency pulsation percentage values.

Table 1 Test parameters used in static and semi-dynamic bioassays with ephyrae jellyfish

	Conditions	
Parameters	static	semi-dynamic
Container	multi-well plates	beaker
Aeration	no	yes, constant
Density of organisms	1 ephyra/well	10 ephyrae/beaker
Temperature (°C)	20	20
Photoperiod	No, full dark	No, full dark
Exposure (hours)	24-48	24-48

2.5. Recovery test

Recovery test was performed exposing the new ephyrae jellyfish (not used for the toxicity test) to fluorescent green MPs in semi-dynamic condition according to toxicity tests (Table 1). In detail, 10 ephyrae, collected immediately after strobilation, were incubated in a glass beaker filled with 100 mL of FNSW with serial MP concentrations (0-0.01-0.1-1-10 mg/L) with aeration for 48 hours. Three replicates were prepared for each dilution and control. A total of 150 ephyrae were used for this test. After this time, for each concentration of MP tested, the organisms were washed three times with fresh FNSW to remove MP bound to the gelatinous body ***and*** then ***were*** placed in new containers filled with clean FNSW under the same experimental conditions ***following the semi-dynamic exposure (Table 1). At different exposure times for each concentrations and the control, the pulsation made by each ephyrae were measured by SBR as described in paragraph 2.4. Then the organisms exposed to MPs were exposed in new FNSW. The recovery test was stopped when ephyrae were considered completely recovered*** as soon as they are again able to perform an optimal number of pulsations – i.e. not different from controls – in clean FNSW, after exposure to different MP concentrations. ***For the control were considered the pulsations made by each ephyrae exposed in FNSW during this test. In addition, the presence of MPs***

attached on the body ephyrae ephyrae was evaluated by epi-fluorescence microscope (Olympus) as described in section 2.3.

2.6. Statistical analysis

The median Effective Concentrations (EC_{50}: concentration of a compound resulting in 50% Immobility, I% or Alteration of Frequency pulsation, % AFp) effect in the exposed ephyrae and related 95% Confidence Limits (CL) were calculated using Trimmed Spearman–Karber analysis (Finney, 1978) after 24 and 48 h of MP exposure.

Significant differences between controls and treated samples were identified using one-way analysis of variance (ANOVA) followed by Tukey test. When data failed to meet the assumption of normality, non-parametric Kruskal Wallis test and Mann Whitney test were used to compare individual treatments. For AFp, statistical analysis was performed using Frequency pulsation data. ***For the recovery test the statistical analysis was performed comparing the Frequency pulsation between ephyrae collected immediately after the toxicity test to different MP concentrations (namely "0" in the x-axis; Fig. 4) and the same organisms placed in clean FNSW for a different time of recovery.***

The Lowest Observed Effect Concentration for both endpoint, ($LOEC_I$ and $LOEC_{AFp}$) were deducted by ANOVA results. Data were considered significantly different when $p < 0.05$. SPSS statistical software (Statistical Package for the Social Sciences, Version 20) was used for data analysis.

3. Results

3.1 Ingestion

Microscopy observations showed that ephyrae ingested polyethylene MP at all tested concentrations (Figure 1). Fluorescent green microspheres were found attached onto the body surfaces of ephyrae, as well as around the mouth and lappets after 48 hours (Figure 1 A), as confirmed by confocal (Figure 1 B-C) observations. Regarding lappets, MP were localized closed to the rhopalia (Figure 1 C), the motor nerve of Scyphozoa jellyfish. Moreover, MP internalization by the organism body was assessed using holotomography. ***Figure 2 shows a 3D reconstruction of ephyrae in which the extracellular matrix is colored in yellow, nematocysts are depicted in purple, and MP fluorescence is reported in green. From the 3D representation, PE MPs (with a refractive index of 1.5) are localized inside the ephyra jellyfish body (Figure 2), among the nematocysts (refractive index: 1.355-1.412).***

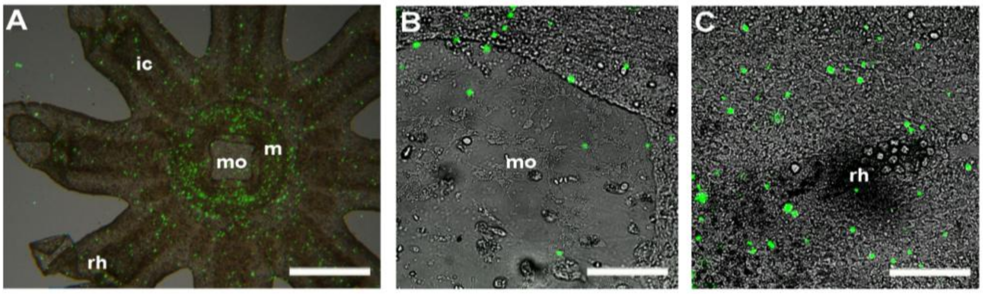
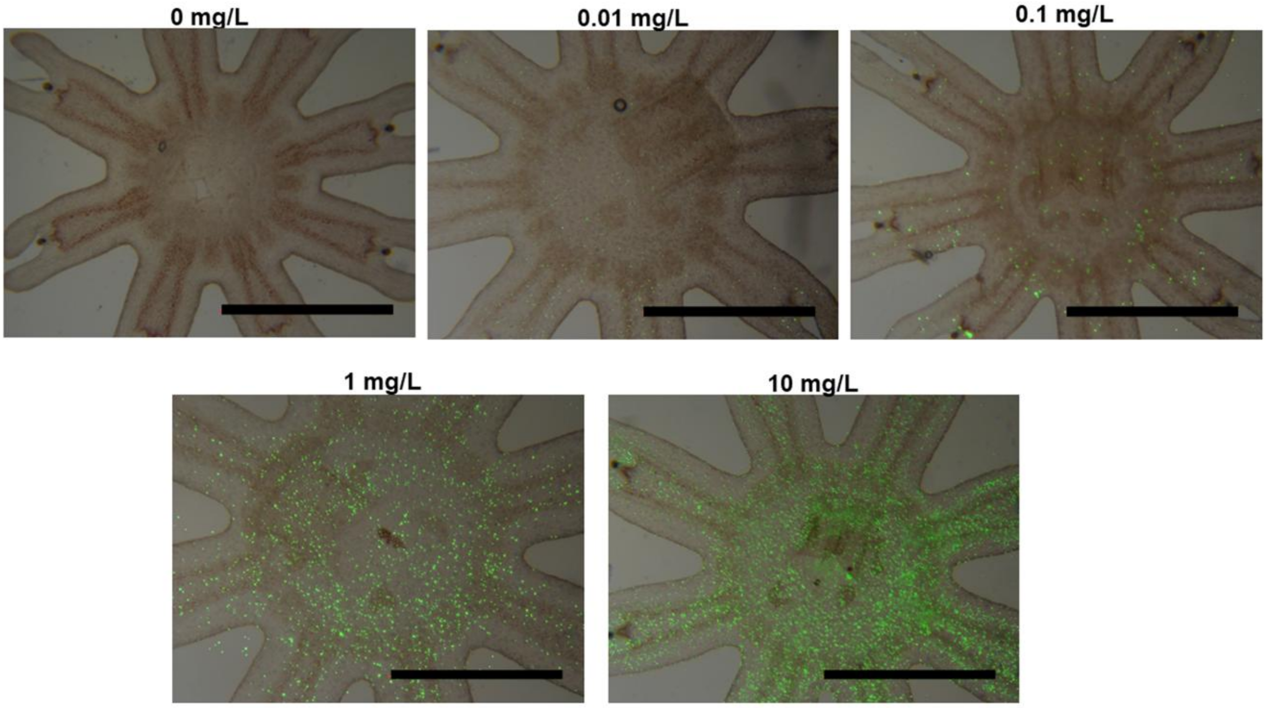


Figure 1. Ephyra of *Aurelia* sp. exposed in static conditions to 10 mg/L of 1-4 µm MP for 48 hours. The MP result to be (A), around the mouth (B) and on the arms, closed to the rhopalia (C). mo=mouth; m=manubrium; a=arm; ic=inter-radial canal; rh=rhopalia A: epifluorescence image, bar scale=1 mm; B-C: CLSM images showing the overlay of green fluorescence channel on the transmitted one; bar scale= 64 µm.



Suppl. Fig 1. Ephyra of Aurelia sp. exposed in static conditions to different concentration (0-0.01-0.1-1-10 mg/L) of 1-4 µm MP for 48 hours. The amount of fluorescent MPs attached on the ephyrae jellyfish body, increased in a dose dependent manner. Bar scale=1 mm

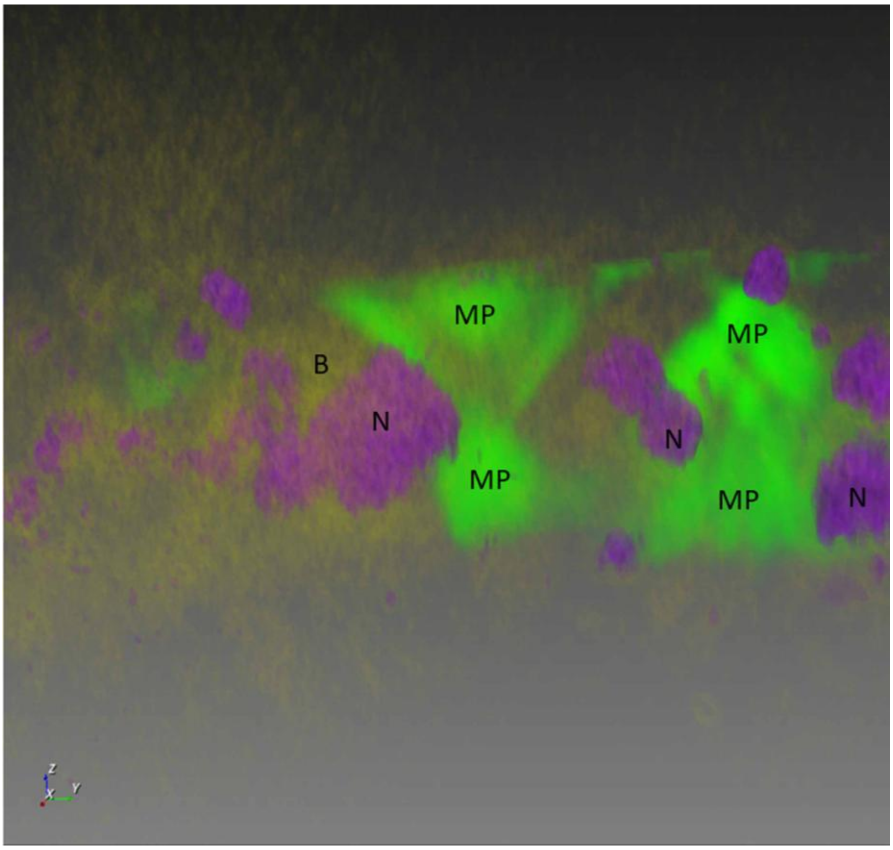


Figure 2. Epi-fluorescence of MP in *Aurelia* sp. ephyrae jellyfish acquired together with holotomogram. MP (green color representing the fluorescence channel, refractive index 1.5) are localized inside the gelatinous body (yellow regions indicate the body (refractive index range 1.355-1.378), around the nematocystes (refractive index range 1.398-1.412). B: body; N: nematocysts; MP: microplastics.

3.2. Toxicity test

Results of *Aurelia* sp. ephyrae exposed to different 1-4 µm MP following static and semi-dynamic exposure are reported in Figure 3. ***A relatively little significant difference in terms of effects (immobility, AFp) among treatments was observed, even though there was a thousand-fold increase from lowest to highest concentration of spheres. It can be noted a difference in sensitivity among the endpoints in terms of LOECI-AFp and EC_{50} (Table 2) after 24 hours. These results show that the behavioural endpoint (AFp) was more sensitive than the acute one (Immobility) (LOECAFp: 0.01 mg/L versus LOECI 0.1 mg/L) for all exposure conditions. Conversely, after 48 h, MP significantly affected ($p < 0.05$) both endpoints already at the lowest tested concentration, since LOECAFp was 0.01 mg/L for Immobility and AFp".*** In addition, a toxic effect could be observed only for Immobility in terms of EC_{50}s, in both exposure times and independently of exposure conditions (Table 2). Overall, the behavioral endpoint (AFp) was very sensitive, since a significant effect was observed already at the lowest tested concentration (0.01 mg/L).

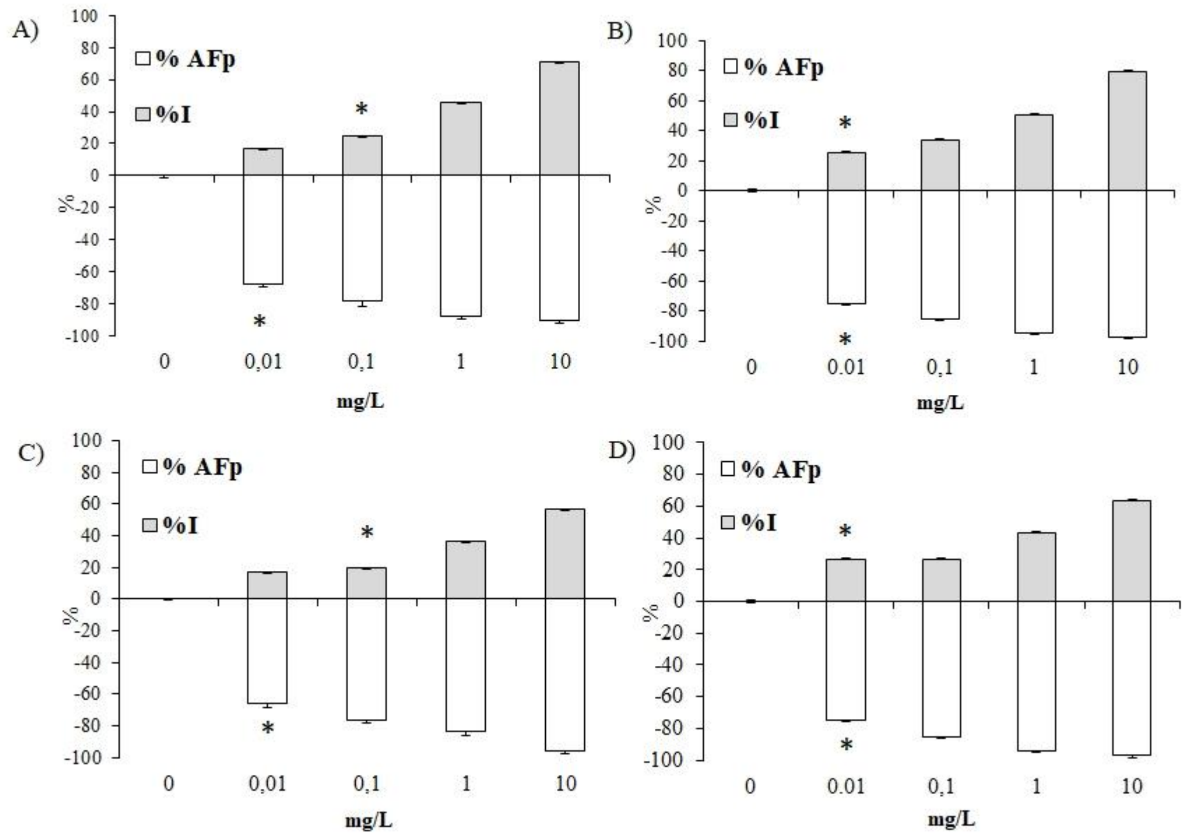


Figure 3. Immobility (% I) and Alteration of Frequency pulsation (% AFp) of *Aurelia* sp. ephyrae after 24 h and 48 h of static (A, B) and semi-dynamic (C, D) exposure at increasing 1-4 µm MP concentrations (M ±SE, n = 3). *= $p < 0.05$ (one-way ANOVA).

Table 2 EC_{50} values at 24- and 48-h with 95% confident limits derived from Immobility percentage (% I) and Alteration of Frequency pulsation(%AFp) of *Aurelia sp.* ephyrae exposed to 1-4 µm polyethylene microplastics (MP) and n.c. (not calculable) Cadmium nitrate.

Reference compound	**Exposure**	**Endpoint**	**24h-LOEC**	**24h- EC_{50} and confident limits (C.L.)**	**48h-LOEC**	**48h-EC_{50} and confident limits (C.L.)**
Polyethylene MP	Static	Immobility	0.1	1.36 mg/L (0.73-2.55)	0.01	0.53 mg/L (0.27-1.04)
		AFp	0.01	< 0.01 mg/L (n.c.)	0.01	< 0.01 mg/L (n.c.)
	Semi-dynamic	Immobility	0.1	4.16 mg/L (1.90-9.09)	0.01	3.16 mg/L (1.73- 5.79)
		AFp	0.01	< 0.01 mg/L (n.c.)	0.01	< 0.01 mg/L (n.c.)
$Cd(NO_3)_2$	Static	Immobility	0.5	0.40 mg/L (0.35-	0.1	0.23 mg/L (0.20-0.28)

			0.46)		
	AFp	0.1	0.13 mg/L (0.10-0.15)	0.05	0.06 mg/L (0.05-0.07)
Semi-dynamic	Immobility	5	>5 mg/L (n.c.)	1	2.99 mg/L (1.86-4.80)
	AFp	0.01	0.10 mg/L (0.070.13)	0.01	0.05 mg/L (0.04-0.07)

3.3. Recovery test

Ephyrae immediately observed after the toxicity test (0 recovery time, Figure 4) show a frequency of pulsation ranging from 1% to 16%. Conversely, (i.e. after 27-72 hours in clean FNSW) they showed a frequency of pulsation comparable to controls (dashed black line, Fig. 4) with the increase of the recovery time (Figure 4). Significant recovery compared to ephyrae immediately exposed for 48 hours at different MP concentrations (0 recovery time, Figure 4). was observed already after 30 minutes for exposure to environmental concentration MP (0.01 mg/L), while for higher concentrations the recovery time went up to 24 hours (*p<0.05). ***In addition, after recovery test a few MP particles were found attached on the body ephyrae jellyfish and around the mouth (Fig. 5)***

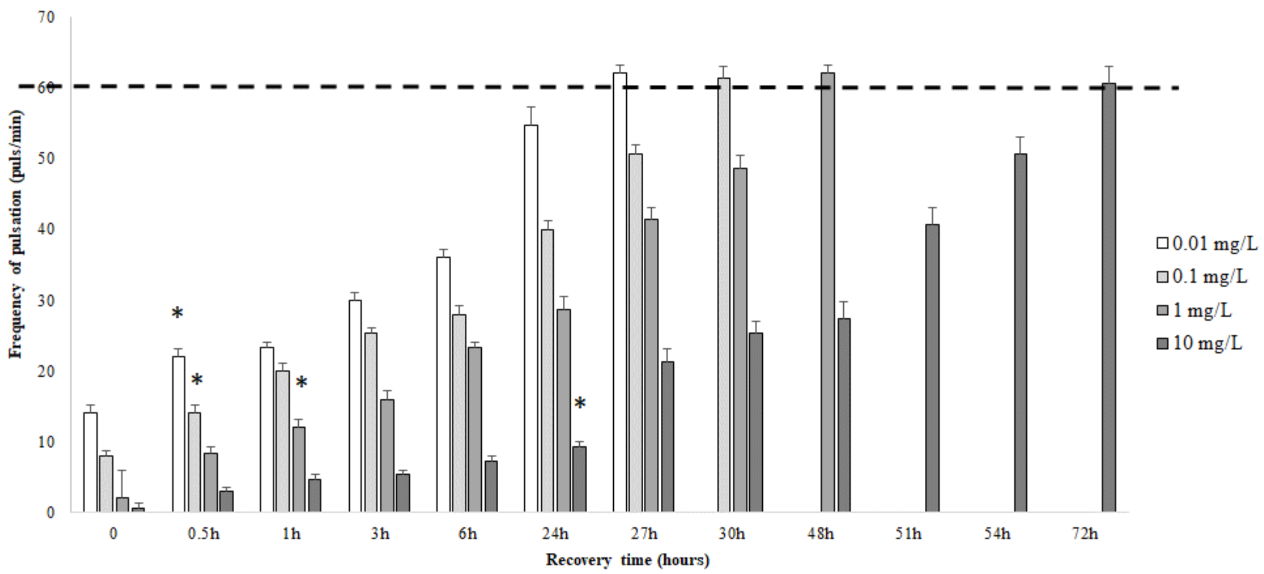


Figure 4. Recovery test with *Aurelia* sp. ephyrae in filtered natural sea water (FNSW) after 48h exposure to different MP dilutions (0-0.01-0.1-1-10 mg/L). Significant recovery was observed from 0.5 to 24 hours for ephyrae exposed to environmental (0.01 mg/L) and high (10 mg/L) MP concentrations (M ±SE, n = 3) ***compared to ephyrae immediately observed after the toxicity test (0).*** * p < 0.05 (one-way ANOVA). ***Dashed black*** line represents controls (Ctr), namely the Frequency pulsation made by each ephyra ***exposed in cleaned FNSW after recovery time.***

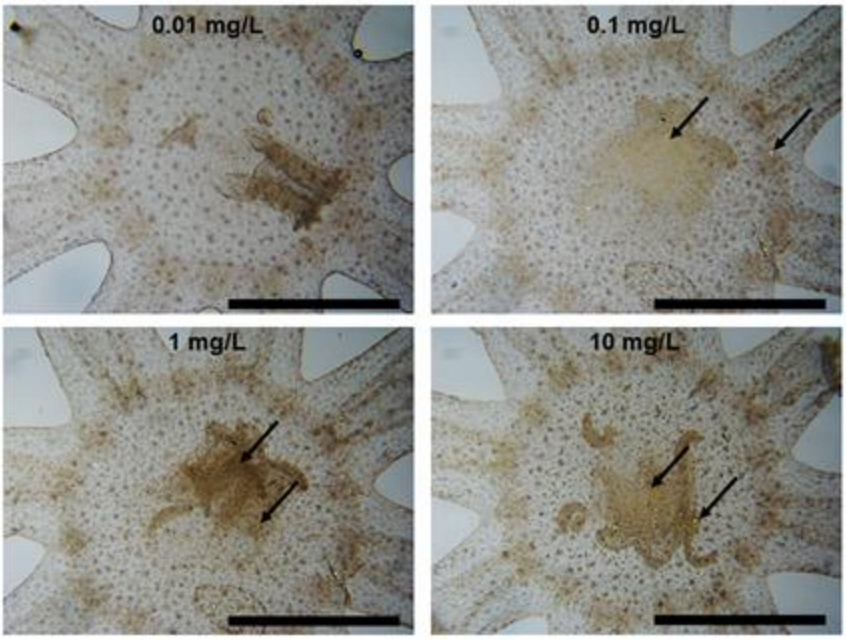


Figure 5. Ephyra of Aurelia sp. exposed in static conditions to different concentration (0.01-0.1-1-10 mg/L) of 1-4 µm MP for 48 hours and observed after recovery time. Only a few MP particles were attached on the jellyfish body and around the mouth (blach arrow). Bar scale=1 mm.

4. Discussion

In this study, the ***correspondence*** between MP ingestion and ecotoxicological effects has been reported for the first time in the juvenile stage of a gelatinous zooplankton species (Cnidarian jellyfish). Cnidarians are known to ingest MP, but to date any evidence has been reported only in corals (Hall et al. 2015; Taylor et al. 2016) while considering gelatinous species only in the adult jellyfish species *Pelagia noctiluca* (Macali et al. 2018; Sun et al. 2017). In our study, the uptake of MP in *Aurelia* sp. – the most common Scyphozoan jellyfish in world oceans and temperate coastal waters (Scorrano et al. 2016; Takao et al. 2014) – has been investigated through several microscopic approaches. With traditional and confocal microscopic techniques external particles can be identified that have attached to the jellyfish surface, probably due to the mucous that these organisms secrete under different conditions, including stress to clean their surface, to defend themselves from predators, and to trap particles (Hanaoka et al. 2001; Patwa et al. 2015). However, these microscopic approaches were not able to clarify whether MP were also inside the organisms and therefore whether any toxic effect was due to contact or even internalization/ingestion. To verify MP internalization/ingestion in *Aurelia* ephyrae jellyfish, an innovative interferometric technique (tomographic microscope) has been used, which measures the 3-D refractive index distribution of optical samples such as cnidarians and plastic particles (Soto et al. 2017). As to confocal microscopy, it is a technique used to reconstruct relevant structural features of the ephyrae body, like cnidocytes containing a structure called nematocysts, which are important to gather information about MP position inside the gelatinous body (Figure 2). Thus, 3-D imaging has the potential to investigate MP in gelatinous zooplankton, for the first time providing valuable information to reveal any MP ingestion in biological samples.

Jellyfish have only one external opening for food intake, waste disposal and gamete discharge (Katsuki and Greenspan, 2013). They capture foods by oral arms and lappets, that pass through the mouth. Likewise, they can use the same mechanism to handle MPs, wrongly recognized as food. This assumption could explain MP in the mouth and lappets, being the main parts of jellyfish body involved in the prey capture (Sullivan et al. 1997). The mechanism of prey capture in Aurelia sp. ephyrae has been well documented by Sullivan et al. (1997) that highlighted how all the many preys were captured by fluid flows around swimming ephyrae. Then, the prey in the proximity of an ephyra were entrained in these flows and directed toward capture surfaces, primarily the lappets, subumbrella, and mouth of the ephyra. Likewise, also the MP particles could be captured by lappets and oral arms and to pass through the mouth. Jellyfish ephyrae feed on food particles, including other zooplankton (i.e. crustaceans, Hansson et al. 2005) and are predators of fish or free-swimming invertebrates (Waggoner and Speer, 1996). Intense predation by jellyfish on certain preys – such as other zooplankton – can change the trophic structure of the pelagic community as a result of trophic cascading (Purcell and Grover 1990; Stibor et al. 2004; Sullivan, 1997). Since MP ingestion has been documented in zooplankton and fish (Beiras et al. 2018), MP transfer may occur in the food chain, affecting different trophic levels. Further investigations on the accumulation of MP along a trophic food web (i.e. crustaceans and jellyfish ephyrae) are necessary to confirm this hypothesis.

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In this work, the ***correspondence*** between MP ingestion and ecotoxicological effects has been observed in jellyfish ephyrae. Many studies conducted so far report MP uptake in marine zooplankton, but in few cases ingestion is likely to be the cause of acute toxicity (Anbumani and Kakkar, 2018). In this regard, PE MP affect ephyrae survival and behavior, inducing toxic effects both at ***concentrations below the highest MP concentration estimated for marine water (< 0.5 mg L^{-1} Koelmans et al., 2015) and at high concentrations (up to 10 mg L^{-1})***, contrary to what has been reported for other zooplankton models exposed to the same MP (Table 3, Beiras et al. 2018).

Although the nauplii of the crustacean *Tigriopus fulvus* exposed to MP showed LC_{50} values in the range of those found to cause jellyfish Immobility, LOEC levels support the hypothesis that among marine invertebrates jellyfish ephyrae are the most sensitive species to MP (Table 3). In this regard, a significant effect of MP on ephyrae Immobility and Frequency pulsation was found at concentrations lower by 2-4 orders of magnitude than those observed in other organisms, such as crustaceans, rotifers and mussels (Beiras et al. 2018). Comparing the two endpoints, jellyfish behavioral response after MP exposure resulted to be more sensitive than the acute one (Immobility) as indicated by EC_{50} values (<0.01 *vs.* 3.16 mg/L). The results are in line with literature data (Faimali et al. 2014) and confirm that our findings in both static and semi-dynamic conditions are sound.

Table 3. 48 h-LC_{50} (median Lethal Concentration) and EC_{50} (median Effective Concentration) with 95% confidence limits reported in the literature for marine zooplankton exposed to 1-4 µm polyethylene microplastics (MP).

Marine organisms	**Species**	**Stage**	**Endpoint**	**EC_{50}-LC_{50} (mg/L)**	**LOEC**	**References**
Cnidarians	*Aurelia* sp.	Ephyra	%Immobility	3.16 (1.73- 5.79)	0.01	This study
			% Alteration of Frequency pulsation	<0.01	0.01	
Crustaceans	*Tigriopus fulvus*	Nauplii	% Mortality	1.82 (1.34-2.48)	1	Beiras et al. 2018
Rotifers	*Brachionus plicatilis*	-	% Mortality	>10 (nc)	1	
			% Immobility	>10 (nc)	0.01	
Mussels	*Mytilus galloprovincialis*	embryos	% developmental anomalies	>100 (nc)	>100	

No difference in Alteration of Frequency pulsation was found in ephyrae exposed to cadmium nitrate and MP after static or semi-dynamic conditions. Conversely, Immobility was different between the two exposure conditions: ephyrae were more severely affected in static rather than semi-dynamic conditions. As a matter of fact, under static exposure, the concentration of the bioavailable test substance (toxicants, MP) fraction in the exposure water can be depleted by processes such as volatilization, sorption at the surface of the exposure container, degradation, precipitation or coalescence into droplets, and accumulation by the test organism (Landrum et al. 2013). ***However, the absence of aeration for constant mixing of the microsphere and for a little volume of seawater used in static condition (2 ml), could be lead to the formation of aggregates in sea water causing an obstruction reducing motility and pulsation of ephyrae jellyfish.***

Moreover, the particles with different density such as MP settle with different rates; hence, performing tests under static conditions would not provide stable exposure levels (Gerdes et al. 2018). On this basis, the semi-dynamic exposure reported in this study has proved to be successful and more realistic to assess MP toxicity in jellyfish, since aeration may facilitate MP suspension and also ephyrae body contraction preventing sinking to the bottom (Fossette et al. 2015; Rakow et al. 2006;).

Behavioral responses integrate biochemical and physiological processes, reflecting changes at higher ecologically relevant levels of organization (Faimali et al. 2017). This may account for the high sensitivity of the behavioral response compared to Immobility. Behavioral responses can provide initial health condition indications before the organism death (Calfee et al. 2016). Accordingly, there is growing emphasis in marine ecotoxicology on examining behavioral changes in response to exposure to MP. In this regard, MP affect feeding and swimming behavior of blue mussels, oysters, crustaceans, rotifers, sea urchins and fish (Barboza et al. 2018; Cole et al. 2013; Gambardella et al. 2017, 2018; Sussarellu et al. 2016; Wegner et al. 2012).

The relatively primitive architecture and behavior of jellyfish provide an opportunity to address how sensory inputs and internal information are integrated to produce coordinated motor output. ***Zooplankton can use a combination of chemo-and mechano receptors in response to stress or under natural conditions to select appropriate prey*** (Kjorboe, 2011). Jellyfish have a full battery of molecular machinery for neurotransmission and neuromodulation (i.e. sensory receptors) allowing them to respond to various stimuli (Katsuki and Greenspan, 2013). Detection of pressure disturbances within the water due to MP may generate sensory responses in jellyfish, modulating pulse frequency. MP were mainly found in proximity of the arms (lappets), where the main sensory organs are located (Katsuki and Greenspan, 2013). The latter are known as rophalia: they are a specific structure of the nervous system, provided with mechanoreceptors and containing ocelli – chemosensory pits – and statocysts (Ambrams et al. 2015). They are protected by lappets, the marginal segments of 'rhopalar arms' (Nakanishi et al. 2009). MP attached on rhopalia could directly affect Frequency pulsation, since electrical impulses trigger spontaneous contractions of the 'rhopalar arms' in this organism (Nakanishi et al. 2009). Therefore, jellyfish could modulate pulse frequency, delaying contraction in response to MP stress and resulting in Alteration of Frequency pulsation, as previously demonstrated for light changes (Faimali et al. 2014; Katsuki and Greenspan, 2013) and other contaminants (Costa et al. 2015). However, this alteration is temporary, as demonstrated by the recovery test performed in this study. ***Indeed, after a 24 hours stay in clean water, jellyfish, that show a low amount of MP (Figure 5), pulse frequency fully resumed the same level of uncontaminated ephyrae (Figure 4).*** According to these findings, it can be assumed that MP adhesion on ephyra's oral arms is likely to burden the body, thus preventing muscular contraction. Lappets closed around the mouth cause a loss in radial symmetry, which, in this organism, is responsible for jellyfish propulsion, food capture, and orientation in the water column (Abrams and Goentoro, 2016; Fossette et al. 2015; Sattarlie, 2002; Spencer and Arkett, 1984). Jellyfish have a self-repair mechanism, not only capable of regenerating lost appendages, but also of reorganizing existing limbs to become symmetrical again after an injury (Abrams et al. 2015). Thus, the arms, lopsided and shriveled due to MP attached on the body may inhibit propulsion power. Since MP effect is temporary, jellyfish radial symmetry can be resumed with increasing contraction of arms and lappets when the ephyrae are placed back in clean sea water, after MP exposure

5. Conclusions

This study provides some initial data that are very important to understand and explain any potential impact of MP contamination on gelatinous zooplankton and, in particular, cnidarian jellyfish. By using a new approach based on 3-D imaging, we demonstrated that environmentally relevant and high levels of polyethylene MP are ingested by ephyrae. MP ingestion temporarily affected the sub-lethal responses of ephyrae – namely Immobility and Frequency pulsation – with unknown consequences on the food chain. Finally, the acute and behavioral effects observed may be related to some mechanical disturbance which in ephyrae jellyfish causes a loss of radial symmetry, although further investigations are required to support this assumption.

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Conflict of interest statement

The authors represent that there are no conflicts of interest.

Ethical approval:

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (prot. CNR n. 0067798/2019)

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Table

Table 1 Test parameters to be used in static and semi-dynamic bioassays with ephyrae jellyfish

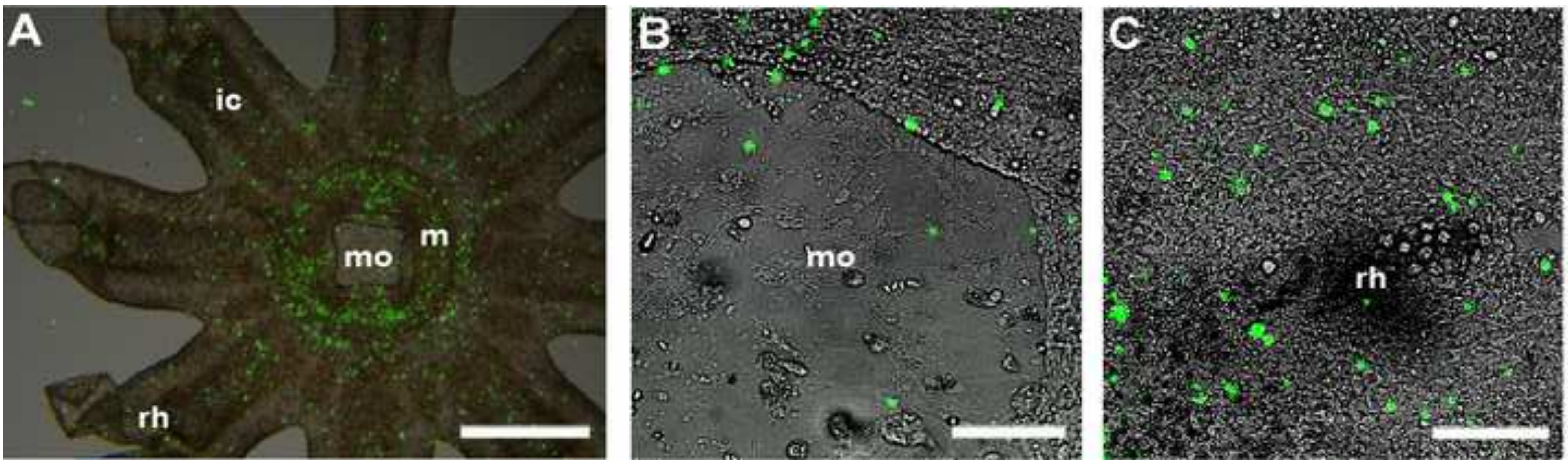
	Conditions	
Parameters	static	semi-dynamic
Container	multi-well plates	beaker
Aeration	no	yes, constant
Density of organisms	1 ephyra/well	10 ephyrae/beaker
Temperature (°C)	20	20
Photoperiod	No, full dark	No, full dark
Exposure (hours)	24-48	24-48

Table 2 EC_{50} values at 24- and 48-h with 95% confident limits derived from Immobility percentage (% I) and Alteration of Frequency pulsation(%AFp) of *Aurelia sp.* ephyrae exposed to 1-4 µm polyethylene microplastics (MP) and n.c. (not calculable) Cadmium nitrate.

Reference compound	**Exposure**	**Endpoint**	**24h-LOEC**	**24h- EC_{50} and confident limits (C.L.)**	**48h-LOEC**	**48h-EC_{50} and confident limits (C.L.)**
Polyethylene MP	Static	Immobility	0.1	1.36 mg/L (0.73-2.55)	0.01	0.53 mg/L (0.27-1.04)
		AFp	0.01	< 0.01 mg/L (n.c.)	0.01	< 0.01 mg/L (n.c.)
	Semi-dynamic	Immobility	0.1	4.16 mg/L (1.90-9.09)	0.01	3.16 mg/L (1.73- 5.79)
		AFp	0.01	< 0.01 mg/L (n.c.)	0.01	< 0.01 mg/L (n.c.)
$Cd(NO_3)_2$	Static	Immobility	0.5	0.40 mg/L (0.35-0.46)	0.1	0.23 mg/L (0.20-0.28)
		AFp	0.1	0.13 mg/L (0.10-0.15)	0.05	0.06 mg/L (0.05-0.07)
	Semi-dynamic	Immobility	5	>5 mg/L (n.c.)	1	2.99 mg/L (1.86-4.80)
		AFp	0.01	0.10 mg/L (0.070.13)	0.01	0.05 mg/L (0.04-0.07)

Table 3. 48 h-LC_{50} (median Lethal Concentration) and EC_{50} (median Effective Concentration) with 95% confidence limits reported in the literature for marine zooplankton exposed to 1-4 µm polyethylene microplastics (MP).

Marine organisms	**Species**	**Stage**	**Endpoint**	**EC_{50}-LC_{50} (mg/L)**	**LOEC**	**References**
Cnidarians	*Aurelia* sp.	Ephyra	%Immobility	3.16 (1.73- 5.79)	0.01	This study
			% Alteration of Frequency pulsation	<0.01	0.01	
Crustaceans	*Tigriopus fulvus*	Nauplii	% Mortality	1.82 (1.34-2.48)	1	Beiras et al. 2018
Rotifers	*Brachionus plicatilis*	-	% Mortality	>10 (nc)	1	
			% Immobility	>10 (nc)	0.01	
Mussels	*Mytilus galloprovincialis*	embryos	% developmental anomalies	>100 (nc)	>100	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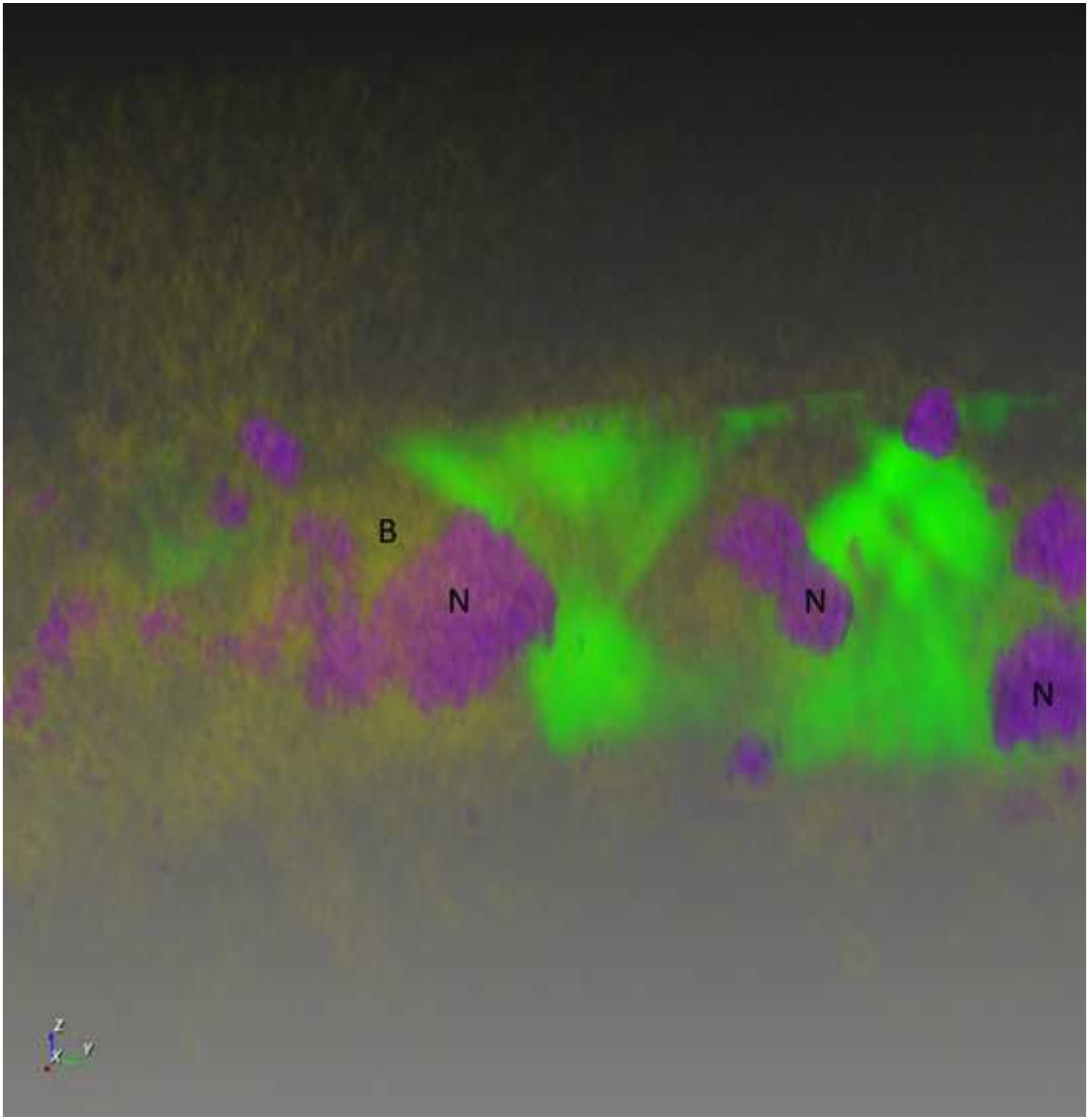
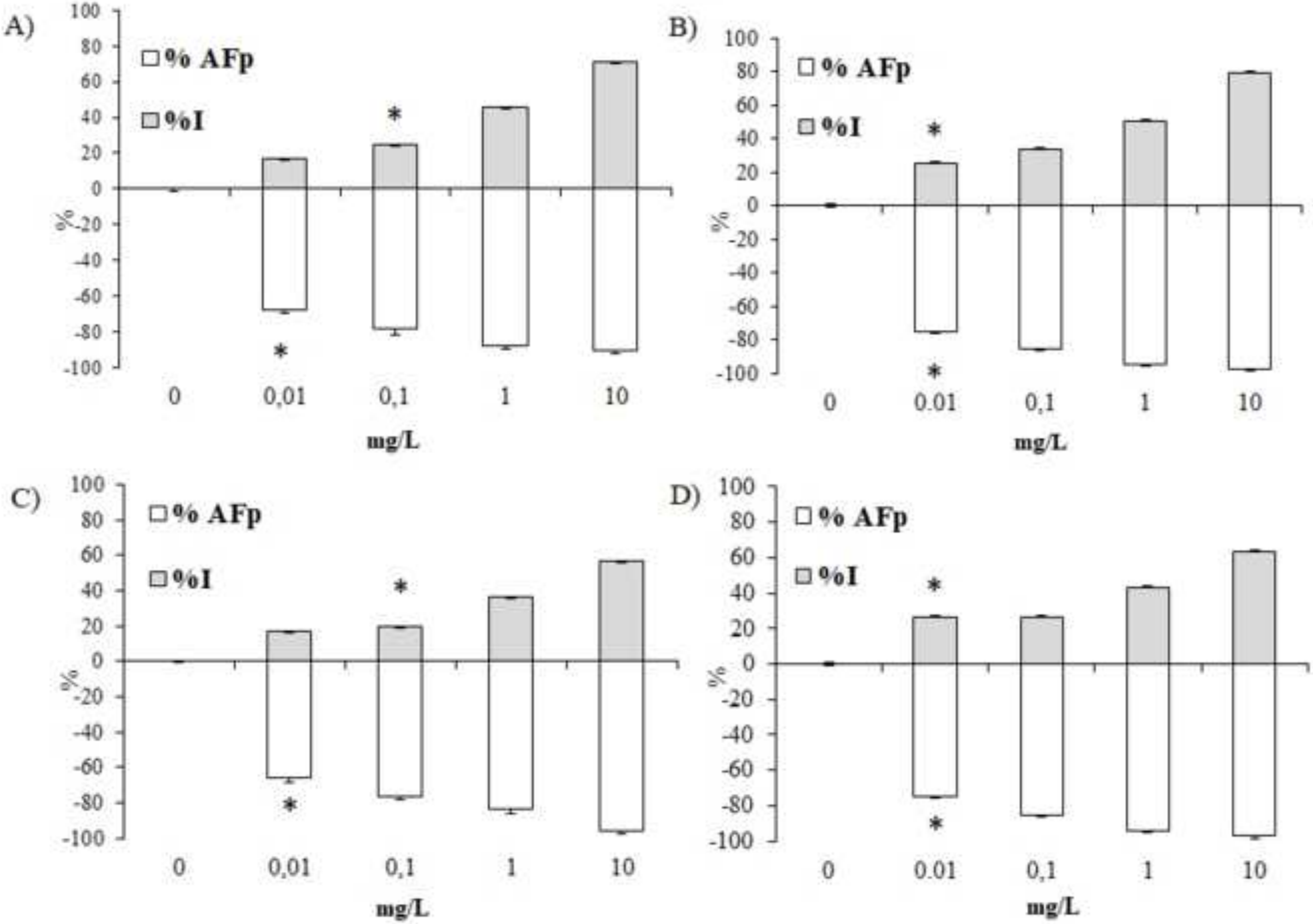
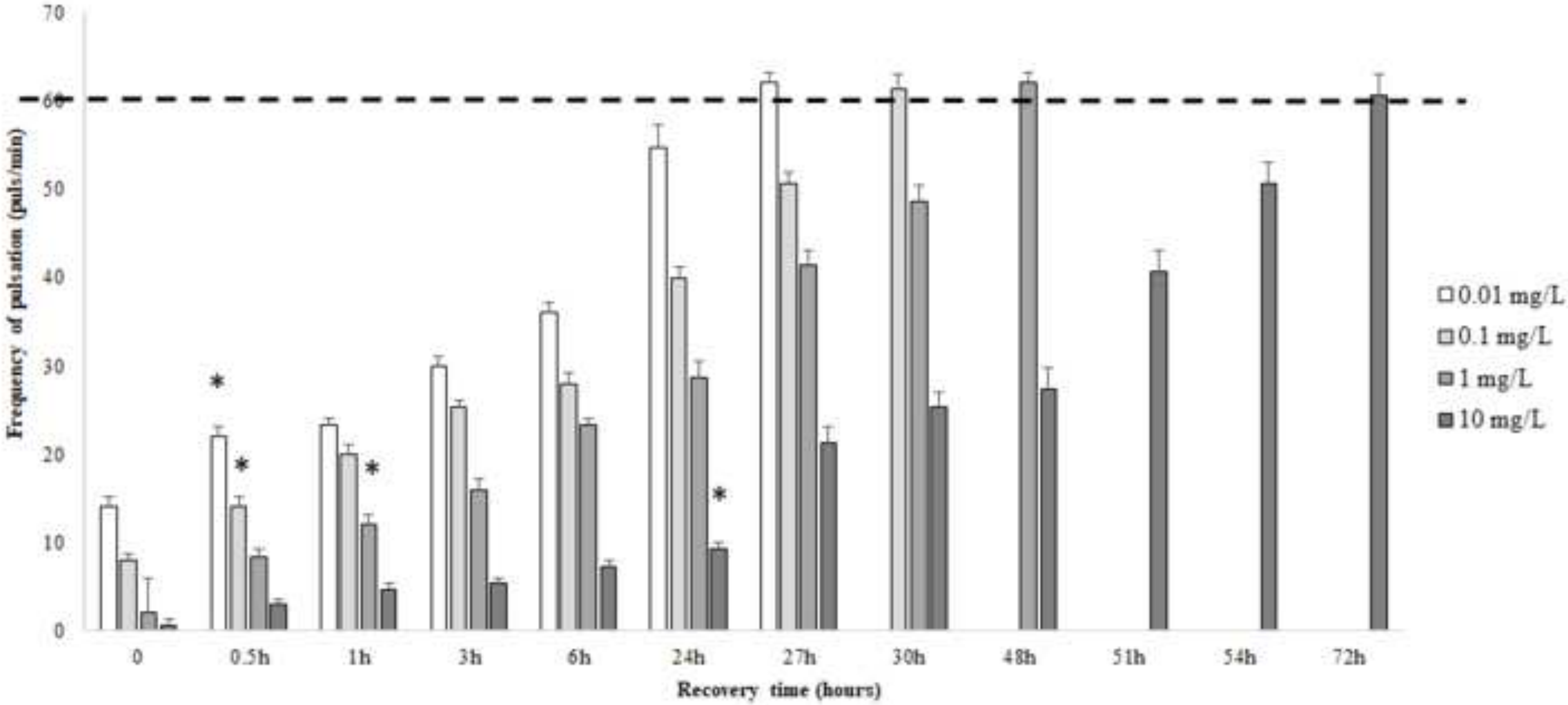
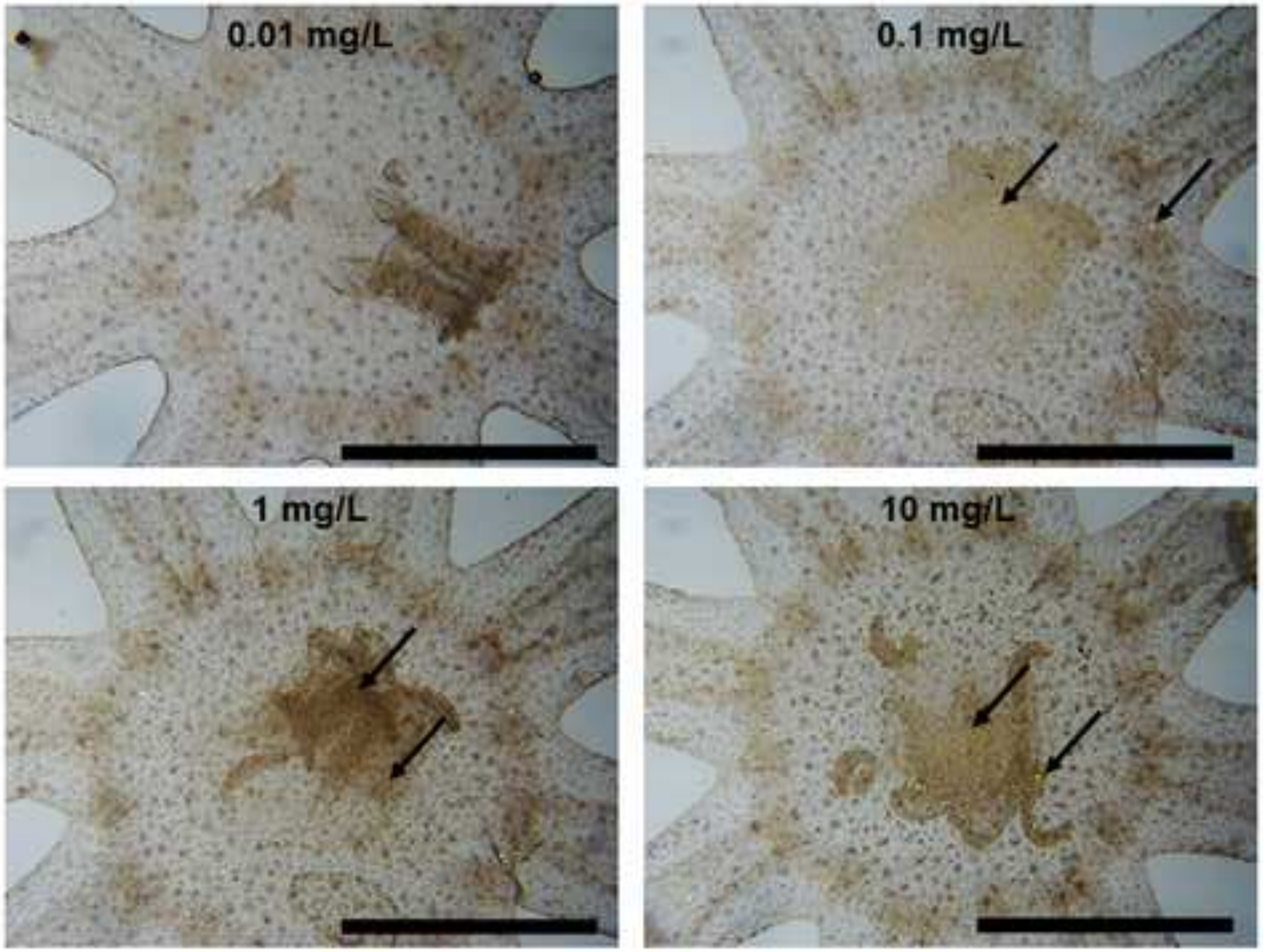
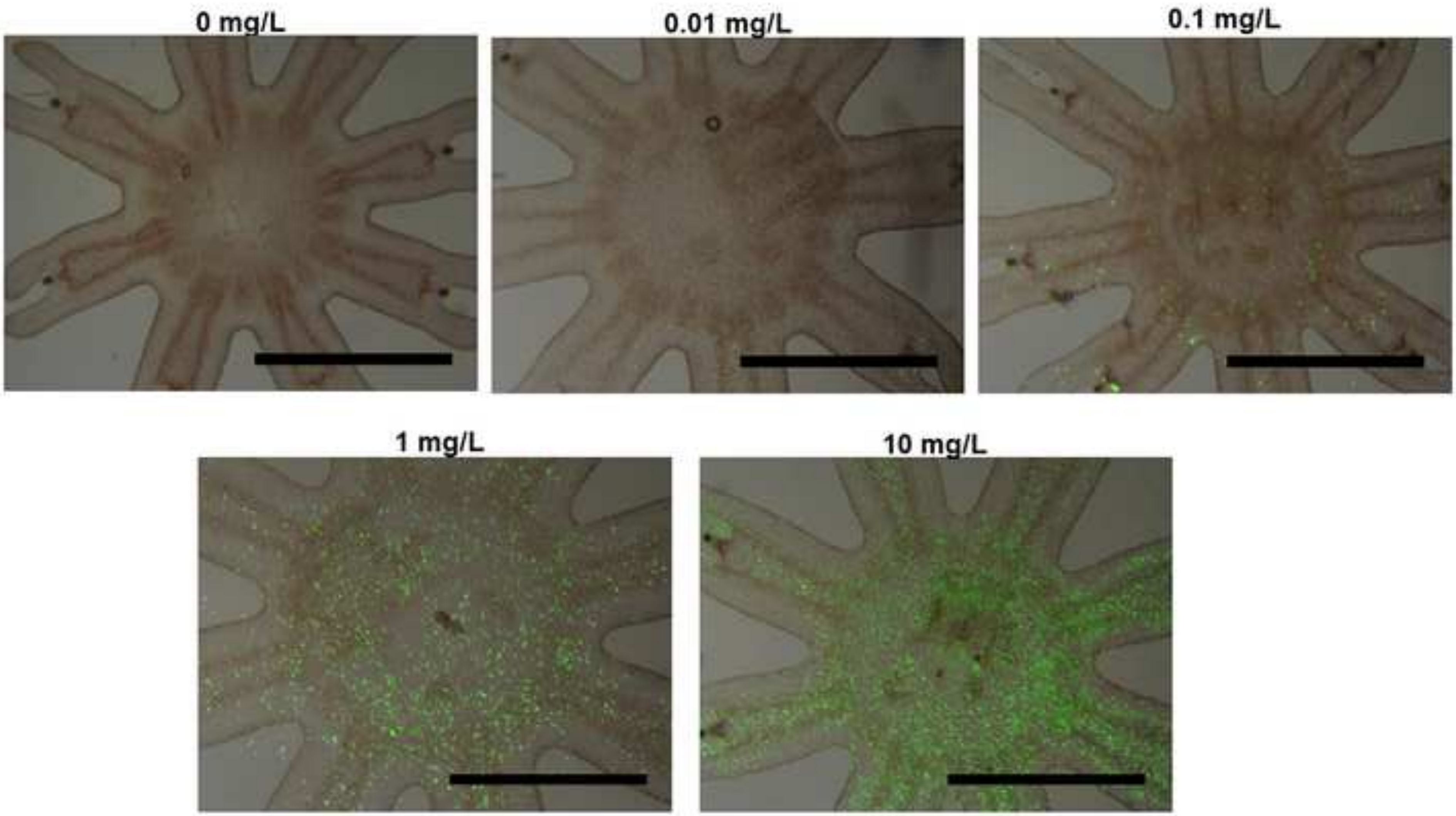

B
N
N
N
Z
Y
X


A)
□% AFp
%I
%
mg/L
B)
□% AFp
%I
%
mg/L
C)
□% AFp
%I
%
mg/L
D)
□% AFp
%I
%
mg/L


70
60
50
40
30
20
10
0
Frequency of pulsation (puls/min)
*
*
*
*
0
0.5h
1h
3h
6h
24h
27h
30h
48h
51h
54h
72h
Recovery time (hours)
0.01 mg/L
0.1 mg/L
1 mg/L
10 mg/L


0.01 mg/L
0.1 mg/L
1 mg/L
10 mg/L


0 mg/L
0.01 mg/L
0.1 mg/L
1 mg/L
10 mg/L

Ministero delle politiche agricole alimentari e forestali

DIPARTIMENTO DELLE POLITICHE COMPETITIVE, DELLA QUALITA'AGROALIMENTARE , IPPICHE E DELLA PESCA
DIREZIONE GENERALE DELLA PESCA MARITTIMA E DELL'ACQUACÒLTURA
PEMAC I

Roma,

A tutte le Direzioni marittime
(elenco in allegato)

Alla Soc. COSTA EDUTAINMENT S.p.A.
Area porto antico
Ponte Spinola
16128 GENOVA

Oggetto: *Rinnovo* dell'Autorizzazione a svolgere campagne di pesca per la raccolta di organismi marini a scopi espositivi.

Si trasmette copia della nota pervenuta dall'Istituto in indirizzo, intesa ad ottenere l'autorizzazione al proseguimento dell'attività scientifica per l'acquario di Genova, l'acquario di Livorno, l'acquario di Cattolica e l'acquario Oltremare.

Al riguardo, considerato l'elevato interesse scientifico, didattico ed educativo dell'attività svolta nell'ambito della pesca marittima, si autorizza il proseguimento, con le modalità richieste, della pesca scientifica **fino al 31 dicembre 2019**.

Il personale scientifico e acquaristico che prenderà parte alla raccolta, per conto dei citati acquari di Genova, Cattolica, Oltremare e Livorno, sarà esclusivamente quello indicato nella nota trasmessa dall'Istituto.

L'attività di ricerca dovrà essere subordinata, altresì, all'osservanza delle seguenti prescrizioni:

- imbarco del personale conformemente a quanto previsto dal D.P.R. 9 giugno 1976, n.1057;
- diretta ed esclusiva responsabilità del personale autorizzato anche su tutte le operazioni necessarie allo svolgimento dell'attività di pesca;
- comunicazione preventiva alle Capitanerie di porto in indirizzo delle modalità operative dell'attività di pesca (ad es: giorni, orari, personale imbarcato, unità impiegata).

Il Dirigente
Roberto Cherubini
Firmato digitalmente ai sensi del CAD

Il Funzionario Responsabile
Stingo

Gazzetta n. 7 del 11 gennaio 2011 (vai al sommario)

MINISTERO DELL'AMBIENTE E DELLA TUTELA DEL TERRITORIO E DEL MARE

DECRETO 10 novembre 2010

Rilascio della licenza di giardino zoologico all'Acquario di Genova.

IL MINISTRO DELL'AMBIENTE
E DELLA TUTELA DEL TERRITORIO
E DEL MARE

di concerto con

IL MINISTRO DELLA SALUTE

e

IL MINISTRO DELLE POLITICHE AGRICOLE
ALIMENTARI E FORESTALI

Vista la direttiva 1999/22/CE relativa alla custodia degli animali selvatici nei giardini zoologici;
Visto il decreto legislativo 21 marzo 2005, n. 73, e successive modificazioni, recante attuazione della direttiva 1999/22/CE;
Visto in particolare l'art. 4, comma 1 del decreto legislativo n. 73/2005, il quale prevede che la licenza di giardino zoologico e' rilasciata con decreto del Ministro dell'ambiente e della tutela del territorio e del mare, di concerto con il Ministro della salute e con il Ministro delle politiche agricole, alimentari e forestali, sentita la Conferenza Unificata, previa verifica del possesso dei requisiti indicati dall'art. 3 dello stesso decreto legislativo n. 73/2005;
Vista la nota del 15 novembre 2005 con la quale l'Acquario di Genova ha inoltrato la domanda per il rilascio della licenza di cui all'art. 4, comma 1, del decreto legislativo n. 73/2005, cosi' come previsto all'Allegato 4, punto A), del decreto legislativo n. 73/2005;
Considerata la rispondenza della documentazione inviata alle indicazioni di cui al predetto Allegato;
Viste le note prot. DPN/1D/2006/24557 del 28 settembre 2006 e prot. DPN/2008/0001170 del 17 gennaio 2008, con cui si chiedeva al Ministero della salute e al Ministero delle politiche agricole, alimentari e forestali, la designazione degli esperti preposti all'ispezione presso la struttura Acquario di Genova per l'accertamento del possesso dei requisiti prescritti dall'art. 3 del decreto legislativo n. 73/2005, cosi' come previsto dall'art. 6 e dall'Allegato 4, punto B) dello stesso decreto legislativo;
Vista la nota del Ministero delle politiche agricole, alimentari e forestali prot. n. 200602759 pos. 2/B del 16 ottobre 2006, con cui e' stata designata la dott.ssa Cecilia Ambrogi, quale esperto per lo svolgimento del sopralluogo presso la struttura;
Vista la nota del Ministero della salute, prot. n. 0012941 - P del 14 giugno 2008, con cui sono stati designati, quali esperti per lo svolgimento dell'ispezione presso la struttura, la dott.ssa Rosalba Matassa e la dott.ssa Cristina Zacchia;
Vista la nota della Direzione per la protezione della natura, prot. DPN-2008-0028579 del 2 dicembre 2008, con cui la commissione di esperti - composta, oltre che dai suddetti componenti, dal sig. Sergio Scacco del Ministero dell'ambiente, per la tutela del territorio e del mare - e' stata incaricata di effettuare il sopralluogo presso l'Acquario di Genova il giorno 18 dicembre 2008;
Considerato che la prescritta ispezione si e' svolta alla predetta data e che dal verbale redatto dalla commissione, trasmesso con nota prot. n. DPN-2009-004384 del 27 febbraio 2009, l'Acquario di Genova risulta essere in possesso dei requisiti di cui dall'art. 3 del decreto legislativo n. 73/2005;

Considerata la sussistenza di tutte le condizioni richieste dal decreto legislativo n. 73/2005 ai fini del rilascio della licenza di giardino zoologico;
Visto il parere favorevole espresso dalla Conferenza Unificata in data 29 ottobre 2009;

Decreta:

Art. 1

E' rilasciata la licenza di giardino zoologico, di cui all'art. 4, comma 1 del decreto legislativo n. 73/2005, all'Acquario di Genova sito in area Porto Antico - Ponte Spinola Genova.
Il presente decreto sara' pubblicato nella Gazzetta Ufficiale della Repubblica italiana.
Roma, 10 novembre 2010

Il Ministro dell'ambiente
e della tutela del territorio
e del mare
Prestigiacomo

Il Ministro della salute
Fazio

Il Ministro delle politiche agricole
alimentari e forestali
Galan

Author Contributions Section

Elisa Costa and Chiara Gambardella carried out the experiments and wrote the manuscript. Massimo Vassalli and Francesca Sbrana have supported the confocal and tomographic microscope analysis during all experiment activities. All authors discussed the results and contributed to the final manuscript. Both Francesca Garaventa and Marco Faimali contributed to the final version of the manuscript. Silvia Lavorano (Supervisor of tropical laboratory of the Acquario of Genoa) supported this study during the polyps and ephyrae jellyfish collection.