RESEARCH ARTICLE

REVISED Natural sea water and artificial sea water are not equivalent in plastic leachate contamination studies [version 2; peer review: 1 approved, 1 approved with reservations]

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Abstract

Background

Plastic contamination is one of the concerns of our age. With more than 150 million tons of plastic floating in the oceans, and a further 8 million tons arriving to the water each year, in recent times the scientific community has been studying the effects these plastics have on sea life both in the field and with experimental approaches. Laboratory based studies have been using both natural sea water and artificial sea water for testing various aspects of plastic contamination, including the study of chemicals leached from the plastic particles to the water. We set out to test this equivalence, looking at the leaching of heavy metals form plastic particles.

Methods

We obtained leachates of PVC plastic pre-production nurdles both in natural and artificial sea water and determined the elements in excess from untreated water by Inductively coupled plasma – optical emission spectrometry. We then used these different leachates to assess developmental success in the tunicate *Ciona intestinalis* by treating fertilised eggs through their development to hatched larvae.

Results

Here we report that chemical analysis of PVC plastic pre-production

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pellet leachates shows a different composition in natural and artificial sea water. We find that the Zn leaching from the plastic particles is reduced up to five times in artificial sea water, and this can have an effect in the toxicological studies derived. Indeed, we observe different effects in the development of *C. intestinalis* when using leachates in natural or artificial sea water. We also observe that not all artificial sea waters are suitable for studying the development of the tunicarte *C. intestinalis*.

Conclusions

Our results show that, at least in this case, both types of water are not equivalent to produce plastic leachaetes and suggest that precaution should be taken when conclusions are derived from results obtained in artificial sea water.

Keywords
Plastic leachates, Zinc, Natural sea water, Artificial sea water, Development, Ciona intestinalis

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REVISED **Amendments from Version 1**

This version has been modified according to the reviewers' comments. The main difference is the addition of a new water chemical analysis to show that not other metals other than Zn were leached from the plastics, as observed by reviewer 2. With this analysis we have also statistically assessed the differences of Zn in "clean" samples of the three artificial sea waters as suggested by reviewer 1.

Any further responses from the reviewers can be found at the end of the article

Introduction

Plastic pollution is ubiquitous and persistent. In marine ecosystems, it has emerged as a significant concern: large plastic items can entangle marine animals, cause physical injuries, and alter or disrupt habitats; smaller plastic items can be ingested by organisms, and even be transferred to internal organs and cells[1,2]. Plastics also can contain chemical additives which are added to the polymers during the manufacturing process or that can be adsorbed and accumulated from the environment[3,4]. These pollutants can then be transferred from the plastic into the aquatic systems[3–9]. Multiple reports have studied the effect of plastic leachates in several aquatic organisms. Laboratory experiments have shown that plastic leachates increase oxidative stress[9–11] and impair embryo and larval development[5,6,8,9,12–16].

These studies used leachates from different types of plastic items: farming gear, pre-production plastic pellets, plastic toys, plastic bags, as well as virgin plastics and others. The protocols for lixiviation of these different studies vary slightly, and recently a study has been published to provide a unified protocol for further studies[17]. Variations include lixiviation time, rotation speed of the samples during lixiviation, and plastic concentrations. However, the source of seawater used to obtain the leachates, provided it is not contaminated, has not been suggested as one of the variables to take into account, as both artificial seawater (ASW) and natural filtered seawater (FSW) have been accepted as equivalent ecologically relevant media for ecotoxicity tests with marine organisms[17,18]. As such, different studies use either ASW[5,7,15,19] or FSW[8,9,11,13,14,16] to obtain their leachates.

We have previously used pre-production PVC pellet leachates in toxicology studies in sea urchin adults and embryos[8,9,11] and chemical analysis of the leachates by inductively coupled plasma – optical emission spectrometry (ICP-OES) has shown that they leach high amounts of zinc into natural seawater. These leachates inflicted very important developmental abnormalities in a large number of invertebrate taxa larvae[8,9,20]. However, we wanted to know if the composition of the leachates would be similar when using artificial sea water, to test whether ASW and FSW are equivalent in laboratory tests. Here we obtain leachates of pre-production PVC pellets in three types of ASW as well as in FSW and in double deionised water (ddW) to compare their elemental content, as well as their effect on developing embryos of the tunicate *Ciona intestinalis* to confirm if they are equivalent media for obtaining heavy metal leachates of plastic particles and to perform ecotoxicology studies. In this study, we did not test for the leaching of other molecules, including organic molecules, which are known to leach from plastic pellets[3,4].

Methods

PVC plastic leachate preparation

Commercial PVC plastic pre-production nurdles were purchased from Northern Polymers and Plastics Ltd. (UK). Leachates from these plastic pellets were obtained as described in Rendell-Bhatti *et al*., 2020 with small modifications. In brief, PVC plastic pellets were added to natural seawater, artificial seawater or distilled water at a concentration of 6.5% (w/v), equivalent to 10% (v/v). Pellets were left to leach in each type of water in a glass bottle on a platform shaker at 60 rpm at 18°C in the dark for 72 h. Leachates were obtained by filtering the treated waters through filter paper in order to remove particles. Working solutions were obtained by diluting the leachates to the desired concentration with the relevant type of water.

Natural sea water was obtained from Falmouth bay, UK, and filtered using a 0.22 µm filter, with a salinity of 32 PSU.

Three brands of artificial sea water were used, prepared to a salinity of 32 PSU:

Aquarium Systems Instant Ocean Sea Salt (AS) (https://www.aquariumsystems.eu/sea-salt-instant-ocean-uk-c2x31922668)

TMC reef salt (RbT) (https://www.tropicalmarinecentre.com/en/tmc/aquarium/water-chemistry/salt/reef-salt-10kg300l-bucket)

iQuatics Ocean Reef Pro Coral Salt (OR) (https://www.iquaticsonline.co.uk/product/iquatics-ocean-reef-pro-coral-aquarium-salt-20kg/)

Chemical analysis of plastic leachates by Inductively Coupled Plasma – Optical Emission Spectrometry (ICP-OES)

Previous research has shown elevated Zn concentrations, but not of other heavy metals, in PVC plastic pre-production nurdles using the same pellets as we use here[9]. Zn concentrations were quantified using an Agilent 5110 VDV Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) using the ICPExpert software version 7.6.2.12331 to control the instrument. Other selected elements were assessed to corroborate the absence of other heavy metals (see below). Analytical routines were followed as described in 9 with minimal modifications. All dilutions of chemical solutions used for analysis were done using 2 % v/v HNO_3 prepared from concentrated Fisher Scientific General purpose grade nitric acid for which sufficient purity for ICP analysis was previously established. Dilution of the nitric acid was carried out using MilliQ water (18.2 MΩ) in a ratio of 1 part concentrated nitric acid in 32 parts MilliQ.

Zn concentrations were quantified using an acid blank and four calibration solutions made from a certified 1,000 µg/g Zn single element plasma standard (Fisher Scientific Specpure™). Calibrations were done before and after sample analysis to verify consistency of the calibration curves. Zn calibration solutions were prepared to nominal concentrations of 5.21 ng/g, 10.30 ng/g, 20.72 ng/g, and 52.22 ng/g using an intermediate stock solution of 1 µg/g that was prepared via another intermediate stock solution of 30 µg/g from the original certified standard. All stock solutions and standards were prepared gravimetrically to ensure optimal precision. Internal consistency of the measurements was ascertained using a synthetic Zn solution prepared to 25.94 ng/g which reproduced to 26.05 ng/g (n = 2) during the analytical sequence.

Potential biases resulting from seawater matrix were previously falsified using a suite of calibration solutions of the same standard in seawater matrix. Signals for all wavelengths were obtained in radial mode in six blocks of 30 seconds each, resulting in total signal integration of 180 s per sample. The peak intensity in counts per second was derived using the two nearest pixels to the expected peak centre with automatic baseline fitting applied. The Zn signal was quantified using the 213.857 nm and the Zn 202.548 nm lines.

In addition to quantitative Zn measurements, in additional analysis (presented in the Extended data, Chemical analysis 2) a series of metals were monitored and concentrations determined using a mixture of Inorganic Ventures multi element standards IV-ICPMS-71A and IV-ICPMS-71B resulting in nominal concentrations of 21.64 ng/g for Ba, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb and V, and 21.23 ng/g for Sb and Sn. Zn calibration solutions were prepared to nominal concentrations of 2.22 ng/g, 5.37 ng/g, 10.80 ng/g, and 21.29 ng/g using an intermediate stock solution of 1 µg/g that was prepared via another intermediate stock solution of 30 µg/g from the original certified standard. All stock solutions and standards were prepared gravimetrically to ensure optimal precision. Internal consistency of the measurements was ascertained using two synthetic Zn quality control solutions prepared to 4.31 ng/g and 8.58 ng/g which reproduced to 4.20 ng/g (n = 3) and 8.50 ng/g (n = 6) during the analytical sequence. Signals for all wavelengths were obtained in axial mode in five blocks of 40 seconds each, resulting in total signal integration of 200 s per sample. The peak intensity in counts per second was derived using the two nearest pixels to the expected peak centre with automatic baseline fitting applied. The Zn signal was quantified using the 213.857 nm line. The detection limit for Zn was determined as six times the standard deviation of the concentration estimate for the blank solution which was nominally devoid of Zn and found to be 0.13 ng/g, equivalent to c. 30 ng/g in the unknown solutions, accounting for an average dilution factor of c. 200 for the unkowns. Detection limits for the other elements were calculated as six times the standard deviation of concentrations obtained for the Zn quality control solutions which are nominally devoid of these metals. Wavelengths used for this quantification were Ba 455.403 nm; Cd 214.439 nm and 226.502 nm; Co 228.615 nm, 230.786 nm and 238.892 nm; Cr 205.560 nm and 267.716 nm; Cu 327.395 nm; Fe 259.940 nm; Mn 257.610 and 259.372 nm; Ni 231.604 nm and 231.604 nm; Pb 220.353 nm; Sb 206.834 nm and 217.582 nm; Sn 189.925 nm; V 292.401 nm. Where multiple wavelengths were used for quantification, the concentration estimates of individual analyses were pooled before averaging to improve detection limits. Resulting detection limits accounting for the average dilution factor of c. 200 for the unknown solutions convert to Ba = 4 ng/g; Cd = 20 ng/g; Co = 30 ng/g; Cr = 40 ng/g; Cu = 30 ng/g; Fe = 120 ng/g; Mn = 2 ng/g; Ni = 70 ng/g; Pb = 340 ng/g; Sb = 370 ng/g; Sn = 390 ng/g; V = 150 ng/g.

For all analysis, intensities of Ca (317.933 nm, 422.673 nm), Mg (279.553 nm, 280.270 nm, 285.213 nm), Na (589.592 nm), Sr (407.771 nm, 421.552 nm), S (181.972 nm) and Ba (455.403 nm) were monitored to verify the consistency of the sample dilution.

Plastic leachates were gravimetrically diluted c. 1:200 using 50 mL tubes in 2% nitric acid in order to adjust the sample matrix for optimal analytical conditions. Three independent leachate events for each type of water were analysed.

Ciona intestinalis treatments

C. intestinalis were collected from Mylor Yacht Harbour (UK) in October 2023, transported to the lab within 30 minutes of collection and kept in clean natural seawater with aeration at 18 ºC, with constant light to inhibit spawning. After a week, gametes were retrieved from both male and female gonads sequentially to avoid self-fertilisation. Crosses were done using single matings from male and female gonads from different animals. Eggs from each gonad were placed in 15 ml of FSW and dry sperm was diluted 15 µl to 10 ml of FSW, and 100 microliters of this dilution were used to fertilise the eggs. Fertilised eggs were left in a rotating platform for 10 minutes, after which they were washed once in FSW to prevent polyspermy. Washed zygotes were immediately transferred to the treatment solutions at a rate of 20 embryos per ml. Treatment solutions were each of the seawaters used, and PVC leachates in each type of seawater at a concentration of either 1% or 5% (v/v). Experiments were performed in triplicate using embryos from different parents for each replica. Images of 22 hours post fertilisation (hpf) larvae were obtained using a Leica M165 C scope and a Leica DFC 295 camera.

Image processing

Images in Figure 2 were individually adjusted for color balance, tone and exposure to obtain similar background colors using the levels tool in Photoshop CS 5.1. The current version of this program can be found at https://www.adobe.com/products/photoshop.html. A free alternative of this software can be used such as Fiji, which can be found at https://imagej.net/software/fiji/.

Results and discussion

Zn content in PVC plastic leachates

We monitored the zinc concentrations in 10% PVC nurdle leachates in normal sea water, double distilled water, and the three commercial brands of artificial sea water (Figure 1; Extended data, Chemical Analysis 1 and 2) using inductively coupled plasma – optical emission spectrometry. Other metals were assessed quantitatively (Extended data, Chemical Analysis 2). We found that all three artificial sea waters used contained significantly less zinc originating from the plastics than filtered sea water or double distilled water.

We have previously shown high amounts of Zn leaching from PVC plastic pre-production nurdles into filtered sea water[9]. Here, we repeat those experiments to find similar concentrations to the ones reported in that work (Paganos *et al.*, 2023: n = 4, range = 0.78 to 1.18 µg/g, this work: n = 3, range 0.81 to 1.03 µg/g). These Zn concentrations are in average about one

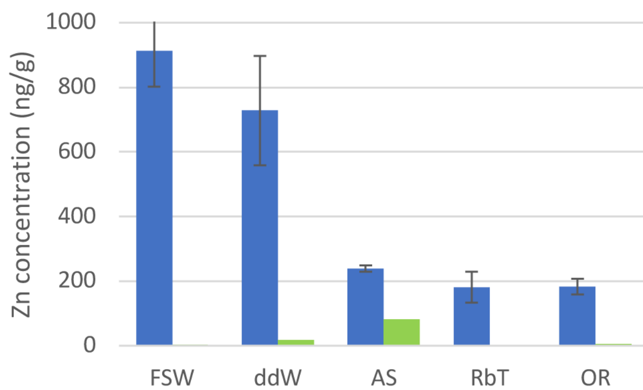

Figure 1. Zn concentrations in water. Concentration of Zn in 10% pre-production PVC nurdle leachates obtained in different natural and artificial waters used to treat *C. intestinalis* embryos (blue) and in their clean (with no pellets added) counterparts (green). FSW: filtered sea water; ddW: double distilled water; AS: Instant Ocean (Aquatic Systems) artificial sea water; RbT: Reef by TMR artificial seawater; OR: Ocean Reef artificial sea water. See Methods for full artificial sea water information.

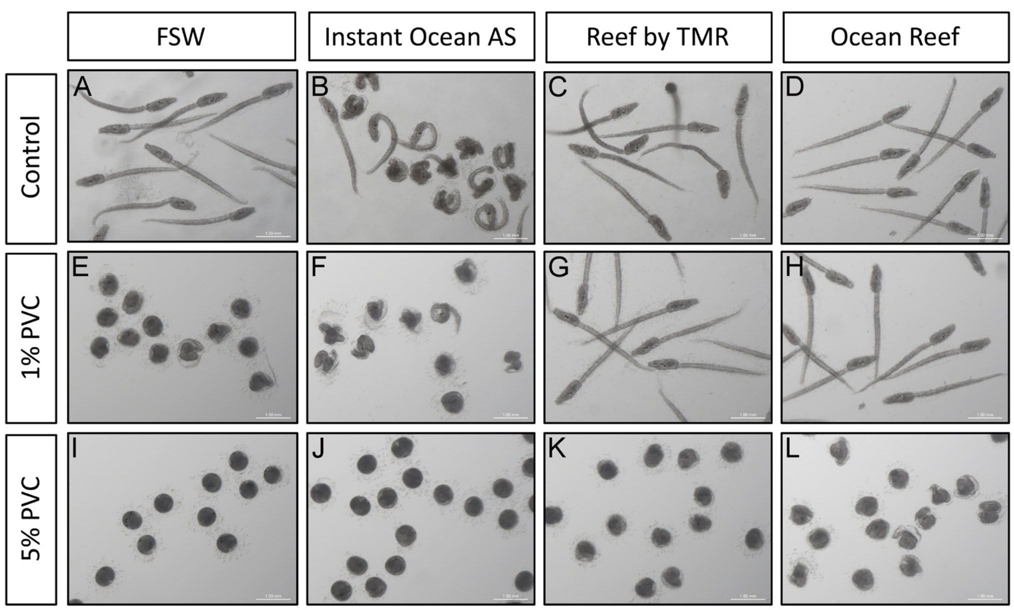

Figure 2. Effect on *C. intestinalis* development. 22 hpf larvae of *C. intestinalis* grown in four types of clean seawater (**A**–**D**) and in 1% (**E**–**H**) and 5% (**I**–**L**) PVC leachates obtained adding PVC pellets to those waters. Scale bar is 1 mm. All animals depicted come from the same batch. Individual panels have been level balanced to unify background colors.

thousand times higher than that found naturally in seawater[21]. However, the concentration of Zn due to PVC leachates found in the water is at least five times less when using any of the ASWs (Figure 1, Extended data, Chemical analysis 1). Of the metals other than Zn that were monitored, the majority (Cd, Co, Cr, Ni, Pb, Sb, Sn, V) was found to be consistently below detection limit in all tested solutions (Extended data, Chemical analysis 2). A few tested solutions showed elevated levels of Cu (n=2; 50 ng/g, 392 ng/g) and Fe (n = 3; 270 ng/g; 330 ng/g; 2,400 ng/g) which is attributed to contamination, as these values are not consistent between solutions previous and after the leaching experiment. An increase of Ba linked to Zn increase has previously been related to the use of Zn-Ba mixed metals as liquid stabilisers. Indeed, the amount of Ba in PVC leachates increases as well in all water samples, except in AS where the baseline Ba content is unusually high. Differences between pre- and post-leaching concentrations are statistically significant at the 95 % confidence level for all treatments apart from AS, indicating that Ba is leached from plastic pellets during the experiment (Extended data, Chemical analysis 2). Water composition for the elements analysed is similar in all seawaters, expect for Mn. where levels above detection limit (2 ng/g) were observed in the majority of solutions but not in samples of de-ionised water. These correlate strongly ($r^2 = 0.99$) between pre- and post-leaching concentrations with a relationship of $Mn_{after\ leaching} = 0.995 * Mn_{before\ leaching}$. This indicates that Mn was introduced via the salt rather than leached from plastic pellets during the experiment (Extended data, Chemical analysis 2). We do not relate this increase to the differences in Zn contents in our different seawater samples. Interestingly, AS has an increased baseline of Zn of at least ten times higher than any of the other sea waters (Figure 1 (green bars)).

Leachates from PVC pre-production nurdles in FSW and ASW cause different intensity of developmental abnormalities in the tunicate *C. intestinalis*

We have previously shown that embryos from several species of marine invertebrates fail to grow properly when left to develop in these pre-production PVC pellet leachates[20]. The tunicate *Ciona robusta* is particularly sensitive and displays aberrant phenotypes at lower concentrations of plastics than other animals. Here, we use the sister species *Ciona intestinalis* to see the effect the leachates in different waters have on embryo development.

C. intestinalis larvae exhibit two primary structures: the trunk, housing the adhesive organ, brain vesicle with pigmented sensory organs (otolith and ocellus), endoderm, and mesenchyme; and a straight tail for locomotion, containing the neural tube, notochord, endodermal strand, and muscles, all enveloped by the larval tunic. All control water treatments except AS displayed a typical trunk and straight tail and vacuolated notochord cells (Figure 2. A, C, D). However, larvae grown in AS ASW displayed strong abnormalities (Figure 2. B): they developed shorter, coiled tails, and abnormal trunks, with misshaped adhesive organs and deformed sensory vesicles. We believe these abnormalities could be due to the high concentration of Zn found in this water (more than 80 ng/g; Figure 1, green bars). Other authors have found similar phenotypes when treating *C. intestinalis* with zinc chloride[22]. Indeed, 1% PVC leachates in FSW displayed aberrations equivalent, if more intense, to the ones described for AS water before (Figure 2. E): the tails were totally coiled, and most larvae had not managed to hatch from the chorions. Despite this, malformed trunks displayed sensory organs, demonstrating that morphogenesis had, to a certain extent, proceeded. These phenotypes were equivalent of those seen before in PVC leachate treated *C. robusta* larvae[20]. 1% PVC leachate treatments in TMR or OR did not give rise to any aberrations in the larvae (Figure 2. G, H), showing that the concentration of Zn in these leachates (of around 18 ng/g) is not enough to create aberrations. Once the concentration of PVC particles increased to 5%, larvae failed to form in all treatments (Figure 2. I–L), resulting in unhatched round individuals with pigmented spots, hinting at otolith and ocellus structures. Despite the strong malformations, larvae grown in 5% PVC leachates in TMR or OR ASW displayed marginally less aberrant structures (Figure 2. K, L).

The aberrations observed in this context follow a pattern of increased malformations with increased concentration of Zn in the water (Figure 1, Figure 2). Hence, the malformations clearly correlate with the amount of Zn present in the leachates. We show that, if artificial sea water is used to obtain the leachates, the phenotypes observed can be less severe than in FSW, putatively leading to false negative results. Moreover, in highly sensitive animals like *C. intestinalis*, variations in the artificial sea water composition, like the one observed for AS which contains increased amounts of Zn, can cause developmental problems that are not due to the treatments but to the experimental setup, potentially leading also to false positive results.

Consequences for plastic contamination research

Extensive efforts are being made to catalogue the consequences of plastic contamination in different animals species, at different life cycle points (adults[11,23–25], embryos[5,6,8,9,13]), using different toxicity routes (ingestion[26–29], leachates[5,8,11,13,16,30–32], contact[33,34]) and physiological states (immunology[11,23,35–38], reproduction[11,24,39], embryology[5,8,9,12,13,16,31] or behaviour[40–42]). A substantial part of this research is laboratory based, and scientists have so far been using indistinctively natural sea water and artificial sea water (formalised in Almeda *et al.*, 2023[17]). Our results here show that research groups studying plastic toxicology may need to carry out additional tests to ensure that the water they are using reflects either environmental conditions or, at least, does not hinder their results and the conclusions they reach. If utilising artificial sea water can change the concentration of Zn retrieved from plastic preproduction pellets, like we demonstrate here, it may have other behaviours that may also change the dynamics of action of plastic leachates for other chemicals, including molecules known to leach from plastics such as PAH, phthalates, PCBs and others[4]. In the case of plastic leachates, if what researchers intend is to mimic environmental conditions, it would be important to ascertain the chemical composition of such waters, and if this is artificial sea water, to be able to contrast it with the composition of leachates obtained in natural sea water. We cannot state the reach of our findings for other types of experiments, for instance when the studied interaction is using other types of plastic particles or nanobeads, but our results would suggest that comparisons between ASW and FSW leachates should be performed when using these other particles in ASW.

Conclusions

Artificial and natural sea water have different behaviours when leaching heavy metals from pre-production PVC particles. This may be extendable to other chemicals leaching from these particles, and also to other particles or types of plastic material. The leachates obtained in artificial and natural seawater using these plastics do not have the same effects on the development of the tunicate *C. intestinalis*. Moreover, some artificial water compositions may not be ideal for embryo-toxicology tests and may lead to increased aberrations. Our findings suggest that artificial and natural sea water types are not comparable, emphasizing the need for caution or additional tests when drawing conclusions from data obtained in artificial seawater.

Ethics and consent

C. intestinalis were collected from Mylor Yacht Harbour (UK) with permission of the company. *C. intestinalis* is not covered under EU Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes nor in the Animals Scientific Procedures Act (ASPA) (UK). Animal handling was in accordance with the guidelines of our academic institutions. The least number of animals were used to perform the experiments while still permitting to have sufficient replicas to validate the results.

Data availability

Figshare: "Extended data for Natural sea water and artificial sea water are not equivalent in plastic leachate contamination studies", https://doi.org/10.6084/m9.figshare.25127069[43].

This project contains the following underlying data:

- Chemical analysis 1 spreadsheet data
- Raw, unedited, uncropped images
- Rebuttal chemical analysis of leachates (Chemical analysis 2)

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Open Peer Review

Current Peer Review Status: ? ✔

Version 2

Reviewer Report 11 September 2024

https://doi.org/10.21956/openreseurope.19843.r42797

?

Atsuko Sato
Department of Biology, Ochanomizu University, Tokyo, Japan

The author's replies are all good and my previous suggestions and comments are all responded. However one thing I still do not understand is why, in Fig 2, B looks very different from A, C and D. According to the author, embryos are collected from the same adults and A-D should look similar as control experiments.
If we get a convincing answer to the difference or better pictures from two other replicates, I will approve this manuscript.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 11 Sep 2024

Eva Jimenez Guri

We thank the reviewer for their positive comments, and would like to clarify their doubts about Figure 2B. Indeed, all larvae in the Figure 2 are from the same fertilisation event. The fact that panel B (control for AS water) does not look like the other controls is because we have found that AS salt has an increase in Zn content as compared with other artificial seawaters and filtered seawater. Because of this, we believe the malformations seen in the AS seawater when no leachates are used is because of this higher concentration of Zn. This is stated in the results when explaining Figure 2 and the phenotypes observed in *C. Intestinalis* larvae:

- However, larvae grown in AS ASW displayed strong abnormalities (Figure 2. B): they developed shorter, coiled tails, and abnormal trunks, with misshaped adhesive organs and deformed sensory vesicles. We believe these abnormalities could be due to the high concentration of Zn found in this water (more than 80 ng/g; Figure 1, green bars).

At the end of this section we also comment on this observation with this discussion:

- Moreover, in highly sensitive animals like *C. intestinalis*, variations in the artificial sea water composition, like the one observed for AS which contains increased amounts of Zn, can cause developmental problems that are not due to the treatments but to the experimental setup, potentially leading also to false positive results.

And in the conclusions we refer to the fact that some artificial seawaters (like AS in the case of this report) may not be suitable for developmental experiments as their salt composition may lead to aberrations that are not linked to the experiment but to the salt used:

- Moreover, some artificial water compositions may not be ideal for embryotoxicology tests and may lead to increased aberrations.

We hope that this clarifies the reviewer's doubts.

Competing Interests: No competing interests were disclosed.

Reviewer Report 06 August 2024

https://doi.org/10.21956/openreseurope.19843.r42798

✔ **Amitava Mukherjee**
Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

My concerns with the previous submission are well addressed by the authors in the revised version. The revised version is acceptable.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Nano ecotoxicology; Toxicloogy of Micro nano plastics;

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 17 June 2024

https://doi.org/10.21956/openreseurope.18495.r41337

? **Amitava Mukherjee**
Centre for Nanobiotechnology, Vellore Institute of Technology, Vellore, Tamil Nadu, India

The problem statement and hypothesis:
Whether the authors exclude the possibility of leaching any organic additives in the current study design and hypothesis should be spelled out. Whether the medium have any role in releasing the organic additives too? If they restrict the study to the effects of heavy metal alone, this should be clearly stated in the title, abstract, and introduction too. "Leachate' term may be used in a wider context and this may be misleading to the readers.

Environmental relevance:
The environmental relevance of the work, and how the differences between three different seawater media would reflect real environmental scenarios and eco-toxicological context can be briefly added. They added a section later in the report and a few lines can be added in the introduction and abstract on this point too.

Methods:
The authors should add briefly the leaching protocol in this study unless the reader is forced to go back to their previous publications.

Results and discussion:
Why did the authors select Zn alone in the results principally? As per their previous study, Zn predominantly came from the PVC, whether changing the ASW mediums had any impact on the leachability of other inorganics. Please comment on this aspect.
In the case of toxicity tests with *Ciona robusta,* whether the authors have used a Zn salt control (standard addition to the medium) with equivalent concentrations to drive home the point.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and does the work have academic merit?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Nano ecotoxicology; Toxicloogy of Micro nano plastics;

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Jul 2024

Eva Jimenez Guri

Reviewer 2 Comment:
The problem statement and hypothesis:
Whether the authors exclude the possibility of leaching any organic additives in the current study design and hypothesis should be spelled out. Whether the medium have any role in releasing the organic additives too? If they restrict the study to the effects of heavy metal alone, this should be clearly stated in the title, abstract, and introduction too. "Leachate' term may be used in a wider context and this may be misleading to the readers.

Author Response: The reviewer is right in this comment. We have focused on the leaching of heavy metals since our previous experiments clearly showed that Zn (and no other heavy metals) were strongly released from these particles onto filtered sea water. Checking for organic and other compounds was not available to us at the moment of this research, but finding this difference on the Zn content already was telling us that there was a difference in the leaching of compounds between ASW and FSW. We have stressed that we focused our experiment on the leachates of heavy metals in the abstract and introduction and emphasised that we have not ruled out the possibility of other molecules, including organic additives, can be leached from the plastics, despite not having measured them in this case.

Reviewer 2 Comment:
Environmental relevance:
The environmental relevance of the work, and how the differences between three different seawater media would reflect real environmental scenarios and eco-toxicological context can be briefly added. They added a section later in the report and a few lines can be added in the introduction and abstract on this point too.

Author Response: We do not believe that the different artificial sea waters could reflect natural environmental scenarios. We wanted to report that artificial sea water in experimental settings working on plastic leachates may not be always reflecting what natural sea water is doing. We have clarified this in the abstract and the introduction as suggested by the reviewer.

Reviewer 2 Comment:
Methods:

The authors should add briefly the leaching protocol in this study unless the reader is forced to go back to their previous publications.

Author Response: We have extended the explanation for the leaching protocol.

Reviewer 2 Comment:
Results and discussion:
Why did the authors select Zn alone in the results principally? As per their previous study, Zn predominantly came from the PVC, whether changing the ASW mediums had any impact on the leachability of other inorganics. Please comment on this aspect.

Author Response: We were conducting experiments on animals reared in ASW and on animals reared in FSW. Only on those animals where the leachates were done in ASW were not presenting clear disruptive phenotypes, whereas when using FSW leachates we did see aberrant phenotypes. We then used different ASW, and the results were different again, where both controls and treated had problems. We then decided to check if this could be due to differences between in the ASW and the FSW, and between different ASWs. We looked at the presence of other heavy metals and other elements in the PVC leachates (Ba, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb, Sn, V, Zn) and did not find any significant difference in the composition for any other than Zn (and Ba and Mn as discussed in the manuscript). However, we did not record all those measurements. We have now looked at the presence of those elements in the different waters analysed (preparing leachates of the PVC pellets in six new independent aliquots of water from each type of salt). We provide the measurements in a new table in the Extended Data, Chemical Analysis 2. We see the same trend as we discussed in the manuscript, except for trace amounts of coper in some of the samples, very close to the limit of detection of the instrument. Moreover, individual aliquots show a spike in some elements (only Fe and Co clearly over the limit of detection of the instrument) for a few samples. These spikes are not uncommon and are attributed to punctual contamination during the manipulation of the samples in the ICP-OES instrument, as these values are not consistent between solutions previous and after the leaching experiment. We have added this information in the manuscript, as well as providing the new analysis in the Extended data, Chemical analysis 2

Reviewer 2 Comment:
In the case of toxicity tests with Ciona robusta, whether the authors have used a Zn salt control (standard addition to the medium) with equivalent concentrations to drive home the point.

Author Response: We have not conducted this experiment. However, there is other literature showing the effects of Zn on *Ciona* showing equivalent phenotypes (Gallo et al 2011, Marine Ecology). This reference is now added to the manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Report 28 April 2024

https://doi.org/10.21956/openreseurope.18495.r39551

?

Atsuko Sato
Department of Biology, Ochanomizu University, Tokyo, Japan

This work is very important in the research field concerning the impact of plastics to the oceans. The results presented were unexpected, and should be made aware to other researchers in the field.

This work presents the results in a qualitative as well as a quantitative way; however, the way of replicates should be clarified. Experiments on embryonic development were performed triplicate; whether this was done with the same batch (cross from eggs from the same mother and sperm from the same father) or different three batches, were not clear. Presumably there are individual differences in susceptibility. In Figure 2, it should also mentioned in the legend whether the pictures are from the same batch or not (it should be the same batch, because of the reason as above). Since the control phenotype in AS is apparently different from TMR or Reef, I was not sure whether we could compare the difference in the impact of leachates between AS and TMR or AS and OR. The large difference in the impact between AS and TMR or AS and OR might be due to difference in the individual batches.

Also, to conclude whether difference in the concentration of Zn affected the results, differences in Zn concentration between AS, RbT and OR needs to be assessed statistically.

Also I should point out that there is a very minor mistake: page 5 left column, 'crosses were done using single mattings' should probably be 'single matings'.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and does the work have academic merit?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Jul 2024

Eva Jimenez Guri

Reviewer 1 Comment:
This work is very important in the research field concerning the impact of plastics to the oceans. The results presented were unexpected, and should be made aware to other researchers in the field.

Author Response: We thank the reviewer for their positive comments about our article.

Reviewer 1 Comment:
This work presents the results in a qualitative as well as a quantitative way; however, the way of replicates should be clarified. Experiments on embryonic development were performed triplicate; whether this was done with the same batch (cross from eggs from the same mother and sperm from the same father) or different three batches, were not clear. Presumably there are individual differences in susceptibility.

Author Response: The triplicates were performed using different adult animals for each replica. This has been clarified in the methods section.

Reviewer 1 Comment:
In Figure 2, it should also mentioned in the legend whether the pictures are from the same batch or not (it should be the same batch, because of the reason as above).

Author Response: The pictures are all from the same batch of animals. This is now stated in the figure legend.

Reviewer 1 Comment:
Since the control phenotype in AS is apparently different from TMR or Reef, I was not sure whether we could compare the difference in the impact of leachates between AS and TMR or AS and OR. The large difference in the impact between AS and TMR or AS and OR might be due to difference in the individual batches.

Author Response: All exposures were done in triplicate with three different batches of eggs and sperm from different adults. The three batches showed the same phenotype, the same susceptibility of AS as compared to the other artificial sea waters or FSW. Therefore we are confident the differences we see are because of three different type of water rather than because of susceptibility of the individual batches.

Reviewer 1 Comment:
Also, to conclude whether difference in the concentration of Zn affected the results,

differences in Zn concentration between AS, RbT and OR needs to be assessed statistically.

Author Response: We have done the analysis of Zn concentrations in the artificial sea waters again, preparing six independent aliquots of water from each type of salt. The Zn concentrations were determined using the 213.857 nm line, as described in the Materials and Methods. The limit of detection was 13 ng/g, so anything below that limit was not quantified and values have been set to zero. The readings are provided in the table below, and the differences between the Zn concentration in the samples of each artificial sea water with no plastic added is significantly different between AS and RbT or OR with a p value of 0.003355 using an ANOVA test. Type of water Zn in AS (ng/g) Zn in RbT (ng/g) Zn in OR (ng/g) Aliquot 1 78 0 0 Aliquot 2 59 0 0 Aliquot 3 0 0 0 Aliquot 4 92 0 0 Aliquot 5 49 0 0 Aliquot 6 0 0 0   We do not want to alter Figure 1 to add these results, as those measurements were done in the waters used for the exposure experiments in the *C. intestinalis* embryos and the bars of the “clean” water correspond to the same water as was used for the preparation of the leachates. Also, note that two of the readings of AS were below the limit of detection. We believe this is because the Zn particles in the salt mix are punctual, so some aliquots of salt may stochastically contain more Zn than others.

Reviewer 1 Comment:
Also I should point out that there is a very minor mistake: page 5 left column, 'crosses were done using single mattings' should probably be 'single matings'.

Author Response: This was indeed a mistake and has now been corrected.

Competing Interests: No competing interests were disclosed.