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Bioaccumulation, release and genotoxicity of stainless steel particles in marine bivalve molluscs

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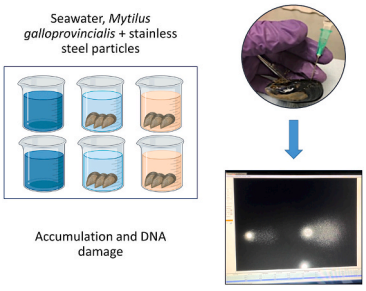
HIGHLIGHTS

- Tissue specific stainless steel particle (SSP) uptake noted in marine mussels.
- SSPs accumulate predominately in the digestive gland and after 5-h exposure.
- Little dissolution of elemental components of steel observed.
- No genotoxic effects on mussel haemocytes detected.
- Further research using tritiated SSPs can be performed to study radiotoxicity.

GRAPHICAL ABSTRACT



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ABSTRACT

During the decommissioning and removal of radioactive material in nuclear facilities, fine, tritiated dusts of stainless steel, cement or tungsten are generated that could be accidently released to the environment. However, the potential radio- and ecotoxicological effects these tritiated particles may have are unknown. In this study, stainless steel particles (SSPs) representative of those likely to be tritiated are manufactured by hydrogenation and their tissue-specific bioaccumulation, release (depuration) and subsequent genotoxic response have been studied in the marine mussel, *Mytilus galloprovincialis,* as a baseline for future assessments of the potential effects of tritiated SSPs. Exposure to 1000 μg L^{-1} of SSPs and adopting Cr as a proxy for stainless steel revealed relatively rapid accumulation (~5 h) in the various mussel tissues but mostly in the digestive gland. Over longer periods up to 18 days, SSPs were readily rejected and egested as faecal material. DNA strand breaks, as a measure of genotoxicity, were determined at each time point in mussel haemocytes using single cell gel electrophoresis, or the comet assay. Lack of chemical genotoxicity was attributed to the rapid processing of SSP particles and limited dissolution of elemental components of steel. Further work employing tritiated SSPs will enable radio-toxicology to be studied without the confounding effects of chemical toxicity.

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1. Introduction

The fate of tritium, an isotope of hydrogen (half-life 12.3 y), in the human body, as tritiated water (HTO) or organically bound tritium (OBT), is well understood, with inhaled HTO translocated quickly into blood and distributed uniformly (Di Pace et al., 2008; Nie et al., 2021). Furthermore, tritium-induced biological effects have been extensively explored in aquatic biota under a range of radiation dose rates (Jha et al., 2005, 2006; Adam-Guillermin et al., 2012; Jaeschke and Bradshaw, 2013; Dallas et al., 2016a,b; Gagnaire et al., 2017; Festarini et al., 2019; Arcanjo et al., 2020). However, there is a gap in understanding related to the environmental fate and subsequent radiotoxicity of tritiated products. Tritiated materials are generated during the normal operation, decommissioning and/or dismantling of nuclear facilities (fusion and fission) (Decanis et al., 2018; Liger et al., 2018). During fusion energy generation, a high amount of tritiated dust can also be formed within the reaction chamber where severe conditions (high temperatures and particle expulsions) erode surrounding walls (Zinkle and Was, 2013; El-Kharbachi et al., 2014; Peillon et al., 2017). From a radiotoxicity perspective, particulates such as tritiated stainless steel, cement or tungsten are a potential hazard to both humans and non-human biota following accidental exposure (ingestion, inhalation or skin contamination).

The first step in understanding potential biological consequences of radioactive particles would be to determine any toxicity of their non-tritiated analogues or carrier particulates while analysing their characteristics using a range of analytical techniques. Many properties of stainless steel, including ready manufacture and high corrosion, impact and heat resistances make it well suited for use in the nuclear industry (Lo et al., 2009; Zinkle and Was, 2013; Fan et al., 2020). Varying forms of stainless steel (such as 304 L and 316 L grades) are predominantly used within containment vessels holding radioactive water or gas (e.g., reactor cooling system pipes and welds, reactor pressure vessel cladding and internal components, and reactor coolant pumps) (Cattant et al., 2008). In terms of environmental impact, and during operational or dismantling procedures, tritiated stainless steel particles (hereafter referred to as SSPs) may enter the atmosphere and hydrosphere via accidental release (Liger et al., 2018).

Comprising mainly iron and chromium (additional components include nickel, copper and molybdenum), SSPs themselves have the potential to cause adverse health impacts when ingested or inhaled, or via exposure to separate elemental components when dissolved in surrounding media (e.g., seawater) (Ortiz et al., 2011; Gissi et al., 2018; Wang et al., 2020). Bivalve molluscs, like *Mytilus galloprovincialis*, are prominent as study species in ecotoxicological and radiobiological research because of their (a) ecological relevance within coastal and marine habitats, (b) sessile nature and ability to rapidly accumulate particulates/contaminants from surrounding media, (c) importance as a major food resource for both humans and non-human biota, and (d) ability to act as a surrogate for investigating vertebrate model (e.g., human and mammalian) toxicity response (Beyer et al., 2017). Consequently, *Mytilus* species have been widely used for ecotoxicological and environmental monitoring studies of various contaminants, including radionuclides (Hagger et al., 2005a,b; Jha et al., 2005; ,Jaeschke and Bradshaw, 2013; Dallas et al., 2016a; Pearson et al., 2018; Vernon et al. 2018, 2020).

In the present study, the bioaccumulation, depuration and toxicity of SSPs was determined in marine mussels (*M. galloprovincialis*) in order to evaluate whether any damage induced by tritiated SSPs in future exposures (as part of a broader programme of research) is attributable to radio- or chemical toxicity. To this end, SSPs were produced and characterised and exposed to *M. galloprovincialis* under controlled conditions. The principal elements in the SSPs were measured in different tissues of the mussel and in filtered and unfiltered aliquots of the exposure medium and DNA damage was assessed using single cell gel electrophoresis, or the comet assay, in mussel haemocytes.

2. Materials and methods

2.1. Chemicals and materials

SSP particles similar in size and characteristics to those generated by cutting using a reciprocating saw (stainless steel 1.4404, AISI 316 L powder) were purchased from Goodfellow Cambridge Ltd, UK. To mimic the tritiation protocol for SSPs developed at CEA Saclay (French Alternative Energies and Atomic Energy Commission), particles were hydrogenated in two steps. Firstly, the thin oxide layer on the surface was reduced at 450 °C for 2 h under an excess of $^1H_2$, and secondly particles were loaded with $^1H_2$ at 450 °C for 4 h. All other chemicals and reagents used in the study were purchased from Fisher Scientific, Anachem, Sigma-Aldrich, VWR or Greiner Bio-One unless stated otherwise.

2.2. Characterisation of SSPs

Characterisation of the hydrogenated SSPs was performed by scanning electron microscopy (SEM; JSM-6010-LA from JEOL), optical microscopy (Malvern® Morphologi G3), X-ray fluorescence (XRF) spectrometry (Niton GOLDD+ XL3t) and a TSI® Aerosizer powder sizer. Microscopy revealed that all hydrogenated particles were spherical, with diameters ranging from 1 to 8 μm, and sizing of about 60,000 particles showed that the distribution could be fitted by a lognormal function with a volume median diameter equal to 4.7 μm and a geometric mean and standard deviation of 4.2 ± 1.35 μm. XRF analysis of about 5 g of SSPs of approximately 5 mm in depth and contained in a polyethylene specimen bag yielded elemental concentrations on a dry weight basis shown in Table 1. The composition of the original particles provided by the manufacturer was: 68.8% Fe, 17% Cr, 10% Ni and 2% Mo: and the two measures are, therefore, very close.

2.3. Modification of SSP elemental composition during exposures

In order to establish whether any chemical changes to the SSPs occurred during the experimental exposures, five 1-L samples of filtered (<10 μm) seawater were spiked with 1000 μg L^{-1} of particles under the conditions described below. The concentration was selected based on projections for further experiments in which SSPs would be tritiated. After 2 d, 6 d and 10 d, four 10 mL aliquots were abstracted: 2 x via pipette and 2 x using a 10 mL syringe attached to a 0.20 μM filter (Fisherbrand™ Sterile PES Syringe Filter). Filtered and unfiltered seawater samples were added to 40 mL aqua regia for digestion and after at least 48 h were analysed using an iCAP RQ ICP-MS Qtegra software (Thermo Fisher Scientific, UK). The instrument was calibrated externally using matrix matched standards and internally by the addition of 115-indium and 193-iridium to all samples and standards.

2.4. Exposures of mussels to SSPs

M. galloprovincialis (shell length ~ 45 mm) were collected and maintained in accordance with other studies from our laboratory (Dallas

Table 1
Concentrations of the different elements present in the SSPs analysed by XRF spectroscopy. Data are presented as mean (%) ± 2 standard deviations.

Element	Concentration ± 2σ
Fe	68.6 ± 0.11
Cr	14.9 ± 0.055
Ni	13.1 ± 0.087
Mn	1.50 ± 0.046
Mo	1.29 ± 0.090
Cu	0.463 ± 0.026
V	0.0850 ± 0.006
W	0.0600 ± 0.0170

et al., 2016b; Pearson et al., 2018). In brief, mussels were maintained in UV-treated, filtered, aerated, natural seawater (salinity = 31.8, pH = 7.9) under a 12:12 h photoperiod at 15 °C and were fed a solution of *Isochrysis galbana* algae (8×10^5 cells mL^{-1}, Reed Mariculture, Campbell, CA, USA).

Twenty, 2 L beakers containing 1.5 L of seawater and three mussels each were used for the exposures. Five different periods of exposure were selected: 5 h, 3 d, 7 d, 11 d and 11 d plus a 7-d period of depuration. For each period, two beakers (or six mussels in total) were dosed with SSPs to a nominal concentration of 1000 μg L^{-1} (and derived from a stock of 1 g L^{-1} in deionised water), and two (with six mussels in total) served as controls. An additional two beakers with three mussels each, and including one control, were prepared for 11-d exposures and subsequent electron microscopic analysis (see below). Water parameters (pH, salinity, temperature, dissolved oxygen) were measured every alternate day and water changes (50%) were performed on days 3, 5, 7, 9, 11 and following the 11 d exposures on days 13, 15 and 17. Mussels were fed (as above) 1 h before each water change and appropriate quantities of SSP were added to maintain the nominal concentration.

2.5. Mussel tissue sampling and analysis

Tissue-specific bioaccumulation and genotoxicity in the haemocytes were measured for the different exposure periods. Haemolymph was extracted from mussels in each beaker by inserting a 25-gauge needle attached to a 1 mL syringe (BD Microlance, Fisher Scientific Ltd.) into the adductor muscles. Haemolymph was stored in a tube on ice until experimental use. Mussels were then dissected into individual tissues (gill, mantle, digestive gland, adductor muscle, foot and other soft tissue), with samples blotted on paper to remove excess water and stored in a series of 50 mL Fisher Scientific Falcon tubes. Meanwhile, seawater from each beaker was passed through a 250 μm sieve (Fisherbrand, ISO 3310/1) and faeces and pseudofaeces retained were transferred to a series of Falcon tubes.

The SSP content in mussel faecal matter and mussel tissues was evaluated by measuring the concentrations of elements found in the SSPs (Table 1). Dissected tissues and faecal samples were dried at ~70 °C for 48 h before being weighed and added to 5 mL aqua regia (1:3, HNO_3 and HCl) in a series of 50 mL Falcon tubes along with six procedural blanks and a certified reference material (TORT-2, lobster hepatopancreas). Samples were boiled in a water bath at 70 °C until tissues were fully digested. Once cooled, digests were diluted to 20 mL with Milli-Q water and stored at room temperature before being analysed by ICP-MS or ICP-OES as above.

2.6. Genotoxic response: Comet assay

Prior to performing the genotoxic assay, cellular viability was determined using the Trypan Blue exclusion dye assay (Strober, 2001; Vernon and Jha, 2019). Ten μL of haemolymph was gently mixed with 1 μL Trypan blue (0.40%) before the solution was smeared onto a microscope slide and a coverslip applied. Cells examined under light microscopy (x 40, 100 cells per individual), revealed >90% viability (data not shown), allowing DNA damage to be determined using the comet assay.

The procedure to prepare slides for analysis of DNA strand breaks has been described elsewhere in detail (Dallas et al., 2013; Vernon et al., 2020). Briefly, 150 μL haemolymph from six individuals was centrifuged (775 g, 2 min), with the supernatant subsequently removed and the remaining cellular pellet (~10 μL) used for the assay. Being a known reference genotoxic agent, hydrogen peroxide (H_2O_2) was employed as a positive control, where 150 μL haemolymph collected from six healthy mussels was exposed to 500 μM H_2O_2 in phosphate buffer saline (1 h and in the dark) and subsequently processed as above. Cells were stained with GelRed® (10X, Cambridge Bioscience) and scored using an epifluorescent microscope (DMR; Leica Microsystems, Milton Keynes, UK). One hundred cells per slide (50 cells per microgel) were quantified using the Comet IV imaging software (Perceptive Imaging, Bury St Edmunds, UK). The software provides results for different parameters, with % tail DNA considered the most reliable to present the results (Kumaravel and Jha, 2006).

2.7. TEM, STEM and EDS analysis in digestive gland and gill tissue

The characteristics (e.g., distribution, size, chemical properties) of accumulated particles within specific mussel tissues were investigated by transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS). Due to logistical constraints, analysis was limited to the digestive gland and gill tissues collected from six mussels on day 11 of the exposure period.

Small tissue sections were immersion-fixed in glutaraldehyde (2.5% pH 7.2, 0.1 M) for 15 min and stored at 4 °C until use. Samples were rinsed in duplicate in sodium cacodylate buffer (pH 7.2, 0.1 M, 15 min) and post-fixed with osmium tetroxide (1%, in buffer, pH 7.2, 0.1 M, 1 h). Tissues were dehydrated through an ethanol series (30, 50, 70, 90 and 100%, 15 min per series), with the final step repeated twice. Ethanol was replaced with Agar low viscosity resin at increasing concentrations (3:7, 5:5, 7:3, 10:0 resin: ethanol, 12 h per series), with the final step repeated twice. Tissue samples were placed in Beem capsules and put into an embedding oven and the resin polymerised at 60 °C overnight. Resulting blocks were sectioned with a Leica Ultracut E ultra microtome using a diatome diamond knife (80 nm). Ultrathin sections were collected on copper grids (200 mesh thin bar; Agar Scientific) and placed in a DEBN STEM holder and examined in a JEOL 7001 FEG-SEM using a DEBN STEM detector, with EDS data collected using an Oxford Instruments spectrometer and Aztec software.

2.8. Statistical analysis

Statistical analyses were performed using R (RStudio, R 3.4.3 GUI 1.70 El Capitan build (7463), https://www. r-project.org/). Data normality was tested using the Shapiro- Wilk test and homogeneity of variances using Levene's test. Differences between controls and treatment samples and between tissues were determined using ANOVA with a Tukey's post hoc test or a Wilcoxon rank sum test with Holm-Bonferroni correction, and the strengths of relationships between variables were defined by Pearson's correlation coefficients. For all tests, significance levels were set at $\alpha = 0.05$, 0.01 or 0.001, and data are presented as mean ± standard deviation or mean ± standard error.

3. Results

3.1. Measured elemental concentrations in the exposure medium

Table 2 shows the nominal concentrations of the different elemental constituents of the SSPs used in the exposures (but in the absence of mussels) and based on information given in Table 1, along with measured concentrations in filtered and unfiltered aliquots of the seawater exposure medium. Measured concentrations of Fe, Cr, Ni and Mn are close to nominal concentrations in unfiltered aliquots in some cases, but overall, measurements are variable within and between different time periods. It should be noted, however, that inter-element ratios are more consistent throughout the time course in most cases (for example, the ratio of Fe to Cr is about 4:1 to 5:1). These observations may reflect some particle settlement and adherence to container walls, although measured concentrations exceeding nominal concentration in many cases suggests it is more likely associated with the non-homogenous distribution of SSPs within the exposure medium or imperfect subsampling from the containers. For Mo, measured concentrations are substantially lower than the nominal concentration. This could reflect a lower recovery of Mo from suspended SSPs by aqua regia than other elements, or an overestimation of the Mo content by the XRF.

Table 2
Nominal and measured concentrations (in μg L^{-1}) of different elements in the exposure medium (1000 μg L^{-1} SSPs in filtered seawater) but in the absence of mussels at three time intervals. Errors are standard deviations about the means of five independent determinations.

Element		Day 2		Day 6		Day 10	
		Unfiltered	Filtered	Unfiltered	Filtered	Unfiltered	Filtered
Fe	Nominal	686	0	686	0	686	0
	Measured	826 ± 49.3	25.6 ± 1.7	605 ± 217	0.2 ± 0.0	1190 ± 217	28.0 ± 10.8
Cr	Nominal	149	0	149	0	149	0
	Measured	153 ± 16.4	2.6 ± 0.8	123 ± 68.6	<0.1 ± 0.0	300 ± 62.6	3.0 ± 0.5
Ni	Nominal	131	0	131	0	131	0
	Measured	126 ± 33.5	1.7 ± 0.2	76.0 ± 8.4	<0.1 ± 0.0	132 ± 25.2	2.2 ± 0.7
Mo	Nominal	129	0	129	0	129	0
	Measured	24.4 ± 3.8	0.7 ± 0.2	20.2 ± 1.93	3.8 ± 2.5	31.3 ± 6.60	0.1 ± 0.6
Mn	Nominal	15.0	0	15.0	0	15.0	0
	Measured	25.5 ± 6.56	0.7 ± 0.2	20.0 ± 8.8	0.7 ± 0.2	24.9 ± 4.7	0.8 ± 0.3

Regarding the filtered aliquots, nominal (or theoretical) concentrations in seawater are 0 μg L^{-1} for conservative and non-dissolving particles. Measured concentration, which are <3% of unfiltered measured concentrations in most cases, indicate that there is some elemental dissolution from SSPs into ionic form or some contamination from small (colloidal) SSPs that are not captured by the filtration process.

3.2. Tissue-specific accumulation of SSPs

During the exposure periods, no spawning or mortality were observed and mussels appeared to be actively feeding following dosing with SSPs. This suggests that mussels were not subject to stress under the experimental conditions that could have confounded interpretation of biological measurements and responses.

Based on the observations above, Cr was selected as a proxy element for the accumulation of SSPs by the mussels, and results of Cr analysis in the different tissues of the animal are shown in Fig. 1. Concentrations of Cr in the exposure were significantly greater than corresponding concentrations in the controls for each tissue type only after the initial sampling (5 h), with subsequent times revealing a reduction in concentration to levels no different from the controls. At 5 h, the greatest, but most variable, accumulation was noted in the digestive gland (mean ~ 180 μg g^{-1} dry weight). When compared with corresponding controls, the greatest increase was noted in the foot (>70-fold increase) with gill tissue exhibiting the smallest increase (about two-fold). After the 7-day depuration period, mean Cr concentrations were amongst the lowest measured and were similar to or below the corresponding controls in all tissues.

For logistical reasons, the characteristics of accumulated SSPs within the gill and digestive gland was investigated by TEM-EDS on day 11 of the exposure rather than after 5 h and when accumulation appeared to be greatest. Imagery and spectra revealed no detectable SSP in either exposed or control samples.

3.3. SSPs in faeces and pseudofaeces

Chromium concentrations in marine mussel faecal matter are presented in Fig. 2. After 5 h, no increase relative to the controls was noted. However, significant increases were observed at all other time-points, with mean Cr concentrations in the 1000–1700 μg g^{-1} range in the exposures compared with concentrations below 100 μg g^{-1} in the controls.

3.4. Genotoxic response: Comet assay to determine DNA strand breaks

The % tail DNA damage in the haemocytes of *M. galloprovincialis* following exposure to SSPs at multiple time-points is presented in Fig. 3. Hydrogen peroxide (H_2O_2) used as a concurrent positive control induced significant ($p < 0.01$) damage at all time-points (note, however, that the control was lost on day 3). Although no significant differences were evident between the (negative) controls and SSP treatments at all time-points, mean DNA strand breaks increased relative to the controls on

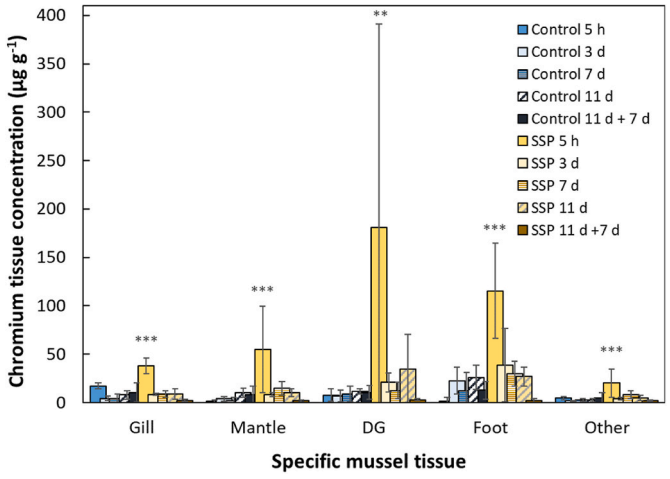

Fig. 1. Tissue specific accumulation of Cr in *M. galloprovincialis* over multiple time points (5 h, 3 d, 7 d, 11 d and plus 7 d) in control and exposed treatments. Errors are standard deviations about the mean of five independent determinations. Asterisks (*, ** or ***) indicate significant differences ($p < 0.05$, 0.01 or 0.001, respectively) from the corresponding controls.

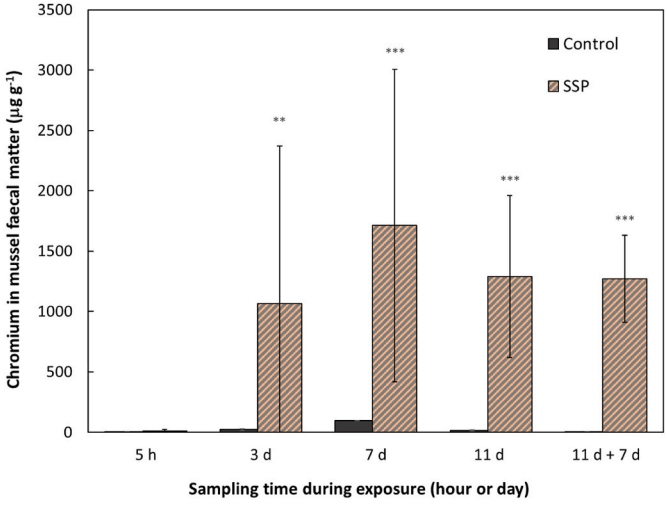

Fig. 2. Concentrations of Cr in *M. galloprovincialis* faecal matter (dry weight) over multiple time points (5 h, 3 d, 7 d, 11 d and 11 d plus 7 d depuration) in control and exposed treatment. Errors are standard deviations about the mean of five independent determinations. Asterisks (*, ** or ***) indicate significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding controls.

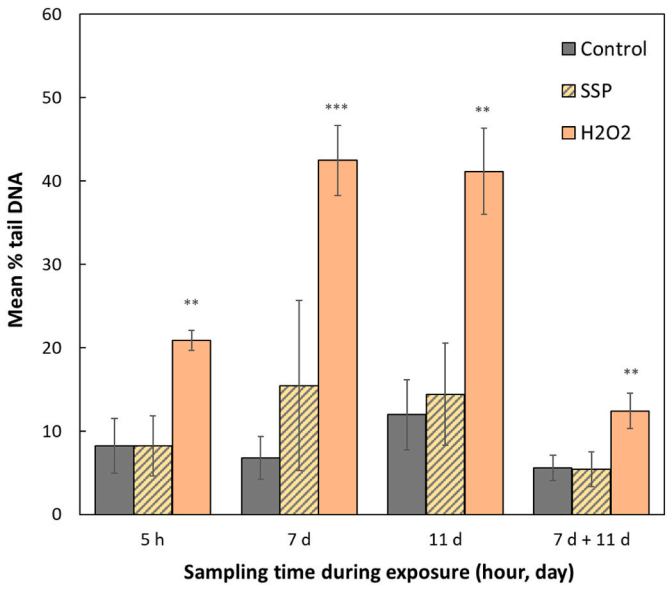

Fig. 3. Genotoxic effects in *M. galloprovincialis* haemocytes at multiple time-points following exposure to 1000 μg L^{-1} SSPs and 500 μM hydrogen peroxide (H_2O_2). Errors are standard deviations about the mean of six independent determinations. Asterisks (*, ** or ***) indicate significant differences ($p < 0.05$, 0.01 or 0.001, respectively) from the corresponding controls.

days 7 and 11 (averaging 15 and 14% tail DNA, respectively).

4. Discussion

Our results show that uptake of SSPs, using Cr as a proxy, occurs soon after exposure with a spike in tissue concentrations after 5 h (Fig. 1). The highest concentrations observed in the digestive gland and relatively low concentrations in the gill suggest that ingestion is the primary route of assimilation (Gagnon et al., 2006; Naimo, 1995). These observations also suggest that Cr (and other elements) are largely taken up in particulate form (Rivera-Hernández et al., 2019), consistent with relatively little dissolution of SSPs during the experiment. Note that, presumably, concentrations observed in the foot and mantle after 5 h reflect the adherence of fine particles to these tissues.

In bivalves, particles in the gill are trapped in a mucous layer and transported to the labial palps, where they are captured and ingested or rejected and released as pseudofaeces (Ward et al., 2003). Previous studies have also shown that mussels may feed selectively and sort particles by size and surface properties (e.g., wettability and charge) before ingestion (Beck and Neves, 2003; Rosa et al., 2013). Lack of accumulation of SSPs beyond the first time point, evident in our Cr results and TEM-EDS analyses on day 11, and a subsequent dramatic increase in SSPs in faeces and pseudofaeces suggests that captured particles are readily rejected or egested after ingestion. Fernández and Albentosa (2019) recently noted that *M. galloprovincialis* exposed to microplastics of a similar size range to the SSPs (2 μm–10 μm) began to eliminate particles within a few hours. SSPs still evident in faecal matter after a 7-d period of depuration, however, suggests rejection or egestion proceeds over extended times. Because previous studies have noted that heavier, denser particles (the density of stainless steel is about 8 g cm^{-3}) are more likely to fall onto the sorting digestive tracts and ciliary gutter and be rejected faster than lighter ones (Brillant and MacDonald, 2000; Fernández and Albentosa, 2019), it is assumed that the egestive route for SSPs is the lengthier process. It is worth noting that accumulation of SSPs may have also taken place after each water change and SSP dosing, since mussels were actively feeding throughout the exposure period, but detection may have been evaded by the lower temporal resolution of sampling beyond 5 h.

The individual and, likely, combined elemental components of SSPs have the potential to induce a toxic response in aquatic biota (Naimo, 1995; Punt et al., 1998; Ortiz et al., 2011; Singh et al., 2019). However, levels observed in the filtered samples in the present study, as either colloids or ions, are well below toxic concentrations in aquatic systems, and the oxidation state of Cr (III) derived from corroding stainless steel is not as harmful as the higher oxidation state (VI) (Walsh and O'Halloran, 1997). Specifically, maximum measured filterable concentrations for Fe, Cr, Ni, Mo and Mn are 28, 3.0, 2.2, 3.8 and 0.8 μg L^{-1}, respectively, while toxic responses to mussels from these and more toxic metals are observed at concentrations at least an order of magnitude greater (Kraak et al., 1994; Timpano et al., 2022). There may be some additional, localised solubilisation in the mildly acidic guts of the mussel (Griscom and Fisher, 2004) but any dissolution does not appear to cause any genotoxic response. More generally, SSPs and their constituents are known to be of very low bioavailability and toxicity, characteristics that partly results from formation of a chromium (III) oxide passivation layer (Santonen et al., 2010).

In demonstrating that small, dense steel particles are not inherently toxic towards marine mussels, further studies employing tritiated steel particles representative of those derived during the operation, decommissioning and/or dismantling of nuclear facilities will enable radiotoxicity (rather than chemical toxicity) to be ascertained. To this end, impacts on various levels of biological organisation, from individual, to population to ecosystem can be studied. Based on the physical observations of the present study, we would anticipate any radio-toxicity from particulate tritium to occur over relatively short periods of time because particles are rapidly taken up and processed, with further toxicity arising from exposure to any tritium solubilised in the seawater medium or the digestive conditions of the animal.

Credit statement

EV: conceptualization; methodology; investigation; formal analysis; writing – original draft; writing – review and editing. AJ: conceptualization; writing - review and editing; project management. MFF: methodology; investigation. DS: writing – review and editing. VM: writing – review and editing; project management. CG: project management. MP: methodology; investigation. AT: conceptualization; methodology; investigation; formal analysis; writing – original draft; writing – review and editing; project management.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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