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# Bioaccumulation of particles

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**Summary**

This report describes the studies performed with hydrogenated (untritiated) steel and cement particles (H-SSP and HCP respectively). Particle behaviour and characterisation of these particles in seawater evidenced the presence of nanosized products. The tissue specific bioaccumulation for these particles in the marine bivalve, Mytilus galloprovincialis was analysed for acute (5 h) and chronic (11 days) exposure scenarios. Exposures to H-SSP and H-CP showed that particles accumulate mainly in the digestive gland (DG). Both particles induced DNA damage as determined by single cell gel electrophoresis (SCGE) or comet assay in DG cells after 5 h and 11 days exposures. Particle bioaccumulation was also analysed in algae (Isochrysis galbana, food for mussels) using transmission electron microscopy (TEM) and inductively coupled plasma mass spectrometry (ICP-MS). Whilst the accumulation of these particles in the algal cells using ICP-MS was not evident, the TEM studies suggested that these particles have the potential to accumulate in the algal cells, indicating the possibility of biomagnification through the food chain.

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# Abbreviation and Acronyms

| Acronym | Description |
|---|---|
| WP | Work package |
| H-SSP | Hydrogenated stainless steel particles |
| H-CP | Hydrogenated cement particles |
| AM | Adductor muscle |
| DG | Digestive gland |
| ICP-MS | Inductively coupled plasma mass spectrometry |
| F | Filtered |
| NF | Non-filtered |
| NTA | Nanoparticles tracking analysis |
| TEM | Transmission electron microscopy |

# Executive Summary

This report describes the studies performed with hydrogenated (untritiated) steel and cement particles (H-SSP and HCP respectively). Particle behaviour and characterisation of these particles in seawater evidenced the presence of nanosized products.

The tissue specific bioaccumulation for these particles in the marine bivalve, *Mytilus galloprovincialis* was analysed for acute (5 h) and chronic (11 days) exposure scenarios. Exposures to H-SSP and H-CP showed that particles accumulate mainly in the digestive gland (DG). Both particles induced DNA damage as determined by single cell gel electrophoresis (SCGE) or comet assay in DG cells after 5 h and 11 days exposures. Particle bioaccumulation was also analysed in algae (*Isochrysis galbana*, food for mussels) using transmission electron microscopy (TEM) and inductively coupled plasma

mass spectrometry (ICP-MS). Whilst the accumulation of these particles in the algal cells using ICP-MS was not evident, the TEM studies suggested that these particles have the potential to accumulate in the algal cells, indicating the possibility of biomagnification through the food chain.

## Keywords

Marine mussels; hydrogenated stainless steel; hydrogenated cement; DNA damage; bioaccumulation; algae.

## Introduction

Tritium (3H) is present in nuclear energy production system such as fission and fusion nuclear power reactors. Its release in the environment and potential impact on humans and non-human biota represents an increasing concern due to the advancement of nuclear technology(Ferreira et al., 2023; Liger et al., 2018). During the decommissioning and normal operation of nuclear facilities, dusts from building materials such as stainless steel and cement are generated and subsequently dispersed in the environment. Often these materials can be associated with tritium producing tritiated stainless steel (T-SSP) and cement particles (T-CP) (Lamartiniere et al., 2022; Liger et al., 2018; Mentana et al., 2022; Smith et al., 2022) . Concerns are for aquatic biota as nuclear power plants are often located near water bodies (Bondareva et al., 2022; Ding et al., 2019; Khamis & Kavvadias, 2012) and cooling water is considered to be one of the major sources of 3H (Dallas et al., 2016). Bivalve molluscs, like *Mytilus galloprovincialis*, are important group of marine organisms. They are prominent species in ecotoxicological and radiobiological studies 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 mammals) toxicity

response (Beyer et al., 2017). Consequently, *Mytilus* species have been widely used for ecotoxicological and environmental monitoring studies of various contaminants, including radionuclides (Dallas et al., 2016; Hagger et al., 2005; Jaeschke & Bradshaw, 2013; Jha et al., 2005; Pearson et al., 2018; Vernon et al., 2020a,b; Vernon et al., 2018).

In this context, the task 3.3 "Potential biological effects of tritiated particles in mussels: individual to population level effects and food chain transfer" of the TITANs project aims to assess the potential impact of these particles on the environment. The study evaluated responses at different levels of biological organisations (i.e. tissue-specific bioaccumulation, DNA damage) in *M. galloprovincialis* under defined laboratory conditions. Characterisation of these particles (i.e. steel and cement) including their elemental composition, behaviours in seawater were also assessed through different techniques (ICP-MS, NanoSight, Electron microscopy).

These data will be essential to model food chain transfer and determine potential population level effects on mussels.

# 1 Particles characterisation

## 1.1 Particle composition (ICP-MS)

In order to establish whether any chemical changes to the particles occurred during the experimental exposures, (three) 10 mL samples of filtered (level of filtration: < 0.2 µm) seawater were spiked with 1000 µg $L^{-1}$ of hydrogenated stainless steel (H-SSP) and hydrogenated cement particles (H-CP). The concentration was selected based on projections for further experiments in which H-SSPs would be tritiated. Four 10 mL aliquots were collected after 5h and 48h, considering the minimum time of exposure and the time between water changes respectively. Two aliquots were collected *via* pipette and two using a 10 mL syringe attached to a 0.20 µm filter (Fisherbrand™ Sterile PES Syringe Filter). Filtered and unfiltered samples (10 mL) were transferred to falcon tube (50mL), digested (48h) with aqua regia

(1:3, $HNO_3$ and HCl) and then diluted to a final volume of 50 mL. These samples 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 Indium (115In) and Iridium (193Ir) to all samples and standards.

The results suggested that the H-SSP leached very little into solution and that the number of elements found in stock/seawater were in line with previous results (Table 1). Chromium (Cr) was selected as a H-SSP tracer for bioaccumulation studies according to the particle's elemental composition and its low background level in seawater. Conversely, H-CP showed a rapid dissolution and elements leaching into solution. Titanium (Ti) was selected as a tracer for bioaccumulation studies according to the particle's elemental composition and its low background level in seawater (Table 1).

**Table 1. Measured concentrations (in μg L-1) of different elements in the exposure medium (1000 μg $L^{-1}$, hydrogenated stainless steel particles [H-SSP] or hydrogenated cement particles [H-CP] in filtered seawater) but in the absence of mussels after 48h. Errors are standard deviations (SD) for the means of three independent determinations. NF= Non-filtered samples; F=Filtered (0.20 μm) samples; %D: Percentage of dissolution.**

| | | 5 h | | 48 h | |
|---|---|---|---|---|---|
| H-SSP | | Cr | Ni | Cr | Ni |
| | NF | 209.69 ± 21.31 | 33.0197 ± 2.35 | 167.094 ± 3.68 | 104.42 ± 0.66 |
| | F | 3.545 ± 0.87 | 6.920 ± 4.21 | 0.45 ± 0.45 | 3.55 ± 2.24 |
| | % D | 1.69 | 20.96 | 0.27 | 3.40 |
| H-CP | | Ti | Al | Ti | Al |
| | NF | 7.85 ± 4.42 | 64.48 ± 9.82 | 11.34 ± 7.21 | 88.50 ± 13.70 |
| | F | 6.40 ± 1.050 | 54.36 ± 4.82 | 10.074 ± 4.86 | 78.36± 15.80 |
| | % D | 81.48 | 84.31 | 88.80 | 88.54 |

## 1.2 Nanoparticle Tracking analysis (NTA, Nanosight)

In order to characterise and determine the hydrodynamic diameter of particles, stock solutions of the particles (i.e., H-SSP and H-CP) were prepared and analysed through Nanoparticle Tracking Analysis (NTA, Nanosight, LM10, Version 2.2).

A stock solution of H-SSP (1 mg $mL^{-1}$) was prepared with 10 mg of H-SSP in 10 mL deionised water in Falcon tubes. The stock solution of H-CP (200 mg $L^{-1}$ ) was prepared in seawater and HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) buffer was added to a final concentration of 10 mM to stabilise the pH. Non-filtered and 1 mL filtered water samples (0.45 mm) from each stock solution were processed in duplicate.

The analysis of the H-SSP filtered stock solution showed that the most abundant particle diameter was ~0.140 µm.  In the non-filtered stock solution, however, two particle populations centred around ~0.250 µm and ~1.500 µm (Figure 1 A). In the H-CP stock solution, different particle sizes were detected between 0.05 µm and 0.4 µm. Particles of ~0.190 µm diameter presented the highest concentration in the stock solution.

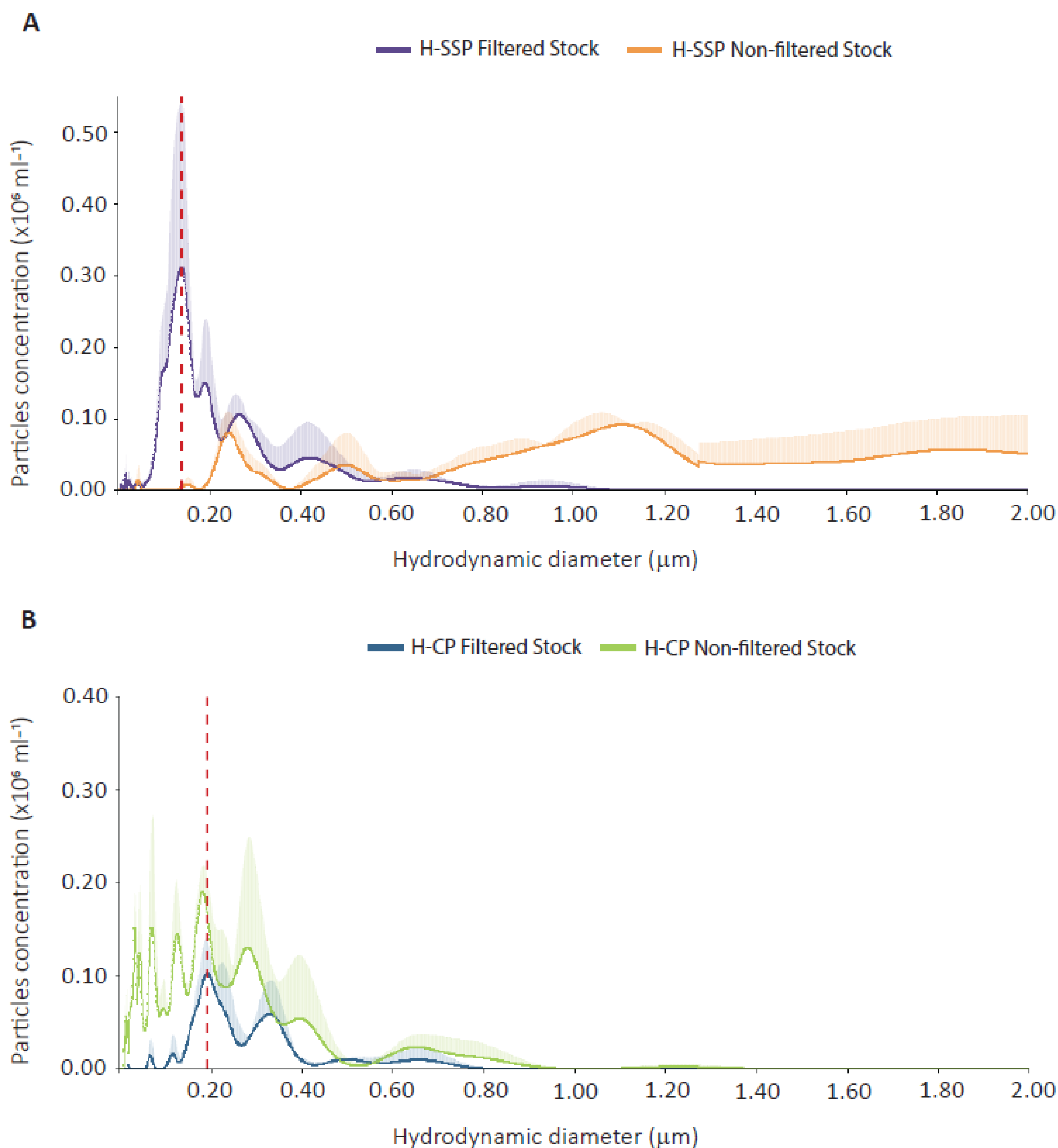


**Figure 1. The hydrodynamic diameter of (A) hydrogenated stainless steel particles (H-SSP) and (B) hydrogenated cement particles H-CP filtered (0.45 µm) and non-filtered stocks solutions in seawater measured with Nanoparticle Tracking Analysis (Nanosight). The filled area corresponds to the standard deviation of the measurements and the dashed line shows the most abundant particle size in the H-SSP and H-CP filtered stocks solutions (~0.140 µm and 0.190 µm, respectively).**

# 2 Particles bioaccumulation in mussels (ICP-MS)

## 2.1 Mussels exposure to hydrogenated stainless steel particles (H-SSP)

Mussels, *M. galloprovincialis* (shell length ~ 45 mm), were collected and maintained in accordance with previous studies from our laboratory (Dallas *et al.*, 2016; Pearson *et al.*, 2018). In brief, mussels were maintained in UV-treated, filtered (<10 µm), 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 (1 x $10^5$ cells $mL^{-1}$, Reed Mariculture, Campbell, CA, USA). In order to examine the tissue-specific bioaccumulation of H-SSP, different exposure scenarios were selected. Mussels were exposed 5 h (acute exposure) and 11 days (chronic exposure) to a nominal concentration of 1 mg $L^{-1}$ of H-SSP (derived from a stock solution of 1 g $L^{-1}$ of the H-SSP in MiliQ water).

Two beakers (or six mussels in total) were used for each treatment and a seawater control was run alongside. Water parameters (pH, salinity, temperature, dissolved oxygen) were measured every alternate day. In the 11-days exposure, water changes (100%) were performed on days 3, 5, 7, 9. Mussels were fed (as above) 1 h before each water change and appropriate quantities of H-SSP were added to maintain the nominal concentration (1 mg $L^{-1}$). No spawning of the mussels occurred during the exposure period indicating that the mussels were not stressed.

Water samples at the beginning of the 5h-exposure showed that Cr concentration was 117.2 ± 17.37 µg $L^{-1}$, which is in line with the expected Cr concentration considering H-SSP particle composition (Slomberg et al., 2023). At the end of the 5h exposure, water samples showed a concentration of 31.53 ± 3.10 µg $L^{-1}$ and suggest that most of the particles have been up taken by mussels. This was later confirmed by the tissue bioaccumulation

results (Figure 2A). In the present study, particle settlement at the bottom of the beakers (i.e. sedimentation) was not measured as the objective was to assess the particles bioaccumulation in the mussel tissues. The influence of this phenomenon in particle uptake cannot be excluded bearing in mind some of them could also be attached to the glass walls.

Results of water samples during the 11 days experiment are presented in Table 2. Using Cr as a tracer of the H-SSP, it is evident that the concentration decreases after each water change (Table2).

**Table 2. Cr concentration (µg L-1) in filtered (F, 0.2 µm) and non-filtered (NF) water samples during the 11 days experiment, at the beginning (day 1), before and after each water change (days 3,5,7,9) and at the end of the experiment (day 11).**

| Time point | Sample type | Cr concentration | |
|---|---|---|---|
| Day 1 | NF | 158.33 ± 48.3 | |
| | F | 6.76 ± 5.09 | |
| Time point | Sample type | Before | After |
| Day 3 | NF | 13.69 ± 5.58 | 183.90 ± 53.70 |
| | F | 11.83 ± 7.05 | 7.25 ± 1.00 |
| Day 5 | NF | 10.72 ± 1.90 | 220.01 ± 22.6 |
| | F | 12.26 ± 6.24 | 9.58 ± 1.52 |
| Day 7 | NF | 81.73 ± 6.81 | 154.30 ± 22.80 |
| | F | 26.53 ± 5.11 | 4.71 ± 0.70 |
| Day 9 | NF | 18.037± 2.55 | 136.78 ± 37.80 |
| | F | 2.91 ± 6.04 | 2.76 ± 3.62 |
| Time point | Sample type | Cr concentration | |
| Day 11 | NF | 3.68 ± 1.28 | |
| | F | 2.81 ± 5.54 | |

Bioaccumulation results showed that H-SSP accumulate in the digestive gland as evidenced by the higher concentration of Cr (H-SSP tracer) in DG compared to the controls (Figure 2A).

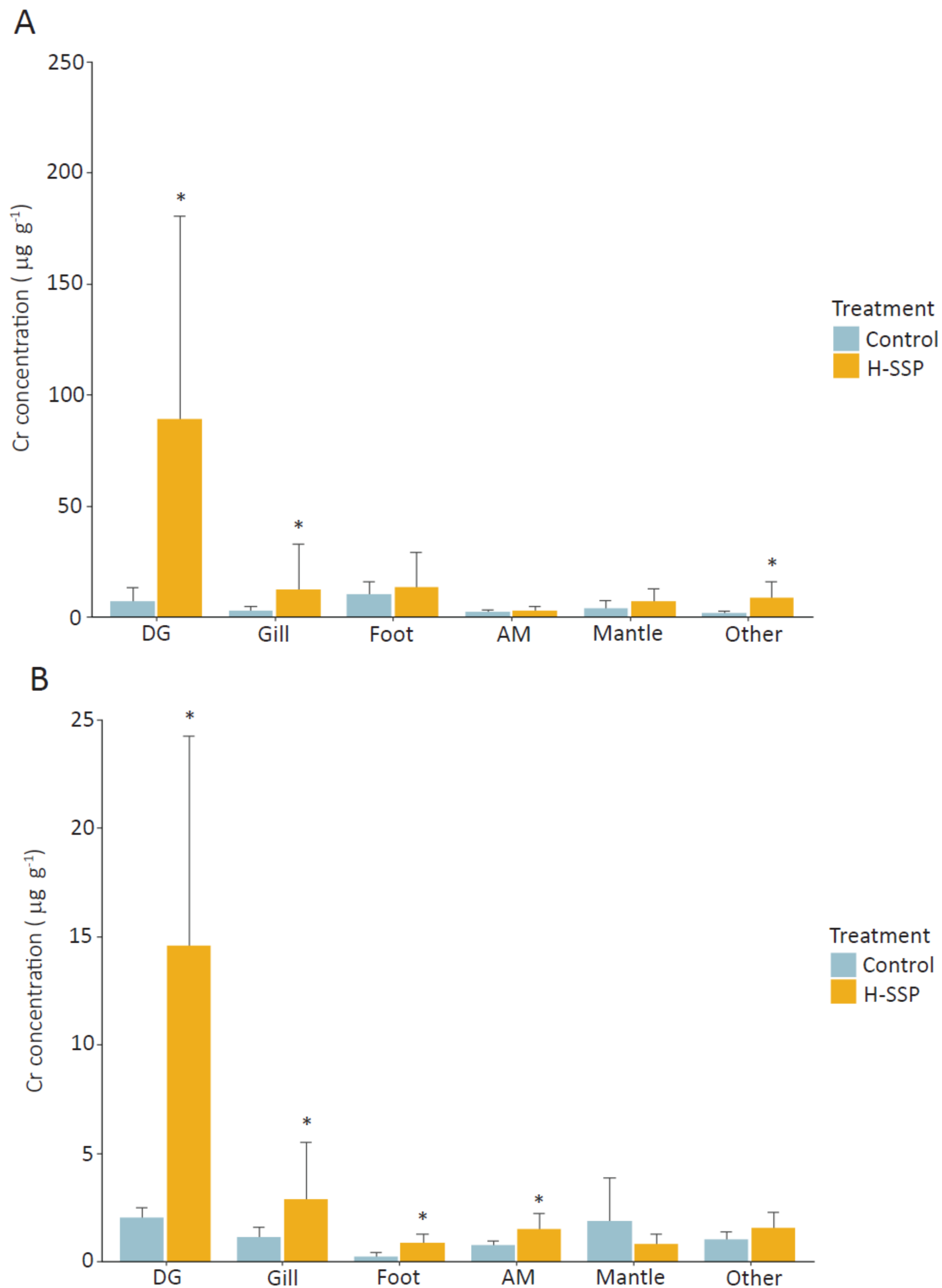


**Figure 2. Tissue-specific bioaccumulation of Cr in *Mytilus galloprovincialis* after (A) 5h and (B) 11 days exposure to hydrogenated stainless steel particles (H-SSP). Bars represent standard deviation about the mean for each group. Asterisks (*) in top of each bar indicate significant differences (ANOVA, p<0.05) from the corresponding negative control in different tissue. AM: adductor muscle.**

## 2.2 Mussels exposure to hydrogenated cement particles (H-CP)

Mussels collection and acclimatisation were performed as described in section 2.1. Following an approach similar to the study of H-SSP bioaccumulation, mussels were exposed 5 h (acute exposure) and 11 days (chronic exposure) to a nominal concentration of 1 mg $L^{-1}$ of HCP. Stock solution (200 mg $L^{-1}$) of the H-CP was prepared in seawater and 10 mM HEPES was added to stabilise the pH of the solution. A negative (seawater) was run alongside. Water parameters (pH, salinity, temperature, dissolved oxygen) were measured every alternate day. Water changes were performed on days 3, 5, 7, 9 and mussels were fed 1 h before each water change.

Using Ti as a bioaccumulation tracer element, results showed that H-CP accumulate in the digestive gland (Figure 3) at both sampling times. After 11 days, bioaccumulation was also observed in foot and adductor muscle.

No differences were observed between Ti concentration in treated media (1000 µg $L^{-1}$ H-CP) and seawater only (controls), and therefore, H-CP were not detected during the experiments (5h and 11 d) using Ti as a tracer. This could be due to particles filtration by mussels and considering the low concentration of Ti in particles composition (6%, .A. Jha et al., 2022).

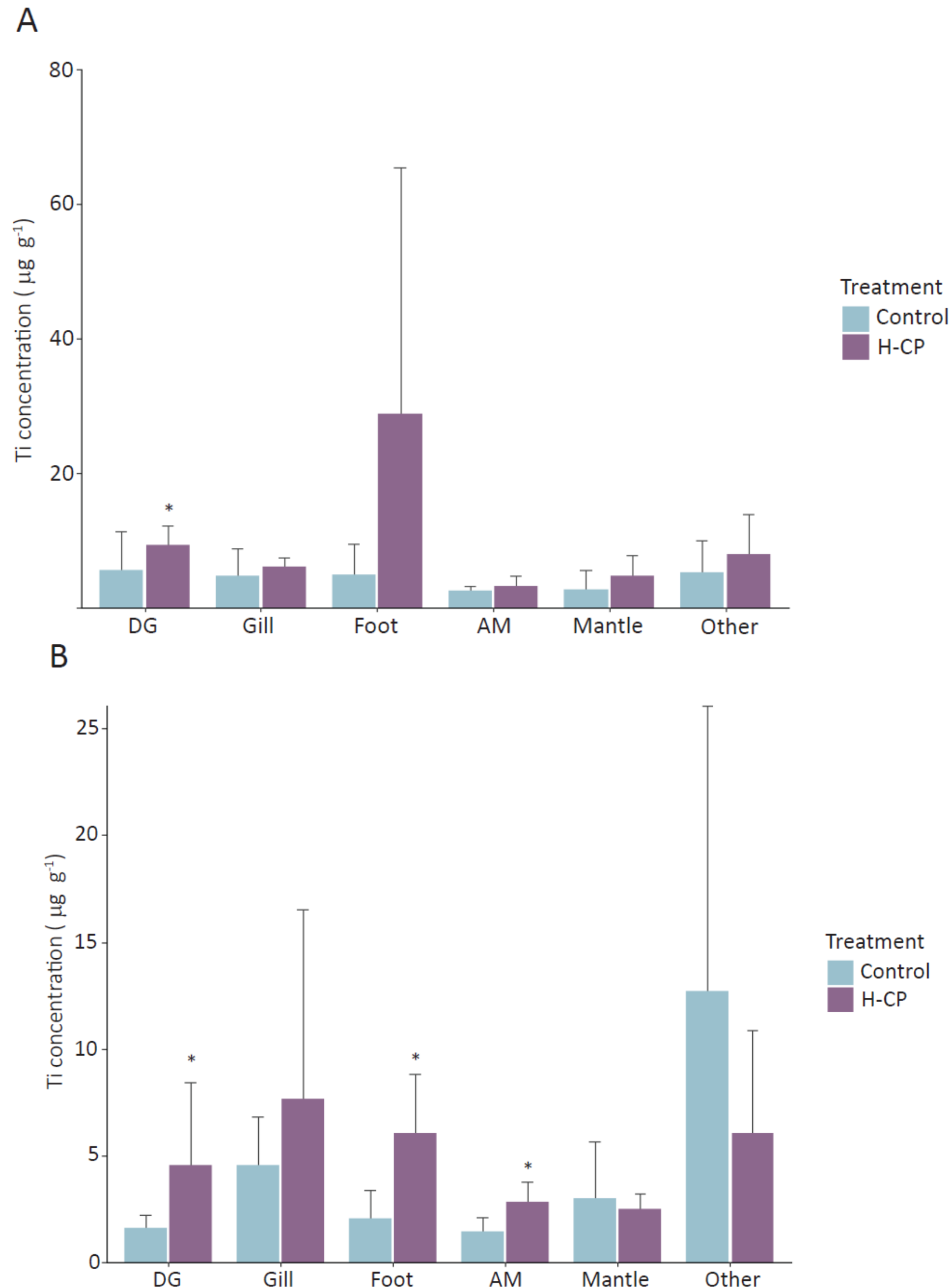


**Figure 3. Tissue-specific bioaccumulation of Ti in *Mytilus galloprovincialis* after (A) 5h and (B) 11 days exposure to hydrogenated cement particles (H-CP). Bars represent standard deviation about the mean for each group. Asterisks (*) in top of each bar indicate significant differences (ANOVA, p<0.05) from the corresponding negative control in different tissue.**

# 3 Genotoxic effects of the particles (Comet assay)

Digestive gland samples collected during the experiments detailed in section 2 were analysed through the comet assay to assess potential DNA damage in mussel's digestive gland cells. Prior to conducting comet assay, as a prerequisite, health status in terms of cellular viability was determined using the Trypan Blue exclusion dye assay (Strober, 2001). Ten μL of digestive gland lysate was gently mixed with 1 μL Trypan blue (0.40 %, Sigma), the solution was smeared onto a microscope slide and coverslip was applied. A total of 100 cells per individual were examined under light microscopy (x 40). Samples showed >90% viability (data not shown) and were subsequently used to determine DNA damage using the comet assay. The procedure to prepare slides for analysis of DNA strand breaks using the comet assay has been described elsewhere in details (Dallas et al., 2013; Vernon et al., 2020a). Briefly, 150 μL of the digestive gland lysate from six individuals was centrifuged (775 g, 5 min), and supernatant were used for the assay according to previous studies (Vernon et al., 2020b; Vincent-Hubert et al., 2011). In addition, a modified comet assay to assess oxidative DNA damage was also performed adding an incubation step with the bacterial enzyme formamidopyrimidine DNA glycosylase (FPG, New England Biolabs) to target oxidized purine bases as described in detail elsewhere (Dallas et al., 2013; Pearson et al., 2018).

As a known reference genotoxic agent, hydrogen peroxide ($H_2O_2$) was utilised as a positive control, where digestive gland lysate (150 μL, as above) collected from healthy mussels (n = 6) was exposed to 500 μM H2O2 in phosphate buffer saline (1 h, in dark) and processed with control and H-SSP-exposed samples. This 1 concentration of $H_2O_2$ as positive control was used based on previous validation experiments (Dallas et al., 2013). Cells were stained with GelRed® (10X, Cambridge Bioscience) and scored using an

epifluorescent microscope (DMR; Leica Microsystems, Milton Keynes, UK). 100 cells per slide (50 cells per microgel) were quantified using the Comet IV imaging software (Perceptive Imaging, Bury St Edmunds, UK) software. The software provides results for different parameters, % Tail DNA was considered the most reliable to present the results (Kumaravel & Jha, 2006). Our results showed significant DNA damage when compared with samples from the (negative) controls and mussels exposed to particles (i.e., H-SSP, H-CP) at all time-points. After H-SSP exposure, mussels gland cells presented DNA strand breaks after 11 days but oxidative DNA damage after 5h and 11 days (Figure 4A). After H-CP exposures (5h and 11 days), only oxidative DNA damage was observed in the digestive gland in mussels (Figure 4B).

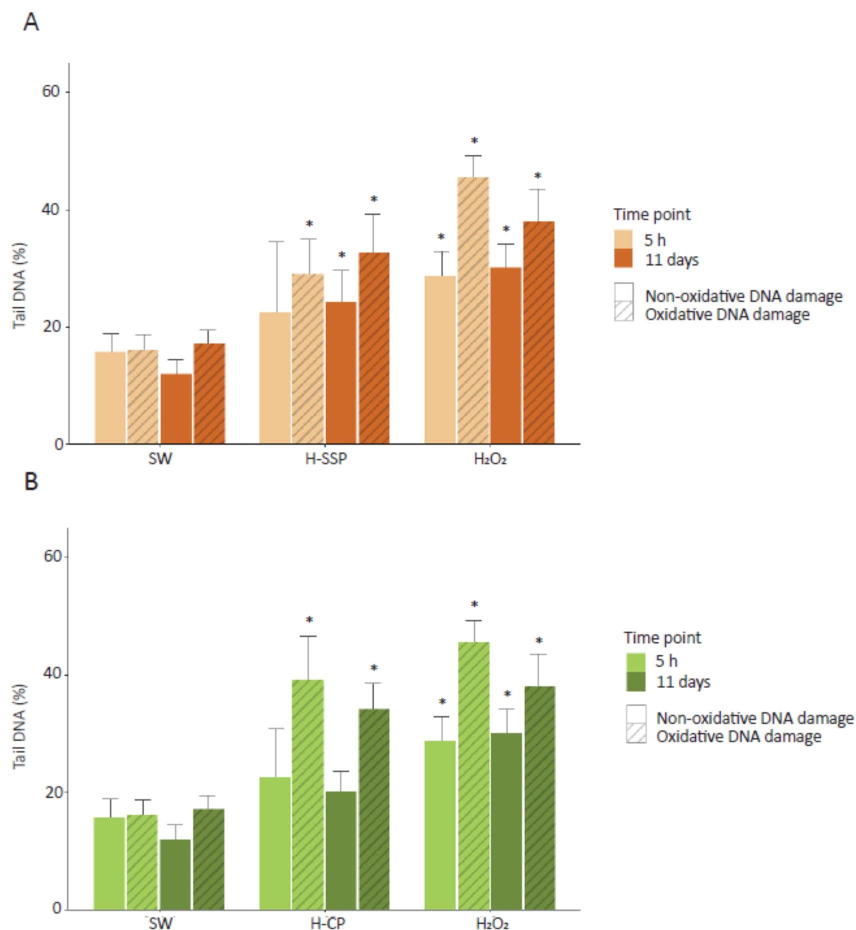


**Figure 4. DNA damage in *M. galloprovincialis* digestive gland cells after exposure to (A) hydrogenated stainless steel particles (H-SSP) and (B) hydrogenated cement particles (H-CP). Hydrogen peroxide ($H_2O_2$, 500 $\mu$M) as a positive control for the comet assay is also shown. Asterisks (*) on top of each bar indicate significant differences (ANOVA, $p<0.05$) from the corresponding negative control (SW; sea water).**

# 4 Bioaccumulation in algae (ICP-MS, TEM)

*Isochrysis galbana* algae was cultured with natural filtered sea water (SW), additionally filtered (0.20 µm) and autoclaved, under 18/6 h light/dark cycle at 20C. Cultures of *I. galbana* ($1 \times 10^5$ cells $mL^{-1}$) were exposed in triplicate to 1 mg $L^{-1}$ H-SSP and H-CP and a SW control for 60 days to let the culture reach its stationary phase and determine particles bioaccumulation. After the exposure periods, 10 mL of the culture was filtered (0.45 µm, Whatman), and the filter was then treated with aqua regia (5 mL). After digestion samples were diluted to a final volume of 25 mL to determine the presence of the particles through ICP-MS analysis.

The exposed culture was also analysed in duplicate through transmission electron microscopy (TEM, JEOL 1400) to determine particles uptake. Analyses of particle bioaccumulation by algae was conducted on samples fixed with glutaraldehyde (2.5%), and at least 50 cells were analysed per sample.

Particles were not detected through ICP-MS, which could be due to the low concentration of the particles incorporated by algae. However, TEM analysis revealed the presence of nanoparticles bioaccumulated by the algae cell (Figure 5).

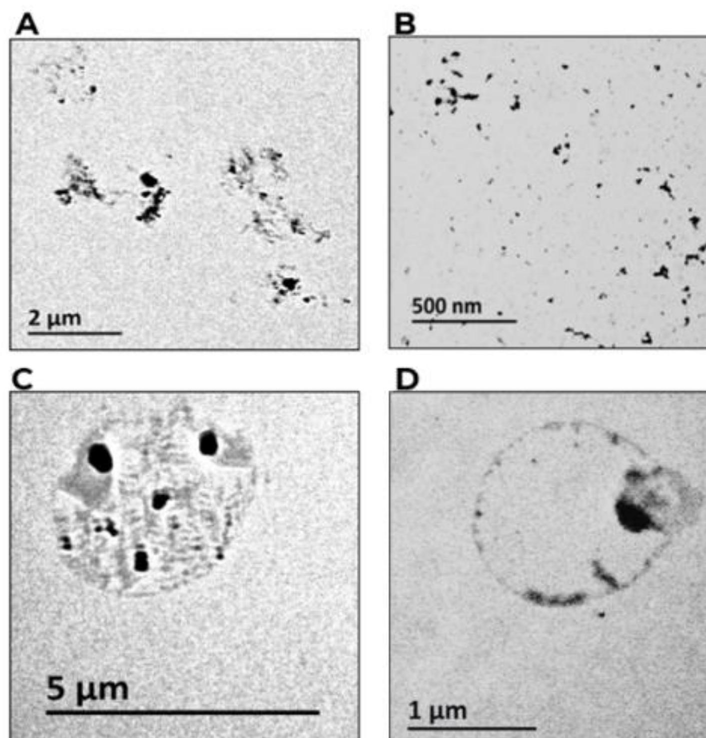


**Figure 5. Image of (A) hydrogenated stainless steel particles (H-SSP) stock solution (B) hydrogenated cement particles (H-CP) stock solution; (C) algae cell exposed to H-SSP and (D) H-CP particles.**

# 5 Bioaccumulation in mussel tissues in the presence of algae as food (ICP-MS)

In order to determine if particles accumulation is influenced by the presence of food (i.e., algae), mussels were exposed to the H-SSP (1 mg $L^{-1}$) and H-CP (1 mg $L^{-1}$) as described in section 2.1. but in presence of *Isochrysis galbana* (1 x $10^5$ cells $mL^{-1}$). The duration of the exposure was 5 h considering that the highest levels of H-SP and H-CP bioaccumulation was observed after 5h.

Water samples collected during the experiment showed that the concentration of H-SSP in exposure media decrease after 5h (Table 3). This was expected considering particles uptake by mussels and tissue specific bioaccumulation results (Figure 6 A). Interestingly, H-SSP concentration remained similar after 5h when algae were present (Table 3). This is

consistent with the lack of tissue bioaccumulation observed in mussels when exposed to this condition (H-SSP + Algae) (Figure 6 A).

Table 3 also shows that Ti concentration was similar in all experimental conditions including controls (mussels in seawater). Therefore, it was not possible to detect the presence of H-CP in water samples during the experiment using Ti as a tracer, as previously mentioned in section 2.2.1.

**Table 3. Cr and Ti concentration (µg L-1) in filtered (F, 0.20 µm) and non-filtered (NF) water samples from the different experimental conditions during the 5h-experiment.**

| | | | Beginning | End (5h) |
|---|---|---|---|---|
| **Cr** | H-SSP | NF | 117.27 ± 17.38 | 36.54 ± 15.69 |
| | | F | 5.33 ± 2.11 | 3.33 ± 0.16 |
| | H-SSP + ALG | NF | 145.68 ± 19.16 | 146.18 ± 23.88 |
| | | F | 4.17 ± 0.84 | 3.55 ± 0.41 |
| | SW | NF | 4.01 ± 0.57 | 3.55 ± 0.22 |
| | | F | 3.52 ± 0.99 | 3.66 ± 0.22 |
| **Ti** | H-CP | NF | 11.12 ± 0.63 | 10.56 ± 0.69 |
| | | F | 11.95 ± 0.70 | 11.19 ± 0.509 |
| | H-CP + ALG | NF | 12.48 ± 0.47 | 12.28 ± 0.87 |
| | | F | 12.72 ± 0.84 | 12.02 ± 0.99 |
| | SW | NF | 11.89 ± 1.00 | 11.22 ± 0.52 |
| | | F | 11.27 ± 0.47 | 11.31 ± 0.75 |

Tissue samples showed no significant bioaccumulation between controls and mussels exposed to H-SSP or H-CP in presence of algae (Figure 6). Particles uptake by an algae cell can take more than 5 hours (Corsi et al., 2022; Zhang et al., 2020), and this could explain the lack of bioaccumulation by mussels after 5 h exposure. Moreover, it has been reported that the presence of microalgae can retard the filtration of nanoparticles in mussels, indicating that mussels might be able to distinguish nutritive foods and unusable suspended particles (Wang et al., 2021a).

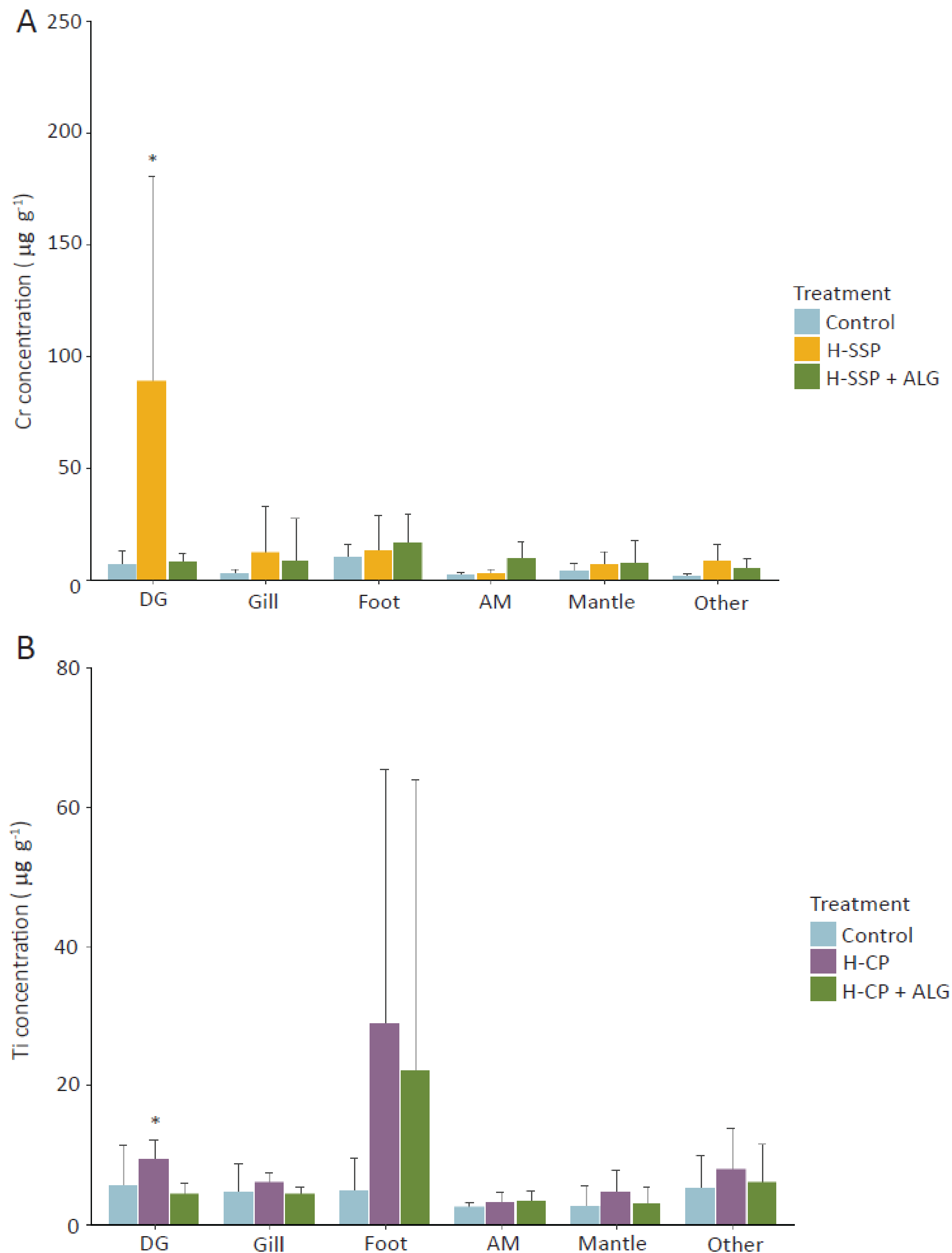


**Figure 6. Tissue-specific bioaccumulation in *M. galloprovincialis* after 5h to (A) hydrogenated stainless steel particles (H-SSP) and (B) hydrogenated cement particles (H-CP) in presence of food (*Isochrysis galbana*). Bars represent standard deviation about the mean for each group. Asterisks (*) in top of each bar indicate significant differences (ANOVA, $p<0.05$) from the corresponding negative control in different tissue.**

# 6 Conclusion

The potential bioaccumulation and ecotoxicological effects of untritiated (H-SSP and H-CP) particles were studied in the marine bivalve *Mytilus galloprovincialis*, as well as potential biomagnification through the food chain.

Exposures to H-SSP and H-CP showed that particles accumulate mainly in the DG after 5 h. After 11 days, H-CP bioaccumulation was also observed in foot and adductor muscle in addition to DG.

Both particles induced DNA damage in DG cells after 5h and 11 d exposures. In particular, mussels exposed to H-SSP presented oxidative DNA damage after 5h and 11 days of exposure, but only non-oxidative DNA damage (i.e., DNA strand breaks) after 11 days of exposure. On the contrary, H-CP induced only oxidative DNA damage after 5h and 11 days.

Particle bioaccumulation was also observed in algae (*Isochrysis galbana*), as revealed by TEM studies (but not using ICP-MS) indicating the possibility of particles biomagnification through the food chain. This was not, however, evidenced in mussels after 5h exposure to both algae and particles (H-SSP, H-CP). This could indicate differential uptake of particles, as previously observed in mussels exposed to other particles (Harris & Carrington, 2020; Wang et al., 2021b; Zhang et al., 2024). Bioaccumulation (by means of particle filtration) could be independent of particle uptake by the algal cells. The potential biomagnification of the particles through algae (i.e. food) may not therefore be evidenced due to short exposure period (i.e. 5 h). Future experiments with tritiated particles will, therefore, require longer exposure periods to determine particles bioaccumulation and biomagnification through the food chain.

# Bibliography

Bondareva, L., Kudryasheva, N., & Tananaev, I. (2022). Tritium: Doses and Responses of Aquatic Living Organisms (Model Experiments). *Environments - MDPI*, *9*(4), 1–17. https://doi.org/10.3390/environments9040051

Corsi, I., Desimone, M. F., & Cazenave, J. (2022). Building the Bridge From Aquatic Nanotoxicology to Safety by Design Silver Nanoparticles. In *Frontiers in Bioengineering and Biotechnology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2022.836742

Dallas, L. J., Bean, T. P., Turner, A., Lyons, B. P., & Jha, A. N. (2013). Oxidative DNA damage may not mediate Ni-induced genotoxicity in marine mussels: Assessment of genotoxic biomarkers and transcriptional responses of key stress genes. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *754*(1–2), 22–31. https://doi.org/10.1016/j.mrgentox.2013.03.009

Dallas, L. J., Bean, T. P., Turner, A., Lyons, B. P., & Jha, A. N. (2016). Exposure to tritiated water at an elevated temperature: Genotoxic and transcriptomic effects in marine mussels (*M. galloprovincialis*). *Journal of Environmental Radioactivity*, *164*, 325–336. https://doi.org/10.1016/j.jenvrad.2016.07.034

Ding, X., Tian, W., Chen, Q., & Wei, G. (2019). Policies on water resources assessment of coastal nuclear power plants in China. *Energy Policy*, *128*(April 2018), 170–178. https://doi.org/10.1016/j.enpol.2019.01.008

Ferreira, M. F., Turner, A., Vernon, E. L., Grisolia, C., Lebaron-Jacobs, L., Malard, V., & Jha, A. N. (2023). Tritium: Its relevance, sources and impacts on non-human biota. *Science of The Total Environment*, 162816. https://doi.org/10.1016/j.scitotenv.2023.162816

Hagger, J. A., Atienzar, F. A., & Jha, A. N. (2005). Genotoxic, cytotoxic, developmental and survival effects of tritiated water in the early life stages of the marine mollusc, *Mytilus edulis*. *Aquatic Toxicology*, *74*(3), 205–217. https://doi.org/10.1016/j.aquatox.2005.05.013

Harris, L. S. T., & Carrington, E. (2020). Impacts of microplastic vs. natural abiotic particles on the clearance rate of a marine mussel. In *Limnology And Oceanography Letters* (Vol. 5, Issue 1, pp. 66–73). John Wiley and Sons Inc. https://doi.org/10.1002/lol2.10120

Jaeschke, B. C., & Bradshaw, C. (2013). Bioaccumulation of tritiated water in phytoplankton and trophic transfer of organically bound tritium to the blue mussel, *Mytilus edulis*. *Journal of Environmental Radioactivity*, *115*, 28–33. https://doi.org/10.1016/j.jenvrad.2012.07.008

Jha, A., Maria, M., Ferreira, F., Vernon, E., & Turner, A. (2022). *Report on ecotoxicological and genotoxicological impact on tritiated and untritiated particles on marine bivalves*. https://transat-h2020.eu/

Jha, A. N., Dogra, Y., Turner, A., & Millward, G. E. (2005). Impact of low doses of tritium on the marine mussel, *Mytilus edulis*: Genotoxic effects and tissue-specific bioconcentration. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *586*(1), 47–57. https://doi.org/10.1016/j.mrgentox.2005.05.008

Khamis, I., & Kavvadias, K. C. (2012). Trends and challenges toward efficient water management in nuclear power plants. *Nuclear Engineering and Design*, *248*, 48–54. https://doi.org/10.1016/j.nucengdes.2012.03.039

Kumaravel, T. S., & Jha, A. N. (2006). Reliable Comet assay measurements for detecting DNA damage induced by ionising radiation and chemicals. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *605*(1–2), 7–16. https://doi.org/10.1016/J.MRGENTOX.2006.03.002

Lamartiniere, Y., Slomberg, D., Payet, M., Tassistro, V., Mentana, A., Baiocco, G., Rose, J., Lebaron-Jacobs, L., Grisolia, C., Malard, V., & Orsière, T. (2022). Cyto-Genotoxicity of Tritiated Stainless Steel and Cement Particles in Human Lung Cell Models. *International Journal of Molecular Sciences*, *23*(18). https://doi.org/10.3390/ijms231810398

Liger, K., Grisolia, C., Cristescu, I., Moreno, C., Malard, V., Coombs, D., & Markelj, S. (2018). Overview of the TRANSAT (TRANSversal Actions for Tritium) project. *Fusion Engineering and Design*, *136*(August 2017), 168–172. https://doi.org/10.1016/j.fusengdes.2018.01.037

Mentana, A., Lamartinière, Y., Orsière, T., Malard, V., Payet, M., Slomberg, D., Guardamagna, I., Lonati, L., Grisolia, C., Jha, A., Lebaron-Jacobs, L., Rose, J., Ottolenghi, A., & Baiocco, G. (2022). Tritiated Steel Micro-Particles: Computational Dosimetry and Prediction of Radiation-Induced DNA Damage for in Vitro Cell Culture Exposures. *Radiation Research*, *199*(1), 25–38. https://doi.org/10.1667/RADE-22-00043.1

Pearson, H., Dallas, L. J., Comber, S. D. W., Braungardt, C. B., Worsfold, P. J., & Jha, A. N. (2018). Mixtures of tritiated water, zinc and dissolved organic carbon: Assessing interactive bioaccumulation and genotoxic effects in marine mussels, *Mytilus galloprovincialis*. *Journal of Environmental Radioactivity*, *187*(December 2017), 133–143. https://doi.org/10.1016/j.jenvrad.2017.12.018

Smith, R., Ellender, M., Guo, C., Hammond, D., Laycock, A., Leonard, M. O., Wright, M., Davidson, M., Malard, V., Payet, M., Grisolia, C., & Blanchardon, E. (2022). Biokinetics and Internal Dosimetry of Tritiated Steel Particles. *Toxics*, *10*(10). https://doi.org/10.3390/TOXICS10100602

Strober, W. (2001). Trypan Blue Exclusion Test of Cell Viability. *Current Protocols in Immunology*.

Vernon, E. L., Bean, T. P., & Jha, A. N. (2020). Assessing relative biomarker responses in marine and freshwater bivalve molluscs following exposure to phosphorus 32 (32P): Application of genotoxicological and molecular biomarkers. *Journal of Environmental Radioactivity*, *213*(July 2019), 106120. https://doi.org/10.1016/j.jenvrad.2019.106120

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Vernon, E. L., Moore, M. N., Bean, T. P., & Jha, A. N. (2020). Evaluation of interactive effects of phosphorus-32 and copper on marine and freshwater bivalve mollusks. *International Journal of Radiation Biology*, *0*(0), 1–14. https://doi.org/10.1080/09553002.2020.1823032

Vernon, E. L., Smith, J. T., & Jha, A. N. (2018). Relative comparison of tissue specific bioaccumulation and radiation dose estimation in marine and freshwater bivalve molluscs following exposure to phosphorus-32. *Journal of Environmental Radioactivity*, *192*(April), 312–320. https://doi.org/10.1016/j.jenvrad.2018.07.005

Vincent-Hubert, F., Arini, A., & Gourlay-Francé, C. (2011). Early genotoxic effects in gill cells and haemocytes of *Dreissena polymorpha* exposed to cadmium, B[a]P and a combination of B[a]P and Cd. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, *723*(1), 26–35. https://doi.org/10.1016/j.mrgentox.2011.03.008

Wang, S., Zhong, Z., Li, Z., Wang, X., Gu, H., Huang, W., Fang, J. K. H., Shi, H., Hu, M., & Wang, Y. (2021a). Physiological effects of plastic particles on mussels are mediated by food presence. *Journal of Hazardous Materials*, *404*. https://doi.org/10.1016/j.jhazmat.2020.124136

Wang, S., Zhong, Z., Li, Z., Wang, X., Gu, H., Huang, W., Fang, J. K. H., Shi, H., Hu, M., & Wang, Y. (2021b). Physiological effects of plastic particles on mussels are mediated by food presence. *Journal of Hazardous Materials*, *404*. https://doi.org/10.1016/j.jhazmat.2020.124136

Zhang, J., Xiang, Q., Shen, L., Ling, J., Zhou, C., Hu, J., & Chen, L. (2020). Surface charge-dependent bioaccumulation dynamics of silver nanoparticles in freshwater algae. *Chemosphere*, *247*. https://doi.org/10.1016/j.chemosphere.2020.125936

Zhang, J., Xu, M., & Yang, Y. (2024). Assessing the effects of environmental factors on filtration rate of golden mussel (*Limnoperna fortunei*). *Ecological Indicators*, *158*. https://doi.org/10.1016/j.ecolind.2024.111544