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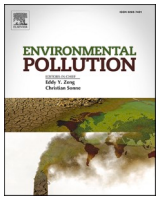

High resolution visualisation of tiemannite microparticles, essential in the detoxification process of mercury in marine mammals☆

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ABSTRACT

The North Sea is an ecologically rich habitat for marine wildlife which has also been impacted by industrial developments and anthropogenic emissions of contaminants such as mercury. Marine mammals are particularly susceptible to mercury exposure, due to their trophic position, long lifespan, and dependence on (increasingly contaminated) aquatic prey species. To mitigate impact, marine mammals can detoxify methylmercury by binding it to selenium-containing biomolecules, creating insoluble mercury selenide granules. Here, liver, kidney, muscle, and brain samples from an adult male bottlenose dolphin (*Tursiops truncatus*) with known elevated mercury concentrations were analysed through scanning electron microscopy (SEM). Tiemannite (HgSe) deposits were identified in all organs, ranging from 400 nm to 5 μm in diameter, with particle size being organ-dependent. Although reported in other studies, this is the first time that the three-dimensional nature of tiemannite is captured in marine mammal tissue.

1. Introduction

Marine mammals are key species within their ecosystems, with complex environmental and social interactions (P. S. Ross, 2000). Although not all marine mammals share phylogeny, all species are well adapted to life in the aquatic realm and are dependent on a functioning ecosystem for survival (Moore, 2008), thus are considered sentinel species for aquatic ecosystem health (Aguirre & Tabor, 2004; Bossart, 2011). However, due to their feeding ecology they are increasingly exposed to an elevated concentration of anthropogenic contaminants accumulated in prey species (P. S. Ross, 2000). Heavy metals are among the contaminants of concern for marine mammals (Frodello et al., 2000), as they enter the ocean through various pathways, where they are then bioaccumulated by organisms and biomagnify through the foodweb (Jitar et al., 2015). One example for this is the naturally occurring heavy metal mercury, which speciates under environmental conditions and can be biologically methylated into the highly neurotoxic methylmercury (Gonzalez-Raymat et al., 2017; Hamdy & Noyes, 1975). Once ingested, it accumulates in tissues and organs over time due to its slow elimination rate (Kerper et al., 1992). In the body, methylmercury irreversibly binds to sulphydryl and organoseleno groups, leading to the denaturing of proteins and subcellular structures (Kageyama et al., 1986; Ralston et al., 2012). Interestingly, marine mammals have developed a detoxification mechanism based on the essential trace element selenium, a component of various selenoproteins involved in e.g., reproduction, thyroid hormone metabolism, and oxidative stress protection (Sunde, 2012). In organs such as the liver, methylmercury is demethylated and bound to selenium to form inert and insoluble crystalline mercury selenide (HgSe), otherwise known as tiemannite (Caurant et al., 1996; Wagemann et al., 1998). Although this process is known to occur, the factors governing the formation and size of crystals is not fully understood (Gajdosechova et al., 2016). However, both the exposure to methylmercury, and the depletion of selenium levels resulting from prolonged reliance on the detoxification mechanism, can lead to primary neurotoxic effects and other health impairments (Dietz et al., 2022; López-Berenguer et al., 2020).

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One cetacean species of particular interest is the common bottlenose dolphin (*Tursiops truncatus*), due to their separation into inshore and offshore populations (Díaz López, 2019). Inshore/coastal populations are considered resident, and as such can be valuable indicators for the health of local ecosystems(Moore, 2008; P. S. Ross, 2000). Bottlenose dolphins are fully mature from around 7 years of age and live for up to 50 years (Venn-Watson et al., 2011). They require a large volume of prey, due to their high metabolic rates and local/regional abundance (Greller et al., 2021). These characteristics make them particularly vulnerable to direct contact with coastal anthropogenic stressors (Díaz López, 2019; Pinn et al., 2018), and they are an Annex II European protected species (JNCC, 2018).

In August of 2020, a locally well-known adult male bottlenose dolphin known as 'Spike' was found dead stranded on the Scottish coastline. It was estimated that he was at least 37 years old, as he was first observed in the North Sea in 1989, already being an adult at that time. He was part of a monitored bottlenose dolphin population from the Moray Firth Special Area of Conservation (NatureScot Moray Firth SAC). Spike was found in the River Tay, and his body was recovered by the Scottish Marine Animal Stranding Scheme (SMASS, part of the University of Glasgow). As part of routine examination, brain, liver, kidney, and muscle tissue samples were collected and stored for assessment. Subsamples of these tissue samples were provided by SMASS, to determine the heavy metal burden. As part of the analysis, tiemannite particles were observed through scanning electron microscopy (SEM).

2. Materials and method

Bottlenose dolphin brain, muscle, kidney, and liver samples were provided by the Scottish Marine Animal Stranding Scheme (SMASS, part of the University of Glasgow, NatureScot Licence Nr. 187976), collected during a routine postmortem examination, following an internationally standardised protocol (ASCOBANS/ACCOBAMS, 2019). All samples were stored at −20 °C at the SMASS freezer archive (University of Glasgow), the sub-samples were stored at −80 °C until processing. After lyophilisation, samples were stored at −20 °C.

Glass vials and screw caps (30 ml volume) were soaked in a 5 % Nitric acid (HNO_3, trace metal grade, 70%, CAS: 7697-37-2) solution overnight, rinsed thoroughly with deionised water and left to dry at 60 °C. Between 5 and 10 g wet weight (ww) per sample were chopped into ~1 mm cubes before placing in the cleaned glass vials and covered with a piece of aluminium foil pierced ~15 times with a taxidermy needle. The samples were lyophilised in a Modulyo 4 K Freeze Dryer (Edwards High Vacuum International, Sussex, UK) for 55 h at −50 °C. Samples were then ground in a ceramic pestle and mortar for 2 min. If a fine powder consistency was reached sooner, the grinding process was not continued. The samples were then stored in aluminium foil-wrapped glass vials. The sample grinding was conducted in line with accepted protocols for heavy metal analysis (Calderón et al., 2013). For SEM analysis, this was further the most suitable sample preparation.

The SEM measurements were performed with a Zeiss GeminiSEM 300 system (Zeiss, Oberkochen, Germany). Lyophilised and manually pulverised liver, kidney, muscle, and brain tissue samples were mounted on stainless steel specimen stubs using double sided tape, and carbon coated for surface investigations. Screening for particles was done with a working distance of ~10.5 mm, 15 kV electron high tension (EHT), and using the backscatter function. Elemental analysis was performed using an additional Aztec Energy energy-dispersive x-ray spectroscopy (EDS) analysis system with an XMax 80 detector (Oxford instruments, UK) with manufacturer provided software suite. High magnification images were taken at a working distance of ~7.7 mm, 5 kV EHT, and using the InLens function. The images shown below do not indicate that the grinding process has negatively impacted the size or shape of the tiemannite structures. However, we appreciate that future research would benefit from comparing different methods for tiemannite determination, to ensure that sample preparation has no impact.

3. Results and discussion

The imaging presented here was conducted as part of a pilot study for various heavy metals in marine mammal organ samples collected since 2015 along the Scottish coastline. The data obtained from the metal analysis is preliminary and thus not presented here in detail. However, the SEM images obtained here are, as far as we are aware, the first of their kind and should thus be published as stand-alone communication. The mean total mercury concentrations measured in the above-stated samples ranges from 2 mg/kg wet weight (ww) in blubber samples to more than 500 mg/kg ww in liver samples, with similar organ-dependent trends observed in selenium measurements (2–240 mg/kg ww). The following water contents were computed after lyophilisation: 82 % in the brain, 78 % in the kidney, 73 % in muscle, and 69 % in the liver.

Considering the spectra information (SI 1-SI 4), the observed particles were identified as tiemannite, a mineral containing mercury and selenium. Tiemannite was observed in all analysed organs (Fig. 1). The particles ranged between 400 nm and 5 μm in size, with smaller particles observed in brain and muscle samples, and larger ones in the liver and kidney. All tiemannite particles were embedded within the tissue matrix, but the shape differed between the organs. In the liver, large multi-nodular particles were observed (Fig. 1 A, B), to a lesser extent in the kidney (Fig. 1 E, F). Tiemannite in muscle (Fig. 1 C, D) and brain (Fig. 1 G, H) samples was markedly smaller, and less nodular, in comparison to the particles in the liver and kidney.

Fig. 2 is a close-up of tiemannite in the liver, providing a more detailed view of the multi-nodular shape of the particle. As the particles observed in the remaining tissue types were partially covered by residual tissue fibres no clear images comparable to Fig. 2 could be obtained. The particle visible in the figure is approximately 1400 nm, although other tiemannite particles in the liver were up to 5 μm in size.

The calculation of the mercury-selenium ratio has been accepted as a determinant for the detoxification of methylmercury in marine mammals (Cáceres-Saez et al., 2013; Capelli et al., 2008; Endo et al., 2006; Sakamoto et al., 2015; Yang et al., 2007). This is based on the knowledge that selenium, under normal circumstances, would undergo homeostatic regulation and show no accumulation. This approach, however, has the potential to overestimate the protective function of selenium by assuming a bonding ratio of 1:1 for mercury to selenium (Gajdosechova et al., 2016). Thus, a shift towards more accurate determination of analyte presence has been suggested, such as measuring the presence of tiemannite itself. Martoja & Viale (1977) were one of the first to measure tiemannite in liver samples of various odontocete species, ranging from 1 to 5 μm in diameter, irregularly shaped, within the connective tissue of the portal vessels. Similar findings were made in further studies (Nigro, 1994; Nigro & Leonzio, 1996) which, although produced at lower magnifications, are in line with present observations.

Although the presence of tiemannite has been measured in marine animals for many decades, the mechanisms underlying its formation remain less clear. A study of long-finned pilot whales (*Globicephala melas*) determined that tiemannite nanoparticles formed in the liver and brain attached to selenium-rich structures acting as a point for nucleation, thus leading to the granular structure formation of larger tiemannite nodes (Gajdosechova et al., 2016). They hypothesised that the Se-protein P formed the backbone of these aggregates, being one of the most common selenoproteins in plasma (Yoneda & Suzuki, 1997). A study conducted on mercury detoxification in human hepatic cell lines determined that tiemannite granules formed and accumulated exclusively in intracellular lysosomal-like structures through aggregation of smaller primary particles with a diameter of 5–10 nm (Tanaka et al., 2021). It was further observed that the acidic conditions in lysosomes facilitated the precipitation of soluble Hg–Se complexes. They reported a positive correlation between the number of primary tiemannite particle formed and the concurrent mercury and selenium exposure. Tanaka et al. (2021) also showed that extracellular tiemannite nanoparticles

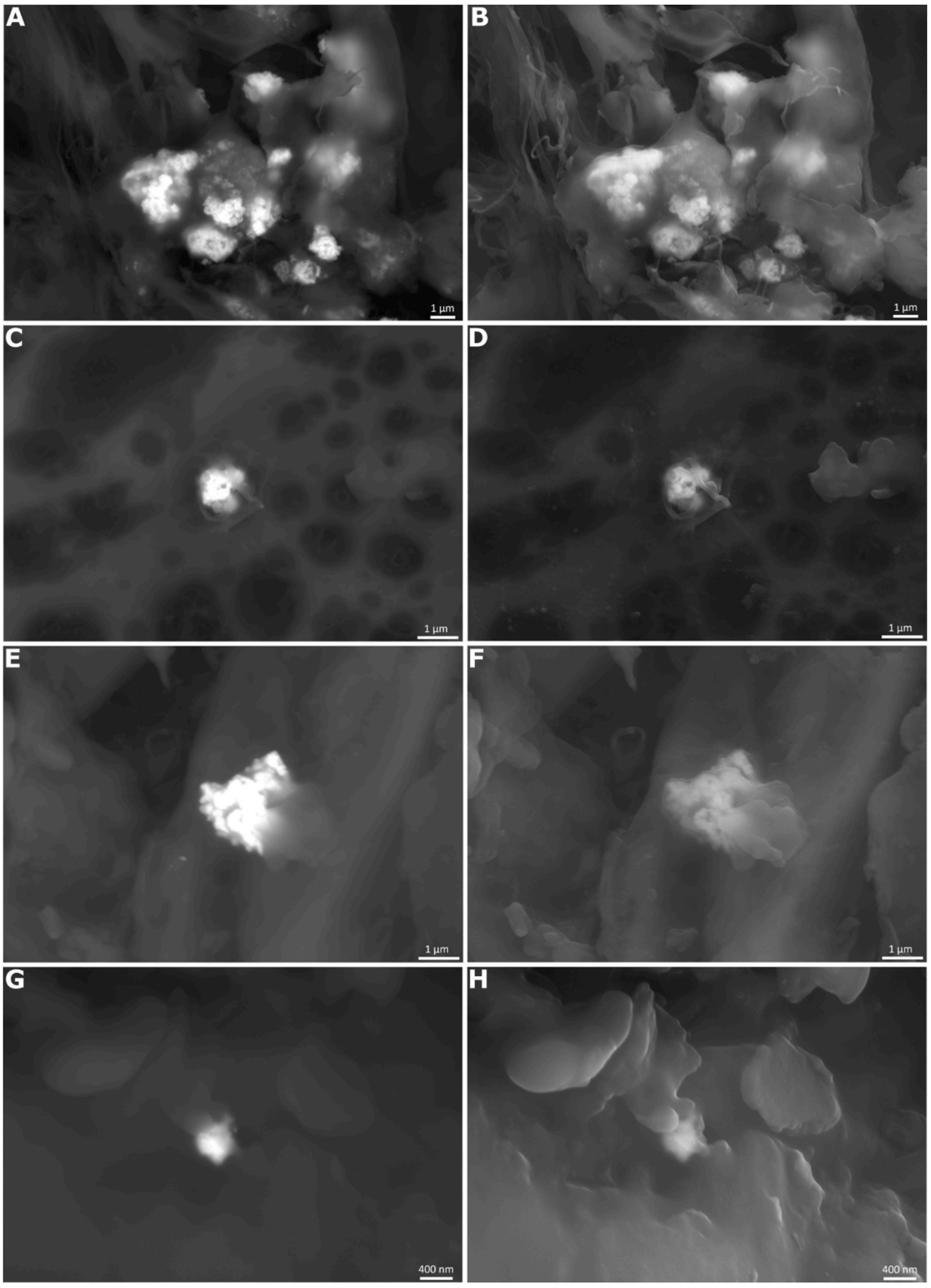

Fig. 1. Tiemannite (HgSe) particles in liver (A, B), muscle (C, D), kidney (E, F), and brain (G, H) samples of a North Sea bottlenose dolphin. A, C, E, and G: Electron backscatter mode. B, D, F, and H: SEM mode (spectra can be found in SI 1 - SI 4). Error bars: 1 µm (A–F), 400 nm (G, H). Images were acquired on a Zeiss GeminiSEM 300 system.

were taken up by endocytosis but stored in different organelles, thus differing from biogenic tiemannite particle accumulation patterns. Moreover, an excretion of tiemannite particles was noted in the study, further indicating that transport between formation site and other tissues is possible. Whilst this does not conclusively answer whether the observed large tiemannite particles in the present study are in fact aggregates of primary particles, it strongly supports the hypothesis. Further research, however, is required to conclusively determine such

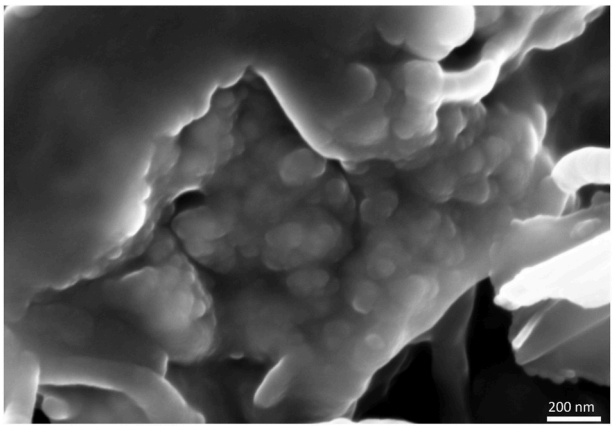

Fig. 2. SEM image of a tiemannite (HgSe) particle in the liver of a North Sea bottlenose dolphin. Image taken with Zeiss GeminiSEM-300 in InLens mode.

mechanisms.

Tiemannite has been observed in Kupffer cells, indicating that crystals produced in other tissues could accumulate in the liver by Kupffer cells through the blood stream (Lailson-Brito et al., 2012), thus supporting the assumption that methylmercury detoxification takes place in multiple organs (Aschner & Aschner, 1990; Bridges & Zalups, 2010; Gajdosechova et al., 2016). Previous research determined that association between sulphur and mercury could be related to the binding of mercury in Kupffer cells, should a selenium deficiency have occurred (Marumoto et al., 2022). We believe, however, that the sulphur peaks in the EDS spectra are an artifact of the automated identification, resulting from the overall of the M and K peaks of mercury and sulphur, respectively (see e.g., Fellowes et al., (2011)). Other works, such as Lancaster et al., (2022) and Gajdosechova et al. (2016), have used additional analysis techniques to confirm the Hg:Se equimolarity of the observed particles. The present findings also determined the presence of tiemannite in all examined organs. To the best of our knowledge, the here presented SEM images are the first to show the three-dimensional macroscopic structure of naturally *in vivo* formed tiemannite in marine mammals. SEM offers a unique visualisation opportunity for acquiring good quality images and spectra of tiemannite in chemically unprocessed samples. Previous work examined HgSe and other metal nanoparticles in sea bird tissue, using formic acid to rapidly digest tissue. Initial findings indicated that treatment had no effect on the overall structure of the particles (El Hanafi et al., 2023). However, differences in aggregation density of primary particles were visible in published figures, leading to the conclusion that although SEM imaging can be considered stochastic at best, it offers the only solution to visualising naturally precipitated tiemannite through sample processing without chemical reagents. It must further be noted that the present findings are an initial assessment of subsampled tissue sections of a single individuals. Further research is required, before statements can be made, regarding the detoxification potential of different organs.

4. Conclusion

To the best of our knowledge, the here presented SEM images are the first to show the three-dimensional structure of tiemannite in marine mammal tissue. Although the presence of these crystals and their role in mercury detoxification has been discussed since the late 1970s, their macroscopic structure has so far not been elucidated. The here presented images lead to questions about the development of large tiemannite structures (local formation *versus* aggregation) and how mechanical tissue damage may play a role in aging marine mammals as hypothesised by others. By addressing the question of what factors govern the formation and size of tiemannite particles, insights could be gained into the detoxification potential of different organs, as well as assessing whether tiemannite accumulates homogenously within each organ. The here presented short communications on SEM imaging to determine tiemannite in bottlenose dolphin tissue samples thus proves a vital starting point for more focussed efforts in addressing the mercury exposure and accumulation in marine mammals. Future research would benefit from including SEM imaging techniques with quantification methods for determining mercury speciation, e.g., through mercury and selenium concentrations obtained from mass spectrometry.

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CRediT authorship contribution statement

Rebecca von Hellfeld: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing, Formal analysis. **Christoph Gade:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - review & editing. **Mariel ten Doeschate:** Resources, Writing - review & editing. **Nicolas J. Davison:** Resources, Writing - review & editing. **Andrew Brownlow:** Conceptualization, Funding acquisition, Resources, Writing - review & editing. **Lenka Mbadugha:** Supervision, Writing - review & editing, Funding acquisition. **Astley Hastings:** Funding acquisition, Supervision, Writing - review & editing. **Graeme Paton:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

Data availability

No data was used for the research described in the article.

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CG and RvH contributed equally to this manuscript. The authors would like to thank Dr Hedda Weitz and the laboratory technicians of the School of Biological Sciences at the University of Aberdeen (UoA) for their support. We also thank John Still (ACEMAC facility, UoA) for the assistance in SEM analysis. Thank you also to our colleagues at the University of Aberdeen's Lighthouse Field Station whose long-term individual based study of bottlenose dolphins on the east coast of Scotland provided life history information and images on ID#49 Spike. We also thank the National Museums Scotland for the provision of further images of Spike, as seen in the graphical abstract. We also acknowledge funding and in-kind support from the Net Zero Technology Centre and the University of Aberdeen through their partnership with the UK National Decommissioning Centre.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.123027.

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