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Tissue-specific incorporation and genotoxicity of different forms of tritium in the marine mussel, *Mytilus edulis*

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Tritium from tritiated glycine demonstrates greater accumulation and persistence in tissues and enhanced genotoxicity in haemocytes of marine mussels, compared to tritium from tritiated water.

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ABSTRACT

Marine mussels (*Mytilus edulis*) were exposed to seawater spiked with tritiated water (HTO) at a dose rate of 122 and 79 μGy h^{-1} for 7 and 14 days, respectively, and tritiated glycine (T-Gly) at a dose rate of 4.9 μGy h^{-1} over 7 days. This was followed by depuration in clean seawater for 21 days. Tissues (foot, gills, digestive gland, mantle, adductor muscle and byssus) and DNA extracts from tissues were analysed for their tritium activity concentrations. All tissues demonstrated bio-accumulation of tritium from HTO and T-Gly. Tritium from T-Gly showed increased incorporation into DNA compared to HTO. About 90% of the initial activity from HTO was depurated within one day, whereas T-Gly was depurated relatively slowly, indicating that tritium may be bound with different affinities in tissues. Both forms of tritium caused a significant induction of micronuclei in the haemocytes of mussels. Our findings identify significant differential impacts on *Mytilus edulis* of the two chemical forms of tritium and emphasise the need for a separate classification and control of releases of tritiated compounds, to adequately protect the marine ecosystem.

1. Introduction

Tritium (^{3}H, T), a radioisotope of hydrogen (half-life 12.32 years), is discharged into the marine environment in relatively large quantities. For example, during 2008 approximately 1800 TBq of tritium was released to the UK marine environment from nuclear installations mainly as tritiated water (HTO). In addition, ca. 14 TBq was discharged due to commercial radiochemical production of tritiated organic compounds (RIFE, 2009). Also, significant increases in the concentrations of tritium have been found in the North Sea over the last two decades (MARINA II, 2003). Tritium emits beta particles, with a mean energy of 5.7 keV (max. 18.6 keV), which have a penetration of about 5 μm in water. Consequently, the majority of radiation dose experienced by an organism is through ingestion. Once within the cells, beta particles from tritium might induce genetic damage which could lead to detrimental effects on biota (Jha, 2004, 2008). Epidemiological and experimental studies indicate that low energy, beta particles from tritium might be more toxic than some X-rays or γ-rays, raising concerns that current environmental and human risk factors for tritium may be underestimated (Bridges, 2008; Little and Lambert, 2008; Little and Wakeford, 2008).

Because tritium is discharged mainly as HTO, it is assumed that it will rapidly mix and dilute in natural waters and is, therefore, thought to have minimal impact on biota. However, laboratory investigations (Hagger et al., 2005; Jha et al., 2005) have demonstrated a significant toxicological impact on marine mussels, *Mytilus edulis*, of HTO doses lower than internationally-recommended exposure limits (IAEA, 1992). Concentration factors for biological materials, *CF* (defined as the ratio of the activity concentration of tritium in the organism, A_O Bq kg^{-1} dry or wet weight, to that in seawater, A_S Bq l^{-1}), for tritiated species are generally unknown (IAEA, 2004). Elevated *CF* values of the order 2×10^4 (based on dry weights) for mussels and flounders in the Severn Estuary (UK) were attributed to the release, and subsequent uptake of various bioavailable tritium-labelled organic radiochemicals rather than HTO (Hunt et al., 2010; McCubbin et al., 2001). In this context, fish and shellfish consumption is identified as one of the most significant exposure pathways to humans following radioactive discharges (Little and Wakeford, 2008; RIFE, 2009).

Tritium in biological tissues is present in three forms (a) tissue free water tritium (TFWT), i.e. tritium in the water trapped within the tissues; (b) exchangeable organic tritium (EOT), i.e. tritium weakly bonded to oxygen, sulphur, nitrogen or phosphorus atoms

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within the tissues which leads to a reduction in the biochemical mobility of tritium and an increase in its biological half-life compared to TFWT; and (c) organically bound tritium (OBT) which involves stronger carbon–tritium bonds and which confers a longer biological half-life for OBT over both EOT and TFWT (Baumgaertner et al., 2009; Diabaté and Strack, 1993; Pointurier et al., 2003). It is assumed that tritiated compounds in seawater rapidly attain a 1:1 activity concentration ratio with the TFWT in an organism. However, it is not clear that equilibration takes place, even in the natural environment where timescales are longer than those employed in laboratory experiments (Baeza et al., 2009).

The differences in behaviour between the various forms of tritium in organisms have been identified as a matter of concern when modelling their fate in the environment (Ciffroy et al., 2006; IAEA, 2008), although very little experimental testing of such differences exists and there is scant evidence for potential bio-transformation of TFWT into EOT and/or OBT (Baumgaertner et al., 2009). Also, the impact of these various internalised forms of tritium on the genetic integrity of organisms has not been resolved, particularly if tritium is incorporated into DNA (Baumgaertner and Kim, 2000) where, potentially, there would be a greater genotoxic and ecotoxic risk.

The edible marine mussel, *Mytilus edulis*, is commonly used in 'pulse-chase' type incubation studies of bioaccumulation of various substances, including HTO (Jha et al., 2005; Le Bris and Pouliquen, 2004; Punt et al., 1998; Wang et al., 1996). However, there is insufficient information on the tissue-specific bio-accumulation and biological effects following exposure to environmentally-relevant radionuclides in natural biota (ERICA, 2010; Jha et al., 2005; Pentreath, 2009). Thus, this study aimed to examine the DNA and tissue-specific bio-accumulation and depuration of tritium from HTO and the tritiated amino acid glycine (T-Gly), by *Mytilus edulis*. Glycine was selected as the test organic compound because free glycine is actively taken up through the gills of mussels, from where it may be dispersed to other tissues (Cheney et al., 2007; Kube et al., 2007). Also, glycine is a major component of proteins, particularly the structural protein collagen, and it plays an important role in the synthesis of DNA *via* the *de novo* pathway (Berg et al., 2007). The genotoxicity of tritium was assessed using the micronucleus (MN) assay on haemocytes (Canty et al., 2009; Jha et al., 2005; Jha, 2008). To our knowledge this is the first time that an integrated approach has been adopted to elucidate the uptake, tissue-specific bio-accumulation, DNA activity concentration and genotoxic effects of two different forms of an environmentally-relevant radionuclide in a representative marine species.

2. Materials and methods

2.1. Mussel collection, acclimatisation and monitoring

Adult mussels (shell length 40–60 mm) were collected in early spring from a reference site in Whitsand Bay (Cornwall, UK) (Jha et al., 2005). Mussels were collected and experiments were performed at the same time of the year, thus avoiding seasonal influences that have been suggested to cause variations in biological responses including MN inducibility (Canty et al., 2009; Jha, 2004, 2008; Wrisberg and Rhemrev, 1992).

Five mussels were placed in aerated glass tanks (10 l) each containing 5 l of filtered seawater (<5 μm pore size). Experimental tanks were kept in a temperature controlled room (14 °C ± 1), with a 12:12 h light: dark cycle and water quality parameters (i.e. salinity, dissolved oxygen and pH) were monitored at regular intervals. The tanks were covered to restrict any potential evaporation of the radionuclide. Prior to exposure, the mussels were acclimatised for seven days, during which they were fed Instant Algae Isochrysis 1800 (Reed Mariculture Inc., USA) and the water was changed every two days. Throughout the experiments water quality parameters were within acceptable ranges and no spawning or mortality occurred in any of the exposure scenarios.

2.2. Concentrations of tritiated compounds and dose calculations

An established method (Hagger et al., 2005; Jha et al., 2005) was used to investigate the uptake and tissue-distribution of tritium in the form of tritiated water (HTO; 99.96% purity; 185 MBq ml^{-1}; GE Healthcare, Amersham, UK) spiked into two separate exposures with activity concentrations of 37 MBq l^{-1} and 24 MBq l^{-1}. In a separate experiment the uptake of tritium from tritiated glycine (T-Gly; H_2NCT_2COOH; >97% purity; 37 MBq ml^{-1}; GE Healthcare, Amersham, UK) spiked into seawater at an activity concentration of 1.48 MBq l^{-1} was determined.

The dosage to organisms from internalised tritium was estimated assuming that the tritium spiked into the seawater rapidly attains 1:1 equilibrium within the tissues as TFWT and that the tritiated species are dispersed homogeneously within the tissues (Strand et al., 1977):

$$D_\beta = 0.58 \times 10^{-6} \times E_\beta \times A_S \quad (1)$$

where D_β is the dose rate (μGy h^{-1}), E_β is the mean beta particle energy of tritium (0.0057 MeV), A_S is the activity concentration of tritium in seawater (Bq ml^{-1}) and the numerical constant is a unit conversion factor.

The dose rates for HTO exposures were estimated using the appropriate activity concentrations and were 122 and 79 μGy h^{-1}, respectively, and the total doses administered to each mussel for the 7 and 14 day HTO exposure periods were 20.5 mGy and 26.5 mGy, respectively. It is not known whether the T-Gly activity concentration in the tissues reached equilibrium with the surrounding media because T-Gly is suggested to be actively taken up by the organisms (Cheney et al., 2007). However, to a first approximation the T-Gly dose rate was 4.9 μGy h^{-1} or a total of 0.81 mGy over 7 days of exposure (see Section 2.4.), assuming equilibrium conditions. The activity concentrations above were comparable with those experienced in the vicinity of discharges or during relatively short timescales after an acute release (Rowe et al., 2001). Such activity concentrations represent doses substantially lower than the limit of 400 μGy h^{-1}, below which no significant detrimental effects on aquatic organisms at the population level are expected (IAEA, 1992). Furthermore, this dose range was based on our earlier studies using this organism (Hagger et al., 2005; Jha et al., 2005).

2.3. Exposure of mussels to tritiated compounds

Exposure 1: Seven 10 l tanks each containing five mussels and 5 l of seawater were spiked with HTO to an activity concentration of 37 MBq l^{-1} for 7 days (Hagger et al., 2005; Jha et al., 2005). Mussels were not fed during the exposure period and at the end of the experiment the HTO-spiked seawater was replaced with clean seawater to allow depuration. At each point in the depuration period (i.e. 0, 1, 3, 7, 10, 14 and 21 days after exposure), mussels from one tank were taken for analysis and the water in the remaining tanks was replaced with clean seawater. During the depuration period mussels were fed as described in Section 2.1.

Exposure 2: In order to establish the uptake of tritium into DNA a second HTO exposure was carried out using the same conditions, except that the activity concentration of the HTO spike was 24 MBq l^{-1} and the exposure lasted for 14 days. Immediately after the exposure, the mussels were sampled for analysis.

Exposure 3: Five 10 l tanks each containing five mussels were spiked with 1.48 MBq l^{-1} of T-Gly for 7 days. The mussels were not fed during the exposure but feeding was resumed, as described in Section 2.1, during the depuration period. At each point in the depuration, i.e. 0, 1, 7, 14 and 21 days after exposure, mussels from one tank were taken for analysis and the water in remaining tanks was replaced.

Negative controls: Two 10 l tanks each containing five mussels were left untreated in clean seawater, one in parallel with exposure 1, the other parallel to exposures 2 and 3. Mussels were sampled at the end of the exposure periods to give values of activity concentration in tissues and DNA and MN frequency.

2.4. Analyses of tritium incorporation and genotoxicity

2.4.1. Determination of induction of micronuclei (MN)

Induction of MN as a measure of genotoxicity is routinely used in our laboratory and has been validated against reference chemicals and HTO (Canty et al., 2009; Jha et al., 2005). Briefly, a sample of haemolymph (300 μl) was extracted from the posterior adductor muscle of each mussel using a 21-gauge hypodermic needle into a 1 ml syringe containing 300 μl of chilled (4 °C) physiological saline (Peek and Gabbot, 1989) to prevent cell clumping (Al-Subiai et al., 2009; Jha et al., 2005). Before counting the frequency of MN, the viability of the haemocytes was also checked using eosin-Y staining. Eosin-Y stain (2 μl of 2% solution; Sigma–Aldrich, Poole, UK) was added to a subsample of haemolymph (40 μl) in an Eppendorf tube, mixed gently, and placed in a haemocytometer. Using a light microscope (final magnification ×400) the cell density and percentage of unstained (viable) cells were determined.

To determine MN frequency, 100 μl of the haemolymph from each mussel was centrifuged (470*g* for 5 min) using a cytospin centrifuge (Thermo Shandon Ltd., Cheshire, UK) onto a microscope slide. The slides were allowed to air dry (24 h) and were then fixed by immersing in a Coplin jar containing absolute ethanol for 15 min. Fixed slides were then stained with (5%) Giemsa stain (BDH, Poole, UK) for 20 min before mounting with DPX adhesive with a cover glass. The slides were allowed to dry and harden for 48 h before 1000 cells per slide were scored for the induction of MN under a light microscope using specific criteria described by Venier et al. (1997).