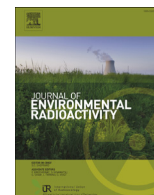
Journal of Environmental Radioactivity xxx (2017) 1–8

Contents lists available at ScienceDirect

Journal of Environmental Radioactivity

journal homepage: www.elsevier.com/locate/jenvrad



Aquatic live animal radiotracing studies for ecotoxicological applications: Addressing fundamental methodological deficiencies

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ARTICLE INFO

Article history:
Received 18 December 2016
Received in revised form
8 May 2017
Accepted 31 May 2017
Available online xxx

Keywords:
Metal
Method
Nanomaterial
Radiotracing
Invertebrate
Vertebrate
Live-counting

ABSTRACT

The use of live animal gamma radioisotope tracer techniques in the field of ecotoxicology allows laboratory studies to accurately monitor contaminant biokinetics in real time for an individual organism. However, methods used in published studies for aquatic organisms are rarely described in sufficient detail to allow for study replication or an assessment of the errors associated with live animal radioanalysis to be identified. We evaluate the influence of some important methodological deficiencies through an overview of the literature on live aquatic animal radiotracer techniques and through the results obtained from our radiotracer studies on four aquatic invertebrate species. The main factors discussed are animal rinsing, radioanalysis and geometry corrections. We provide examples of three main techniques in live aquatic animal radiotracer studies to improve data quality control and demonstrate why each technique is crucial in interpreting the data from such studies. The animal rinsing technique is also relevant to non-radioisotope tracer studies, especially those involving nanoparticles. We present clear guidance on how to perform each technique and explain the importance of proper reporting of the validation of each technique for individual studies. In this paper we describe methods that are often used in lab-based radioecology studies but are rarely described in great detail. We hope that this paper will act as the basis for standard operating procedures for future radioecology studies to improve study replication and data quality control.

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1. Introduction

Live animal radiotracer techniques (i.e. using gamma-emitting isotopes) present important tools for ecotoxicology, providing opportunities to study the uptake/depuration kinetics within living individuals in a non-destructive manner. These techniques also help to understand the physiology of live animals by quantifying the time integrated transfer of essential elements at natural levels from different media (water, food and/or sediment in the case of aquatic organisms). Although these techniques have been in use since the 1970s (e.g. Fowler et al., 1971), their application has increased in recent years (Cresswell et al., 2014; Gillis and Wood, 2008; Fowler et al., 2004; King et al., 2005; Metian et al., 2010; Wang, 2001; Warnau and Bustamante, 2007) with advances in radiation detector technology and autoradiography techniques (Cresswell et al., 2015, 2017; Lacoue-Labarthe et al., 2011; Lanctôt et al., 2017; Renaud et al., 2014; Rouleau et al., 2001). Given that live animal radiotracer techniques have been used for nearly half a century, it is surprising that the methodological descriptions in resultant publications often lack sufficient information on the steps taken to ensure the accuracy and precision of the data presented. Considerations such as the rinsing of aquatic organisms to remove adsorbed contamination, the effect of live animal movement on the resultant signal received and effective geometry correction are seldom described in detail. Without attention to these aspects of the method, counting errors arise that may make statistically significant differences between treatments difficult to detect, skew data and cause mis-interpretation of results. While many journals have strict word limits that may result in authors not reporting detailed methods, the majority of publications now include a

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http://dx.doi.org/10.1016/j.jenvrad.2017.05.017
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Supporting Information section. This section allows for extra methodological details to be described at length, following a briefer mention of the methods in the main text of a paper.

Incomplete reporting of methodological information has been identified as an issue in bioscience generally and guidelines have been developed to ensure that data reported from live animal studies are "fit for purpose" (Kilkenny et al., 2010). Studies into dietborne metal bioavailability and toxicity have specifically been identified as having inconsistencies in methodological reporting (Clearwater et al., 2002). With national and international initiatives promoting a reduction in the use of animals in scientific research (e.g. Burden et al., 2015), there is a need to demonstrate the scientific advantages of live animal radiotracer studies (i.e. use of fewer animals and non-invasive measurement techniques) and to optimise the data quality obtained with the technique.

In this paper we evaluate the influence of selected methodological factors through a critique of the relevant literature and through the results obtained from radiotracer studies undertaken by the authors on four aquatic invertebrate species and two vertebrate species (Table 1). These factors are classically encountered in live aquatic animal radiotracer techniques; namely animal rinsing, radioanalysis and geometry.

2. Live animal radiotracer techniques

The following sections describe data collected from several lab-based live-animal biokinetic studies conducted using gamma-emitting isotopes and detected using coaxial detectors (LaBr, NaI and HPGe) housed within lead shields and connected to PCs equipped with spectra analysis software. To ensure that propagated counting errors were less than 5%, animals were presented to the detector for 2–10 min; the counting time used was a function of the task being conducted, animal welfare and the measurement time interval.

2.1. Rinsing of animals prior to radioanalysis during aqueous radioisotope exposures

The majority of radiotracer studies conducted in the laboratory for aquatic organisms require the animal to be exposed to aqueous radioisotopes to determine uptake and loss kinetics of elements from solution (i.e. accumulation at permeable respiratory surfaces such as the gill and, to a lesser extent, from the stomach from imbibing ambient water). As this exposure involves the animal being submerged in a solution of ionic or labile species of the element of interest, adsorption of the radiotracer to the specific structure of the organism in direct contact with the exposure media is inevitable (i.e. cuticle of soft and hard tissues). The primary aim of live animal radioanalysis during solution exposures is to determine the activity of the element that has been bioaccumulated/bioconcentrated from waterborne exposure (i.e. assimilated into the internal tissues of the animal). Therefore, it is important to either remove the adsorbed radiotracer from the cuticle via rinsing, or account for the relative contribution of the cuticle-adsorbed activity to the whole animal activity. These rinse conditions are rarely described in much detail in the methodological sections of published radiotracer studies, with validations of such rinse techniques often absent. While this section mainly focusses on animals used for solution exposures, the same rinse techniques can also be used during dietary exposures (e.g. pulse-chase) to ensure uneaten food or disassociated dissolved isotope are removed from the cuticle of the animal prior to radioanalysis.

Rinsing an aquatic organism or removing the surface adsorbed fraction of radioactivity is best achieved via the physical action of moving the animal within the rinse media (Cresswell et al., 2015) or spraying the animal with rinse media (Golding et al. in prep). This not only acts to dilute any surface adsorbed radiotracer with non-radioactive forms of the element or another element via displacement, but it also acts to physically disassociate the radiotracer from the cuticle. The rinse media can be either the standard medium the organism is exposed to (i.e. freshwater or saltwater without the addition of the radiotracer) and/or a pH-matched chelating solution (e.g. ethylenediaminetetraacetic acid; EDTA), which, due to its ability to sequester cationic elements, should increase the removal of weakly-adsorbed radiotracers from the cuticle. It is important to limit the time each animal spends within the EDTA to minimize ingestion of the solution and potential adverse effects associated with EDTA. Therefore, optimisation of EDTA exposure time and concentration is required prior to conducting the actual experiment. Regardless of the type of media used for animal rinsing, studies should report data to validate the efficacy of the rinse method used, or at least describe preliminary tests used to determine that the activity measured reflects the bioconcentrated activity. This reporting is essential in order to confirm that reported whole-animal activities are those that represent the true bioaccumulated element and not the bioaccumulated plus cuticle-adsorbed radioactivity.

In a study investigating the bioaccumulation of insoluble cerium dioxide nanoparticles, freshwater snails (*Potamopyrgus antipodarum;* New Zealand mud snail) were exposed to ionic or nanoparticulate (nominal size of <25 nm) forms of ^{141}Ce (cerium(III) sulphate and cerium dioxide respectively). All radioisotope sources were produced in the OPAL reactor of the Australian Nuclear Science and Technology Organisation (ANSTO) using thermal

Table 1
Aquatic species used in the live animal radiotracer studies reported in this paper.

Common name	Genus and Species	Length (mm)	Width (mm)	Depth (mm)	Mass (g)	Distribution
New Zealand Mud Snail	*Potamopyrgus antipodarum*	5	1.5	1.5	0.0042†	Native to New Zealand; invasive in Asia, Europe, North America
Freshwater Prawn	*Macrobrachium australiense*	43	7.5	7.5	0.72‡	Eastern Australia
Spotted Smooth Shore Crab	*Paragrapsus laevis*	17	21	15	6.6‡	Temperate eastern Australia
Zebrafish	*Danio rerio.*	75	55	8	12‡	Native to Himalayan region; Global experimental (common model in ecotoxicological studies on fish)
Sydney Rock Oyster	*Saccostrea glomerata*	78	53	23	55‡	Australia and New Zealand
Turbot	*Scophthalmus maximus*	35	5	7.5	0.294‡	Western Atlantic and Mediterranean Sea

†dry weight; ‡ fresh weight.

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neutron bombardment. These exposures were conducted in synthetic, medium hardness water based on a USEPA recipe (USEPA, 2002) and in filtered (0.45 μm) local river water. Both exposure media were at comparable pH, hardness, total dissolved solids and alkalinity. To determine the cuticle-only adsorption of ionic cerium, nine empty snail shells (to ensure no bioaccumulation) were exposed to 82 μg Ce(III) in 10 mL of each media (i.e. synthetic water and river water; $n = 9$ in each) for 2 h. The shells were then removed from the exposure solutions, individually transferred to 6 mL polycarbonate vials and radioanalysed. The shells were subsequently rinsed with one of the following rinse media (representing 1 rinse cycle) using wash bottles: 1) Milli-Q rinse, which consisted of 5 × 2.5 mL flushings of Milli-Q water; 2) 50 μM EDTA rinse, which consisted of 3 × 1.5 mL flushings of 50 μM EDTA and 2 × 1.5 mL flushing of Milli-Q water; 3) 100 μM EDTA rinse conducted as per the 50 μM EDTA rinse. Rinse solution was removed from the 6 mL vial between each successive rinse. The rise procedure for one cycle took approximately 10 s. The rinsed shells were radioanalysed in clean 6 mL polyethylene vials and the rinse procedure was repeated until the radioactivity did not change between successive rinses (i.e. the loosely-bound radiotracer had been removed). This rinse trial made the assumption that the capacity of the inside of the snail shell to bind ionic cerium is the same as the outside surfaces of the shell.

Fig. 1 shows the reduction in ionic ^{141}Ce radioactivity in empty shells after a cumulative number of rinses of shells exposed to either synthetic or natural freshwaters. For the shells exposed in synthetic freshwater, the rinse media type did not have an effect on the removal of ionic cerium. However, over three rinse cycles (i.e. 15 rinses), there was a reduction in the radioactivity associated with the shells, suggesting physical removal/dilution of ionic ^{141}Ce dominated. These shells were subsequently rinsed through a total of six cycles (i.e. 30 individual rinses) and the resulting radioactivity had not reduced significantly relative to shells that had gone through three cycles (Student's *t*-test; $p = 0.12$). The most efficient rinse procedure was therefore three rinse cycles (i.e. 15 individual rinses) of Milli-Q of each live snail prior to radioanalysis. Fig. 1 also shows the effects of three and six rinse cycles for shells exposed in natural freshwater. The data demonstrates a slight reduction in shell activity between a Milli-Q rinse and a 100 μM EDTA rinse that was not apparent for the shells exposed in synthetic water, suggesting the chelating effect of the EDTA was assisting in the removal of the radiotracer from the shell. However, there was no significant difference between the amount of radioactivity removed from the shells after six rinse cycles compared with three rinse cycles of the Milli-Q rinse and the 100 μM rinse (Student's *t*-test; $p = 0.12$ and 0.28 respectively). This suggests that there was a stronger binding of the ionic cerium with the shell in the presence of organic ligands in the natural river water such that physical removal (i.e. continuous rinsing) had a negligible impact on radioisotope removal.

A similar rinse test was conducted for live snails exposed to radioactive nanoparticles (nominal size of <25 nm) of cerium dioxide in the same study. Radioactive nanoparticulate $^{141}CeO_2$ were suspended in synthetic freshwater and filtered onto a 0.025 μm membrane (47 mm diameter). The membrane was placed in a polystyrene Petri dish (of a similar diameter) and 10 mL of synthetic freshwater was transferred to the Petri dish. Ten live *P. antipodarum* were transferred to the Petri dish and allowed to traverse the filter membrane for 2 h. Each snail was subsequently removed and transferred into a clean 6 mL vial and radioanalysed without rinsing. Following radioanalysis, five snails were rinsed with 5 × 2.5 mL flushings of Milli-Q water from a wash bottle (representing 1 rinse cycle) and radioanalysed. The remaining five snails were left in their dry vials and were not rinsed as controls that represented the egestion of any nanoparticles over time. The five

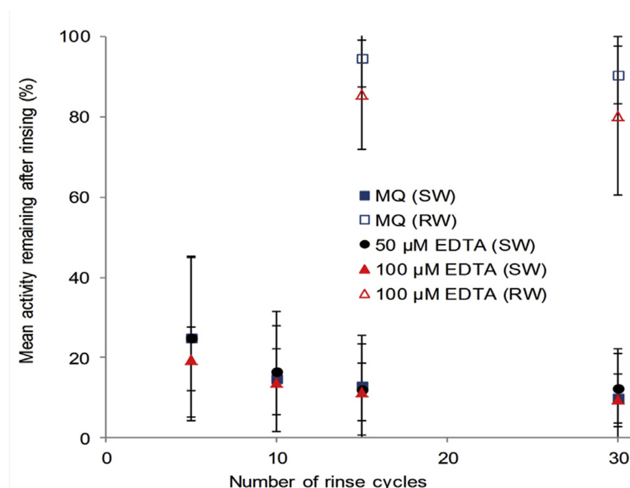

Fig. 1. Effect of different rinses on reducing the ionic ^{141}Ce activity measured in empty shells of freshwater snails. *P. antipodarum* were exposed to ^{141}Ce (as Ce^{3+}) in synthetic water (SW) and river water (RW). MQ – Milli-Q water. EDTA – Ethylenediaminetetraacetic acid. Error bars represent one standard deviation, $n = 3$.

rinsed snails were subjected to repeat rinse cycles of up to a total of 30 cumulative rinses and radioanalysis until radioactivity no longer decreased. The five live snails that had gone through six rinse cycles were transferred to new 6 mL vials with 4 mL of synthetic water and not fed for 96 h to allow for any ingested nanoparticles to be egested.

Fig. 2 shows the reduction of radioactive nanoparticles after successive rinse cycles for five live snails relative to controls that were not rinsed. The non-rinsed snails did not lose any radioactive nanoparticles suggesting minimal desorption or egestion of any ingested nanoparticles while out of water. The rinsed snails continued to lose radioactivity up to three rinse cycles (i.e. 15 cumulative rinses), after which, there was no significant reduction in ^{141}Ce radioactivity up to six consecutive rinse cycles (Student's *t*-test; $p = 0.45$). This suggests that three rinse cycles was sufficient to remove the nanoparticles adsorbed to the cuticle. Interestingly, six rinse cycles did not remove the same proportion of radioactivity associated with nanoparticles as for the ionic cerium trials conducted with empty snail shells. This was likely due to the ingestion of $^{141}CeO_2$ nanoparticles by the live snails. After 24 h of depuration, the activity in the live snails had reduced from 80% to 12% and by 96 h, the nanoparticle activity remaining was 3% (Fig. 2), thus suggesting that the snails had egested nanoparticles. This follows previous work conducted by Forbes and Forbes (1997) who demonstrated that the gut passage time of *P. antipodarum* is approximately 3 h. The data demonstrates that the three rinse cycles (i.e. 15 rinses) were sufficient to remove the majority of nanoparticles associated with the external shell surface of live snails, allowing accurate quantification of the internal ^{141}Ce activity.

The rinsing techniques described above can also be used for studies on non-radioactive nanoparticles and aquatic organisms where the exposure is via solution. If the aim of the study in this situation is to quantify the amount/concentration of nanoparticles bioaccumulated by an organism (i.e. tissue concentration), organisms should be sufficiently rinsed or dissected to ensure that nanoparticles associated with the cuticle do not contribute to the tissue concentration. However, if the aim of the study is to determine effects of food chain transfer of nanoparticles, it is not necessary to rinse the organism when quantifying the total

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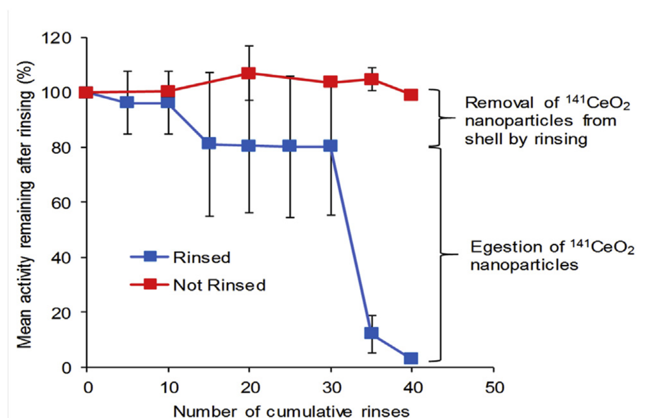

Fig. 2. Effect of rinsing on reducing nano ^{141}Ce activity measured in live freshwater snails. *P. antipodarum* were rinsed with either Milli-Q or not rinsed. Rinses >30 for rinsed snails represent depuration in synthetic water for 24 and 96 h respectively. Error bars represent one standard deviation, $n = 5$.

amount/concentration of nanoparticles associated with prey organisms as a non-rinsed organism more accurately reflects natural situations in the field.

A recent study was conducted using a dissolved radio-cadmium (^{109}Cd) exposure with the freshwater prawn *Macrobrachium australiense* (Cresswell et al., 2017). Three live prawns were exposed to 50 kBq/L (0.76 µg Cd/L) in synthetic river water (SRW) for 24 h before being radioanalysed without rinsing (excess liquid was removed with a lint-free tissue). The prawns were then rinsed using a method based on that reported by Cresswell et al. (2015) whereby prawns were immersed in 400 mL of rinse solution in 4 consecutive containers; 1 × fresh SRW, 2 × 100 µM EDTA solution (adjusted to pH 7.2) and 1 × fresh SRW. The prawn was raised up and down in each solution using an internal polyethylene basket 10 times (for a total period of approximately 10 s) before being transferred with the basket to the next rinse solution. This represented 1 rinse cycle. The prawns were radioanalysed before undergoing a second and third rinse cycle with radioanalysis after each cycle. The whole-body radioactivity of the prawns remaining after the first rinse cycle was 87 ± 9% and 74 ± 3% after the second with no significant reduction in radioactivity between the second and third rinse cycles (Student's *t*-test; $p > 0.05$). Based on these data, the rinse method used the equivalent of 2 rinse cycles (i.e. raising and lowering 20 times in each rinse solution) prior to radioanalysis.

While this method was successful at removing loosely-bound ^{109}Cd tracer solution from the cuticle of freshwater prawns, the autoradiography conducted by the study and by Cresswell et al. (2015) demonstrated that a small proportion of ^{109}Cd remained adsorbed to the cuticle following rinsing. This must be taken into account when interpreting and reporting whole-animal activities, as the total activity detected associated with a rinsed animal may possibly represent the bioaccumulated activity plus any residual cuticle activity.

Separate exposures to aqueous radiotracers have also been conducted in our facilities using the Sydney rock oyster (*Saccostrea glomerata*) with ^{65}Zn (Lee et al., 2015) and the spotted shore crab (*Paragrapsus laevis*) with ^{85}Sr and ^{134}Cs (unpublished results; T. Cresswell). Both studies demonstrated a clear requirement to rinse the radiotracer solution from the cuticle prior to radioanalysis (as a small portion of the radioactivity remained associated with the cuticle).

While considering aqueous radiotracer experiments it is worth noting that, as with all aqueous contaminant studies, feeding of organisms must be conducted outside of the exposure solution to delineate main uptake pathways (i.e. dissolved uptake at respiratory surfaces vs. uptake in the alimentary tract following ingestion). In many cases, it is appropriate to remove the animal from the exposure solution, rinse the animal using a method discussed above and transfer the organism to a clean container with non-active/non-contaminated media and allow feeding for a brief period of time (e.g. 1 h). Other studies such as those with freshwater prawns (Cresswell et al., 2014, 2015, 2017) and with estuarine crabs (unpublished results; T. Cresswell) fed the animal in the counting chamber during radioanalysis. This method enabled the feeding of the organism outside the exposure solution and reduced the movement of the animal during radioanalysis, thus reducing the error associated with live-animal radioanalysis.

2.2. Errors associated with live animal radioanalysis

Radioanalysis of any active object is subject to errors when the subject is mobile. As a radioactive source moves across the scintillation crystal, the solid angle subtended by the detector changes(Knoll, 2010) and the signal amplitude received by the multichannel analyser will change, therefore affecting the resultant radioactivity reported during the count period. It is crucial that the organism remains as immobile as possible during radioanalysis and, as such, it may be appropriate to anaesthetise the animal. However, providing the correct dose of anesthetic to each animal to immobilise for a short period of time (ideally 5–10 min) can be problematic and potentially affect the physiology of the animal post counting, therefore affecting bioaccumulation kinetics.

2.2.1. Aquatic invertebrates

Reducing the animal's movement during radioanalysis by physical means can be achieved using a variety of containers. For example, the New Zealand mud snails (*Potamopyrgus antipodarum*) investigated in our laboratory were initially radioanalysed in 6 mL polyethylene vials with 3 mL of non-active exposure media. However, following radioanalysis, the snails were found to have moved vertically within the vial, therefore reducing the counts reported. The snails were subsequently radioanalysed within empty dry vials, which resulted in the snail closing its operculum (to prevent internal moisture loss) and hence immobilizing the animal. Radioanalysis of freshwater prawns was conducted in square 60 mL Nalgene™ bottles with a Kimwipe™ tissue in the bottle's neck (Cresswell et al., 2015). The internal dimensions of the bottles were approximately the same length and width as the prawns, therefore restricting their movement. Shore crabs were radioanalysed in cylindrical 250 mL polystyrene vials, which were the same diameter as the detector head (unpublished results; T. Cresswell). The crabs were immobilized using a sponge cut to a slightly greater diameter than the vial, and pressed down onto the shell of the crab (Fig. 3). As mentioned above, changes in subject alignment with the detector head can cause variability in the number of photon-crystal interactions recorded by gamma detector. It is therefore crucial to not only immobilise the animal being radioanalysed but to also ensure that the alignment of the animal with the detector head is consistent across all counts to allow for comparison among radioanalyses.

It is possible to successfully radioanalyse animals that are a greater diameter than the detector head such as oysters. Five live Sydney rock oysters were used in aqueous exposures to ^{65}Zn (Lee et al., 2015) and were radioanalysed once with the digestive organs of the animal located over the center of the detector and once with the gill over the center of the detector. There was no

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significant difference (Student's *t*-test; $p = 0.63$) between the counts of the oysters in the different positions, suggesting that small variations in animal orientation over the detector had a negligible effect on the radioactivity received by the detector. When the five oysters were orientated in the same position and radioanalysed four separate times each, the mean relative standard deviation of the counts was 3% (1.1–4.6% range; Table 2), demonstrating that reproducible results can be achieved if the animal is radioanalysed in the same orientation relative to the detector head over consecutive counts. These data suggest that, for ^{65}Zn in a marine oyster, the animal position over the detector did not significantly affect the radioactivity received by the detector. However, it is important to note that this phenomenon is likely organism- and radionuclide-dependent and we recommend that the effects of organism orientation and repeat radioanalysis measurements be quantified and reported for each study.

2.2.2. Fish

The above examples of errors associated with live animal radioanalysis deal with either sessile invertebrates or organisms that are able to be immobilized out of water. A greater challenge is the accurate radioanalysis of constantly-moving organisms such as those studies performed with fish (Jeffree et al., 2006; Mathews and Fisher, 2008; Pouil et al., 2015; Reinardy et al., 2011). This section summarizes the combination of strategies that need to be employed to properly radioanalyse mobile vertebrates and limit the associated errors as much as possible. Techniques are usually developed case-by-case and, as previously mentioned, methodologies used are not always well described. Nevertheless (Reinardy et al., 2011), provide a good example with a short description on how they performed the radioanalysis of zebrafish *Danio rerio* exposed to aqueous and dietary gamma-emitters.

In order to reduce the fish movement during radioanalyses (physical means), small pieces of plastic tubing are cut (length adapted to counting vials) and halved along their length, in order to create a tunnel-like structure that sufficiently limits movement without creating any detrimental effect on the fish (Reinardy et al., 2011, Fig. 4). In contemporary aquatic radioecology studies, it is not recommended to use anesthetic for immobilizing fish for radioanalysis. As previously mentioned, providing the correct dose of anesthetic can be problematic and can potentially affect the physiology being studied (e.g. gill ventilation rate affecting uptake rate of contaminant from solution vial gills). Some fish species are easier to work with in terms of limiting their movement. For example, flat fish such as turbots *Scophthalmus maximus* are ideal for live counting due to calm behaviour, if not manipulated too much prior the counting (e.g. Pouil et al., 2015). The size/age of the fish will be important and large fish may not fit into regular vials used on coaxial detectors (typical germanium detector diameter is 85 mm). In this case, fish can be placed in vials filled sufficiently with non-contaminated seawater (150 mL; diameter: 80 mm, height: 50 mm) but no 'tunnel' or other devices are required in the case of the turbot. The fish do not move considerably during the radioanalysis and there is no hyperventilation observed. Animal welfare is monitored to ensure that the animals appear to be unstressed and water conditions stay adequate for the time of the radioanalysis (stable temperature and dissolved oxygen levels; the latter only decreasing by a few percent compared to original levels; personal observation).

Obviously, each live animal radioanalytical method used must be validated by preliminary tests to determine the error associated with small variations in fish orientation over the detector relative to the effect on the photon-crystal interactions detected. Such validation data generated from preliminary tests should be reported along with the main quality control data. In addition, Pouil et al. (2015) carried out radioanalytical simulations where turbot were placed in the counting vials in the dark and the dissolved oxygen (DO) of the surrounding water was measured using a probe and fish behaviour regularly observed. The DO probe indicated 100% saturation for the duration of the radioanalysis (25–60 min). Furthermore, before experiments, fish were acclimated to the handling process (from aquarium to the counting box and then to the counting chamber) in order to minimize the stress caused by handling and thus limit the motion of the fish during γ-counting.

The reporting of the reproducibility of consecutive radioanalyses of the same individual is crucial for interpreting live animal radioanalysis data. Table 2 shows the variability associated with counting the same individuals when the animal was restricted in its movement and when the animal was found to have moved during the radioanalysis. The data demonstrates the requirement to re-analyse any animal found to move during analysis or to calibrate the detector with a standard in various positions prior to conducting the study. We recommend that future live animal radiotracing studies quantify and report live animal radioanalysis reproducibility data to determine the errors associated with such analyses.

Fig. 3. Pictures of the set-up especially made for gamma-counting spotted smooth shore crabs, *Paragrapsus laevis*. Photos courtesy of Tom Cresswell.

2.3. Accounting for irregular geometries of live animals to determine sample efficiency

To convert a count per second (CPS) of a signal obtained from a gamma spectrum to a Becquerel (Bq) activity, the efficiency of the sample must be determined for each detector in a given geometry (Knoll, 2010). This is achieved by radioanalysing a certified or in-house standard of a known activity of each isotope of interest in a set geometry. This relative efficiency is then applied to samples in the same geometry to calculate Bq from CPS. This conversion is important to standardise across data reported from multiple experiments and studies.

If one were to change the geometry of the standard while maintaining the radioactivity, the detector would return a different CPS. For example, if a homogenous 10 mL solution of 100 Bq of ^{60}Co was radioanalysed in a 20 mL liquid scintillation vial with a coaxial detector and the integration of the resulting gamma spectrum returned 1 CPS, the relative efficiency would be 1%. If the same solution was transferred to a 90 mm diameter petri dish, the reported activity would likely increase to around 2–3 CPS as there would be a greater proportion of the sample closer to the detector crystal. In this case, the relative efficiency would increase to 2–3%,

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Table 2
Examples of variance (% relative standard deviation) of recorded radioactivity between consecutive counts (>4) of the same individual organism when immobile and mobile. Typical live to digested ratios used for geometry/efficiency corrections.

Organism	% RSD of counts of immobile organism[a]	% RSD of counts of mobile organism[a]	Typical live to digested ratio[b]	Study ref
New Zealand Mud Snail	5% (4–6%; n = 4)	n/a	0.54 (n = 6) for ^{141}Ce	Golding et al. in prep
Freshwater Prawn	5% (1–9%; n = 12)	9% (5–13%; n = 12)	0.74 (n = 3) for ^{65}Zn and ^{109}Cd	Cresswell et al., 2014, 2015
Spotted Shore Crab	2% (1–7%; n = 6)	10% (1–23%; n = 22)	0.70 (n = 5) for ^{134}Cs and ^{85}Sr	Cresswell et al. in prep
Sydney Rock Oyster	3% (1–5%; n = 7)	n/a	1.09 (n = 15)[c] for ^{65}Zn	Lee et al., 2015

[a] Mean % relative standard deviation (% RSD range; number of individuals radioanalysed).
[b] Digested ratio = live counts/whole organism digested counts in standard geometry (10 mL solution in 20 mL liquid scintillation vial) for the isotopes listed.
[c] Digested ratio for soft tissue only.

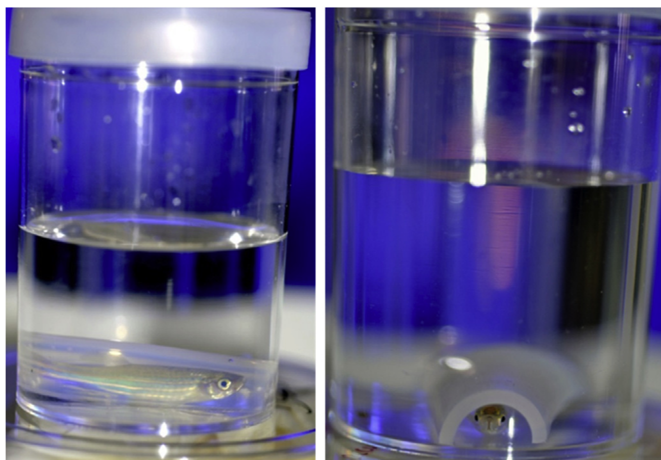

Fig. 4. Pictures of the set-up especially made for gamma-counting zebrafish, *Danio rerio* as described by Reinardy et al. (2011). Photos courtesy of Jean-Louis Teyssié.

thus affecting the conversion of CPS to Bq.

Many radiotracer studies conducted with aquatic organisms report that samples were calibrated against radionuclide standards with identical geometry and sample mass as the whole organism (e.g. Alquezar et al., 2007), that efficiencies were estimated using matrix and geometric matched standards (e.g. Creighton and Twining, 2010) or that whole animal or animal organs were compared with standards of known activity and appropriate geometry (e.g. Hervé-Fernández et al., 2010). However, there is often no mention of what these standards were and how they were prepared. Without the explicit description of these standards, it is very difficult for the reader to understand how the CPS were converted to a meaningful value (i.e. Bq or mass of element).

To ensure that an appropriate geometry correction has been applied to live animal counting data, we have used two approaches. The first is to construct phantoms using the organisms from the study and the second is to perform digestions in a standard liquid geometry on a sub-set of exposed organisms.

2.3.1. Phantoms

Constructing phantoms is the most commonly used geometry correction approach in radioecology. The so-called phantom corresponds to a “standard organism” bearing a known activity of one or several radioelements and it is used to simulate, in the most optimised way, the geometry (shape and the density) of the studied organism. This approach is key to accurately measuring the activity of radioisotopes accumulated in a living organism, but it is also important to properly calibrate the instrument efficiency with an independent standard. However, these detailed methodologies for preparation and quality assurance of phantoms for whole organisms are rarely described within published manuscripts.

For a given organism and prior to the exposure, an individual is sampled then euthanized and internal tissues (mainly viscera) are removed but care is taken to maintain the structure and exterior appearance (especially facing the detector) in the best condition as possible in order to closely mimic the shape (geometry) of a living individual. The next step is to guarantee the same state of this biological material for the entire duration of the exposure. Therefore, the organism is soaked in a bath of a diluted formalin solution (approx. 4%) overnight (>12 h).

When removed from the formalin solution, tissue samples are exposed to air in order to evaporate the excess of formalin. Then, an absorbent support (e.g. paper towel/tissue) is placed strategically within the organism. The organism is then placed in a counting tube, identical to the one that will be used to count the living organisms, and is ready for the spike of a small volume of solution of known activity (verified by radioanalysis against a certified standard solution) of radioelement of interest. The absorbent support is first imbibed with a solution of weak acid (usually 2 M of HCl) which facilitates the diffusion of the radioelements (good internal distribution). If a specific organ is the major storage place of a radioelement (e.g. the hepatopancreas of a decapod crustacean in the case of most transition metals) the absorbent support will be positioned in the area where this organ is located. While radioanalysis of live fish necessitates counting in a volume of clean water, to avoid loss of radioisotope from the phantom into the surround medium, phantoms aren't normally submerged in water. Therefore, it is important to be aware that photon attenuation in water may affect the counts received from live fish and the extent of attenuation will be dependent on the isotope; i.e. isotopes with a low energy such as ^{109}Cd (88 keV) will attenuate more so than those with a higher energy such as ^{65}Zn (1.1 MeV) by the surrounding water.

Below are some complementary recommendations that need to be used to optimise as much as possible the phantom quality when applied to the above approach:

(1) Ensure the radioactive spike is completely dry on the absorbent support to avoid migration.
(2) Dedicate at least one phantom per experiment.
(3) The relative size of the phantom to the samples is very important. Therefore, if studies on allometry (biological scaling) are undertaken a series of phantoms are recommended.
(4) Radioactivity of the phantom needs to be high enough to reduce the counting time to 5–10 min, while remaining comparable to the activity the organism will accumulate during the exposure.
(5) The spike for the experiment and for the phantom standard should originate from the same source to avoid differences in

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the resultant peak characteristics within the gamma spectrum.

(6) The lifetime of phantoms depends on what they are composed of. Fish phantoms will often only be used for the duration of an individual experiment due to the fact that some organic matter is still present and will degrade with time. Conversely, liquid standards can serve for a very long period (e.g. months to years depending on isotope half-life) due to the fact that they are often preserved in dilute acid. Of course, evaporation over time could occur if they are not properly stored, which can affect the geometry of liquid standards and resultant detected activity.
(7) Maintain the phantom in the same location and orientation on the detector during the calibration and also during the daily check of the activity during the experiment.
(8) Test statistically the effect of phantom position after the calibration – for example the rotation on the center of the detector or the translation (left/right or forward/backward) compared to the initial selected position. This will assist in accounting for errors associated with live animal radioanalysis as discussed above.
(9) In the context of using a coaxial detector, it is preferable to position the phantom and samples as close to the detector as possible (i.e. limiting the distance to the crystal) and as centrally on the detector as possible to maximize interaction of emitted photons with the detector crystal.

2.3.2. Standard liquid geometries

The second approach of geometry correction involves digestion and analysis of a sub-set of exposed organisms. When aqueously exposed organisms have accumulated enough activity to result in <5% counting error for a 5 min count, a subset of at least three organisms is selected which represent the range of sizes of animals within the study. Each selected organism is radioanalysed three times, euthanized, dried and completely digested in concentrated reverse aqua regia (3:1 nitric acid to hydrochloric acid). This may require microwave digestion depending on the organism; for oysters we digested only the soft tissues assuming that this was where the significant majority of accumulated ^{65}Zn was present (Lee et al., 2015). This may not be appropriate for other isotopes in such organisms. The digestant is then made up to 10 mL with Milli-Q water and transferred to a plastic 20 mL liquid scintillation vial. The solution is then radioanalysed on the same detector and the CPS for the digested sample is compared to the CPS for the live animal to calculate a live to digested ratio. Table 2 lists the typical live-to-digested ratios for organisms considered in this paper. Once the live animal CPS have been converted to digested CPS, a standard efficiency can be applied based on solutions prepared in-house with known activities of the isotopes of study in exactly the same geometry (i.e. 10 mL homogenous solution in a 20 mL liquid scintillation vial). This then allows the conversion of digested animal CPS to Bq, which can be applied to the animals remaining in the exposure.

This method can be used for animals of all shapes and sizes. Due to the specificity for different isotopes to be accumulated in different organs (e.g. Cd in hepatopancreas and gills; Cresswell et al., 2015) it is preferable that the geometry correction method is undertaken for the specific isotopes being studied. This could be conducted from a multi-isotope exposure of at least three animals rather than having to expose each isotope individually. It must be noted that the internal location of an accumulated isotope within an animal may change over time. For example, aqueous ^{65}Zn is accumulated by prawns via the gills, transferred to the hepatopancreas and subsequently transferred to the antennal gland for excretion over a period of weeks (Cresswell et al., 2015). If the animal is maintained in the same location and orientation on the detector during this period, the transfer of the radioisotope from more centrally-located organs through to the extremities of the organism may change the solid angle subtended by the detector and reduce the detection efficiency as a result (Knoll, 2010). However, the differences in detection efficiency from this phenomenon are likely to be negligible in most cases, as demonstrated by the example of the oyster gill vs. gut radioanalysis discussed in section 2.2.

3. Concluding remarks

Although non-lethal methods have increasingly been used for viral and DNA studies in wildlife, there have been relatively few attempts to use non-lethal methods for determination of contaminant burdens. This is largely due to limitations in analytical resolution and in the understanding of contaminant behaviour within organisms (e.g. Wood et al., 2011). However, there have been significant advances in pollution ecology and contaminant analysis in recent years (e.g. Yankovich et al., 2010; although that paper focused on lethally-harvested tissues) and there is a renewed opportunity for interdisciplinary research to develop non-lethal methods for measuring contaminant burdens in wildlife. Initiatives such as the National Centre for Replacement, Refinement & Reduction of Animals in Research (NC3Rs; www.nc3rs.org.uk), are strongly promoting a move away from the destructive use of animals in research (Cook and Robinson, 2007) towards the development of new research techniques and approaches (Burden et al., 2015). This has been supplemented by international initiatives, such as the formation of the International Union of Radioecology (IUR) Task Group on Non-lethal Methods (http://goo.gl/GbgSnG).

Live-animal radioanalysis using gamma-emitting isotopes of contaminants or essential elements allows for a more detailed analysis of element bioaccumulation using fewer animals and non-destructive techniques. Furthermore, changes in an individual organism's physiology (e.g. moulting in decapod crustaceans) and the impacts of these changes on element bioaccumulation and depuration can be studied in great detail using this technique. While the production of phantoms and standard liquid geometries described above are destructive techniques, the general use of live-animal radioanalysis greatly reduces the amount of lethal sampling being undertaken.

With advancements in positron emission tomography (PET) and single-photon emission computed tomography (SPECT) imaging techniques, methods often used to study radioelement organ distribution *in vivo* in mammals may soon be applicable to a wide range of aquatic organisms, further reducing the requirements for destructive procedures in the lab. The use of live animals in radioisotope tracing studies combined with post mortem autoradiographic imaging of the biodistribution of accumulated radioisotopes from the same individuals maximises the data generated from each animal (Cresswell et al., 2015; Lacoue-Labarthe et al., 2009; Rouleau et al., 2001).

In this paper we have described methods that are often used in lab-based radioecology studies but are rarely described in great detail. We hope that this paper will act as the basis for standard operating procedures for future radioecology and radiotracer-based ecotoxicology studies to standardise such methods and improve the accuracy, efficiency of measurements and optimise quality control of the data. Beyond laboratory applications, there is a move in field radioecology towards live-monitoring of wildlife, enabling whole-body activity concentrations to be determined in situ, thereby minimising the impact of such studies on wild populations (Bondarkov et al., 2011; Wood et al., 2011). We anticipate that the

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important methodological developments discussed here for laboratory studies will also provide a basis for the future development of field live-monitoring procedures.

Acknowledgments

This paper has been developed through collaborations formed within the International Union of Radioecology (IUR) Task Group on Non-lethal Methods in Radioecology, which is chaired by M.D. Wood. The contributions of M.D. Wood were supported by the TREE project (www.ceh.ac.uk/TREE), which is funded by the Natural Environment Research Council, the Environment Agency and Radioactive Waste Management Ltd. The contributions of L.A.Golding were supported by an Australian Institute of Nuclear Science and Engineering (AINSE) Research Award (ALNGRA13055). The IAEA is grateful for the support provided to its Environment Laboratories by the Government of the Principality of Monaco. The authors declare no conflict of interest relating to this paper.

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