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The suitability of gallium as a substitute for aluminum in tracing experiments

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Abstract Aluminum is a toxic metal whose complex aquatic chemistry, mechanisms of toxicity and trophic transfer are not fully understood. The only isotope of Al suitable for tracing experiments in organisms—^{26}Al—is a rare, costly radioisotope with a low emission energy, making its use difficult. Gallium shares a similar chemistry with Al and was therefore investigated as a potential substitute for Al for use in aquatic organisms. The freshwater snail, *Lymnaea stagnalis* was exposed to either Al or Ga (0.0135 mM) under identical conditions for up to 40 days. Behavioural toxicity, metal accumulation in the tissues, and sub-cellular partitioning of the metals were determined. Al was more toxic than Ga and accumulated to significantly higher levels in the soft tissues ($P < 0.05$). The proportion of Al in the digestive gland (DG; detoxificatory organ) relative to other tissues was significantly lower than that of Ga ($P < 0.05$) from day 14 onwards. There were also differences in the proportions of Al and Ga associated with heat stable proteins (HSPs) in the digestive gland, with significantly more HSP present in the DGs of snails exposed to Al, but significantly less Al than Ga associated with the HSP per unit mass protein present. From this evidence, we conclude that Ga may be of limited use as a tracer for Al in animal systems.

Keywords Metal toxicity · *Lymnaea stagnalis* · Aluminum · Gallium · Bioaccumulation · Freshwater snail · Aquatic toxicology

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Introduction

The aluminum (Al) ion is profoundly toxic, affecting cellular secondary messenger systems and increasing the production of reactive oxygen species (ROS) (Rengel 2004). Fortunately, Al in the lithosphere is predominantly associated with hydroxides and silicates and is therefore largely unavailable to biota. In the aquatic environment at circum-neutral pH and in simple solution, Al is predicted to be precipitated as aluminum hydroxides such as gibbsite (Martin 1986); these species are considered inert and hence non-toxic. The bioavailability of aquatic Al does vary, however, increasing as pH decreases, and is also affected by the presence of different ligands (Walton et al. 2009). Anthropogenic effects such as freshwater acidification and nitrogen deposition also contribute to an increase in Al bioavailability (Courtijn et al. 1990; Bowman et al. 2008). Furthermore, different species accumulate and are affected by Al toxicity to

differing extents, and there is an increasing body of evidence that Al is bioavailable and toxic to some organisms even at circum-neutral pH. The aquatic snail *Lymnaea stagnalis*, for example, accumulates Al from water at neutral pH (Elangovan et al. 1997), and also exhibits significant behavioural and physiological toxicity (Campbell et al. 2000a, b; Dobranskyte et al. 2004). The mechanism and rate of Al accumulation, its speciation and action in the tissues, together with excretion mechanisms, are not fully understood and require further investigation.

Radioactive tracers and stable isotopes are both used in environmental studies to investigate the flux and cellular partitioning of metals, and also their trophic transfer (Croteau et al. 2007; Metian et al. 2009). In the case of naturally occurring Al, over 99% is present as its only stable isotope, ^{27}Al. The only radioactive isotope with a half life of more than a few minutes and hence of use in tracing studies is ^{26}Al, a positron emitter with a low specific activity (7×10^8 Bq g^{-1}). Techniques such as low level liquid scintillation spectrometry (Bjornstad et al. 1992) allow its measurement. However, its low activity, coupled with high cost, mean ^{26}Al is rarely considered practical in ecological tracing studies (an exception being Oughton et al. 1992).

Due to their chemical similarities, gallium (Ga) may be of use as an alternative to Al isotopes in tracing experiments. The hydrated ion of Ga, like Al, is a Pearson hard acid, forming complexes with hydroxide, phosphate, sulphate and fluoride (Wood and Samson 2006). In aqueous systems Ga forms silicate species analogous to Al–Si complexes (Pokrovski et al. 1996, 2002), and is known to substitute for both Fe and Al ions in rock-forming minerals (Burton 1978). However, Al may well complex with organic compounds more readily than Ga (Shiller and Frilot 1996). In biological systems, at equilibrium, both Ga and Al associate with transferrin (Harris and Pecoraro 1983; Harris and Sheldon 1989) with both preferentially associating with the N-terminal site of the protein (Andre and Macke 2003), indicating that they may possess some shared transport mechanisms. Their similar chemistry may also lead to comparable mechanisms of toxicity, with both Ga and Al stimulating an increase in the production of ROS through the disruption of Fe metabolism (Rengel 2004; Beriault et al. 2007). Researchers have therefore investigated the suitability of Ga as a substitute for Al in mammalian in vivo and cell culture studies. Results have been varied, with some studies concluding that Ga is an effective substitute for Al (Dobson et al. 1998), whereas others indicate differences in Al and Ga transport (Radunovic et al. 1998).

To establish whether Ga might be a suitable tracer for Al in aquatic invertebrates, we compared rates and amounts of accumulation, together with the cellular localisation of Al and Ga in *L. stagnalis*. Snails were exposed to the same molar concentrations of the metals over periods of up to 40 days. Sub-lethal toxicity of the two metals was assessed relative to control snails and metal accumulation in the soft tissues was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES). Sub-cellular fractionation followed by metal quantification allowed a comparison of the cellular location of Al and Ga in the digestive gland, which is the main detoxificatory organ in the snail.

Materials and methods

Mature *Lymnaea stagnalis* (shell length 2–2.5 cm) were obtained from unpolluted freshwater ponds in Surrey, UK and acclimatized to laboratory conditions for 2 weeks prior to use in exposure experiments. Snails were maintained in aerated simulated, defined pond water (SDPW) (White et al. 2008), at 14°C and pH 7.1 ± 0.5 in a 12 h/12 h dark/light regime, and fed on lettuce ad libitum. Solutions were prepared using ultra-high purity (UHP) water; all reagents were high purity (Sigma, UK or VWR, UK) unless otherwise stated, and HNO_3 was ultrapure grade (Romil, UK). Polypropylene plastic-ware was used throughout, acid washed (10% HNO_3) for 24 h, rinsed with UHP water and dried prior to use.

Exposure conditions

Three groups of 50 snails were used. One group was kept in control conditions (SDPW only) whilst the other groups were exposed to either Al or Ga at concentrations of 0.0135 mM in the water column. These levels are comparable to Al concentrations used our previous studies, e.g. (White et al. 2008; Walton et al. 2009), and within the range found in the environment (Boult et al. 1994; Dixon and Gardner 1998). Stock solutions of 0.0135 M $Al(NO_3)_3$ (pH 3)

or $Ga(NO_3)_3$ (pH 3) (Alfa Aesar), were added to the tanks following which the SDPW was readjusted to pH 7 using NaOH. Snails were exposed for up to 28 days for toxicity assessment, tissue accumulation measurements and for 40 days for sub-cellular fractionation. Snails were fed lettuce ad libitum, and were moved to clean tanks containing SDPW (plus metals) every 2 days. Water samples (10 ml) were collected from the exposure tanks prior to addition of the snails, acidified with 100 µl HNO_3 and stored for metal quantification to check that levels in the water column were as expected.

Toxicity assessments

Behavioural toxicity was measured for a cohort of ten snails per tank every 2–3 days up to day 30. Snails were placed in SDPW-filled Petri dishes on a mirrored surface and left to recover from handling for 5 min. Each snail was then given a ranked score based on objective activity criteria observed over a 2-min time period (Tuersley and McCrohan 1987; Dobranskyte et al. 2004). These behavioural state scores (BSS) were analyzed by a non-parametric analysis of variance (Kruskall–Wallis test) followed by Mann–Whitney *U*-tests (SPSS) with a modified Bonferroni correction procedure to adjust for multiple comparisons (Hochberg 1988). Nominal alpha was set to 0.05.

The effects of Al and Ga on the fertility and fecundity of snails were also assessed. Egg masses were collected from the different exposure groups from 2 to 28 days after the start of metal exposure, and kept in clean SDPW until hatching. The number of egg masses and total eggs were counted, as was the number of eggs that hatched from each exposure group. Proportions of hatched eggs were converted to frequencies, and the probability of Al and Ga affecting the fertility of *L. stagnalis* was assessed using a X^2 test.

Tissue accumulation

Snails ($n = 7$) from both exposure groups and controls were sampled for metal analysis at days 0, 7, 14, 21 and 28 as described previously (Walton et al. 2009). In brief, snail tissue was dissected into digestive gland (DG) and rest of soft tissues (RST), with the gut removed. Samples were rinsed in UHP water, air-dried at 60°C and then digested in 2 ml of 70% (wt/vol) HNO_3 for 24–48 h followed by 2 ml 30% (wt/vol) H_2O_2. Digest blanks were also analyzed.

Samples were analyzed for Al and Ga using ICP-AES (Perkin-Elmer Optima 5300) and data were analyzed using ANOVA (in SPSS).

Sub-cellular fractionation

Analysis of the sub-cellular distribution of Al and Ga in the DG was based on methods described previously (Steen Redeker et al. 2007), using snails that had been exposed to Al or Ga for 40 days. Control snails were also analyzed to assess the proportions of total and heat stable proteins (HSP) in the DG. DGs were removed from the snails ($n = 6$ in each group) and homogenized manually on ice with eight strokes of a glass homogenizer in 6× their volume of ice cold homogenization buffer (10 mM TRIS; 250 mM sucrose, pH 7.4) relative to DG wet mass.

Ten percent of the volume of each homogenate was removed for total metal quantification, and the remainder was centrifuged at 1,500*g* for 10 min at 4°C. The supernatant was removed and stored on ice prior to further fractionation; the pellet was then resuspended in 400 µl dH_2O and heated for 4 min at 100°C. An equal volume of 1 M NaOH was added to this suspension, the samples mixed thoroughly and then incubated at 65°C for 1 h. Following incubation samples were centrifuged at 10,000*g* for 30 min, yielding a supernatant containing digested cellular debris, and a pellet containing metal-rich granules.

The supernatant from the initial centrifugation step was ultracentrifuged at 100,000*g* for 90 min at 4°C in a Sorvall RC 28S supraspeed centrifuge using a F28/13 rotor and Sorvall polypropylene ultra bottles (Thermo Electron, USA). The resultant pellet contained the organelle (mitochondria, lysosomes and microsomes) fraction, whereas the supernatant contained the cytosol. Five hundred microliter from each supernatant was taken and stored at 20°C for cytosolic total protein quantification. The remainder was heated at 80°C for 10 min and then cooled on ice for 1 h to denature and precipitate heat-sensitive proteins. These samples were then centrifuged at 21,000*g* for 15 min to separate the heat stable proteins, which would remain in the supernatant, from the heat denatured proteins. Five hundred microliter of the supernatant was removed and stored at −20°C for protein quantification.

Pellets from the different centrifugation steps were resuspended in dH_2O, and these suspensions and the supernatants were transferred to pre-weighed vials and dried prior to acid digestion and metal quantification, as described above; owing to the smaller mass of the samples analyzed, 750 µl of acid and H_2O_2 were used to digest the samples.

Protein measurement

Total and heat-stable cytosolic protein concentrations were quantified using Bradford's method (Bradford 1976). A standard curve was generated from 0 to 60 µg ml^{-1} dilutions of bovine serum albumin made up in homogenization buffer. Samples for analysis were diluted appropriately in homogenization buffer following initial measurements to yield concentrations within the range of the standard curve.

Two hundred microliter of standards and appropriate dilutions of samples were mixed with 800 µl of Bradford reagent (Sigma), mixed, and the absorbance of light at 595 nm read after 5 min in an Aquamate Thermo Spectronic UV–VIS spectrophotometer. The standard measurements were fitted to a quadratic curve ($y = 4 \times 10^{-3}\ x^2 + 0.0055x,\ R^2 = 0.9962$), and protein concentrations of the samples were calculated from this.

Results

Toxicity of Al and Ga

Both Al and Ga induced behavioural toxicity in the snails (Fig. 1). Al-exposed snails exhibited significantly lower behavioural state scores between days 4 and 7 ($P < 0.05$), when compared to controls, followed by a period of recovery, and then a further drop in activity which lasted from day 21 to the end of the behavioural assessments ($P < 0.05$ versus controls). Ga-exposed snails did not exhibit the same initial drop in behavioural activity as the Al-exposed snails, and were significantly more active than Al-exposed snails on day 7 ($P < 0.05$); this was the only time point at which behavioural activity was significantly different between the Ga- and Al-exposed snails. Ga-exposed snails showed depressed behaviour on days 12, 16, 24, 27 and 28, compared to controls ($P < 0.05$).

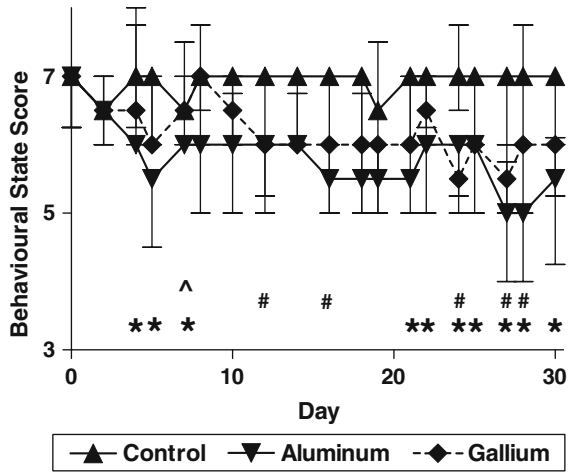

Fig. 1 Behavioural state scores from control, Al-exposed and Ga-exposed *Lymnaea stagnalis*. Data are median values ± interquartile ranges; $n = 10$. The connecting line from the Ga-exposed group is *dashed*; the other two group lines are *solid*. * $P < 0.05$ Control versus Al; # $P < 0.05$ Control versus Ga; ^ $P < 0.05$ Al versus Ga

The number of eggs laid by Al- and Ga-exposed snails was higher than controls: 1,739 eggs (in 48 egg sacks) in the control group compared to 2,103 (in 65 sacks) and 2,629 (in 74 sacks) eggs in the Al- and Ga-exposed groups, respectively (Fig. 2). However, the percentage of eggs which hatched was lower for the metal exposed groups: 63% in the control group compared to 39% of eggs from the Al-exposed group and 53% of eggs from the Ga-exposed group. Hatching rates from metal exposed groups were significantly lower than expected, compared to the

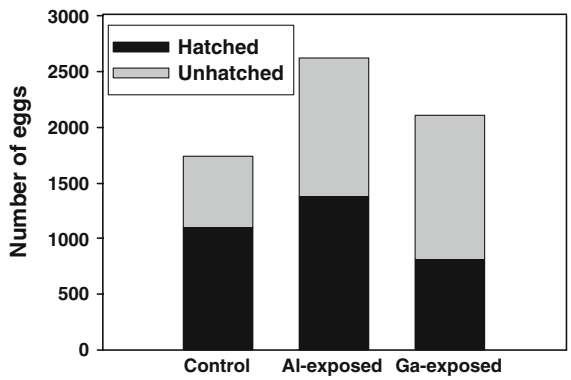

Fig. 2 Total numbers of hatched snails and unhatched embryos from eggs laid over a 28 day period from control *Lymnaea stagnalis* and those exposed to Al and Ga ($n = 50$ at start, decreasing to $n = 22$ at day 28)

control rate of hatching ($P < 0.005$ in both cases; X^2). Numbers of live young were 1,101 for the control group, 1,385 for the Ga-exposed group, and 818 from the Al-exposed group.

Accumulation of Al and Ga in the soft tissues

Neither snails exposed to Al nor control snails contained any detectable amounts of Ga in their soft tissues; Al levels in the Ga-exposed snails were very low and comparable to control values. The dry mass of tissues (DG, RST and total soft tissues) did not differ significantly between the three groups of snails at any sampling time point ($P > 0.05$).

The concentrations of accumulated Al in the total soft tissues of Al-exposed snails were significantly higher than levels of Ga in equivalent tissues of the Ga-exposed snails from day 14 onwards ($P < 0.05$). In the soft tissues without the DG (i.e. RST), accumulation of Al in Al-exposed snails was significantly higher than levels of Ga in equivalent tissues of the Ga-exposed snails, from day 7 onwards ($P < 0.05$) (Fig. 3a). Concentrations of the two metals in the DG were not different on any day ($P > 0.05$).

When the total amount of metal accumulated was considered, the amount of Ga in the DG was significantly higher on day 14, and significantly lower on days 21 and 28 in Ga-exposed snails, compared to the total amount of Al in the DG of Al-exposed snails. Total Ga accumulated in both the remaining soft tissues, and also total soft tissues (DG + RST) was also significantly lower than Al on days 21 and 28 ($P < 0.05$; accumulation measured in μM). Whilst mean masses of snails in the three groups were not significantly different, they did vary, and so the relative distribution of the total metals between the DG and the RST was calculated. In this case, the proportion of Al or Ga in the DG relative to the RST was significantly higher for Ga in the Ga-exposed group from day 14 onwards when compared to Al distribution in the Al-exposed group ($P < 0.05$) (Fig. 3b).

Subcellular localization of Al and Ga

The subcellular fractionation of DG samples yielded five different fractions: cellular debris (CD), granules (mainly inorganic material), cellular organelles, heat denaturable proteins (HDP) and heat stable proteins

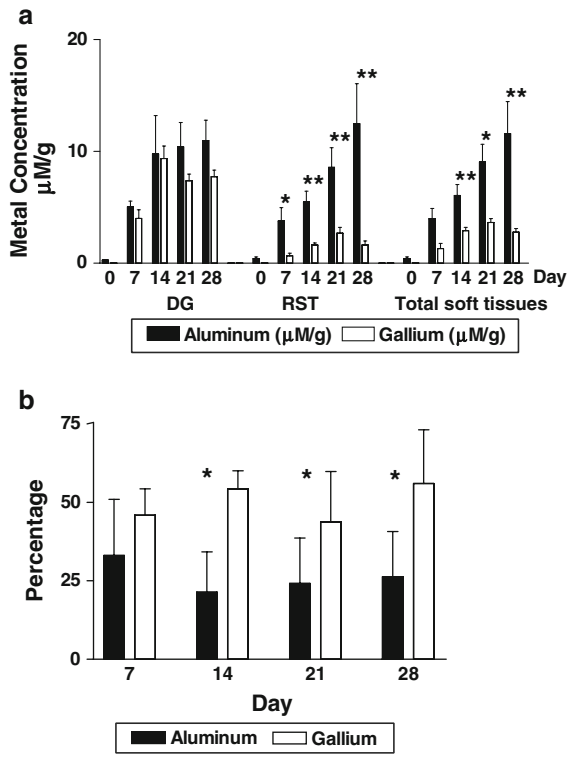

Fig. 3 **a** Accumulation of either Al or Ga in the soft tissues of *Lymnaea stagnalis* per unit dry mass over 28 days. *DG*, digestive gland; *RST*, remaining soft tissues(-DG). Data are mean values ± SEM, $n = 7$. * $P < 0.05$; ** $P < 0.01$. **b** Percentage of Al and Ga accumulated in the digestive gland of *Lymnaea stagnalis* relative to the total amounts of metal accumulated in the soft tissues. Data are mean values ± SEM, $n = 7$. * $P < 0.05$

(HSP). The cellular debris would comprise a mix of the various other fractions in different proportions that were incompletely homogenized.

Ten percent of the initial homogenate was not centrifuged but was used to estimate total Al and Ga levels in the samples. Total metal yields from the five fractions accounted for, on average, 95% (range 78–115%) of the total metal present in the initial homogenate; these differences were probably due to cumulative uncertainties arising from pipetting, dilution and measurement. The levels of Al and Ga in the total DG homogenates from Al- and Ga-exposed snails were not significantly different from each other ($P > 0.1$).

Owing to the different sizes of the DGs analyzed, variations in levels of Al and Ga in the different fractions could have been related to initial DG wet weight, so percentage distributions were used to compare Al and Ga localisation in the different

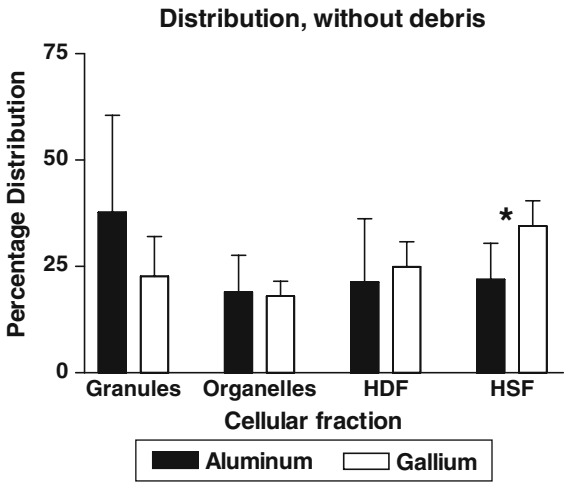

Fig. 4 Distribution of Al and Ga in different sub-cellular fractions of digestive gland, when cellular debris is discounted. *HDF*, heat denaturable protein fraction. *HSF*, heat stable protein fraction. Data are mean values ± SEM, $n = 5$. * $P < 0.05$

cellular fractions. A majority of both metals was located in the cellular debris fraction, and this proportion was significantly higher for the Ga-exposed group ($P < 0.05$). As this fraction was likely to contain mainly unhomogenized tissue, this difference would have artificially skewed the proportions of Al and Ga in the other fractions, with lower percentage distributions in the Ga-exposed group. Therefore percentage distributions were re-calculated from the metal levels in the remaining fractions. In this case, a significantly higher proportion of Ga associated with the heat stable protein fraction than did Al ($P < 0.02$) (Fig. 4); there were no differences in the proportional distribution in the other fractions.

Quantification of Al and Ga in protein fractions

Average protein levels in the cytosol of the DG tissue per unit wet mass are summarized in Table 1; there were no significant differences between the three groups ($P > 0.5$).

Table 1 Protein concentrations in the cytosol of the digestive gland of *Lymnaea stagnalis*

	Metal exposure group (mg g^{-1})		
	Control	Al	Ga
Total protein	4.25	4.30	4.26
Heat stable protein	0.73	1.08	0.70

When the Al-exposed snails' levels were compared to both control snails and Ga-exposed snails the quantity of HSP in each sample relative to the total protein present was significantly different ($P < 0.01$), with HSP in the Al-exposed snails comprising 25% of the total protein in the DG cytoplasm, whereas HSP contributed to 16.8% of total DG protein in both the control and Ga-exposed snails.

When the Al and Ga associated with the HSP were quantified in terms of metal per unit mass of protein, almost twice as much Ga was associated with the HSP fraction as Al (20 nanomoles Ga per mg protein compared to 11 nanomoles Al per mg protein; $P < 0.05$). There was no significant difference in the amounts of Al and Ga associated with the HDP per unit mass protein when HDP was taken to be the total protein less the HSP fraction.

Discussion

The aim of this study was to establish whether or not Ga represents a suitable substitute tracer metal for Al in tissue accumulation and flux studies. Our findings suggest that, although some similarities were present, the different rates at which Al and Ga accumulated in the snails, and the different protein associations indicate that Ga is not a good substitute for Al.

Al-induced behavioural toxicity in *L. stagnalis* followed a similar pattern to that observed in previous studies (e.g., Dobranskyte et al. 2004), including initial toxicity around days 4–7, followed by apparent recovery and then a further behavioural depression. Ga was also toxic to the snails, although the initial onset of toxicity was slower, commencing around day 12. There was also evidence that Ga did not affect the fertility of the snails to the same extent as Al. Similarly, Al is more toxic than Ga to the giant alga *Chara coralline* (Reid et al. 1996), whilst Al and Ga exhibit similar levels of toxicity in yeast (Ritchie and Raghupathi 2008). There are few comparative data regarding the toxicity of Al and Ga in animals, but the observations from our study, as well as those above, indicate that the toxicity of Ga is at least no higher than that of Al and so would not differentially affect snail behaviour in any metal tracing experiment.

When the accumulation of Al and Ga in soft tissues is compared, Al was accumulated to at least three times the levels of Ga at any time point, rising to almost eight times by day 28. This greater accumulation is the most likely cause of the faster onset of behavioural toxicity observed in the Al-exposed snails. A more rapid uptake rate of Al compared to Ga is possible since Al has a smaller ionic radius—0.54 Å compared to 0.62 Å (Shannon 1976; Henderson 1982).

Speciation modelling carried out using Visual MinTeq Version 2.60 (Gustafsson 2009) over a range of pH values from 3 to 10, at 14°C, and with the same components present as are added to SPDW, demonstrated that hydrated Ga^{3+} and Al^{3+} ions are not present at pH 7 in SDPW (Fig. 5). This modelling also indicates that the presence of other counter ions such as Cl^- will not affect the speciation of Al or Ga in SDPW. The species present are consistent with

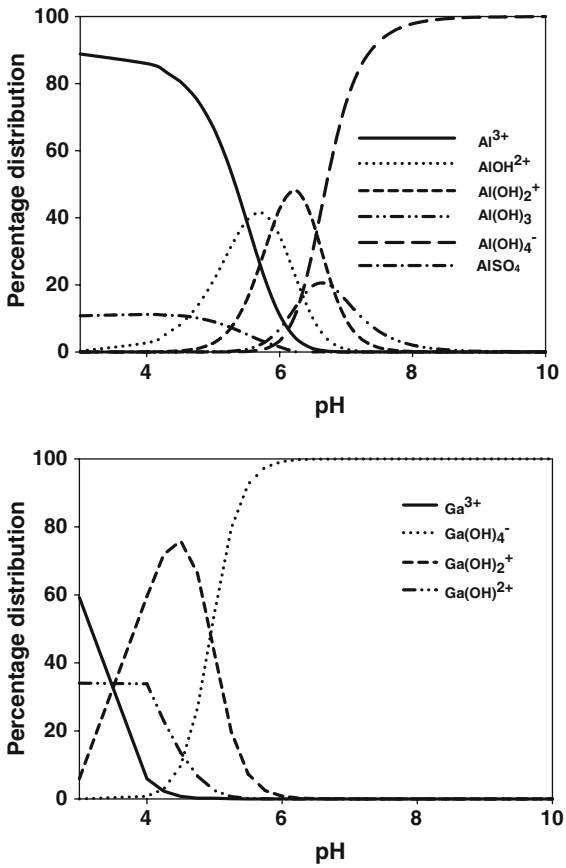

Fig. 5 Distribution of Al and Ga species in SDPW over a range of pH values, as calculated using VisualMinTeq

those predicted in simple solution (Wood and Samson 2006; Martin 1986). The gut of the snail is slightly acidic, around pH 6.25–6.5 (Walton, unpublished observations), and this could therefore affect Al and Ga absorption. At this lower pH, Ga is present mainly as $Ga(OH)_4^-$, whereas Al species comprise a mix of $Al(OH)^{2+}$, $Al(OH)_2^+$, $Al(OH)_3$ and a small quantity of Al^{3+} (Fig. 5). The smaller, more highly charged species, particularly the Al^{3+} ion, are likely to be more readily absorbed than the $Ga(OH)_4^-$ ion, so depletion of the Al^{3+} concentration by absorption, together with maintenance of chemical equilibrium, could contribute to an increased rate of uptake of Al. The free ion will also interact with carrier ligands such as citrate that are known to facilitate the passage of Al across cell membranes (Yokel et al. 1999). In contrast, previous studies have demonstrated that Ga is accumulated at approximately twice the rate of Al in cell cultured neuroblastoma cells (Dobson et al. 1998). Similarly, Al- and Ga-accumulating bacteria remove Ga from their growth medium far more rapidly than Al (Gascoyne et al. 1991), whilst the membrane flux in the alga *Chara* is also higher for Ga than it is for Al (Reid et al. 1996). In rats, the permeation of Al and Ga through the blood brain barrier is different, both in terms of quantity, and also localisation of the metal within the brain (Allen and Yokel 1992). In mice, amounts of tissue accumulation of Al and Ga are comparable in the liver, spleen and brain, but Al accumulates to a greater extent than Ga in the renal cortex and bone (Radunovic et al. 1998). In both these cases, Al and Ga were administered through intravenous infusion, and so any effects of gut pH can be discounted in these instances, indicating that passage into tissues from the blood are also different and therefore the differences in accumulation rates in the snail may not simply be due to the differences in metal speciation in the snail gut. It appears likely that, whilst Ga may in some instances accumulate to broadly the same levels as Al, the variability in the rate of accumulation means that Ga could not be used to quantitatively assess the flux of Al in animal systems with accuracy.

To date, there are few comparative data available with regard to tissue accumulation of Al and Ga in aquatic higher organisms. Studies in carp indicate that Ga is toxic and accumulates on the gill surface (Yang and Chen 2003), as does Al in salmon (Oughton et al. 1992; Teien et al. 2004) and crayfish

(Ward et al. 2006). However, other metals such as copper (Karan et al. 1996) and cadmium (Wong and Wong 2000) also accumulate here, causing similar damage and immune response, with a majority of effects being extracellular. The co-localisation of Al and Ga on the gill is therefore not strong enough evidence to support the use of Ga specifically as an Al tracer, as interaction with the gills is a common phenomenon for a range of metals. *L. stagnalis* is a pulmonate, and therefore the main site of toxicity of Al and Ga will be different from that in fish. When considering the tissue distribution of Al and Ga, both accumulate to similar levels in the DG. It is possible that the two metals share a comparable detoxificatory mechanism, as this is the major organ for metal accumulation and detoxification in molluscs (Marigomez et al. 2002). However, as other toxic metals also accumulate here, the comparable rates at which Al and Ga accumulate in the DG could be due to non-specific metal detoxification, and again, as with fish gills, there is insufficient evidence to conclude that Ga and Al are detoxified in a similar, unique manner that would give credence to the idea that Ga is an effective tracer for Al. Coupled with this, the proportions of Al and Ga in the DG relative to the remaining soft tissues were significantly different. This would indicate that either Ga was more effectively directed to the DG, detoxified and excreted, or that Al accumulated more rapidly than Ga in the soft tissues, but its rate of influx into the DG was limited.

The higher levels of HSP present in the cytosol, but with lower levels of metal association in the Al-exposed snails, imply that Al and Ga do not interact with proteins in the same way. The metallothioneins (MTs), a group of low molecular weight metalloproteins that have been implicated in metal detoxification for over 30 years (Cherian and Goyer 1978), are a family of metal-inducible HSP expected to be present in the DG. However, there is little evidence that MTs are utilized in the detoxification of Al. In Zn-displacement assays, Al did not associate with MT at all, even at concentrations of 1 mM (Waalkes et al. 1984). Al does induce the production of a MT-like protein in rat liver and kidney, yet less than 5% of the cytosolic Al associates with this protein, indicating that the induction and binding of MT to Al would not be sufficient protection from its toxicity (Jeffery et al. 1987). It is possible that Al does induce the production of a MT-like protein in the snail DG, but does not bind effectively to it. A range of other stress-induced HSP may also be present in the DG. Heat shock protein 70 and catalase, for example, are induced in *Dreissena polymorpha* introduced into metal polluted sites (Contardo-Jara and Wiegand 2008). As Al associates readily with protein and also induces toxicity that could induce a non-specific stress response, it is probably not surprising that Al was associated with both protein fractions and may have induced up-regulation of some HSP. Further investigation of the interactions between Al and cytosolic proteins would be of interest. Our results showed that Ga associates with the HSP fraction more effectively than Al but does not cause the same changes in proportion of HSP to HDP. This could be related to the lower observed toxicity of Ga and differences in metal-specific induction compared to Al, but these results also demonstrate at least a partially different mechanism for protein association.

In summary, although Al and Ga induce similar toxicity in *L. stagnalis*, their differences in tissue distribution and rate of accumulation, coupled with the variation in protein association, indicate that Ga is unlikely to be an effective tracer for Al in whole animal studies.

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