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Amelioration of behavioural toxicity of aluminium by oligomeric silicic acid and humic acid

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ABSTRACT
Despite its limited solubility at neutral pH, aluminium is significantly accumulated by the freshwater snail *Lymnaea stagnalis,* leading to behavioral toxicity. Both organic (humic acid) and non-organic (oligomeric silicic acid) ligands have been shown to reduce Al accumulation probably owing to their binding affinity for Al. This study examined the effect of these ligands on behavioral responses to Al over a 30-day exposure period. Both behavioral state score (a measure of general activity) and feeding behaviors were initially (days 2-4) depressed in the presence of 500 μgl^{-1} added Al, followed by apparent tolerance (days 8-16) and subsequent depression (day 30). In the presence of either oligomeric Si (molar ratio Al:Si 1:40) or humic acid (10 mgl^{-1}) behavioral responses to Al were completely abolished. Neither ligand alone had any effect on behavior except for a slight increase in biting activity in the presence of humic acid on days 2 -4. These results suggest that the presence of complexing ligands may affect toxicity of Al in the natural environment at neutral pH.

KEYWORDS: Aluminium, humic acid, silicon, *Lymnaea stagnalis*, behaviour

INTRODUCTION

Aluminium has been considered largely unavailable to the biota in neutral freshwater systems, owing to its limited solubility at neutral pH (Driscoll & Schecher 1989). This explains its widespread natural occurrence and its extensive use in industrial process, in water treatment, in drugs, food additives, and in food containers. It thus represents a major environmental contaminant to which the general, widespread population is exposed. However, any entry of this metal into the food chain could have significant implications since it is known to be highly toxic to plants (Taylor 1988), fish (Driscoll *et al.* 1980) and man (Kerr *et al.* 1992). Despite its limited solubility, Al is significantly accumulated into the soft tissues of the freshwater snail *Lymnaea stagnalis* (Elangovan *et al.* 1997), when snails are exposed to environmentally relevant concentrations (Dixon & Gardner 1998) of the metal. Accumulation occurs largely via ingestion through grazing on Al bound to extracellular polysaccharides (EPS), including mucus, in the substrate biofilm (Jugdaohsingh *et al.* 1998). Snail mucus has been shown to avidly bind polyhydroxy colloids of Al, present under neutral conditions and it is in this form that it is thought to enter the snail. Thus, grazing macro invertebrates, together with filter feeders and shredders, which are also exposed to polyhydroxy Al in their diet, are potential carriers of significant quantities of Al into the food chain. Concentration factors of Al in snail tissues as high as 10^4 have been measured (Elangovan *et al.* 1997). The accumulated Al is also toxic. Both snails (Truscott *et al.* 1995; Campbell *et al.* 2000) and freshwater bivalves (Kadar *et al.* 2001) show marked sublethal behavioral changes alongside Al accumulation in exposure experiments. Behavioral responses in snails included changes in locomotory activity and decreased feeding activity.

The chemical speciation of Al in natural freshwater systems is complex, partly owing to the presence of organic and inorganic ligands, which bind the metal, potentially keeping it in solution (Driscoll & Schecher 1989). This is likely to influence its bioavailability and hence toxicity. The inorganic ligand oligomeric silicic acid has a strong affinity for Al (Taylor *et al.* 1997) and we have recently shown that addition of oligomeric Si greatly reduces accumulation of Al by snails (Desouky *et al.,* unpublished data); this appears to be due to the prevention of precipitation of Al out of the water column as hydroxy colloids, thus making it less available to the grazing snails. Similarly, humic acid, a major source of organic carbon in freshwaters, reduced accumulation of Al by snail tissues, though to a much lesser extent than Si, and this was paralleled by a slight reduction in Al precipitation, presumably owing to formation of Al-humate (Desouky *et al.,* unpublished data).

The aim of this study is to determine whether a reduction in accumulation of Al in the presence of competing ligands would be accompanied by a reduction in behavioral toxicity. The hypothesis was that the reduction in accumulation would be closely linked to behavioral responses.

MATERIALS AND METHODS

L. stagnalis (shell length 25-35 mm) were obtained from suppliers (Blades Biological, Kent, UK) and maintained in acid-washed, 30 litre, high-density plastic tanks containing 20 liters standard snail water (SSW; Thomas *et al.* 1975). Before addition of Al, snails were individually labeled and acclimatised for 10 days at 8-12^{0}C under a 12:12 hr light: dark regime. Groups of snails (n=10) in three tanks were exposed for 30 days to SSW to which was added either 0 (control), 10 mgl^{-1} humic acid (Aldrich, Gillingham, Dorset, UK), or 740 μMl^{-1} oligomeric silicic acid. These three tank conditions were replicated in a further three tanks to which 500 μgl^{-1} Al (as $Al(N0_3)_3$) was also added. Oligomeric Si was initially prepared from sodium silicate at 42 mM and adjusted to pH 7.2 using HCl. This 42 mM solution was incubated at room temperature for 24 hr prior to dilution in the tanks to ensure that the majority of the Si is in the oligomeric form (Taylor *et al.* 1997). A molar ratio 40:1 (Si:Al) was used because this was found to sequestrate all Al in the solution (Taylor *et al.* 1997).The concentration of humic acid used was within the range found in natural waters. The water in the tanks was renewed every 48 hr and re-dosed with metal and ligands as required. After 30 days exposure, snails were transferred to uncontaminated SSW for a further 20 days as a recovery period. Behavioral measurements on individual snails were made just prior to addition of Al, Si and/or humic acid, one hr after addition (BSS only) and then on days 2,4,8, 16,30 and 50*,* between 10.00 and 18.00 hrs. Snails were starved for 2 days prior to each test in order to minimise individual variation due to differences in recent food consumption. At all other times, snails were fed lettuce *ad libitum.* Behavioral parameters measured were as follows.

Behavioral state score (BSS): This is an established behavioural score giving a sensitive measure of overall activity (Tuersley & McCrohan 1987; Campbell *et al.* 2000). Points are awarded for different activities: fully retracted into shell — 0 pt, withdrawn —1 pt; withdrawn plus exploratory tentacle movements — 2 pt, head and foot extended and active with no locomotion — 4 pt, locomoting —5 pt. Additional points are awarded for actively attaching to the substrate (1), floating (2), biting (2), twisting/shrugging of shell (1), mouthing with no obvious radula movements (1). Each snail was observed for 1 min and awarded the maximum BSS that it achieved during this time.

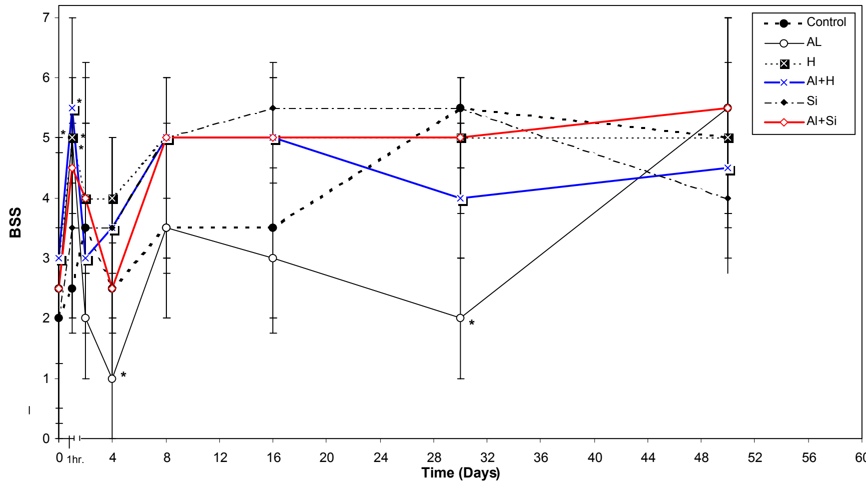
Feeding behavior: After BSS testing, snails were removed from the experimental tanks and placed individually in a Petri dish containing 10 ml SSW, mounted over a mirror so that radula movements could be observed. They were left for 10 min. to recover from handling. Three

feeding measures were made. (1) The total number of spontaneous bites was recorded for 2 min. (2) A 1 ml 4% sucrose solution was pipetted gently 2 cm from the snail's head; this is an effective phagostimulant. The latency to first bite (LTB) after the stimulus was recorded. Snails that failed to respond within 2 min. were arbitrarily awarded an LTB of 120 sec. (3) Moreover, the total number of bites in the 2 min following presentation of sucrose stimulus was recorded.
Data were analysed by non-parametric analysis of variance (Kruskal-Wallis test) followed by a non-parametric comparison test (Mann-Whitney test) (Minitab Inc., Pennsylvania, USA).

RESULTS

Behavioral state score*:* Prior to addition of Al, Si and humic acid, there was no significant difference in BSS between experimental groups; median BSS was between 2 and 3. Figure 1 plots medians and interquartile ranges for BSS measurement taken during and following exposure. After only 1 hr exposure, groups exposed to Al alone, humic acid alone, Al plus humic acid and Al plus Si all showed significantly ($P<0.05$) elevated BSS compared to control, with medians ranging from 4.5 to 5.5. The group exposed to Si alone was not significantly different from control. At day 2 there was no significant difference between the groups but by day 4 BSS of snails exposed to Al alone was significantly ($P<0.01$) depressed, to a median score of 1 (Fig. 1). Other groups did not differ significantly from control at this or any subsequent time point. The Al-exposed group recovered to control levels by days 8 and 16, suggesting tolerance to continued Al exposure. However, by day 30, BSS for this group was again significantly ($P<0.01$) depressed. By day 50 (20 days recovery) this group had recovered to control BSS levels (Fig. 1).
Spontaneous biting activity: Prior to Al exposure, there were no significant differences in spontaneous biting activity between the groups; median number of bites in 2 min. was between 6.5 and 8. At days 2 and 4, snails exposed to Al alone showed a significant ($P<0.05$) reduction in spontaneous biting (Fig. 2). Biting activity returned to control levels on days 8 and 16. However, it dropped significantly ($p<0.01$) again by day 30 to a median of 1 bite in 2 min. By day 50 (20 days recovery) biting activity returned to control level. When Si or humic acid was also present, the effect of Al on biting activity was completely abolished with activity not significantly different from control at any time point (Fig. 2). Si alone had no effect on biting but, interestingly, the presence of humic acid alone caused a slight but significant ($p<0.05$) elevation of biting activity at days 2 and 4 only, when compared to control (Fig. 2).
Latency to bite: Median LTB for all groups prior to exposure was similar at between 7 and 15.5 sec. At 2 days, snails exposed to Al alone showed a significant ($P<0.05$) increase in LTB compared to control, to a median of 32.5 sec. LTB then declined again at days 4 and 8. However, after this it arose to become significantly ($P<0.01$) greater than control by day 16 and reached a peak of 41 sec by day 30. By day 50*,* complete recovery of LTB was seen. None of the other experimental groups showed any difference from control at any time point, confirming that the presence of Si or humic acid completely reversed the effect of Al on this behavioral parameter and that neither ligand had any effect on its own.
Total number of bites in 2 min. following application of sucrose: At day (zero), there was no significant difference between the experimental and control group. The number of bites in response to sucrose was significantly ($P<0.05$) reduced on day 2 and then returned to control level by day 4 up to day 16. After 30 days of Al exposure, the number of bites within 2 minutes was significantly ($P<0.01$) reduced again. The exposure of the snails to Al in presence of oligomeric Si or humic acid was found to significantly increase the number of bites at day 2 ($P<0.05$) and day 30 ($P<0.01$) of exposure compared to Al exposure. It was also noted that the

exposure of snails to humic acid significantly (P<0.05) increased the number of bites after 2 days of exposure. The increases at day 8 and 16 of humic acid exposure or day 2 up to 16 of Si exposure are insignificant. At the end of the recovery period (day 50), no significant difference was found between any of the experimental groups and control.



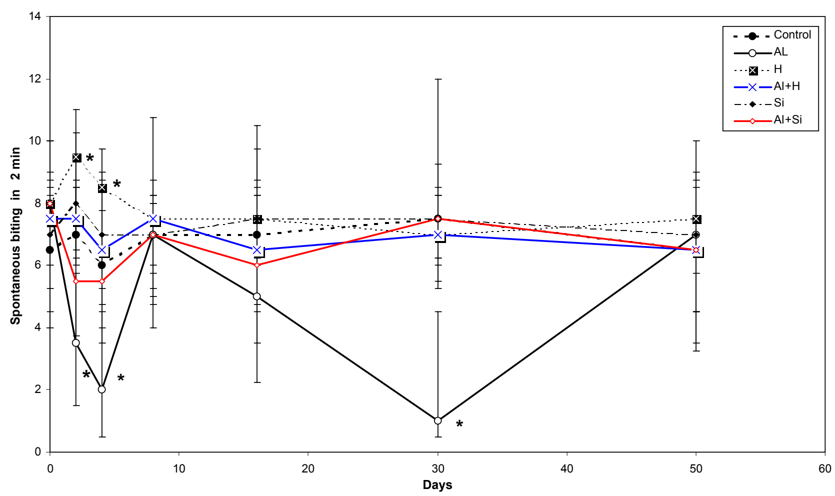


Figure 2. Number of spontaneous bites in two minutes (median ± interquartile range) generated by *L. stagnalis* exposed to 500 µgl^{-1} added Al alone (-O-) and in the presence of oligomeric Si (molar ratio 1:40) (-◊-) and humic acid (10 mgl^{-1}) (-X-), oligomeric Si alone (-♦-), humic acid alone (-X-), and control (-●-), for 30 days, followed by 20 days in clean standard snail water. * indicates where biting activity is significantly different from control (p<0.05)

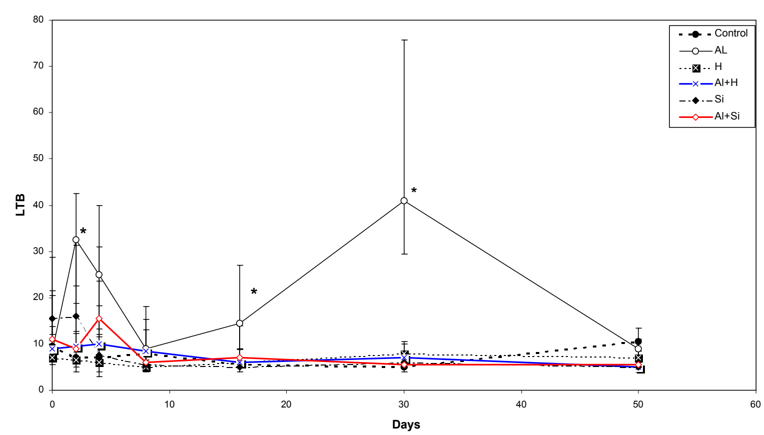


Figure 3. Latency to first bite (LTB) (median ± interquartile range) by *L. stagnalis,* following presentation of a sucrose stimulus. Groups were exposed to 500 µgl^{-1} added Al alone (-O-) and in the presence of oligomeric Si (molar ratio 1:40) (-◊-) and humic acid (10 mgl^{-1}) (-X-), oligomeric Si alone (-♦-), humic acid alone (-X-), and control (-●-) for 30 days, followed by 20 days in clean standard snail water. * indicates where LTB is significantly different from control (p<0.05).

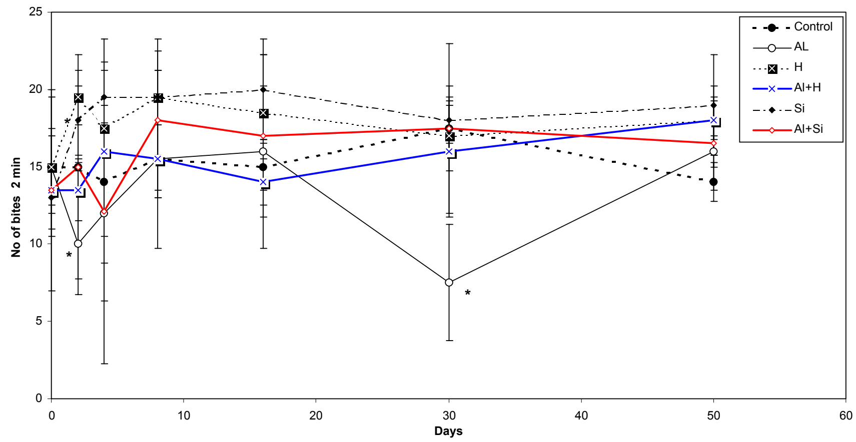


Figure 4. Numbers of bites in two minutes (median ± interquartile range) by *L. stagnalis,* following presentation of a sucrose stimulus. Groups were exposed to 500 µgl^{-1} added Al alone (-O-) and in the presence of oligomeric Si (molar ratio 1:40) (-◊-) and humic acid (10 mgl^{-1}) (-X-), oligomeric Si alone (-♦-), humic acid alone (-⊠-), and control (-●-) for 30 days, followed by 20 days in clean standard snail water. * indicates where number of bites is significantly different from control (p<0.05).

DISCUSSION

The major finding reported here is the complete amelioration of behavioral effects of 500 µgl^{-1} added Al in the presence of oligomeric Si or humic acid. This supports the hypothesis that total or partial amelioration of tissue accumulation of Al in the presence of these ligands, reported previously (Desouky *et al.,* unpublished data), could be responsible for a reduction or abolition of sub lethal behavioral toxicity. The case for Si is clear. However, amelioration of Al accumulation by humic acid is much less marked (Desouky *et al.,* unpublished data), probably because considerably less Al is retained in the water column by humic acid than by oligomeric Si. The total body burden of Al was reduced by addition of humic acid, but the amount in the digestive gland was unaffected (Desouky *et al.,* unpublished data). It is possible therefore that at least some of the ameliorating effects of humic acid at the behavioral level may be due to some other mechanism independent of effects on uptake of the metal.

Behavioral state score is an effective measure of the state of 'arousal' of the snail and has been shown to be sensitive to motivational factors (Tuersley & McCrohan 1987). Feeding activity is an important ongoing behavior in this grazing animal, taking up a large proportion of the animal's time, and again provides a useful measure relating to general activity. The snail feeds by rhythmic protraction and retraction of the radula. The whole feeding cycle consists of four phases: inactive or resting state, protraction, rasping and swallowing. The feeding process involves the action of radula, odontophore and the muscular system (46 muscles) of the buccal mass. It is of particular interest because it is a relatively complex, brain related and easily observed behavior (Cheng 1994). In addition, measures of spontaneous biting and LTB give an indication of both appetitive and responsive behaviors, respectively.

Although the digestive gland appears to act as a 'sink' for accumulated Al, Al is also found at not inconsiderable levels in other soft tissues (Elangovan *et al.* 1997) and therefore could exert its behavioral effects through direct actions on motor centres in the central nervous system and/or by causing muscular dysfunction (Finnegan *et al.* 1986).

The time course of changes in behavior in snails exposed to Al alone is worthy of discussion. The initial depression in activity around days 2-4, followed by apparent tolerance to continued Al exposure around days 8-15 were reported by Campbell *et al.* (2000). However, the latter study did not continue beyond day 14. In this study, behavior was again depressed between days 20 to 30, suggesting that any tolerance mechanisms could not cope with continuous Al exposure over this longer period of time. It has been suggested that the tolerance observed earlier is due to sequestration of Al in a non-toxic form in lysosomal granules of the digestive gland (Elangovan *et al.* 2000). It is possible that this system has limited capacity owing to imbalance between sequestration and excretion of granules. Depression of activity in response to pollutants may have some survival value since it could reduce uptake and hence toxicity. However, previous studies confirm that accumulation of Al by the snail over similar period of time is rapid and substantial (Elangovan *et al.* 1997).

Interestingly, both Al and/or humic acid caused an elevation in BSS following 1 hr of exposure. This may represent an active avoidance mechanism following direct contact with these chemicals. Similar stimulating effects on activity have been reported for other pollutants (e.g. Reish & Carr 1978; Eldon *et al.* 1980; Mona *et al.* 1994). Si alone did not elicit the same effect. Humic acid may itself act as a form of phagostimulant when added alone it increased biting activity at days 2 and 4.

The results demonstrate the importance of Al speciation in determining its toxicity to freshwater invertebrates at neutral pH. In the absence of organic and inorganic ligands, which bind Al, toxicity would be enhanced. Levels of Si vary considerably in standing waters depending on the presence of diatoms, which utilize Si in construction of the frustules. Similarly, humic acid concentration is dependent on soil composition and extent of run-off into the water.

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الملخص العربى

إستخدام حمض عديد السلسيك وحمض الهيوميك فى إزالة سمية الألومنيوم على السلوك الحيوانى

محمود الدسوقي

قسم علم الحيوان - كلية العلوم - جامعة الزقازيق – الزقازيق - مصر

من المعروف أن أملاح الألومنيوم شحيحة الذوبان فى الوسط المتعادل. وعلى الرغم من ذلك فقد وجد أن قوقع اللمنيا (*Lymnaea stagnalis*) له القدرة على تجميع تركيزات عالية من الألومنيوم داخل أنسجته عند تعرضه لهذا العنصر فى الوسط المائى ويؤدى ذلك إلى أعراض تسمم سلوكيه لهذا القوقع. فى هذا البحث تمت دراسة النشاط العام والسلوك الغذائى لقوقع اللمنيا بعد تعريضه معملياً لتركيز 500 ميكروجرام/لتر من الألومنيوم لمدة 30 يوماً حيث لوحظ وجود تغيرات سلوكية للحيوان أثناء فترة التعرض وقد تمثلت هذه التغيرات فى المراحل التالية:

1- زاد النشاط العام للحيوانات بعد ساعة واحدة من التعرض للألومنيوم فى محاولة لمقاومة التأثير السام لهذا العنصر.

2- تأثر كل من النشاط العام والسلوك الغذائي للحيوانات سلبياً بعد 2-4 أيام من بداية التجربة.

3- تلى ذلك مرحلة تأقلم (بعد 8-16 يوم) حيث زالت أعراض التسمم السلوكى وقد يرجع ذلك إلى وجود آلية للتخلص من الألومنيوم المتراكم داخل الغدة الهاضمة أثناء هذه الفترة (بحث آخر للمؤلف).

4- بعد ذلك تدهور النشاط العام والسلوك الغذائى للحيوانات المدروسة حتى وصل أدنى مستوى له فى نهاية فترة التعرض (30 يوم) حيث فقد الحيوان القدرة على التأقلم مع طول فترة التعرض.

وفى محاولة للتخلص من التأثيرات السامة للألومنيوم تم استخدام مركبين هما حمض عديد السلسيك (مركب غير عضوى) وحمض الهيوميك (مادة غير عضوية) وهما من المواد المتواجدة طبيعياً فى المياه العذبة وتتميزان بمقدرتهما على الارتباط بالعناصر مما يجعلها فى صورة غير سامة.

وعند تعريض القواقع لمحلول الألومنيوم فى وجود أى من هذين المركبين فقد وجد أن التأثيرات السامة للألومنيوم على القواقع قد اختفت تقريباً وعاد النشاط العام والسلوك الغذائى للحيوانات إلى معدله الطبيعى. على الجانب الآخر فإنه قد وجد أن تعريض الحيوانات إلى أى من المركبين بمفرده غير ذات أثر مهم على السلوك فيما عدا بعض التأثيرات الطفيفة لحمض الهيوميك على بعض أوجه السلوك الغذائى مما يؤكد دور هذان المركبان فى إزالة سمية الألومنيوم.