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Histopathology of gills and gut of the edible cockle *Anadara Antiquata* (Bivalvia: Arcidae) exposed to acute level of Ammonium sulfate

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Abstract
Toxicity studies with ammonium sulfate were scanty. Based on standard procedures, we estimated the 96 h acute toxicity of this agrochemical on the edible cockle, *Anadara antiquata* Linnaeus (Bivalvia: Arcidae), in a static *in vitro* bioassay at mean pH 8, temperature 28±1°C, salinity 33% and DO 6 mg/L. The LC_{50} was estimated at 270 mg/L±0.3 and mortality was 33.08% (4.56 probits), both at 95% significance level. Although the exposure was not acutely toxic, some uncontrolled massive deformities evidenced in the victims over 336 h. These post-exposure injuries promoted mortality to 61.6% (5.3 probits) in which all samples exposed to 200 mg/L and above died. Stress manifestations involved excessive shell gaping, foot protrusion, mucus secretion coupled by necrosis, hyperplasia, cilial shortening, lamellar shrinkage, abscess formation and eventual epithelial ruptures and proliferations in the gills and gut. Prior to death, the victims lost mobility and sensitivity and their shell gaps were abnormally expanded. However, around 38.4% of all specimens survived this acute exposure, and the overall injury ranged between acute and chronic levels hence was subchronic. These results are indicating that *A. antiquata* has great potential of biomonitoring the toxicity of ammonium sulfate. A necessity for handling this agrochemical conscientiously is also justified.

Keywords: Acridae, acute toxicity, Ammonium sulfate, *Anadara antiquata*, bivalvia.

INTRODUCTION

Ammonium sulfate is a chemical of considerable attention mainly due to its tendency to elevate the ammonia load in the aquatic bodies following its agricultural application (Banerjee and Paul, 1996; Zwaan *et al*., 2001; UNEP, 2003). The toxicity of ammonium solutions does not usually cause problems for humans and other mammals because a special mechanism prevents ammonia build-up in the bloodstream: ammonia (NH_3) is converted to carbamoyl phosphate by the enzyme carbamoyl phosphate synthase, and then enters the urea cycle to be either incorporated into amino acids or excreted in the urine. However, fish, amphibians and most aquatic invertebrates lack this mechanism; they can usually eliminate ammonia from their bodies by direct excretion (Zwaan, 1992; Campbell and Reece, 2002; Tilak *et al.*, 2002). At pH 9.25, any local production of NH_3 will immediately remove any free H^+ thereby increasing the local pH; however, if NH_3 is excessive, the pH can be significantly raised to damaging levels. In addition, the ammonium radical (NH^{4+}) inhibits oxidative phosphorylation (electron transport) by transporting across mitochondrial membranes and breaking down the H^+ gradient that is critical for electron transport to occur. Furthermore, because the reaction $NH_3 + H_2O \rightarrow NH^{4+} + OH^-$ is a redox reaction, the production of NH_3 interferes with redox balance (e.g. the reactions involving NAD^+ and $NADP^+$, and in oxidative metabolism). Hence, even at dilute concentrations, ammonium solutions are toxic to aquatic animals (Holleman and Wiberg, 2001; Campbell and Reece, 2002; Tilak *et al.*, 2002; UNEP, 2003). However, little is known of toxicity of ammonium sulfate to *A. antiquata* although it is a trendy subject of being exposed to this agrochemical.

Toxicity of ammonium sulfate to aquatic species has been reported by a number of workers. Banerjee and Paul (1993) reported massive destructions in the epidermis of the freshwater catfish *Heteropneustes fossilis* exposed to 4,000 mg/L of ammonium sulfate,

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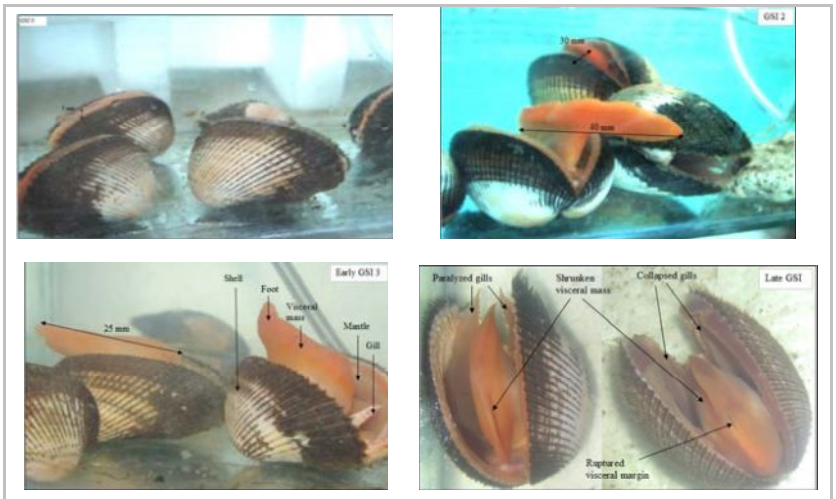


Figure 1: Morpho-behavioral alterations of *A. antiquata* upon a 96 h exposure to ammonium sulfate (GSI 0, 2 and 3), January-February 2006.

but the toxic effects of this chemical to many other aquatic animals are not very well addressed. Phillips (1980) showed that sedentary bivalves could be efficient bioindicators of aquatic pollution; however, the delayed effects of this chemical remain largely unknown (Zwaan *et al*., 2001, DCNA, 2003; PMI, 2008). Sufficient quantification of toxicity of ammonium sulfate could help adopt proper handling of this agrochemical. The present paper examines obvious toxic effects in *A. antiquata* when acutely exposed to ammonium sulfate. It is according to the study we conducted in year 2006 at University of Dar es Salaam, Tanzania. LC_{50}, stress and mortality are elucidated; and obvious stressful alterations that occur in the gills and gut of the exposed bivalves are highlighted.

MATERIALS AND METHODS

A. antiquata sampling and acclimation

We investigated acute toxicity of ammonium sulfate on *A. antiquata* chiefly based on the guidelines of the United States Environmental Protection Agency (USEPA, 1996). *A. antiquata* for the study was collected at Kaole shore located at 39°25′ E and 6° 34′ N, some 3 km southeast of Bagamoyo town (43 km north of Dar es Salaam). These bivalves were obtained at the mean depth of 25 cm, temperature 28°C, pH 8, salinity 33‰, DO 6 mgl^{-1} and ammonia 0.1 mgl^{-1}. A total of 409 specimens (measuring 31-40 mm in shell-length) were collected from their natural habitat (the soft seashore sediments) in three sessions in January-February 2006. These samples were taken to the laboratory of the Department of Zoology and Wildlife Conservation (University of Dar es salaam) where the 336 h acclimation, 96 h ammonium sulfate (99% pure, manufactured by May and Baker Ltd., England) exposure, and 336 h health monitoring exercises were run. Aquaria were first sterilized in strong saltwater for 12h; then were rinsed with seawater of the sampling site. A loading density of 1 cockle per 2.5L of the water medium was maintained both in the acclimation and exposure sets. Oxygen was supplied from the atmosphere using an electric air pump. This was fitted with a plastic tubing (measuring 1 cm in diameter) which was directly connected into the air-stone. Seawater and exposure solutions were replenished after every 48 h. The samples were fed only during acclimation and after running a 96 h ammonium sulfate exposure. Before feeding, the test cockles were starved for 24h so as to render their guts empty. Every 10 cockles were fed twice daily (morning and evening), with 2.5g of a special yeast meal per 25L of seawater. The acclimation was generally successful because it was accompanied by only 2.2% mortality inasmuch as only 9 out of 409 *A. antiquata* individuals died.

Ammonium sulfate exposure

Immediately after acclimation, a total of 16 specimens (4 specimens in each of the four aquaria containing 200, 400, 600 and 800 mg/L of ammonium sulfate) were subjected in the 96 h range-finding exposure. Only the set with 600 mg/L caused 100% mortality, and because the dose separation factor between subsequent concentrations had not to exceed 1.8 (USEPA, 1996), we decided the exposure series to comprise 0 (the parallel control) and nominal concentrations of 50, 80, 130, 200, 300, 400, and 600 mg/L, each with 8 cockles as recommended by Stephan (1977). One bioassay was run to estimate the LC_{50} and the other one was run for histopathological analysis. Each of these tests was conducted in three replicates hence consumed 192 cockles (i.e., 8 cockles x 8 aquaria x 3 replicates). The mean shell-length of the experimental *A. antiquata* was 39.3±0.36 mm (SD), and the mean body weight was 61±0.34g (SD). Stress was monitored by morphological and behavioral manifestations of the cockles. Mortality was recorded every 6 h in which all dead bivalves (recognized by their failure to move when gently prodded) were recorded and removed immediately to avoid fouling of the test medium.

Histopathology

Just after the exposure exercise, we excised the gills and intestine from the body of the test cockles and processed them for optical microscopy, which we conducted according to the standard histological procedures. Tissues of the gills and intestine were cut into 1 cm pieces, fixed in Bouin's fluid (for 24 h) and

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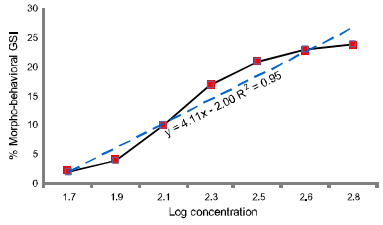


Figure 2: Variation of morpho-behavioral condition of *A. antiquata* with the ammonium sulfate exposure concentration, Jan.-Feb., 2006.

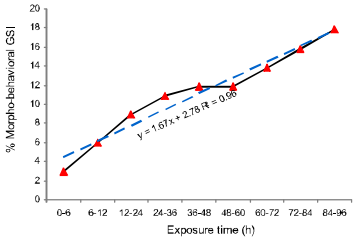


Figure 3: Relationship between morpho-behavioural deformity of *A. antiquata* and the ammonium sulfate exposure time, Jan.-Feb., 2006.

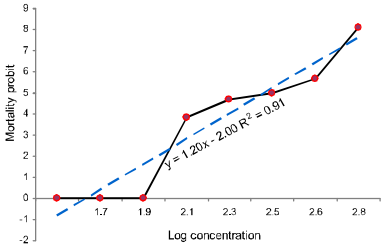


Figure 4: *A. antiquata* mortality increases with ammonium sulfate exposure concentration.

dehydrated through 70, 80, 90, to 95% ethanol. These were cleared in xylene (for 15 min), impregnated in paraffin wax of melting point 55-60°C, embedded in moulds of dimensions 25x25x25 mm, sectioned at 7µ slides, stained with Ehrich's haematoxylin and then counterstained with Eosin. The cover slips were mounted with DPX, and the abnormalities were examined under a Leica DMLB2 light microscope fitted with MPS 30 camera unit.

Data analysis

The morphological alterations in the test cockles were assessed using the Graduated Severity Index (GSI) previously described by Norcross *et al.* (1996). The test cockles were monitored for abnormality over the period of 336 h including the 96 h when they were on exposure to ammonium sulfate. LC_{50} (the concentration at which 50% or 5 probits of the bivalves were killed by the exposure) was computed based on the mortality probits using the Arithmetic Graphic Method. Data were analyzed using the Microsoft Office Excel 2003 software. Stress and deformity significances were analyzed using Chi-square Test of Contingency Table in which the *Observed probits* were compared with the *Expected probits* (Zar, 1999).

RESULTS

We graduated the resultant behavioral and morphological alterations into four levels, namely GSI 0, GSI 1, GSI 2 and GSI 3 (Fig. 1). GSI 0 level designated the status of the control cockles, in which the mean shell-gaping diameter was 5 mm and the mean shell closure rate on gentle prod was 2 mm/sec. The cockles at this level had no significant foot protrusion, no mucus discharge, or necrosis. In addition, there was no hyperplasia or tissue shrinkage. Cockles influenced at GSI 1 level had the mean shell-gaping diameter of 15 mm, foot protrusion length of 15 mm, and shell closure rate of 0.5 mm/sec. These cockles had 25% hyperplasia, red necrosis and a slight mucus discharge. These cockles had been exposed to 50 and 80 mg/L of ammonium sulfate. The GSI 2 level occurred in 130 and 200 mg/L exposures. In this case, the cockles had the mean shell-gaping diameter of 30 mm, foot protrusion of 40 mm, and shell closure rate of 0.1 mm/s. These bivalves had 100% hyperplasia and were with a whitish necrosis. Furthermore, mucus prevalently discharged at their gills. Specimens stressed at GSI 3 level had been exposed to 300, 400 and 600 mg/L of ammonium sulfate. These cockles had the mean shell-gaping diameter of 35 mm, foot protrusion of 25 mm, and shell closure rate of 0 mm/s. They also had whitish necrosis, body shrinkage, collapsed gills, and uncontrolled mucus accumulation on the mantle edges. Values of morphological and behavioral GSI of *A. antiquata* increased with the concentration of ammonium sulfate exposure (Table 1 *[Supplementary data]*). As illustrated in Fig. 2, this relationship gave a straight line of squared regression 0.95. In addition, the morphological and behavioral GSI increased linearly (from 0 to 3) with the exposure time (from 0 to 96 h) giving a squared regression of 0.96 (Fig. 3).

Patterns of gill alterations are partly shown in Fig. 5. Hyperplasia and necrosis were overt in the gills at GSI 1. The GSI 2 level comprised obvious abscesses at epithelium. In addition, the gills comprised swollen lamella, shrunken water tubes, loose inter-lamellar junctions, unlimited mucus secretion, and a 12.5% decrease in cilial length. During GSI 3 most abscesses had ruptured open and the lamellar junctions were markedly broken down. The gills were paralyzed; the inter-lamellar junctions, air spaces and water control mechanisms were extremely disrupted and proliferated. About 12.5% of cilia were detached off and their lengths had averagely reduced by 25%. The mobility and sensitivity of the victims were impaired prior to death, and while unstressed cockles died shell gaps closed, the stressed cockles died shell gaps excessively expanded. At GSI 1 of the intestine, the epithelial cells were bulged to 600 µ (from the usual value of 200-300 µ) in thickness and were detached from the muscular layer and basement membrane as abscesses compounded on serosa. The GSI 2 level of the intestine was accompanied by substantial shrinkage of villi. Also, there were significant abscess formations particularly at the squamous epithelium of the serosa. At GSI 3, the

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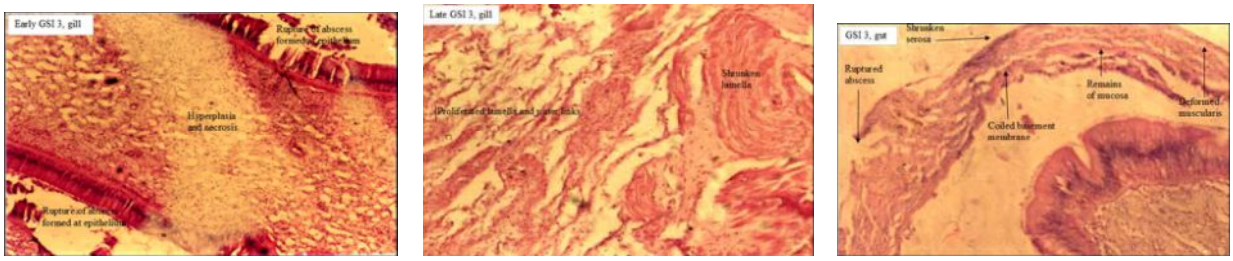


Figure 5: Alterations in the gill and gut of *A. antiquata* upon a 96 h exposure to ammonium sulfate, January-February 2006 (GSI 3).

epithelial cells were conspicuously detached from the smooth layer of the mucosa; the basement membrane was largely coiled; the mucosal epithelium was broken down, which evidently coincided with the rupture of the abscesses. All these observations marked an obvious dysfunction of the gut.

LC_{50} occurred at 282 mg/L (i.e. log concentration 2.45) within 99.2% confidence level, bearing in mind that the exposure comprised 8 individuals per aquarium (Stephan, 1977). Hence, at 95% confidence level the LC_{50} was 282x95/99.2=270 mg/L (SD=0.3, SE=0.17). The 95% confidence interval for population LC_{50} = Mean LC_{50} + 1.96 x SE. Thus the population LC_{50} of this test = 270+(1.96 x 0.17)= 270.33 mg/L for the upper confidence limit (UCL), and 270 - (1.96 x 0.17) =269.7 mg/L for the lower confidence limit (LCL). The computed LC_{50} (i.e. 270 mg/L) of the present bioassay lied within this confidence interval and it was therefore a true value of the population.

Mortality of *A. antiquata* was found also to increase with the concentration of ammonium sulfate exposure. Within 96 h of this acute exposure, no mortality occurred among the control (unexposed) cockles and in those exposed to 50 and 80 mg/L of ammonium sulfate (Table 2 *[Supplementary data]*). Conversely, mortality increased progressively in 130, 200, 300, 400 to 600 mg/L exposures, and this was 12.5, 37.5, 50, 75 and 100%, respectively. A plot of mortality probits versus ammonium sulfate log concentration (Fig. 4) also provided a linear relationship of approximately the same squared regression (0.91) as was observed with the entire body stress (morpho-behavioral manifestations). The acute *A. antiquata* mortality due to the 96 h ammonium sulfate exposure was 33.08%±0.3 (i.e. 4.56 probits) at 95% confidence level. However, some delaying effects (including massive deformities reported above), which persisted in the victims for 336 h, promoted this mortality to 61.6%±0.3 (i.e. 5.3 probits) where all cockles that were exposed at 200 mg/L or higher died. The overall survivals (i.e. 38.4%) were only those cockles which were exposed to 130 mg/L and lower; however, when observed up to 336 h these cockles showed complete remission from the toxic symptoms and this inferred that the toxic effects were not chronic.

The null hypothesis of this study was that the 96 h acute ammonium sulfate exposure caused significant toxic effects in the cockles. The Chi-square values (χ^2) of differences between the observed and expected GSI probits were 4.42 for the general stress, 4.052 for the gills, and 4.07 for the gut. All these values were much less than the critical (tabulated) value (i.e.14.067) at DF 7 hence $p>0.05$. Therefore, the 96 h acute exposure of ammonium sulfate significantly injured the gills, gut and the general body of the test cockles. The χ^2 probits of mortality within 96 h was 19.46 hence greater than the critical value of 14.067 (i.e. $p<0.05$). Therefore, the 96 h ammonium sulfate exposure did not prompt significant mortality to the cockles. On the other hand, the χ^2 probits of the post-exposure mortality (i.e. from 96 h to 336 h) was 9.791 hence less than the critical value (i.e. $p>0.05$). Therefore, during this period mortality was significant. Furthermore, the χ^2 value for the overall 366 mortality was 10.17 hence lower than the tabulated value (i.e. $p>0.05$). Therefore, up to 336 h when *A. antiquata* symptoms went constant the 96 h acute ammonium sulfate exposure had caused a significant mortality in the cockles.

The NOEC (No Observed Effect Concentration) of this acute exposure was 80 mg/ whilst the LOEC (Lowest Observed Effect Concentration) was 130 mg/L at 99.2% confidence level (Table 2). Thus, at 95% confidence level the NOEC was 80x95/99.2= 76.92 mg/L, and the LOEC was 130x95/99.2= 125 mg/L. The 95% confidence interval of NOEC was 76.92-(1.96x0.17)= 76.59 mg/L for LCL, and 76.92+(1.96 x 0.17)= 77.25 mg/L for UCL, evidencing that 76.92 mg/L was a true NOEC value of the population. On the other hand, the 95% confidence interval of LOEC was 125-(1.96x0.17)= 124.67 mg/L for the LCL, and 125+(1.96x0.17)= 125.33 mg/L for the UCL, indicating that 125 mg/L was a true LOEC value of the population.

DISCUSSION

Acute toxicity data were widely available for fish; little data were found for invertebrates although this was reported to be the most sensitive group. Animals with the significantly lower value of LC_{50} than that of *A. antiquata* (270 mg/L) upon a 96 h exposure to ammonium sulfate at pH 8-9 and temperature 28-33°C

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include (LC_{50} in mg/L provided in parentheses) the freshwater catfish *H. fossilis* (200) (Banerjee and Paul, 1996); the green mussel *Perna viridis* (47.7 for the juveniles and 95 for the adults); the seawater fingerlings of *Labeo rohita* (67); a seawater fish *Agonus cataphractus* (210); freshwater fishes *Pimephales promelas* (>100), *Catla catla* (48), *Cirrhinus mrigala* (62), *Cyprinus carpio* (141) and *Tilapia mossambica* (50); the rainbow trout *Oncorhychus mykiss* (38.2); Crustaceans *Daphnia magna, Asellus intermedius, Gammarus fasciatus* (>100 for each); *Daphnia pulicaria* (39.4), and the aquatic insect of the genus *Notonecta* (154). On the other hand, animals with the significantly higher LC_{50} than that of *A. antiquata* include the freshwater fishes *Leuciscus idus* (681), *Brachydanio rerio* (480), and *Lebistes reticulates* (592). The LC_{50} of *A. antiquata* is notably much lower than that of the terrestrial higher animals. For example, it is around 11 times lower than that (3,000) of the rat, and 5.6 times lower than that (1,500) of man. However, it is worthwhile that the LC_{50} of *A. antiquata* compares favorably with those of the closest species, e.g. the mussels *Indoplanorbus exustus* (248); *Lymnaea leuteola* (255), and *Viviparous bengalensis* (248) (Tilak *et al,* 2002; UNEP, 2003; DCNA, 2003; USEPA, 2006; PMI, 2008).

The toxic effects of the 96 h acute exposure of *A. antiquata* to ammonium sulfate generally showed to be subchronic, ranging between acute and chronic levels. However, due to its low LC_{50} (i.e. 270 mg/L) and high sensitivity, *A. antiquata* indicated to be a useful biomonitor of ammonium sulfate toxicity. The results are sensitizing us to handle this chemical cautiously. The dosage of this agrochemical should be carefully designed to avoid causing injury to the targeted crops and the neighboring organisms. Allowable distances between agricultural fields and the water bodies should be revisited in order to protect aquatic life from ammonium sulfate exposure. Ammonium sulfate containers should be carefully designed to avoid its spillage which might accidentally occur during storage and transportation. The delaying effects caused by ammonium sulfate exposure on varieties of organisms should be critically investigated. Further studies are also needed to establish more toxic effects of this chemical. This will enable manufacturers and users to adopt better ways of handling this chemical, and of protecting organisms from its contamination.

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