Effects of ammonia toxicity on stream biota in north Queensland

Thesis submitted by

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ABSTRACT

Lethal and sublethal effects of ammonia toxicity to two north Australian fish species and one invertebrate species were investigated under laboratory conditions following the OECD guidelines for testing of chemicals. Acute toxicity was tested in a static non-renewal system at pH 9.0 and temperature around 29 °C. 96-hour LC_{50} values for the two fish species were 1.31 mg NH_3 – N L^{-1} for barramundi (*Lates calcarifer*) and 1.99 mg NH_3 – N L^{-1} for the eastern rainbowfish (*Melanotaenia splendida splendida*). The 96-hour LC_{50} value for freshwater shrimp (*Caridina nilotica*) was 1.53 mg NH_3 – N L^{-1}. The acute values indicate that barramundi, in particular, is sensitive to ammonia toxicity, comparable to salmonid species. Acute values for the freshwater shrimp suggest moderate sensitivity, given that invertebrates in general are more resistant to ammonia toxicity; the lethal values obtained were comparable to values reported for sensitive invertebrates. The acute values for rainbowfish indicated medium sensitivity, comparable to published values for a range of non-salmonids. There was no unequivocal evidence for gill damage resulting from acute ammonia exposure in the two fish species studied. Changes in gill structure in these fish are therefore not a strong indicator of exposure to short-term ammonia toxicity. A three-week post-exposure experiment on surviving individuals from the acute toxicity test, in uncontaminated water, indicated that exposure to acute concentrations up to 1.5 mg NH_3 – N L^{-1} did not have any significant effects on growth in either barramundi or rainbowfish. It was possible that this was a result of compensatory growth in higher concentrations.

ETHICS APPROVAL

This project was undertaken in compliance with James Cook University Ethics Approval number A796_02.

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1. INTRODUCTION

In recent years there has been growing concern about the effects of anthropogenic activities on the health of aquatic ecosystems. Lowland streams in rural areas of tropical Queensland experience recurrent fish kills, especially following heavy rain (Pearson et al. 2003). Typically these incidents are reported from streams adjacent to broad-scale agriculture. In these streams major changes to water quality are recorded, typically involving decreased concentrations of dissolved oxygen (DO), altered pH, increased sediment loads, and increased concentrations of nitrogen and phosphorus (Congdon and Lukacs 1995). Several studies (Bramley and Roth 2002; Hunter et al. 2001; Pearson et al. 2003) have shown that higher levels of nutrients, and therefore increased probability for ammonia toxicity, are most likely to occur in streams subjected to drainage from fertilised fields, such as sugar cane plantations.

Ammonia is a natural product of bacterial mineralisation and of the excretion by living animals. However, anthropogenic sources such as fertilisers, industry and urban run-off may lead to levels of ammonia that are toxic to the aquatic biota. Harmful effects of ammonia are believed to arise at the intracellular level (Smith and Piper 1975) and can be either lethal or sublethal (reduction in hatching success, growth rate and morphological development as well as pathological changes in gill, liver and kidney tissue) depending of length and magnitude of exposure (WHO 1986). Ammonia has been reported to be lethal to fish at concentrations from 3.9 to 190.54 mg TAN[1] L^{-1} at pH 8. Invertebrates generally exhibit a wider range of sensitivities, with acute values ranging from 1.3 to 1466 mg l^{-1} TAN at pH 8 (US EPA 1999; ANZECC/ARMCANZ 2000). Sublethal effects usually arise at much lower concentrations than the lethal threshold (ANZECC/ARMCANZ 2000).

This project investigated ammonia toxicity in tropical stream fauna. It stemmed from a recent study on the effects of cane field drainage on stream ecology in the Herbert River area (Pearson, et al. 2003), which included laboratory based experiments as well as field observations. That study recognised two situations under which ammonia toxicity is most likely to occur:

[1] TAN = Total ammonia ($NH_3 + NH_4$) , total ammonia as a fraction of N.

1) When run-off from adjacent farmland drains into streams in a non-flooding rain event occurring soon after nitrogen-rich fertilisers have been applied. These events are often of short duration, but may be detrimental to aquatic organisms inhabiting the streams. However, pH levels are often relatively low during these events, thus reducing the risk of acute ammonia toxicity (further discussed in Section 2.2.4).

2) During the falling hydrograph, as nutrient-rich water is retained and low flow leads to increasing stagnation of the water mass, setting the stage for increased biological activity and thus oxygen-demanding mineralisation. Under these conditions, diurnal fluctuations in pH may vary immensely in productive waters as photosynthesis during the day lowers the CO_2 concentration and respiration increases it at night. As long as ammonia-rich and deoxygenated waters from the deeper layers of the water mass remain unmixed, the organisms inhabiting the epilimnion may be unaffected. However, if ammonia-rich water mixes with the upper water mass, then its inhabitants are likely to suffer hypoxia and ammonia toxicity.

The characteristics of tropical freshwater ecosystems may vary substantially from their counterparts in other climate regions. Although aquatic organisms in the tropics have the ability to acclimatise to relatively high temperatures, harmful effects may develop if heating occurs at such a rate to cause overshoot metabolic reactions (Hawker and Connell 1992). Obviously if this coincides with high levels of ammonia, a synergistic relationship between the two stressors should be expected, so elevated temperature would indirectly increase the toxicity of ammonia. In addition, dissolved oxygen concentration generally decreases with temperature in smaller water bodies, as a result of increasing respiration rates in the biota (Cole 1994). Thus, these factors may in combination present a worst-case scenario for ammonia toxicity. Such a scenario is most likely to occur at low flow levels as a result of in-stream biophysical processes as well as the effect of stagnation itself. However, during and after heavy rainfall when temperatures are within the non-detrimental range, higher concentrations of ammonia, often in combination with low levels of dissolved oxygen, may create conditions toxic to the biota (Pearson et al. 2003).

Pearson et al. (2003) and Pearson and Connolly (1998) have shown that much of the fauna of tropical streams is adapted to periodically unfavourable conditions, such as low levels of dissolved oxygen, enhanced nutrient levels and extreme temperatures. Nevertheless, concentrations up to 5 mg TAN L^{-1} have been recorded from waterways in north Queensland

(Watershed 2002: Pearson et al. 2003), which according to results from laboratory experiments may be acutely toxic to both fish and invertebrates (Pearson et al. 2003) and are much higher than recommended upper limits for protection of aquatic life (ANZECC/ARMCANZ 2000).

Since aquatic organisms in Australian fresh waters are adapted to a nutrient-poor environment, they may be more sensitive to increased levels of nutrients and ammonia toxicity compared to freshwater organisms in parts of the world with naturally higher nutrient concentrations. The majority of data on ammonia toxicity has been derived from tests using species inhabiting temperate regions of the northern hemisphere (US EPA 1999) and the derivation of trigger values for ammonia toxicity in the Australian and New Zealand water quality guidelines (ANZECC/ARMCANZ/ 2000) does not include any north Australian species. Research indicating that Australian species may be less resistant to acute ammonia toxicity (Pearson et al. 2003) suggests the need for inclusion of more endemic species in the derivation of future guidelines. Further research is therefore needed to determine the effects of different concentrations of ammonia on the tropical stream biota, in order to allow appropriate water quality guidelines to be set.

This project aimed to provide preliminary data on the sensitivity to ammonia toxicity to selected Australian tropical freshwater species. The data may provide a supplement to refining the ANZECC/ARMCANZ (2000) guidelines, as well as be applied in the future development of site-specific guidelines. Specific aims were:

1. To document lethal concentrations of ammonia for freshwater shrimp *Caridina nilotica*, barramundi *Lates calcarifer* and eastern rainbowfish *Melanotaenia splendida splendida*.

2. To investigate post-exposure effects of acute ammonia exposure on survival, growth and histopathology for the two fish species

It was beyond the scope of this study to investigate the combined effects of ammonia and other stressors, such as low dissolved oxygen and extreme temperatures. Thus, the effects of ammonia explicitly were prioritised, since little information existed on ammonia toxicity to local freshwater species. Its objectives therefore were to document the effects of ammonia toxicity on selected fish and invertebrates from streams of tropical north Queensland.

Acute effects of ammonia on the three test species were investigated in short-term (96-hour) static non-renewal laboratory experiments that involved a strict pH and temperature regulating system. The two fish species were investigated for post-exposure effects, such as changes in growth and histopathology of gills, in a three-week follow up experiment, using uncontaminated water and ambient pH.

This thesis is arranged as follows:

- Chapter 2 reviews the general world literature on ammonia toxicity in fresh waters, with particular reference to tropical and Queensland systems.
- Chapter 3 describes the general methodology used in this study.
- Chapter 4 investigates acute toxicity of ammonia on the test species.
- Chapter 5 investigates sublethal and secondary effects of acute toxicity.
- Chapter 6 discusses the results of this study in relation to previous knowledge
- Chapter 7 conclusions from this study and suggestions for future research and management needs

2. LITERATURE REVIEW

2.1 Nutrient contamination in rivers of north Queensland

2.1.1 Introduction

Nutrients (e.g., C, N, P, trace metals) are essential to the growth of all living organisms (Welch et al. 1998). The natural distribution of nutrients is strongly influenced by biological as well as chemical and physical processes (McClain et al. 1998). Nutrients are either inorganic (e.g., NH_4^+, NO_3^-, PO_4^{3-} and SO_4^{2}) or organically bound (e.g., dissolved organic nitrogen); both forms can be either available or not available for utilisation by living organisms (Campbell et al. 1999).

While the nutrient budget of a healthy riverine ecosystem is a fine balance between the input and output (McClain et al. 1999), natural and anthropogenic disturbances, such as fire, climate change, logging, fertiliser application and urbanisation, may disrupt the nutrient cycles by altering the nature of the controlling processes (McClain et al. 1998). Studies of small forested catchments have led to the introduction of the term nitrogen saturation (Gundersen & Bashkin 1994) which refers to an ecosystem where the capacity to absorb nitrogen in soil and biomass has been exceeded. The natural result of such saturation is the leaching of nitrate and ammonia into adjacent waterways, causing major environmental problems (Steinman & Mulholland 1995). Increased levels of nutrients (e.g., phosphorous and to some extent nitrogen from anthropogenic sources such as sewage and fertilisers are generally regarded as the sources of cultural eutrophication (Cole 1994). This process is partly characterised by increased primary production and breakdown of organic matter leading to a reduction in oxygen concentration (Cole 1994). Furthermore, this may alter the composition of the biotic community, usually with a temporary increase in abundance and lowered diversity (Hynes 1961).

Even though eutrophication is traditionally regarded a problem in lakes, slow flowing rivers may become eutrophic (Williams 1984) when the input exceeds the systems capacity to recycle nutrients (Boulton & Brock 1999). The sources of this eutrophication are usually upstream as higher flow rate and low biological activity may lead to a downstream export. Effects of anthropogenic activities on lotic ecosystems (e.g., eutrophication) can be modified

by measures such as replanting logged areas and protecting or re-establishing riparian vegetation (McClain et al. 1999). In a study on the effects of forest fertilisation on streams in the Mohun basin, Vancouver Island, Perrin et al. (1984) found that streams without riparian vegetation had nitrogen concentrations 20 times higher than streams with riparian vegetation.

Approximately 100,000 tonnes of nitrogenous compounds and 10,000 tonnes of phosphorus are discharged through Australia's sewage system each year (White 1997). Much of these nutrients end up in the marine environment, either directly through marine outfalls or indirectly through rivers and creeks. In Queensland, where agriculture accounts for the main volume of nutrient run off (White 1997), 77,000 tonnes of nitrogen and 11,000 tonnes of phosphorus enters the Coral Sea annually, eventually ending up in the Great Barrier Reef lagoon (Eyre 1993). These estimates indicate a four-fold increase in the amount of sediments, nitrogen and phosphorus ending up in the GBR-lagoon compared to pre-settlement times (White 1997). However, Furnas & Mitchell (2001) argue that the available empirical data from river sampling is insufficient evidence to strongly support the reported magnitude of the increase. Much of Australia's fresh water has high salinity, especially in the more arid regions, but is naturally poor in limiting nutrients such as nitrogen and phosphorus (Rutherford and Gippel 2001).

A substantial amount of data on riverine nutrients and particulates (Mitchell et al. 1991) and water quality and nutrient/sediment fluxes (Mitchell et al.1996) has been collected in rivers of north Queensland over the past two decades. The duration of these studies varies from three years in the lower Herbert River (Bramley and Roth 2002) to thirteen years in the Tully River (Mitchell et al. 2001). Even so, there are no apparent parallels between length and significance of a study, since the sampling frequency and numbers of sampled parameters do not directly correlate with duration. For example, the Herbert River study (Bramley et al. 1994; Bramley and Johnson 1996), in which 19 stations were sampled at monthly intervals for most of the forms of nitrogen and phosphorus in addition to total suspended sediment (TSS), is regarded a major study. Nevertheless, the majority of the studies on nutrient loading and water quality in north Queensland have concentrated more on the magnitude of nutrient loading to the marine environment and less on the environmental effects on the freshwater ecosystems in the region.

2.1.2 Seasonality of nutrient loading

By world standards, Australian rivers have extremely variable flow rates and, atypically, variability increases with catchment size (Rutherford and Gippel 2001). Even rivers of the humid zone experience more varied flow rates, with the occasional cease-to-flow period, compared to rivers in similar areas overseas (Boulton & Brock 1999). A study on the water quality in the Johnston River Basin, which is in one of the wettest regions in Australia, revealed an increase in flow rates of over 700% in the wet compared to the dry season. As a consequence, the nutrient concentrations vary with flow, generally with a correlation between increased flow rate and particulate nitrogen (PN) and particulate phosphorus (PP) concentration (Boulton & Brock 1999). Concentrations of the dissolved inorganic and organic forms of nitrogen and phosphorus have been found to be less dependent on discharge rates (Furnas & Mitchell 2001).

In general, elevated nutrient concentrations occur in the wet season with increased water flow (Mitchell et al.1991; Bramley et al. 1994; Bramley & Johnson 1996; Furnas et al. 1995). However, increased nutrient concentrations have been recorded from periods of low rainfall and stream flow, which, according to Mitchell et al. (1991), is likely to originate from the irrigation drainage of agricultural lands. Furnas et al. (1995) use the term first flush-flow events to explain the typical scenario that occurs during the beginning of each wet season where increased flow leads to the highest nutrient loading in rivers of north Queensland. This probably applies to a greater extent to the semi-arid tropics compared to the wet tropical region where monsoonal rainfall is characterised by bursts of short duration followed by a number of discrete discharge events (Mitchell et al. 1996).

Several studies have concluded that dissolved inorganic and organic nitrogen and phosphorus are less dependent on discharge rate and sediment load than particulate nitrogen and phosphorus (Bramley et al. 1994; Bramley and Johnson 1996; Hunter et al. 1997). Concentrations of dissolved inorganic nutrients (NO_2, NO_3, NH_4^+, PO_4) have been found to increase with small river flow peaks but are diluted during high flow events (Mitchell et al. 1991). Later studies also reported that dissolved inorganic nitrogen (DIN) concentrations might be short-lived and therefore undetected during high flow as a result of dilution (Mitchell et. al 1996; Furnas and Mitchell 2001). DIN concentrations that remain elevated for

a longer period following first flow event levels may reflect continuing exchange to the river from groundwater (Mitchell et al. 1996).

2.1.3 Effects of land use

A number of studies have examined the impact of land use on water quality in the rivers draining coastal areas of north Queensland (e.g., Hunter et al. 1997; Furnas and Mitchell 2001; Bramley and Roth 2002). The land use in catchments of this region is typically dominated by cropping, predominantly sugar cane production in the lower reaches, and grazing in the upper parts (Mitchell et a1.1996; Bramley and Roth 2002). Currently there is an apparent consensus among investigators that the main sources of riverine nutrient contamination in North Queensland are non-point sources such as cropping (banana and cane farming) and grazing (animal wastes and erosion). There is evidence to suggest that the nutrient levels in creeks draining sugarcane land are higher than in any other land use (Bramley and Roth 2002, Table 1.). However, since grazing covers a greater area, it is likely that this activity is the most important contributor of nutrients to the GBR-lagoon (Bramley and Roth 2002).

Table 1. Mean, median, maximum and minimum values for the range of nitrogen and phosphorus fractions ($\mu g\ L^{-1}$).

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Table 1. Data presented in table are selected from Bramley and Roth (2002), where 37 sites in the Herbert River floodplain were investigated for the effects of land use on water quality. As the table indicates, sugarcane is the land use resulting in the highest nutrient concentrations.

Bramley and Roth (2002) reported that elevated total dissolved nitrogen (TDN) values typically occurred in smaller streams on the floodplain where sugarcane and banana cropping dominate, which agrees with the elevated nitrate concentrations recorded from such areas (Hunter et al. 2001).

The study on nitrogen levels in the Tully River (Mitchell et al. 2001) revealed increasing levels of both particulate nitrate and nitrate in the 13-year period of the sampling program (see fig. 2). In the same period (1987-1999) there was an expansion of sugarcane and banana cropping in the area and the sale of nitrogenous fertiliser in the Tully region increased by 130%. The authors proposed that a link between the increasing levels of nitrogen in the river and an expansion in cropping land must be assumed, and stated that this trend is likely to occur elsewhere in catchments adjacent to the Great Barrier Reef lagoon where agricultural activity has expanded.

Figure 1. Nitrate (a) and particulate nitrogen (b) concentrations with concurrent discharge (c) in Tully River 1987-2000. Dotted lines show trend at low discharge periods; solid line is trend line through all data points. The figure indicates a gradual increase in nitrate and particulate nitrogen over the study period and that the peak concentrations for both nitrogen compounds coincide with high discharge concentrations (Mitchell et al. 2001).

Not surprisingly, higher levels of dissolved inorganic phosphorus (DIP) are recorded from areas with agricultural and urban activity (Furnas and Mitchell 2001), with concentrations as high as 0.18 mg PO_4^{3-} – P L^{-1} in the Proserpine River during the wet season (Mitchell et al.

1991). These levels are likely to cause eutrophication in lakes or slow moving waters (Vennerød 1984). In the Johnston Basin, the annual average export of total phosphorus (TP) from areas dominated by sugarcane cropping and areas with banana plantations were 6.6 and 6.8 kg/ha respectively (Hunter et al. 2001). In contrast, the annual average export of total phosphorus from the rainforest in this catchment was 2.3 kg/ha. Similarly, the total nitrogen (TN) exported from the rainforest was less than a third of the export from either banana or sugar cane growing. Results from the Johnston Basin (Hunter et al. 2001) support the idea that sugarcane growing is the greatest contributor of nitrate, where 48% of the nitrate exports in the catchment originated from land dominated by cane farming, which occupies only 13% of the area. In the Herbert River, nitrate concentrations were consistently much higher downstream than upstream (Furnas and Mitchell 2001), coinciding with the fact that the major land uses in the lower parts of the catchment is sugarcane cropping.

Results from a recent study on the effects of cane field drainage on stream ecology in the Herbert River area (Pearson et al. 2003) agree with the growing consensus that the main source of nutrient contamination in north Queensland are agricultural non-point sources, especially regarding the suggested increase in nutrient concentrations (e.g., inorganic nitrogen) in cane-farm run-off. Interestingly, the highest median values for most water quality parameters (e.g., NO_3, NH_4^+ and PO_4) recorded from the studies by Hunter et al. (2001) and Bramley and Roth (2002) are consistently lower when compared to the highest median values for these parameters recorded by Pearson et al. (2003). The difference may be due to Pearson et al. (2003) distinguishing between the different flow phases –that is, the rising and falling hydrograph, base flow and no flow – whereas Hunter et al. (2001) and Bramley and Roth (2002) recorded water quality values as annual medians consisting of an over-representation of data from base flow periods, which generally have lower values than do events and the falling hydrograph. Several studies on water quality in north Queensland will therefore often reflect the chronic levels of nutrients and suspended solids rather than acute values. However, when median levels are recorded over a number of years, they may be indicative of trends in nutrient loading and overall water quality (Pearson et al. 2003).

2.1.4 Effects on stream biota

According to Pearson et al. (2003), wetland areas of the coastal plain of north Queensland are of a much more heterogeneous character compared to the large homogeneous water systems, which are the focus of most studies of anthropogenic sources of nutrients. Attention has typically been directed at the relationship between nutrients and the occurrence of phytoplankton blooms. In contrast, the biophysical character of tropical wetlands means that macrophytes, benthic algae and heterotrophic micro-biota are the dominant primary producers, and are therefore the major players in oxygen distributing processes. Furthermore, Pearson et al. (2003) stress that many of the streams on the coastal floodplain of rivers such as the Herbert and Burdekin drain away from the main river. This implies that water quality data collected in the region (e.g., in larger rivers) in many cases does not reflect the water quality at sub-catchment levels. It is also pointed out that degradation of the wetlands in the area may have substantially reduced their capacity to retain or filter nutrient- and sediment-enriched waters.

Unlike many of the previous investigations on water quality in the region (e.g., Mitchell et al. 1996; Hunter et al. 2001), Pearson et al. (2003) investigated not only the connection between land use and water quality, but also the effects on aquatic organisms through a combination of field investigations and laboratory experiments. The result provided unequivocal indications that reduced dissolved oxygen level is the major water quality issue in tropical wetlands of this region, and the main trigger for a fish kill recorded from one of the creeks investigated (Lagoon Creek, November 1999). The water quality data from Lagoon Creek, sampled during and after a major rainfall in February 2002, revealed severely hypoxic conditions in the receiving water for more than one month. Despite the high re-aeration rates that would have occurred during the peak/rising stages of the hydrograph, dissolved oxygen levels did not rise above 10% saturation throughout the water column. This was attributed to several probable factors, such as elevated biological oxygen demand (BOD), through-flow of hypoxic waters from upstream, water hyacinth weed mats, and inhibition of photosynthesis due to cloudy weather and turbid water. Elevated biological oxygen demand, in this and other cane-field-dominated areas, has been found to be caused mainly by runoff containing highly biodegradable substances such as sugar, from cane juice, shortly after harvesting, with concentrations as high as 400 mg L^{-1} reported (Rayment and Bohl 2002).

Recordings of chronically low dissolved oxygen levels during the falling hydrograph and into the ambient water levels may be attributed to increased primary production as a result of the high nitrate concentrations in the run off (Pearson et al. 2003). Nitrate concentrations as much as 1000 times higher than what may be expected in unimpacted surface water were recorded. Furthermore, nitrate in the presence of low dissolved oxygen would naturally stimulate the growth of denitrifying bacteria. Since these bacteria are facultative heterotrophs (they use oxygen when present but nitrate when it is absent) high nitrate concentrations may lead to the build-up of substantial populations of these bacteria, which may hinder recovery to pre-disturbance levels of oxygen. Results from laboratory experiments indicated that the low dissolved oxygen levels recorded were potentially lethal to some species of fish and macroinvertebrates. A five-day experiment on the survival of local fish species (rainbowfish, glass perch, fly-specked hardyhead and empire gudgeons) to low dissolved oxygen resulted in 100% mortality for all species in oxygen concentrations below 10% saturation. Furthermore, a 24-hour experiment on survival of rainbowfish and barramundi, by Pearson et al. (2003) revealed a fine line between survival and mortality. Rainbowfish were able to survive dissolved oxygen levels down to 13 % saturation for the duration of the test, whereas 12 % saturation was lethal to 90 % of the fish. Similarly, for barramundi, at 17 % saturation all fish survived while 15 % saturation resulted in 70 % mortality. The results from the laboratory experiments supported field observations following low-dissolved oxygen events. The results indicate that local fish species, even though remarkably tolerant to low oxygen levels (when compared to fish from temperate regions) have a sharp transition between lethal and sublethal levels of dissolved oxygen (Pearson et al. 2003).

The macroinvertebrates sampled from 26 creeks with low to high cane farm input in the Herbert region did not produce any significant correlation between numbers of taxa and level of disturbance, so diversity was not a good indicator of disturbance (Pearson et al. 2003). However, when multivariate methods were used to compare distribution of taxa and environmental conditions, effects of cane runoff on the invertebrate community became apparent by the absence of sensitive species. The data from a 5-day laboratory experiment on the effects of low dissolved oxygen on survival and emergence of macroinvertebrates was in agreement with this result, where typical sensitive taxa, such as Leptophlebiidae (Ephemeroptera) declined rapidly in numbers at oxygen concentrations of 10-20% saturation. These results indicate that there is a narrow limit in dissolved oxygen that diverges between survival and death for most of the species included in the study.

Pearson et al. (2003) found that both fish and invertebrates displayed avoidance behaviour at dissolved oxygen levels well above the critical thresholds, indicating that even though low dissolved oxygen levels may not result in acute mortality, they may be harmful over a length of time, resulting in reduced growth and reproduction. The authors concluded that there was good scope for the development of a biomonitoring program applicable to the region.

2.1.5 Water quality management

Currently, no studies of water quality in north Queensland meet all the steps required to develop a water quality management framework. The study from the Johnstone Basin (Hunter et al. 2001) identifies and discusses these key requirements. The study provides an assessment of much of the available water resource information necessary for further development of a water quality management strategy for the Johnstone Basin. The authors concluded that the water quality in the Johnstone River Basin generally was good, with median concentrations within the levels for protection of aquatic life in the Australian and New Zealand guidelines for fresh and marine water quality (ANZECC/ARMCANZ 2000). They suggested the possibility of applying this experience to other parts of the wet tropics as well as other parts of Australia. There are some possible flaws to this conclusion: for example, detecting high levels of ammonia in a routine monitoring program may statistically be a rigorous task (section 2.2.3), even though sampling effort might be intensified during major flow periods, as in this case, with up to 36 samples a day at five of the twenty sites (Hunter et al. 2001). These five sites were draining smaller sub-catchments of single or mixed land uses, which are in agreement with other studies as being more likely to retain higher levels of nutrients compared to the main waterways (Bramley and Johnson 1996; Pearson et al. 2003). This should increase the statistical possibility of detecting harmful levels of nutrients such as ammonia. However, during the low flow period sites were monitored much less frequently and with highly variable sampling effort per site, possibly because of the idea that the low flow period generally exhibits lower concentrations of nutrients (Mitchell et al. 1991; Bramley et al. 1994; Bramley & Johnson 1996; Furnas et al.1995). In periods of increasing water stagnation, either in the form of isolated pools or as slow-flowing river stretches, increasing nutrient concentrations in combination with a range of physico-chemical factors may create conditions harmful to the inhabitants (Pearson et al. 2003). Despite periodically high sampling intensity, both spatially and temporally, there is a

possibility that such conditions may have been overlooked, thus making some of the conclusions less reliable.

2.2 Ecotoxicology with special reference to the toxicity of ammonia

2.2.1 Introduction

Ecotoxicological studies have mainly focused on the effects of contaminants from human sources, such as industry, sewage and agriculture. The overall goals of studies on toxicity have been to identify effects of pollution to (1) regulate discharge, (2) compare toxicants, animal sensitivities or factors affecting toxicity, and (3) predict environmental effects (Buikema et al. 1982; Buikema & Voshell 1993). Bodou and Ribeyre (1989) divide ecotoxicology into two major fields – ecotoxicological models and monospecific approaches. The primary aim of ecotoxicological models is to identify effects of interspecific relationships on the transfer of contaminants and their effects using linear transfer models (experimental trophic chains) and interactive models (experimental ecosystems and microcosms), while monospecific approaches use 'tools' borrowed from toxicology, physiology, biochemistry and other sciences. In the literature, the term ecotoxicology is also referred to simply as toxicity studies. Buikema and Voshell (1993) approach the field from a historic point of view, with toxicology studies being the precursor to what they refer to as toxicity studies. Toxicity studies range in scale and complexity from laboratory-based tests on single or multiple species, where the environmental parameters are completely controlled, to outdoor enclosures (either artificial or part of a ecosystem) where the environmental conditions are less controllable, to whole ecosystem manipulations where only the manipulations induced upon the ecosystem can be controlled.

Single-species tests (acute or chronic) may be carried out in indoor enclosures, (microcosms < 10 m^3), whereas multiple species tests are conducted in both microcosms and in outdoor mesocosms (> 10 m^3). Acute or chronic single-species tests are the most frequently used toxicity tests (Buikema and Voshell 1993; Hickey 2000). The majority of the trigger values for toxicants in the Australian and New Zealand guidelines for fresh and marine water quality (ANZECC/ARMCANZ, 2000) are based on data from acute toxicity tests, either directly or through extrapolation methods. Single-species tests have been criticised for being environmentally unrealistic and artificial (Boyle 1983; Buikema and Voshell 1993). Clements

et al. (1989) stress that single-species tests will not reveal indirect effects of a pollutant (e.g., less predation or competition from more sensitive species) and are therefore possibly limited in their ability to predict responses under natural conditions (Kimball and Levin 1985; Hickey 2000). Multi-species tests, on the other hand, can to some degree mimic natural conditions and predict the effects of a contaminant at the community level. Even so, even larger mesocosm studies are limited as they are not able to mimic natural conditions such as stream meanders and lake stratification, factors that may influence an organism's response to a contaminant (Cooper and Barmuta 1993). Results from such studies should therefore only be extrapolated to field conditions with great caution (Cooper and Barmuta 1993; Hickey 2000). Apparently, environmental realism and the applicability to extrapolate to the field increase with the ability to mimic the real world in the experimental environment. However, the results can be difficult to interpret because of the complexity of such studies and the wide range of factors that may affect the organisms (ANZECC/ARMCANZ 2000; Hickey 2000).

At present the need for integrated approaches to solve water management issues are recognised by scientists and regulatory agencies. The integrated approach, which ideally relies on resources from multiple disciplines, includes chemical-specific guidelines coupled with water quality monitoring, direct toxicity assessment and biological monitoring (ANZECC/ARMCANZ, 2000). According to Hickey (2000), the application of integrated approaches is better able to attribute cause-effect relationships than any one technique used on its own.

2.2.2 Sources of ammonia

As a part of the nitrogen cycle (figure 1), ammonia exists in the aquatic environment in the ionised form, the ammonium ion (NH_4^+) and the unionised form (NH_3). The two forms are in equilibrium, which changes with pH (for more details see section 2.2.4). Ammonium ion is nitrogen in its most reduced form, and is readily assimilated by photosynthesising organisms. A major source is bacterial mineralisation, where decomposing organisms produce ammonia as a by-product of the breakdown of organic matter (Cole 1994; Spiro and Stigliani 1996; Campbell et al. 1999). While the main source of ammonia in the hypolimnion is from bacterial mineralisation (ammonification), its contribution to ammonia levels is less evident in the epilimnion where ammonia excretion is a contributing factor (Cole 1994).

Aquatic animals produce ammonia, either directly as a waste product of metabolism or in the form of urea (NH_2CONH_2), which is hydrolysed to carbon dioxide and ammonia by urease enzymes. For freshwater fish most of this production (≈ 90%) is carried out through diffusive excretion across the gills, predominantly in the form of unionised ammonia, NH_3 (Ip et al. 2001). Nitrogen- fixating bacteria release ammonia as a waste product in the reduction of dinitrogen (N_2) to unionised ammonia. This process is carried out (aerobically or anaerobically) by either free-living bacteria, such as *Azotobacter* or photosynthesising organisms, such as blue-green algae, in particular the family Nostocaceae. Natural edaphic sources such as runoff of ammonia from nitrogen-fixing plants (e.g., *Acacia*) may locally increase the nitrogen concentration in the water (Cole 1994), but most of this nitrogen will be in the form of nitrate as a result of oxidation. Nitrogen - fixation, nonetheless, contributes only a tiny fraction to the nitrogen - pool, since most of the nitrogen is recycled locally by decomposition and reassimilation. Under natural conditions, only a smaller proportion of the nitrogen (5-10%) enters the ecosystem through atmospheric deposition as ammonium ion and nitrate (NO_3) (Campbell et al. 1999).

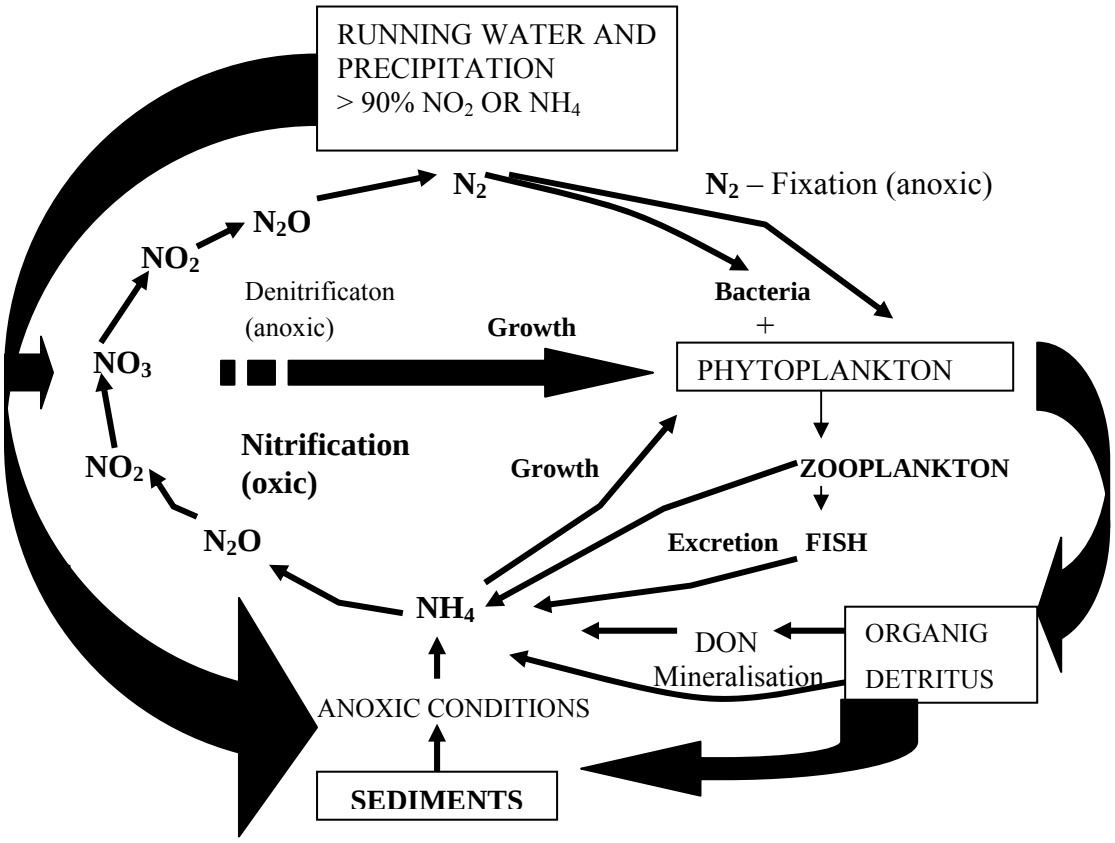

Figure 2. The nitrogen cycle.

Higher levels of ammonia often coincide with low levels of oxygen, while oxygen is utilised in the bacterial breakdown of organic nitrogenous compounds. Ammonia, therefore, accumulates in oxygen-starved conditions (e.g., hypolimnion) where nitrification does not occur (Cole 1994). The nitrification process, where ammonia is oxidised to nitrite (NO_2) and later nitrate may also add to the oxygen depletion of a water body.

While only a small fraction of the available dinitrogen gas in the atmosphere enters the nitrogen - pool naturally through biological fixation, the cycle is easily distorted by anthropogenic activities. A major man-made contributor to the nitrogen - cycle is the application of nitrogenous fertilisers (e.g., ammonium nitrate, calcium nitrate and urea) produced through the Haber process, where nitrogen is artificially fixated (Kolenbrander 1977; Spiro and Stigliani 1996). Currently artificial (synthetic) fertilisers have widely replaced the use of natural fertilisers, such as animal manure (Spiro and Stigliani 1996). Nitrogen is released at slower rates from natural fertilisers in comparison to artificial fertiliser, which already is in the bio-available forms (e.g., NH_4 and NO_3). This suggests that the natural fertilisers are generally less subject to losses into neighbouring environments (Muchovej and Rechcigl 1994). Currently, urea (NH_2CONH_2) is a widely used form of nitrogen - fertiliser (Muchovej and Rechcigl 1994; Freney et al. 1994). Depending on the local conditions (e.g., pH of soil, temperature, humidity, soil texture and cation exchange capacity), the nitrogenous fraction of urea is either volatilised into ammonia gas (NH_3) or undergoes microbially mediated changes with the formation of ammonium and nitrate. In a study on urea application on cane trash in the Herbert Valley, North Queensland, Pramannee et al. (1988) found that more than 40% of the urea was lost by ammonia volatilisation (Freney et al. 1994). Legg and Meisinger (1982) reported that as much as 70% of the nitrogen - fertiliser might be lost through volatilisation. Atmospheric decomposition of ammonia and oxidised nitrogen has increased dramatically in urban areas as a result of agricultural activities and increased effluents from combustion processes (Paycna 1989). In Europe a 50% increase in NH_3 emissions occurred from 1950 to 1980 (ApSimon et al. 1987).

Most of the surface run-off of nitrogen has been reported to be as nitrate in both agricultural and forested catchments where fertilisers are applied (Gundersen & Bashkin 1994). This follows as a result of the rapid uptake of ammonium by plants as well as the oxidation of NH_4 to the more mobile nitrate. However, during heavy rainfall, either before the assimilation of the ammonium or oxidation into nitrate it is evident that nitrogen may enter the local

freshwater systems in its most reduced form, ammonium ion. Ammonium ion leaching is also found in areas with extensive ammonia pollution (Nilsson and Grenfelt, 1988). A study on the effects of fertiliser on the nitrogen fluxes and ecological processes in the upper reaches of the Desna River, Ukraine, revealed that ammonium ion entered the rivers mainly in surface runoff while nitrate was the dominant form in the groundwater (Gundersen & Bashkin 1994). Other major non-point sources of ammonia, besides fertiliser, are urban runoff, animal wastes spread on the soil and precipitation (Ip et al. 2001). Evidently inorganic nitrogen (e.g., NH_4, NH_3 and NO_3) originating from the application of fertilisers (natural and synthetic) enters fresh waters either locally through leaching, or both locally and from more distant sources as a result of volatilisation. In addition to these non-point sources, point sources such as effluents from industry and sewage may increase the concentration of ammonia in the waterways, thus creating toxic conditions to aquatic life (Welch 1992). Effluents from sewage are currently the greatest source of organic pollution in fresh waters in many industrialised countries (Ip et al. 2001; Mason 2002): in the United States, for example, more than 95% of the aquatic point source emissions of ammonia are from sewage treatment plants (Ip et al. 2001). As effluents from a sewage treatment plant enter the stream, ammonia is often present but will normally be oxidised to nitrate further downstream (Mason 2002). This indicates that the peak of ammonia often occurs relatively close to the effluent outlet. Sewage is often high in BOD and this along with oxidation, leads to oxygen depletion in the receiving waters (Mason 2002).

2.2.3 Local sources of ammonia

There are two conditions under which enhanced levels of ammonia occur in fresh waters: 1) where ammonia-enriched water enters the aquatic environment, through point or non-point sources, and 2) under hypoxic conditions, where organic nitrogen is decomposed by microbial activity.

The major sources for nutrient contamination in riverine systems in north Queensland are non-point sources such as fertiliser run-off from farming areas (Furnas and Mitchell 2001; Hunter et al. 2001; Bramley and Roth 2002). Pearson et al. (2003) reported that high concentrations of ammonia in the creeks coincided with increased water flow and were generally much higher in areas receiving run-off from cane farming. High concentrations of

ammonia in farm run-off are most likely to be present when rainfall occurs soon after fertilisers have been applied, before significant assimilation has taken place, and the highest concentrations will most likely occur during and after events when the catchment is already waterlogged. This is explained by the reduced nitrification as a result of hypoxic conditions in the pore water, hence ammonia is not oxidised into the less harmful nitrate. When soil is aerated and moist, some of the ammonia will usually be lost through volatilisation (as NH_3). The highest concentrations recorded (up to 4.5 mg TAN L^{-1}) may be acutely toxic to aquatic life (Pearson et al. 2003) and are higher than the Australian and New Zealand water quality guideline trigger values for protection of 80% of the species at pH 8 (ANZECC/ARMCANZ 2000). Higher concentrations of ammonia occur in water quality data (e.g., Watershed, Department of Natural Resources and Mines; Mitchell et al. 1991), but are relatively rare. This rarity of data can be explained by disproportionately low sampling activity during events where the higher concentrations are most likely to occur (chapter 2). Pearson et al. (2003) show, for example, that at a site that is subjected to one six-hour ammonia toxification event per year, the probability of a random sample coinciding with such an event is only 1 in 1460. This means that it would be necessary to collect several thousand samples per year in order to be reasonably confident that the frequency of occurrence is known and even more if the concentrations maintained during the event need to be quantified with statistical rigour. The majority of existing ambient water quality datasets contain no more than 12 values per site per year so the probability of detecting a six-hour event (for example) is only 1 in 120 at best.

An evaluation of water quality data collected in rivers in north Queensland over the last few decades (Watershed, a database from the Department of Natural Resources and Mines in Queensland) supports this contention: the data revealed that higher concentrations of ammonia were positively correlated with sampling frequencies. For example, the highest number of samples analysed for TAN were collected in the Haughton River basin, with 150 samples in 1993 and 269 samples in 1999, and accordingly produced the highest maximum concentrations recorded in the database, with 4.3 mg L^{-1} and 5 mg L^{-1} respectively.

Most likely as a result of in-stream biophysical processes (i.e., ammonification under hypoxic conditions), enhanced levels of ammonia were recorded on the falling hydrograph following a storm event in Lagoon Creek, with samples indicating that ammonia levels may have been above 0.2 mg TAN L^{-1} for at least ten days (Pearson et al. 2003). Concentrations above 0.2 TAN mg L^{-1} may cause sublethal effects, especially when coinciding with low dissolved

oxygen. On-going studies indicate that increasing levels of ammonia with decreased water levels are not limited to areas receiving cane-farm run-off. For example, in areas where livestock congregate around isolated waterholes, waste products rich in urea may lead to excessive levels of ammonia in these periodically stagnant waters (pers. Comm. B. Butler 2003). Thus, even though it appears that sugarcane farming contributes a greater load of nutrients per unit area on a regional scale, livestock (grazing) may locally be just as detrimental to the freshwater communities.

2.2.4 Speciation and toxicity of ammonia

Ammonia exists in aqueous solution primarily in the two forms, the ionised form (NH_4^+), also referred to as the ammonium ion, and the unionised form, unionised ammonia (NH_3). Consequently, total ammonia consists of the two forms ($[NH_4^+] + [NH_3]$), however when further specification is not relevant it is simply referred to as ammonia in the literature (and hereafter, unless otherwise is warranted). In aqueous solution, the two forms are in equilibrium with each other according to the following equations (Equation 1-4, US EPA 1999):

$$NH_4^+ \leftrightarrow NH_3 + H^+$$

$$K = \frac{[NH_3] + [H^+]}{[NH_4^+]}$$

As the equilibrium constant K is dependent on temperature this relationship can be described as follows:

$$Pk = 0.09018 + \frac{2729.92}{273.2 + T}$$

Where $Pk = -\log_{10} K$ and T is temperature in degrees Celsius.

Furthermore this gives the following equation to calculate the fraction of the two forms from total ammonia:

$$f_{NH_3} = \frac{1}{1 + 10^{Pk - pH}}.$$

$$f_{NH_4^+} = \frac{1}{1 + 10^{pH - Pk}}.$$

$$f_{NH_3} + f_{NH_4^+} = 1$$

At 25° C and pH 7.5 (estimated Pk of 9.24) the fractions of unionised ammonia and ammonium ion are 0.015 for the unionised form and 0.985 for the ionised form. With a five-degree increase in temperature, still at pH 7.5, the fraction of the unionised to the ionised form is 0.024 to 0.975. If the pH increases by one unit at the same time, the fraction of unionised ammonia increase to 0.45. This indicates that pH has a much greater effect than temperature on the individual fractions of the two forms; the ratio of unionised ammonia to ammonium ion increases 10-fold per pH unit rise and approximately 2-fold for each 10° C rise in the 0-30° C range (Erickson, 1985). An estimate suggests unionised ammonia to be 50 times more toxic to aquatic life than ammonium ion (Thurston et al. 1979). This explains why there is a strong correlation between increasing pH and toxicity of total ammonia (figure 2).

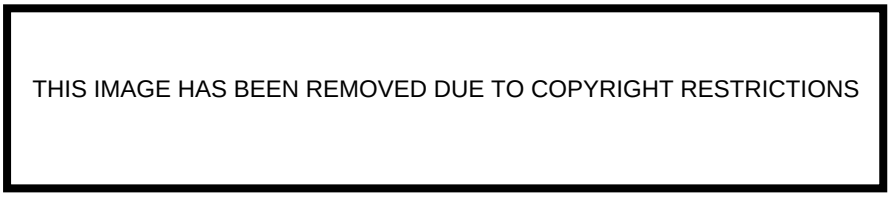

Figure 3. The relationship between pH and toxicity of ammonia (US EPA 1998). As the figure indicate, the toxicity of total ammonia increases with pH as a result of the increasing fraction of the unionised ammonia, which is the more toxic of the two forms.

Unionised ammonia is the most toxic of the two forms, as it consists of neutral molecules and therefore diffuses through the epithelial membranes of aquatic animals more readily than the charged ammonium ion (US EPA 1998). It is evident, however, that there is a combined effect of the two forms, in particular at lower pH levels where the fraction of unionised ammonia is minute (Schubauer et al. 1995). Consequently, at pH levels less than 7.0, toxicity occurs at much higher concentrations of total ammonia than at higher pH. This relationship

explains why LC_{50}'s (concentration lethal to 50% of test animals), in terms of unionised ammonia, decrease with decreasing pH (Schubauer et al. 1995; US EPA 1998); the increasing fraction of ammonium ion makes it appear as though unionised ammonia toxicity increases. However, toxicity of total ammonia increases with increasing pH as a result of the increasing fraction of unionised ammonia relative to the less toxic ammonium ion. Besides the obvious effect on the speciation of ammonia and toxicity, pH may at extreme levels have physiological effects on the organisms, indirectly affecting the toxicity of ammonia (US EPA 1998).

Erickson (1985) reported a four-fold increase in toxicity for fish from unionised ammonia at 5 °C compared to at 25 °C. A possible explanation for the increasing toxicity of unionised ammonia with decreasing temperature is that the relative concentration of ionised ammonia increases with decreasing temperature, thus adding to the increasing toxicity of the total ammonia. When toxicity is described as a function of total ammonia, temperature appears to have less effect (US EPA 1998; Ip et al. 2001). However, even though there are many exceptions, in general toxicity increases with temperature (Mason 2002), partly as a result of the increased metabolism in an organism thus increasing intake of any contaminant in its environment.

When unionised ammonia enters the bloodstream of an organism it is rapidly converted to ammonium ion as a result of the lower pH of the blood (Ip et al. 2001); thus intriguingly, while it is the unionised ammonia that is of greatest concern in the environment, it becomes internally toxic to organisms in the ionised form. It has been suggested that the pH effect on ammonia toxicity by being measured as a product of pH in the bulk water does not reflect the ammonia toxicity to fish, since the pH is different at the gill surface compared to that of the bulk water (Lloyd and Herbert 1960). The theory behind this is that the release of carbon dioxide through respiration lowers the pH at the gill surface and, therefore, depending on the alkalinity and pH of the bulk water, alters the relative concentration of unionised ammonia, and thus the toxicity of the total ammonia.

Erickson (1985) concluded that there were too many uncertainties concerning the chemical speciation of the inorganic carbon released and its effects of gill surface pH to strongly suggest that the effects of pH at the gill surface have anything but a minor impact on the pH dependency of ammonia toxicity. On the other hand, elevated calcium levels have been

found to have ameliorating effects on ammonia toxicity (Randall and Wicks, 2000), possibly by reducing gill permeability to ions (Lin and Randall, 1995). Wilson et al. (1998) suggested that calcium might indirectly reduce toxicity of ammonia because calcium may reduce cortisol production, which is a response to stressful situations (Yesaki and Iwama, 1992), and because cortisol stimulates protein catabolism this leads to a decreased production of ammonia. Given that increased hardness in alkaline waters is positively correlated with increased concentration of calcium, the results from Lin and Randall (1995) and Wilson et al. (1998) suggest a link between increased hardness of water and reduced toxicity of ammonia. However, Schubauer et al. (1995) reported no significant effects of hardness in the 42 to 270 mg/kg (as $CaCO_3$) range, when testing the influence of pH on ammonia toxicity to the oligochaete *Lumbriculus variegatus* and the larval midge *Chironomus tentans* in four-day acute toxicity tests (see also section 2.2.5).

Ammonia toxicity has been found to decrease with increasing salinity up to approximately 30% of seawater (Herbert and Shurben, 1965; Harader and Allen, 1983). In addition, an increase in ionic strength reduces the fraction of unionised ammonia in the water (Ip et al. 2001). However, the effects of salinity have been found to be relatively modest: a change from fresh to marine water at any temperature between 5 and 35 °C will only increase the Pk value by about 0.114, which is approximately one-third of the effect caused by a temperature change of 10 °C (Ip et al. 2001).

The effects of hypoxia are generally not considered in studies on toxicity of ammonia. The focus is rather on keeping oxygen within the non-detrimental range, > 40% saturation (e.g., Smith and Piper 1975; Schubauer et al. 1995; Ankley et al. 1996; Richardson 1997). Nevertheless, several studies conclude that reduced levels of dissolved oxygen increase the toxicity of ammonia (Allan et al. 1990; Wajsbrot et al. 1991). Pearson et al. (2003) found that most of the mortality (80%) to juvenile eastern rainbowfish (*Melanotaenia splendida*) that were exposed to 0.57 – 0.75 mg (NH_3 – N)[1] L^{-1} occurred when dissolved oxygen levels fell to approximately 15%. No mortality in controls was recorded, thus suggesting a combined effect of low dissolved oxygen and ammonia. It should be noted, however, that the low dissolved oxygen levels resulting in this mortality was close to the dissolved oxygen levels (12%) reported (Pearson et al. 2003) to be lethal to 90% of adult rainbowfish.

[1] NH_3 – N = unionised ammonia as a fraction of nitrogen (N)

In the study by Pearson et al. (2003) mortality to juvenile rainbowfish was not tested, making direct comparisons unfeasible; however, it appears that only a brief exposure to near lethal levels of dissolved oxygen in combination with high ammonia may be detrimental. In contrast, Thurston et al. (1981) found no correlation between oxygen levels and ammonia toxicity to fathead minnows (*Pimephales promelas*). US EPA (1998) bases its criteria for ammonia toxicity only on data from tests conducted within a preferred dissolved oxygen range, where no additional effects of hypoxia occur (e.g., above 40% saturation).

According to Ip et al. (2001), the US EPA (1998) criteria for ammonia toxicity are based on the most extensive data collection on ammonia toxicity in the world. The criteria are widely used as a reference in related studies (Richardson 1997; Hickey et al. 1999) as well as in the Australian and New Zealand Water Quality Guidelines (ANZECC/ARMCANZ 2000). US EPA (1999) operates with two different criteria, the maximum concentration (CMC) and the continuous concentration (CCC) for protection of aquatic life. The CMC, which is derived from mean acute values from 48 North American (dominated by Atlantic) species of macroinvertebrates (19) and fishes (29), is defined as half of the final acute value for the most sensitive species in the data set or lethal to less than 50% of the individuals in the fifth percentile genus (particularly sensitive genus). Acute values are identical to either LC_{50} or EC_{50} (effective concentrations 50%). The CCC is developed from chronic values for nine different genera of North American macroinvertebrates and fish. The chronic values are based on EC_{20}'s (effective concentration 20%) from life cycle tests, partial life-cycle tests and tests on early life stage where ecotoxicological variables such as survival, fry survival, reproduction, growth and embryo production and hatchability were obtained. The CCC consequently was set to a level where no more than 20% reduction in these variables occurred for the most sensitive species tested. According to the US EPA (1999), aquatic life should be protected, except possibly for very sensitive species, if the following criteria are satisfied:

1. The one-hour average concentration in mg TAN L^{-1} does not exceed, more than once every three years on the average the CMC calculated using site pH in the following equation:

$$CMC = \frac{0.275}{1 + 10^{7.204 - pH}} + \frac{39.0}{1 + 10^{pH - 7.204}}.$$

Alternatively, at sites where salmonids do not occur, the CMC may be calculated using the following equation (and pH):

$$CMC = \frac{0.411}{1 + 10^{7.204 - pH}} + \frac{58.4}{1 + 10^{pH - 7.204}}$$

2. The 30-day average concentration of TAN (in mg l^{-1}) does not exceed, more than once every three years on the average, the CCC calculated using site pH and temperature in the following equation:

When fish early life stages are present:

$$CCC = \left(\frac{0.0577}{1 + 10^{7.688 - pH}} + \frac{2.487}{1 + 10^{pH - 7.688}} \right) \cdot MIN\,(2.85, 1.45 \cdot 10^{0.028 \cdot (25 - T)})$$

When fish early life stages are absent:

$$CCC = \left(\frac{0.0577}{1 + 10^{7.688 - pH}} + \frac{2.487}{1 + 10^{pH - 7.688}} \right) \cdot MIN\,(2.85, 1.45 \cdot 10^{0.028 \cdot (25 - MAX\,(T,7))})$$

3. The highest four-day average within the 30-day period does not exceed twice the CCC.

The CMC for non-salmonids at pH 9 is 1.32 mg TAN L^{-1} and the CMC with salmonids present at the same pH is 0.885 mg TAN L^{-1}. The CCC at the same pH level and at 30 ° C for early fish life stages absent is 0.442 mg TAN L^{-1}. For early fish life stages present the CCC at the same pH and temperature is 0.179 mg TAN L^{-1}. The Australian and New Zealand Water Quality Guidelines (ANZECC/ARMCANZ 2000) according to the authors base the derivation of trigger values for ammonia toxicity on methods used in the US EPA (1998). Instead of specific criteria for long-term and short-term toxicity, as in the US EPA (1998), ANZECC/ARMCANZ (2000) advises at what threshold concentrations of TAN 80, 90, 95, and 99% of the species are protected. The ANZECC/ARMCANZ water quality guidelines derive their trigger values (concentrations that should trigger management reactions) from NOEC (no observed effect concentrations) data from recent New Zealand and Australian studies. Only chronic data from five species were eventually used in the derivation of the trigger values for protection of freshwater life. The trigger value for protection of 95% of

all aquatic life has been set to 0.18 mg TAN L^{-1} at pH 9, which is similar to the North-American CCC at the same pH level, even though, according to the authors, the temperature dependency in the updated US EPA (1999) version was not taken into account. All data used in the derivation of both the American criteria (US EPA 1998) and the guideline trigger values in the ANZECC/ARMCANZ (2000), were converted to TAN at pH 8 for ease of comparison. Therefore, any ammonia toxicity value referred to in this review has been converted to TAN at pH 8 (for acute values: $AV_{t\ 8}$ and for chronic values: $CV_{t\ 8}$), using methods from the American guidelines (US EPA 1998).

According to Ip et al. (2001), the following factors should be considered when applying the guidelines from the US EPA (1998) criteria for ammonia toxicity: 1) the tests are conducted under such conditions (i.e., resting, starved and stress-free animals) that internal ammonia levels are at a minimum making the animals less susceptible to ammonia toxicity compared to natural conditions (the results from a study on ammonia toxicity to larvae of three odonate species support this idea, where results indicated between 1.2 and 3.7 times greater tolerance of ammonia toxicity for starved compared to fed larvae (Beketov 2002)); and 2) because of the tests conditions, additional factors such as increased toxicity of ammonia with increased activity, for example in migrating fish, are not taken into account – Ip et al. (2001) concluded from several studies that the US EPA (1998) ammonia criteria would not protect migrating salmon.

2.2.5 Acute effects of ammonia toxicity

Acute toxicity is defined as relatively short-term lethal or other effect usually occurring within four days for fish and macroinvertebrates and shorter times (two days) for organisms with shorter lifespan (Meister and Van den Brink 2000). Effects of acute toxicity in fish are hyperventilation, hyper-excitability, convulsions, loss of equilibrium, coma and death. In tests on invertebrates, immobility, besides death, is a common measure of acute toxicity (WHO, 1986).

The US EPA (1998) reports acute toxicity values at pH 8 for fish between 6.38 mg TAN L^{-1} (mountain whitefish, *Prosopium williamsoni*) and 190.54 mg TAN L^{-1} (fathead minnow, *Pimephales promelas*). Invertebrates were generally more tolerant, with acute values at pH 8

spanning from 15.48 mg TAN L^{-1} (cladoceran, *Daphnia magna*) to an extreme high of 1466 mg TAN L^{-1} (crayfish, *Oronectes immunis*).

The ANZECC/ARMCANZ (2000) acute values from 15 species of freshwater fish at pH 8 range from 3.9 mg TAN L^{-1} to 169 mg TAN L^{-1} and for invertebrates 1.3 mg TAN L^{-1} (rotifer, *Brachionus rubens*) to 282.4 mg TAN L^{-1} (Appendix 1).

Richardson (1997) tested the acute toxicity of ammonia on one indigenous New Zealand shrimp (*Paratya curvirostris*) and seven indigenous fish (banded kokopu *Galaxias fasciatus*, inanga *G. fasciatus*, common bully *Gobiomorphus cotiadanus*, redfin bully *G. huttoni*, common smelt *Retropinna retropinna*, longfin eel *Anguilla diffenbachi* and shortfin eel *A. australis*). The 96-hour LC_{50} for the fish at pH 8.1 ranged from 0.75 mg NH_3 L^{-1} (AV_{t8} = 21 mg TAN L^{-1}) for the banded kokopu juveniles and common smelt to > 1.80 mg NH_3 L^{-1} (AV_{t8} = 50.1 mg TAN L^{-1}) for the longfin eel. Surprisingly, the shrimp was more sensitive than most of the fish, with the 96-hour LC_{50} at pH 8.1 being 0.75 mgL^{-1} NH_3 ($AV_{t\,8}$ = 21.5 mg TAN L^{-1}). Richardson compared the acute values from this study with the US EPA (1985) criterion for acute toxicity of ammonia and established that the criterion appeared to be protective of New Zealand species. However, the author pointed out that the results are only indicative, since only a selection of all lowland stream species or life stages were tested. The updated criterion for acute ammonia toxicity (US EPA 1998) has not undergone any major changes since the 1985 criterion (US EPA 1985), thus suggesting that such a comparison may still be valid.

Schubauer-Berigan et al. (1995) tested ammonia sensitivity at different pH levels to two sediment dwelling invertebrates, the midge larva, *Chironomus tentans,* and the oligochaete, *Lumbriculus variegatus* in ten days acute toxicity tests. In agreement with other studies (e.g., Thurston et al. 1979; US EPA 1998), toxicity of total ammonia increased with pH and the LC_{50} values for unionised ammonia were positively correlated with increasing pH, suggesting joint toxicity of the two forms. The 10-day LC_{50}s for *L. variegatus* and *C. tentans* were 0.77 mg NH_3 - N L^{-1} (AV_{t8} = 12.23 mg TAN L^{-1}) and 4.61 mg NH_3 - N L^{-1} (AV_{t8} = 106.37 mg TAN L^{-1}) respectively. Schubauer-Berigan et al. (1995) also tested the effect of hardness on ammonia toxicity. These tests were conducted in Lake Superior water with a hardness of 42 mg L^{-1} as $CaCO_3$ and in reconstituted water with a hardness of 270 mg L^{-1} as $CaCO_3$. The

authors concluded that acute values of ammonia toxicity for the two test species at pH 6.1 and 8.1 did not vary significantly with water hardness.

When Ankley et al. (1996) evaluated ammonia toxicity in pore water to benthic macroinvertebrates in organic sediments, the 10-day LC_{50} for *C. tentans* and *L. variegatus* indicated the toxicity to be approximately 30% and 50% less than in the water-only tests for the two species. The higher LC_{50} concentration in the sediment tests was explained by behaviour, with increasing avoidance behaviour as ammonia concentrations increased. The LC_{50} values for the water-only tests in this experiment were positively comparable to the LC_{50} values reported by Schubauer et al. (1995), as described above.

Pearson et al. (2003) investigated the effects of cane-field drainage on stream biota in north Queensland (see section 2.1.4 and 2.2.3) and tested lethal and sublethal effects of ammonia on local species, by imitating a potential scenario for ammonia toxicity in a tropical environment. The tests were conducted at high pH (pH 9) and temperature (27.7-29.4 ° C) with daily cycling of dissolved oxygen (from 20-30% saturation to 50- 60% saturation) in 48-hour laboratory experiments on invertebrates (shrimp *Caridinia nilotica*, snail *Physa* sp., trichopterans and a zygopteran) and fish (barramundi *Lates calcarifer* and eastern rainbowfish *Melanotaenia splendida splendida*). Mortality in juvenile (34-40 mm) rainbowfish was observed at concentrations between 0.57 and 0.75 mg NH_3 - N L^{-1} (suggests $AV_{t8} < 11.34$ mg TAN L^{-1}) and higher. For juvenile barramundi (42-48 mm) lethal effects were recorded from the 0.56 – 0.67 NH_3 - N treatment concentration (70% mortality) and higher. These results suggest that the LC_{50} for juvenile barramundi may be lower than 0.67 mgL^{-1} NH_3 - N ($AV_{t8} < 10.13$ mg TAN L^{-1}). This is much lower than the 96-hour LC_{50} value for the most sensitive fish recorded by Richardson (1997) from New Zealand, as well as lower than the species mean acute values (SMAV) for all salmonid species recorded in the US EPA (1998). However, daily cycling of oxygen may be a contributing factor in this relatively low LC_{50} for barramundi and rainbowfish.

2.2.6 Sublethal effects of ammonia toxicity

Chronic effects of ammonia may include a reduction in hatching success, reduction in growth rate and morphological development, and pathological changes in gill, liver and kidney tissue

(WHO 1986). According to Smith and Piper (1975), harmful effects of ammonia are believed to arise at the intracellular level and may become manifested when (1) normal detoxification processes are impaired by disease, (2) ammonia is introduced to rapidly, (3) when quantities of ammonia are excessive, or (4) the form of ammonia introduced is highly toxic. There is some disagreement in the literature concerning the definitions of chronic toxicity and sublethal concentrations. Buikema et al. (1993) defined 'chronic toxicity' as being caused by very low doses of a toxicant or an effluent over a long period of time and may, furthermore, be either lethal or sublethal (not causing death). On the other hand, Svobododva et al. (1994) stated that mortality of fish is not generally recorded in chronic toxicity tests. Furthermore they defined 'chronic' from a toxicological point of view, by referring to chronic toxicity tests as being conducted in the range below those concentrations found to induce mortality in acute toxicity tests. ANZECC/ARMCANZ (2000) defines chronic as a situation lasting for a substantial portion of an individual's lifetime or including several generations; depending on the species this will in many cases indicate weeks to months or more. Several scientists refer to sublethal concentrations at the individual level, being effects on individuals surviving acute toxicity, yet others refer to it at the population level, being long-term concentrations not causing significant mortality (Müller and Lloyd 1994). However, a number of scientists have defined sublethal concentrations as those that do not cause rapid or significant mortality (Twitchen and Eddy 1994; Svobododva et al. 1994; Frances et al. 2000; Mason 2002).

ANZECC/ARMCANZ (2000) reported average chronic NOEC and EC_{20} (growth and survival) for nine species of fish to range from 1.350 to 19.729 mg TAN L^{-1} at pH 8 (species listed in appendix 1). Mean chronic NOEC and EC 20 (7 days – 10 weeks experiments on reproduction) for four species of freshwater crustaceans were reported from 1.450 to 19.770 mg TAN L^{-1} at pH 8. Chronic NOEC (29 days reproduction) for two freshwater insects was found to be 1.790 and 4.4 mg TAN L^{-1} at pH 8.

Frances et al. (2000) evaluated the effect of ammonia on the short-term growth in juvenile silver perch (*Bidyanus bidyanus*) in a 39-day experiment. Five concentrations (0.03, 0.04, 0.07, 0.14 and 0.36 mg NH_3 - N L^{-1}) at pH 8 (equivalent to 0.29, 0.5, 0.8, 1.7 and 4.3 mg TAN L^{-1}) were tested in a flow-through regime with survival and gill histopathology as additional endpoints. Only the highest concentration (0.36 mg NH_3 - N L^{-1}) had a modest effect on survival, with two casualties in one of the three tanks two days prior to the finalisation of the

experiment. The food conversion rate (FCR) was not negatively affected by increasing ammonia concentrations, in contrast to both specific growth rate (SGR) and wet weight gain. However, only the highest concentration (0.36 mg NH_3 - N L^{-1}) resulted in a significant reduction in wet weight gain and SGR. The authors suggested that this was an indication of energy being expended on metabolic maintenance rather than growth in the higher concentrations of ammonia. Even though the tested concentrations did not inflict any severe pathological damage to gills, a correlation between increasing ammonia concentrations and the percentage of filaments with epithelial lifting was observed. Epithelial lifting increases the diffusion distance across the membrane and thereby the capacity for gas exchange (Smart 1976). The authors concluded that their results indicated that the sensitivity of silver perch was within the range established for other cultured temperate species, but less sensitive than rainbow trout when compared to results from Smith and Piper (1975). There are some uncertainties in firmly concluding that there are no effects in concentrations below 0.36 mg NH_3 - N L^{-1}, since there is a relatively large gap between the highest and the next to the highest concentration, 0.14 mg NH_3 - N L^{-1}.

An assessment of the susceptibility to ammonia toxicity to the early life stages of carp (*Cyprinus carpio*) and roach (*Rutilus rutilus*) revealed that newly hatched larvae and fry were more sensitive to ammonia than eggs and older fish (Mallet and Sims 1994). For both species the highest concentrations tested (0.66 mg NH_3 - N L^{-1} and 0.31 mg NH_3 - N L^{-1} for carp and roach respectively) had no significant effect on hatching success. On the other hand, survival of newly hatched carp was significantly reduced with increasing concentrations of ammonia. There was less correlation between increasing concentrations and reduced survival of post-hatch stage roaches. Growth for carp was not drastically reduced below 0.35 mg NH_3 - N L^{-1}. Given that the results indicated that sensitivity decreased with age, this suggests that the sensitivity of ammonia to carp is comparable to the results reported by Frances et al. (2000) for silver perch (*Bidyanus bidyanus*). Furthermore, the authors concluded that the roach were relatively resistant to ammonia toxicity, as confirmed by the results indicating only significant negative effects on growth at the highest concentrations (0.31 mg NH_3 - N L^{-1}) after 130 days of exposure (indicates a CV_{t8} of 12.86 mg TAN L^{-1}).

Chronic exposure of ammonia to juvenile rainbow trout (*Oncorhynchus mykiss*) was studied in a flow-through experiment lasting for one year (Smith and Piper 1975). Three different

concentrations of total ammonia (0.5, 1.0, 1.5 mg L^{-1}) were obtained by varying number of fish and flow through rate in the tanks (i.e., fish were exposed to ammonia of metabolic origin). The experiment was performed at pH 7.75 and temperature around 10 °C which indicates NH_3 concentration for the three treatments to be 0.0051, 0.01037 and 0.0155 mg L^{-1} respectively. The authors reported no effect on growth at any of the tested concentrations when compared to the controls within the first four months. However, after six months there was significant reduction in growth in the highest treatment (CV_{t8} of 2.75 mg TAN L^{-1}). Similarly there was little effect on histopathological endpoints such as gill damage after four months of exposure; only mild scattered hypertrophy to secondary lamellae was found at the higher concentrations. After six months of exposure, signs of toxification were apparent in the high concentration treatments: some individuals were lethargic and emaciated and had swollen gill filaments, extended behind the opercula. Overall condition of fish in the lower treatments (0.5, 1.0 mg total ammonia L^{-1}) was good, and only mild hyperplasia and hypertrophy to secondary lamellae was observed in some of the individuals after 12 months exposure.

In the highest treatment, however, not only was pathological damage to gills common after 12 months, but also all of the 13 fish examined had lesions in the liver. Nevertheless, when the authors compared their results with data from similar studies, they concluded that these studies in many instances might have overestimated the chronic toxicity of ammonia. Furthermore, they stressed the importance of keeping the oxygen levels above detrimental levels (5 mg L^{-1} or more) in such studies, apparently to avoid data being biased by the additional effects of low dissolved oxygen levels.

In a study of the effects of chronic toxicity of ammonia to New Zealand freshwater invertebrates, Hickey et al. (1999) tested a range of ammonia concentrations on macroinvertebrate communities in a 29-day mesocosm experiment. Macroinvertebrate communities (beetles, mayflies, dobsonflies, caddisflies, stoneflies, dipteran flies, snails and midges) collected in a nearby stream (Waikato, NZ) were established in the artificial stream channels with the intention of mimicking natural community interactions with predators present. The results indicated that the test concentrations, below 8.5 mg TAN L^{-1} at pH 8.4, had no significant impact on taxon richness. However, there was a significant reduction in abundance of mayflies (*Deleatidium* sp. and *Coloburiscus humeralis*) in the higher treatment (mean = 6.253 mg TAN L^{-1} at pH 8.4). For the mayfly *Deleatidium* sp., which was found to

be the most sensitive species, the 29-day EC_{50} for total and NH_3 - N at pH 8.4 and 16 °C) was 2.15 mg L^{-1} and 0.145 mg L^{-1} respectively. Furthermore the EC_{20} for the same species under identical conditions was 0.53 mg TAN L^{-1}, which is 21% below the CCC of the US EPA (1998). The authors concluded that the US EPA (1998) CCC does not sufficiently protect this species. An EC_{50} and an EC_{20} based on the median concentrations may even be an underestimate as a result of the relatively high range in the within-treatment concentrations of both total and NH_3 - N. When compared to the ANZECC/ARMCANZ (2000) trigger value for protection of 95% of aquatic life, which is 0.480 mg TAN L^{-1} at pH 8.4, the most sensitive species in this study appears adequately protected by these guidelines.

Single-species tests will not predict indirect effects of a pollutant, such as less predation or competition from more sensitive species (Kimball and Levin 1985; Clements et al. 1989; Hickey 2000). Hedtke et al. (1978) studied the relationship between largemouth bass (*Micropterus salmoides*) and its prey, the mosquitofish (*Gambusia affinis*), under the influence of sublethal concentrations of ammonia. The results showed that the growth of bass was negatively affected with increasing ammonia concentrations and prey densities. The negative correlation between prey densities and growth in bass, could, according to the authors, be explained by the increased harassment by the mosquitofish, which were more resistant to the ammonia contamination. These results indicate that sublethal concentrations of ammonia may be detrimental to some species due to disturbance of natural interspecific interactions.

3. GENERAL METHODS

Experiments were conducted under laboratory conditions following the OECD guidelines for testing of chemicals no. 203.

3.1 Test animals

Three species were selected to assess the lethal and sublethal effects of ammonia: two fish, barramundi (*Lates calcarifer*) and eastern rainbowfish (*Melanotaenia splendida splendida*), and one crustacean (*Caridina nilotica*). All were tested for their LC_{50} (estimated concentration lethal to 50% of the tested organism) and behavioural responses to lethal and sublethal levels of ammonia. Changes in growth and histopathology of the gills were tested for barramundi and eastern rainbowfish.

The barramundi is a warm-water species that inhabits northern coastal drainages of Australia, from about 24 °S on both sides of the continent. It is also found in coastal areas of East Africa and Asia. The life cycle is catadromous with adult fish migrating from fresh waters into estuaries to spawn in the wet season (September to March). Juvenile fish migrate upstream within the first year of life (size *ca*. 20 cm) and spend the next three to four years in the freshwater environment before returning to saline waters, typically mangrove areas. Its diet consists of fish and crustaceans and, in its juvenile stages, insects (Allen et al. 2002). Barramundi are fast growing under optimal conditions (28.5 ° C) and can reach a maximum size of 180 cm (Primary Industries and Resources SA 1999).

The barramundi was chosen as one of the test species because it meets many of the criteria of an ideal test organism (Rosenberg and Resh 1993): it is common to the area, ecologically and economically important, occupies a trophic position that leads directly to humans, is easily obtained and has adequate background data available (e.g., physiology, genetics, taxonomy, ecology). For practical reasons only juvenile barramundi (1.25 ± 0.009 g) were tested, obtained from a local supplier (Bluewater Barramundi, Cardwell). The fish were stocked in 1000-L cattle tanks (250 fish per tank) and acclimated to laboratory conditions for two weeks with daily feeding of protein-rich pellets up to 24 hours before experiments commenced.

The eastern rainbowfish is a purely freshwater species and is widely distributed in Pacific coastal drainages of eastern Queensland between Central Cape York Peninsula in the north and Gladstone in the south (23° S). It inhabits rivers, swamps, marshy lagoons, lakes and reservoirs. It feeds on invertebrates and algae. It is a small species, reaching a maximum length of 14 cm (Allen et al. 2002). This species was chosen because it also meets many of the criteria of an ideal test organism (Rosenberg and Resh 1993): it is common in the region (Allen et al. 2002; Williams 1980) and is ecologically important (being an important food for larger fish species such as barramundi); it occupies a trophic position that leads indirectly to humans and it has adequate background data. It is also easily maintained in the laboratory. Test specimens were collected from a local stream; Campus Creek (19°22'S, 146°45'E), with a seine net and then acclimated to laboratory conditions for a minimum of two weeks. The fish were fed daily with Nutrafin complete flake food (minimum crude protein content 44%) during the acclimation period and up to 24 hours before experiments commenced. All fish were juveniles (0.62 ± 0.025 g).

Caridina nilotica is a common tropical shrimp species. It is small (10-30 mm), transparent and quick moving animal often found congregating amongst aquatic vegetation (Williams 1980). *Caridina nilotica* was tested because of its wide distribution, its ease of maintenance in the laboratory and its abundance, which suggests that it has an important trophic position. It therefore meets some of the criteria of an ideal test organism (Rosenberg and Resh 1993). The shrimps were collected from the Ross River (19°22'S, 146°45'E) with a dip net and then acclimated in the laboratory for two days prior to testing. Shrimps ranged in size from 10 to 20 mm. No food was offered during the acclimation period.

During the acclimatisation period commercial test kits were routinely applied to monitor water quality to assure that ammonia and nitrite levels were kept below harmful levels. A series of "Raindance" filters (5µm sediment, 1 µm sediment and 0.5 µm carbon) were used to filter tap water, used as both culture and later test water. Laboratory tests by the Australian Centre for Freshwater Research confirm the efficiency of these filters to remove sediments, chlorines, heavy metals and bacteria (pers. Comm. B. Butler 2001).

3.2 Physico - chemistry of test water

The acute toxicity tests were conducted at pH 9.0 and temperatures ranging from about 28.0 to about 30.0° C firstly because of the need to keep pH and temperature stable in order to ensure minimal fluctuations in ammonia toxicity (see section 2.2.4), and secondly to mimic a worst-case scenario for ammonia toxicity (see chapter 1). Controls at ambient pH around 7.5 were included to investigate any negative effects on test animals from high pH separately. Test water was buffered to pH 9 by adding a 1-molar solution of sodium carbonate (Na_2CO_3). To avoid pH reduction via exchange of atmospheric CO_2, tanks were covered with plastic lids sealed with adhesive tape. The in-going airline passed through a solution of sodium hydroxide (NaOH) and calcium oxide (CaO) to absorb atmospheric carbon dioxide. Any additional negative pH drift was stabilised by adding sodium carbonate as required. dissolved oxygen was kept close to 100% saturation in order to reduce the risk of additional effects of lowered oxygen concentrations, such as build-up of NO_2 or increased ventilation rates, that could cause increased uptake of the toxicant. Room temperature where experiments were conducted was stable to within +/- 0.2° C. The concentrations used in the pilot test were based on the LC_{50} concentrations in similar studies, ranging from 0.25 to 5.0 mg unionised ammonia per litre. All ammonia samples were analysed using automated phenate method with membrane diffusion at the Australian Centre for Tropical Freshwater Research, James Cook University.

The post exposure effects study (chapter 5), was a follow-up experiment on surviving fish from the acute toxicity experiment to investigate secondary effects of short-term exposure. These were conducted in ambient water quality, (i.e., no ammonium chloride (NH_4Cl) was added to the water), thus rendering the strict pH control system in the acute toxicity experiments unnecessary. Water quality was monitored regularly for any nitrogen build up (NH_3, NO_2 and NO_3) with commercial test kits. Temperature, pH and dissolved oxygen were maintained within ambient levels and between 20 and 30% of the test water was renewed every 24 hours. Samples for analysis of total suspended solids and alkalinity were collected at the end of the experiments.

3.3 Preservation and histology

Fish were preserved initially in 4% phosphate-buffered formaldehyde; the most commonly used multipurpose fixative (Leong 1994). Subsequently, because of tissue tearing during sectioning, FAACC (formaldehyde acetic acid calcium chloride), a fixative with more softening properties, was used. The calcium chloride ingredient had a stabilising effect on the tissue and therefore reduced the chance of shrinkage and hardening of tissue during later embedding in paraffin wax (Winsor 1994). An incision was made in the dorsal and anal-pelvic region of all the fish before fixation, to allow the fixative to reach the internal parts of the body. The fish that did not die during the experiments were sacrificed by cold euthanasia in ice water. The shrimp were fixed by direct immersion in 70% ethanol. Vertical segments of whole fish were dehydrated, cleared and impregnated in an automatic tissue processor (Shandon, Hypercenter XP). This process included a series of increasing ethanol concentrations for dehydration, xylene for clearing and paraffin for impregnation. Tissues were embedded in paraffin wax (Paraplast) the following day. Blocks were further softened in a 10% ammonia solution before being cut in 5-µm sections and mounted on microscope slides. After being oven dried for at least 30 minutes in 60 ° C, the sections were stained using Mayer's Haematoxylin and Young's Eosin and mounted with DPX. Sections were examined by light microscopy.

3.4 Pilot study

48 hour unreplicated range finding tests served as a pilot study for the acute toxicity experiments (chapter 4), and applied the same experimental set up as described above and below, except for the lack of replication. Results on physicochemical parameters from the range finding tests indicated that the experimental set up functioned satisfactory, with little variation in pH, temperature and test concentrations. The exposure-response result from the acute range finding test for barramundi was successful with an evenly increasing ratio of dead with increasing ammonia concentration, the same was the case with the shrimp, however, the greatest response was only 50 % (in the highest test concentration). For the rainbow fish on the other hand the, the range finding test was not a success as there was no clear trend in exposure/response. Nominal test concentrations were based on results from comparable studies (e.g. Richardson (1997) and Pearson et al. (2003).

Measured test concentration range in the range finding tests were, for barramundi; 0.17 – 0.38 – 0.52 – 0.93 – 1.46 – 1.92 – 2.31 mg NH_3 – N L^{-1}, for eastern rainbow fish; 0.20 – 0.56 – 0.87 – 1.4 – 1.87 mg NH_3 – N L^{-1}, for shrimp; 0.20 – 0.56 – 0.93 – 1.5 – 1.8 – 2.53 – 3.17 – 3.88 mg NH_3 – N L^{-1}.

This resulted in an estimated LC_{50} of 1.8 mg NH_3 – N L^{-1} for barramundi, 3.83 mg NH_3 – N L^{-1} for the shrimp. Unfortunately the results from the range finding test for rainbowfish was not applicable for calculation of acute values (e.g. LC_{50}).

Materials and methods used in the post exposure experiments (chapter 5) were based on a three-week pilot study only on barramundi (on survivors from the 48 hour range finding test), due to the limited time frame of the study. Analyses of histopathological endpoints were not included as part of the pilot study due to time limitations.

4. ACUTE TOXICITY OF AMMONIA

4.1 Introduction

Concentrations of ammonia inducing lethal effects vary greatly among species and, in general, invertebrates are more tolerant to acute levels than fish (US EPA 1998). The testing of acute values is a standardised method in aquatic ecotoxicology and provides a value (e.g., LC_{50}) at the extreme acute end of a concentration gradient inducing a range of effects on exposed organisms. This study aimed firstly to determine the lethal concentrations of ammonia for selected species. It was expected from the Australian and New Zealand water quality guidelines (ANZECC/ARMCANZ 2000) and laboratory experiments on local tropical species (Pearson et al. 2003) that the species targeted in this project would be more sensitive to acute ammonia toxicity than species overseas.

Secondly, I aimed to determine whether short-term exposure to potentially lethal doses of ammonia lead to abnormal pathology of gills in selected fish species. Even though abnormal pathology of gills from short-term exposure on a range of toxicants is reported in several studies (Mallat 1985), the time frame for abnormal pathology from ammonia toxicity to develop is unclear.

4.2 Materials and methods

The acute toxicity experiments were conducted in a static non-renewal system, in which no water changes were made during the experiments.

The shrimp (*Cardinia nilotica*) were tested in an acute toxicity test lasting for 96 hours. The experiment consisted of seven different treatments of NH_3 - N at pH 9.0. There were two replicates for each treatment and four controls, two at pH 9 and two at ambient pH (approximately pH 7.5 – 8). It was decided with background in the results from the pilot study that a widest possible test range was needed on the expense of replication. Each replicate included ten animals (10 to 20 mm). For the fish species, barramundi and rainbowfish, the 96-hour acute toxicity experiments consisted of five different treatments, with three replicates for each treatment as well as six controls, three at pH 9 and three at

ambient pH levels (pH around 7.5). Each replicate consisted of ten animals for barramundi and seven per replicate for rainbowfish. All fish were weighed prior to the commencement of the acute toxicity experiment. The fish were lightly anaesthetised using 20 mg benzocaine L^{-1} before being weighed and measured (standard and total length). Loadings (i.e. volume of water per unit weight animal) for barramundi were approximately 2.5 litres per 1 grams fish and for rainbowfish approximately 5 litres per 1 gram of fish. The acute toxicity experiments were conducted in 30-L aerated glass tanks filled with filtered tap water covered with plastic lids. The tanks subjected to treatment were sealed and pH regulated (section 3.2). Aeration either passed through the pH stabilising solution, in tanks with treatments, or was pumped directly into tanks via plastic 5-mm tubing. The tanks were placed side by side on racks in two heights. Paper screens were attached to the sides of tanks to prevent visual contact between neighbouring tanks. Tanks were randomly assigned to treatments, as were the test animals. The photoperiod was set to 14/10-hr light/dark with automatic timers. To reduce build-up of metabolic ammonia, no food was offered to test animals for the duration of the acute toxicity experiment. Failure to respond to prodding with a plastic rod was used as an indicator of mortality in shrimp. The fish remaining motionless when removed determined fish mortality. These checks were performed continuously for the three first hours and every five hours thereafter until the termination of the experiment. In addition to the fish that died from exposure, two fish from each treatment replicate were sacrificed by cold euthanasia for subsequent analysis of histopathological changes in gills.

4.2.1 Oxygen, pH, temperature and salinity

In preliminary range-finding tests for the acute toxicity experiment, the pH was found to decline up to 0.1 pH units in every five hours. This variation generated a negative deviation in unionised ammonia exposure of up to 13.1% at 28 ° C and pH range 8.90 to 9.0 (Thurston et al. 1979) and was within acceptable variability for testing of chemicals (OECD 1992) as well as within the exposure range in comparable studies (e.g., Schubauer et al. 1995). Consequently, pH was monitored every five hours together with dissolved oxygen and temperature, using an electronic pH/oxygen meter (WTW multiline 340i). Where negative pH drift occurred, sodium carbonate was added to bring pH back to the targeted test level (section 3.2). Samples for analysis of total suspended solids and alkalinity were collected at the end of each experiment.

4.2.2 Ammonia test solutions

Target unionised ammonia concentrations were determined from unreplicated range-finding tests, with the highest concentration being higher than the calculated LC_{50} value and the lowest concentration being equal to the concentration where there were no observed effect. Nominal test concentrations were calculated using the aqueous ammonia equilibrium (Thurston et al. 1979) where percentage of unionised ammonia is a function of pH and temperature. Ammonium chloride, which consists of approximately 32% total ammonia, was used as the test solution and the following equation was applied to calculate quantity of ammonium chloride to attain chosen target test concentrations:

1. $\frac{\%\ \text{TAN of } NH_4Cl}{\%\ \text{UAN}^1 \text{ of TAN}} = \text{Conversion factor}$

2. Target test UAN in mg L^{-1} x Conversion factor = Product x $\frac{NH_4Cl \text{ in grams}}{\%\ \text{TAN of } NH_4Cl} = \text{Fixed factor}$

3. Target test UAN in mg L^{-1} x $\frac{\text{fixed factor}}{\%\ \text{UAN of TAN}} = \text{Quantity of } NH_4Cl \text{ (mg } L^{-1}\text{) to add}$

For example, if using 1500 mg NH_4Cl L^{-1} at pH 9 and temperature 30 °C as a starting point and the target test concentration chosen is 0.125 mg $NH_3 – N$ L^{-1} the equation was calculated as follows:

1. $\frac{32\% \text{ of } 1500 \text{ mg } NH_4Cl \text{ } L^{-1}}{44.6\% \text{ of } 490 \text{ mg TAN } L^{-1}} = \frac{490}{218.5} = 2.24$

2. 0.125 mgL^{-1} UAN x 2.24 = 0.28 x $\frac{1500}{490}$ = 3.06 (Fixed factor)

3. 0.125 mgL^{-1} UAN x $\frac{3.06}{0.446^2}$ = 0.858 NH4Cl (mg/L) to add

Nominal NH_3 - N test concentrations in mg l^{-1} for the three acute toxicity experiments were as follows: Shrimp: 1.5 - 2 - 2.5 - 3 - 3.5 - 4 - 4.5; Barramundi: 0.5 - 1 - 1.5 - 2 - 2.5; Eastern rainbowfish: 0.5 - 1 - 1.5 - 2 - 2.5

[1] UAN = unionised ammonia nitrogen, elsewhere in text as $NH_3 – N$

Duplicate water samples were collected from each tank at the start of the experiments, after 48 hours and at the conclusion of the experiments (96 hours). Samples were analysed using automated phenate method with membrane diffusion at the Australian Centre for Tropical Freshwater Research, Townsville, to confirm the nominal test concentrations. The results from these analyses were later compared with the calculated target NH_3 – N concentrations and, when required, exposure concentrations were adjusted according to test results by applying the ammonia equilibrium (Thurston et al. 1979) as explained above. All acute values are therefore based on measured concentrations.

4.2.3 Statistical analysis

The LC values with 95% confidence limits were calculated by the Probit Program Version 1.5 (USEPA 1988). Pooled data for each treatment were used. The LC_1, LC_{10} and LC_{50} values are defined as the concentrations that were lethal to 1, 10 and 50% respectively. The Probit Program also calculated the Chi-square statistics for heterogeneity, and tested it against a critical value for how well the data fit the model. Regression analyses were carried out with the SPSS 11.0 statistical package for Windows.

4.2.4 Observations of test animals – sublethal effects

Observations of abnormal behaviour (i.e., different behaviour between treatment and control fish) served as a qualitative measure of sublethal exposure-related stress. Characteristics of abnormal behaviour were unnatural body position, unusual swimming behaviour, increased ventilation rate and active surface respiration (ASR). Any signs of disease, such as white spots and fin rot, were recorded as possible stress reactions.

4.2.5 Histopathology of gills

All fish that died in the acute toxicity experiments and two from each treatment replicate and control replicate were examined for histopathological changes in gills. All features that clearly differed from the ideal healthy fish gill were recorded. One obvious feature, epithelial

lifting, was used as an index of stress (Frances et al. (2000) found epithelial lifting to be a valid indicator of stress in fish subjected to chronic ammonia exposure, see section 2.2.6) for subsequent analyses. Ten secondary lamellae from five randomly chosen primary lamellae were examined for degree of lifting (percentage of epithelial layer clearly elevated from main structure of the secondary lamellae). Each secondary lamella was given a score according to its condition (table 1). The summarised scores for each of the primary lamellae were applied in a ranked Mann-Whitney test to determine if there were any statistically significant differences in degree of lifting in exposed fish compared to fish from controls . In all, ten fish from the controls and nine fish from higher treatments, in which mortality occurred, were subjected to this analysis, which for barramundi were fish from the two highest concentrations (pooled) and for rainbow fish from the highest concentration only. Only recently dead fish (where death occurred less than one hour from removal) were used in the statistical testing.

Table 2. Method for scoring degree of epithelial lifting of secondary lamellae.

Degree of epithelial lifting	Score
Minor degree of lifting (<10%)	1
Medium degree of lifting (10-25%)	2
High degree of lifting (25-50%)	3
Very high degree of lifting (>50%)	4

4.3 Results – acute toxicity

4.3.1 Physico-chemical variables

Only minor variations in pH and temperature occurred during the acute toxicity experiments (Tables 3 - 5). The test animals were therefore subjected to a mainly stable exposure regime. The greatest within-treatment exposure variation occurred in two of the treatments in the acute toxicity test for barramundi, with about 20 % variation. There were greater variations in the test concentrations for the two fish compared to in the test concentrations for shrimp. A build up of metabolic ammonia in the tanks could possibly explain the slight increase in test concentrations toward the end of the experiments for fish. The greatest variation was found in

the 1.54 mg NH_3 – N L^{-1} treatment for barramundi, which was about 15% higher than the average exposure concentration. However, the test concentrations were lower than the nominal test concentrations assigned to each of the treatments, probably due to natural volatilisation of NH_3 gas to the atmosphere. Concentrations of ammonia were less than 50 µg L^{-1} (TAN) in all control replicates during the tests. Dissolved oxygen (DO) levels varied little and stayed close to saturation, except in the shrimp acute test were supersaturation occurred in some tanks. Salinity was at or below 50 mg L^{-1} (total suspended solids) in all tanks.

Table 3. Water quality in the acute toxicity experiment on shrimp.
All values are means of replicate tanks ± s.e.

NH_3 - N (mg L^{-1})	Temp. (°C)	pH	DO % saturation	DO (mg L^{-1})	Salinity (‰)	Total alkalinity (mg L^{-1} $CaCO_3$)
Control, pH	27.74 ± 0.06	8.16 ± 0.06	117,6 ± 2,7	9.7 ± 0.2	0.05 ± 0.0	15 ± 0.0
Control, NH_3	27.77 ± 0.04	8.99 ± 0.004	117.8 ± 2.6	9.2 ± 0.2	0.05 ± 0.0	15 ± 0.0
1.1 ± 0.03	27.77 ± 0.05	8.96 ± 0.008	116.7 ± 2.5	9.1 ± 0.2	0.05 ± 0.0	15 ± 0.0
1.57 ± 0.03	27.71 ± 0.04	8.98 ± 0.005	111.9 ± 2.7	8.9 ± 0.2	0.05 ± 0.0	15 ± 0.0
1.90 ± 0.03	27.78 ± 0.05	8.98 ± 0.006	120.5 ± 2.4	9.6 ± 0.1	0.05 ± 0.0	15 ± 0.0
2.35 ± 0.06	27.86 ± 0.07	9.01 ± 0.003	123.3 ± 2.3	9.6 ± 0.1	0.05 ± 0.0	15 ± 0.0
2.47 ± 0.14	27.76 ± 0.03	9.00 ± 0.003	116.9 ± 2.7	9.1 ± 0.2	0.05 ± 0.0	15 ± 0.0
3.18 ± 0.07	27.84 ± 0.08	8.99 ± 0.006	122.1 ± 2.3	9.6 ± 0.1	0.05 ± 0.0	15 ± 0.0
3.45 ± 0.08	27.76 ± 0.06	8.98 ± 0.005	113.7 ± 2.5	9.1 ± 0.2	0.05 ± 0.0	15 ± 0.0

Table 4. Water quality in the acute toxicity experiment on barramundi.
All values are means of replicate tanks ± s.e.

NH_3 - N (mg L^{-1})	Temp. (°C)	pH	DO % saturation	DO (mg L^{-1})	Salinity (‰)	Total alkalinity (mg L^{-1} $CaCO_3$)
Control, pH	29.1 ± 0.1	8.22 ± 0.02	100.7 ± 3.2	7.87 ± 0.2	0.04 ± 0.0	13 ± 0.0
Control, NH_3	29.1 ± 0.08	8.94 ± 0.01	100.6 ± 2.3	7.85 ± 0.2	0.04 ± 0.0	13 ± 0.0
0.41 ± 0.02	29.2 ± 0.09	8.93 ± 0.01	100.9 ± 2.6	7.88 ± 0.2	0.04 ± 0.0	13 ± 0.0
0.76 ± 0.03	29.3 ± 0.08	8.93 ± 0.01	101.6 ± 2.9	7.97 ± 0.2	0.04 ± 0.0	13 ± 0.0
1.16 ± 0.02	29.2 ± 0.08	8.89 ± 0.02	102.1 ± 2.9	7.99 ± 0.2	0.04 ± 0.0	13 ± 0.0
1.54 ± 0.17	29.2 ± 0.08	8.93 ± 0.01	101.8 ± 2.5	7.96 ± 0.2	0.04 ± 0.0	13 ± 0.0
1.97 ± 0.12	29.5 ± 0.08	8.96 ± 0.01	102.4 ± 3.7	8.00 ± 0.3	0.04 ± 0.0	13 ± 0.0

Table 5. Water quality in the acute toxicity experiment on eastern rainbowfish. All values are means of replicate tanks ± s.e.

NH_3 - N (mg L^{-1})	Temp. (°C)	pH	DO % saturation	DO (mg L^{-1})	Salinity (‰)	Total alkalinity (mg L^{-1} $CaCO_3$)
Control, pH	27.84 ± 0.04	8.20 ± 0.02	95.8 ± 0.3	7.47 ± 0.3	0.05 ± 0.0	15 ± 0.0
Control, NH_3	27.77 ± 0.03	8.95 ± 0.007	94.4 ± 0.4	7.73 ± 0.2	0.05 ± 0.0	15 ± 0.0
0.44 ± 0.01	27.75 ± 0.02	8.95 ± 0.008	96.5 ± 0.2	7.76 ± 0.1	0.05 ± 0.0	15 ± 0.0
0.69 ± 0.02	27.76 ± 0.04	8.95 ± 0.07	95.3 ± 0.5	7.76 ± 0.5	0.05 ± 0.0	15 ± 0.0
1.05 ± 0.01	27.69 ± 0.02	8.95 ± 0.006	97.1 ± 0.2	7.58 ± 0.2	0.05 ± 0.0	15 ± 0.0
1.48 ± 0.02	27.73 ± 0.02	8.97 ± 0.006	94.9 ± 0.5	7.41 ± 0.3	0.05 ± 0.0	15 ± 0.0
1.88 ± 0.02	27.78 ± 0.03	8.98 ± 0.005	96.0 ± 0.3	7.49 ± 0.2	0.05 ± 0.0	15 ± 0.0

4.3.2 Acute toxicity – freshwater shrimp (*Caridina nilotica*)

No shrimp died within the first three hours of exposure. In concentrations from 2.35 mg NH_3 - N L^{-1} and upwards, 10% of the test animals (excluding controls where no mortality occurred) died within the first 12 hours of exposure. In addition, one individual died in the lowest concentration, 1.1 mg NH_3 -N L^{-1}. However, this individual could be seen as an outlier, since no further mortality occurred in this or in the two following treatments (1.57 and 1.90 mg NH_3 - N L^{-1}) during this period. After 24 hours of exposure, 25.71% of the total numbers of exposed test animals were dead. The greatest number of dead animals occurred in the three highest treatments (2.47, 3.18 and 3.45 mg NH_3 - N L^{-1}), with between 50 and 60% mortality (figure 3). This gave an estimated 24-hour LC_{50} value of 3.05 (± (95% C.L) 2.67-3.82) mg NH_3 - N L^{-1}.

There was an obvious increase in mortality with time (figure 4), which at the end of the experiment (96 hours) was up to 100% in the four highest treatments (concentrations of 2.35 mg NH_3 - N L^{-1} and above). Furthermore, mortality increased comparatively more in the lower treatments, with an increase of up to approximately 300% compared to 24 hour observations. Clearly, ammonia becomes more toxic over time. This is reflected by the estimated 96-hour LC_{50} value of 1.53 (± (95% C.L) 1.37-1.66) mg NH_3 - N L^{-1} being close to half the 24-hour LC_{50} value.

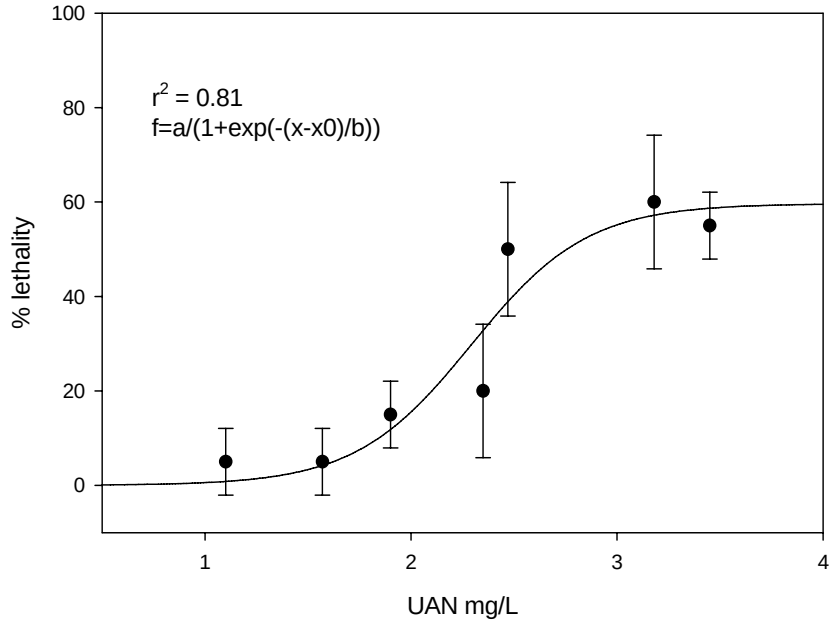

Figure 4. Percentage mortality (mean ± s.e.) of shrimp after 24 hours' exposure to increasing concentrations of NH_3 – N (UAN).

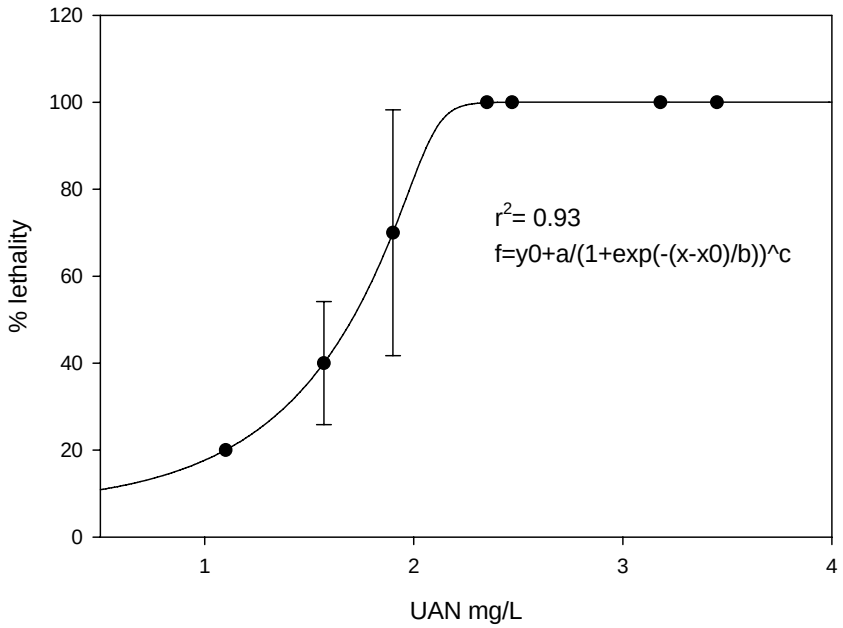

Figure 5. Percentage mortality of shrimp (mean ± s.e.) after 96 hours' exposure to increasing concentrations of NH_3 – N.

At sublethal levels, behavioural changes were observed in all treatments. These changes were most frequent in the higher concentrations, in which the highest numbers of deaths also

occurred. Typically, inactivity was a precursor of changes in normal body position and, where the latter occurred, death usually followed soon after. However, in the lower concentrations, distinct changes of body position were recorded in a number of animals that survived the experiment, and can therefore possibly be seen as indicator of toxicity at a sublethal level. No mortality or behavioural changes were observed in any of the four control replicates.

4.3.3 Acute toxicity – barramundi (*Lates calcarifer*)

Lethality occurred in the highest concentration (1.97 mg NH_3 - N L^{-1}) within the first three hours of the experiment. Twelve hours into the experiment, 22.6% of the total number of test animals (excluding controls) had died, presumably from ammonia exposure. At 24 hours, the number of dead fish was 44, 29.3% of the total. In the highest treatment 80% of the fish were dead (figure 5), evenly distributed among the replicates. The 24-hour LC_{50} estimate was 1.59 (± (95% C.L) 1.48-1.73) mg NH_3 - N L^{-1}.

Of the 150 test animals exposed to ammonia, 40% were dead at the conclusion of the experiment (96 hours). This relatively modest increase in mortality from 24 to 96 hours (figure 6) is reflected by only a moderate decrease in the LC_{50} estimate. The estimated 96-hour LC_{50} value for barramundi was 1.31 (± (95% C.L) 1.22 – 1.39) mg NH_3 - N L^{-1}. The response was spread among the three highest treatments, with an evenly increasing number of dead with increasing concentrations of ammonia. None of the test animals survived the highest exposure concentration. No mortality was recorded from the two lowest concentrations (≤ 0.76 mg NH_3 - N L^{-1}) or in any of the controls. The result therefore fits the ideal treatment/response curve (figure 4) closely ($r^2 \approx 0.99$), forming an asymptotic curve, which reflects a gradual increase from no response to 100 % response.

Signs of sublethal stress were recorded in all treatments except in the lowest (0.41 mg NH_3- N L^{-1}), and involved hyper-excitability and unnatural swimming behaviour. These behaviours occurred in the treatments where no death was recorded, as well as in the higher treatments where mortality increased with time. In higher treatments (≥ 1.16 mg NH_3 - N L^{-1}), these sublethal stress signs were typically followed by more intense indicators of toxification such as increased ventilation rate, ASR, convulsions, loss of equilibrium, coma and finally death.

In addition, fungal infections were observed in fish in treatments at 0.76 mg NH_3 - N L^{-1} and above.

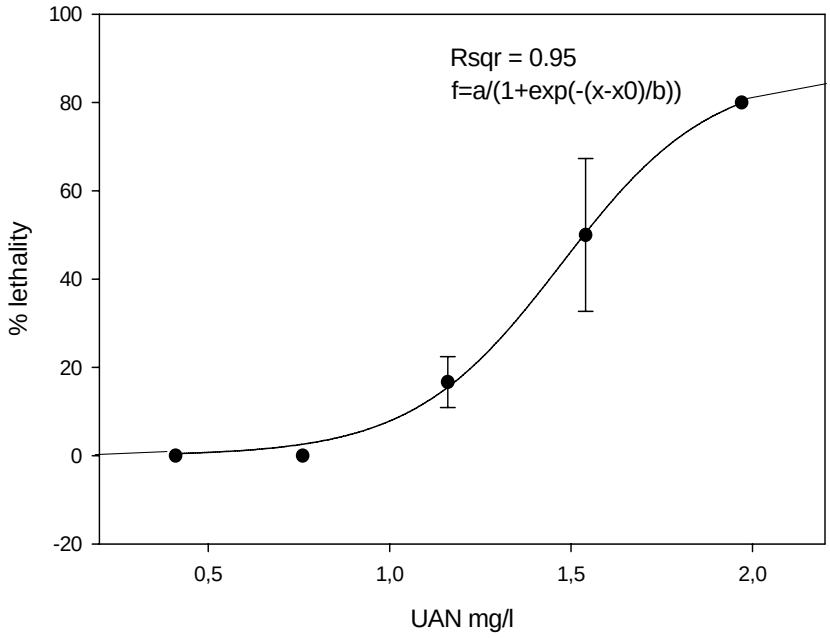

Figure 6. Percentage mortality of barramundi (mean ± s.e.) after 24 hours' exposure to increasing concentrations of NH_3 – N.

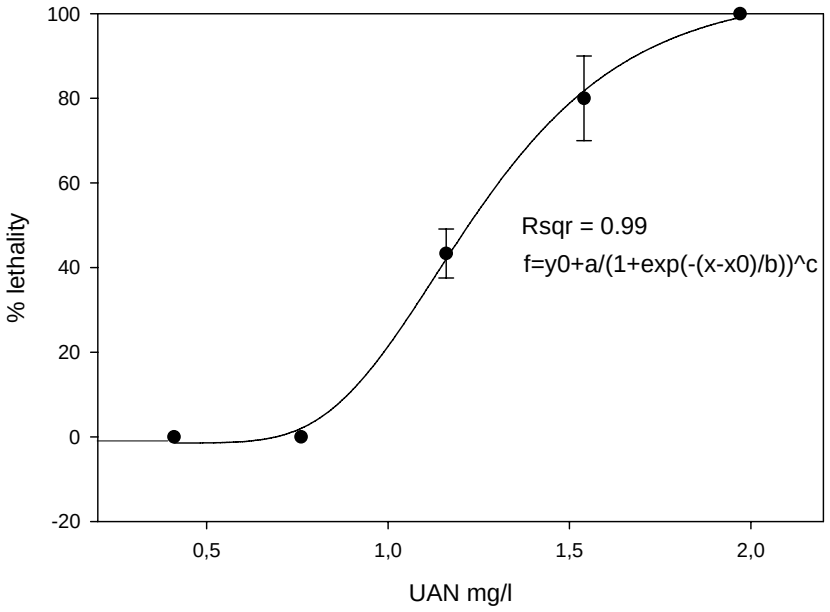

Figure 7. Percentage mortality of barramundi (mean ± s.e.) after 96 hours' exposure to increasing concentrations of NH_3 – N.

4.3.4 Acute toxicity – rainbowfish (*Melanotaenia splendida splendida*)

No animals died within the first three hours of the experiment in any of the treatments. After eight hours' exposure, mortality was recorded in the highest concentration (1.88 mgL^{-1} NH_3 - N), with two dead fish in one of the replicates. After 12 hours there was a total of four dead fish in the same replicate. No mortality was recorded at this stage in the two other replicates of this treatment. Lethality was recorded in concentrations 0.69, 1.05 and 1.48 mg NH_3 - N L^{-1}, with one dead fish in each treatment. Mortality increased very little in the next 12 hours, that is, after 24 hours of exposure. Only one more fish died in the highest treatment (1.88 mg NH_3 - N L^{-1}) and two in the next to the highest (1.48 mg NH_3 - N L^{-1}). Curiously, one of the replicates in the highest treatment had no mortality after 24 hours. The number of dead fish constituted only 9.52 % of the total number of exposed fish; this small response meant that the 24-hour LC_{50} estimate of 3.49 (± (95% C.L) 2.16-124.94) mg NH_3 - N L^{-1} for rainbowfish is statistically weak (figure 7).

Halfway through the experiment (48 hours), mortality increased only in the highest treatment with four deaths in one of the replicates, and no deaths in other treatments. Total number of dead fish at this point was 14, or 13.3 % of the total number of exposed fish. The 48-hour LC_{50} value estimate was 2.33 (± (95% C.L) 1.81-5.50) mg NH_3 - N L^{-1}. The low response is reflected by a relatively wide confidence interval. At the conclusion of the experiment (96 hours) only a minor increase in mortality in the highest treatment had taken place, with three more dead fish, giving a total of 17 dead fish, which constituted only 16.19 % of the total number of exposed fish. The 96-hr LC_{50} estimate for rainbowfish was 1.99 (± (95% C.L) 1.66-3.08) mg NH_3 - N L^{-1} figure 8).

Despite the limited response in mortality, there were obvious signs of stress in concentrations from 0.69 mg NH_3 - N L^{-1} upward early in the experiment. ASR was recorded early in the experiment. Other signs of sublethal stress were disorientation, hyper-excitability and unnatural swimming behaviour in 0.69 mg L^{-1} and above.

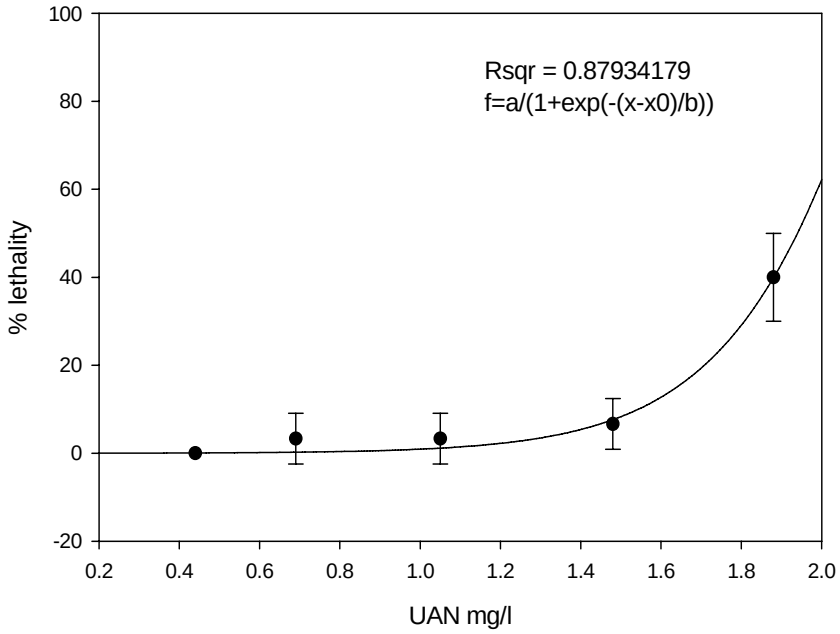

Figure 8. Percentage mortality of rainbow fish (mean ± s.e.) after 48 hours' exposure to increasing concentrations of NH_3 - N.

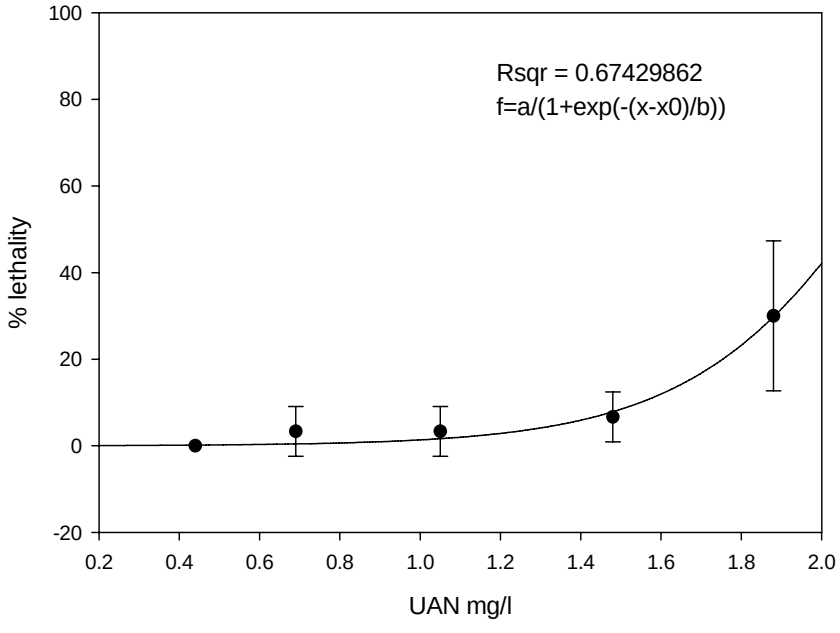

Figure 9. Percentage mortality of rainbow fish (mean ± s.e.) after 96 hours' exposure to increasing concentrations of NH_3 – N.

4.3.5 Summary from acute toxicity tests

Table 4 summarises the results from the acute toxicity tests for all three species. The lethal concentrations mostly decreased with time, as the animals presumably became more susceptible to the ammonia toxicity over time. The concentration-response relationship follows an upward trend, as a higher dose is required to be lethal to an increasing proportion of animals. The AV_{t8} was calculated to allow comparison with results from similar studies, to be discussed below. Clearly, barramundi was the most sensitive of the three species to acute ammonia toxicity by having the lowest acute values overall as well as the lowest AV_{t8}. The shrimp, while being more resistant to ammonia at an early stage of exposure, became more sensitive to ammonia at a longer period of exposure when compared to the rainbowfish.

Table 6. Summary of results from acute toxicity tests

Acute value	*Caridina nilotica*	*Lates calcarifer*	*Melanotaenia s. splendida*
24 H LC_1 [a]	0.90 (0.43-1.22)	0.88 (0.64-1.04)	0.51 (0.01 - 0.83)
48 H LC_1	1.35 (1.01-1.60)	0.78 (0.56-0.92)	0.61 (0.15-0. 90)
96 H LC_1	0.82 (0.58-0.99)	0.87 (0.69-0.98)	0.67 (0.25-0-91)
24 H LC_{10}	1.55 (1.09-1.84)	1.15 (0.96-1.37)	1.22 (0.61- 1.75)
48 H LC_{10}	1.48 (1.23-1.65)	1.03 (0.85-1.15)	1.12 (0.68-1.36)
96 H LC_{10}	1.09 (0.87-1.24)	1.04 (0.90-1.13)	1.09 (0.72-1.30)
24 H LC_{50}	3.05 (2.67-3.82)	1.59 (1.48-1.73)	3.49 (2.16-124.94)
48 H LC_{50}	2.11 (1.94-2.27)	1.47(1.35-1.59)	2.33 (1.81-5.50)
96 H LC_{50} [b]	1.53 (1.37-1.66)	1.31 (1.22-1.39)	1.99 (1.66-3.08)
$AV_{t\,8}$ [c]	24.32	18.63	30.51

[a-b] Concentrations where 1, 10 and 50% mortality occurred at 24, 48 and 96 hours' exposure. All LC values are as mg NH_3-N L^{-1} with 95% confidence limits.

[c] LC_{50} at 96 hours transformed to AV_{t8} (Acute toxic value at pH 8 for mg TAN L^{-1}) following the procedures from US EPA (1998).

4.3.6 Histopathology of gills

Gills from fish that died in the acute toxicity experiments revealed an array of lesions. The most frequently observed lesion in both barramundi and rainbowfish was epithelial lifting. The degree of epithelial lifting of secondary lamellae varied from approximately 10 to 100%. This variation was mainly between individuals, but the variation was high within some individuals as well.

Infiltration of leucocytes in the epithelial cavity was found in some samples (from both species) with high degree of epithelial lifting. Congestion of blood cells occurred both in secondary lamellae and in the filaments, the latter apparently from a rupture of the basal lamina. This feature appeared more frequent in samples from the rainbowfish.

Curiously, in one of the samples from the highest treatment in the acute tests for barramundi (fish that died) major hypertrophy of epithelial cells as well as chloride cells was found, but there was only minor epithelial lifting. Fusion of secondary lamellae occurred in a minority of the samples for both species, mainly resulting from hypertrophy and on rare occasions from rupture of the epithelia. However, three samples with normal histopathology were found in barramundi that died during the acute toxicity test.

There appeared to be little difference in pathology of gills between fish that died in the acute toxicity tests and the fish that survived (figure 9 vs. figure 10). This was also the case with the gill samples from the control treatments.

There were no significant differences (Mann-Whitney: $U_{(1)\ 9,10} = 66$, $P > 0.05$) in degree of epithelial lifting between controls and fish that died in the acute experiments for either barramundi or rainbowfish. Since there appeared to be little variation in the samples over the range of treatments for epithelial lifting, further statistical testing was considered unnecessary. Results from scoring of epithelial lifting are listed in appendix 2.

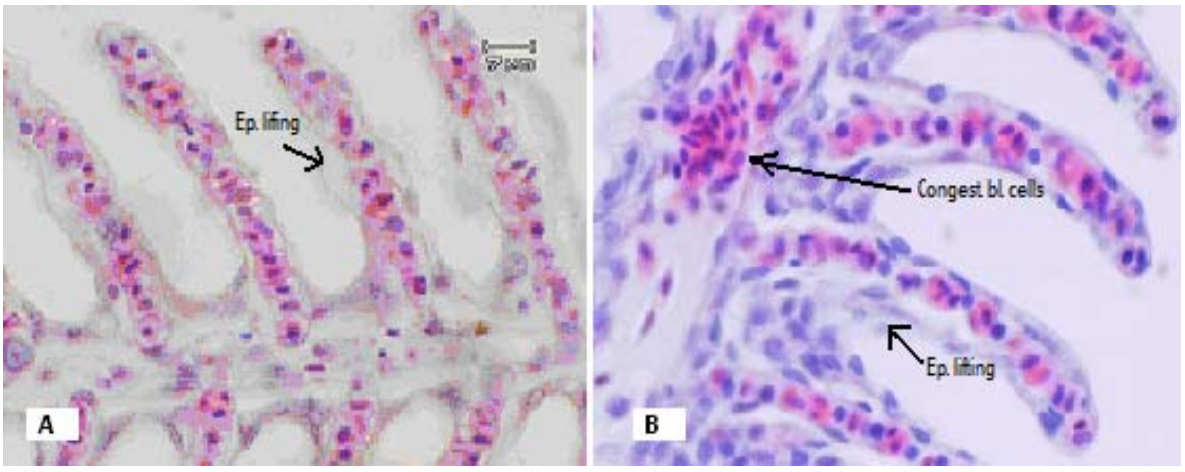

Figure 10. Sections of secondary lamellae from barramundi (A) and rainbow fish (B) that died in the acute toxicity tests. Arrows indicate areas with epithelial lifting and congested red blood cells in the filaments.

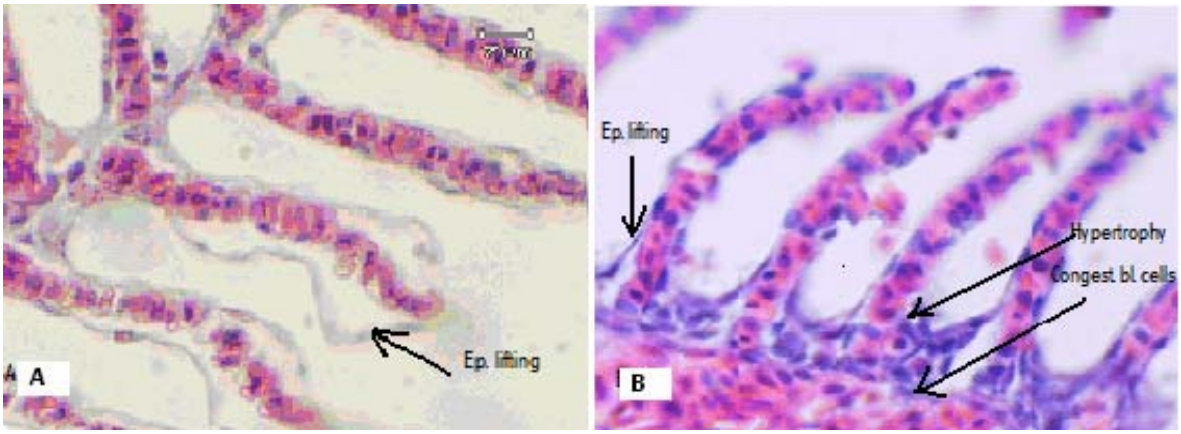

Figure 11. Sections of secondary lamellae from barramundi (A) and rainbow fish (B) that survived the acute toxicity tests. Arrows indicate areas with epithelial lifting, hypertrophy and congested red blood cells in the filaments.

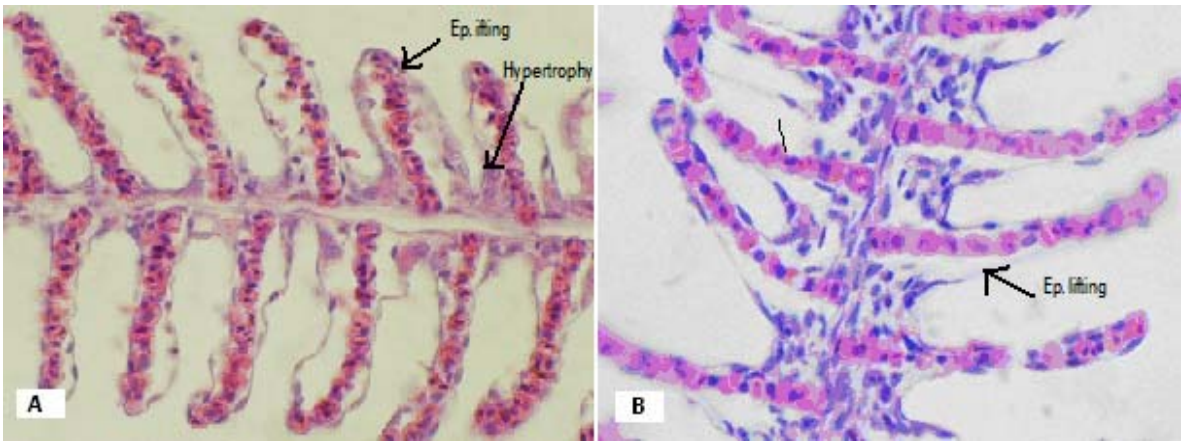

Figure 12. Sections of secondary lamellae from barramundi (A) and rainbow fish (B) from the control treatments of the acute toxicity tests. Arrows indicate areas with hypertrophy and epithelial lifting.

4.4 Discussion of acute toxicity results

4.4.1 Overview

The results from the acute toxicity experiments indicated that barramundi was the most sensitive of the three test species to ammonia toxicity; with lethal values comparable to highly sensitive species such as salmonoids (US EPA 1998). Both the American (US EPA 1998) and the Australian (ANZEC 2000) water quality guidelines appear to be protective of the species tested in this study. Behavioural changes such as ASR, convulsions and loss of equilibrium were observed at concentrations where no mortality occurred. This study did not produce any unequivocal evidence for gill damage from acute ammonia exposure.

4.4.2 Test conditions

4.4.2.1 Test concentrations

The test animals were subjected to mainly stable concentrations of ammonia in the acute toxicity tests. Only in one of the treatments for rainbowfish and in two for the barramundi, did the within-treatment exposure range vary with as much as 20%. The highest variation was found in the 1.54 mg NH_3 - N L^{-1} treatment replicates for barramundi, where the highest measured concentrations were about 15% higher than the average exposure concentration. While this may have caused overestimates of the toxicity of ammonia by bringing the LC estimate down, the results indicate that this spike did not last for the full duration of the test, nor did it have any significant effects on survival.

4.4.2.2 pH

It has been theorised that fish actively excrete protons to form an acid layer next to the gill surface (Wright et al. 1989; Lin and Randall, 1995) thereby augmenting ammonia excretion. Nevertheless, at high pH (pH > 9), the ability to excrete ammonia is reduced as a result of the absence of ion trapping (Wright et al. 1989) in alkaline waters. As the tests in this study were conducted at high pH (≈ 9), with approximately 40% of total ammonia in the form of NH_3 – N being relatively stable, it is likely that ammonia excretion was low. However, the transepithelial exchange of sodium (Na^+) and ammonium ion enhances the excretion of

ammonium ion and thus reduces the toxicity of ammonia (Payan et al. 1978; Wright and Wood 1985). This could explain the slight increase of total ammonia towards the end of the acute tests for the two fish species. However, the low ammonia levels in the pH controls as well as the NH_3 controls suggest that excretion was at a natural low (tests were conducted on starved animals) and had little to do with the ability to excrete ammonia as an effect of the chemical properties of the water. Nevertheless, as the aim of these tests was to mimic a worst-case scenario, including high pH, a further elaboration on the influence of these factors was not important to the outcome of this study.

4.4.2.3 Temperature

Obviously aquatic animals in the tropics are adapted to tolerate higher temperatures than their relatives in cooler water (Wootton 1990), which the American and Australian and New Zealand guidelines (derived from only species outside the tropical region) are based upon. However, when temperature approaches the thermal tolerance for a species the increased metabolism may result in a synergistic relationship between temperature and any toxicant present due to an increased uptake of the toxic material (Mason 2002). The temperatures experienced by the test animals in this study, however, were within the temperature range normally tolerated by these species (Williams 1980; Allen et al. 2002) and should therefore not be considered an additional stressor.

4.4.2.4 Dissolved oxygen

The US EPA water quality criteria for ammonia (1998) did not include any tests conducted at dissolved oxygen below 40% saturation and thereby set a standard for the preferred dissolved oxygen range for testing of ammonia toxicity. This indicates that low dissolved oxygen increases the toxicity of ammonia, which is supported by results from other studies (e.g. Allan et al. 1990; Wajsbrot et al. 1991). Possible synergistic effects between hypoxia and high ammonia concentrations are further discussed in chapter 6. The dissolved oxygen levels in this study were kept close to saturation and should not have had any confounding effects on the reported acute values. Although total gas pressures in excess of 115 % over a few hours has been reported to cause lethality in fish (Fickeisen and Schneider 1976), it appears that the supersaturation experienced in both the barramundi and shrimp experiments did not have any negative effects on the wellbeing of the test animals, while neither lethality nor abnormal behaviour occurred in any of the controls.

4.4.2.5 Salinity

A decrease in ammonia toxicity has been reported in salinity levels up to about 30% of sea water (Herbert and Shurben 1965; Harader and Allen 1983). Given that the effect is relatively modest within the salinity levels normally encountered in fresh waters (section 2.2.4) and that salinity, measured as total anions and cations, was minute in all tests, this should not have had any ameliorating effects on the ammonia toxicity in the current study. Similarly, it has been found that toxicity of ammonia is reduced in highly alkaline waters (Yesaki and Iwama 1992), when alkalinity is caused by calcium bicarbonate. Calcium stimulates the ammonia excretion via a sodium-related exchange in the branchial epithelia and thereby reduces the internal ammonia levels. In this study however, sodium bicarbonate was used as a buffer solution and therefore would not have had any such reducing effects on the toxicity of ammonia. It is also more preferable to use a sodium-based rather than a calcium-based buffer, since the tested species inhabit sodium-dominated waters.

4.4.3 Test animals

All animals were acclimated to laboratory conditions before tests. However, it is possible that animals collected in the field are more resistant to stressors, if the population has been subjected to unfavourable conditions prior to collection (Meister and Van den Brink 2000). Water quality at the collecting site for eastern rainbowfish (Campus Creek) and shrimp (Ross River) was within the ANZECC/ARMCANZ (2000) trigger values for physical and chemical stressors (pH slightly above) as well as toxicants in guideline values over the three months prior to collection (Citiwater Laboratory Services, Townsville). Barramundi were obtained from a fish farm and were kept in uncontaminated water at all times before being shipped (Bluewater Barramundi, Cardwell). As the tests in this study were conducted on juvenile animals it could be that the acute values for older and adult animals would be higher. However, reports on size/age dependence on sensitivity to ammonia toxicity are somewhat conflicting: Richardson (1997), for example, found no significant differences in sensitivity to ammonia for juvenile and adult individuals of common bully (*Gobiomorphus cotidianus*); conversely, Solbé and Shurben (1989) reported early life stages of rainbow trout (*O. mykiss*) to be considerably more sensitive to ammonia toxicity than older stages; while Mallet and Sims (1994) reported that older fish and eggs of both carp (*Cyprius carpio*) and roach (*Rutilus*

rutilus) were more sensitive to ammonia than newly hatched larvae and early fry. According to Rand and Petrocelli (1985), younger and smaller individuals are generally more sensitive to toxicants because of their larger surface-to-volume ratio, which promotes higher relative intake of the tested chemical.

Thurston and Russo (1983), on the other hand, reported decreasing sensitivity to acute ammonia toxicity in rainbow trout (*O. mykiss*) with increasing weight in the 0.06-2.0 g range and that sensitivity gradually increased above that weight range. Apparently there is considerable variation between species. In addition, little information regarding size-dependent sensitivity to ammonia toxicity exits for the species tested in this study. However, findings from Pearson et al. (2003), on eastern rainbowfish (*Melanotaenia splendida splendida*), showed that sensitivity to acute ammonia exposure was negatively correlated with size – that is, smaller individuals had less resistance. This suggests that the lethal values obtained for rainbowfish in the current study should be expected to be lower for smaller/younger individuals. Currently, no information exists on the effects of ammonia toxicity to different sizes of barramundi, so it is unknown if the acute values for larger and smaller individuals would differ significantly from those found in this study.

4.4.3.1 Freshwater shrimp

The acute value for *Caridina nilotica* (AV_{t8} = 24.32) indicates relatively high sensitivity to ammonia toxicity when compared with North American invertebrates. Mean SMAV (species mean acute value at pH 8) for invertebrates in the USEPA (1998) database is 188.9 mg TAN L^{-1}. All 17 invertebrate species listed had a higher SMAV than the acute value recorded for *Caridina nilotica.* Only two of the eleven tests performed on the most sensitive species (*Daphnia magna*) in this database produced a lower acute value than that obtained for *Caridina nilotica.* The result is comparable with that of Richardson (1997), who reported the lowest acute value for the New Zealand shrimp *Paratya curvirostris,* a relative of *Caridina,* to be 0.75 mg NH_3 L^{-1} at pH 8.1 (AV_{t8} = 21.5). *P. curvirostris*, which was the only invertebrate species tested by Richardson (1997) was found to be as sensitive as the most sensitive of the seven fish species tested. This suggests relatively high sensitivity to ammonia toxicity for the Atyidae family, especially given that invertebrates generally are regarded as more resistant to ammonia toxicity than fish (USEPA 1998; ANZECC/ARMCANZ 2000).

4.4.3.2 Barramundi

The most sensitive of the three species tested in this study was clearly the barramundi. The asymptotic exposure/response curve (figure 6), reflects the appropriateness of the chosen exposure range and the strength of the estimate is supported by the narrow confidence intervals. The estimated AV_{t8} for barramundi (18.63) is equal to the 25th percentile most sensitive North American fish species in regards to the SMAV (USEPA 1998) and indicate relatively high sensitivity to acute ammonia toxicity. The mean SMAV for salmonid species (range from 11.23 to 42.07) in the USEPA database is 21.33 (SD = 7.14) and is higher than the estimated AV_{t8} for barramundi. Salmonid species are generally thought to be among the most sensitive to ammonia toxicity of all fish (US EPA 1985). Barramundi also appear to be more sensitive to short-term ammonia exposure than native New Zealand fish species (Richardson 1997), where the most sensitive of the seven fish species tested, *Galaxius fasciatus*, had a final LC_{50} value of 0.75 mg $NH_3 - N\ L^{-1}$ at pH 8.1 (AV_{t8} = 21.5). Pearson et al. (2003) reported 70% mortality in juvenile barramundi (42-48 mm) in the 0.56 – 0.67 mg $NH_3 - N\ L^{-1}$ range ($AV_{t\ 8} < 10.13$ mg TAN L^{-1}). The pH and temperature range was comparable to that of this study, but the cycling of dissolved oxygen in their study precludes direct comparisons. Nevertheless, since the barramundi tested in these two studies were similar in size and from the same commercial hatchery (Bluewater Barramundi, north Queensland), this may indicate that lowered dissolved oxygen, even though well above the lethal threshold for barramundi (see section 2.1.4) may more than halve the ammonia concentration needed to induce the same lethal response as without cycling of dissolved oxygen.

4.4.3.3 Rainbowfish

The uncertainty of the acute value estimates for rainbowfish was clear in the exposure/response curve and the relatively wide confidence intervals. The unreplicated range-finding test, which the treatment range in this study was based upon, was not successful as there was considerable mortality in the controls as well as a homogeneous response in the increasing exposure range. It is possible that the rainbowfish were inadequately acclimated before the range finding test or that the fish were unfit or infected with disease, although there were no external signs to confirm this. The exposure range in the acute toxicity experiment

was therefore based on previous investigations (Pearson et al. 2003) but, clearly, higher exposure concentrations were required to induce a greater response. Unfortunately due to the time limitations in this study another range finding test could not be initiated. The outcome of the acute toxicity experiment on rainbowfish indicates greater resistance to ammonia toxicity than barramundi or the shrimp. The low response in all but the highest treatments may indicate that rainbowfish can survive concentrations up to ca. 1.5 mg NH_3 - N L^{-1} for at least four days. The acute values (AV_{t8} = 30.51) for rainbowfish suggests medium to high resistance to acute ammonia toxicity when compared to the mean SMAV's for the 29 species of fish (range: AV_{t8} 11.23 to 51.73, SD = 11.51) in the US EPA Updated Ammonia Criteria (US EPA 1998). Given the moderately positive skewness ($SMAV_{mean}$ = 28.64, $SMAV_{median}$ = 26.10) in the US EPA (1998) data, the result could indicate relatively high resistance to ammonia toxicity for the rainbowfish (equals the 60th percentile North American species). Moreover, the LC_{50} for rainbowfish in this study is comparable to that recorded from fish with medium sensitivity to acute ammonia toxicity in New Zealand (Richardson 1997). When the lethal values for rainbowfish in this study are compared to the results from Pearson et al. (2003), it appears, as with the results for barramundi, that the sensitivity to ammonia toxicity in juvenile rainbowfish increase with low dissolved oxygen levels. The 60% mortality in juvenile rainbowfish (34-40 mm) in the 0.97-1.08 mg NH_3 - N L^{-1} concentration range reported by Pearson et al. (2003) suggest the 48-hour LC_{50} to be less than 1 mg NH_3 - N L^{-1}, which is more than two-fold lower than obtained in this study, thus suggesting the magnitude of the influence of low dissolved oxygen levels.

4.4.4 Histopathology of gills

Gills of fish subjected to ammonia toxicity showed great variation of frequency and nature of abnormal pathology. No clear trends, such as higher frequency of lesions with increasing exposure were found, and there were no significant statistical differences in degree of epithelial lifting between fish that died, presumably from exposure, and control fish. The fact that epithelial lifting was encountered at all levels of treatment, suggests that this apparent abnormality may be an artefact of the subsequent processing. Mallat (1985) stated that some authors have claimed that the widely reported gill alterations are primarily artefacts. Abnormal pathology may have resulted from the use of cold euthanasia, whether through temperature stress or change in osmotic balance, as the filtered test water would have had

lower salinity than the water used for euthanasia (L. Owens, pers. comm.). Nevertheless, epithelial lifting also was encountered in fish that died in the experiment and were not subjected to euthanasia.

Samples of gills with normal morphology in fish that died (from the highest treatment) suggests that short term exposure to potentially lethal concentrations of ammonia do not lead to considerable gill damage in these two species. It seems that damage to gills from acute exposure to ammonia has been little studied and the results are equivocal. Herbert and Shurben (1965) and Smart (1976) reported gill lesions in the rainbow trout (*Salmo gairdneri*) resulting from acute ammonia exposure. Waisbrot et al. (1990), on the other hand, found no evidence of pathological changes in the examined organs (gills, liver and kidneys) of juvenile gilthead seabream (*Sparus aurata*) that died from exposure to 1.27 mg NH_3 - N L^{-1} in a 96 hours acute toxicity test. It appears that ammonia, unlike a number of other toxicants (Mallat 1985), generally takes time to be damaging to the gills; so chronic levels are more likely to induce abnormal pathology (e.g., Smith and Piper 1975; Thurston et al. 1984; Frances et al. 2000). This theory agrees with Heath (1995) who stated, «Acute exposure to some substances such as ammonia causes relatively little gill damage, but death still takes place. So gill histopathology may or may not indicate "cause of death", depending on the extent of lesions encountered». It is unlikely that the gill damage reported in this study would be the cause of death, as some of the fish that died in the highest treatments had little or no abnormal pathology. Similarly, Daoust (1984) found that the gill lesions in rainbow trout exposed to acute lethal levels of copper and mercury did not appear to be a cause of death. In the present study, no examination of the chemistry at the intracellular level was conducted, so an exact cause of death cannot be established. Nevertheless, given the evidence from the histopathological examinations, as well as the general view that gill damage is usually modest and even absent in gills from fish exposed to short- term (≤ 96 hours) acute ammonia toxicity (Waisbrot et al. 1990; Heath 1995), it is likely that the cause of death in the test animals was at the intracellular level.

5. POST-EXPOSURE EFFECTS

5.1 Introduction

Post-exposure effects from acute ammonia toxicity were investigated in a follow-up study to the previous experiments, using surviving fish from the acute toxicity experiments on barramundi and rainbowfish. Firstly, this study aimed to determine if short-term exposure to ammonia had any effects on subsequent growth for selected species. I predicted that sublethal effects might appear at much lower concentrations than those inducing lethal effects (e.g., Frances et al. 2000; Hickey et al. 1999; Pearson et al. 2003; US EPA 1998; ANZECC/ARMCANZ 2000). The effect on growth from short-term ammonia exposure has not been reported before; however, observations of depressed feeding in fish after range-finding tests suggest that short-term exposure may have a negative effect on growth. Secondly, given that acute toxicity of ammonia might lead to abnormal pathology of gills, I aimed to investigate recovery from gill damage during the three-week follow-up period. The recovery potential of many of the pathological changes reported from ammonia exposure in fish is great (Smith and Piper 1975; Thurston et al. 1984 b). The time frame for recovery to take place, however, is uncertain (USEPA 1998).

5.2 Materials and methods

5.2.1 Experimental set-up

The experimental set-up was as for the previous experiments (Chapter 4), except that water in all the tanks was replaced with fresh and uncontaminated water at ambient pH levels (ca. pH 7.5). The photoperiod was set to 14 hours' light/ 10 hours' dark, similar to the length of daylight during the wet season (November – February). The experiment, using barramundi (survivors from the three lowest treatments and controls) and eastern rainbowfish (survivors from the four lowest treatments and controls), lasted for three weeks. Changes in behaviour and growth were recorded with death potentially as the final endpoint.

Fish were fed to satiation each afternoon and remaining food was siphoned from tanks approximately two hours after feeding and collected in 75-ml specimen jars. Before feeding, all faeces were siphoned out of the tanks in order to avoid any bias in later calculations of food conversion rate (FCR). This resulted in a daily water change of between 20-30% in all tanks, thus resulting in good water quality. Commercial test kits were used regularly to ensure that there was no build up of nitrogenous wastes. Temperature, pH and dissolved oxygen were monitored regularly using a WTW pH/Oxi 340i meter. Dead animals were removed and preserved on phosphate-buffered 4% formaldehyde or FAACC (formaldehyde acetic acid calcium chloride). Histological examination of gills was performed as described previously (section 3.3).

Fish were examined for short-term growth changes. All fish were weighed prior to the commencement of the acute toxicity experiment and after the conclusion of this experiment. Initial weight data were found to be significantly homogeneous for barramundi ($p < 0.05$). Even though the individuals were assigned to replicates so that the variation among treatments and replicates was minimised, the initial weight data for rainbow fish were not significantly homogenous ($p > 0.05$), as reflected by the greater standard errors. The fish were lightly anaesthetised using 20 mgL^{-1} benzocaine and had excess surface water removed before being weighed.

5.2.2 Growth analyses

Total feed was recorded for each treatment concentration and for the controls. Uneaten remains were oven dried at 90°C for 24 hours and weighed. Food Conversion Ratio (FCR) was calculated by subtracting uneaten food from total amount of food and divided by total wet-weight gain in each of the replicates. Specific Growth Rate (SGR) (average percentage wet-weight gain per day) was calculated, using the following formula (Frances et al. 2000):

$$SGR = \{\ln(\text{initial weight}) - \ln(\text{final weight})/\ \text{number of days}\} \times 100$$

Weights of dead or sacrificed fish in the acute experiment were subtracted from initial wet-weight means. These adjusted means were applied in all of the growth calculations. When fish were sacrificed, two per replicate, fish that were visually different from the average were

removed. This procedure evened out some the initial variation, particularly in the rainbowfish weights. All the growth data reflects means per replicate.

5.2.3 Statistical analysis of growth results

All data were log-transformed to homogenise variance and normalise the distribution. Data that met the assumptions of normality and homogeneity of variances were analysed for significant effects with a one-way ANOVA. Where results indicated significant main effects ($p < 0.05$), pair-wise multiple comparison (post-hoc Tukey) tests were carried out to examine which treatments differed significantly. When homoscedascity in data was not confirmed (Levene's statistic), a non-parametric Kruskall-Wallis test was used. This test only indicates that two or more treatments are significantly different, not which ones. In addition, regression analysis was performed to investigate the linear relationship between growth and exposure to ammonia; significance was attributed at $P \leq 0.05$. SPSS 11.0.1 (LEAD technologies inc. 2001) was used in all statistical analyses of growth data.

5.2.4 Histopathology of gills

Gill samples from sacrificed fish that survived in the acute toxicity test were compared to gill samples from fish at the end of the post-exposure experiment to investigate the potential for recovery from gill damage. Epithelial lifting, which was the most observed sign of abnormal pathology in the acute tests, was measured as previously. A ranked Mann-Whitney U-test was used to determine any statistically significant difference in the degree of epithelial lifting in secondary lamellae in fish at the end of the post-exposure experiment compared to those at the end of the acute tests. Data from treatments with low mortality where epithelial lifting was frequent in the acute tests were pooled and compared to pooled data from the respective treatments at the conclusion of the post-exposure experiment. In all ten samples from treatments and ten samples from controls were subjected to statistical testing (following methods of section 4.2.5).

5.3 Results – Post-exposure effects

5.3.1 Water quality

Temperatures were stable in all tanks (28.5 ± 0.5º C). Tanks were continuously aerated and dissolved oxygen was kept at or close to saturation. pH was maintained at 8.10 ± 0.1. Regular monitoring indicated no harmful levels of nitrate, nitrite or ammonia (< 50 μg L^{-1}).

5.3.2 Feeding behaviour

Spitting and regurgitating of food and overall lack of interest in feeding was frequently observed in higher treatments. It appeared that this abnormal feeding behaviour in general subsided after a few days, with fish from the lower treatments returning to normal feeding behaviour first, followed by fish from the medium exposure treatments and then finally fish exposed to the highest concentrations. The latter were returning to normal feeding behaviour after approximately 3-4 days into the post-exposure experiment. This varied somewhat between individuals as well as replicates; nevertheless, there was a clear tendency for the time to return to normal feeding behaviour to be directly related to the previous exposure concentration. Two of the surviving barramundi from one of the 1.16 mg NH_3 - N L^{-1} treatment tanks and one from the 1.54 mg NH_3 - N L^{-1} tanks died within a week of the post-exposure trial. These fish were not feeding and were infected by white fungal disease. No mortality occurred in the post-exposure experiment for rainbow fish.

5.3.3 Changes in growth

Growth effects were calculated using replicate means. For barramundi only the three lower ammonia treatments were applied in this analysis, because too few fish survived in the higher treatments. For rainbow fish there were sufficient survivors for all treatment levels except the highest (1.88 mg NH_3 - N L^{-1}) to be applied in the subsequent calculations. Food conversion ratio (FCR) for two of the replicates in the highest treatment (1.16 mg NH_3 -N L^{-1}) for barramundi tested for changes in growth could not be calculated due to the unfeasibility of quantifying the amount of food consumed by the fish that died.

5.3.3.1 Barramundi

All fish survived in the controls and two lower treatments (0.44 and 0.79 mg NH_3-N L^{-1}). For the higher treatment (1.16 mg NH_3-N L^{-1}) the numbers of fish in the three tanks were 6, 5 and 3 at the beginning of this experiment. At the conclusion of the experiment the numbers of fish were 6, 3 and 2 respectively, as fish died in two of the three tanks. Previous exposure to ammonia had no significant effect on wet weight gain (ANOVA: $F_{(4,14)} = 0.07$, $P > 0.05$). The highest overall wet weight gain was recorded for one of the replicates in the highest treatment (1.16 mg NH_3-N L^{-1}) where no fish died during the three-week post exposure experiment (table 6). Growth data as specific growth rate (SGR) were significantly heterogeneous ($P < 0.05$) and therefore did not meet the assumption of the ANOVA, and the linear regression indicated no relationship ($P > 0.05$) between previous exposure range and growth as percentage weight gain per day (figure 12, 13). The nonparametric test indicated no significant difference (Kruskall-Wallis: $\chi^2_2 = 1.99$, $P > 0.05$). No significant differences over the treatment range were found in either food conversion ratio (figure 14) (ANOVA: $F_{(3,11)} = 0.22$, $P > 0.05$) or food consumption (figure 15) (ANOVA: $F_{(3,11)} = 0.94$, $P > 0.05$).

Table 7. Final weight, wet weight gain, specific growth rate (SGR), food conversion ratio (FCR) and food consumption of barramundi (*Lates calcarifer*) 24 days after a 4-day exposure to increasing concentrations of NH_3 - N.

Treatment:	C1	C2	0.41	0.76	1.16
I.W	1.20 ± 0.02	1.30 ± 0.03	1.27 ± 0.02	1.24 ± 0.02	1.29 ± 0.02
F.W	2.56 ± 0.09	2.62± 0.08	2.60 ± 0.08	2.55 ± 0.07	2.62 ± 0.24
Growth	1.36 ± 0.08	1.32 ± 0.05	1.33 ± 0.04	1.32 ± 0.03	1.33 ± 0.23
SGR (% day^{-1})	3.08 ± 0.5	2.90 ± 0.2	2.97 ± 0.07	2.96 ± 0.02	3.04 ± 0.39
FCR	0.85 ± 0.007	0.91 ± 0.01	0.86 ± 0.02	0.87 ± 0.03	N/A
Total food fed	1.18 ± 0.05	1.15 ± 0.009	1.14 ± 0.006	1.13 ± 0.009	N/A

I.W = Initial wet weight (g), weight before acute experiment. F.W = Final wet weight (g), Growth = Wet weight gain (g $fish^{-1}$), SGR = specific growth rate, FCR = food conversion ratio, Total food fed = Total food consumption (g[dry weight] $fish^{-1}$. C1 = control NH_3, C2 = control pH. Values are expressed as means ± s.e.

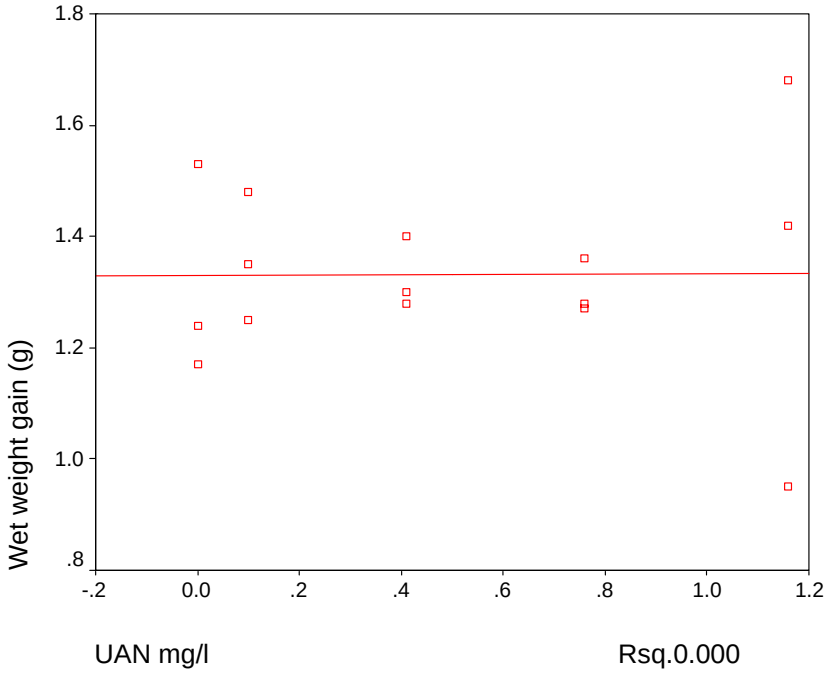

Figure 13. Increase in wet weight gain for juvenile barramundi three weeks after 96 hours' exposure to increasing concentrations of NH_3 - N. Data points are replicate means.

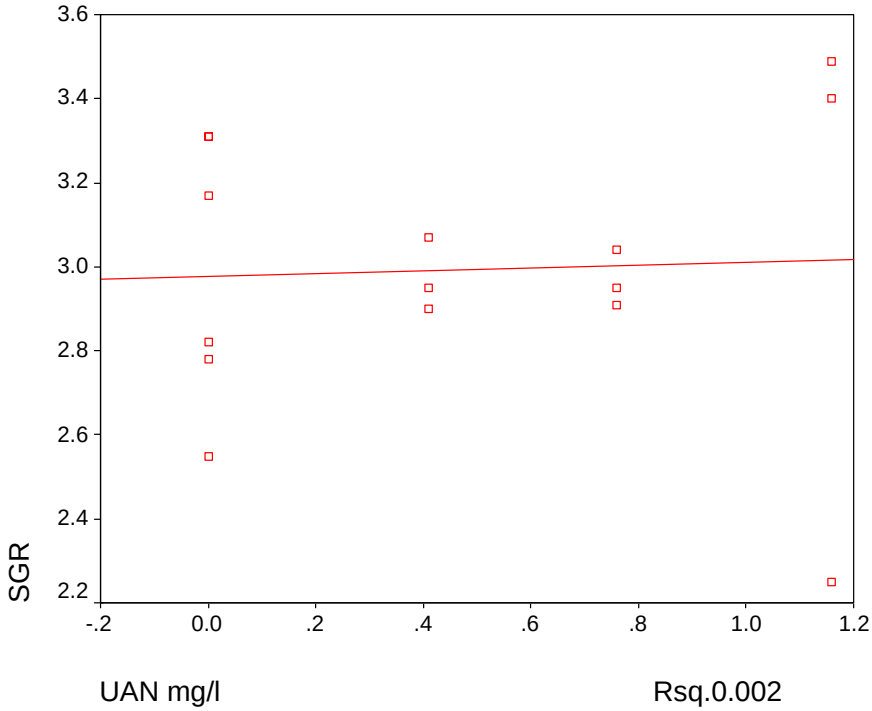

Figure 14. Specific growth rate for juvenile barramundi during the three-week post exposure experiment. Data points are replicate means.

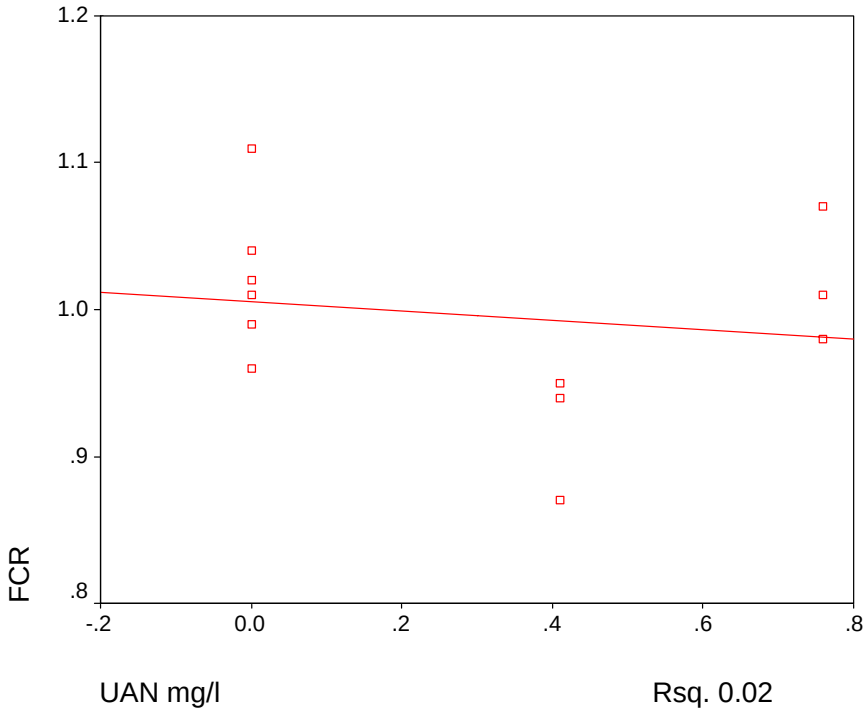

Figure 15. Food conversion rates (FCR) for juvenile barramundi three weeks after exposure to increasing concentrations of ammonia. Data points are replicate means.

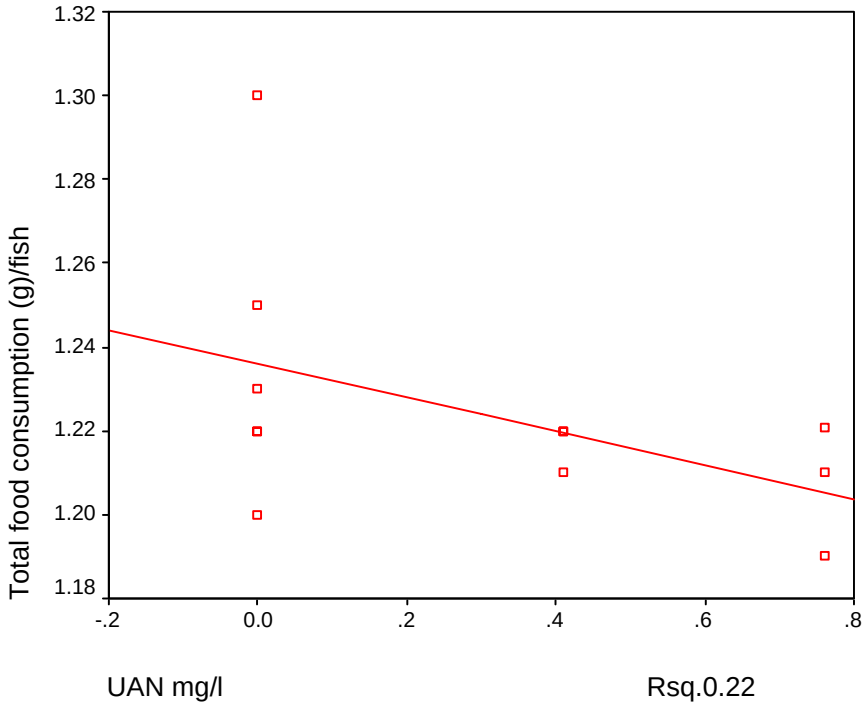

Figure 16. Total food consumption for juvenile barramundi during the three-week post exposure experiment. Data points are replicate means.

5.3.3.2 Rainbowfish

All fish survived all treatments. There was no significant effect from exposure to increasing concentrations of ammonia on average wet weight gain (ANOVA: $F_{(5,17)} = 0.23$, $P > 0.05$). Because of the great within-treatment variation in the specific growth rate (SGR) and food conversion ratio (FCR) data (table 7), the assumptions of homogeneity of variance in the ANOVA were not met. The non-parametric test, nevertheless, indicated no significant difference in specific growth rate (Kruskall-Wallis: $\chi^2_2 = 7.92$, $P > 0.05$) or food conversion ratio (Kruskall-Wallis: $\chi^2_2 = 4.47$, $P > 0.05$). As with the results for food conversion ratio in barramundi, the greatest variation occurred in the highest treatment (1.48 mg L^{-1}). There may have been a slight increase in food consumption in the higher treatments (table 7). This, however, was not statistically significant (Kruskall-Wallis: $\chi^2_2 = 1.22$, $P > 0.05$). The linear regression indicates that there is a weak trend, although non-significant ($P > 0.05$), between food consumption and increasing exposure (figure 19).

Table 8. Final weight, wet weight gain, specific growth rate (SGR), food conversion ratio (FCR) and food consumption of rainbowfish (*Melanotaenia splendida splendida*) 24 days after a 4-day exposure to increasing concentrations of NH_3 - N.

Treatment:	C1	C2	0.44	0.69	1.05	1.48
I.W	0.54 ± 0.03	0.62 ± 0.05	0.59 ± 0.06	0.56 ± 0.03	0.54 ± 0.05	0.55 ± 0.06
F.W	0.67 ± 0.05	0.77± 0.06	0.76 ± 0.06	0.73 ± 0.05	0.68 ± 0.04	0.73 ± 0.05
Growth	0.15 ± 0.03	0.15 ± 0.01	0.16 ± 0.02	0.17 ± 0.04	0.14 ± 0.01	0.18 ± 0.05
SGR (% day^{-1})	1.01 ± 0.15	0.93 ± 0.08	1.11 ± 0.2	1.16 ± 0.3	1.04 ± 0.17	1.27 ± 0.35
FCR[1,3]	4.83 ± 0.5	4.57 ± 0.5	3.82 ± 0.6	4.43 ± 1.2	4.80 ± 0.3	4.90 ± 1.8
Total food fed	0.69 ± 0.04	0.64 ± 0.003	0.64 ± 0.01	0.69 ± 0.05	0.70 ± 0.05	0.74 ± 0.04

I.W = Initial wet weight (g), weight before NH_3 exposure. F.W = Final wet weight (g), Growth = Wet weight gain (g $fish^{-1}$), SGR = specific growth rate, FCR = food conversion ratio, Total food fed = Total food consumption (g[dry weight] $fish^{-1}$. C1 = control NH_3, C2 = control pH. Values are expressed as means ± s.e.

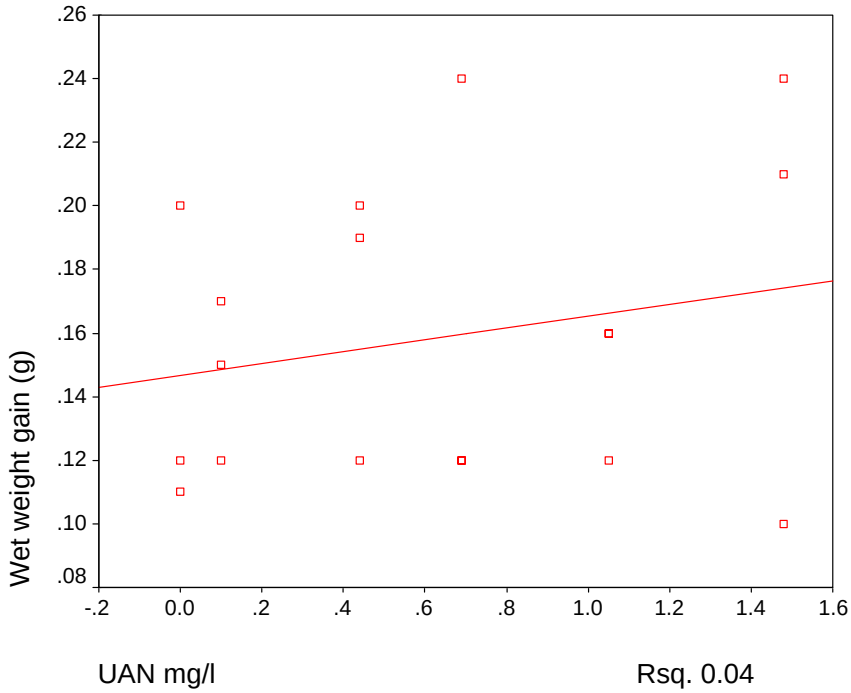

Figure 17. Increase in wet weight gain for juvenile rainbow fish three weeks after the 96 hours' exposure to increasing concentrations of ammonia. Data points are replicate means.

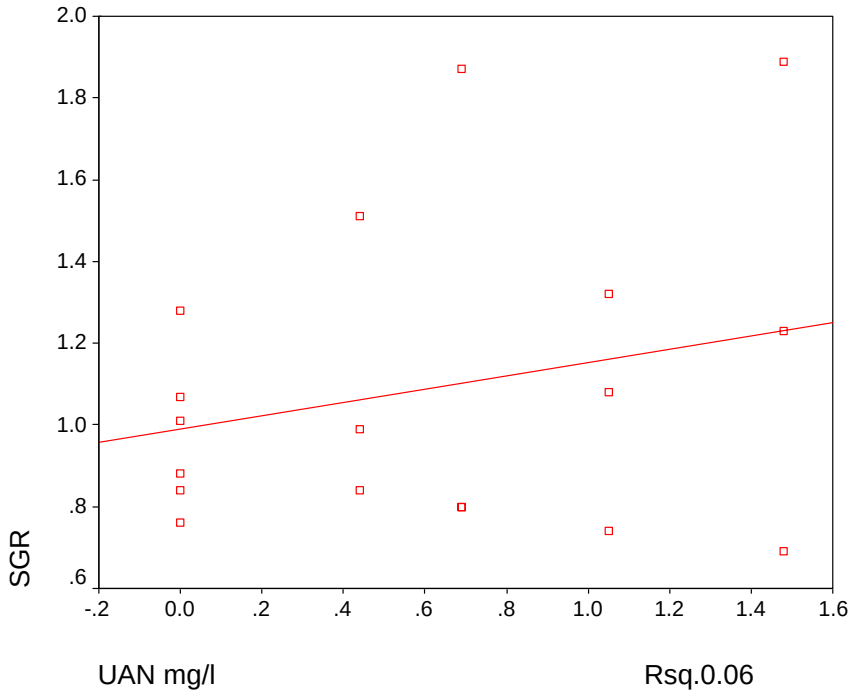

Figure 18. Specific growth rate for juvenile rainbow fish during the three-week post-exposure experiment. Data points are replicate means.

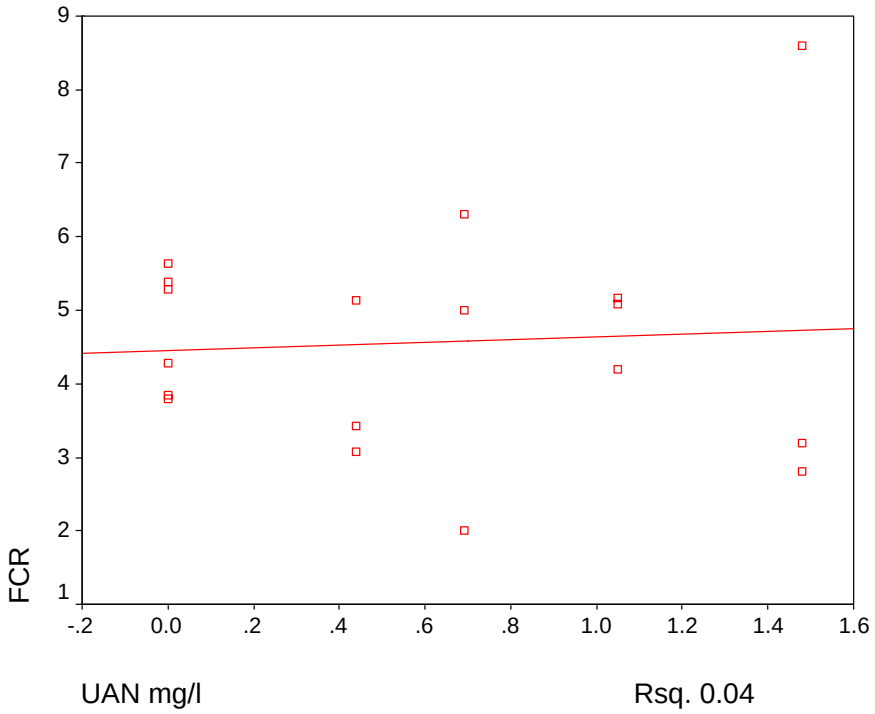

Figure 19. Food conversion rates (FCR) for juvenile rainbowfish three weeks after exposure to increasing concentrations of ammonia. Data points are replicate means.

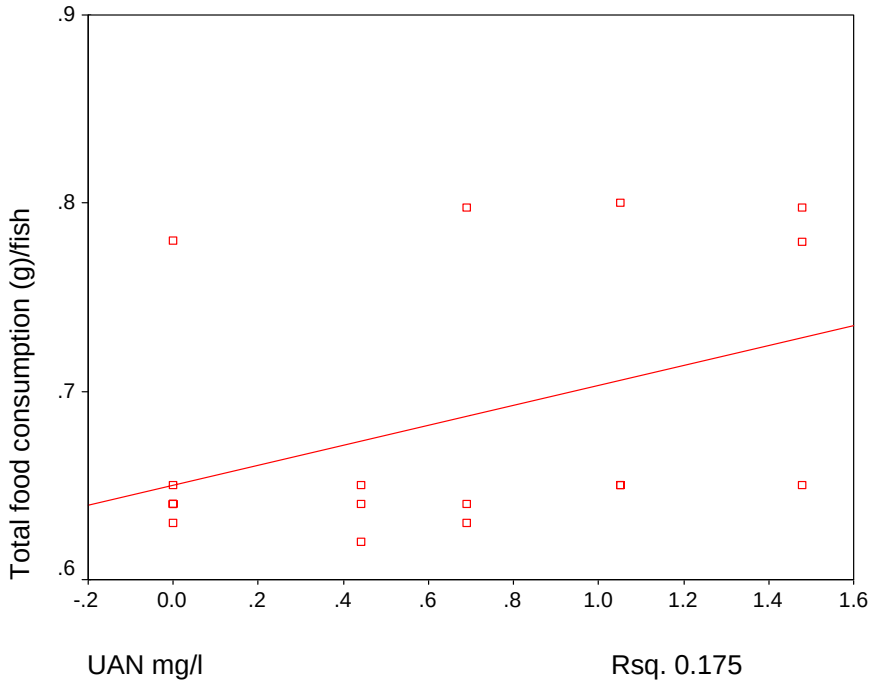

Figure 20. Total food consumption for juvenile rainbow fish during the three-week post-exposure experiment. Data points are replicate means.

5.3.4 Recovery from gill damage

Many of the same lesions found in the analysis of gills after exposure to acute toxicity were found in the samples from the two fish species over the range of treatments after the three-week post-exposure experiment. As with the results from the acute test, epithelial lifting of secondary lamellae was found to be the most frequent lesion in both barramundi and rainbow fish. As in the samples from the acute test, degree of epithelial lifting of secondary lamellae varied from approximately 10 to 100%. There was no statistical significant difference (Mann-Whitney: $U_{(1)\ 10,10} = 73$, $P > 0.05$) in degree of epithelial lifting of secondary lamellae in either fish species for the respective treatments at the end of the post-exposure experiment compared to those at the end of the acute tests. Results from scoring of epithelial lifting in appendix 3.

5.4 Discussion of post exposure results

5.4.1 Overview

Exposure to chronic ammonia has been found to have negative effects on growth in fishes (Smith and Piper 1975; Mallet and Sims 1994; Frances et al. 2000). There is no published information on the effects on growth in fish during or following acute ammonia exposure. This study indicates that there are no significant effects on wet weight gain, specific growth rate, food conversion ratio or food consumption for either barramundi or eastern rainbow fish following acute ammonia exposure.

Since individual growth rate varies for both fish species (Primary Industries and Resources SA, 1999; Humphrey et al. 2003), it is possible that dead and sacrificed fish would have influenced the calculated growth parameters, by having either atypically high or low growth rate. The removal of over-average-size fish may have affected the growth positively in the remaining fish since some fish would have grown faster than they would in the presence of bigger fish, a phenomenon observed by Trneny (2002), when studying growth in housed nightfish (*Bostockia porosa*). Ali et al. (2003) cautioned against changing numerical abundance and density of fish groups in studies of compensatory growth. Whether this advice is based on the theory that higher density may affect growth negatively, or that changes in

numerical abundance during an experiment would confound the results with the unfeasibility of calculating food consumption and food conversion ratios is unclear. Loading rate just below one gram of fish per litre test water has been reported to have a negative effect on growth in carp (*Cyprinus carpio*) during exposure to chronic ammonia (Mallet and Sims 1994). However, the optimal loading rate of 25 g fish L^{-1} commonly applied in barramundi farming in Australia (Primary Industries and Resources SA, 1999), suggests that the maximum loading rate of approximately 0.7 g fish per litre in this study most likely had no negative effects on growth in barramundi. In addition the OECD (2000) recommends a loading rate for rainbow trout (*O.mykiss*) of between 1.2 and 2 grams wet weight for fish per litre. For rainbow fish, two of the initial seven fish were removed in the lower treatments. Because of the greater variance in initial weights for rainbow fish, even though the outliers were removed, the possible influence of this adjustment could be somewhat greater than for the barramundi.

5.4.2 Barramundi

Growth and food conversion efficiency of barramundi was high at all treatment levels, reflected by both high specific growth rate and low food conversion ratios. The growth reported in the current study appears to be within the natural variability that can be expected in barramundi. In a study on the effects of dietary fish-oil replacement on growth and carcass composition of juvenile barramundi, Raso and Anderson (2003) reported food conversion ratios as low as 0.6 and specific growth rates up to 3.7, while Tian and Quin (2003) reported specific growth rates to vary from about 1.7 to about 3.3 and food conversion from approximately 75 to 100% in a study on compensatory growth in juvenile barramundi. In the study by Tian and Qin (2003), where juvenile barramundi were subjected to a single phase of food deprivation lasting from one to three weeks, starved fish had higher specific growth rates when feeding commenced compared to fish that had been continuously fed. This led to full growth compensation in fish that were deprived of food for one week. In the current study, all the barramundi had been subjected to a six-day period of complete food deprivation when food was offered. Suppressed feeding in the higher treatments, even though this did not have any significant effects on growth, suggests that hyperphagia lasted for a longer period when feeding resumed. This agrees with Ali et al. (2003), who reported that the length of food depression increased the length of the hyperphagic phase. It is therefore likely that increasing

ammonia concentrations indirectly led to a longer period of hyperphagia and that this resulted in a conversion of growth trajectories and thus a reduction of variance in growth over time. The specific growth rates are also close to those reported by Tian (2003) for barramundi deprived of food for one week, which further supports the suggestion of compensatory growth in barramundi surviving exposure to acute ammonia toxicity.

A number of studies conclude that increased food utilisation efficiency plays an important role as well when full growth compensation occurs after a period of food deprivation (Koppe et al. 1992; Zhu et al. 2001; Guerney et al. 2003). The overall low food conversion ratios obtained in this study suggest that little energy was spent on metabolic maintenance, which indicates relatively fast recovery following short-term exposure of ammonia up to 0.76 mgL^{-1} NH_3-N. Both the lowest and the highest wet weight gain were recorded in the highest treatment (1.16 mg NH_3-N L^{-1}). This result, however, is unclear because of the deaths that occurred, but may be an indication of an exposure threshold where variance in fitness level greatly influences subsequent recovery and growth.

The results from this study show that feeding is suppressed following exposure to concentrations above 0.41 mg NH_3-N L^{-1}, with, as pointed out above, increasing time for feeding to resume to be positively correlated with degree of exposure. Pearson et al. (2003) reported that feeding was depressed for several days in juvenile barramundi (42-48 mm) following exposure to 0.08-0.19 mg NH_3-N L^{-1}. While that result appears inconsistent with the observations made in this study, where suppression of feeding occurred at concentrations approximately three fold lower, the toxicity experiments by Pearson et al. (2003) involved oxygen cycling and lasted for half the time of the acute experiments in the current study (see also section 6.3). Interestingly, the extent of difference between the concentrations where mortality occurred and the concentrations where feeding was suppressed is essentially the same when results from the two studies are compared. This indicates that the synergistic effects of low dissolved oxygen and ammonia exposure, not only have an influence on survival, but also on the subsequent feeding behaviour in surviving individuals as well.

5.4.3 Rainbow fish

Growth in rainbow fish was slow and food conversion efficiency low as reflected by the low specific growth rates as well as the high food conversion ratios. This result is inconsistent with that of Humphrey et al. (2003), where eastern rainbowfish grew from approximately 32 mm to approximately 35 mm in standard length in seven days, which is equal to the entire gain in standard length for fish in this study (including controls). This indicates that growth measured as length increase was less than 1/3 of that reported by Humphrey et al. (2003). It is therefore likely that growth of rainbowfish in this study was considerably below maximum for the species. An explanation for this could be that the feeding regime offered below-optimal food intake and that feeding should have been more frequent. In addition, a more varied diet may affect the growth efficiency positively (Wootton 1990). Humphrey et al. (2003) applied a highly varied diet consisting of live *Artemia* nauplii, ground up adult *Artemia* and ground commercial flake food in larvae older than 14 days (and up to 87 days). Nevertheless, the food offered in the current study should have been of adequate nutritional quality (crude protein content of 44%) for optimal growth, which agrees with the statement from Humphrey et al. (2003) that rainbowfish, probably referring to older stages, will develop on standard commercial food. It is therefore possible that the quantity rather than quality of the food would have caused the low growth rates. Direct comparisons between these two studies may, however, be somewhat ambiguous, while Humphrey et al. (2003) studied laboratory-bred fish in contrast to the wild caught fish in the present study.

Ali et al. (2003) described different levels of growth compensation, ranging from no compensation to overcompensation, where the degree of compensatory growth response seems closely related to length and severity of food deprivation. For example, in European minnows (*Phoxinus phoxinus*) four days of food deprivation was not enough to induce compensatory growth (Russell and Wotton 1992), while after seven days of food deprivation, minnows displayed compensatory growth sufficient to catch up with growth in control fish (Zhu et al. 2001). It is possible that a stronger response occurred in fish from higher treatments, where previous exposure to ammonia indirectly depressed feeding for a longer period, so fish caught up with growth in controls, but that the group as a whole (including controls) did not compensate fully, meaning that they did not achieve full growth compensation and thus had below optimal growth. Furthermore, it is possible that had food been offered more frequently there would have been higher growth, and thus full

compensation would have occurred. This, however, is speculative due to the lack of feeding controls. Nevertheless, the results indicate that exposure to increasing ammonia concentrations up to 1.48 mg NH_3-N L^{-1} did not significantly affect subsequent growth in surviving fish. As for the barramundi, the greatest variation in weight gain occurred in the highest treatment, consistent with the suggestion of an exposure threshold where subsequent growth varies more, reflecting individual variation in fitness level (section 5.4.2).

5.4.4 Histopathology of gills

The lack of significant difference in epithelial lifting in the gills of both species following the post-exposure experiment when compared to samples from the acute tests, suggests that no recovery occurred. These results are inconsistent with the high recovery potential observed in studies of chronic ammonia toxicity, where many of the same lesions were reported (Smith and Piper 1975; Thurston et al. 1984 b). This supports the suggestion of the damage resulting from tissue processing (Mallat 1985). However, the fact that some of the fish that died in the acute tests had abnormal pathology, while others had normal pathology, suggests that gill damage occurs in some fish in higher concentrations and not in others. This indicates that natural variations in fitness may induce lesions in weakened animals. Nevertheless, since no firm conclusions could be made on the effects of acute ammonia toxicity on histopathology of the two fish species tested, a serious assessment of the recovery potential from gill damage becomes unfeasible. Given the evidence in this study as well as the findings by others (e.g., Smith and Piper 1975; Thurston et al. 1984 b) it is probable that gill damage is not a realistic indicator of acute ammonia toxicity, and that gill damage observed in this study is predominantly an artefact of subsequent processing.

6. GENERAL DISCUSSION

6.1 Overview

This study investigated the lethal and sublethal effects of ammonia toxicity on selected freshwater biota in north Queensland. The results indicate considerable variation in sensitivity to acute ammonia toxicity for the three species investigated. Barramundi was found to be the most sensitive, followed by freshwater shrimp and eastern rainbowfish. The derived acute toxicity values for barramundi were comparable to published lethal values for highly sensitive species such as salmonoids. Therefore, the results support the conclusion that typical warm-water species, previously thought to be more resistant, can be as sensitive as fish inhabiting cooler, less turbid waters (USEPA 1985). The lethal values for the freshwater shrimp indicated relatively high sensitivity, in agreement with results for New Zealand freshwater shrimp (Richardson 1997), in contrast with a commonly held view that invertebrates are more resistant to ammonia toxicity than fishes. The lethal values for rainbowfish indicate medium sensitivity to acute ammonia toxicity when compared to other studies (Richardson 1997; US EPA 1998). Suppressed feeding in barramundi that had been subjected to intermittent sublethal levels of ammonia was consistent with the findings of Pearson et al. (2003). However, the similar result for rainbowfish in this study was a new finding. Nevertheless, growth was not suppressed in either of the fish species during a three-week post exposure period in uncontaminated water, possibly as a result of a compensatory growth reaction, mainly caused by hyperphagia (Ali et al. 2003). The result is consistent with Tian et al. (2003) who reported that juvenile barramundi showed full growth compensation following one week of food deprivation. This study found no unequivocal evidence for pathological damage to gills being an indicator of acute ammonia toxicity in rainbowfish and barramundi.

6.2 Applicability of results

Whether the results from this study reflect true acute values for the test species, depends on a several considerations. For example, the level of exposure inducing a response, be it a sublethal or a lethal endpoint, may differ significantly between a laboratory-based experiment and a field experiment because of the many factors that might affect toxicity in the natural

environment (Hickey 2000; Meister and Van den Brink 2000). Furthermore, organisms may display avoidance behaviour to harmful conditions and thus reduce a species' contact with harmful conditions such as high ammonia concentrations and hypoxia when gradients exist (Richardson et al. 2001; Pearson et al. 2003). There is, however, great inconsistency in how different species react to these stressors, as some clearly display avoidance to harmful conditions while others seem to be attracted to them (Richardson et al. 2001; Pearson et al. 2003).

During the experiments, the physico-chemical test parameters were kept stable and the toxicant concentrations were held within accepted levels of variation. However, the responses varied between the species, reflected by both the shape of the exposure/response curves and confidence intervals of the estimated acute values. The estimated acute values for barramundi and *C. nilotica* were more robust than that of the rainbowfish. Nevertheless, the results from laboratory tests on the same species may vary considerably due to differences in test populations, test conditions and experimental design (Meister and Van den Brink 2000). For example, the acute value for rainbow trout (*O. mykiss*), the most tested fish in the USEPA (1998) database, vary more than 600% from the lowest to the highest estimate. The acute values for the tested species in this study also diverged somewhat from the results of the preliminary 48-hour range-finding tests. For example, the 48-hour LC_{50} for barramundi (table 4) was approximately 20% lower than that obtained in the range-finding test. Such differences were most likely a natural variation in test populations rather than differences in test conditions as identical methods were used in both tests. Nevertheless, the results from the current study should be interpreted with caution, as repeated testing is needed to firmly conclude the sensitivity to ammonia toxicity in the tested species. However, the fact that the CMC (acute criterion) for ammonia toxicity (USEPA 1998) is set below the SMAV (species mean acute value) for the most sensitive species, which happens to be the most tested species (*O. mykiss*), reflects the precautionary attitude applied.

It appears that the species tested in this study should be adequately protected by both the USEPA (1998) CMC as well as the ANZECC/ARMCANZ (2000) guideline trigger values for protection of 95 % of the species (figure 20). However, a direct comparison between the values obtained from this study and ANZECC/ARMCANZ (2000) can only be tentative, since the guideline values are derived from chronic NOEC (no observed effect concentration) data only. Higher ammonia concentrations may often be of short duration (section 2.2.3) and

therefore difficult to detect in a typical water quality monitoring programme. Thus, in a situation of acute short-term ammonia toxicity it could be perceived that the CMC (USEPA 1999) is a more realistic guideline than ANZECC/ARMCANZ (2000), since the latter targets chronic situations (time of exposure > 4 days) rather than intermittent episodes of high exposure.

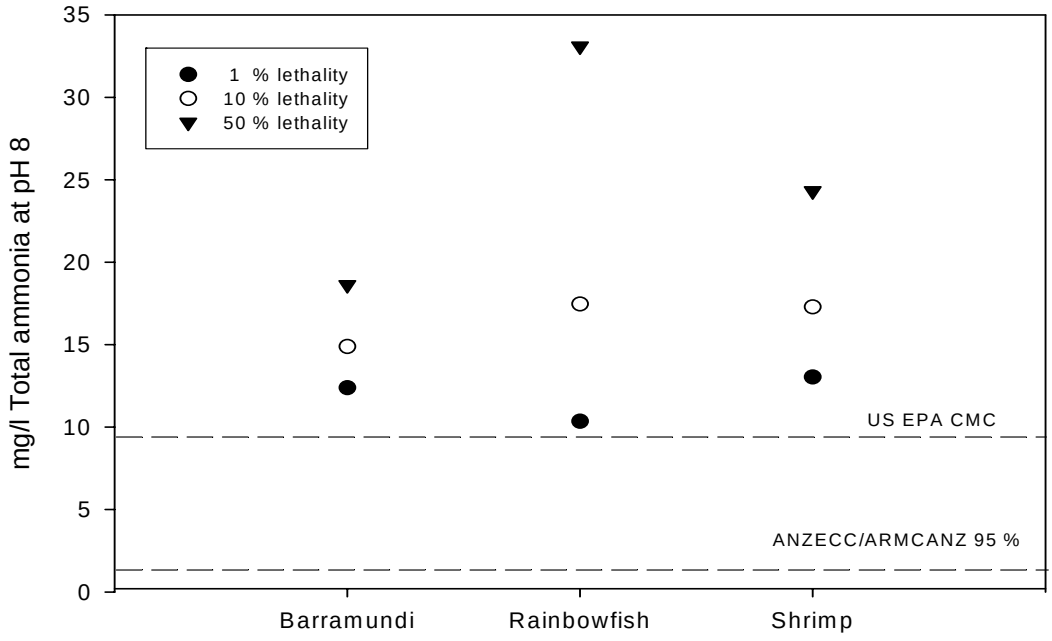

Figure 21. Acute toxicity values for tested species in relation to upper protection guideline values from the USEPA (1998) and ANZECC/ARMCANZ (2000).

However, by using the procedures in ANZECC/ARMCANZ (2000) to convert from acute to chronic toxicity (ACR = 10), as well as the assessment factor (the laboratory to field factor for essential elements = 2), the AV_{t8} for the most sensitive species in the current study is similar to the trigger value for protection of 95% of all life (ANZECC/ARMCANZ 2000). There is a caveat in applying this calculation since the procedure was used on data to calculate a moderate reliability guideline (ANZECC/ARMCANZ 2000), while all the trigger values for ammonia toxicity were derived from data satisfying the calculation of high reliability trigger values. Nonetheless, the procedures may indicate that the most sensitive of the species tested, barramundi, is protected in a chronic exposure situation when applying the guidelines. This, however, can only be validated through chronic bioassays.

6.3 Combined effects of ammonia and other stressors

The results from this study indicated that both the American and Australian/New Zealand water quality guidelines would be adequately protective of the species tested in this study. However, additional stressors, such as low dissolved oxygen and high temperatures, may increase sensitivity to ammonia, and thereby bring the lethal values obtained below protection levels. Water quality guidelines in general should not be applied without some caution, as there are certain factors that need to be considered: 1) always expect adverse effects of a pollutant to occur at lower concentration in the field than predicted by toxicity tests because of the complexity of factors affecting the transfer of a pollutant through an ecosystem (Hickey 2000); and 2) be aware that long-term water quality monitoring may reflect measures of central tendency instead of extremes, possibly leading to Type II errors.

In relation to 1) above it should be acknowledged that studies have identified some populations of aquatic animals to be able to develop resistance to metal pollution (e.g. Klerks and Weis 1987; Klerks and Levinton 1989), either through physiological adaptation or by the selection of tolerant genomes (Luoma 1996). However, adaptability to nutrient contamination appear to be less well understood, in fact from the available literature the consensus seem to be that of a lowered biodiversity being the result of enhanced levels of nutrients (e.g. Cole 1994). Whether natural populations of freshwater biota in north Queensland, subjected to increasing levels of nutrient contamination, are adapting to unfavourable conditions (compared to populations in pristine areas) is at this stage unknown. A precautionary approach is therefore still justifiable in relation to management decisions.

The coastal lowland freshwater systems (e.g., slow-flowing streams and billabongs) of north Queensland are prone to periods of stagnation (Pearson et al. 2003), with typically a rise in temperatures, increasing photosynthetic activity and bacterial mineralisation which in turn leads to increasing ammonification and decreasing levels of dissolved oxygen. In concert, these conditions will likely influence ammonia toxicity, and studies on these combined effects are warranted.

Temperature and pH affect the speciation of the total ammonia, with the NH_3 increasing with pH and temperature (Section 2.2.4). However, there are inconsistent reports on the effects of temperature on the toxicity of total ammonia. The US EPA (1998) reported some evidence of

temperature dependency on toxicity of total ammonia by some of the fish species in the temperature range tested (5-30 °C), but this dependency was weak or absent in most species. For invertebrates, the trend was more consistent with increasing acute ammonia toxicity with temperature. Even though a stronger trend of temperature dependency was present in the acute toxicity data for invertebrates this did not affect the CMC – that is, no temperature dependency to the acute ammonia criteria was added, while the most sensitive species was still adequately protected regardless of this temperature dependency. However, because of lack of data on temperature dependency in chronic toxicity to invertebrates, an assumption was made that there would also be some temperature dependence in chronic toxicity. This assumption was based on observations in the relationship between acute and chronic toxicity data for fish. This resulted in a revision of the CCC (chronic criterion), which in the updated version (USEPA 1999) includes temperature as well as pH as the factors for site-specific derivation of trigger values. ANZECC/ARMCANZ (2000) did not follow this procedure and accordingly did not adjust their trigger value from this temperature dependency relationship. Nevertheless, their trigger values display uniformity with the CCC trigger values in the updated water quality criteria for ammonia toxicity (USEPA 1999), even though according to their procedures the temperature dependency is not accounted for. In practice, however, this suggests that the Australian guidelines (ANZECC/ARMCANZ 2000) should be protective of sensitive species that display increasing sensitivity to ammonia toxicity with increasing temperature.

Increasing nutrient contamination has been found to correlate with agricultural activity in north Queensland, such as sugar cane production (Furnas and Mitchell, 2001), further increasing the potential for harmful levels of these stressors. Pearson et al. (2003) reported lowered dissolved oxygen to occur together with high ammonia levels during the falling hydrograph. Wajsbrot et al. (1991) reported a two-fold increase in ammonia toxicity (following a linear relationship) for juvenile gilthead seabream *Sparus aurata* when dissolved oxygen was reduced from about 95% to 40%. Below this level toxicity increased two-fold below the linear relationship in the above dissolved oxygen range. Thus the synergistic effect of the two stressors followed a 50/50 pattern down to an oxygen level (<40%) beyond which the continued lowering of dissolved oxygen had a greater effect on the combined toxicity of the two stressors. The results from the current study agree with the trends reported by Wasjsbrot et al. (1990) and Pearson et al. (2003). Pearson et al. (2003) reported 70% mortality in juvenile barramundi in the 0.56-0.67 mg NH_3-N L^{-1} range and 60% mortality in

juvenile to adult rainbowfish in the 0.97-1.08 mg NH_3-N L^{-1} range when cycling of dissolved oxygen was involved (12 h: 80-90% saturation/12 h: 20-30% saturation). The dissolved oxygen levels applied were well above the lethal threshold while no mortality for either of the species when subjected to comparable oxygen levels for 24 hours were reported. Whilst the acute values in the current study were close to two-fold higher for the same species, it becomes apparent that a synergistic relationship exists between ammonia and low levels of dissolved oxygen. The rainbowfish and barramundi tested by Pearson et al. (2003) were comparable in size and from the same sources as in the current study, so the results suggests that lowered dissolved oxygen, even though well above the lethal threshold for barramundi and rainbowfish, may more than halve the ammonia concentration needed to induce the same lethal response as without cycling of dissolved oxygen. In addition, when the results from this study is compared with those of Pearson et al. (2003), it appears that lowered oxygen levels in combination with ammonia also have a negative effect on feeding behaviour in exposed fish (section 5.4.2). Allan et al. (1990) reported a near three-fold increase in mortality to the leader prawn *Penaeus monodon* at approximately 1.60 mg NH_3 – N L^{-1}, when dissolved oxygen levels were reduced from 5.7 mg L^{-1} to 2.2 mg L^{-1}. The authors did not include any controls, but Seidman and Lawrence (1985) found neither survival nor growth was affected in *Penaeus monodon* at dissolved oxygen levels of 2.2 mg L^{-1} and above. This indicates that the obtained acute value for the freshwater shrimp *Caridina nilotica* also may be considerably lower during the influence of low levels of dissolved oxygen.

Salinity has been found to have an ameliorating effect on the toxicity of ammonia up to 30% of seawater (Herbert and Shurben 1965; Harader and Allen 1983), at higher salinity levels ammonia toxicity increases, however modest (as discussed in section 2.2.4). One of the species tested in this study, barramundi, is a catadromous species with its early life stages occuring in estuaries and its juveniles usually migrating upstream to freshwater reaches at a length of approximately 20-30 cm (Allen et al. 2002). This indicates that the species does not normally encounter fresh waters at the early juvenile stages tested in the current study. Thus it may be that the test conditions are somewhat unrealistic; however, the effect of salinity has been found to be minute (Ip et al. 2001), and the lethal values obtained in this study should not be expected to diverge much from acute values under the influence of higher salinity concentrations.

Although the ANZECC/ARMCANZ (2000) water quality trigger value for protection of 95 % of all life appear to be protective of the species in this study during chronic ammonia exposure, when considering that dissolved oxygen may influence toxicity of ammonia by at least one order of magnitude; the guidelines may not sufficiently protect the species in the current study during chronic ammonia exposure when dissolved oxygen levels are at a low. Nevertheless, if the dissolved oxygen default trigger value of 85% saturation in the guidelines for physical and chemical stressors (ANZECC/ARMCANZ 2000) is incorporated in a local water management programme, the guidelines should offer adequate protection for this species. However, application of the ANZECC/ARMCANZ (2000) recommended dissolved oxygen trigger value of 85% saturation is unrealistic, as many freshwater bodies in north Queensland, even when virtually undisturbed, maintain dissolved oxygen values of less than 80% saturation for prolonged periods (Pearson et al. 2003).

7. CONCLUSIONS AND FUTURE RESEARCH NEEDS

The aims of this study were to provide preliminary data on the sensitivity to ammonia toxicity on Australian tropical freshwater species. Three species, freshwater shrimp *Caridina nilotica*, barramundi *Lates calcarifer* and eastern rainbowfish Melanotaenia *splendida splendida* were selected to investigate individual sensitivities to acute ammonia toxicity through laboratory experiments. In addition the two fish species were investigated for post-exposure effects of acute ammonia exposure on survival, growth and histopathology. The results indicated substantial variation in tolerance to acute ammonia in the three species. Barramundi was the most sensitive, with acute values comparable to those reported for sensitive species such as salmonids (US EPA 1999). Growth was not suppressed in either barramundi or rainbowfish during the three-week post exposure period in uncontaminated water, it was speculated that this could be the result of a compensatory growth reaction, mainly caused by hyperphagia (Ali et al. 2003).

The Australian (ANZECC/ARMCANZ 2000) water quality guidelines appear to be protective of the species tested in this study. However, firm conclusions on whether the ANZECC/ARMCANZ (2000) guidelines offers adequate protection to the tropical freshwater biota of Australia can not yet be made, for the following reasons:

1) The species in this study make up only a small fraction of the diverse aquatic fauna in freshwater ecosystems of north Queensland and the susceptibility to ammonia toxicity in animals not assessed can only be surmised.
2) The guidelines are derived from chronic values for only five species, none of which are typical warm-water species.
3) The combined effects of ammonia and other stressors, such as low dissolved oxygen and high temperature, are highly complex and can be difficult to separate from the toxic effects caused by ammonia alone.

Apparently further research is required to gain a broader understanding of the impacts of ammonia toxicity to the diverse freshwater biota of north Queensland. Future research need to apply a range of techniques; data derived from both toxicity studies and biological monitoring needs to be coupled with an ongoing water quality monitoring in order for realistic site

specific guidelines to be developed. The following research and management actions are therefore suggested:

1) Repeated testing is needed to firmly conclude the sensitivities to ammonia toxicity in the species tested in this study and at the same time it is imperative that more tropical species are included in toxicity testing for appropriate local water quality guidelines to be set. Testing of long-term effects of chronic ammonia exposure to tropical freshwater biota, at both the organism as well as at the community level, on endpoints such as growth and reproduction should also be evaluated. Such studies may produce research data with a higher degree of environmental realism.

2) Since the stressors discussed above (3) should be expected to coincide (Cole 1994; Pearson et al. 2003), more research into their synergistic relationship is needed. This research should include a variety of species tested at a range of physico-chemical stressors including different concentrations of ammonia, dissolved oxygen and temperature. Thus it could be conceived that guidelines should consist of an incorporation of all these stressors with different trigger values for the different levels of stressors in question.

3) Detecting intermittent episodes of potentially harmful levels of ammonia may be highly unrealistic in a routine monitoring program (Pearson et al. 2003). Water quality programs designed to monitor periods and areas where harmful levels of contaminants such as ammonia are likely to occur should therefore be considered.

4) Fertiliser application protocols should be assessed to find realistic minimum levels of fertilisers to be applied, as it is known that the majority of sugarcane farmers in north Queensland apply fertilisers in excess of plant growth requirements (Pearson et al. 2003).

5) The natural wetlands and riparian vegetation on the north Queensland floodplains have been extensively cleared since pre-European settlement (Russell et al. 1996) and therefore no longer perform the buffering to the aquatic environment they once provided (Johnson et al. 1999; Pearson et al. 2003). Riparian vegetation can act as a sink for nutrients carried in suspension via overland flow during flood periods (Johnson et al. 1999) and belts of riparian vegetation have been found to greatly reduce the input of nutrients from adjacent fertilised areas (Perrin et al. 1984). A program for restoration of riparian vegetation to reduce the input of nutrient-rich runoff should therefore also be assessed as a preventative to harmful levels of nutrients such as ammonia.

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Appendix 1. Species assessed for derivation of ammonia toxicity trigger values in the New Zealand and Australian water quality guidelines.

Appendix 2.

Table x. Results from scoring of degree of epithelial lifting in barramundi.

A) Fish nr.	Total score/ ten secondary lamellae	Mean score
1	40 – 40 – 30 – 33 – 30	35.8
2	40 – 40 – 40 – 40 – 40	40
3	15 – 25 – 24 – 15 – 10	17.8
4	29 – 15 – 32 – 17 – 14	21.4
5	40 – 40 – 40 – 40 – 30	38
6	21 – 10 – 19 – 23 – 30	20.4
7	40 – 40 – 40 – 40 – 40	40
8	20 – 40 – 40 – 20 – 30	30
9	24 – 17 – 32 – 28 – 40	28.2
B) Fish nr.	Total score/ ten secondary lamellae	Mean score
1	40 – 40 – 40 – 40 – 40	40
2	10 – 30 – 10 – 10 – 17	15.4
3	24 – 11 – 28 – 24 – 11	21.6
4	40 – 40 – 40 – 40 – 40	40
5	40 – 40 – 40 – 40 – 30	38
6	40 – 40 – 40 – 40 – 30	38
7	10 – 20 – 30 – 40 – 40	20.8
8	20 – 40 – 40 – 20 – 30	30
9	25 – 17 – 30 – 30 – 40	28.4
10	10 – 20 – 30 – 30 – 40	26

A) Fish that died from exposure (1.54 and 1.97 mg l^{-1} NH_3 - N treatments)
B) Fish from controls (euthanasied). The scores indicate that there is little variation in the degree of epithelial lifting between fish from treatments and controls.

Table x. Results from scoring of degree of epithelial lifting in rainbowfish.

A) Fish nr.	Total score/ ten secondary lamellae	Mean score
1	40 – 20 – 30 – 30 – 30	24
2	40 – 25 – 40 – 30 – 40	35
3	24 – 30 – 28 – 30 - 40	30.4
4	32 – 17 – 38 – 18 – 25	26
5	24 – 10 – 34 – 28 – 40	27.2
6	30 – 15 – 26 – 32 – 18	24.2
7	30 – 40 – 40 – 40 – 38	37.6
8	18 – 25 – 30 – 15 –17	21
9	40 – 20 – 40 – 30 – 30	32
B) Fish nr.	Total score/ ten secondary lamellae	Mean score
1	40 – 40 – 40 – 40 – 40	40
2	40 – 40 – 30 – 20 – 40	34
3	14 – 24 – 28 – 30 – 18	22.8
4	40 – 40 – 30 – 40 – 40	38
5	20 – 20 – 10 – 15 – 17	16.4
6	20 – 20 – 30 – 30 – 40	28
7	30 – 40 – 30 – 30 – 18	29.6
8	25 – 32 – 30 – 38 – 28	30.6
9	20 – 20 – 30 – 30 – 40	30
10	20 – 25 – 22 – 15 – 19	20.2

A) Fish that died from exposure (1.88 mg l^{-1} NH_3 - N treatment)
B) Fish from controls (euthanasied). The scores indicate that there is little variation in the degree of epithelial lifting between fish from treatments and controls.

Appendix 3.

Table x. Results from scoring of degree of epithelial lifting in barramundi.

A) Fish nr.	Total score/ ten secondary lamellae	Mean score
1	30 – 40 - 35 – 30 – 40	35
2	25 – 28 – 30 – 22 - 24	25.8
3	40 – 30 – 40 – 40 - 40	38
4	32 – 25 – 34 – 22 – 19	26.4
5	40 – 40 – 40 – 40 – 30	38
6	40 – 40 – 40 – 40 – 40	40
7	40 – 25 – 40 – 30 – 40	35
8	20 – 30 – 24 – 20 – 30	24.8
9	15 – 24 – 18 – 22 - 18	19.4
10	28 – 30 – 26 – 28 – 32	28.2
B) Fish nr. 1	28 – 32 – 24 – 26 - 26	27.2
2	40 – 40 – 35 - 40 - 40	39
3	24 – 22 – 24 – 15– 18	20.6
4	40 – 40 – 40 – 40 – 40	40
5	40 – 40 – 36 – 40 – 30	37.2
6	40 – 40 – 40 – 40 – 30	38
7	10 – 20 – 30 – 40 – 40	20.8
8	20 – 20 – 10 – 30 – 30	22
9	22 – 18 – 30 – 30 – 30	26
10	35 – 25 – 30 – 30 – 28	29.6

A) Sacrificed fish that survived the acute experiment (treatments: 0.41, 0.76 and 1.16 mg l^{-1} NH_3 - N)
B) Fish (euthanasied) from the respective treatments as above at the end of the post exposure experiment.

Table x. Results from scoring of degree of epithelial lifting in rainbowfish.

A) Fish nr.	Total score/ ten secondary lamellae	Mean score
1	18 – 20 – 16 – 19 - 22	19
2	30 – 30 – 35 – 40 – 38	34.6
3	24 – 30 – 28 – 30 - 40	30.4
4	40 – 40 – 40 – 40 – 40	40
5	35 – 40 – 38 – 35 - 30	35.6
6	30 – 15 – 26 – 32 – 18	24.2
7	40 – 40 – 40 – 38 - 40	39.6
8	30 – 20 – 35 – 30 – 30	29
9	15 –18 – 14 – 20 - 22	17.8
10	20 – 22 - 28 – 24 - 26	24
B) Fish nr. 1	34 – 22 – 25 – 28 - 24	26.6
2	40 – 40 – 40 – 40 - 40	40
3	30 – 27 – 28 - 25 - 32	28.4
4	24 – 30 – 16 – 20 - 18	21.6
5	32 – 38 – 36 – 35 - 28	33.8
6	24 - 15 – 20 – 14 - 17	18
7	38 – 40 – 35 – 35 - 40	37.6
8	30 – 32 – 26 – 34 – 28	30.8
9	32 – 34 – 20 – 24 – 18	25.6
10	30 – 30 – 40 – 40 - 30	34

A) Sacrificed fish that survived the acute experiment (0.44, 0.69 and 1.05 mg l^{-1} NH_3 - N)
B) Fish (euthanasied) from the respective treatments as above at the end of the post exposure experiment. The scores indicate little variation in the degree of epithelial lifting between fish (for both species) at the end of the four-day exposure period and after the three-week post exposure period.