TOXICITY OF AMMONIA TO THE LARVAE OF *PENAEUS INDICUS* H. MILNE EDWARDS*

P. JAYASANKAR AND M. S. MUTHU
Central Marine Fisheries Research Institute, Cochin-18.

ABSTRACT

Short-term experiments revealed a progressive increase in tolerance of the larval stages of *Penaeus indicus* to ammonia. 24-h LC_{50} values for nauplius, protozoea and mysis were 3.58 mg ammonia N/l (0.29 mg NH_3-N/l), 17.86 mg ammonia-N|1 (0.95 mg NH_3-N) and 46.01 mg ammonia-N|1 (3.17 mg NH_3-N/l). respectively. 48-h LC_{50} value for protozoea was 16.8 mg ammonia-N per liter (1.18 mg NH_3-N/l. Long-term chronic-toxicity experiments on nauplius to postlarva-1 showed that the 9-day incipient LC_{50} level was 11.99 ppm of ammonia-N|l (0.93 ppm NH_3-N). The EC_{50} (the concentration at which the percentage of larvae that metamorphosed to postlarvae was reduced to 50% of that of the controls) for ammonia was 3.23 ppm ammonia-N|l (0.25 ppm NH_3-N). "Safe level" of ammonia that should be maintained in the sea water used for larval rearing was calculated on the basis of incipient LC_{50} value as 1.2 mg ammonia-N/l or 0.093 ppm NH_3-N/l. More sensitive estimate of "safe level" based on EC_{50} value was 0.32 ppm ammonia-N|l or 0. 25 ppm NH_3-N.

INTRODUCTION

Ammonia toxicity is one of the common causes of death during fish and shellfish culture. In intensive culture systems, ammonia, which is the major end product of protein catabolism in aquatic animals (Campbell 1973), needs to be carefully monitored and controlled. The problem of ammonia accumulation is particularly intensified in systems where recircluated water is used. Kinne (1976) has reviewed the considerable amount of work which has been done on the toxicity of ammonia to fishes, but very little information is available on the effect of ambient ammonia concentrations on crustaceans (Anderson 1944; Shaw 1960; Mangum et al 1976; Wickins 1976; Delistraty et al 1977; Catedral et al 1977 a, b; Armstrong et al 1978). These latter workers have shown that the crustaceans are able to tolerate higher levels of ammonia than the fishes and that ammonia tolerance varies with the ontogenic development of the animals.

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In the present study, experiments on ammonia-exposure, prolonged exposure to stduy the chronic toxicity and short-term exposure to study acute toxicity, were conducted with larvae of *Penaeus indicus*, a prawn in great demand for culture in coastal impoundments.

Material and Methods

The larvae of both wild and eye-ablated spawners of *Penaeus indicus* obtained from the Narakkal Prawn Culture Laboratory of the Central Marine Fisheries Research Institute were used in the experiments. The test solutions were made by dissolving requisite amounts of analar grade NH_4Cl as the source of ammonia. The experiments were conducted in 1-litre capacity Corning beakers containing 800 ml of test solution. Each beaker contained 10 larvae and was aerated by an air-stone. The larvae were fed with a mixed culture of phytoplankton, dominated by *Chaetoceros* sp. and *Thalassiosira* sp. To ensure uniform feeding conditions in all the beakers, filtered seawater was mixed with the phytoplankton culture in the ratio of 8:1 in a 50-l capacity plastic bin, from which all the test solutions were prepared. The test solutions in the beakers were renewed daily.

Experiments

Prolonged-exposure experiments

These experiments were conducted to study the long-term effects of ammonia concentrations on (1) the survival of the larvae and (2) the rate of larval development, two aspects that are of importance in hatchery operations. Two experiments using two separate broods were carried out. In experiment I, larvae were obtained from a wild spawner and were exposed to eight concentrations of ammonia, viz. 0.5, 1.0, 2.5, 5.0, 7.0, 10.0 30.0 and 50.0 mg ammonia-N|l. The experiments were started with nauplius-1 (except for concentrations 5.0. and 7.0 which were started with protozoea-1) and continued for 10 days till they reached mysis-3 stage. Then the experiment had to be terminated due to unavoidable circumstances. For experiment II the larvae were obtained from an eye-ablated spawner. This experiment was started when the larvae were in a late nauplius (N5 or N6) stage and lasted for 9 days when the larvae in the control beakers metamorphosed to postlarva-1. The concentrations used were 1.0, 2.0, 4.0, 6.0 and 8.0 mg ammonia-N|1. The percentage of postlarvae obtained in the various experimental media in 9 days is taken as an index of the relative rate of larval development in different concentrations.

Each concentration was tried in triplicate. On the first day, observations on the condition of the larvae and mortality were made once in 12 h and thereafter once in 24 h. Initial and final pH and ammonia values in the beakers were measured every day. The larvae were examined under a stereoscopic microscope every day at the time of changing the medium to see if there are any morphological deformities.

Short-term exposure to study acute toxicity

Since penaeid larval development is rapid and punctuated by frequent metamorphosis into morphologically distinct larval stages which are likely to tolerate progressively higher concentrations of toxins, short-term (24-h and 48-h) LC_{50} experiments were also done. Larvae obtained from wild spawners were used in these experiments. Each larval stage was exposed to different concentrations of ammonia taken in geometric progression. Each concentration was duplicated.

Statistical analysis

The short-term and long-term LC_{50} values (defined as the concentrations at which 50% of the population is killed) were estimated by weighted probit analysis, i.e. the iteration method (Finney 1952). The EC_{50} value, defined as the concentration at which 50% of the larvae did not metamorphose into postlarvae, was also estimated for experiment II by probit analysis. Effect of various concentrations of ammonia on the larval survival was tested using Analysis of variance. The effect of ammonia concentrations on the percentage of postlarvae I (of the survivors) obtained at the end of 9-day period was tested by analysis of co-variance.

Chemical analysis

The pH was measured with a digital pH meter with combination electrode, and total ammonia by the method of Solorzano (1969). Un-ionised portion of total ammonia was calculated according to Whitfield (1974).

Abbreviations of different forms of ammonia-nitrogen used in the present study:

Ammonia-N	=	Total ammonia
NH_3-N	=	Un-ionised ammonia
NH_4-N	=	Ionised ammonia

Environmental conditions during the experiments

	Chronic toxicity experiments	*Acute toxicity experiments*
Temperature	33° ± 1°C	30° ± 2°C
Salinty	33-34 ‰	33-34 ‰
pH	7.69-8.26	7.90-8.40
Photoperiod	12D : 12L	13D : 11L
Light intensity	200-700 lux	150-650 lux

RESULTS

Long-term exposure experiment

Experiment I with larvae of wild spawners: In the higher concentrations, viz., 10, 30 and 50 mg/l ammonia-N the nauplii died before they could transform

into protozoea (Fig. 1). At the end of 12 h, when the fiirst observation was made, the nauplii appeared distinctly weak in these concentrations. In lower concentration (1.0 mg|1 ammonia-N) some nauplii and protozoea had deformed furcal setae. The effect of various concentrations on the survival of the

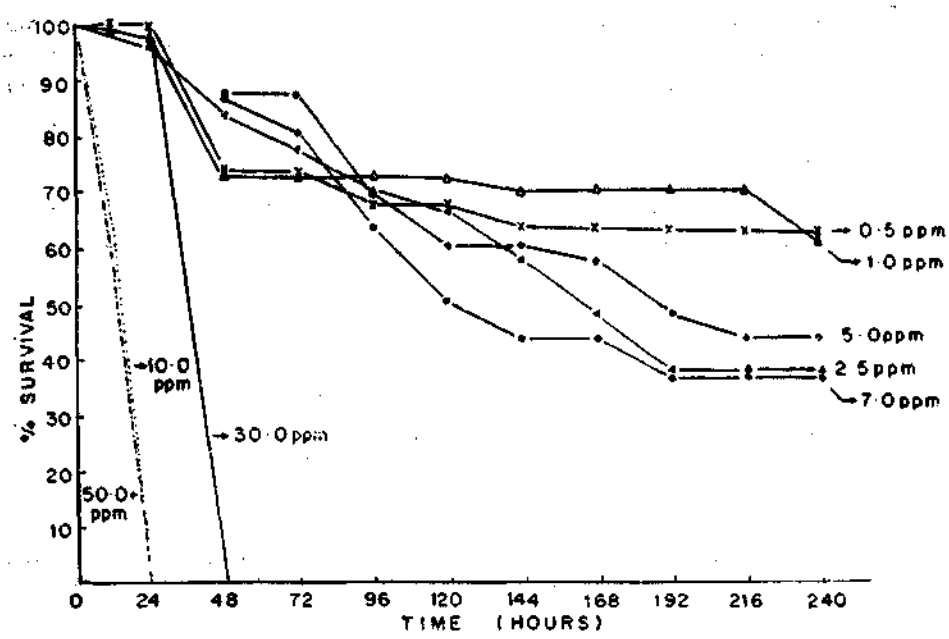

FIG. 1. Survival of *Penaeus indicus* larvae from nauplius to mysis-3 in various concentrations of ammonia during prolonged exposure (Experiment I) (* concentrations started from protozoea 1).

larvae was found to be highly significant ($P \leq 0.01$). The 10 day LC_{50} was estimated as 1.45 mg|1 ammonia-N. As the experiment could be continued only up to the mysis-3 stage, the effect of different concentrations on the percentage of larvae that metamorphosed into postlarvae could not be studied.

Average ammonia (estimated total ammonia and calculated un-ionised ammonia) values in the control and experimental beakers at the beginning and end of the 24-h period are given in Table-1. In the control the total ammonia was less than 0.1 ppm in all the cases. Generally a slight decrease in the concentration of ammonia was noticed in all the beakers (except the controls) at the end of 24 h.

Experiment II with larvae of eye stalk-ablated spawners: Since there was total mortality of nauplii in ammonia concentrations of 10 ppm and above, in this experiment concentrations less than 10 ppm were tried. These larvae from the eye stalk-ablated spawner performed better (Fig. 2) than the larvae from the

TABLE 1. *Ammonia concentration at the beginning and end of a 24-h period in the test solutions (Experiment I).*

Initial values (ppm)			At the end of 24-h period		
Total ammonia (ammonia-N)	Un-ionised ammonia (NH_3-N)	pH	Total ammonia (ammonia-N)	Un-ionised ammonia (NH_3-N)	pH
Control					
0.064	0.0065	8.21	0.07	0.0038	7.92
Test concentrations					
0.50	0.03	7.83	0.43	0.025	7.89
1.0	0.09	8.14	0.97	0.07	7.90
2.5	0.22	8.15	2.10	0.19	8.01
5.0	0.47	7.98	4.47	0.43	8.15
7.0	0.64	8.16	5.82	0.45	8.02
10.0	0.65	8.00	9.38	0.93	8.20
30.0	1.23	7.79	30.00	2.45	8.11
50.0	2.19	7.82	46.38	3.00	8.00

wild spawner used in the experiment I. The survival was better in all the concentrations and setal deformities were rare. The 9 day LC_{50} was calculated as 11.99 mg|1 ammonia-N (0.93 mg|1 NH_3-N at pH 8.1) as against 1.45 mg|1 in experiment I. The effect of different concentrations on larval survival was highly significant ($P \leq 0.01$).

The percentage of larvae that metamorphosed into postlarvae I on the 9th day in different concentrations of ammonia were as follows:

Control	1.0 ppm	2.0 ppm	4.0 ppm	6.0 ppm	8.0 ppm
100	84.6	73.7	31.3	23.5	12.5

The effect of amomnia levels on larval metamorphosis was analysed statistically and was found to be highly significant ($P \leq 0.01$) by Analysis of Co-variance. From the above data EC_{50}, i.e. the ammonia concentration at which 50% of the larvae did not metamorphose into postlarvae, was calculated by probit analysis as 3.23 mg|1 ammonia-N, which is equivalent to 0.25 mg|1 NH_3-N at pH 8.1.

The average ammonia concentrations (ammonia-N and NH_3-N) at the end of 24-h period in all the beakers except the controls (Table-2) showed a decline over the initial values, as in experiment I.

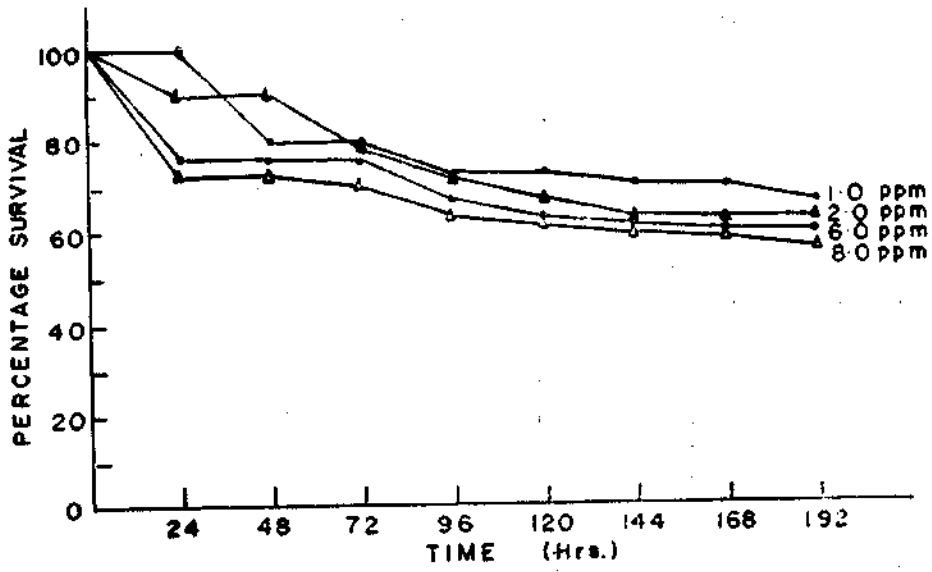

FIG. 2. Survival of *Penaeus indicus* larvae from nauplius to postlarva I in various concentrations of ammonia during prolonged exposure (Experiment II)

Short-term exposure experiments

a) *Nauplius*: Late nauplii (stage V or VI) were exposed to 1.0, 2.0. 4.0, 8.0 and 16.0 mg|1 ammonia-N and 24-h LC_{50} was estimated as 3.5 mg|1 ammonia-N (Fig.3). At pH 8.17 and temperature 29°C this was equivalent to 0.29 mg|1 NH_3-N.

b) *Protozoea*: Protozoea I were exposed to 2.0., 4.0, 8.0, 16.0 and 32.0 mg|l ammonia-N. The 24-h LC_{50} was 17.80 mg|l ammonia-N (0.95 mg|l NH_3-N at pH 8.04 and temperature 27°C), while the 48-h LC_{50} came down to 16.80 mg|1 ammonia-N (1. 18 mg|1 NH_3-N at pH 8.17 and temperature 27°C) Fig. 4 and 5).

c) *Mysis*: Mysis I larvae were exposed to 2.0, 4.0, 8.0, 16.0, 32.0 and 64.0 mg|l ammonia-N. The 24-h LC_{50} was found to be 46.01 mg|l ammonia-N (3.17 mg|l NH_3-N at pH 8.12 and temperature 28°C) (Fig. 6).

Discussion

The larvae of *Penaeus indicus* pass through three larval stages, namely, nauplius, protozoea and mysis, before they metamorphose into postlarvae. Since

TABLE 2. *Ammonia concentrations at the beginning and end of a 24-h period in the test solutions (Experiment II).*

Initial values (ppm)			At the end of 24-h period		
Total ammonia (ammonia-N)	Un-ionised ammonia (NH_3-N)	pH	Total ammonia (ammonia-N)	Un-ionised ammonia (NH_3-N)	pH
Control					
0.06	0.0042	8.04	0.11	0.005	7.82
Test concentrations					
1.0	0.12	8.00	0.95	0.04	7.72
2.0	0.14	8.01	1.59	0.07	7.75
4.0	0.27	7.99	3.31	0.14	7.76
6.0	0.39	7.97	4.44	0.21	7.75
8.0	0.50	7.96	6.51	0.26	7.71

the duration of these larval stages is very short (2-4 days) the LC_{50} values were calculated for each larval stage on the basis of 24-h or 48-h experiments. These experiments revealed that tolerance to ammonia increased progressively as the nauplius metamorphosed to protozoea and then to the mysis stage. The 24-h LC_{50} value was 3.58, 17.86 and 46.01 mg|l ammonia-N for the nauplius, protozoea and mysis stages, resptectively. The nauplius is the most sensitive stage, the weakest link in the series of larval stages. So any deterioration in water quality will affect the nauplius stage first and greatly influence the ultimate survival rate. While monitoring ammonia levels in seawater used in hatcheries, the requirements of the nauplii should be borne in mind.

Sprague (1969) opined that the short term LC_{50} (especially 24-h LC_{50}) values can be very misleading and recommended that toxicity should be described in terms of incipient LC_{50} or lethal threshold concentrations, defined as "the level of toxicant which is lethal for 50% of the individuals exposed for periods sufficiently long that the acute lethal action has ceased." Incipient LC_{50} is also defined as "that level beyond which 50% of the population cannot live for an indefinite time." According to Sprague (1969) "if this cannot be estimated, the 4-day LC_{50} is a useful substitute and often its equivalent." Wickins (1976) has used the long-term (4-weeks) toxicity experiments to calculate incipient LC_{50} Armstrong et al (1976 and 1978) have demonstrated how the LC_{50} values decline steeply from 24 h to 96 h and then stabilize to reach an asymptote; their 144-h or 192-h LC_{50} values can be considered equivalent to the incipient LC_{50}

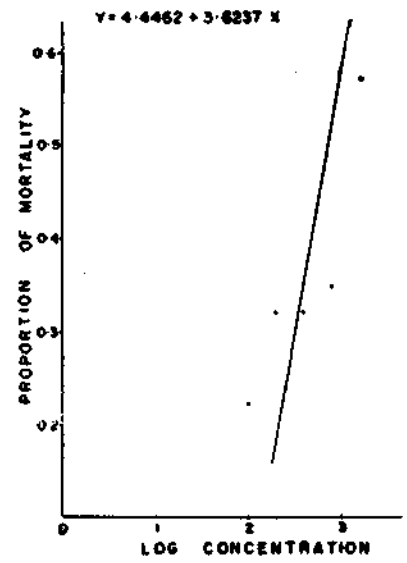

FIG. 3. Regression of mortality on ammonia concentration in 24-h acute toxicity experiment on nauplius of *P. indicus*.

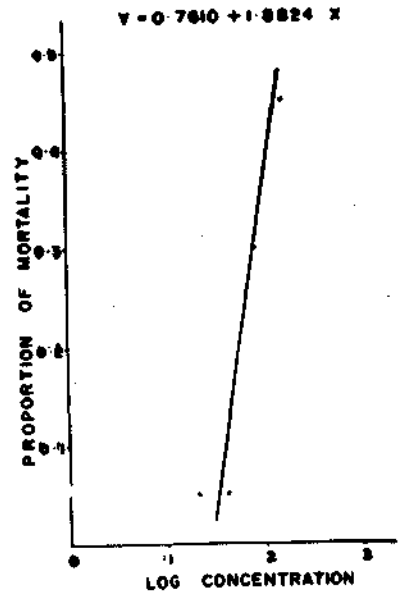

FIG. 4. Regression of morality on ammonia concentration in 24-h acute toxicity experiment on protozoea of *P. indicus*.

Following the recommendations of Sprague (1969) the incipient LC_{50} values of ammonia toxicity for the two broods were calculated using the data obtained in the long-term (9|10 days) chronic-toxicity experiments. The incipient LC_{50} value was 1.45 mg|l ammonia-N (0.11 ppm NH_3-N) for the larvae from the wild spawner in experiment I. It was 11.99 mg|l ammonia-N (0.93 ppm NH_3-N) for the larvae from the eye-ablated spawner used in experiment II. This shows that tolerance to ammonia varies between different broods and that the larvae of eye-ablated spawners are in no way inferior to the larvae from wild spawners. The very low ammonia tolerance of the brood from wild spawner was also associated with the occurrence of many deformed nauplii among them and a slower rate of development (the larvae did not metamorphose into postlarve even after 10 days). The larvae from the eye-ablated spawner were more tolerant of ammonia, the incidence of abnormalities was negligible and they transformed into postlarvae in 9 days.

The LC_{50} values including the incipient LC_{50} are based on mortality rates and measure quantal (all or none) responses; the sub-lethal effects of the toxins on the larvae are not revealed. A more sensitive index, LC_{50} or the Median Effective Concentration (i.e. the concentration at which 50% of the

population showed any sub-lethal response, i.e. retardation of growth etc.) has been used by some workers (Wickins 1976) to study sub-lethal effects. In the present study, the EC_{50} is taken as the concentration at which the percentage of larvae that metamorphosed to postlarvae is reduced to 50% of that observed in the controls in 9 days. This index is related to the rate of larval development

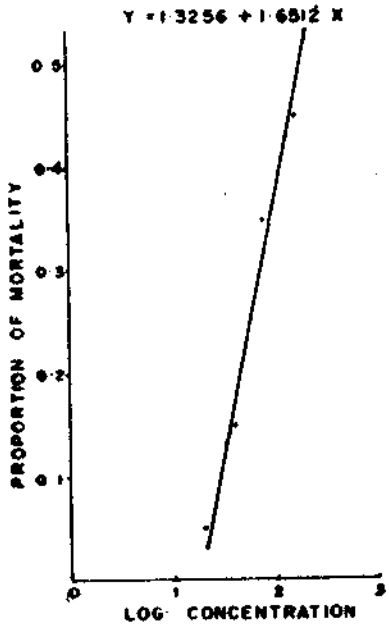

FIG. 5. Regression mortality on ammonia concentration in 48-h acute toxicity experiment on protozoea of *P. indicus*.

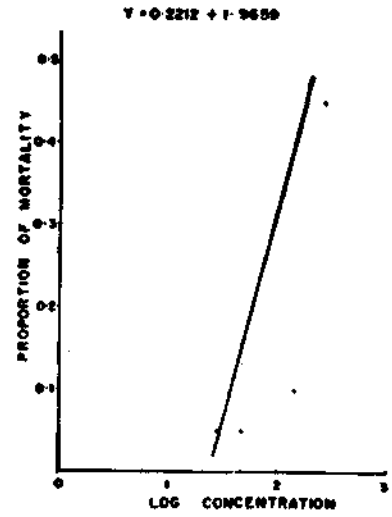

FIG. 6. Regression of mortality of ammonia concentration in 24-h acute toxicity experiment on mysis of *P. indicus*.

and is very relevant in the context of rearing of the larvae in hatcheries, where it is advantageous to have larvae metamorphosing quickly and synchronously. The EC_{50} value calculated on the basis of experiment II was 3.23 ppm ammonia-N(or 0.25 ppm NH_3-N at pH 8.1). This value is lower than the incipient LC_{50} calculated for the same experiment and gives a more sensitive estimate of the toxicity of ammonia to the larvae.

Besides the present study, the only other work dealing with the lethal effect of ammonia on penaeid larvae is that of Catedral et al (1977 a,b). Working with the larvae of *Penaeus monodon*, they determined the concentration of ammonia at which 60% of the larvae survived. The value was 11-12 ppm ammonia for protozoea, 60 ppm for mysis and 50 ppm for postlarva (P3). These estimates for protozoea and mysis were derived from the experiments in

which protozoea and mysis were respectively reared up to the postlarval stage and the experiments with postlarvae lasted for 10 days. Further, it is not clear whether ammonia concentrations expressed as ppm refer to NH_4Cl or NH_4-N or total ammonia. So their values cannot be directly compared with the results obtained in the present study.

Toxicity of ammonia to juvenile penaeids was studied by Wickins (1976). He found that the 48-h LC_{50} was 1.28 mg|1 NH_3-N. Compared to that value, the present data indicate that the protozoea of *Penaeus indicus* (48-h LC_{50} 1.18 ppm NH_3-N) are only a little more sensitive to NH_3-N than the juveniles, whereas the nauplii (24-h LC_{50} 0.29 ppm NH_3-N) are very highly vulnerable to NH_3. For the 3-8 day old larvae of *Macrobrachium rosenbergii* Armstrong et al (1978) estimated the 24-h LC_{50} at pH 8.34 as 37 ppm ammonia-N (3.58 ppm NH_3-N) which is very similar to the 24-h LC_{50} value of 3.17 ppm NH_3-N, obtained during the present study for the mysis larvae of *P. indicus*.

The incipient LC_{50} for the stage-IV larvae of the lobster *Homarus americanus* and the 3-8 day old larvae of the freshwater prawn *Macrobrachium rosenbergii* was calculated as 1.4 ppm NH_3-N and 1.35 ppm NH_3-N, respectively, by Delistraty et al (1977) and Armstrong et al (1978). *Penaeus indicus* larvae, for which the incipient LC_{50} value was estimated as 0.93 ppm NH_3-N during the present study, appear to be more sensitive than the larvae of lobster and freshwater prawns to NH_3.

Using growth as a sensitive gauge, Armstrong et al (1978) estimated that 32 ppm ammonia-N retarded the growth of *Macrobrachium rosenbergii* larvae at pH 6.83 (0.11 ppm NH_3-N) and pH 7.6 (0.63 ppm NH_3-N). For juvenile penaeids Wickins (1976) calculated the 3 week EC_{50} (i.e the concentration that reduced the growth by 50% of that of the controls) as 0.45 ppm NH_3-N. These values are higher than the EC_{50} value of 3.23 ppm ammonia-N (0.25 ppm NH_3-N at pH 8.1) calculated during the present study on the basis of the percentage of larvae that metamorphosed to postlarvae on the 9th day in different concentrations of ammonia, indicating that the larvae of *P. indicus* are more susceptible to the sublethal toxic effects of ammonia.

The toxicity of ammonia to aquatic animals is generally credited to the NH_3 molecules (Spotte 1970, Hampson 1976) despite the evidence that NH_4+ adversely affects some physiological functions (Shaw 1960; Maetz 1972; Campbell 19(3). Armstrong et al (1973) showed that toxicity of ammonia was not due solely to the NH_3 molecules; in solutions of different pH and equal concentrations of NH_3, survival of *Macrobrachium rosenbergii* larvae was greatly reduced as NH_4+ levels increased. They propose that at higher pH (8.4) toxicity results from copious diffusion of NH_3 into the larvae, while at lower

pH (6.8) toxicity is due to competitive inhibition of Na^+ transport by NH_4+. The latter may be true for freshwater animals, for Na^+ has to be taken up from the external medium to maintain the osmotic concentration of the body fluids. But competitive inhibition of Na^+ transport by NH_4+ may not be relevant to marine animals where there is, in fact, a need to prevent Na^+ from entering the body. In seawater which normally has a pH of 8.2 the un-ionised ammonia fraction will be higher and toxicity is more likely to be caused by the rapid inward diffusion of NH_3 when the ammonia level is increased in the ambient medium. High external concentration of ammonia may also hamper free diffusion of excretory ammonia from the body into the external medium leading to accumulation of toxic ammonia in the body of the animal. The slight increase of ammonia in the control beakers at the end of the 24-h period (Tables 1 and 2) may perhaps be due to the free excretion of ammonia by the larvae as the ambient ammonia level was low in the control beakers. This increase was not noticed in the other beakers where the concentration of ammonia was increased by the addition of NH_4Cl (Tables 1 and 2), which might have inhibited ammonia excretion by the larvae. In fact, there was a slight decrease in ammonia concentration at the end of 24-h period, most probably due to the oxidation of ammonia to nitrite by the nitrifying bacteria which multiply rapidly making use of the NH_4Cl added to the test solutions. Filtered seawater was kept in beakers without larvae or added NH_4Cl and ammonia concentrations were estimated at the beginning and end of the 24-h period, but no change was noticed in the concentrations because no larvae were present to increase the ammonia level, nor could sufficient nitrifying bacteria have developed in the absence of added ammonia.

Finally, some discussion of the results in relation to water-quality requirements for hatchery rearing of penaeid larvae is warranted. Sprague (1969) while discussing the "safe levels" of toxicants for aquatic animals, points out that the incipient LC_{50} is a very important parameter in this connection. Safe levels have been calculated from incipient LC_{50} values by using an empirical "application factor" which varies from 0.05-0.3. Sprague (1969) recommends 0.1 of incipient LC_{50} as the safe level of most of the toxicants. Accordingly, the safe level of ammonia for rearing of the larvae of *Penaeus indicus* has been calculated as 1.20 mg/l ammonia-N or 0.093 mg/l NH_3-N at pH 8.1 (i.e. 0.1 of the estimated incipient LC_{50}). Since EC_{50} was found to be a more sensitive index of toxicity which takes into consideration the sub-lethal effects as well, it is proposed that the safe level could be taken as 0.1 of EC_{50} Based on the EC_{50} value obtained during the present study the safe level of ammonia for rearing of larvae of *Penaeus indicus* is estimated as 0.32 ppm ammonia-N or 0.025 ppm of NH_3-N at pH 8.1. The ammonia concentration in the controls during the present study did not exceed 0.11 ppm ammonia-N or 0.005 ppm NH_3-N, i.c it was well within the "safe level".

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