Vol. 3: 159–165, 1987 | DISEASES OF AQUATIC ORGANISMS Dis. aquat. Org. | Published December 31

Experimental investigations on the toxicity of ammonia: effects on ventilation frequency, growth, epidermal mucous cells, and gill structure of rainbow trout *Salmo gairdneri*

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ABSTRACT: Juvenile rainbow trout *Salmo gairdneri* Rich. were experimentally exposed to 0.25 mg l^{-1} of NH_3 for 4 wk. This concentration lies between those causing acutely lethal and sublethal effects. At first, significant effects from the ammonia were recorded: greatly increased ventilation frequency, reduced food intake and an associated decrease in weight gain. Subsequently, the fish adapted considerably to the toxicant. Ventilation frequency decreased, although it remained above that of controls. Food was taken up completely and there was only a minor difference in growth between exposed trout and unexposed controls. No change from exposure to the pollutant could be detected in the number of mucous cells in the epidermis. Examination of the gill structure at the end of the experiment revealed only a slight, but significant, tissue proliferation. Unexpectedly, the comparatively high ammonia concentration produced few chronic effects. Nevertheless, results indicate that the well-being of the experimental fish was disturbed.

INTRODUCTION

Intensive commercial production of marketable fishes usually results in the contamination of the water with nitrogenous waste products due to high stocking densities, low rates of water exchange, and the use of artificial feeds with high protein content.

Especially important is the increase in the ammonia concentration resulting mainly from fish excretion and the bacterial decomposition of faeces and food remains in the water. High ammonia levels are looked upon as a limiting factor in fish production (Westers & Pratt 1977, Redner & Stickney 1979, Sadler 1981, Soderberg et al. 1984).

Numerous references to the toxicity of ammonia to fishes have appeared in the literature, but the critical concentrations reported have varied greatly. Haywood (1983), summarizing the results of diverse authors, concluded that the lowest LC_{50} value for salmonids exposed for 24 to 96 h is in the range from 0.2 to 0.3 mg l^{-1}, and sublethal effects may be expected at concentrations greater than 0.002 mg l^{-1}.

Sublethal effects of NH_3 that are frequently reported include gill damage, reduction in the growth rate, and an increased susceptibility to pathogens (Kuhn & Koecke 1956, Burrows 1964, Reichenbach-Klinke 1967, Flis 1968b, Larmoyeaux & Piper 1973, Smith & Piper 1975, Smart 1976, Schreckenbach & Spangenberg 1978, Colt & Armstrong 1979, Soderberg et al. 1983, Peters et al. 1984, Thurston et al. 1984, Klontz et al. 1985, Mallatt 1985).

In the investigation described here, young rainbow trout *Salmo gairdneri* Rich. were exposed experimentally to a mean NH_3 concentration of 0.25 mg l^{-1}. This concentration was selected because it lies in the range between the occurrence of lethal and sublethal effects, and therefore significant reactions to this pollutant by the fishes could be expected.

As indicators for physiological disturbances of the fishes, we investigated changes in behavioural

0177-5103/87/0003/0159/$ 03.00

patterns, in ventilation frequency, and weight gain. Since it has been demonstrated that mucus production can be influenced by the presence of harmful environmental factors such as stress and pollution (Christie & Battle 1963, Jakowska 1963, Eisler 1974, Daye & Garside 1975, 1976, Pickering & Macey 1977, Zuchelkowski et al. 1981), we also recorded the structure and number of epidermal mucous cells. Additionally, we looked for proliferations of the gill tissue.

The goal of the experiment was to obtain information about the intensity and pathological sequence of NH_3 damage. It should be investigated whether a comparatively high concentration of the toxicant, approaching lethal levels, results in a constant or increasing degree of damage or whether an adaptation of the fishes to the ammonia can occur.

MATERIAL AND METHODS

For the experiment, 40 rainbow trout with an average initial weight of 20 g each were used. They were first allowed to adapt to the experimental facilities for 3 wk. During this period and throughout the entire experiment, individuals were kept alone in order to eliminate any influence from battles for social rank on their reactions to the toxicant.

The trout were fed commercial feed pellets once daily to 1 % of their body weight, recalculated weekly.

For a period of 4 wk, 20 of the 40 trout were exposed to a NH_3 solution while the remaining 20 were kept as a control group in aerated tap water (total hardness 1.2 mmol l^{-1}; 2.2 mg l^{-1} free CO_2; 11.0 mg l^{-1} Cl^-; pH 7.8; 8.7 mg l^{-1} O_2). Mean water temperature was 14.2 °C.

Every 12 h, 50 % of the water was exchanged. Before and after this procedure, the relevant water chemistry parameters were determined.

The experimental containers were ten 40 l glass aquaria, each divided by PVC walls with small holes into 4 compartments of equal size. In 5 of the containers, selected ammonia concentrations were obtained by the addition of ammonium chloride (NH_4Cl) to the tap water used in the experiment.

The amount of NH_4Cl required was calculated after each water exchange on the basis of the water temperature and the pH value determined in the single test aquaria. The total ammonium content was determined photometrically by the Hypochlorid-Indophenol method. From the results, together with water temperature and pH, the NH_3 concentrations were calculated (Trussel 1972).

On the assumption that changes in the NH_3 concentration between water exchanges occurred linearly, mean values were calculated from the NH_3 levels detected before and after each water exchange. Using these values, the median of the entire exposure time was determined:

exposed group: 0.250 ± 0.0050 mg l^{-1} NH_3
control group: 0.006 ± 0.0004 mg l^{-1} NH_3

However, we are aware that the actual ammonia exposure of the experimental fishes, if integrated over time, was certainly different from the assumed median concentration. Since continuous determination of ammonia and pH over a 1 d cycle was not possible during this experiment, we intend to verify the integrated exposure level through experimental modelling at a later date.

The ventilation frequency of experimental and control trout was determined by counting the number of opercular movements per minute.

To determine the growth rate, the unanaesthetized trout were placed in a pail with a predetermined volume of water and weighed individually on a balance every 7 d. The fish had been accustomed to this procedure during the period of adaptation before the start of the experiment.

For examination of the epidermis, fish were anaesthetized in neutralized MS 222, and a square piece of skin (ca 1 cm^2) was cut from the left side of each fish between the dorsal fin and the lateral line. Some of the samples were fixed in Bouin's fluid, and the rest were preserved in 4 % buffered formalin in 0.6 % NaCl. Sections 8 µm thick were cut with a microtome, stained with haematoxylin and eosin, and examined microscopically at 250×. Using an ocular micrometer, the number of mucous cells in the epidermis per 1 mm of basal membrane was calculated. For each section several random counts were made, and their arithmetic means and standard deviations were calculated. The skin formalin-fixed samples were treated according to a modified method of Pickering (1974). This involved rinsing the samples and then staining them for 5 min in Alcian blue (pH 2.5). This treatment results in a staining only of those mucous cells on the surface of the epidermis that are open to the outside but not completely emptied. The samples were placed in cavity slides, and the stained cells were counted under 250× magnification. Using an ocular micrometer grid, the number of cells per 1 mm^2 of surface area could be determined. For each sample, 12 random counts were made, and their arithmetic means and standard deviations were calculated.

Diagnosis of the gill structure was performed by examining preparations of the gill arches, either fresh or fixed in 4 % buffered formalin, at 6 to 50× magnification after staining with methylene blue (5 %). The degree of hyperplastic lesions was determined using a calculated index of proliferation:

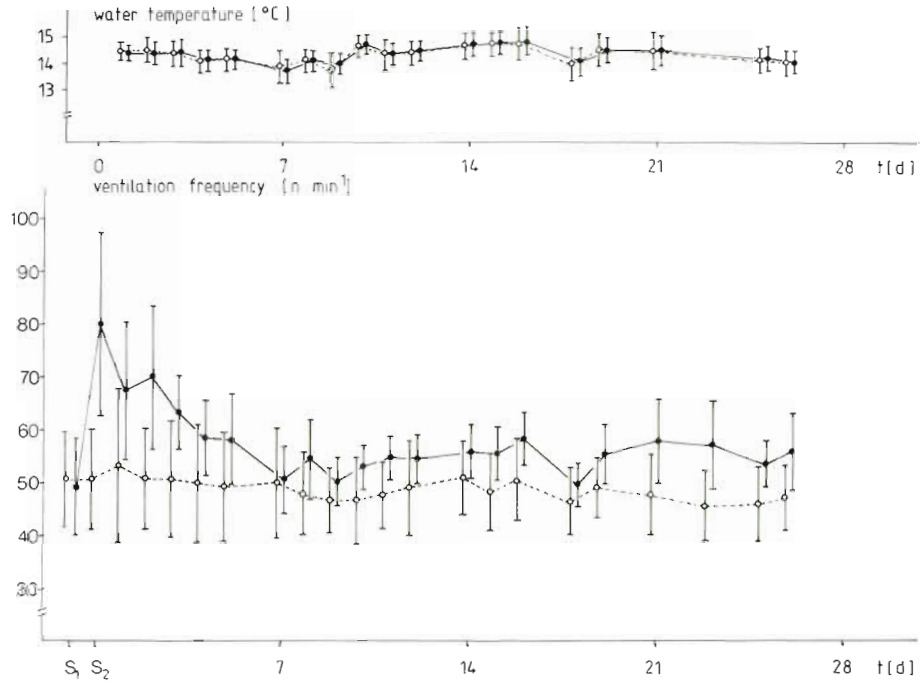

Fig. 1. *Salmo gairdneri*. Changes in the ventilation frequency ($\bar{x} \pm$ SD) of trout exposed to 0.25 mg l^{-1} NH_3 (solid line; n = 16) compared to controls (broken line; n = 20) and water temperature (above: $\bar{x} \pm$ SD) during the 28 d experiment. S_1 = 30 min before, S_2 = 60 min after the first application of NH_3

0 = no proliferations
1 = proliferations at the tips of the primary lamellae, secondary lamellae overgrown by proliferating tissue up to 1/3
2 = secondary lamellae overgrown 1/3 to 2/3
3 = secondary lamellae overgrown 2/3 to completely

The indices of proliferation were expressed as arithmetic means and standard deviations.

Statistical evaluation of the results was performed using the Mann-Whitney U-test.

RESULTS

The first clearly recognizable reactions of the fishes to the pollutant were detected immediately after the start of the experiment. These included a darkening of their skin and an increased sensitivity to external disturbances, which manifested itself as hectic swimming reactions and attempts to flee. The dark coloration became less intense during the first week, but in comparison to the controls, the fishes tended to remain darker throughout the entire experimental period. In contrast, the excitability of the trout had returned to its normal level after a few days.

Ventilation frequency

During the 3 wk period of adaptation the mean ventilation frequency of the trout settled down at a fairly constant level of approximately 50 opercular movements min^{-1} (Fig 1; S_1), without significant differences between the 2 experimental groups. The control fish remained at this level throughout the entire experiment. The mean ventilation frequency of the trout exposed to NH_3 increased immediately after administration of the toxicant, to a significantly elevated level (58 %) compared with the control group ($p < 0.001$) (Fig. 1; S_2). As the experiment continued, the breathing rate decreased rather steadily until the 7th day. Thereafter, it levelled off and remained almost constant for the remaining 3 wk of exposure. Over the entire period, the daily average calculated for the experimental fish lay above the value for the controls. Except for Day 7, 9, 12, 14, and 18 the mean ventilation frequency of the exposed trout was significantly higher than that of the unexposed fishes ($p < 0.05$).

The fluctuations of the mean ventilation frequency in both the exposed and the unexposed trout generally corresponded to the daily changes in mean water temperature depicted in Fig. 1.

Growth

In Table 1 the absolute and the relative weight gains of exposed and unexposed trout are listed. Neither at the start of the experiment nor at its end could significant differences in the mean body weight between the experimental groups be detected. However, looking at the mean percent growth rate over the entire experimental period in both groups, the exposed trout exhi-

Table 1. *Salmo gairdneri*. Changes in body weight, weight gain, and percent growth rate of rainbow trout exposed to 0.25 mg l^{-1} NH_3 (n = 20) compared to controls (n = 16)

Time	Body weight (g) Control $\bar{x} \pm SD$	Body weight (g) NH_3 $\bar{x} \pm SD$	Weight gain (g wk^{-1}) Control $\bar{x} \pm SD$	Weight gain (g wk^{-1}) NH_3 $\bar{x} \pm SD$	Growth rate (% wk^{-1}) Control $\bar{x} \pm SD$	Growth rate (% wk^{-1}) NH_3 $\bar{x} \pm SD$
Start	23.00 ± 1.84	23.74 ± 0.97	– ± –	– ± –	– ± –	– ± –
1 wk	24.70 ± 2.20	24.52 ± 1.38	1.70 ± 0.48	0.79 ± 0.90	7.29 ± 1.97	3.32 ± 3.77••
2 wk	26.24 ± 2.20	26.03 ± 1.40	1.54 ± 0.36	1.51 ± 0.32	6.31 ± 1.77	6.18 ± 1.30
3 wk	28.21 ± 2.25	27.68 ± 1.49	1.77 ± 0.30	1.58 ± 0.24	6.79 ± 1.19	6.10 ± 0.96•
4 wk	30.41 ± 2.25	30.05 ± 1.67	2.40 ± 0.99	2.31 ± 0.41	8.55 ± 0.84	8.37 ± 1.47
Overall	–	–	7.41 ± 0.99	6.25 ± 1.03	32.22 ± 3.44	26.32 ± 4.15••

Level of significance: •• $p < 0.001$; • $p < 0.01$

bited a significantly slower growth rate compared with the control fishes ($p < 0.001$).

If the growth rate is separated into weekly percentages of weight gain (Fig. 2), there is evidence that the reduced growth rate of the exposed trout resulted mainly from the significantly lower weight gain during the first week of exposure ($p < 0.001$). In the following weeks a significant difference between the exposed and unexposed fishes could be determined only in the third week ($p < 0.01$).

These results correspond well with observations on feeding behaviour during the experiment. Trout exposed to NH_3 showed an initial loss of appetite, while the controls continued to rapidly consume their feed rations as usual. None of the exposed fish consumed any of their feed on the first day after the administration of the NH_3. On the following days the exposed trout took food only with hesitation and irregularly. The feed was actually consumed only by some of the fish, and all of it was not eaten. However, from the fifth day on, all of the fish consumed their feed completely. Thus, differences in feeding behaviour between the exposed fishes and the controls could no longer be determined.

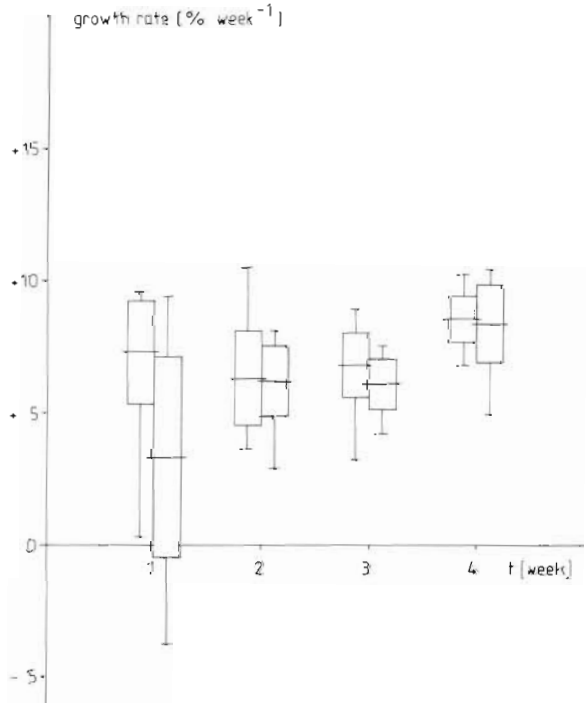

Fig. 2. *Salmo gairdneri*. Weekly increase of body weight of rainbow trout exposed to 0.25 mg l^{-1} NH_3 (stippled bars; n = 16) compared to controls (open bars; n = 20). Horizontal lines: $\bar{x}$; bars: SD; vertical lines: total range

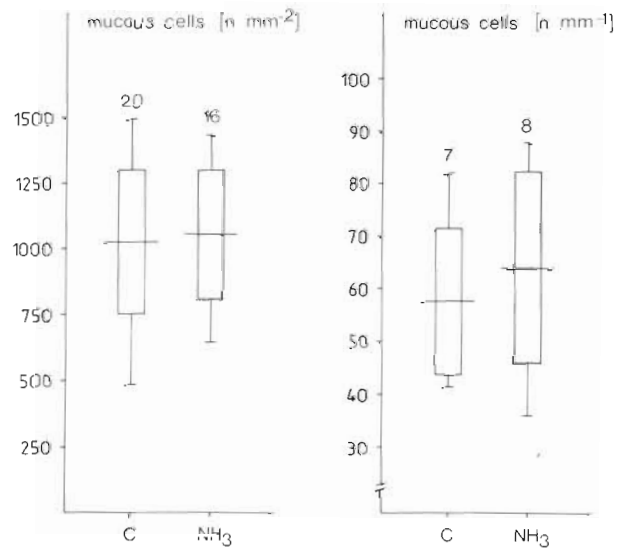

Fig. 3. *Salmo gairdneri*. Number of mucous cells in the epidermis of rainbow trout after 28 d of exposure to 0.25 mg l^{-1} NH_3 (stippled bars) and controls (open bars). Left: no. of open mucous cells mm^{-2} of the epidermis surface; Right: no. of mucous cells mm^{-1} of basal membrane in sections of the epidermis. Horizontal lines: $\bar{x}$; bars: SD; vertical lines: total range; above: no. of fish (n)

Mucous cells

The results of the quantitative analysis of the mucous cells are shown in Fig. 3. No significant differences in the number of mucous cells open on the surface or within the epidermis could be detected. Additionally, microscopical examinations on the structure of the epidermis sections and the surface of the skin revealed no alterations due to the exposure to ammonia.

Gill structure

At the end of the experiment, in half of the trout exposed to NH_3 the gill tissue was proliferated in a clearly recognizable way, whereas in 19 of the 20 control fish no atypical proliferations could be found. The mean index of proliferation (Fig. 4) differed significantly between the 2 groups ($p < 0.05$). Slight hyperplasias affected only the distal parts of the primary lamellae, whereas more severe ones were determined as tissue swellings at the whole primary lamellae. There were remarkable individual differences in the degree of proliferation.

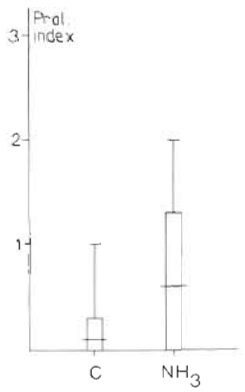

Fig. 4. *Salmo gairdneri*. Indices of proliferation of gill tissue of rainbow trout exposed to 0.25 mg l^{-1} NH_3 (stippled bars; n = 16) compared to controls (open bars; n = 20) after 28 d of experiment. Horizontal line: $\bar{x}$; bars: SD; vertical lines: total range

DISCUSSION

Increases in the ventilation frequency of fish after exposure to NH_3 have been reported by Flis (1968b), Smart (1975, 1978), Sousa & Meade (1977), Hillaby & Randall (1979), and Klontz et al. (1985). Smart (1978) also reported that the increase in the ventilation frequency was accompanied by increases in the ventilation volume and the amount of oxygen consumed. Possible causes discussed include: an NH_3-induced effect on the O_2 binding ability of hemoglobin; a decrease in the number of circulating erythrocytes; an increased energy consumption for the maintenance of the osmotic balance; and a general disturbance of the cellular metabolism (Brockway 1950, Reichenbach-Klinke 1967, Smart 1978).

Our findings revealed that after the initial increase of the ventilation frequency a quite rapid decrease occurred indicating that the trout were able to acclimatize to the toxicant to a certain degree.

Changes in weight gain by rainbow trout attributed to NH_3 have been observed to occur at concentrations as low as 0.12 and 0.03 mg l^{-1} (Larmoyeaux & Piper 1973, Klontz et al. 1985), and these were blamed in part on poor food utilization (Schreckenbach & Spangenberg 1978). Our results indicated however, that the significantly slower growth of the exposed trout in the first week, was probably due more to reduced and irregular food consumption than to poorer feed utilization. Thereafter, the differences in weight gain declined even though the NH_3 concentration applied was relatively high compared with the investigations mentioned above.

The increase in growth rate after the first week of exposure corresponded well with the decrease in ventilation frequency. This supports the assumption of an adaptation of the fish to the ammonia.

The structure of the rainbow trout epidermis and the quantitative data yielded by the mucous cell counts provided no evidence of NH_3 having an influence on mucus production, as described by such authors as Flis (1968b) and Schreckenbach & Spangenberg (1978). Possibly, the fishes had adapted their formation and release of mucus to the NH_3 level during the course of the experiment. A similar adaptation was suggested by Pickering & Macey (1977), who conducted experiments on the effects of stress (from repeated handling) on the mucous cells of *Salvelinus alpinus* (L.). In their experiments a significant increase in the number of mucous cells on the skin surface was detected after 1 wk. Thereafter, the number decreased until, after 1 mo, there was no longer any difference from the controls, even under continued stress.

However, it has to be kept in mind that our methods of investigation permitted only a determination of the momentary condition of the skin. It is possible that the number of recognizable mucous cells in the epidermis always remains constant, but that the time required for the formation and discharge of the mucous cells varies under the influence of NH_3. In this way, the amount of mucus discharged onto the body surface during a given period could change without being detectable by counting the number of mucous cells. In order to find a solution to this methodical problem, it would be more appropriate to determine the quantity of mucus actually secreted onto the skin.

The higher number of trout exhibiting gill proliferations in the exposed group indicated a possible effect of

NH_3 comparable to that reported by other authors (Burrows 1964, Flis 1968b, Larmoyeaux & Piper 1973, Smart 1976, Smith & Piper 1975, Peters et al. 1984, Soderberg et al. 1984, Klontz et al. 1985). However, the gill lesions observed in our experiment have to be considered as minor damage compared with the degree of gill proliferations described by the authors mentioned above although we applied much higher concentrations of the toxicant than they did. One possible cause for the different reactions of the gill tissue might have been different experimental designs used when maintaining the fish. Static systems, recirculating units, and systems with extremely low levels of water exchange may lead to accumulation of other metabolic products besides ammonia, which may act in an additive or synergistic way together with NH_3. These kinds of systems are also known to run the risk of developing elevated CO_2- or lowered O_2-levels which have both been proved to intensify the toxicity of NH_3 (Downing & Merkens 1955, Lloyd & Herbert 1960, Lloyd 1961).

In general, the effects of the pollutant that were recorded in this study could only be classified as minor, even though the NH_3 concentration used was approaching documented acutely lethal levels. Observed effects consisted chiefly of changes in the indicator parameters of weight gain and ventilation frequency. Gill damage occurring in some of the trout never reached levels reported by other authors or those observed in northern German aquaculture facilities (Peters et al. 1984). The results of the present study reveal that under the given experimental conditions, the fish were apparently capable of a relatively rapid adaptation to the toxic substance. Nevertheless, they must be considered stressed, as revealed by the continually elevated ventilation frequency, the slight decrease of the growth rate, and the slight degree of gill proliferation. However, results suggest that NH_3 alone probably cannot be responsible for the structural and metabolic disturbance of cultured fishes so frequently reported to be associated with the presence of this pollutant.

Acknowledgements. We thank Mrs S. Tollgreef for valuable technical help. The research was supported by Grant No. MFE 0528 of the Bundesminister für Forschung und Technologie. The authors are responsible for the contents. The study includes parts of the Ph. D. theses of T. Lang, R. Hoffmann and E. Meyer

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Editorial responsibility: Managing Editor; accepted for printing on December 11, 1987