The effects of surface access and dissolved oxygen levels on survival time of a water-breathing and an air-breathing fish species exposed to a plant toxin (Croton tiglium, Euphorbiaceae, seed extract).

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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GENERAL ABSTRACT

Brachydanio rerio (Cyprinidae) a water-breathing fish and *Clarias macrocephalus* (Clariidae) an air-breathing fish were subjected to 4 and 10 mg L^{-1}, respectively, of the extract of the *Croton tiglium* (Euphorbiaceae) seeds with and without surface access at different dissolved oxygen levels (0.8, 1.1 and 8.1 mg $O_2.L^{-1}$ for *Brachydanio*; and 0.7 and 8.1 mg $O_2.L^{-1}$ for *Clarias*) to investigate (a) whether the action of toxin is affected by level of dissolved oxygen and (b) whether fish exposed to toxin increase their survival time by breathing at the surface. For *Brachydanio*, the mortality occurred more quickly at lower than at higher levels of dissolved oxygen; under hypoxic conditions mortality also occurred more quickly when surface access was prevented than when access was provided. For *Clarias* with surface access in toxin, both air-breathing frequency and survival time were higher in hypoxia than in normoxia; when surface access was denied, survival time in toxin was greatly reduced under both hypoxia and normoxia.

RÉSUMÉ GÉNÉRAL

Brachydanio rerio (Cyprinidae), un poisson à respiration aquatique, et *Clarias macrocephalus* (Clariidae), un poisson à respiration aérienne, ont été soumis respectivement à 4 et 10 $mg.L^{-1}$ d'un extrait de graines de *Croton tiglium* (Euphorbiaceae) avec et sans accès à la surface de l'eau et ce, sous différentes concentrations d'oxygène (0.8, 1.1 et 8.1 mg $O_2.L^{-1}$ pour *Brachydanio*; 0.7 et 8.1 mg $O_2.L^{-1}$ pour *Clarias*) de façon à vérifier a) si l'effet de la toxine est affecté par la teneur en oxygène dissous et b) si les poissons exposés à la toxine peuvent accroître leur chance de survie par la respiration aérienne. Dans le cas de *Brachydanio* la mortalité fut plus rapide aux faibles concentrations d'oxygène. Sous ces mêmes conditions, la mortalité fut tout aussi rapide lorsque l'accès à la surface n'était pas permis. Dans les cas de *Clarias*, la fréquence de respiration aérienne de même que le temps de survie à la toxine, furent plus élevés en hypoxie qu'en normoxie lorsque l'accès à la surface était possible. Cependant, lorsque l'accès à la surface n'était pas possible, le temps de survie à la toxine fut grandement réduit et ce, sous les deux conditions d'oxygénation.

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PREFACE

This thesis consists of two papers, each presented as a chapter according to the regulations of the Faculty of Graduate Studies, which states:

> The candidate has the option, subject to the approval of the department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication.

Both the first and second paper presented will be submitted to the Canadian Journal of Fisheries and Aquatic Sciences. These papers are united as a thesis by a general conclusion. Dr. D.L. Kramer is the second author on each paper, because of his role throughout their development and his significant contribution to the final versions of the manuscripts. As primary author, I assume responsibility for validity of the data and theory presented in these papers.

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GENERAL INTRODUCTION

Several studies on water-breathing species of fish show that the toxicity of waterborne substances increases at low levels of dissolved oxygen (Southgate et al. 1933, Downing 1954, Lloyd 1961, Pickering 1968, Hicks and DeWitt 1971, Smith and Oseid 1972, Voyer et al. 1975, Thurston et al. 1981, Gupta et al. 1983 and Verma et al. 1985). Gupta et al. (1983) and Verma et al. 1985 also showed such an effect using bimodally respiring species at 5 mg $O_2.L^{-1}$.

All these studies on the effect of oxygen on toxicity have failed to consider the impact of surface access and its interaction with dissolved oxygen on toxicity. This appears to be a serious lapse because it is well established that surface access under hypoxia is important for the survival and activity of fish. Under hypoxia, water-breathing species rise to the water surface to respire at the well oxygenated thin surface film (Lewis 1970), a response termed as aquatic surface respiration (ASR) by Kramer and Mehegan (1981). During ASR, fish can reduce the frequency of gill ventilation significantly over what would be required if using the water column (Gee et al. 1978). ASR in hypoxic water improves oxygen levels in blood (Burggren 1982) and increases survival and activity (Kramer and Mehegan 1981, Kramer and McLure 1982). Fish with bimodal respiration also depend on surface access. These species are capable of respiring both dissolved and atmospheric oxygen, and during air breathing, gill ventilation may be reduced (Johansen 1970). Bimodal respiration characterizes a diverse array of species from the tropics (Jhingran 1975, p. 790-800) which are notable for their resistance to environmental hypoxia and other stresses (Dehadrai and Tripathi 1976).

These findings in respiratory physiology and behaviour suggest that in a toxic medium, by resorting to surface use (ASR or air breathing), fish can prolong their survival by reducing the rate of gill ventilation to lower the toxin uptake through the respiratory water current. Support for such a change in respiratory mode to reduce toxin uptake comes from anecdotal evidence for the voluntary surfacing of fish when exposed to different kinds of toxins, including rotenone (Bhuyan 1968, Konar 1970, Hickling 1971, p. 95, Chakraborty et al. 1972, Davies and Shelton 1983, Tiexeira et al. 1984, Hegen 1985) and copper sulfate (Kulakkattolickal personal observations on grass carp, Ctenopharyngodon idella, 1983).

There is experimental evidence that bimodal species of fish also adjust to the increased oxygen demand on exposure to toxins, by depending more on air breathing than on water breathing. Bakthavathsalam and Reddy (1983) showed that on exposure to lindane, Anabas testudineus showed a significant increase in the rate of oxygen uptake from air with relatively little increase in the rate of oxygen uptake from water. Natarajan (1981) found a similar effect for Channa striatus exposed to metasystox. Although both these works failed to relate the survival time of fish to oxygen uptake from air, it seems that such an increased dependency on air breathing rather than on water breathing to meet the increased oxygen demand produced by exposure to toxin, was to limit the toxin uptake as much as possible, through the respiratory water current. In addition to the studies on air-breathing fish already cited, Smatresk and Cameron (1982) have demonstrated that the gar (Lepisosteus oculatus) increased the use of atmosphere and decreased the use of dissolved oxygen on exposure to hyperosmotic solutions, and Burggren (1978) reported similar responses to dissolved carbon dioxide. So far no study has been conducted to establish the survival value of either air breathing or ASR in fish exposed to toxins in hypoxic water.

Based on the anecdotal and experimental studies already cited, I felt the need to establish the importance of surface access on survival time of fish exposed to toxins. Therefore, the main goals of this investigation were to quantify: (1) the effect of dissolved oxygen levels on toxicity of a waterborne toxin (Croton tiglium seed extract) to a water breathing species Brachydanio rerio, the zebrafish, and a bimodal species Clarias macrocephalus, (2) the role of surface access as a modifying factor of toxicity at different levels of dissolved oxygen for both species, and (3) the effect of toxin on the rate of air breathing in Clarias macrocephalus.

The original contributions of this study are as follows:

1) This study provides the first experimental evidence for the effect of surface access on toxicity for both air-breathing and a water-breathing species of fish.

2) This study gives the first experimental evidence that toxicity under hypoxia is magnified for a water-breathing species of fish when surface access is denied.

3) As an extension of previous studies showing that toxicity is increased at lower levels of dissolved oxygen, this study gives the first evidence of such an effect for a proteinaceous plant toxin on a water-breathing species of fish.

4) This study provides the first evidence for a decrease in toxicity at very low levels of dissolved oxygen for an air-breathing fish with surface access.

I believe that these results have applications in aquaculture and sampling of wild fish populations. When applying toxins to eradicate unwanted fish from aquacultural ponds (Bhuyan 1968), selecting a time of day with high or low dissolved oxygen levels as required and preventing surface access can increase the rate of action of toxins on fish. This should reduce the quantity of toxin applied and hence should result in increased cost-effectiveness and reduced environmental contamination.

The surfacing behaviour of fish in natural water bodies in response to rotenone has been used for sampling wildfish populations (Shireman et al. 1981). On the basis of the results of this study it seems that such surfacing behaviour is the toxin-induced aquatic surface respiration (for water-breathing species of fish) and perhaps air breathing (for air-breathing species of fish). If this is established through further research, this might result in better ways of manipulating the toxin-induced respiratory behaviour of fish for sampling wild fish populations.

LITERATURE CITED

BAKTHAVATHSALAM, R., AND Y.S. REDDY. 1983. Changes in bimodal oxygen uptake of an obligate air breather Anabas testudineus (Bloch) exposed to lindane. Water Res. 17: 1221-1226.

BHUYAN, B.R. 1968. A note on the use of Croton tiglium Linn. seed as a fish poison in ponds. J. Bombay Nat. Hist. Soc. 65: 236-240.

BURGGREN, W.W. 1978. Biomodal gas exchange during variation in environmental oxygen and carbon dioxide in the air breathing fish Trichogaster trichopterus. J. Exp. Biol. 82: 197-213.

BURGGREN, W.W. 1982. Air gulping improves blood oxygen transport during aquatic hypoxia in goldfish, Carassius auratus. Physiol. Zool. 55: 327-334.

CHAKRABORTY, D.P., A.C. NANDY, AND M.T. PHILIPOSE. 1972. Barringtonia acutangula (L.) Gaertn. as a fish poison. Indian J. Exp. Biol. 10: 78-80.

DAVIES, W.D., AND W.L. SHELTON. 1983. Sampling with toxicants, p. 119-213. In L.A. Nielsen and D.L. Johnson [ed.] Fisheries Techniques. Amer. Fish. Soc., Bethesda, Maryland.

DEHADRAI, P.V., AND S.D. TRIPATHI. 1976. Environment and Ecology of freshwater air-breathing teleosts, p. 39-74. In Respiration of amphibious vertebrates. G.M. Hughes (ed). Academic Press, London.

DOWNING, K.M. 1954. The influence of dissolved oxygen concentration on the toxicity of potassium cyanide to rainbow trout. J. Exp. Biol. 31: 161-164.

GEE, J.H., R.F. TALLMAN, AND H.J. SMART. 1978. Reactions of some great plains fishes to progressive hypoxia. Can. J. Zool. 56: 1962-1966.

GUPTA, S., R.C. DALELA, AND P.K. SAXENA. 1983. Influence of dissolved oxygen levels on acute toxicity of phenolic compounds to fresh water teleost, Notopterus notopterus (Pallas). Water Air Soil Pollut. 19: 223-228.

HEGEN, H.E. 1985. Use of rotenone and potassium permaganate in estuarine sampling. N. Am. J. Fish. Manage. 5: 500-502.

HICKS, D.B., AND J.W. DEWITT. 1971. Effects of dissolved oxygen on kraft pulp mill effluent toxicity. Water Res. 5: 693-701.

HICKLING, C.F. 1971. 2nd Edn, Fish culture. Faber and Faber, London. 317 p.

JHINGRAN, V.G. 1975. Fish and Fisheries of India. Hindustan Publishing Corporation (India), Delhi. 954 p.

JOHANSEN, K. 1970. Air breathing in fishes, p. 361-411, In W.S. Hoar and D.J. Randall [ed.] Fish Physiology. Vol. IV. Academic Press, New York.

KONAR, S.K. 1970. Nicotine as a fish poison. Prog. Fish-Cult. 32: 103-104.

KRAMER, D.L., AND M. MCLURE. 1982. Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. Env. Biol. Fish. 7: 47-55.

KRAMER, D.L., AND J.P. MEHEGAN. 1981. Aquatic surface respiration, an adaptive response to hypoxia in the guppy, Poecilia reticulata (Pisces, Poeciliidae). Env. Biol. Fish. 6: 229-313.

LEWIS, W.M., Jr. 1970. Morphological adaptations of cyprinodontoids for inhabiting oxygen-deficient waters. Copeia 1970: 319-326.

LLOYD, R. 1961. Effect of dissolved oxygen concentrations on the toxicity of several poisons to rainbow trout (Salmo gairdneri Richardson). J. Exp. Biol. 38: 447-455.

MATLOCK, G.C., J.E. WEAVER, AND A.W. GREEN. 1982. Sampling nearshore estuarine fishes with rotenone. Trans. Amer. Fish. Soc. 111: 326-331.

NATARAJAN, G.M. 1981. Changes in the bimodal gas exchange and some blood parameters in the air-breathing fish, Channa striatus (Bleeker) following lethal (LC50/48 Hrs.) exposure to Metasystox (Demeton). Curr. Sci. 50: 40-41.

PICKERING, Q.H. 1968. Some effects of dissolved oxygen concentrations upon the toxicity of zinc to the bluegill, Lepomis macrochirus, Raf. Water Res. 2: 187-194.

SMATRESK, N.J., AND J.N. CAMERON. 1982. Respiration and acid-base physiology of the spotted gar, a bimodal breather. III. Response to a transfer from fresh water to 50% sea water, and control of ventilation. J. Exp. Biol. 96: 295-306.

SMITH, L.L., AND D.M. OSEID. 1972. Effects of hydrogen sulfide on fish eggs and fry. Water Res. 6: 711-720.

SOUTHGATE, B.A., F.T.K. PENTELOW, AND R. BASSINDALE. 1933. CXXVII The toxicity to trout of potassium cyanide and p-cresol in water containing different concentrations of dissolved oxygen. Biochem. J. 27: 983-985.

TEIXEIRA, J.R.M., A.J. LAPA, C. SOUCCAR, and J.R. VALLE. 1984. Timbós: Ichthyotoxic plants used by Brazilian Indians. J. Ethnopharmacol. 10: 311-318.

THURSTON, R.V., G.R. PHILLIPS, R.C. RUSSO, AND S.M. HINKINS. 1981. Increased toxicity of ammonia to rainbow trout (Salmo gairdneri) resulting from reduced concentrations of dissolved oxygen. Can. J. Fish. Aquat. Sci. 38: 983-988.

VERMA, S.R., R. CHAND, AND I.P. TONK. 1985. Effects of environmental and biological variables on the toxicity of mercuric chloride. Water Air Soil Pollut. 25: 243-248.

VOYER, R.A., P.O. YEVICH, AND C.A. BARSZCZ. 1975. Histological and toxicological responses of the mummichog, Fundulus heteroclitus (L.) to combinations of levels of cadmium and dissolved oxygen in a freshwater. Water Res. 1069-1074.

CHAPTER I

The Effect of Surface Access and Oxygen Concentration on the Toxicity of *Croton tiglium* (Euphorbiaceae) Seed Extract to *Brachydanio rerio* (Cyprinidae)

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ABSTRACT

Zebrafish (Brachydanio rerio, Cyprinidae), a water-breathing fish, were tested in a 4 mg · L^{-1} extract of Croton tiglium (Euphorbiaceae) seeds at 0.8, 1.1 and 8.1 mg $O_2.L^{-1}$, with and without surface access. Equilibrium loss occurred more quickly at lower levels of dissolved oxygen than at higher levels. Under hypoxic conditions equilibrium loss also occurred more quickly when surface access was prevented than when it was provided. The results suggest that by performing aquatic surface respiration fish with surface access in hypoxic water reduce their ventilation rate and hence their uptake rate of toxins.

INTRODUCTION

A wide range of chemical pollutants are more toxic to fishes at lower than at higher levels of dissolved oxygen. Substances that produce a given mortality rate more rapidly or at lower concentrations under hypoxic conditions include cyanide (Southgate et al. 1933, Downing 1954), ammonia (Lloyd 1961, Thurston et al. 1981), hydrogen sulfide (Smith and Oseid 1972), phenols (Lloyd 1961, Gupta et al. 1983), heavy metals (Pickering 1968, Voyer et al. 1975, Verma et al. 1985) and kraft mill effluent (Hicks and Dewitt 1971). It is likely that hypoxia increases toxicity by its effect on ventilation rate which brings more toxin into contact with the gill epithelium, a major site of toxin absorption (Lloyd 1961). Given this, it is surprising that little attention has been given to the interaction between surface access and hypoxia in toxicological studies. Under hypoxic conditions most fish species rise to the air-water interface where they perform aquatic surface respiration (ASR), inspiring the oxygen-rich surface layer maintained by diffusion (Lewis 1970, Gee et al. 1978, Kramer and Mehegan 1981). ASR increases survival, growth, activity and blood oxygen levels under hypoxic conditions (Lewis 1970, Kramer and Mehegan 1981, Burggren 1982, Weber and Kramer 1983) and probably permits the maintenance of oxygen uptake at lower ventilation rates. Thus, it might be expected that surface access would improve survival in the presence of toxins. However, no studies have experimentally examined the effect of surface access on toxicity, and most studies of the interaction of oxygen concentration and toxicity even failed to record whether or not surface access was provided.

The present study was designed to examine the interaction between surface access and oxygen concentration on the toxicity of a waterborne substance. As a toxin I used an extract of the seeds of Croton tiglium (Euphorbiaceae). Croton seed extract is used in India to harvest wild fish for human consumption (unpublished observations) and has been suggested as a substitute for rotenone to remove predators and competitors before stocking fish ponds (Bhuyan 1968, Jhingran 1975, p. 550-554). If oxygen and surface access do influence toxicity, it may be possible to reduce economic costs and environmental contamination through the judicious choice of method and timing of piscicide application.

MATERIALS AND METHODS

C. tiglium seeds were imported from Kerala, India. The seed coats were removed from the seeds and the remaining portion was used in making the extract. To do this, 90 g of the material was frozen in liquid nitrogen and ground, using a mortar and pestle, until a thick paste was obtained. This was suspended in water in bleached cheese cloth (Fisher grade #50), filtered and made up to 9 L to give a 1% (weight/volume) stock solution. Aliquots of 30 mL were frozen and thawed at room temperature when needed.

As a test species I used 300 zebrafish, Brachydanio rerio (Cyprinidae) purchased from a local aquarium wholesaler ($\bar{x} \pm$ S.D., standard length 29.42 ± 2.43 mm, weight 390.3 ± 105.3 mg). This species is widely used in toxicological testing (Laale 1977) and is known to perform ASR (Lewis 1970).

Tests were carried out in aquaria measuring 76 x 30 x 30 cm deep divided into two equal sections by a plastic screen perforated by 1 mm diameter holes every 5 mm. On one side plexiglass supports were provided for a glass cover that could be positioned to prevent surface access. Adjacent to each aquarium was a 3.5 L jar in which oxygen levels (0.8, 1.1 and 8.1 mg $O_2 \cdot L^{-1}$) were controlled by bubbling in nitrogen or air. Water was pumped from the jar into both sides of the aquarium at a rate of 4 $L.min^{-1}$ and siphoned back into the jar. The entire apparatus contained a volume of 64 L. Tests using dyes verified that the water was well-mixed. Differences in oxygen levels between the two sides of the aquarium were corrected by manipulating the position of the water inlet tubes. Siphons in each half of the aquarium were used to collect water samples into a 250 mL graduated cylinder for oxygen measurement using a Yellow Springs Instruments Model 57 Oxygen Meter. For hypoxic trials the cylinder was filled with nitrogen gas before use to reduce oxygenation of the sample. All readings were corrected for changes in dissolved oxygen levels occurring during collection, using a previously determined calibration curve relating aquarium dissolved oxygen to cylinder reading. A detailed summary of oxygen variations is given in Appendix I. A two-way analysis of variance (Sokal and Rohlf 1981) revealed no significant effect of surface access or toxin treatments or their interaction on oxygen level. Variation among trials in oxygen level was relatively small: very hypoxic 0.8 ± 0.1 mg $O_2 \cdot L^{-1}$ (0.6 - 1.0 mg $O_2 \cdot L^{-1}$); moderately hypoxic 1.1 ± 0.1 mg $O_2 \cdot L^{-1}$ (1.0 - 1.3 mg $O_2 \cdot L^{-1}$); normoxic 8.1 ± 0.2 mg $O_2 \cdot L^{-1}$ (7.7 - 8.3 mg $O_2 \cdot L^{-1}$) $\bar{x}$ ± S.D., (range). Water temperature was maintained at 25 ± 0.6°C.

Five trials were conducted at each nominal oxygen concentration. Each trial involved one tank with and one tank without toxin, each tank having one section with and one without surface access. Two days before each trial fish were fin-clipped to permit individual identification. The experimental tanks were set up on the evening before the trial and filled with aged tap water. On the morning of the trial, five marked fish were randomly allocated to each half of both tanks and left (with surface access) for 4-5 h until the desired oxygen level was achieved. Then 12.8 mL of the stock solution of toxin was added to each side of the experimental aquarium to provide a 4 mg $\cdot$ L^{-1} concentration of toxin, and the glass covers were put in place. The fish were observed continuously for the next 5 h and the equilibrium loss time (ELT) of each individual was noted. Equilibrium loss was defined by the failure of fish to maintain their dorso-ventral body axis in the vertical plane without even a brief recovery. It was used as an indicator of mortality because of the difficulty of making more detailed observations of opercular or other movements on so many individuals. In preliminary experiments, fish that reached this stage never recovered upon transfer to fresh water. Tanks were detoxified after each trial by circulating in them an 8 mg $\cdot$ L^{-1} solution of potassium permanganate overnight.

For each treatment in each trial the median ELT in minutes was determined. Then a three-way analysis of variance (Sokal and Rohlf 1981) was performed on the median values of ELT to examine the effects of the presence or absence of toxin, presence or absence of surface access, level of dissolved oxygen, and their interactions. Subsequent comparisons of median values of ELT between particular treatments were made using t-tests.

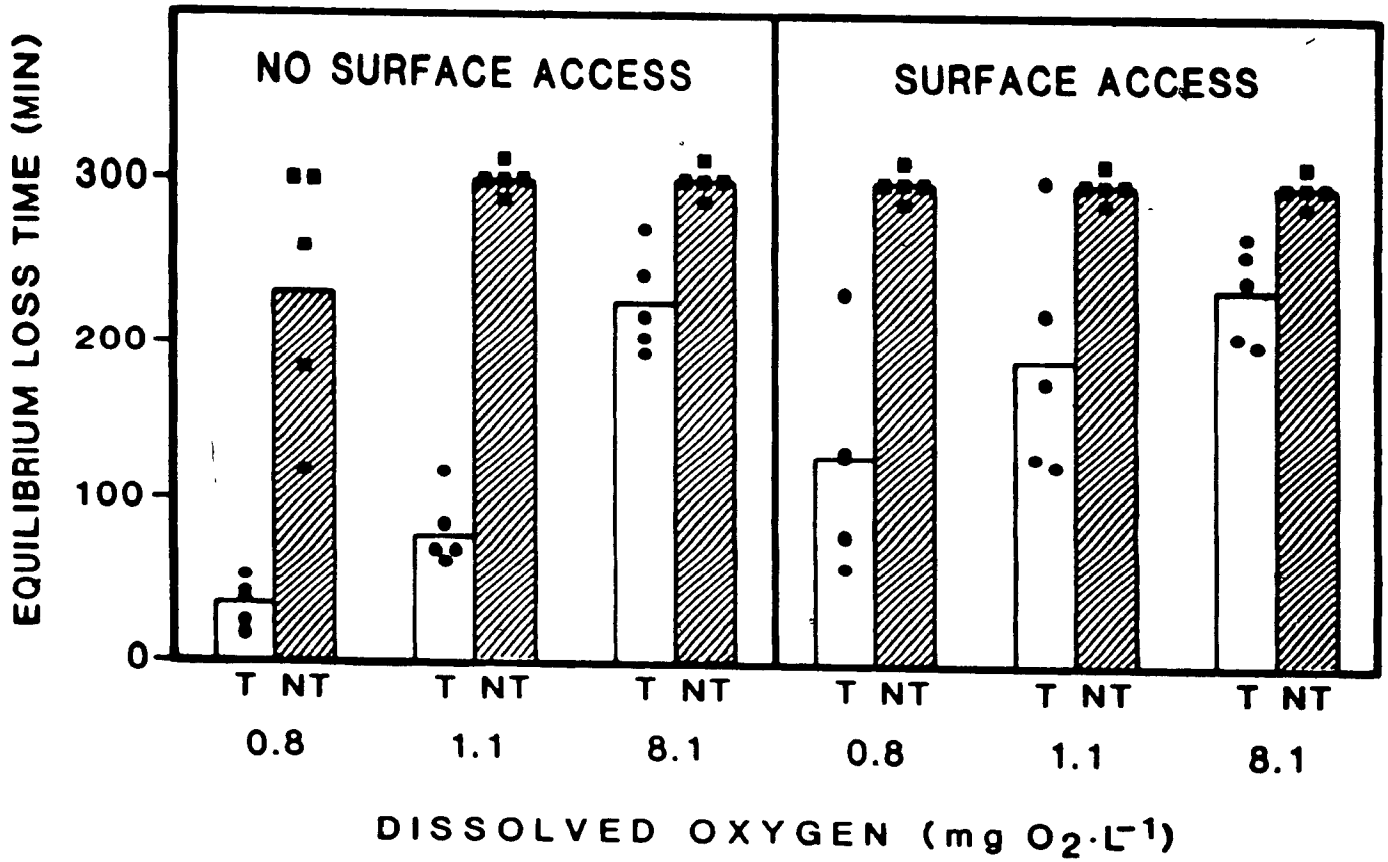
RESULTS

Figure 1 summarizes the results. Toxin, surface access, oxygen concentration and their two-way interactions all had significant effects on ELT (Table 1). With surface access and no toxin the median fish always survived the test period at all oxygen levels. With surface access and toxin the median fish always lost equilibrium before the test finished at all oxygen levels; ELT decreased as oxygen concentration decreased, but two-way comparisons were significant only for 0.8 vs 8.1 mg $O_2 \cdot L^{-1}$ ($p < 0.02$). Without surface access in the absence of toxin, the median fish survived in all trials at 8.0 and 1.1 mg $O_2 \cdot L^{-1}$ but died before the end of three of five trials at 0.8 mg $O_2 \cdot L^{-1}$. Without surface access in the presence of toxin the median equilibrium loss always occurred before the end of the trial and all comparisons of ELT between oxygen levels were highly significant. (0.8 vs 1.1 mg $O_2 \cdot L^{-1}$ $p < 0.0063$, 1.1 vs 8.1 mg $O_2 \cdot L^{-1}$ $p < 0.0001$, 0.8 and 8.1 mg $O_2 \cdot L^{-1}$ $p < 0.0001$) In addition, the ELTs of fish in toxin without surface access were significantly lower than those with surface access at both 0.8 ($p < 0.0175$) and 1.1 mg $O_2 \cdot L^{-1}$ ($p < 0.0115$), but was not at 8.1 mg $O_2 \cdot L^{-1}$. Behavioral observations during the first 30 min of treatment demonstrated high levels of surface use (over 60% of the time in contact with the surface) for fish in both toxin and control treatments at both 0.8 and 1.1 mg $O_2 \cdot L^{-1}$.

Table 1. Analysis of variance for equilibrium loss time of Brachydanio in relation to oxygen, toxin and surface access.

Source	Mean Square	F ratio	d.f.	Significance
Oxygen	41976.867	26.944	2	p 0.0001
Toxin	300900.017	193.142	1	p 0.0001
Access	32994.150	21.178	1	p 0.0001
Oxygen x Toxin	19060.467	12.235	2	p 0.0001
Oxygen x Access	7284.200	4.676	2	p 0.014
Toxin x Access	8906.017	5.717	1	p 0.021
Oxygen x Toxin x Access	3746.467	2.405	2	ns
Residual	1557.925			

Fig. 1. Comparison of equilibrium loss time of Brachydanio in toxin (T, open bars) and no toxin (NT, shaded bars) with and without surface access at three dissolved oxygen levels. Circles and squares represent the median values of survival time of 5 groups of 5 fish in toxin and no toxin, respectively. Each bar represents the mean of the medians; points clustered around the top of the bars indicate that the fish survived for the entire experimental period of 300 minutes.


NO SURFACE ACCESS
SURFACE ACCESS
EQUILIBRIUM LOSS TIME (MIN)
300
200
100
0
T NT
0.8
1.1
8.1
DISSOLVED OXYGEN (mg $O_2 \cdot L^{-1}$)

DISCUSSION

The present experiment confirms earlier findings that toxicity of waterborne substances to fishes increases as oxygen decreases. Previous studies have primarily involved heavy metals and small ions like ammonia and cyanide. Mine is apparently the first record for a plant toxin. The toxins of Croton seeds have not been completely characterized, but include proteins (Kraemer 1915, p. 475-476, Stirpe et al. 1976, Banerjee and Sen 1981). My study also provides the first direct evidence that surface access affects toxicity under hypoxic conditions. Not only did the fish in toxin live significantly longer when they had surface access than when they did not, but the lack of access accentuated the effect of oxygen levels on toxicity. Without surface access, the ELTs in all three oxygen treatments differed significantly from each other, while with access the ELTs in only the extreme levels were significantly different. This magnification, by the lack of surface access of the effect of hypoxia parallels previous studies of the effect of surface access, on growth and activity in hypoxic conditions (Weber and Kramer 1983). My results help to interpret a previous study of an apparent age-related effect of oxygen on toxicity. Graves et al. (1981) reported an effect of oxygen on the toxicity of kraft mill effluent to juvenile sheepshead minnows (Cyprinodon variegatus) although no effect on fry or adults was observed. Interestingly, only the test containers used for juveniles apparently lacked surface access.

My observations tend to support Lloyd's (1961) suggestion that the effect of low levels of oxygen on toxicity results from increased branchial ventilation. The effect of hypoxia on ventilation volume is well

established (e.g. Saunders 1962, Marvin and Heath 1968, Randall 1969). Other factors that increase oxygen demand, and hence ventilation volume, also increase toxicity. These include temperature (MacPhee and Ruelle 1969, Verma et al. 1985) and the presence of cestode parasites (Pascoe and Cram 1977). Rodgers and Beamish (1981) provided additional evidence by showing that with increased levels of activity rainbow trout Salmo gairdneri increased their uptake rate of methylmercury. I suggest that toxicity increases for similar reasons when surface access is prevented. Fish that are unable to perform ASR must ventilate at higher rates to meet their oxygen requirements (Gee et al. 1978). Fish are known to perform ASR when exposed to levels of hypoxia that are not lethal (Weber and Kramer 1983). The evidence from my study supports the conclusion that, in addition to possibly reducing the cost of ventilation, a reduction in toxin uptake may be a benefit of performing ASR. It is very important that future studies of the effect of hypoxia on fishes control and report surface access. In general, laboratory studies preventing surface access are likely to find more severe effects of hypoxia than may occur under natural conditions where fish can perform ASR.

Although Lloyd's (1961) hypothesis is plausible, there may be other reasons why low levels of dissolved oxygen increase the toxicity of substances in the water. Fish are apparently able to metabolize and detoxify or excrete some potential toxins (Lee et al. 1972, Brocksen and Bailey 1973, Thomas and Rice 1975). Because of the depressing effect of hypoxia on metabolic rate, fish may be less able to use such means to protect themselves. Thus, the availability of more oxygen through ASR should result in faster detoxification. The possibility that

oxygen permits detoxification in the test apparatus, for example by chemical oxidation or bacterial activity, also should not be discounted. Finally, there is the simple suggestion that a stressor such as a toxin or physical barrier is bound to have a stronger effect on a fish already under stress (e.g. by hypoxia).

The generality of the effect of oxygen on toxicity and the evidence that this effect is compounded by lack of surface access may have application to aquaculture because many fish rearing ponds show strong diurnal oxygen fluctuations (Hickling 1971, p. 48, Jhingran 1975, p. 356-357, Woynarovich 1975, p. 19). On the one hand, low oxygen levels can exacerbate the effects of contaminants in the water supply. On the other hand, in order to reduce the dose of toxin used to remove unwanted fish from aquaculture ponds before stocking, it may be useful to choose a time at which oxygen levels are minimal. In addition, it may be possible to prevent surface access by using a barrier such as plastic sheeting or even by frightening the fish away from the surface. The interaction of oxygen and surface access is an important process which should be considered in both laboratory and field studies involving toxins.

LITERATURE CITED

BANERJEE, K.K., AND A. SEN. 1981. Purification and properties of a lectin from the seeds of Croton tiglium with hemolytic activity toward rabbit red cells. Arch. Biochem. Biophys. 212: 740-753.

BHUYAN, B.R. 1968. A note on the use of Croton tigium Linn. seed as a fish poison in ponds. J. Bombay Nat. Hist. Soc. 65: 236-240.

BROCKSEN, R.W., AND H.T. BAILEY. 1973. Respiratory response of juvenile chinook salmon and striped bass exposed to benzene, a water soluble component of crude oil, p. 783-791. In Proceedings of joint conference on prevention and control of oil spills. Am. Pet. Inst., Environ. Prot. Agency, U.S. Coast Guard, Wash., D.C.

BURGGREN, W.W. 1982. Air gulping improves blood oxygen transport during aquatic hypoxia in goldfish, Carassius auratus. Physiol. Zool. 55: 327-334.

DOWNING, K.M. 1954. The influence of dissolved oxygen concentration on the toxicity of potassium cyanide to rainbow trout. J. Exp. Biol. 31: 161-164.

GEE, J.H., R.F. TALLMAN, AND H.J. SMART. 1978. Reactions of some great plains fishes to progressive hypoxia. Can. J. Zool. 56: 1962-1966.

GRAVES, W.C., D.T. BURTON, L.B. RICHARDSON, AND S.L. MARGREY. 1981. The interaction of treated bleached kraft mill effluent and dissolved oxygen concentration on the survival of the developmental stages of the sheepshead minnow (Cyprinodon variegatus). Water Res. 15: 1005-1011.

GUPTA, S., R.C. DALELA, AND P.K. SAXENA. 1983. Influence of dissolved oxygen levels on acute toxicity of phenolic compounds to fresh water teleost, Notopterus notopterus (Pallas). Water Air Soil Pollut. 19: 223-228.

HICKLING, C.F. 1971. Fish culture. 2nd Edn. Faber and Faber, London. 317 p.

HICKS, D.B., AND J.W. DEWITT. 1971. Effects of dissolved oxygen on kraft pulp mill effluent toxicity. Water Res. 5: 693-701.

JHINGRAN, V.G. 1975. Fish and Fisheries of India. Hindustan Publishing Corporation (India), Delhi. 954 p.

KRAEMER, H. 1915. Scientific and applied pharmacognosy for students of pharmacy and practicing pharmacists, food and drug analysts and pharmacologists. John Wiley & Sons, Inc. New York. 857 p.

KRAMER, D.L., AND M. MCCLURE. 1982. Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. Env. Biol. Fish. 7: 47-55.

KRAMER, D.L., AND J.P. MEHEGAN. 1981. Aquatic surface respiration, an adaptive response to hypoxia in the guppy, Poecilia reticulata (Pisces, Poeciliidae). Env. Biol. Fish. 6: 229-313.

LAALE, H.W. 1977. The biology and use of zebrafish, Brachydanio rerio in fisheries research. A literature review. J. Fish Biol. 10: 121-173.

LEE, R.F., R. SAUERHEBER, AND G.H. DOBBS. 1972. Uptake, metabolism and discharge of polycyclic aromatic hydrocarbons by marine fish. Mar. Biol. 17: 201-208.

LEWIS, W.M., Jr. 1970. Morphological adaptations of cyprinodontoids for inhabiting oxygen deficient waters. Copeia 1970: 319-326.

LLOYD, R. 1961. Effect of dissolved oxygen concentrations on the toxicity of several poisons to rainbow trout (Salmo gairdnerii Richardson). J. Exp. Biol. 38: 447-455.

MACPHEE, C., AND R. RUELLE. 1969. A chemical selectively lethal to squawfish (Ptychocheilus oregonensis and P. umpquae). Trans. Amer. Fish. Soc. 98: 677-684.

MARVIN, D.E., AND A.G. HEATH. 1968. Cardiac and respiratory responses to gradual hypoxia in three ecologically distinct species of fresh-water fish. Comp. Biochem. Physiol. 27: 349-355.

PASCOE, D., AND P. CRAM. 1977. The effect of parasitism on the toxicity of cadmium to the three-spined stickleback, Gasterosteus aculeatus L. J. Fish. Biol. 10: 467-472.

PICKERING, Q.H. 1968. Some effects of dissolved oxygen concentrations upon the toxicity of zinc to the bluegill, Lepomis macrochirus, Raf. Water Res. 2: 187-194.

RANDALL, D.J. 1970. Gas exchange in fish, p. 253-292. In W.S. Hoar and D.J. Randall [ed.] Fish Physiology, Vol. IV Academic Press, New York.

RODGERS, D.W., AND F.W.H. BEAMISH. 1981. Uptake of waterborne methylmercury by rainbow trout (Salmo gairdneri) in relation to oxygen consumption and methylmercury concentration. Can. J. Fish Aquat. Sci. 38:1309-1315.

SAUNDERS, R.L. 1962. The irrigation of the gills in fishes II. Efficiency of oxygen uptake in relation to respiratory flow activity and concentrations of oxygen and carbon dioxide. Can. J. Zool. 40: 817-862.

SMITH, L.L., AND D.M. OSEID. 1972. Effects of hydrogen sulfide on fish eggs and fry. Water Res. 6: 711-720.

SOKAL, R.R., AND F.J. ROHLF. 1981. Biometry, 2nd Edn. The principles and practice of statistics in biological research. W.H. Freeman and Co., New York. 859 p.

SOUTHGATE, B.A., F.T.K. PENTELOW., AND R. BASSINDALE. 1933. CXXVII. The toxicity to trout of potassium cyanide and p-cresol in water containing different concentrations of dissolved oxygen. Biochem. J. 27: 983-985.

STIRPE, F.A., A. PESSION-BRIZZI, E. LORENZONI, P. STROCCHI, L. MONTANARO, AND S. SPERTI. 1976. Studies on the proteins from the seeds of Croton tiglium and of Jatropha curcas. Toxic properties and inhibition of protein synthesis in vitro. Biochem. J. 156: 1-6.

THOMAS, R.E. AND S.D. RICE. 1975. Increased opercular rates of pink salmon (Oncorhynchus gorbuscha) fry after exposure to the water-soluble fraction of Prudhoe Bay crude oil. J. Fish. Res. Board Can. 32: 2221-2224.

THURSTON, R.V., G.R. PHILLIPS, R.C. RUSSO, AND S.M. HINKINS. 1981. Increased toxicity of ammonia to rainbow trout (Salmo gairdneri) resulting from reduced concentrations of dissolved oxygen. Can. J.Fish. Aquat. Sci. 38: 983-988.

VERMA, S.R., R. CHAND, AND I.P. TONK. 1985. Effects of environmental and biological variables on the toxicity of mercuric chloride. Water Air Soil Pollut. 25: 243-248.

VOYER, R.A., P.O. YEVICH, AND C.A. BARSZCZ. 1975. Histological and toxicological responses of the mummichog, Fundulus heteroclitus (L.) to combinations of levels of cadmium and dissolved oxygen in a freshwater. Water Res. 1069-1074.

WEBER, J.-M. AND D.L. KRAMER. 1983. Effects of hypoxia and surface access on growth, mortality, and behavior of juvenile guppies, Poecilia reticulata. Can. J. Fish. Aquat. Sci. 40: 1583-1588.

WOYNAROVICH, E. 1975. Elementary guide to fish culture in Nepal. Food and Agriculture Organization of the United Nations, Rome. 131 p.

CHAPTER II

The Role of Air Breathing in the Resistance of Bimodally Respiring Fish to Waterborne Toxins

by

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ABSTRACT

Under well oxygenated conditions (8.1 ± 0.4 mg $O_2.L^{-1}$) Clarias macrocephalus (Clariidae), an air-breathing fish, respond to toxic extracts (10 mg L^{-1}) of Croton tiglium (Euphorbiaceae) seeds by increasing their frequency of air breathing. When surface access is denied, survival in the absence of toxin is unaffected, but survival time in toxin is greatly reduced. Under hypoxic conditions (0.7 ± 0.1 mg $O_2.L^{-1}$) both air-breathing frequency and survival time in toxin are increased. These results suggest that air breathing increases the resistance of fish to toxins by permitting a decrease in the rate of gill ventilation and hence the rate at which toxins are absorbed.

INTRODUCTION

Fish with bimodal respiration are capable of using both dissolved and atmospheric oxygen (Johansen 1970). This respiratory mode characterizes a diverse array of species, including a number with economic importance. Some are the objects of subsistence and commercial fisheries, and others are significant in aquaculture, either as cultivated species or as unwanted predators in fish ponds (Dehadrai and Tripathi 1976). Many bimodal species are notable for their resistance to environmental stresses in addition to aquatic hypoxia (Dehadrai and Tripathi 1976). It is possible that air breathing renders fish more resistant to toxins by permitting the reduction of gill ventilation, thereby reducing contact with toxins at a major site of uptake. For water-breathing fish toxicity of a wide range of substances increases as dissolved oxygen decreases (Southgate et al. 1933, Downing 1954, Lloyd 1961, Pickering 1968, Hicks and DeWitt 1971, Smith and Oseid 1972, Voyer et al. 1975, Thurston et al. 1981, Gupta et al. 1983, Verma et al. 1985, Chapter I). Lloyd (1961) proposed that this increase in toxicity could be explained by the increased rate of ventilation in hypoxic water. His proposal is supported by the positive association between toxicity and temperature, which also increases oxygen demand and hence ventilation rate (Verma et al. 1985). Further evidence comes from studies by Rodgers and Beamish (1981) showing that the uptake of methylmercury by trout increases in association with increased swimming speed and oxygen uptake.

If toxicity is affected by ventilation rate we would expect strong hypoxia to decrease rather than increase toxicity in bimodal fishes, because at very low levels of dissolved oxygen these species reduce gill

ventilation and rely primarily upon air breathing (Johansen 1970, Graham et al. 1978, Gee 1980). At higher levels of dissolved oxygen, toxicity should increase when surface access is denied because of the higher rate of gill ventilation under these conditions (Singh and Hughes 1971). Finally, we might expect the fish to respond to toxins by reducing their rate of water breathing and increasing their air breathing to compensate for this. Short-term changes in respiratory partitioning are known in response to other factors which alter the costs of each respiratory mode. These include dissolved oxygen (Johansen 1970, Singh and Hughes 1971, Graham et al. 1977, Burggren 1978), carbon dioxide (Burggren 1979), water depth (Bevan 1986) and the risk of aerial predation (Smith and Kramer in press).

The goals of the present study were to determine how dissolved oxygen concentration and surface access interact to influence resistance of bimodal fish to waterborne toxins, and how the presence of toxins affects the rate of air breathing. It parallels a previous investigation of the effect of dissolved oxygen and surface access on toxicity in a water-breathing fish (Chapter I).

As a study species I chose the Asian catfish Clarias macrocephalus. Although less well studied than its congers C. batrachus and C. lazera (bibliographies by Clay 1977 and Vincke 1982), it too is a continuous, facultative air breather, i.e., it breathes air at all levels of dissolved oxygen but can survive without air breathing at normoxia (Bevan 1986). This species is reared commercially in Asia (Clay 1977, F.A.O. 1984). Members of this genus are also predators in aquaculture ponds where they prey on fry of other species (Jhingran 1975, p. 548-554). In Florida, U.S.A. C. batrachus is a pest which has spread widely following accidental

introduction (Idyll 1969).

I used an extract of Croton tiglium (Euphorbiaceae) seeds as toxin. Seeds of this species are crushed in streams to collect fish for food in Kerala State, India (personal observations), and it has been suggested as a substitute for imported rotenone to clear unwanted species of fish from aquaculture ponds before stocking (Bhuyan 1968, Jhingran 1975, p. 552).

MATERIALS AND METHODS

C. tiglium seeds were imported from Kerala, India. The seed coats were removed, and the remaining portion was used in making the extract. To do this 90 g of the material was frozen in liquid nitrogen and ground, using a mortar and pestle, until a thick paste was obtained. This was suspended in water in a bleached cheese cloth (Fisher grade #50), filtered and made up to 9 L to give a 1% (weight/volume) stock solution. Aliquots of 30 mL were frozen and thawed at room temperature when needed.

Clarias macrocephalus fry from Malaysia were raised in the laboratory. They were initially fed live Artemia nauplii and subsequently trout pellets (0.4 mm diameter, from Martin Feed Ltd.). One hundred and eighty-eight fish with standard length 10.53 ± 1.54 cm and weight 13.52 ± 5.85 g ($\bar{x}$ + S.D.) were fin-clipped for individual recognition and held in separate aquaria for two days before use in experiments.

The test apparatus consisted of aquaria 76 x 30 x 30 cm deep, divided into two equal sections by a plastic screen perforated by holes of 1 mm diameter every 5 mm. Plexiglass supports on one side permitted a glass

cover to be positioned to prevent surface access. Dissolved oxygen levels were adjusted by bubbling air or nitrogen into a 3.5 L jar adjacent to each aquarium from which water was pumped to both sides of the experimental tank at a rate of 4 L. min^{-1}. Siphons returned the water to the jar. The apparatus contained a total of 64 L of water. Dissolved oxygen levels in the two sides of the aquarium were corrected by manipulating the level of the inflow pipes bringing water from the jar. Samples for oxygen determination were obtained from siphons on each side. Water was siphoned into a 250 mL graduated cylinder which contained air (normoxic trials) or nitrogen gas (hypoxic trials). All oxygen readings were corrected for changes in dissolved oxygen levels due to diffusion from the air in the cylinder using a previously determined calibration curve relating aquarium dissolved oxygen to cylinder reading. A two-way analysis of variance (Sokal and Rohlf 1981) revealed no significant effect of access or toxin treatments or their interaction on oxygen concentration. The variation in trial mean oxygen values was: hypoxic 0.7 ± 0.1 mg $O_2.L^{-1}$ (0.6 - 0.9 mg $O_2.L^{-1}$) and normoxic 8.1 ± 0.4 mg $O_2.L^{-1}$ (7.7 - 8.8 mg $O_2.L^{-1}$), $\bar{x}$ ± S.D. (range). A detailed summary of oxygen variations is given in Appendix II. Water temperature was maintained at 25 ± 0.6°C.

Six trials were run under normoxic and six under hypoxic conditions. In each trial one tank received 64 mL of toxin (final concentration: 10 mg L^{-1}) and the other was untreated. Within each aquarium, fish on one side had surface access and on the other side they did not. On the evening preceding each trial four Clarias were randomly assigned to each half of each aquarium. The following morning oxygen level was adjusted while all

fish continued to have surface access. This required approximately 5 h for the hypoxic treatments; fish in normoxic trials were held for an equivalent period of time before the start of the experiments. Then the toxin was added in equal parts to both sides of the treated tank. Fish were watched continuously for the next 9 h, and the time of each death recorded. A fish was considered to have died when all visible movements ceased. For the first 30 min two observers recorded the total number of air breaths for each individual in the aquaria with surface access. In one of the hypoxic control tanks, the cover was accidentally lowered prematurely, reducing the number of trials to five.

The analysis was based on the median survival time for each trial of each treatment. A three-way analysis of variance (Sokal and Rohlf 1981) was performed with respect to the treatments toxin (present or absent), surface access (present or absent) and dissolved oxygen (normoxic or hypoxic). Similarly, median air-breathing frequencies of fish with surface access were analyzed by two-way analysis of variance (Sokal and Rohlf 1981) for the effects of toxin and dissolved oxygen. For both survival and air-breathing, particular treatments were compared by *t* tests.

RESULTS

All three treatments and their interactions had highly significant effects on survival time (Table 1). Without toxin all fish survived for the duration of the experiment except under hypoxic conditions without surface access where the median survival time averaged only 24 min

(Fig. 1). In the presence of toxin the median survival times with surface access averaged 367 min at 8.1 mg $O_2.L^{-1}$, and 471 min at 0.7 mg $O_2.L^{-1}$ (Fig. 1). This difference was statistically significant ($p < 0.0348$). Without surface access the effect of oxygen on survival time in toxin was reversed with median survival averaging 80 min at 8.1 mg $O_2.L^{-1}$ in contrast to 23.4 min at 0.7 mg $O_2.L^{-1}$ ($p < 0.0001$). With surface access survival time was greater than without access both at 8.0 mg $O_2.L^{-1}$ and at 0.7 mg $O_2.L^{-1}$ ($p < 0.0001$).

Both oxygen and toxin and their interaction also had highly significant effects on air-breathing frequency (Table 2). In the absence of toxin the fish took about 4.3 breaths per individual per hour under normoxia (Fig. 2), while under hypoxic conditions this frequency increased to a mean of 18.5 breaths per fish per hour ($p < 0.0048$). However, in the presence of toxin this dramatically increased to about 41.3 breaths per fish per hour (Fig. 2). This increase was significant at both 8.0 mg $O_2.L^{-1}$ ($p < 0.0001$) and at 0.7 mg $O_2.L^{-1}$ ($p < 0.0001$). But there was no effect of oxygen on the breathing rate in toxin ($p > 0.6918$).

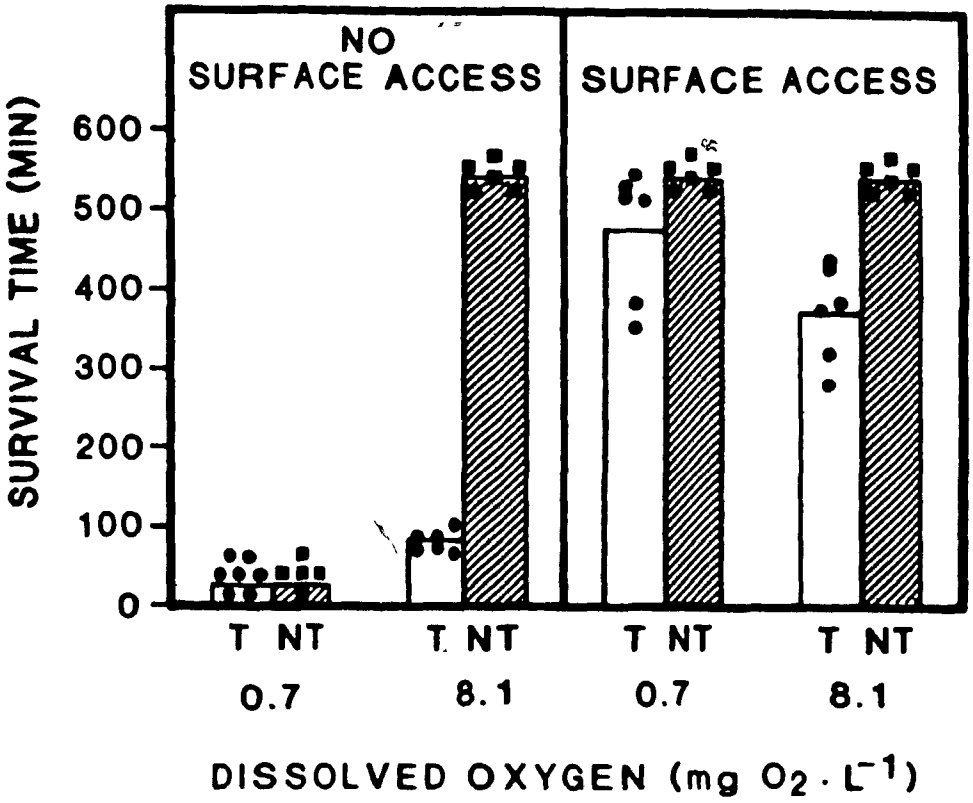
Table 1. Analysis of variance for survival time of *Clarias* in relation to dissolved oxygen, toxin and surface access.

Source	F ratio	d.f.	Significance
Oxygen	98.44	1	0.0001
Toxin	277.57	1	0.0001
Access	766.71	1	0.0001
Oxygen x Toxin	158.30	1	0.0001
Oxygen x Access	229.50	1	0.0001
Toxin x Access	26.75	1	0.0001
Oxygen x Toxin x Access	64.85	1	0.0001

Table 2. Analysis of variance for the number of air breaths per hour of *Clarias* in relation to dissolved oxygen and toxin.

Source	F ratio	d.f.	Significance
Oxygen	4.87	1	0.0390
Toxin	111.51	1	0.0001
Oxygen x Toxin	7.81	1	0.0110

Fig. 1. Comparison of survival time in toxin (T, open bars) and no toxin (NT, shaded bars) of Clarias when there is no surface access and when there is surface access at two dissolved oxygen levels. Circles and squares represent the median values of survival time of fish of each trial in toxin and no toxin, respectively. Each bar represents the mean of the medians of 6 trials (except no toxin at 0.7 mg $O_2 \cdot L^{-1}$ where there were only 5 trials). Six points clustered around the top of the bars indicate that the fish survived for the entire duration of the trial (540 minutes) or more.


NO
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SURFACE ACCESS
SURVIVAL TIME (MIN)
600
500
400
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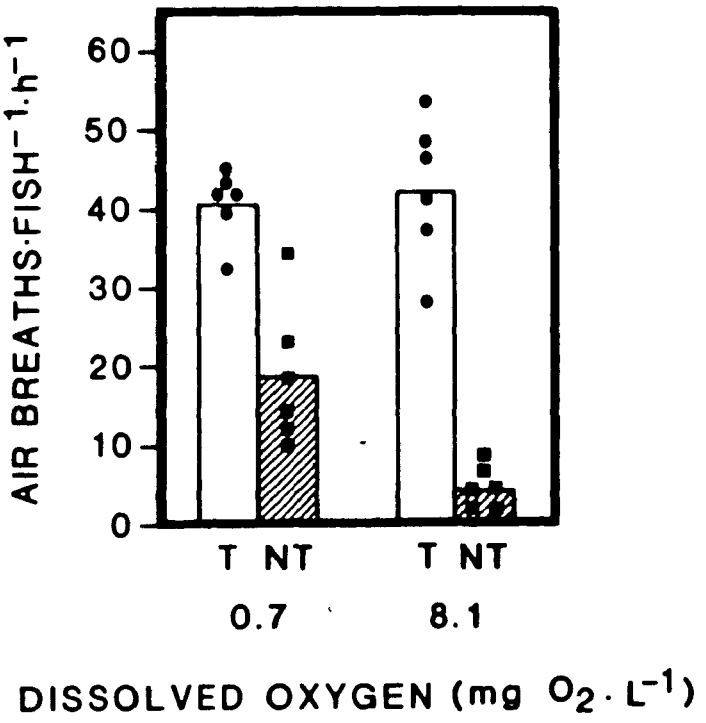
0
T NT
T NT
T NT
T NT
0.7
8.1
0.7
8.1
DISSOLVED OXYGEN (mg $O_2 \cdot L^{-1}$)

Fig. 2. Air breaths per fish per hour in toxin (T, open bars) and no toxin (NT, shaded bars) of Clarias measured at two dissolved oxygen levels for 30 minutes. Circles and squares represent the median values of air breaths per fish per hour in toxin and no toxin, respectively. Each bar represents the mean of the medians of the 6 trials.


AIR BREATHS·FISH^{-1}·h^{-1}
60
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DISSOLVED OXYGEN (mg $O_2 \cdot L^{-1}$)

DISCUSSION

My study shows that both dissolved oxygen level and surface access influence the toxicity of Croton seed extract to Clarias. Numerous previous studies have shown the effect of dissolved oxygen on the susceptibility of fish to toxins. Most report increasing toxicity with decreasing oxygen (Southgate et al. 1933, Downing 1954, Lloyd 1961, Pickering 1968, Hicks and DeWitt 1971, Smith and Oseid 1972, Voyer et al. 1975, Thurston et al. 1981, Verma et al. 1985 and Chapter I). These studies include many different types of toxins and species of fish, although I have found only two studies using a bimodal species (Gupta et al. 1983, Verma et al. 1985). A few report no effect (e.g. Voyer 1975). However, none has reported a decrease in toxicity with decreasing oxygen. Nevertheless, my findings are compatible with Lloyd's (1961) suggestion that hypoxia increases toxicity by increasing exposure of the gill epithelium to toxin. In bimodal species a fall in dissolved oxygen generally induces a rise in both air breathing and water breathing until a threshold point is reached at which water breathing declines sharply (e.g. Graham et al. 1978, Gee 1980). Previous studies on Clarias macrocephalus have demonstrated a peak water breathing frequency at 2.0 mg $O_2.L^{-1}$ while at 0.7 mg $O_2.L^{-1}$ water breathing was less frequent than at normoxia (Bevan 1986). Thus, lowered water breathing rates in my fish at hypoxia probably protected them from the toxin by reducing the amount of water contacting the gill surfaces. In the previous studies of effects of dissolved oxygen on the response of bimodal fish to toxin (Gupta et al. 1983, Verma et al. 1985), the increased susceptibility was probably due to an increase in gill ventilation at the lower dissolved oxygen levels

(near 5 mg $O_2.L^{-1}$ in both cases).

Even though the use of the surface for air breathing or aquatic surface respiration is an important part of the response of fish to hypoxia (Kramer 1983), surface access has been given little attention in studies of the interaction between toxins and dissolved oxygen levels. Sometimes surface access is not reported (e.g. Lloyd 1961, Thurston et al. 1981, Gupta et al. 1983), and it may even vary between experimental groups (e.g. Graves et al. 1981). Surface access is known to affect activity and feeding in hypoxic water (Weber and Kramer 1983). In a previous paper I showed that it affects toxicity of Croton seed extract to the water breather Brachydanio rerio (Chapter I). The present study confirms a similar effect for a bimodal species. Unlike Brachydanio, which was only affected by surface access under hypoxic conditions, Clarias was affected by access at normoxia. This is not surprising since Clarias breathes air under normoxic conditions. In a recent study Bevan (1986) showed that when prevented from air breathing at normoxia, Clarias macrocephalus increased their water breathing by a factor of almost two.

Air-breathing frequency of Clarias increased sharply in the presence of toxins. This suggests that fish can respond facultatively to waterborne contaminants by altering their partitioning of oxygen uptake towards atmospheric oxygen. Similar observations have been made for Channa striatus exposed to metasystox (Natarajan 1981) and Anabas testudineus exposed to lindane (Bakthavathsalam and Reddy 1983). In a study of the control of respiratory partitioning by the gar Lepisosteus oculatus, Smatresk and Cameron (1982) demonstrated an increased use of atmospheric oxygen and decreased use of dissolved oxygen when the gar were transferred

to hyperosmotic solutions. Similar responses to dissolved carbon dioxide have also been reported (Burggren 1978). However, I was unable to measure branchial ventilation frequencies under the conditions of our experiment, and it is possible that some of the increase in air breathing was due to increased oxygen demand. Several previous studies have demonstrated increased oxygen demand in the presence of toxins (Davis 1973, Hughes and Adeney 1977, Dalela et al. 1980, Johnstone and Hawkins 1980). In the studies of bimodal fish exposed to pesticides (Natarajan 1981, Bakthavathsalam and Reddy 1983), the significant increase in oxygen consumption through air breaths apparently functioned to meet the increased oxygen demand while there was a slight increase rather than decrease in oxygen consumption through water breathing. Nevertheless, it seems likely that the presence of potentially harmful substances is one of the factors controlling respiratory partitioning in bimodal fish. The capacity to reduce the uptake of such substances must be added to the list of other advantages to bimodal respiration. These include lowered cost of respiration and the capacity to survive emersion and extreme hypoxia (Kramer 1983). This apparently explains why air-breathing fish have a reputation for being particularly difficult to be controlled by poisoning (Bhuyan 1967, Jhingran 1975, p. 549).

There is a need for additional research on the problem of surfacing behavior of both bimodal and water-breathing fish exposed to toxins. A number of studies on plant toxins including rotenone refer to fish coming to the surface of the treated water (Bhuyan 1967, 1968, Hickling 1971, p. 95, Chakraborty et al. 1972, Matlock et al. 1982, Davies and Shelton 1983, Teixeira et al. 1984). Similarly I (unpublished observations) noted

grass carp (Ctenopharyngodon idella) surfacing in water treated with sub-lethal doses of copper sulfate. However, it is usually unclear whether this represented directed behaviour such as air breathing or aquatic surface respiration or simply the passive floating of dying fishes (but see Konar 1970 and Hegan 1985).

My study has implications for the use of piscicides to remove unwanted fish from aquaculture ponds and other water bodies. It suggests that toxins will be more effective when presented at times when gill ventilation rates are high. This will often occur at intermediate oxygen levels (Gee 1980, Bevan 1986). The effectiveness of the toxins could be increased still further by restricting access to the surface, perhaps even by frightening the fish as they approach. These techniques might not only increase the cost-effectiveness of the application of fish poisons, but reduce the level of environmental contamination as well.

LITERATURE CITED

BAKTHAVATHSALAM, R., AND Y.S. REDDY. 1983. Changes in bimodal oxygen uptake of an obligate air breather Anabas testudineus (Bloch) exposed to lindane. Water Res. 17: 1221-1226.

BEVAN, D.J. 1986. The effect of dissolved oxygen and water depth on respiratory behaviour and growth of Clarias macrocephalus (Pisces, Clariidae). M.Sc. thesis. McGill University Montreal.

BHUYAN, B.R. 1967. Eradication of unwanted fish from ponds by using indigenous plant fish poisons. Sci. and Cult. 33: 82-83.

BHUYAN, B.R. 1968. A note on the use of Croton tiglium Linn. seed as a fish poison in ponds. J. Bombay Nat. Hist. Soc. 65: 236-240.

BURGGREN, W.W. 1979. Bimodal gas exchange during variation in environmental oxygen and carbon dioxide in the air breathing fish Trichogaster trichopterus. J. Exp. Biol. 82: 197-213.

BURGGREN, W.W., AND D.J. RANDALL. 1978. Oxygen uptake and transport during hypoxic exposure in the sturgeon Acipenser transmontanus. Respir. Physiol. 34: 171-183.

CHAKRABORTY, D.P., A.C. NANDY, AND M.T. PHILIPOSE. 1972. Barringtonia acutangula (L.) Gaertn. as a fish poison. Indian J. Exp. Biol. 10: 78-80.

CLAY, D. 1977. Biology of the tropical catfish (Family: Clariidae) with special emphasis on its suitability for culture (including a bibliography of the Clariidae and related topics). Fish. Mar. Serv. Rep. 1458, 1-68.

DALELA, R.C., S.K. BANSAL, A.K. GUPTA, AND S.R. VERMA. 1980. Short-term stress on the oxygen consumption of a freshwater teleost Saccobranchus fossilis, following lethal and sublethal levels of Chlordane, Metasystox and Sevin. Int. J. Environ. Stud. 15: 229-235.

DAVIES, W,D., AND W.L. SHELTON. 1983. Sampling with toxicants, p. 199-213. In L.A. Nielsen and D.L. Johnson [ed.] Fisheries Techniques. Amer. Fish. Soc., Bethesda, Maryland.

DAVIS, J.C. 1973. Sublethal effects of bleached kraft pulp mill effluent on respiration and circulation in sockeye salmon (Oncorhynchus nerka). J. Fish. Res. Board Can. 30: 369-377.

DEHADRAI, P.V., AND S.D. TRIPATHI. 1976. Environment and ecology of freshwater air-breathing teleosts, p. 39-72. In Respiration of amphibious vertebrates. G.M. Hughes (ed). Academic Press, London.

DOWNING, K.M. 1954. The influence of dissolved oxygen concentration on the toxicity of potassium cyanide to rainbow trout. J. Exp. Biol. 31: 161-164.

F.A.O. 1984. Yearbook of fishery statistics. Vol. 54. Food and Agriculture Organization of the United Nations, Rome.

GEE, J.H. 1980. Respiratory patterns and antipredator responses in the central mudminnow, Umbra limi, a continuous, facultative, air-breathing fish. Can. J. Zool. 58: 819-827.

GRAHAM, J.B., D.L. KRAMER, AND E. PINEDA. 1977. Respiration of the air breathing fish Piabucina festae. J. Comp. Physiol. 122: 295-310.

GRAHAM, J.B., D.L. KRAMER, AND E. PINEDA. 1978. Comparative respiration of an air-breathing and a non-air-breathing characoid fish and the evolution of aerial respiration in characins. Phys. Zool. 51: 279-288.

GRAVES, W.C., D.T. BURTON, L.B. RICHARDSON, AND S.L. MARGREY. 1981. The interaction of treated bleached kraft mill effluent and dissolved oxygen concentration on the survival of the developmental stages of the sheepshead minnow (Cyprinodon variegatus). Water Res. 15: 1005-1011.

GUPTA, A.S., R.C. DALELA, AND P.K. SAXENA. 1983. Influence of dissolved oxygen levels on acute toxicity of phenolic compounds to fresh water teleost, Notopterus notopterus (Pallas). Water Air Soil Pollut. 19: 223-228.

HEGEN, H.E. 1985. Use of rotenone and potassium permanganate in estuarine sampling. N. Am. J. Fish. Manage. 5: 500-502.

HICKLING, C.F. 1971. Fish culture. Faber and Faber, London. 371 p.

HICKS, D.B., AND J.W. DEWITT. 1971. Effects of dissolved oxygen on kraft mill effluent toxicity. Water Res. 5: 693-701.

HUGHES, G.M., AND R.J. ADENEY. 1977. The effects of zinc on the cardiac ventilatory rhythms of rainbow trout (Salmo gairdneri, Richardson) and their responses to environmental hypoxia. Water Res. 11: 1069-1077.

IDYLL, C.P. 1969. New Florida resident, the walking catfish. Nat. Geogr. Mag. 135: 846-851.

JHINGRAN, V.G. 1975. Fish and Fisheries of India. Hindustan Publishing Corporation (India), Delhi. 954 p.

JOHANSEN, K. 1970. Air breathing in fishes, p. 361-411, In W.S. Hoar and D.J. Randall [ed.] Fish Physiology. Vol. IV. Academic Press, New York.

JOHNSTONE, A.D.F., AND A.D. HAWKINS. 1980. Changes in the respiration and blood circulation of cod, Gadus morhua L., induced by exposure to pollutants. Scott. Fish. Res. Report. No. 18, 18 p.

KONAR, S.K. 1970. Nicotine as a fish poison. Prog. Fish-Cult. 32: 103-104.

KRAMER, D.L. 1983. The evolutionary ecology of respiratory mode in fishes: an analysis based on the cost of breathing. Environ. Biol. Fishes 9: 145-158.

LLOYD, R. 1961. Effect of dissolved oxygen concentrations on the toxicity of several poisons to rainbow trout (Salmo gairdneri Richardson). J. Exp. Biol. 38: 447-455.

MATLOCK, G.C., J.E. WEAVER, AND A.W. GREEN. 1982. Sampling nearshore estuarine fishes with rotenone. Trans. Amer. Fish. Soc. 111: 326-331.

NATARAJAN, G.M. 1981. Changes in the bimodal gas exchange and some blood parameters in the air-breathing fish, Channa striatus (Bleeker) following lethal (LC50/48 Hrs.) exposure to Metasystox (Demeton). Curr. Sci. 50: 40-41.

PICKERING, Q.H. 1968. Some effects of dissolved oxygen concentrations upon the toxicity of zinc to the bluegill, Lepomis macrochirus, Raf. Water Res. 2: 187-194.

RODGERS, D.W., AND F.W.H. BEAMISH. 1981. Uptake of waterborne methylmercury by rainbow trout (Salmo gairdneri) in relation to oxygen consumption and methylmercury concentration. Can. J. Fish Aquat. Sci. 38: 1309-1315.

SINGH, B.N., AND G.M. HUGHES. 1971. Respiration of an air-breathing catfish Clarias batrachus (Linn.). J. Exp. Biol. 55: 421-434.

SMATRESK, N.J., AND J.N. CAMERON. 1982. Respiration and acid-base physiology of the spotted gar, a bimodal breather. III. Response to a transfer from fresh water to 50% sea water, and control of ventilation. J. Exp. Biol. 96: 295-306.

SMITH, L.L., AND D.M. OSEID. 1972. Effects of hydrogen sulfide on fish eggs and fry. Water Res. 6: 711-720.

SMITH, R.S., AND D.L. KRAMER. 1986. The effect of apparent predation risk on the respiratory behaviour of the Florida gar (Lepisosteus platyrhincus). Can. J. Zool. (in press).

SOKAL, R.R., AND F.J. ROHLF. 1981. Biometry, 2nd Edn. The principles and practice of statistics in biological research. W.H. Freeman and Co., New York. 859 p.

SOUTHGATE, B.A., F.T.K. PENTELOW, AND R. BASSINDALE. 1933. The toxicity to trout of potassium cyanide and p-cresol in water containing different concentrations of dissolved oxygen. Biochem. J. 27: 983-985.

TEIXEIRA, J.R.M., A.J. LAPA, C. SOUCCAR, AND J.R. VALLE. 1984. Timbós: Ichthyotoxic plants used by Brazilian Indians. J. Ethnopharmacol. 10: 311-318.

THURSTON, R.V., R.C. PHILLIPS, AND S.M. HINKINS. 1981. Increased toxicity of ammonia to rainbow trout (Salmo gairdneri) resulting from reduced concentrations of dissolved oxygen. Can. J. Fish. Aquat. Sci. 38: 983-988.

VERMA, S.R., R. CHAND, AND I.P. TONK. 1985. Effects of environmental and biological variables on the toxicity of mercuric chloride. Water Air Soil Pollut. 25: 243-248.

VINCKE, P. 1982. Catfishes of the genus Clarias - a general bibliography. F.A.O. Fish. Circ. No. 739, 38 p.

VOYER, R.A., P.P. YEVICH, AND C.A. BARSZCZ. 1975. Histological and toxicological responses of the mummichog, Fundulus heteroclitus (L.) to combinations of levels of cadmium and dissolved oxygen in a freshwater. Water Res. 9: 1069-1074.

WEBER, J.M., AND D.L. KRAMER. 1983. Effects of hypoxia and surface access on growth, mortality, and behavior of juvenile guppies Poecilia reticulata. Can. J. Fish. Aquat. Sci. 40: 1583-1588.

GENERAL CONCLUSION

This study has successfully shown that for a water-breathing species of fish toxicity is increased at lower levels of dissolved oxygen and that for an air-breathing species, toxicity is decreased towards lower levels of dissolved oxygen. This study has provided the first evidence for the effect of surface access on toxicity proving that toxicity is increased when surface access is blocked both for air-breathing and water-breathing species. This suggests that air-breathing and aquatic surface respiration help to raise survival time in toxic media by reducing toxin uptake through the respiratory water current. For the water-breathing species, the effect of dissolved oxygen on toxicity was magnified when surface access was blocked. On the basis of these results, it can be suggested that selecting a day or time of a day when dissolved oxygen levels are low or high as required, and blocking surface access can speed up the action of toxins on fish and hence has applications to eradicate unwanted fish from aquacultural ponds.

Appendix I: The three nominal dissolved oxygen levels (0.8, 1.2 and 8.0 mg $O_2 \cdot L^{-1}$) and the actual level under which experiments were conducted to determine the equilibrium loss time of zebrafish in toxin (T) and no toxin (NT) with access to the surface (A) and with no access to the surface (NA) at 25 ± 0.6°C.

Nominal dissolved oxygen (mg $O_2 \cdot L^{-1}$) level	Access	Toxin	Trial No	Dissolved oxygen (mg $O_2 \cdot L^{-1}$) on consecutive hours						
				1	2	3	4	5	6	$\bar{x}$
0.8	A	T	1	1.05	0.90	0.70	0.70	0.70	0.70	0.79
0.8	A	T	2	0.77	0.72	0.60				0.70
0.8	A	T	3	0.66	0.95	0.60	0.66	0.72		0.72
0.8	A	T	4	0.83	0.83	0.77	0.72			0.79
0.8	A	T	5	0.83	0.89	0.89				0.87
0.8	NA	T	1	0.9	0.85	0.8	0.7	0.7	0.7	0.78
0.8	NA	T	2	0.72	0.77	0.89				0.79
0.8	NA	T	3	0.66	0.77	0.60	0.66	0.72		0.68
0.8	NA	T	4	0.95	0.83	0.66	0.72			0.79
0.8	NA	T	5	0.83	1.07	1.13				1.01
0.8	A	NT	1	0.90	0.90	0.90	0.60	0.70	0.80	0.80
0.8	A	NT	2	0.72	0.77	0.60	0.60	0.54	0.54	0.63
0.8	A	NT	3	0.83	0.77	0.72	0.60	0.60	0.77	0.72
0.8	A	NT	4	0.83	0.83	0.83	0.77	0.72	0.77	0.79
0.8	A	NT	5	0.83	0.66	0.83	0.83	0.72	0.89	0.79
0.8	NA	NT	1	0.90	0.90	1.00	1.00	0.85	1.10	0.96
0.8	NA	NT	2	0.73	0.72	0.66	0.60	0.60	0.54	0.64
0.8	NA	NT	3	0.73	1.13	0.77	0.54	0.60	0.72	0.75
0.8	NA	NT	4	1.13	0.95	0.83	0.77	0.95	0.95	0.93
0.8	NA	NT	5	0.89	0.72	0.83	0.77	0.77	0.83	0.80

Nominal dissolved oxygen ($mg\ O_2 \cdot L^{-1}$) level	Access	Toxin	Trial No	Dissolved oxygen ($mg\ O_2 \cdot L^{-1}$) on consecutive hours						
				1	2	3	4	5	6	$\bar{x}$
1.2	A	T	1	1.01	1.25	1.07	1.01	1.01	1.01	1.06
1.2	A	T	2	1.13	1.55	1.37	1.13	1.19	1.01	1.23
1.2	A	T	3	1.19	1.55	1.13	1.13	1.55	1.01	1.26
1.2	A	T	4	1.19	1.07	0.89	1.01			1.04
1.2	A	T	5	1.25	1.13	1.07	0.89	0.89	0.13	1.06
1.2	NA	T	1	1.01	1.25	1.07	1.01	1.01	1.01	1.06
1.2	NA	T	2	1.19	1.43	1.43	1.25	1.37	1.01	1.28
1.2	NA	T	3	1.31	1.37	1.37	1.31	1.25	1.01	1.27
1.2	NA	T	4	1.25	1.01	1.01	1.25			1.13
1.2	NA	T	5	1.31	1.31	1.25	1.01	1.25	1.25	1.23
1.2	A	NT	1	1.07	1.25	1.07	1.01	1.07	1.13	1.10
1.2	A	NT	2	1.19	1.49	1.25	1.31	1.13	1.01	1.23
1.2	A	NT	3	1.19	1.43	1.01	1.01	1.37	1.01	1.17
1.2	A	NT	4	1.13	1.25	1.01	1.01	1.13	1.07	1.10
1.2	A	NT	5	1.25	0.95	0.89	0.77	1.01	0.95	0.75
1.2	NA	NT	1	1.01	1.19	1.01	1.01	1.07	1.01	1.05
1.2	NA	NT	2	1.25	1.49	1.25	1.37	1.07	0.95	1.23
1.2	NA	NT	3	1.31	1.37	1.25	0.89	1.13	1.01	1.16
1.2	NA	NT	4	1.19	1.37	1.19	0.95	1.19	1.13	1.17
1.2	NA	NT	5	1.25	1.07	1.01	0.77	1.19	1.01	1.12

Nominal dissolved oxygen (mg $O_2 \cdot L^{-1}$) level	Access	Toxin	Trial No	Dissolved oxygen (mg $O_2 \cdot L^{-1}$) on consecutive hours						
				1	2	3	4	5	6	$\bar{x}$
8.0	A	T	1	8.36	8.25	8.25	8.14	8.14	8.03	8.20
8.0	A	T	2	8.03	8.08	8.25	8.25	8.31	8.20	8.19
8.0	A	T	3	7.59	7.70	7.80	7.14	8.03	7.92	7.70
8.0	A	T	4	7.81	7.81	7.76	7.65	7.81	7.81	7.78
8.0	A	T	5	8.36	8.20	8.20	8.14	8.25	8.25	8.23
8.0	NA	T	1	8.36	8.25	8.25	8.14	8.14	8.03	8.20
8.0	NA	T	2	8.03	8.14	8.25	8.25	8.31	8.20	8.20
8.0	NA	T	3	7.70	7.80	7.80	8.14	8.25	8.25	7.99
8.0	NA	T	4	7.81	7.81	7.81	7.70	7.81	7.81	7.79
8.0	NA	T	5	8.36	8.25	8.25	8.14	8.14	8.25	8.23
8.0	A	NT	1	8.36	8.36	8.36	8.31	8.31	8.25	8.33
8.0	A	NT	2	8.03	8.03	8.20	8.20	8.25	8.20	8.15
8.0	A	NT	3	7.59	7.70	7.81	7.92	8.14	7.92	7.85
8.0	A	NT	4	7.92	7.92	7.50	7.50	7.60	7.70	7.70
8.0	A	NT	5	8.25	8.14	8.14	8.14	8.25	8.03	8.16
8.0	NA	NT	1	8.36	8.36	8.36	8.31	8.31	8.25	8.33
8.0	NA	NT	2	8.14	8.03	8.20	8.20	8.25	8.20	8.17
8.0	NA	NT	3	7.70	7.81	7.81	8.14	8.25	7.92	7.93
8.0	NA	NT	4	7.92	7.92	7.76	7.81	7.81	7.98	7.87
8.0	NA	NT	5	8.25	8.25	8.25	8.14	8.25	8.14	8.21

Appendix II: The two nominal dissolved oxygen levels (0.8 and 8.0 mg $O_2 \cdot L^{-1}$) and the actual level under which experiments were conducted to determine the survival time of Clarias in toxin (T) and no toxin (NT) with access to the surface (A) and with no access to the surface (NA) at 25 ± 0.6°C.

Nominal dissolved oxygen (mg $O_2 \cdot L^{-1}$) level	Access	Toxin	Trial No	Dissolved oxygen (mg $O_2 \cdot L^{-1}$) on consecutive hours										
				1	2	3	4	5	6	7	8	9	10	$\bar{x}$
0.8	A	T	1	0.72	0.72	0.72	0.59	0.59	0.59	0.59	0.54	0.42	0.42	0.59
0.8	A	T	2	0.77	0.72	0.72	0.60	0.83	0.77	0.72	0.72	0.72	0.66	0.72
0.8	A	T	3	0.66	0.84	0.77	0.66	0.54	0.60	0.72	0.72	0.95	0.66	0.71
0.8	A	T	4	0.84	0.89	0.89	0.72	0.77	0.77	0.89	0.60	0.60	0.60	0.76
0.8	A	T	5	0.77	0.48	0.54	0.72	0.60	0.72	0.77	0.83	0.60	0.66	0.67
0.8	A	T	6	0.72	0.66	0.83	0.83	0.72	0.77	0.72	0.72	0.60	0.60	0.72
0.8	NA	T	1	0.72	0.66	0.77	0.59	0.59	0.66	0.59	0.54	0.36	0.36	0.58
0.8	NA	T	2	0.83	0.60	0.54	0.60	0.60	0.66	0.54	0.54	0.54	0.60	0.61
0.8	NA	T	3	0.84	0.95	0.85	0.60	0.60	0.75	0.85	0.80	0.84	0.84	0.79
0.8	NA	T	4	0.89	0.72	0.89	0.66	0.77	0.72	0.60	0.66	0.60	0.54	0.71
0.8	NA	T	5	0.77	0.42	0.42	0.72	0.66	0.72	0.72	0.72	0.66	0.56	0.64
0.8	NA	T	6	0.72	0.54	0.60	0.83	0.77	0.66	0.60	0.42	0.42	0.48	0.60
0.8	A	NT	1	0.72	0.54	0.77	0.83	0.77	0.77	0.77	0.66	0.66	0.66	0.72
0.8	A	NT	2	0.77	0.77	0.77	0.89	0.89	0.72	0.77	0.72	0.60	0.66	0.76
0.8	A	NT	3	0.77	0.77	0.72	0.72	0.72	0.84	0.84	0.84	0.95	0.95	0.81
0.8	A	NT	4	0.89	0.95	0.83	0.77	0.83	0.77	0.72	0.66	0.72	0.66	0.78
0.8	A	NT	5	0.83	0.83	0.83	0.95	0.72	0.77	0.83	0.77	0.72	0.66	0.79
0.8	A	NT	6	0.83	0.66	0.60	0.77	0.77	0.77	0.60	0.72	0.77	0.77	0.73
0.8	NA	NT	1	0.77	0.59	0.77	0.72	0.77	0.83	0.66	0.59	0.54	0.54	0.68
0.8	NA	NT	2	0.83	0.77	0.77	0.95	0.83	0.83	0.89	0.77	0.66	0.72	0.80
0.8	NA	NT	3	0.85	0.90	0.85	0.80	0.80	0.90	0.85	0.90	0.90	0.89	0.86
0.8	NA	NT	4	0.95	0.48	0.84	0.77	0.84	0.72	0.66	0.60	0.66	0.72	0.72
0.8	NA	NT	5	0.83	0.83	0.83	0.83	0.83	0.83	0.89	0.60	0.77	0.72	0.80
0.8	NA	NT	6	0.77	0.42	0.66	0.60	0.77	0.66	0.60	0.66	0.72	0.77	0.66

Nominal dissolved oxygen ($mg\ O_2 \cdot L^{-1}$) level	Access	Toxin	Trial No	Dissolved oxygen ($mg\ O_2 \cdot L^{-1}$) on consecutive hours										$\bar{x}$
				1	2	3	4	5	6	7	8	9	10	
8.0	A	T	1	7.59	7.81	7.70	7.70	7.81	7.81	7.81	7.70	7.70	7.70	7.73
8.0	A	T	2	7.70	7.81	7.59	7.59	7.59	7.59	7.70	7.59	7.59	7.70	7.65
8.0	A	T	3	8.36	8.03	8.25	8.14	8.03	8.14	8.25	8.25	8.14	8.14	8.17
8.0	A	T	4	7.92	7.81	8.14	8.25	8.25	8.25	8.25	8.25	8.25	8.36	8.17
8.0	A	T	5	8.58	8.90	8.69	8.80	8.90	8.69	8.80	8.80	8.80	8.69	8.77
8.0	A	T	6	7.92	8.25	7.48	7.92	7.81	7.70	7.70	7.59	7.70	7.70	7.78
8.0	NA	T	1	7.81	7.81	7.70	7.81	7.70	7.81	7.81	7.70	7.81	7.81	7.78
8.0	NA	T	2	7.81	7.81	7.59	7.59	7.59	7.70	7.59	7.59	7.70	7.59	7.66
8.0	NA	T	3	8.42	8.03	8.25	8.14	8.14	8.14	8.14	8.14	8.25	8.14	8.18
8.0	NA	T	4	7.81	7.81	8.25	8.25	8.14	8.36	8.25	8.25	8.36	8.25	8.17
8.0	NA	T	5	8.58	8.8	8.8	8.8	8.8	8.8	8.8	8.8	8.8	8.8	8.8
8.0	NA	T	6	7.92	8.36	7.59	8.03	7.81	7.81	7.81	7.70	7.81	7.70	7.85
8.0	A	NT	1	7.59	7.59	7.70	7.70	7.81	7.81	7.81	7.70	7.70	7.81	7.72
8.0	A	NT	2	7.81	7.70	7.59	7.59	7.59	7.59	7.70	7.59	7.81	7.81	7.68
8.0	A	NT	3	8.36	8.03	8.14	8.25	8.36	8.25	8.14	8.25	8.25	8.36	8.24
8.0	A	NT	4	7.81	7.92	8.25	8.36	8.25	8.25	8.25	8.25	8.25	8.25	8.18
8.0	A	NT	5	8.69	8.69	8.69	8.69	8.80	8.80	8.69	8.69	8.69	8.69	8.71
8.0	A	NT	6	7.70	8.25	7.59	8.03	7.81	7.81	7.81	7.81	7.81	7.81	7.84
8.0	NA	NT	1	7.70	7.59	7.81	7.70	7.81	7.92	7.81	7.70	7.81	7.92	7.78
8.0	NA	NT	2	7.70	7.70	7.59	7.59	7.70	7.59	7.81	7.59	7.70	7.81	7.68
8.0	NA	NT	3	8.47	8.03	8.14	8.47	8.25	8.25	8.14	8.36	8.36	8.47	8.29
8.0	NA	NT	4	7.92	7.81	8.25	8.36	8.25	8.36	8.36	8.25	8.25	8.36	8.22
8.0	NA	NT	5	8.58	8.69	8.69	8.58	8.90	8.80	8.80	8.80	8.69	8.80	8.73
8.0	NA	NT	6	7.70	8.36	7.59	8.14	7.81	7.92	7.81	7.92	7.92	7.92	7.90