Branchial Na^+-K^+-ATPase Inhibition in a Freshwater Euryhaline Teleost, Tilapia (*Oreochromis mossambicus*), During Short-Term Exposure to Toluene or Naphthalene: Influence of Salinity

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ABSTRACT

The inhibition of branchial Na^+-K^+-ATPase activity in tilapia, Oreochromis mossambicus, *during short-term exposure to toluene or naphthalene at their lethal concentrations was more extensive in salt water than in fresh water. This effect of salinity was seen in fish preacclimated to salt water of 35‰ S before the hydrocarbon exposure at the same salinity, as well as in freshwater acclimated fish exposed to the pollutants in salt water of 20‰ S. The pollution-stressed fish also showed a reduced ability to increase the activity of this enzyme on subsequent transfer to salt water of 20‰ S. This greater enzyme inhibition in salt water probably contributed to the higher pollutant-induced mortality in these fish.*

Significant ($p < 0.05$) in vitro *inhibition by a relatively high concentration of 10 μM of toluene, or naphthalene, was observed in the enzyme activity from both freshwater- and saltwater-acclimated tilapia.*

INTRODUCTION

Due to the significance of Na^+-K^+-ATPase in teleost osmoregulatory mechanism (Epstein *et al.*, 1967; Karnaky *et al.*, 1976*a*), effects of various environmental stressors, all with a capacity to disrupt the functional integrity of this mechanism, on its activity are being extensively investigated. Different types of aquatic pollutants are included among these stressors. Such studies have shown that organic pollutants like DDT and petroleum hydrocarbons and heavy metal pollutants like

Environ. Pollut. Ser. A 0143-1471/86/$03·50 © Elsevier Applied Science Publishers Ltd, England, 1986. Printed in Great Britain

chromium and mercury affect Na^+-K^+-ATPase activity from fish tissues (Davis & Wedemeyer, 1971; Janicki & Kinter, 1971; Kinter *et al.*, 1972; Leadem *et al.*, 1974; Kuhnert *et al.*, 1976; Bouquegneau, 1977; Boese *et al.*, 1982).

The present study deals with effects of exposure of a euryhaline teleost fish to individual petroleum hydrocarbons, namely toluene and naphthalene, generally regarded as the constituents responsible for toxicity of crude and refined oils, and their water-soluble fractions to aquatic organisms (Boylan & Tripp, 1971; Anderson *et al.*, 1974), on the activity of this enzyme in gills, and consequently the ability of such fish to survive the salinity stress. The experimental fish tilapia, *Oreochromis mossambicus*, is a hardy freshwater euryhaline species capable of tolerating saltwater concentrations of up to 60–65‰ S (Assem & Hanke, 1979; Dangé, 1985). It responds to the hyperosmotic stress by increased branchial Na^+-K^+-ATPase activity (Dangé, 1985). Some effects of toluene and naphthalene on the general metabolism of this fish are already known (Dangé & Masurekar, 1981, 1982, 1984; Dangé, 1986*a*).

MATERIAL AND METHODS

Tilapia (40–50 g) were collected and maintained in the laboratory as described earlier (Dangé & Masurekar, 1982). During this period, some of the stock fish were acclimated to salt water of 35‰ S, by a gradual, stepwise process (Dangé, 1985). After a minimum of eight weeks of acclimation at the required salinity level, groups of both freshwater- and saltwater-acclimated fish were exposed to sublethal or lethal concentrations of the hydrocarbons (50% and 100% 96-h LC_{50} levels in fresh water: toluene—45 and 90 mg l^{-1}; naphthalene—3·95 and 7·9 mg l^{-1}) in water of same salinity, according to the static exposure procedure of American Public Health Association (1971), with 24-h water and pollutant replacement, as reported earlier (Dangé & Masurekar, 1982; Dangé, 1986*a*). These hydrocarbon concentrations were selected on the basis of short- and long-term static bioassays conducted previously (Dangé, 1979). The terms sublethal and lethal concentrations refer to their effects on mortality rates in fresh water alone, and will be used henceforth only in this sense. At the end of 24 and 96 h of exposure, the required number of exposed and unexposed control fish were

sacrificed for enzyme assays. The methods for preparation of homogenates of branchial filaments and assay of the specific activity of Na^+-K^+-ATPase are described elsewhere (Dangé, 1985).

In order to study effects of exposure to the combined stress of increased salinity and hydrocarbon pollution, freshwater-acclimated fish were similarly exposed to toluene or naphthalene. However, in this experiment the selected toxicant concentrations were prepared in salt water of 20‰ S. The changes in enzyme activity were measured after 24 and 96 h.

Another set of experiments was conducted to study the ability of pollution-affected fish to adapt to a subsequent hyperosmotic shock. For this purpose, freshwater-acclimated tilapia were subjected to the pollution stress in fresh water for 96 h, and then immediately transferred to salt water of 20‰ S. The branchial Na^+-K^+-ATPase activity was measured 24 and 96 h after this transfer.

Rates of mortality during 24 and 96 h of the specific experimental periods were calculated for control and stressed fish in all these experiments.

For the *in vitro* study, homogenates of gill filaments from unexposed freshwater- and saltwater-acclimated tilapia were used. A series of reaction mixtures was prepared for each homogenate, with each mixture having a different concentration of toluene or naphthalene (0, 0·1, 1, 5 and 10 μM). The rest of the procedure for enzyme assay was the same as above.

Student's *t*-tests for unpaired and paired data were employed to check the significance (at $p < 0{\cdot}05$ level) of *in vivo* and *in vitro* effects, respectively.

RESULTS

The branchial Na^+-K^+-ATPase activity in freshwater-acclimated tilapia was unaffected by exposure to sublethal concentrations of toluene or naphthalene in fresh water for 24 or 96 h (Fig. 1). Even in tilapia exposed to lethal concentrations of the hydrocarbons for 24 h, the enzyme activity was not noticeably different from that in unexposed control fish. However, lethal concentrations caused a significant enzyme inhibition during the more extended exposure period of 96 h—toluene by 16% and naphthalene by 22%.

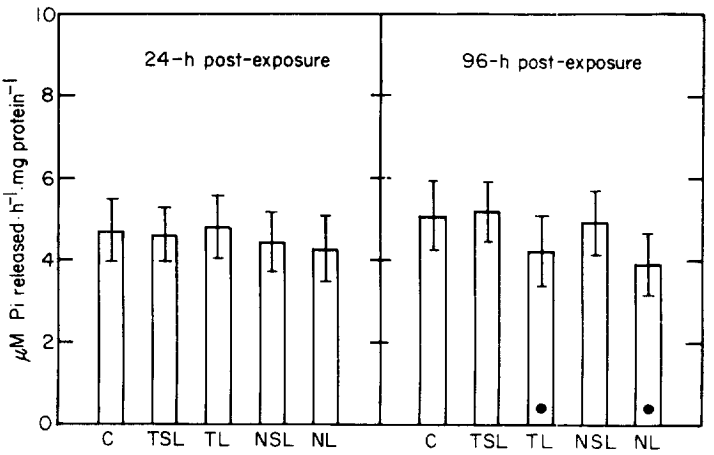

Fig. 1. Effects of short-term exposure in fresh water to toluene or naphthalene on the branchial Na^+-K^+-ATPase activity in freshwater-acclimated tilapia. The values represent means of 10 samples ±SD, for control fish (C) and for fish exposed to sublethal or lethal concentrations of toluene (TSL and TL, respectively) or naphthalene (NSL and NL, respectively). Solid circle within a column indicates statistical significance ($p < 0.05$) of the change from the control value.

A generally similar trend was observed in saltwater-acclimated tilapia exposed to the hydrocarbons in salt water, but in this case the changes were more extensive (Fig. 2). Thus the lethal concentrations of toluene and naphthalene inhibited the enzyme activity by 32% and 37%, respectively, in 96 h.

The changes in enzyme activity in freshwater-acclimated tilapia subjected to the combined pollution and salinity stresses were almost similar to those in fish acclimated to salt water prior to their exposure to the pollutants (Fig. 3). In these fish, the enzyme activity was lowered by 31% and 33%, respectively, by lethal concentrations of toluene and naphthalene for 96 h exposure.

When freshwater-acclimated tilapia were first exposed to the pollution stress for 96 h, and then subjected to the salinity stress, branchial Na^+-K^+-ATPase activity was reduced by 24% and 22% in fish that had been exposed to lethal concentrations of toluene and naphthalene, respectively, at the end of 24 h of salinity stress (Fig. 4). The effects of these concentrations at 96 h after the saltwater transfer could not be assayed due to 100% mortality. At this time, the enzyme activity, in fish which had been only sublethally stressed prior to saltwater transfer,

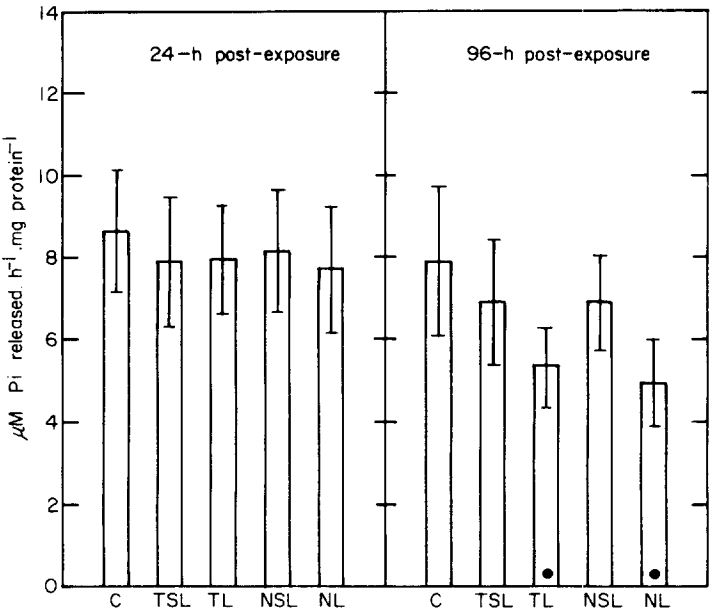

Fig. 2. Effects of short-term exposure in salt water of 35‰ S to toluene or naphthalene on the branchial Na^+-K^+-ATPase activity in tilapia pre-acclimated to the same salinity. The values represent means of 10 samples ±SD, for control fish (C) and for fish exposed to sublethal or lethal concentrations of toluene (TSL and TL, respectively) or naphthalene (NSL and NL, respectively). Solid circle within a column indicates statistical significance ($p < 0.05$) of the change from the control value.

fell by a degree comparable to that seen in freshwater-acclimated tilapia exposed to the lethal toluene concentration in fresh water for 96 h, but the changes were not statistically significant.

In vitro experiments showed that the lower three of the selected concentrations of the two hydrocarbons did not affect the enzyme activity from both freshwater- and saltwater-acclimated tilapia (Figs. 5 and 6). However, the highest concentration of 10 μM inhibited enzyme activity to a comparable degree in both the fish groups, with naphthalene being only marginally more effective than toluene in this respect.

The first two experiments also showed that the fatal effects of the hydrocarbons were salinity dependent (Fig. 7). Both the hydrocarbons seemed to be more lethal in salt water, than in fresh water. The effects were still greater in freshwater-acclimated tilapia exposed simultaneously to the two environmental perturbations—pollution and osmotic.

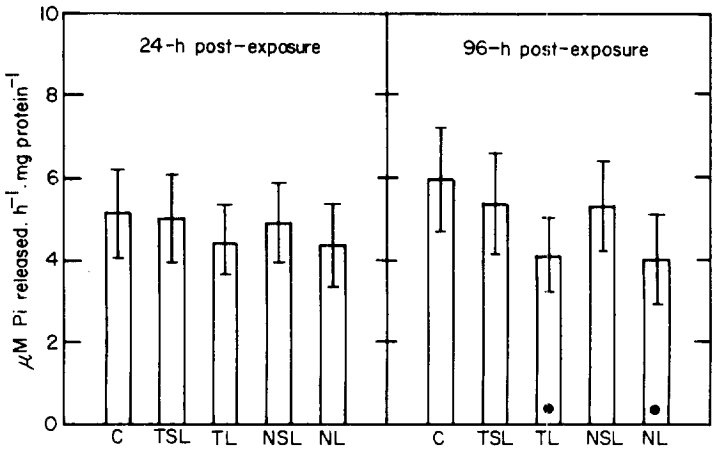

Fig. 3. Effects of short-term exposure in salt water of 20‰ S to toluene or naphthalene on the branchial Na^+-K^+-ATPase activity in freshwater-acclimated tilapia. The values represent means of 10 samples ±SD, for control fish (C) and for fish exposed to sublethal or lethal concentrations of toluene (TSL and TL, respectively) or naphthalene (NSL and NL, respectively). Solid circle within a column indicates statistical significance ($p < 0.05$) of the change from the control value.

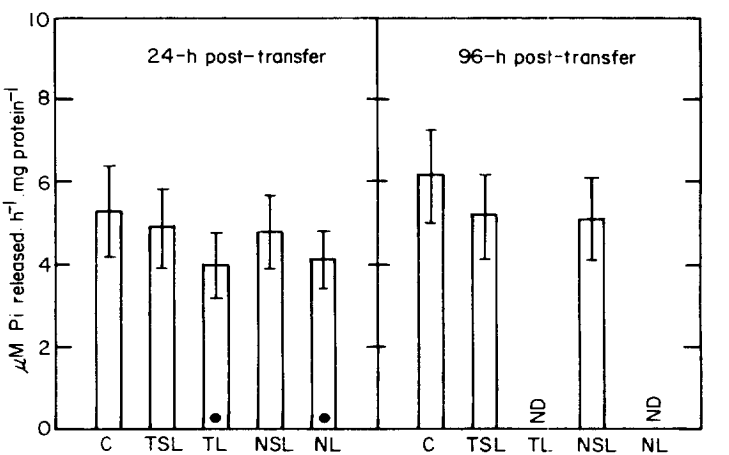

Fig. 4. Effect of transfer to salt water of 20‰ S on the branchial Na^+-K^+-ATPase activity in freshwater-acclimated tilapia pre-exposed to toluene or naphthalene for 96 h. The values represent means of 10 samples ±SD, for control fish (C) and fish exposed to sublethal or lethal concentrations of toluene (TSL and TL, respectively) or naphthalene (NSL and NL, respectively). Solid circle within a column indicates statistical significance ($p < 0.05$) of the change from the control value. ND indicates the values not determined due to 100% mortality.

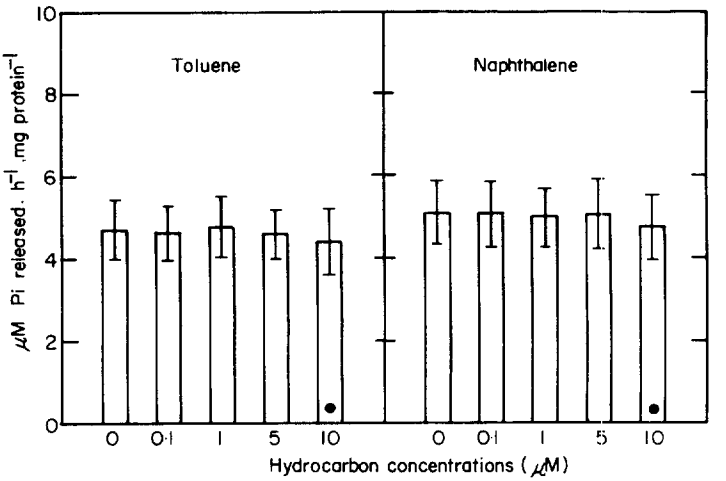

Fig. 5. *In vitro* effects of toluene and naphthalene on the branchial Na^+-K^+-ATPase activity from freshwater-acclimated tilapia. The values represent means of 10 samples $\pm$SD. Solid circle within a column indicates statistical significance ($p < 0{\cdot}05$) of the change from the control (zero concentration) value.

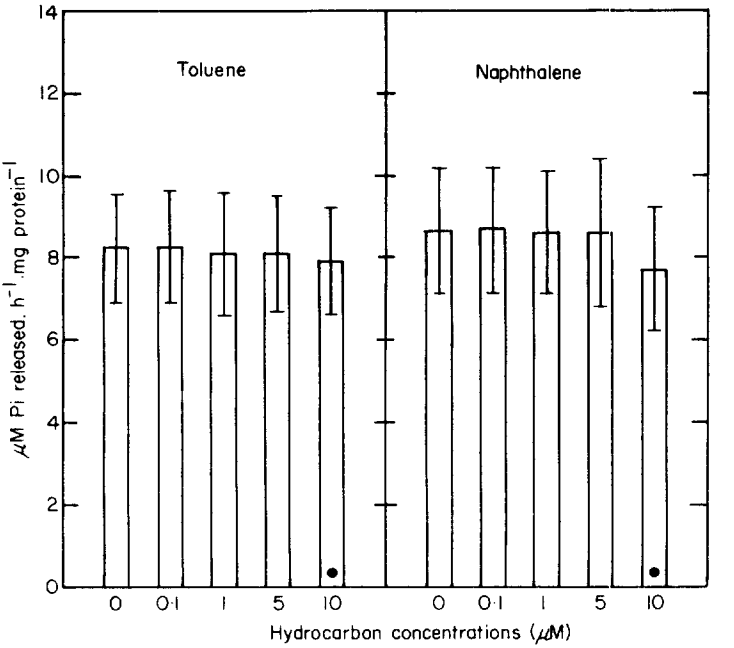

Fig. 6. *In vitro* effects of toluene and naphthalene on the branchial Na^+-K^+-ATPase activity from tilapia acclimated to salt water of 35‰ S. The values represent means of 10 samples $\pm$SD. Solid circle within a column indicates statistical significance ($p < 0{\cdot}05$) of the change from the control (zero concentration) value.

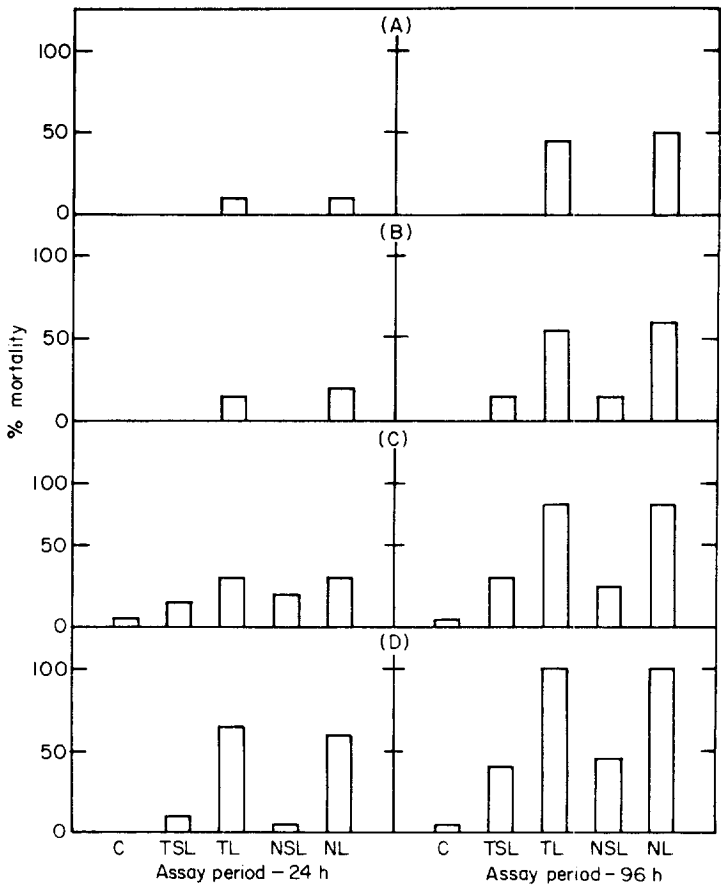

Fig. 7. Effects of the hydrocarbon stress, alone or in combination with the salinity stress, on the mortality rate in tilapia. Each group consisted of 20 fish at the start of the experimental period during which observations on the number of deaths were made. Values are given for control fish (C) and fish exposed to sublethal or lethal concentrations of toluene (TSL and TL, respectively) or naphthalene (NSL and NL, respectively). Experimental regimes are (A) freshwater-acclimated fish exposed to the hydrocarbons in fresh water; (B) saltwater (35‰ S)-acclimated fish exposed to the hydrocarbons in salt water of 35‰ S; (C) freshwater-acclimated fish exposed to the hydrocarbons in salt water of 20‰ S; and (D) freshwater-acclimated fish exposed to the hydrocarbons in fresh water for 96 h and then transferred to salt water of 20‰ S.

The pollution stress also seemed to affect the ability of freshwater tilapia to adjust to a subsequent salinity stress. During 96 h, the salinity stress killed all the fish that had previously survived the lethal hydrocarbon exposure in fresh water. Even in fish that previously had been only sublethally treated, mortality rates almost reached the levels seen in fish exposed to the lethal hydrocarbon concentrations in fresh water.

DISCUSSION

Generally, the branchial Na^+-K^+-ATPase activity in tilapia exposed to toluene, or naphthalene, at their lethal concentrations was lower than that in the unexposed control fish. The results of the present study were thus consistent with the earlier report that the activity of this enzyme in gills of fish is reduced by their exposure to petroleum refinery waste water, especially if the latter contains a high proportion of naphthalene and other solvent extractable organic compounds (Boese *et al.*, 1982). This enzyme inhibition by petroleum hydrocarbons occurred in spite of a possible rise in plasma cortisol levels in the exposed fish, which has been reported for other hydrocarbon stressed fish (DiMichele & Taylor, 1978; Thomas *et al.*, 1980). This hormone is known to stimulate enzyme activity in tilapia (Dangé, 1986*b*).

Though the mechanism of this Na^+-K^+-ATPase inhibition by *in vivo* hydrocarbon exposure is as yet not clear, a few possibilities can be suggested.

(i) Toluene and naphthalene produce significant histopathological changes in the structure of gills in tilapia, even during a short exposure period of 96 h (A. D. Dangé, unpublished observations). These include inflammation of gills with swelling of branchial epithelium, separation of epithelial walls of secondary lamellae from pillar cells, fusion of neighbouring secondary lamellae, and partial to complete erosion, or degeneration, of secondary lamellae, especially those near the tips of branchial filaments. The spread and severity of these changes was directly related to the level and duration of exposure. Such damage to gill tissue could be expected to reduce the number of viable chloride cells, which are known to contain the major portion of branchial Na^+-K^+-ATPase activity in fish (Kamiya, 1972; Karnaky *et al.*, 1976*b*), thereby leading to a fall in the enzyme activity.

(ii) It has been reported that the enzyme is located on the basolateral membranes of chloride cells (Karnaky *et al.*, 1976*b*). It is reasonable to assume that the hydrocarbons, being lipophilic in nature, can bind to such lipid membranes and then act as allosteric inhibitors of the enzyme activity, as suggested for other lipid-soluble pollutants (Kinter *et al.*, 1972; Jackson & Gardner, 1978). It may also be conjectured here that the lack of the expected effect of increased cortisol levels, mentioned earlier, could be due to a loss of necessary receptor sites from the altered surface of such cells.

(iii) The hydrocarbons may also directly inhibit the enzyme activity as suggested by the *in vitro* experiments which showed that the enzyme activity was significantly lowered by the highest selected hydrocarbon concentration of 10 μM. However, at present it is not known whether the gills in tilapia are capable of accumulating hydrocarbons to the relatively high levels required for effective enzyme inhibition. Extrapolative calculations show that a level of 10 μM in the reaction mixture used for enzyme assay would be equivalent to a tissue concentration of 1·84 mg g^{-1} of toluene and 2·56 mg g^{-1} of naphthalene. This, in the lethally exposed tilapia, would amount to concentration factors of 20 and 324, respectively, even if it is assumed that processes like evaporation and microbial degradation were ineffective and that the dissolved hydrocarbon levels in test water remained unchanged over the whole exposure period.

The salinity dependence of effects of pollutants on the enzyme activity is obvious from the results of the present study, as the hydrocarbon-induced inhibition of Na^+-K^+-ATPase activity was more extensive in salt water than in fresh water, almost in the range of an order of magnitude. The reason for this is not yet clear, but it is probably due to the relatively greater uptake of the dissolved hydrocarbons by saltwater-acclimated tilapia, as compared to that by freshwater-acclimated ones, a difference that has been reported for euryhaline fish (Levitan & Taylor, 1979).

The hydrocarbons also interfered with the ability of tilapia to adapt successfully to hyperosmotic environmental conditions. This was clearly evident from differences in the rates of mortality in fish groups exposed to the same hydrocarbon concentrations in different saline media. While

the rates in freshwater-acclimated tilapia exposed to the selected levels of pollution stress in fresh water were in close agreement with the values reported earlier (Dangé, 1979), they increased in tilapia preacclimated to salt water of 35‰ S, and then exposed to the pollution stress in salt water of the same strength. The mortality was still greater in freshwater-acclimated tilapia exposed to the hydrocarbons in salt water of 20‰ S. Increased salinity during exposure to petroleum constituents has also been reported to cause a larger number of deaths in other euryhaline fish (Levitan & Taylor, 1979; Moles *et al.*, 1979).

One of the reasons for the lower chances of survival in salt water during hydrocarbon exposure could be the possible failure of fish tissues to maintain a normal internal hypoosmotic environment, which, at least in part, would be attributable to the reduced activity of branchial Na^+-K^+-ATPase. Though the present study did not deal with the effects of hydrocarbons on tissue and plasma electrolyte levels in tilapia, studies by other investigators (Morrow *et al.*, 1975; Levitan & Taylor, 1979; Stickle *et al.*, 1982) have shown that hydrocarbons like toluene and naphthalene increase plasma osmolality in saltwater fish. It has been suggested that failure of the osmoregulatory system may not be responsible for increased sensitivity of fish to the hydrocarbons at high salinities, and that it is, in fact, only a symptom of other toxic actions of the pollutants (Stickle *et al.*, 1982). However, it may still be argued that osmoregulatory dysfunction, while not being the sole cause of greater hydrocarbon toxicity in salt water, could work in conjunction with other adverse metabolic effects whose severity should also be correlated with the greater and more rapid uptake of hydrocarbons from hyperosmotic media. Mention may be made in this context of such effects of hydrocarbons in tilapia as tissue hypoxia, extensive glycogenolysis and lactic acidosis, indicated by earlier studies (Dangé & Masurekar, 1981, 1982, 1984; Dangé, 1986*a*). Evidence has also been found to support the hypothesis that the rise in toxicity of toluene and naphthalene to fish, with increasing salinity, is due to the lesser capacity of nervous tissue of the saltwater fish to metabolize these pollutants (Thomas & Rice, 1981). The synergistic action of the two stressors, pollution and osmotic, could be an additional factor contributing to the greater susceptibility of fish to hydrocarbons in salt water, especially in the previously freshwater acclimated tilapia.

The mechanisms responsible for the extremely high mortality in telapia exposed to the two stresses sequentially, i.e., first to the

hydrocarbons and then to increased salinity, may be somewhat different. Firstly, the experimental procedure in this case was such that the fish were exposed to stressful conditions for a total of eight days instead of four. Secondly, exposure to the hydrocarbons for four days in fresh water has been reported to produce many metabolic disorders, including those observed during the present study (Dangé & Masurekar, 1982, 1984; Dangé, 1986*a*). These metabolic disturbances could be expected to interfere with the adaptive nature of the entire osmoregulatory machinery, so that a subsequent transfer to salt water would produce an acute osmotic shock, to which the already stressed and probably exhausted fish may succumb easily. In this context, the lowered activity of branchial Na^+-K^+-ATPase, in freshwater-acclimated tilapia transferred to salt water after they had survived the hydrocarbon stress, is probably indicative of the suppressed ability of affected gills to mobilize the necessary biochemical reactions leading to increased enzyme activity.

In summary, the data from the present study could be interpreted to mean that the hydrocarbons caused significant disruptions in the osmoregulatory mechanisms in tilapia, resulting in more severe effects of the pollutants in salt water. Quantitative comparisons also indicated that the levels of the hydrocarbons that could be labelled sublethal in fresh water were highly lethal in salt water. It is thus clear that additional research on the effects of hydrocarbons (and probably other organic and inorganic pollutants) in combination with several natural environmental disturbances like fluctuations in salinity, temperature and pH is required before coming to conclusions regarding the safe levels of these pollutants.

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