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Effects of a sublethal concentration of sodium lauryl sulphate on the morphology and Na^+/K^+ ATPase activity in the gill of the ornate wrasse (*Thalassoma pavo*)

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Abstract

We analysed the morphology and ultrastructure of the gill apparatus of the ornate wrasse, *Thalassoma pavo*, under normal conditions and after exposure to a sublethal concentration of sodium lauryl sulphate (3.5 mg/l, which is one-third of the 96LC99 value). To identify the biochemical mechanisms affected by this pollutant, we evaluated and compared the localisation of Na^+/K^+ ATPase in normal and experimental conditions. Immunocytochemical analysis revealed that this enzyme was active in the chloride cells (CCs), which were distributed in clusters in the interlamellar region of the filament. Ultrastructural analysis revealed conspicuous alterations on the epithelium after 96 and 192 h of exposure to sodium lauryl sulphate: structural features of the surface cells were lost, the appearance of intercellular lacunae changed, and cellular degeneration occurred. Statistical analysis comparing the number and dimensions of CCs in normal conditions and after 96 h of exposure showed that the CC area decreased after exposure to the detergent.
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1. Introduction

Some pollutants can induce alterations to the respiratory apparatus in fish (Mallat, 1985; Van den Heuvel et al., 2000). The gill apparatus and the skin, which are continuously exposed to the aquatic medium, are the principal sites of activity of numerous toxic substances (Van den Heuvel et al., 2000). Anionic detergents represent an important category of pollutants and are still widely used today in the production of many consumer goods because of their surface-active properties. In particular, sodium lauryl sulphate (SLS) is used in many personal care products and cosmetics. These products are disposed off through domestic and industrial waste discharges into the sea where they accumulate in seawater and sediments (Sigoillot and Nguyen, 1992). For example, ~60,000 tons of detergents are poured into the Mediterranean each year (Della Croce et al., 2001); although biodegradation of SLS ranged from 45% to 95% within 24 h, the continuous introduction of SLS into the environment kept the concentration of this pollutant high (Cserhati et al., 2002). A number of researchers have studied the noxious effects of anionic detergents on marine organisms (e.g., Misra et al., 1985, 1991; Zaccone et al., 1985a, b, 1986; Rosas et al., 1988; Roy 1988a, b; Gupta et al., 1989; Ribelles et al., 1995). Such studies highlight the importance of these substances in determining mortality or pathology in marine populations. Anionic detergents have a strong tendency to bind to the lipid component of the membrane, and thus high concentrations can alter the structural arrangement of the membrane and compromise its functionality.

In fish, gills play a fundamental role in gas exchange, and they also represent an important site for osmotic and acid–base regulation (Laurent, 1984; Evans et al., 1999). The structural complexity of the gill apparatus reflects these multiple functions (Franchini et al., 1994; Ribelles

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et al., 1995). Pollutants can affect the gill apparatus and its role as a respiratory and osmoregulatory organ (Mallat, 1985). Following chronic or acute exposure to different pollutants, alterations in gill tissue can occur. These changes represent a response to stressors that have often been interpreted as non-specific (Mallat, 1985; Evans, 1987); in many cases, alterations at the cellular or sub-cellular level are not by themselves diagnostic of a particular type of pollutant. Thus, a combination of morphological analysis, the evaluation of functional activity, and the application of statistical methods might be a better approach to determining a specific response to a particular pollutant (Mallat, 1985).

In this paper, we analysed the morphology and ultra-structure of the gill epithelium of the ornate wrasse, *Thalassoma pavo*, under normal conditions and after exposure to sublethal concentrations of SLS. Our goal was to evaluate the histopathological and sub-cellular alterations induced by exposure to SLS. Furthermore, we used a confocal laser scanning microscope for Na^+/K^+ ATPase immunolocalisation (Witters et al., 1996; Dang et al., 2000) to determine where chloride cells (CCs) reside. We then compared the CC population in the control and treated samples.

2. Materials and methods

2.1. Fish maintenance and holding conditions

The *T. pavo* specimens used in this study were collected from a location on the Tyrrhenian coast (S. Lucido) using baited traps. Adult specimens of both sexes with a mean mass of 9.69 ± 0.48 g were transported to the laboratory and kept in a 150 l aquaria (10 fishes per tank) filled with seawater from the capture site and equipped with filter and oxygenation systems. The fishes were kept in the aquaria for a 5-day acclimatisation period, during which salinity (35‰), density (1.027–1.028 g/cm^3), temperature (18–24 °C), and the nitrite and nitrate concentrations were measured and kept constant. Other conditions included dissolved oxygen at 8.0–8.6 mg/l, hardness 100 mg CO_3Ca/l, and the absence of heavy metals. Throughout the experiment, the animals were maintained under a natural light/dark cycle and fed every 2 days with commercial fish food (Tetramin).

2.2. Exposure to SLS and sampling

The experiments were carried out using a semi-static acute experimental method, meaning that the experimental solution and the samples (i.e., fish) were put in a test chamber (i.e., aquarium). To avoid variations in detergent concentrations, solutions were renewed every 24 h (EPA, 2002). From some preliminary evaluations (not shown) biodegradation levels result to be less than 12% of the initial concentration which is very similar to that obtained in other similar studies (Flores et al., 1980; Ribelles et al., 1995).

The toxicant used in this study was SLS ($CH_3(CH_2)_{11}OSO_3Na$; Sigma, Milano, Italy). The fish were randomly distributed in different concentrations of SLS (2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, and 7 mg/l). For the acute bioassay tests, 15 fishes were used per concentration per replicate. A total of four replicates was used for each dose and for the control group. The number of dead fishes was counted every 12 h, and they were removed immediately from the aquaria.

In this study, the acute toxic effect of SLS on *T. pavo* was determined using Finney's Probit Analysis LC50 Determination Method (Finney, 1971). The computer analysis was performed using LC50 1.00 software developed by the EPA (1999).

The data were also evaluated following the Behrens–Karber method using the following formula (Klassen, 1991; Yilmaz et al., 2004):

$$\mathrm{LC50} = \mathrm{LC100} - \frac{ab + \ldots + ab}{n},$$

where LC50 and LC100 indicate the lethal doses for 50% and 100% of the samples, respectively; a is the difference between the two consecutive doses; b is the arithmetic mean of the mortality caused by two consecutive doses; and n is the number of samples in each group.

After identifying the lethal concentration, we identified the maximum concentration in which all the subjects showed a normal swimming position and normal feeding behaviour, and we carried out two sets of experiments with a sublethal dose (SLS nominal concentration 3.5 mg/l). The gills were removed after 48, 96, and 192 h and control sample gills were removed at the same time. The animals were anaesthetised with 2–4 g/l tricaine methane sulphonate (MS 222, Sandoz, Sigma, St. Louis, MO) and killed by spinal cord transection. The removed gills were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) and for confocal microscopy. Animal manipulation was performed according to Ethical Committee recommendations and under the supervision of authorised investigators.

2.3. Electron microscopy

After removal, the gill samples were fixed using Karnovsky liquid in a cacodylate buffer (pH 7.4). After post-fixation with osmium tetroxide and dehydration in ethanol at increasing concentrations, the samples used for TEM observation were enclosed in epon/araldite. Semi-thin sections (1–2 μm) were stained with Grimley's dyes (toluidine blue method: malachite green and acid fuchsine) and observed with a Leitz Dialux 20 EB light microscope. The ultrathin sections were observed under a Zeiss EM 900 TEM. After dehydration, the samples used for SEM observation were subjected to the progressive substitution of ethanol with hexamethyldisilazane, removed by complete evaporation (Nation, 1983), coated with gold in an Emitech K550 ion sputter unit, and then examined under a Zeiss DSM 940 SEM.

2.4. Immunohistochemistry

The samples were immersed in Bouin liquid, in phosphate-buffered saline (PBS, pH 7.1) for 24 h, and then in graded ethanol; after clarification in xylene, they were embedded in paraffin wax with a mean fusion point of 56 °C. The 10-μm sections were dewaxed and subjected to the indirect immunofluorescence technique (Coons et al., 1955). To block endogenous peroxidase, the sections were immersed in 2% H_2O_2 in 0.1 PBS for 20 min and then incubated for 30 min in a moist chamber with 20% normal goat serum to block non-specific sites. Unwashed sections were then incubated overnight at 4 °C with a mouse monoclonal antibody to Na^+/K^+ ATPase at working dilutions of 1:100 (IgGα5; Developmental Studies Hybridoma Bank, Department of Biological Sciences, The University of Iowa, Iowa City, IA, USA). After several washes in PBS, fluorescein–isothiocyanate-conjugated γ-globulin goat anti-mouse (Sigma) was used as the second antiserum at a dilution of 1:200 for 1 h at room temperature. To verify the specificity of the immunolabelling, the primary antiserum was substituted with non-immune goat serum or with PBS. In addition, propidium iodide was used for visualisation of the general tissue structure (at working dilutions of 1:200); this phenanthrene colourant, when inserted in the DNA, forms a sufficiently stable complex that emits a red fluorescence when excited. The observations were carried out with a Leica TCS SP2 Confocal Laser Scanning Microscope.

2.5. Quantification and statistical analysis

The CCs in the filament and lamellae were identified by Na^+/K^+ ATPase immunohistochemistry (according to Dang et al., 2000) and then

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