

Exposure of rainbow trout (*Oncorhynchus mykiss*) to nonylphenol is associated with an increase in the chloride cell fractional surface area

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Summary

Nonylphenol is a biodegradation product of a widely used group of non-ionic detergents. Because of its ubiquitous distribution and persistence, it is present in surface waters as a pollutant. Little is known about its biological effects at environmentally relevant concentrations other than its action as a xenoestrogen. The goal of the present was to study the trout gill surface epithelium as the major interface between fish and water in view of possible morphological alterations due to exposure to nonylphenol. Rainbow trouts were intermittently exposed to 10 µg/l nonylphenol and gill samples from experimental and control animals were investigated by scanning electron microscopy. Gill surface epithelium was scrutinized for changes in chloride cell density and their status regarding cell surface modifications. In addition, chloride cell fractional surface area (CCFA) was determined by morphometrical methods. Statistical analysis revealed a highly significant increase of CCFA in animals exposed to nonylphenol as compared to control animals ($P = 0.0001$). Semi-quantitative assessment of the other parameters suggested a higher chloride cell density and a larger proportion of chloride cells bearing microvilli. Taken together, these results provide evidence that exposure of trouts to nonylphenol is associated with a substantial increase in the active interface of chloride cells with water. We interpret these findings as being a means to further the fish's capacity for calcium exchange.

Introduction

Nonylphenol is an ubiquitous and persistent biodegradation product of the alkylphenol ethoxylates which are one of the major groups of non-ionic surfactants. Although contamination levels of open waters are improving in Switzerland, concentration of nonylphenolic compounds still exceeds 3 µg/l in the effluents of some Swiss sewage treatment plants (Giger et al., 1998). Because of its effects on reproduction and development in vertebrates including mammals, nonylphenol has attracted considerable attention over the last years (for literature see Talmage, 1994). Besides these interactions with the endocrine system, however, little is known about other reactions at environmentally relevant concentrations. In trout species though, nonylphenol was found to accumulate in the liver, gill, skin, gut, kidney and fat tissue (Coldham et al., 1998; Lewis and Lech, 1996).

In addition, biochemical studies have demonstrated that nonylphenol influences ATPases and affects the calcium metabolism (Michelangeli et al., 1990). This raised our interest since both epithelial cell types lining the fish gill, i. e. the respiratory pavement cell and the ion-regulatory chloride cell, harbour high amounts of ATPases, the latter cell being responsible for calcium uptake in freshwater fish (Laurent and Perry, 1995; Lock et al., 1996). Whereas the pavement cells exhibit a species-specific pattern of microridges at their apical surface, the apex of chloride cells is rather smooth or bears small microvilli. Surface area as well as the number and size of chloride cells, however, are known to depend on environmental factors (for review see Laurent and Perry, 1995). The goal of the present study, therefore, was to scrutinize the trout gill surface epithelium for morphological alterations resulting from exposure to water contaminated with nonylphenol.

Material and Methods

Animal care and treatments

Within a 4 months period, three year old (3+) male rainbow trouts, *Oncorhynchus mykiss*, were intermittently exposed to nonylphenol as described previously (Burkhardt-Holm, Ecotoxicology and Environmental Safety, in press). Nonylphenol was added at a final concentration of 10 µg/l. This experimental procedure was repeated four times for 10 days each. In between, fishes were kept in spring water. Control animals of the same batch were held under similar conditions in the absence of nonylphenol. The fish density was the same for all groups. Five animals each of the exposed and of the control groups were investigated.

Scanning electron microscopy and morphological analysis

Fishes were killed by an overdose of ethylenglycolmonophenylether (Merck, Darmstadt, Germany). Small pieces of gill of all investigated animals were fixed and processed immediately after sampling as described earlier (Burkhardt-Holm et al., 1997). Fixed samples were washed in buffer, postfixed with 1% OsO₄ in 0.1 M cacodylate buffer, dehydrated through an ascending ethanol series and critical point dried in a Bal-Tec CPD 030 (Balzers, Liechtenstein). Immediately thereafter, samples were mounted onto stubs by means of double adhesive conductive tabs (Provac, Liechtenstein) sputtered with approximately 10 nm of gold in a Bal-Tec SCD 004 (Balzers, Liechtenstein) and stored in an exsiccator until examination at 10 to 15 kV accelerating voltage at a working distance of 5 mm in a Zeiss digital scanning electron microscope DSM 982.

Chloride cell fractional surface area (CCFA) was determined by the point counting method (Weibel, 1979). Micrographs showing frontal views of chloride cells were printed out at a final magnification of 16'400x. Micrographs were overlaid with a square grid of test points 1 cm apart. Number of test points counted per chloride cell

were used for statistical analysis of relative CCFA in control animals (50 cells) versus animals exposed to nonylphenol (56 cells). The frequency distribution of obtained values was compiled using the class intervals 1-19, 20-39, 40-59, 60-79, 80-99, 100-119, and 120-139. Furthermore, an estimate of the absolute surface area was computed as $A = n \times d^2/M^2$ where n denotes the median number of points counted per chloride cell, d the width of the square grid and M the magnification. Statistical analysis of collected data included determination of median values with corresponding quartiles, and testing of a hypothetical increase in CCFA after exposure to nonylphenol using the Wilcoxon rank sum test. SEM micrographs were further scrutinized for cell surface modifications of chloride cells.

Results

Quantitative Aspects

Results obtained for the chloride cell fractional surface area (CCFA) in exposed and control animals are compiled in table 1. Histograms and box plots of relative surface areas (number of test points per cell) are shown in Fig. 1. Parting from the median values, average absolute CCFA in exposed trouts versus control animals were computed as $28 \mu\text{m}^2$ and $16 \mu\text{m}^2$, respectively, the ratio being 1.74. According to the Wilcoxon scores (rank sums), the difference in size was highly significant ($P = 0.0001$).

Qualitative and semi-quantitative aspects

Nonylphenol-exposed trouts showed an increased number of chloride cells in the interlamellar space. Scanning electron microscopy also allowed two phenotypically distinct varieties of chloride cells to be distinguished. One type exhibited an almost smooth apical surface (Fig. 2a) whereas the second type displayed short but dense microvilli (Fig. 2b). Although both cell types were observed in control as well as in exposed animals, the ratio of smooth cells to cells bearing microvilli was approximately 6 : 1 in control animals whereas the corresponding value in trouts exposed to nonylphenol was 1 : 1. No statistical analysis was performed on these results since distinction of the two cell types does not rest on quantitative criteria.

Discussion

Enlargement of the overall apical surface of chloride cells basically can be achieved in three ways: by increasing the cell fractional surface area (CCFA), by adding cell surface projections, and by multiplying the number of cells. The present study provides highly significant evidence that exposure of trouts to nonylphenol-contaminated water is linked to a dramatic rise in the average CCFA. This observation is also illustrated by the frequency distribution which revealed that no cells pertaining to the largest size class were observed in control animals whereas representatives of the smallest class were lacking in exposed trouts. Our findings regarding an increased proportion of chloride cells bearing tangible microvilli further suggest that cell surface projections are augmented. In our experiment, nonylphenol-exposed trouts also showed a higher number of chloride cells on their gills as compared to control animals (results not shown). This held particularly true for the base of the secondary lamellae. However, because of poor accessibility of this region to low power micrographs, statistical analysis of chloride cell density was not achievable.

We conclude from these results, that exposure to nonylphenol is associated with an increase in the overall surface area being available for active ion exchange with water. Interestingly, both proliferation of chloride cells and enlargement of their apices have been reported in trouts living in water with a low calcium content (Laurent et al., 1985; Perry, Wood, 1985). However, chronic cortisol treatment also causes a significant increase in the number and apical surface area of trout gill chloride cells (Laurent, Perry, 1990) and similar adaptations have been observed after recovery from hyperoxia (Goss et al., 1994). Thus, the question whether *any* stressor, be it the want of vital electrolytes or the presence of environmental toxicants might lead to the reported morphological changes by an indirect action of cortisol has

not been unequivocally resolved yet. Nonetheless, the interpretation of an increase in the active ion exchange surface area as being a means to further the fish's capacity to take up calcium ions from the water remains attractive. Previous results provide indirect evidence, that inter-lamellar chloride cells might be involved in calcium uptake (Burkhardt-Holm et al.1999). In our material, the proportion of these β -cells was increased in number after nonylphenol exposure, an observation which lends further contingency to the idea that nonylphenol actually generates an increased demand for calcium. In addition, nonylphenol is known to induce the production of vitellogenin (for review see (Arukwe, Goksoyr, 1998). This calcium-rich yolk protein is normally secreted by the liver of adult female oviparous vertebrates. Production of vitellogenin, however, can be induced in males and juveniles by several xenoestrogens including nonylphenol (Arukwe, Goksoyr, 1998). Synthesis of vitellogenin in its turn is highly dependent upon adequate calcium levels and thus promotes the mobilization of calcium from all available sources (Björnsson, Haux, 1985; Carragher, Sumpter, 1991).

In summary, the present study provides unequivocal evidence for a highly significant correlation between exposure of trouts to nonylphenol-contaminated water and a substantial increase in the active interface of chloride cells with water. These data do not warrant a causal connection per se, but taken together with reports in the literature, they strongly suggest a repercussion of exposure to nonylphenol on ion exchange in the fish gill.

Table 1

	Controls	Nonylphenol
Number of observations	50	56
Maximum value	119	131
75% Q3	54	85
50% Median value	43	75
25% Q1	31	57
Minimum value	16	20
Range	103	111
Q3 – Q1	23	28

Figure 1

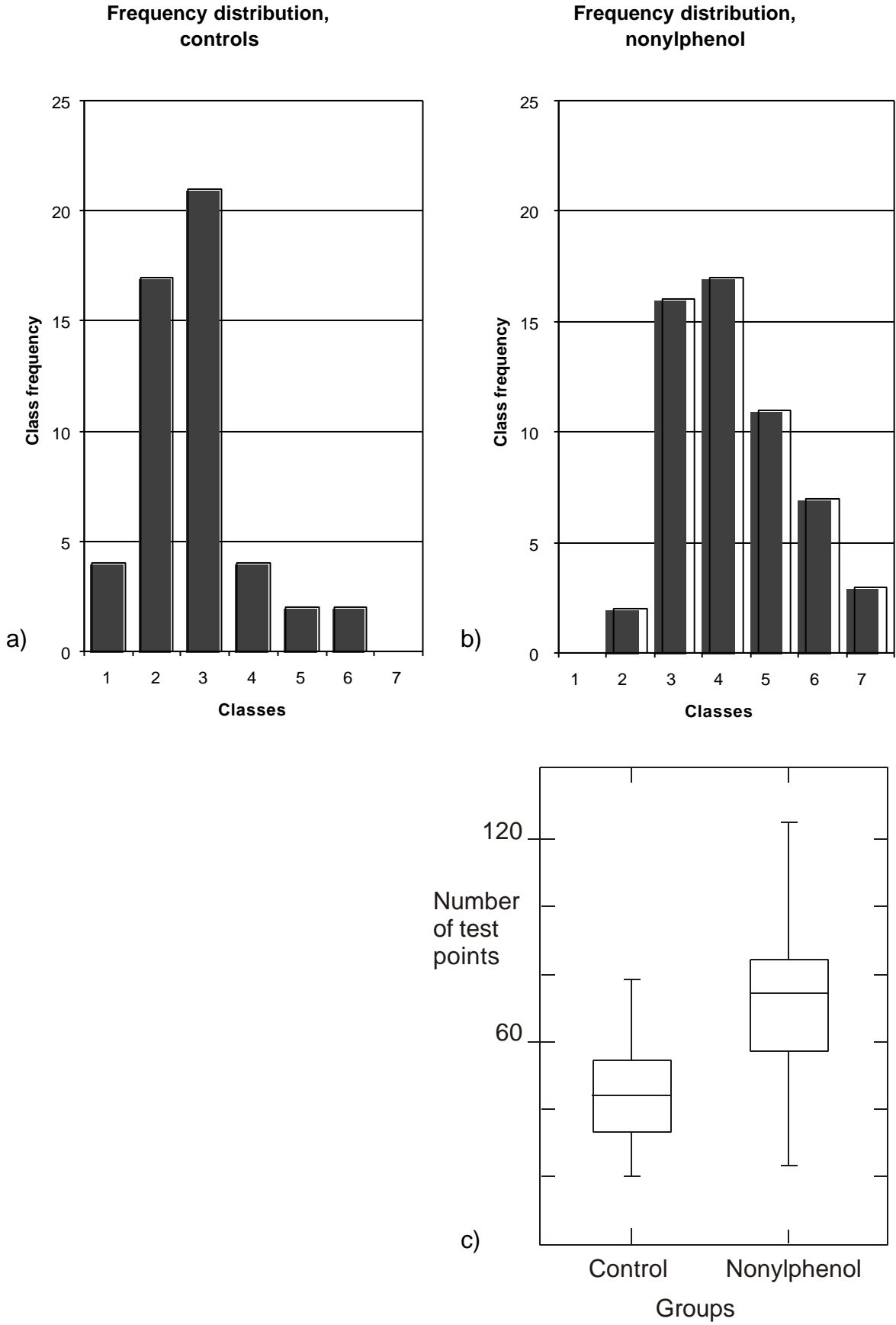


Figure 2

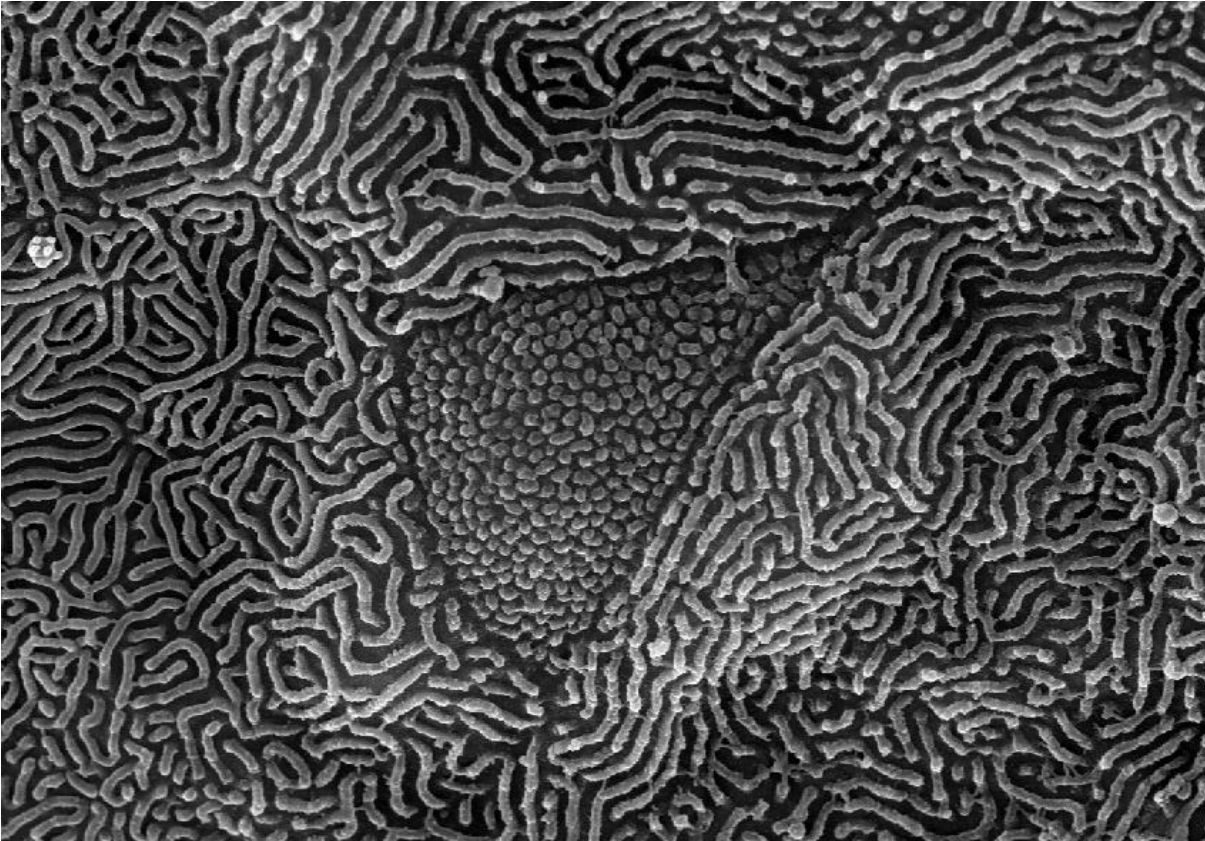
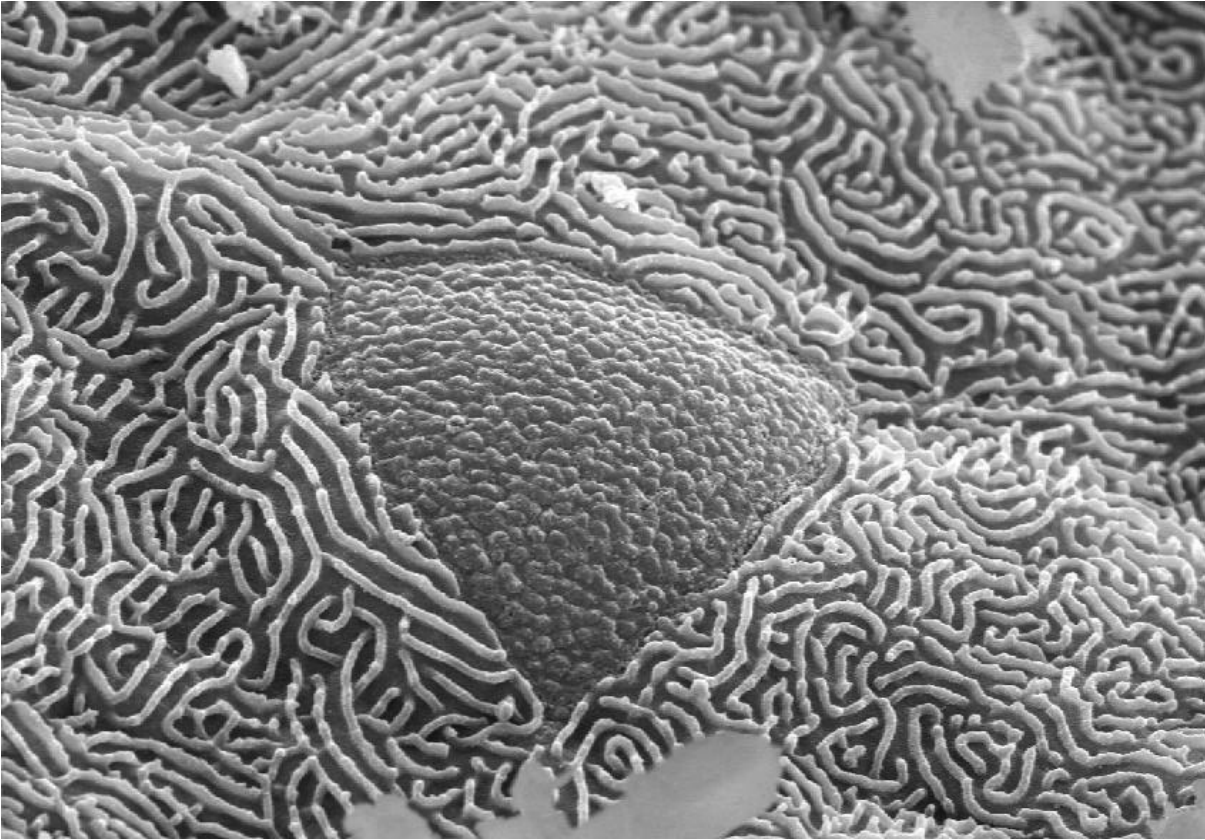


Figure and Table legends

- Table 1 Statistical analysis of relative chloride cell fractional surface area (number of test points per cell).
- Fig. 1 Relative chloride cell fractional surface area (number of test points per cell). Frequency distribution from controls (a) and experimental animals (b), respectively; for class ranges see Material and Methods. (c) Box plots with median values, 25% and 75% quartiles as well as minimal and maximal values.
- Fig. 2 Chloride cells of the smooth type (a) and of the microvilli-bearing type (b) surrounded by pavement cells with typical microridges. Scale bar = 1 μm

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