Exposure of rainbow trout

\textit{(Oncorhynchus mykiss)} to nonylphenol is associated with an increase in the chloride cell fractional surface area

\textit{Michael H. Stoffel}$^1$, \textit{Thomas Wahl}$^2$, \textit{Armin E. Friess}$^1$, and \textit{Patricia Burkhardt-Holm}$^3$

$^1$Department of Veterinary Anatomy, University of Berne Veterinary School, POB 8466, CH-3001 Bern, Switzerland

$^2$Center for Fish and Wildlife Health, University of Berne Veterinary School, POB 8466, CH-3001 Bern, Switzerland

$^3$Swiss Federal Institute for Environmental Science and Technology EAWAG, POB 611, CH-8600 Duebendorf, Switzerland

Correspondence address:

Dr. Michael H. Stoffel Phone: *41 31 631 22 05
Länggass-Strasse 120 Fax: *41 31 631 26 15
CH-3012 Bern e-mail: mstoffel@ita.unibe.ch
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% OsO$_4$ 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 µm² and 16 µm², 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,

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>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Nonylphenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of observations</td>
<td>50</td>
<td>56</td>
</tr>
<tr>
<td>Maximum value</td>
<td>119</td>
<td>131</td>
</tr>
<tr>
<td>75% Q3</td>
<td>54</td>
<td>85</td>
</tr>
<tr>
<td>50% Median value</td>
<td>43</td>
<td>75</td>
</tr>
<tr>
<td>25% Q1</td>
<td>31</td>
<td>57</td>
</tr>
<tr>
<td>Minimum value</td>
<td>16</td>
<td>20</td>
</tr>
<tr>
<td>Range</td>
<td>103</td>
<td>111</td>
</tr>
<tr>
<td>Q3 – Q1</td>
<td>23</td>
<td>28</td>
</tr>
</tbody>
</table>
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
Acknowledgments

The present study was carried out within the frame of a research programme on estrogenic effects of nonylphenol, conducted by the Bavarian State Office for Water Resources Management, Institute for Water Research in Wielenbach, Germany. We thank the members of the scientific and technical staff at the Bavarian State Office for Water Resources Management, namely Drs. R.-D. Negele and J. Schwaiger for conducting the experiments and Dr. W. Kalbfus for the water analysis of nonylphenol. Dr. G. Lamche, Center for Fish and Wildlife Health, University of Berne, is acknowledged for her help with sampling.

The assistance of A. Busato with the statistical analysis is greatly appreciated.
References


Burkhardt-Holm P., T. Wahl, W. Meier Nonylphenol affects the granulation pattern of epidermal mucous cells in rainbow trout, Oncorhynchus mykiss. Ecotoxicology and Environmental Safety, in press

Carragher J.F., Sumpter J.P. (1991): The mobilization of calcium from calcified tissues of rainbow trout (Oncorhynchus mykiss) induced to synthesize vitellogenin. Comparative Biochemistry and Physiology 99A, 169-172

Coldham N.G., Sivapathasundaram S., Dave M., Ashfield L.A., Pottinger T.G., Goodall C., Sauer M.J. (1998): Biotransformation, tissue distribution, and persistence of 4-nonylphenol residues in juvenile rainbow trout (Oncorhynchus mykiss). Drug Metabolism and Disposition 26, 347-353
Spurenanalytik von Nonylphenol-Verbindungen in Abwasser und Gewässern.
EAWAG Info-Tag

Laurent P., Hobe H., Dunel-Erb S. (1985): The role of environmental sodium chloride
relative to calcium in gill morphology of freshwater salmonid fish. Cell and
Tissue Research 240, 675-692

Laurent P., Perry S.F. (1990): Effects of cortisol on chloride cell morphology and ionic
uptake in the freshwater trout, Salmo gairdneri. Cell and Tissue Research 259, 429-442

in fish. Advances in Comparative and Environmental Physiology 22, 91-118

from water in rainbow trout (Oncorhynchus mykiss). Xenobiotica 26, 813-819

to toxicants. Stress mechanisms induced by branchial malfunctioning. In:
Müller, R., Lloyd, R. (eds) Sublethal and Chronic Effects of Pollutants on
Freshwater Fish, University Press, Cambridge pp. 124-134

inhibition of the (Ca$^{2+}$-Mg$^{2+}$)-ATPase by nonylphenol. Biochemistry 29, 3091-
3101

tROUT: Effects of acclimation to various external calcium levels. Journal of
Experimental Biology 116, 411-433