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Changes in apoptotic rate and cell viability in three fish epidermis cultures after exposure to nonylphenol and to a wastewater sample containing low concentrations of nonylphenol

GISELA LAMCHE[1] and PATRICIA BURKHARDT-HOLM[1,2*]

[1] Centre for Fish and Wildlife Health, Institute of Animal Pathology, Laenggassstr. 122, CH-3012 Berne, Switzerland

[2] Interdisciplinary Centre for General Ecology, University of Berne, Falkenplatz 16, CH-3012, Berne, Switzerland. e-mail: patricia.holm@eawag.ch

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Three types of epidermal cultures of fish were used for toxicological investigations, a primary cell culture and a tissue culture prepared from the rainbow trout *Oncorhynchus mykiss* Walbaum and the cell line EPC, derived from a skin tumour of the carp *Cyprinus carpio* L. Two studies were carried out to compare the different culture systems. In the first cultures were incubated with nonylphenol and in the second set of experiments the cell cultures were exposed to a wastewater sample containing low concentrations of nonylphenol (NP). Both cell cultures were similarly sensitive to nonylphenol with respect to the endpoints cell viability (LC_{50} (24 h) 47.1 μM NP (primary cell culture) and 44.2 μM NP (EPC)) values and apoptotic rate (significantly increased apoptotic rate after exposure to 50 μM NP for 24 h, $p < 0.001$ (primary cell culture), $p = 0.008$ (EPC)). The explant culture was slightly less sensitive (increased apoptotic rate after exposure to 50 μM NP for 24 h, but not significant: $p = 0.385$), which could be due to the capabilities of a differentiated tissue, providing more protective repair mechanisms, compared with single cells. All cultures revealed a concentration–response relationship for the endpoint apoptotic rate after the application of nonylphenol for 24 h. After wastewater exposure, a significant decrease in the apoptotic rate was measured in the primary cell culture (dilution wastewater : medium 1:1: $p = 0.018$; dilution wastewater : medium 1:2: $p = 0.003$), whereas the cell line EPC did not reveal any effects. Our results show that the endpoint apoptotic rate is more sensitive than the parameter cell viability for detecting adverse effects of a wastewater sample.

Keywords: epidermis, Oncorhynchus mykiss, cell, *in vitro*, tissue culture, explant, nonylphenol, wastewater.

Introduction

In vitro toxicological tests are of growing interest. One aim is to replace animal tests with cell culture tests (Spielmann and Reinhardt 1996). Additionally *in vitro* systems are easy to handle, of reasonable costs, have a high degree of reproducibility and allow a high number of replicas (Babich and Borenfreund 1987, Rusche and Kohlpoth 1993).

We have recently established a primary culture of fish skin epidermal cells (Lamche *et al.* 1998) and an explant tissue culture of the fish epidermis (Lamche and Burkhardt-Holm submitted) from the rainbow trout *Oncorhynchus mykiss* Walbaum. In comparison to immortal cell lines, the advantage of primary cultures is their greater spectrum of physiological capabilities reflected in a higher degree of

[1] Corresponding author: Patricia Burkhardt-Holm, EAWAG, PO Box 611, CH-8600 Duebendorf, Switzerland.

morphological differentiation as has been found for fish hepatocytes (Zahn *et al.* 1996, Segner 1998a). However, our primary cell culture contains morphologically undifferentiated keratinocytes only (Lamche *et al.* 1998), whereas the tissue culture consists of differentiated keratinocytes as well as mucous cells (Lamche and Burkhardt-Holm submitted). Therefore, we expected differences in the sensitivity of both systems. As the isolation of primary cells is both difficult and time consuming, the studies were also carried out in the fish skin cell line EPC derived from a skin tumour of carp *Cyprinus carpio L.* (Fijan *et al.* 1983).

Nonylphenol (NP) was chosen as an example of a xenobiotic found very commonly in the environment all over the world (Naylor 1992, Talmage 1994, Ding and Tzing 1998, Giger *et al.* 1999, Isobe *et al.* 1999). Nonylphenol belongs to the group of alkylphenols, which are biodegraded products of alkylphenolethoxylates (APEs). Among the APEs the nonylphenolethoxylates comprise about 80 % of the total market volume (Naylor 1992, Staples *et al.* 1998). APEs are widely used as detergents, emulsifiers, solubilizers, wetting agents, dispersants in paints, formulated pesticides and herbicides, personal care products and food packaging (Naylor 1992, Staples *et al.* 1998). Annual usage of nonylphenolethoxylates (NPEs) in Europe was around 75.000 t (Jobling and Sumpter 1993). Most of the APEs are presumed to enter the aquatic environment after use (Naylor 1992).

When discharged into modern sewage treatment plants, APEs can be reduced by up to 79–99 % using aerobic activated sludge treatment (Giger *et al.* 1984, Naylor 1992, Talmage 1994, Staples *et al.* 1998, Isobe *et al.* 1999). However, under anaerobic conditions APEs are degraded only to the alkylphenols, resulting in the formation of greater amounts of the alkylphenol (Giger *et al.* 1984, Naylor 1992).

Due to its high toxicity to invertebrates (EC_{50}, 24 h in *Daphnia magna* is 300 μg l^{-1} NP; Comber *et al.* 1993) and fish (LC_{50}, 96 h in rainbow trout *Oncorhynchus mykiss* Walbaum is 221 μg l^{-1} NP; Brooke 1993, review in Staples *et al.* 1998) and its oestrogenic activity (for review see Tyler *et al.* 1998) NPEs were banned in Switzerland, which led to a 5–10-fold reduction of NP and NPEs in the environment (Giger *et al.* 1999). In effluents from sewage treatment plants NP is currently found in maximum concentrations of 2.4 μg l^{-1} in Switzerland (Giger *et al.* 1999).

One concern of environmental toxicologists is potential additive or synergistic effects, due to the complex mixture of pollutants released in the aquatic systems, for example by wastewater effluents (Altenburger *et al.* 1993). Therefore, we also studied the effects of a mixture of chemicals in a 7-day mixed wastewater sample containing a complex composition of substances, on the two cell cultures. This sample contained low concentrations of 1.63 μg l^{-1} ≈10 nM NP. The selected sewage treatment plant (STW) in Lyss, Switzerland, receives industrial and municipal wastewater and discharges into the river Alte Aare. Downstream of the outlet of the STW increased loads of organic substances are measured occasionally in the river water (VOKOS 1996). Negative effects on the invertebrate community have been seen (Aquaplus 1993). In passive and active biomonitorings a high mortality of fish as well as an impaired health state were observed (Burkhardt-Holm *et al.* 1997, Escher *et al.* 1999). In this study the efficacy of using a cell model *in vitro* has been assessed.

The following endpoints for detecting toxic effects were chosen. First the classical toxicological endpoint 'cell viability' was measured to calculate LC_{50}

values; the latter allow comparisons with other test systems as well as with *in vivo* data. Cell viability was not measured for explant cultures, because the cell viability of the outgrown cells cannot be measured separately from the epidermal fragments which were present.

In order to find a more sensitive parameter we measured the number of cells undergoing apoptosis. Apoptosis, genetically regulated cell death, exerts a homeostatic function in relation to tissue dynamics (Gavrieli *et al.* 1992, Peter *et al.* 1997). Besides several external signals triggering a cell to undergo apoptosis, a disturbance in the balance of regulator molecules is known to alter the apoptotic rate (Mastrangelo and Betenbaugh 1995, Peter *et al.* 1997). Such a disturbance in cellular homeostasis could be evoked by a xenobiotic, because an increase in the number of cells undergoing apoptosis was found in rainbow trout epidermis explant cultures after exposure to cadmium (Lyons-Alcantara *et al.* 1998). Furthermore, increased apoptotic rate in the fish skin was related to environmental stress in several studies (Iger and Wendelaar Bonga 1994, Iger *et al.* 1994, Burkhardt-Holm *et al.* 1997). However, a quantification of apoptotic rate has been rarely utilized as a toxicological endpoint (Janz *et al.* 1997, Lyons-Alcantara *et al.* 1998).

Hence, our aims were (a) to investigate the suitability of our established primary cell culture and of the primary tissue culture from rainbow trout *Oncorhynchus mykiss* Walbaum for toxicity tests, (b) to compare their sensitivity with respect to threshold concentration and time course of the observed effects after exposure to NP with the commercially available cell line EPC (Epithelioma papulosum cyprini) derived from a skin tumour of carp *Cyprinus carpio* L., (c) to study the effects of wastewater exposure on the two cell cultures and (d) to test the parameters 'apoptotic rate' and 'cell viability' for their suitability as biomarkers. Since we found that in our studies the explant cultures did not reveal a significant increase in the apoptotic rate after a 24 h exposure to 50 μM NP, in contrast to both cell cultures, the tissue culture was not used for the investigation of the wastewater sample.

Material and methods

Cell and explant cultures

The cell line EPC (Epithelioma papulosum cyprini, Fijan *et al.* 1983) derived from a skin tumour of carp *Cyprinus carpio* L. was cultured according to standard methods (e.g. Freshney 1994) using Minimal Essential Medium (MEM, Gibco BRL) supplemented with 10% FCS, 13 μg ml^{-1} L-glutamine (Gibco BRL), 10 μg ml^{-1} non-essential amino acids (NEAA, Gibco BRL) and 1 μg ml^{-1} 7.5% sodium bicarbonate (Merck, Darmstadt, Germany) at 22 °C.

Primary fish skin epidermal cells were prepared as described (Lamche *et al.* 1998). Briefly, the epidermis of 4–16 month old rainbow trout *Oncorhynchus mykiss* Walbaum was lifted from the dermis and cut into strips, which were incubated in antibiotic solution and dispase solution. The tissue was disaggregated by warm trypsinization and single cells were seeded into culture flasks. Cells were fed twice a week with William's Medium E (WME, Sigma, St Louis, MO., USA) supplemented with 10% foetal calf serum (FCS, BioConcept, Allschwil, Switzerland), 5 μg ml^{-1} L-glutamine (Seromed, Biochrom, Berlin, Germany), 10 IU ml^{-1} penicillin (Seromed), 10 μg ml^{-1} streptomycin (Seromed), 10 ng ml^{-1} epidermal growth factor (EGF, Gibco BRL, Life Technologies, Gaithersburg, MD, USA), 5 μg ml^{-1} insulin (Sigma), 0.32 μg ml^{-1} hydrocortisone (Sigma), 10 ng ml^{-1} choleratoxin (Sigma) and were cultured at 19 °C, relative humidity 95–98%, 5% CO_2. Under these conditions cells can be passaged several times for harvesting the increased cell numbers, without changes in light microscopical or ultrastructural morphology (Lamche *et al.* 1998). Primary as well as passaged cells were used in the experiments, and in the following sections are referred to as primary cell culture (PCC). Primary fish skin epidermal explant cultures were prepared as described (Lamche and Burkhardt-Holm submitted). The first steps of explant culture preparation are similar to primary cell culture preparation. After

incubation in dispase solution, epidermis pieces were minced, washed and one to several tissue pieces were taken with a pair of tweezers, placed onto a glass cover slip into a well of a 24-well-plate and fed with 1 ml of WME, supplemented as described above and cultured at 19 °C, 5 % CO_2, relative humidity 95–98 %. Outgrown cells were used for toxicological studies, in the earliest instance 5 days after preparation.

Exposure to nonylphenol or to wastewater

A stock solution of 1 g l^{-1} of technical 4-nonylphenol (NP, Sigma) in ethanol was diluted in the appropriate medium for each cell culture. The nominal concentration of the stock solution was measured by extraction/HPLC (Dr M. Sägesser, Water and Soil Protection Laboratory of the district of Berne). For controls only medium (medium control) as well as 1.25 % or 0.025 % of ethanol in medium, corresponding to an ethanol concentration in 50 μM and 1 μM of NP, were used (solvent controls). For preparing NP-solutions glass material was used in order to minimize diffusion of NP into or out of plastic material. When unavoidable, plastic was used, i.e. for applying the NP solutions into 96-wells and the cells were always grown in plastic wells.

For estimating the magnitude of the effective NP concentration cells were exposed to a broad range of NP concentrations for 24 h using cell viability as the toxicological endpoint (table 1). Because the primary cells were expected to be more sensitive, they were exposed to lower nonylphenol concentrations in the first experiment (table 1). Viability measurements were additionally carried out for a 96-h exposure time. In a third viability experiment cells were exposed for 3, 6, 12 and 24 h using the NP concentrations near and above the calculated LC_{50} values (see Results). Cell viability was not measured for explant cultures, because the cell viability of the outgrown cells cannot be measured separately from the present epidermal fragments.

Experiments using the endpoint apoptosis were paralleled by measurements of viability as control for the experimental procedures. EPC, PCC cells and explant cultures were exposed to several concentrations of NP for 24 h (table 1). To study the time course of the change of apoptotic rate, EPC cells were additionally exposed for 3, 6, 12 and 24 h (table 1). In order to find out if an increase in the apoptotic rate after 24 h is due to the exposure time of 24 h, or due to a manifestation time after shorter exposure lengths, the EPC cells were exposed for 3 h to 10 or 50 μM of NP, followed by a 'manifestation time' of 0, 3, 6 or 21 h in medium without NP prior to fixation (table 1).

A 7-day mixed water sample was taken at the outlet of the sewage treatment plant in Lyss, Switzerland in August 1998. The chemical analysis of the sample was carried out by various analytical methods (extraction/GC/MS, headspace/GC/MS, extraction/HPLC, C18/GC/MS; Dr M. Sägesser, Water and Soil Protection Laboratory of the district of Berne). The sample was stored at –20 °C prior to use. Cells were exposed to different dilutions of wastewater in medium for 24 h. The highest concentration of wastewater was in the 1:1 dilution range. For control the medium was diluted 1:1 with tap water. Because of contamination during the first trials, MEM was additionally supplemented with 10 IU ml^{-1} penicillin, 10 μg ml^{-1} streptomycin (Seromed). The experiments were completed with sterile filtered wastewater samples as well as sterile filtered control samples (syringe filters 0.45 μm; Arodisc, Gelman Sciences, Ann Arbor, USA). Exposure experiments to the wastewater sample are summarized in table 2.

The wastewater sample was additionally examined with the *Daphnia* acute toxicity test and the luminescent bacteria toxicity test (microtox™) (DIN 38 412 L 30 and L 34) (data kindly provided from Dr M. Sägesser, Water and Soil Protection Laboratory Berne).

Cell viability

Cells were seeded in 96-wells and grown to confluence. EPC cells were seeded at a density of 1 million cells per well and used on the next day. PCC cells were seeded at a density of 0.1 million cells per well and used after having reached confluence after 7–14 days (Lamche *et al.* 1998).

Cell viability was measured using the neutral red assay according to Borenfreund and Puerner 1985). Briefly, after exposure time the medium was replaced by 50 μg l^{-1} neutral red (3-amino-*m*-dimethylamino-2-methyl-phenazine hydrochloride, Merck) in medium for 3 h at 19 °C for the PCC and at 22 °C for the EPC. Cells were fixed with 1 % formaldehyde – 1 % $CaCl_2$ for 1–2 min and the dye was extracted with 1 % acetic acid – 50 % ethanol for 30 min. Absorbances were measured with a microplate reader model 450 (BioRad Laboratories, Richmond, USA) with a 540 nm filter. The mean of the control values for absorbance was set to 100 % viability. Viability of the cells was expressed as the mean of 8 wells. All measurements for the endpoint cell viability were carried out using 8 wells.

Apoptosis

Cells were seeded on glass cover slips in 24-wells and grown to confluence. EPC cells were seeded at a density of 1 million cells per well and used on the next day. PCC cells were seeded at a density of 0.1 million cells per well and used after having reached confluence after 20–30 days (Lamche *et al.* 1998). Twice as many explants as needed were seeded on glass cover slips and those with the largest outgrowth of cells were selected for the experiment. Explant cultures were used 13 days after seeding.

Table 1. Experimental design for the exposure to nonylphenol (NP) (n = number of wells).

Endpoint (number of replicas) exposure time	Cell line EPC	Primary cell culture PCC	Explant culture
Cell viability ($n \geq 8$)			
24 h	1, 3, 6, 10, 30, 60, 100, 300, 600, 1000 μM NP, medium control, 0.025 % ethanol, 1.25 % ethanol	0.1, 0.5, 1, 5, 10, 50, 100, 500, 1000 μM NP, medium control, 0.025 % ethanol, 1.25 % ethanol	
96 h	1, 5, 10, 50, 100 μM NP, medium control, 1.25 % ethanol	1, 5, 10, 50, 100 μM NP, medium control, 1.25 % ethanol	
3, 6, 12, 24 h	50, 100 μM NP, medium control	50, 100 μM NP, medium control	
Apoptotic rate ($n \geq 4$)			
24 h	0.5, 1, 5, 10, 50 μM NP, medium control, 1.25 % ethanol	0.5, 1, 5, 10, 50 μM NP, medium control, 1.25 % ethanol	0.5, 1, 5, 10, 50 μM NP, medium control, 1.25 % ethanol
3, 6, 12, 24 h	0.5, 1, 5, 10, 50 μM NP, medium control, 1.25 % ethanol		
3 h exposure + 0, 3, 9, 21 h of 'manifestation time'	10, 50 μM NP, medium control, 1.25 % ethanol		

Four glass cover slips were exposed to the same concentration of NP. The cells were fixed with cooled 4% formalin and stored at 4 °C. The cells were stained with the 'In situ cell death detection kit, fluorescein' (Boehringer Mannheim, Mannheim, Germany), following the instructions of the user manual. Samples were examined with a Leitz Laborlux S fluorescence microscope. For each cover-slip four photographs were randomly taken at a final magnification of 200 for PCC and explant cultures and 400 for EPC. All cells on the photographed areas were counted on the basis of the unspecifically stained nuclei (see figure 2). The apoptotic rate was calculated as the number of TUNEL-positive nuclei per total cell number. The mean of the pseudoreplica of the four counts, per cover-slip, was taken as one data point. For quantification of the apoptotic rate in explants the edges of the cell outgrowth were avoided, because nearly all nuclei were TUNEL-stained. This was thought to be an artifact due to the properties of explant cultures and did not represent the reaction of the cells of the tissue culture to NP. All measurements for the endpoint apoptotic rate were carried out in quadruplicate. For PCC and explant cultures the four replica were cells and explants originating from four different fish.

Statistics

Apoptotic rates are given as the mean ± standard deviation (SD). LC_{50} was calculated using probit analysis with 95% confidence intervals. Data were checked for normality (KS-test) and variance homogeneity (Levene-test). Comparisons between exposure and control were calculated using the medium control. Pairwise comparisons were carried out using Student's t-test with assumed or not assumed homogeneity of variances. For multiple comparisons ANOVA was calculated. The Friedman-test was used, if prerequisites for ANOVA were not fulfilled. Correspondingly the Wilcoxon-test replaced the Student's t-test. In the case of *post-hoc* tests, α was corrected according to Bonferroni ($\alpha = \alpha/k$, with k = number of tests). $p < 0.5$ was therefore considered significant. In the case of a correction the significance niveau has been marked in the text. Linear and non-linear regressions as well as all other statistical analysis were calculated using the software program SPSS 8.0.

Table 2. Experimental design for the exposure to a 7-day mixed wastewater sample (dilution in medium) (n = number of wells) (n = number of experiments).

Endpoint (number of replica) exposure time	Cell line EPC	Primary cell culture PCC
Cell viability ($n \geq 8$)		
24 h ($n = 4$)	1:1, 1:2, 1:3, 1:4, 1:6, control — each probe sterile and non-sterile	1:1, 1:2, 1:3, 1:4, 1:6, control — each probe sterile and non-sterile
Apoptotic rate ($n \geq 4$)		
24 h ($n = 1$)	1:1 (sterile and non-sterile), control (non-sterile)	1:1 (sterile and non-sterile), 1:2 (non-sterile), 1:4 (non-sterile), control (sterile and non-sterile)

Results

Nonylphenol

Differences between medium control and solvent control were not detected for any endpoints in any of the experiments.

The LC_{50} values obtained after exposing the cells to NP measured with the neutral red assay for cell viability were similar for both cell cultures. PCC cells revealed a LC_{50} (24 h) = 47.1 (38.2–55.5) μM NP, which decreased slightly for the longer exposure time of 96 h to 41.6 (27.1–52.3) μM NP (lower and upper 95% confidence limits). The LC_{50} values for EPC after 24 h and 96 h exposure were 44.2 (38.5–48.8) μM NP and 42.2 (28.0–52.5) μM NP, respectively.

The cell viability, expressed in % of the control, decreased over the exposure time for EPC and PCC cells (figure 1). The control was set to 100% and is not shown. SD of the control was ± 15.9% for EPC and ± 8.9% for PCC. Cell viability for exposure to 50 μM NP was significantly different from the control after 6 h using EPC (Student's t-test, $t = 4.778$, $p < 0.001$) as well as using PCC (Student's t-test, $t = 2.898$, $p = 0.014$). The data for exposure to 50 μM NP were described by the curve fits $y = 161.5x^{-0.4874}$, $R^2 = 0.74$, $p < 0.001$ for EPC and $y = 153.3x^{-0.4467}$, $R^2 = 0.44$, $p < 0.001$ for PCC (figure 1a,b).

Labelling of apoptotic nuclei occurred as entirely stained nuclei, or in the case of different stages of disruption, as several smaller stained round nuclear fragments (figure 2).

The apoptotic rate, i.e. the number of TUNEL-positive nuclei per total cell number of the randomly selected area, was in general higher in PCC than in EPC, with control values of 0.024 ± 0.018 compared with 0.0046 ± 0.0032. The apoptotic rate was highest in control explant cultures with 0.040 ± 0.029.

Differences in apoptotic rate between exposure and controls after a 24 h exposure time were significant at 50 μM NP for PCC cells (Student's t-test, $t = -7.262$, $p < 0.001$) and EPC cells (Student's t-test, $t = -3.897$, $p = 0.008$), but not for explant cultures (Student's t-test, $t = -0.938$, $p = 0.385$) (figure 3).

A correlation between the increasing apoptotic rate with the rise in the NP concentration was found for all culture systems. In PCC the correlation for the range between 1 and 50 μM NP is best described via a linear correlation $y = 0.001539x + 0.02925$, $R^2 = 0.747$, $p < 0.001$. A calculation for EPC reveals a

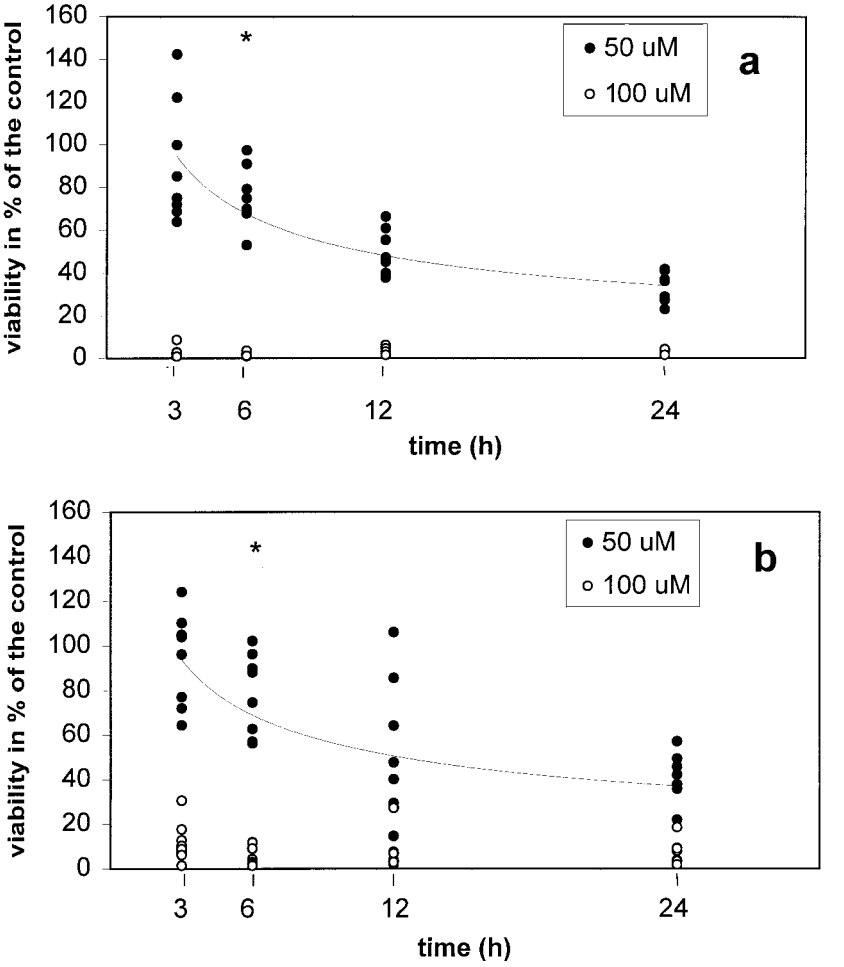

Figure 1. Viability of EPC cells (a) and PCC cells (b) after exposure to nonylphenol for 3, 6, 12 and 24 h measured with the neutral red assay. The mean of the control values was set 100 % (not shown). The lines are the curve fits described for the cells exposed to 50 μM nonylphenol with $y = 161.5x^{-0.4874}$, $R^2 = 0.74$, $p < 0.001$ for the EPC cells and $y = 153.3x^{-0.44667}$, $R^2 = 0.44$, $p < 0.001$ for the PCC cells. Cell viability for exposure to 50 μM of NP was significantly different from the control after 6 h for EPC as well as for PCC (asterisks).

similar correlation $y = 0.001168x - 0.00155$, $R^2 = 0.600$, $p < 0.001$. For the explant cultures the correlation was calculated for the range 5 to 50 μM of NP with $y = 0.00132x + 0.0132$, $R^2 = 0.665$, $p = 0.0012$ (figure 3).

The change of apoptotic rate over time was determined for EPC after 3, 6, 12 and 24 h exposure to NP (figure 4). A continuous increase was measured for cells exposed to 50 μM NP described as $y = 0.001877x + 0.00038$, $R^2 = 0.931$, $p < 0.001$, whereas the apoptotic rate of cells exposed to 10 μM NP did not change. After a 12 h exposure time to 50 μM NP the apoptotic rate was significant higher than in the control (Student's t-test, $t = -8.351$, $p < 0.001$) (figure 4). The experiment investigating the effect of a manifestation time after a 3 h exposure revealed that apoptotic rates were not different between controls and cells exposed to 10 and 50

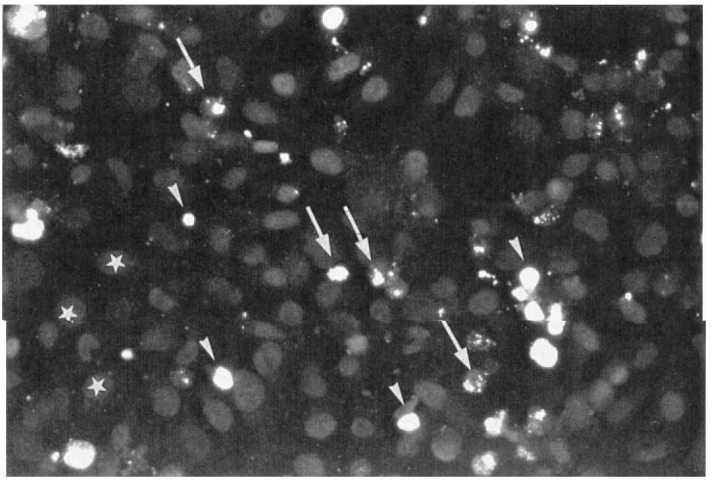

Figure 2. TUNEL-staining of EPC cells after exposure to 50 μM of NP for 24 h. Apoptotic nuclei are stained entirely (arrowheads) or in case of different stages of disruption as several smaller stained round nuclear fragments (arrows). * Points to unspecific stained nuclei. Fluorescence microscopy, magnification 330×.

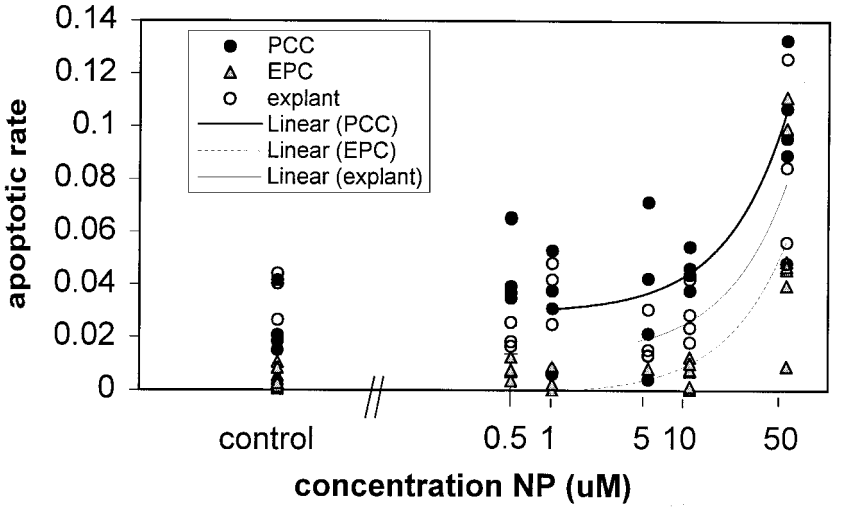

Figure 3. Rate of apoptotic cells of PCC, EPC and explants after exposure to various concentrations of nonylphenol for 24 h. Apoptotic rate, the number of TUNEL-positive nuclei per total nuclei number, of controls is highest in explant cultures, followed by PCC cells and is low in EPC cells. Significant differences to the control are found at 50 μM NP for the cell cultures PCC and EPC, but not for explant cultures. For all cultures the concentration-response relationship can be described with a linear correlation for the range between 1 and 50 μM NP for PCC and EPC and for the range between 5 and 50 μM NP for explants (PCC: $y = 0.001539x + 0.02925$, $R^2 = 0.747$, $p < 0.001$; EPC $y = 0.001168x - 0.00155$, $R^2 = 0.600$, $p < 0.001$, explant culture: $y = 0.00132x + 0.0132$, $R^2 = 0.665$, $p = 0.0012$) (note the logarithmic scale for the concentration).

μM NP at any manifestation time from 0 to 21 h (Friedman-test $\chi^2 = 24.040$, $p = 0.064$) (not shown). Thus, a 3 h exposure time was not sufficient to induce apoptosis or alter cell viability (ANOVA: $F = 1.320$, $p = 0.191$) (see figure 1).

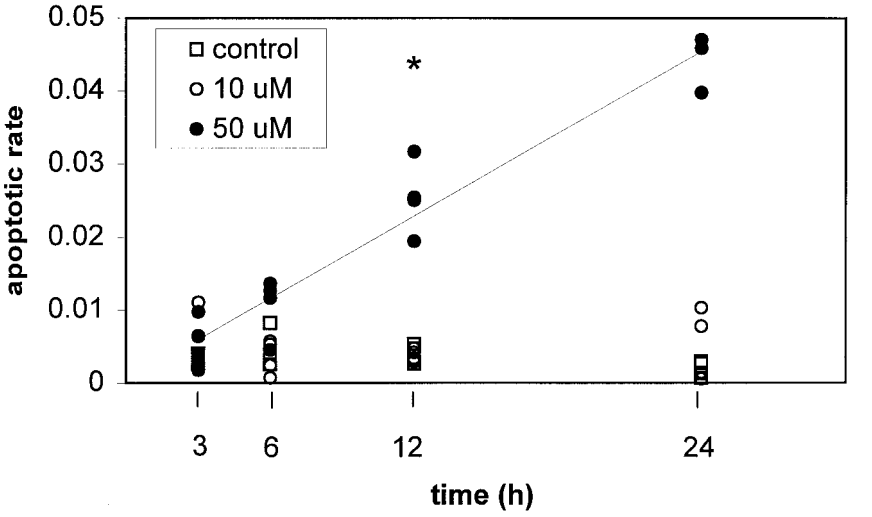

Figure 4. Rate of apoptotic cells for EPC after exposure to nonylphenol for 3, 6, 12 or 24 h. The increase of apoptotic rate for cells exposed to 50 μM NP over time is described as $y = 0.001877x + 0.00038$, $R^2 = 0.931$, $p < 0.001$. After a 12 h exposure time to 50 μM NP the apoptotic rate was significantly higher than in the control (asterisk).

Wastewater sample

No differences between sterile and unsterile controls as well as sterile and unsterile dilutions were found (Student's *t*-test $t = 0.036$, $p = 0.973$ and $t = -0.796$, $p = 0.472$).

Diluted wastewater had no significant effect on the viability of PCC or EPC cells (PCC: Friedman $\chi^2 = 14.778$, $p = 0.039$, *post-hoc:* Wilcoxon, *p*-values 0.011–0.038 > 0.0071 (α after Bonferroni correction); EPC: ANOVA, $F = 1.320$, $p = 0.191$).

Changes in the apoptotic rate were detected in PCC cells after treatment with diluted wastewater (figure 5). Apoptotic rate decreased significantly after dilutions 1:2 and 1:1 were applied (Wilcoxon-test $p = 0.003$ and $p = 0.018$ respectively). At a dilution of 1:4 apoptotic rate did not differ from the control. A correlation between dilution and apoptotic rate was found to be described as $y = 0.00741x + 0.00486$, $R^2 = 0.412$, $p = 0.007$.

In EPC the apoptotic rate did not change when exposed to wastewater diluted 1:1 sterile or non sterile.

Discussion

In this study three *in vitro* systems of the epidermis of fish were tested for their suitability as tools in toxicological studies using cell viability and apoptotic rate as endpoints for detecting adverse effects. The cell cultures were of comparable sensitivity when tested for cell viability and apoptotic rate after exposure to nonylphenol. However, application of wastewater to the cell cultures revealed a higher sensitivity of PCC cells.

Cell viability is an endpoint very commonly used in *in vitro* toxicological studies (Segner 1998b). The LC_{50} values obtained for PCC and EPC after a 96 h exposure

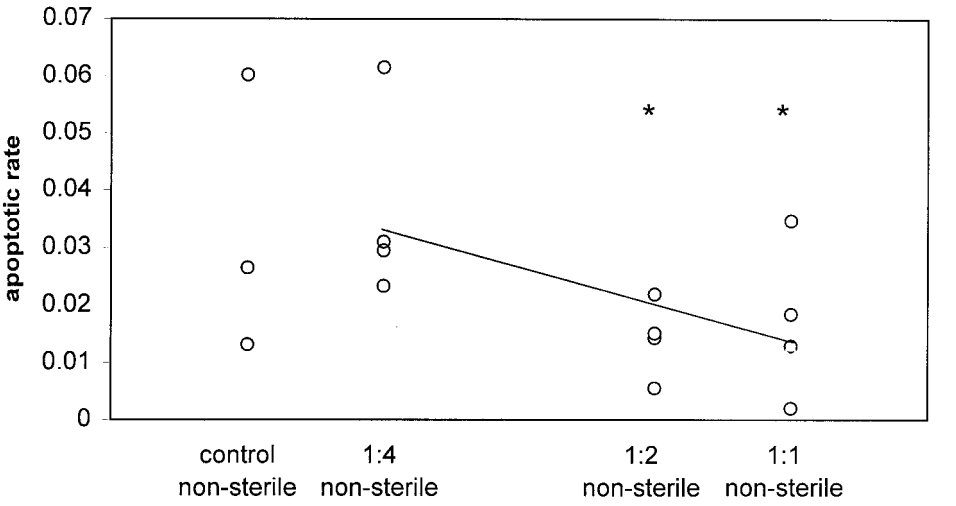

Figure 5. Apoptotic rate for PCC after exposure to a 7-day mixed wastewater sample for 24 h. Apoptotic rate decreased significantly after application of dilutions 1:2 and 1:1 (asterisks). At a dilution of 1:4 apoptotic rate did not differ from the control. The correlation between dilution and apoptotic rate was described as $y = 0.00741x + 0.00486$, $R^2 = 0.412$, $p = 0.007$.

to NP were similar. Cytotoxic effects on primary hepatocyte cultures of rainbow trout *Oncorhynchus mykiss* Walbaum were observed at concentrations > 100 μM of NP (Jobling and Sumpter 1993). Comparably, both cell cultures tested in this study are more sensitive than the primary hepatocytes. However, in comparison to the living organisms our values are nearly two orders of magnitude higher than the LC_{50} value reported for the rainbow trout *Oncorhynchus mykiss* Walbaum of 221 μg $l^{-1} \approx 0.9$ μM of NP (Brooke 1993). Depending on the substances, similar or even larger differences in the absolute sensitivity of cell lines in comparison to the corresponding *in vivo* tests were found (Babich and Borenfreund 1987, Segner and Lenz 1993).

The apoptotic rate increased significantly compared with the control in both cell cultures after exposure to 50 μM NP. For EPC cells a linear correlation of the increase of the apoptotic rate over the exposure time was found. An increase in the percentage of apoptotic cells in fish skin epidermal explants was found after exposure to 50 μM of cadmium (Lyons-Alcantara *et al.* 1998). Apoptotic rate is therefore a suitable biomarker for detecting adverse effects caused by—at least these two—toxicants. The staining pattern of apoptotic cells is similar on all three cultures and corresponds to the pattern described (Zupanc *et al.* 1998).

Considering the effects of NP on the two cell cultures, there is no difference in sensitivity for the two endpoints studied. This is in contrast to studies on primary fish hepatocyte cultures when compared with the hepatocyte-derived cell line R1, where primary cultures were clearly more sensitive than the cell line for a broad range of chemicals tested (Braunbeck 1995).

In explant cultures apoptotic cells were often observed at the edge of the outgrowth, so these regions were excluded from our quantification. The occurrence of more apoptotic cells at the edge was also observed for rainbow trout *Oncorhynchus mykiss* Walbaum epidermal explants in another study (Lyons-Alcantara *et al.* 1998).

The explant culture reacted upon exposure to nonylphenol by a concentration-dependent increase of the apoptotic rate, but this increase of apoptotic rate was not significant compared with the control. This lower sensitivity of the tissue culture may be due to the presence of differentiated cells expressing more genes (Freshney 1994) and providing more protective repair mechanisms for the cell than in cultures of dispersed cells. Explant cultures of rainbow trout epidermis were proven to constitutively express heat shock proteins (hsps) 70 (Lyons-Alcantara *et al*. 1998). Since these proteins take part in repairing and protecting cellular proteins from stressor-induced damage (Hartl 1996, Parsell and Lindquist 1993), cells containing hsps can better cope with a stressor. Thus, the presence of hsps in explant culture cells could contribute to the lower sensitivity. Furthermore the explant culture contains mucous cells. The mucous of fish is known to reduce diffusion rates of substances in general (Shephard 1994). A lower diffusion rate leads to reduced availability, which could also be a reason for the observed slightly lower sensitivity of the tissue culture compared with the cell cultures after a 24 h exposure time.

In an *in vivo* experiment changes of the granulation pattern of rainbow trout mucous cells after exposure to 1 $\mu g\ l^{-1}$ and 10 $\mu g\ l^{-1}$ of NP indicated a change in the chemical composition of the mucous (Burkhardt-Holm *et al*. in press). Exposure to nonoxynol was shown to especially affect the mucous cells in epidermal explants cultures (Dowling and Mothersill in press). Therefore an effect on the mucous cells by nonylphenol or related substances as nonoxynol seemed to occur and could lead to reduced bioavailability of NP for the keratinocytes.

Since it is well known that serum withdrawal leads to apoptosis (Mastrangelo and Betenbaugh 1995), we used serum-containing media. In wastewater toxicity tests it was found that under serum-free conditions a higher sensitivity of the cells than under serum-containing conditions was found in more than half of the tests (Kohlpoth and Rusche 1997), suggesting that the absence of FCS led to a higher bioavailability of toxic substances. In our experiments the possibility of reduced bioavailability due to serum in the media cannot be excluded.

Testing the effects of wastewater from different sources on cell cultures revealed cytotoxic effects in some cases (Vega *et al*. 1994, Schulz *et al*. 1995, Kohlpoth and Rusche 1997, Hollert and Braunbeck 1997), whilst in others no toxic effects of final effluent wastewater samples were found (Castano *et al*. 1994, Schulz *et al*. 1995, Hollert and Braunbeck 1997).

In our cultures exposure to wastewater did not show any effect on cell viability. Analytical data of the used wastewater sample revealed low concentrations of NP (1.63 $\mu g\ l^{-1} \approx 10$ nM) and NPEs as well as some polycyclic musk odorous substances, whereas most of the routinely analysed substances (six polycyclic musk odorous substances, 18 chlorinated carbohydrates, seven pesticides) were below detection limits (Dr M. Sägesser, unpublished). A concentration of 10 nM NP is far too low for inducing effects in our cultures and is also well below NOEC values for any organism tested so far (reviewed in Staples *et al*. 1998).

A decrease in apoptotic rate after exposure to the wastewater sample was observed using the primary cell culture. The observation of apoptosis, compared with nil corresponding observations in the controls, in the epidermis of rainbow trout and brown trout *in vivo* was also reported after cortisol-treatment, exposure to acidified water, exposure to water of the river Rhine and to wastewater (Iger *et al*. 1994, Iger and Wendelaar Bonga 1994, Iger *et al*. 1995, Burkhardt-Holm *et al*. 1997).

One possibility for the decrease of apoptotic rate could be the absence of regulatory systems, e.g. the hormone system, in *in vitro* systems, since it is known that hormones can trigger the apoptotic rate (Mastrangelo and Betenbaugh 1995). Another possibility could be a component in the wastewater inhibiting apoptosis.

Sterile filtration had no effect on apoptotic rate. Hence, in the tested wastewater sample it was obvious that no substances affecting apoptosis were eliminated by the filtration procedure.

The examination with the *Daphnia* acute toxicity test and the luminescent bacteria toxicity test revealed a wastewater dilution of 1:1 to be toxic for *Daphnia* and of 1:2 in the bacterial luminescence test (data kindly provided from Dr M. Sägesser, Water and Soil Protection Laboratory, Berne). Hence the primary cell culture shows a higher sensitivity than the *Daphnia*-test and a similar sensitivity as the luminescence-test, when using apoptotic rate as endpoint.

The endpoint apoptosis is clearly more sensitive than the parameter cell viability. For establishing apoptotic rate as a biomarker in toxicity testing, more studies would be needed to confirm its suitability. Reproducible cell countings by image analysis could provide a possibility for establishing the apoptotic rate as a biomarker in cell tests.

Summarizing the results after testing NP and the wastewater sample, the primary cell culture is more sensitive than the cell line EPC. Both cell cultures were more sensitive than the explant culture after nonylphenol exposure when measuring apoptotic rate.

Conclusion

The three *in vitro* systems tested all revealed suitability to detect adverse effects after exposure to nonylphenol and might therefore be used as tools in toxicological studies.

The two cell cultures were more sensitive than the tissue culture in the nonylphenol experiment, which was suggested to be due to the presence of differentiated cells and mucous in the explant culture.

The experiment exposing the cells to a wastewater sample draws a different picture of the sensitivity of both, the chosen endpoints as well as the tested cell cultures. The primary cell culture is more sensitive than the cell line and the apoptotic rate is more sensitive than cell viability.

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