Biological Activity of Pentachlorophenol on the Digestive Gland Cells of the Freshwater Mussel *Unio tumidus*

Katarzyna Milowska[a], Teresa Gabryelak[a*], Joanna Dudala[a], Magdalena Labieniec[a], and Ekaterina Slobozhanina[b]

[a] Department of General Biophysics, University of Lodz, Bamacha 12/16, 90–237 Lodz, Poland. Fax: (48 42) 635 44 74. E-mail: tgabryl@biol.uni.lodz.pl
[b] Institute of Photobiology, National Academy of Sciences of Belarus, 220072 Minsk, Belarus

* Author for correspondence and reprint requests

Many chlorinated phenols and their derivatives are used extensively as insecticides, fungicides and herbicides by industrial and agricultural users throughout the world. Among these substances, pentachlorophenol (PCP) is a broad-spectrum biocide, which is still used as a wood preservative. In this paper, the digestive gland cells were used to assess the effect of PCP in the range of concentrations 3.75–75 μM (0.01–0.2 ppm) on oxidative DNA damage, fluidity changes and peroxidation activity in the plasma membrane. The toxic property of PCP on DNA strand breakage was studied using the comet assay. The results showed that pentachlorophenol in the range of 37.5–75 μM contributed to these lesions. To demonstrate the changes in the fluidity of plasma membrane we used the spectrofluorimetric method using two fluorescence probes: 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5-hexatriene (TMA–DPH) and 12-(9-anthroyloxy) stearic acid (12-AS). It was shown that PC did not influence the surface of plasma membrane but contributed to the increase in the fluidity of the internal region of the lipid bilayer in the range of concentrations 18.75–75 μM (0.05–0.2 ppm).We also examined the effect of PCP on the lipid peroxidation. To imply its peroxidation properties the spectrophotometry method was used to measure the level of malondialdehyde (MDA), one of the endpoints of the peroxidation of polyunsaturated fatty acids. The obtained results showed that PCP in the used doses did not initiate the formation of lipid peroxides. Thus, our investigation indicates that PCP can behave as a prooxidant agent but its action depends on the used doses and parameters chosen for the research.

Key words: Pentachlorophenol, *Unio tumidus*, Genotoxicity, Peroxidation

Introduction

Freshwater mussels are an ecologically important fauna because they are used as sensitive biomarkers of aquatic ecosystems pollution. Bivalves, such as *Unio tumidus* (Cossu *et al.*, 1997) are stationary, filter-feeding organisms able to bioaccumulate and concentrate most pollutants even if they are present in fairly low concentrations (Niyogi *et al.*, 2001). The measurement of changes in the lipid bilayer and DNA damage in these bivalves are commonly used to diagnose the pentachlorophenol exposure in environmental monitoring studies. Many potentially toxic anthropogenic organic xenobiotics enter the water environment and are taken up by aquatic organisms, thus contributing to the changes in their life stages (Peters *et al.*, 1996). Some reports show that used PCP can be found in phenolic compounds belonging to one of the major classes of pollutants (Choi *et al.*,

1999). PCP, used primarily as a wood preservative, is relatively stable in the natural state and is thus a ubiquitous contaminant in the environment (Klibanov *et al.*, 1980). This agent has been confirmed as a compound toxic to aquatic organisms as well as to mammalian cells (Klobučar *et al.*, 1997; Jansson and Jansson, 1991). Furthermore, it has been found to be genotoxic to higher plant cells (Chand, 1980), and mutagenic to bacteria and mice (Gopalaswamy and Nair, 1992). PCP contamination is generally associated with surface soils from drying areas and with groundwater contaminated from the above source (Kieth and Telliard, 1979). Most chlorophenols, particularly PCP, have been extensively studied regarding their bioeffect and biodegradability in the environment. The aqueous solubility of these substances has become an important parameter in ecotoxicology and biotreatability studies. Aqueous solubility of substances like chlorophenols is useful as an indicator of their hy-

drophobic partitioning from water and, as a result, of the concentration, which they can achieve in biotic phases (Kaiser and Valdmanis, 1982). An extensive literature review on PCP solubility revealed that this agent is a weak acid, only partially dissociates in water and this process depends on the solution's pH. It was noticed that during dissolving of PCP in water, two forms were present: undissociated PCP (PCP°) and a dissociated anionic form – pentachlorophenolate (PCP⁻). Both forms differ in their physico-chemical properties and their toxicity (Arcand *et al.*, 1995). Therefore, it is important to know the solubility behaviour of PCP in water.

There is a possibility that during the degradation of PCP hydroxyl radicals can be generated in Fenton-type reaction (Fahr *et al.*, 1999). Several other mechanisms have been proposed to explain the toxicity of PCP, including

i) uncoupling of oxidative phosphorylation (Weinbach and Garbus, 1969);

ii) metabolic activation by microsomal enzymes resulting in the formation of highly reactive metabolites (Seiler, 1991);

iii) transition metal-rediated reactive oxygen species (ROS) (Rao, 1987).

Reactive oxygen species can directly cause covalent modifications to DNA and they can initiate the formation of lipid hydroperoxides, which contribute to the induction of different kind of damage and diseases.

Chlorophenols affect cell membrane functions by increasing its permeability, as seen by dissipation of transmembrane pH gradients or by efflux of small cellular metabolites such as ATP and K⁺ ions. It was also noticed that the presence of phenols increases the degree of saturation of fatty acids in the membrane, which decreases membrane fluidity (Melin *et al.*, 1988). Those facts explain the importance of the investigation of the influence of PCP on permeability of lipid bilayer. Together with increasing the fluidity of membrane there is a possibility that other toxic chemicals have an easy access to the inside of the cell.

In the present study, we have focused on DNA breaks, changes in the fluidity and peroxidation process in the cell membrane by treatment of the freshwater mussel *Unio tumidus* with PCP, known as an environmental contaminant and toxic sub-stance. The major aim of this work was to assess the biological consequences of using PCP.

Materials and Methods

Chemicals and reagents

Pentachlorophenol (PCP), phosphate buffered saline (PBS), low melting-point (LMP) and normal melting-point (NMP) agarose, 4′,6-diamidino-2-phenyl indole (DAPI), dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), trypan blue, fluorescence labels: 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5,-hexatriene (TMA–DPH) and 12-(9-anthroyloxy)-stearic acid (12-AS) were purchased from Sigma (St. Louis, MO, USA). All other chemicals were from Polish Chemical Reagents (Poland) and were of analytical grade.

PCP solutions were prepared in 1% ethanol solution. The stock solution of lipid probes TMA–DPH and 12-AS was prepared in tetrahydrofuran/H_2O (1:1). All other solutions were made with double-distilled water or purified by the Mili-Q system.

Cell preparations and PCP exposure

Mussels (*Unio tumidus*, 7–10 cm in length) were collected in autumn from the Pilica river (in the central part of Poland). In the laboratory the animals were kept in glass tanks in a flow of dechlorinated, well aerated water (temperature 4–6° C).

Single cells were prepared from the mussel digestive gland. Small pieces of freshly resected digestive gland were treated with trypsin/PBS for 2 h at 14° C. After trypsinization cells were centrifuged at $1500 \times g$ for 10 min and resuspended in PBS. Each sample of *Unio tumidus* digestive gland cells was exposed to a different concentration of PCP (0.375, 3.75, 18.75, 37.5 and 75 μM) for 1 h at 4° C. The control cells were treated by PBS (pH 7.4). After incubation with PCP samples of the cells were separated by centrifugation at $1500 \times g$ for 10 min and washed with PBS.

The cell viability was assessed by trypan blue exclusion and was always above 85% for each tested dose in all experiments of this study.

Comet assay

The comet assay was conducted as described by Singh *et al.* (1988) with a slight modification by Blasiak and Kowalik (2000). Briefly, 30 μl

(~ 30,000 cells) of the cell suspension was mixed with 50 μl 0.75% low melting-point agarose (LMP) at 37° C, spread on a normal agarose (NMP) precoated microscope slide. The slides with cells were covered with a cover slip and were subsequently placed on an ice-cold surface to solidify for about 10 min. The cover slips were removed and the slides were placed in cold lysing solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris (hydroxymethyl aminomethane) pH 10.1% Triton X-100, 10% DMSO; the last two components were added freshly]. Lysis was performed for 1 h at 4° C in total darkness. The slides were then incubated in an electrophoretic buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at the same temperature. Electrophoresis was performed in the same buffer at 0.73 V/cm (280 mA) for 20 min to allow the damaged DNA or fragments to migrate towards the anode. The slides were then washed three times in water and dried by air. Finally, 30 μl DAPI (2 μg/ml) was added to each slide, which was covered with cover slip and kept for 1 h in the dark. Comets were analysed by an Eclipse fluorescence microscope (Nikon, Tokyo, Japan) attached to COHU 4910 video camera (Cohu, San Diego, CA) equipped with UV-1 filter block (an excitation filter of 359 nm and a barrier filter of 461 nm) and connected to a personal computer-based image analysis system Lucia-Comet 4.51 (Laboratory Imaging, Praha, Czech Republic). Tail moment as a measure of DNA damage in the graphic presentation represents the mean ± S. E. M. of 6 individual experiments (100 cells from each slide).

Fluorescence anisotropy measurement

Washed cells (at 5×10^5 cells/cm^3 density) were incubated with the membrane probes: TMA–DPH and 12-AS for 5 min and 15 min at room temperature, respectively. Then, the cells were washed and resuspended in the appropriate buffer, pH 7.4, to a cell density of 2×10^5 cells/cm^3. Fluorescence anisotropy measurements were performed in a Perkin-Elmer luminescence spectrometer (Model LS50B), using the excitation wavelength 358 nm, the emission wavelength 428 nm for TMA–DPH and 360 nm and 471 nm for 12-AS, respectively. The degree of fluorescence anisotropy was calculated according to Shinitzky and Barenholz (1978). Anisotropy values on each

figure are presented as mean ± SD calculated from eight independent measurements.

Lipid peroxidation

Lipid peroxidation was followed by measuring the thiobarbituric acid-reacting products. (Placer *et al.*, 1966). Washed cells were mixed with trichloroacetic acid (TCA) and thiobarbituric acid (TBA), boiled for 20 min. After cooling the mixture was centrifuged and the absorbance at 532 nm measured. All reactions were conducted in the presence of traces of butylated hydroxytoluene (BHT). MDA concentrations are presented in nmol/mg protein from ten independent measurements.

The amount of protein was determined by the method described by Lowry *et al.* (1951).

Statistical analysis

The results are presented as mean ± S. E. M. for the Comet assay and as mean ± SD for fluorescence anisotropy and lipid peroxidation. Statistical evaluation of difference between control and treated group was performed using Student's T-test. P < 0.05 and below was accepted as statistically significant.

Results

Genotoxic effect of PCP

On electrophoresis, fragments of DNA molecules move more rapidly than intact DNA molecules resulting in a "comet with tail" formation. Tail moment one of the best indices of induced DNA damage was calculated by multiplication of the tail length by the amount of DNA in the tail. The level of DNA damaged in cells isolated in the mussels after 1 h of incubation with different concentrations of PCP was studied. The results shown in Fig. 1 were representative of eight independent
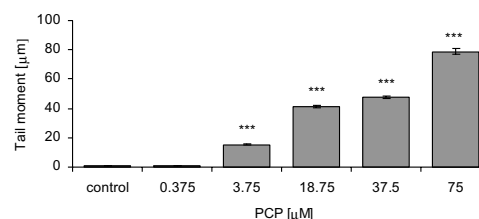

Fig. 1. Mean comet tail moment of exposed for 1 h at 5 °C PCP. The number of cells in each of 6 individual treatments was 100. Error bars denote S. E. M.; *** P < 0.001.

experiments. Lower doses of PCP as 0.375 μM did not induce the formation of single strand breaks, but at higher doses most of the cells showed moderate or strong DNA damaged. A significant increase in tail moment was observed after the exposure in the range of 3.75–75 μM (p < 0.001).

Effect of PCP on fluidity changes

To demonstrate the changes in the fluidity of plasma membrane we used the spectrofluorimetrical method using two fluorescence probes: 1-[4-(trimethylamino)phenyl]-6-phenyl-1,3,5,-hexatriene (TMA–DPH) and 12-(9-anthroyloxy)-stearic acid (12-AS). The lipid probe 12-AS is located relatively deeply in the hydrocarbon interior of the lipid bilayer (Blatt and Sawyer, 1986) and it is a member of the anthroyloxy-fatty acid series used to detect fluidity gradients across the lipid bilayer (Tilley *et al.*, 1979). TMA–DPH, in contrast to 12-AS, is known to be localised in the polar head-group region of the plasma membrane. The results of our study showed (Fig. 2) that PCP did not influence the surface of plasma membrane but contributed to the increase in the fluidity of the internal region of the lipid bilayer in the range of concentrations 18.75–75 μM.

Lipid peroxidation

Lipid peroxidation was measured in the digestive gland cells after incubation at different concentrations of PCP for 1 h. For the assessment of its peroxidation properties the spectrophotometry method was used to measure the level of malonodialdehyde (MDA), one of the endpoints of the
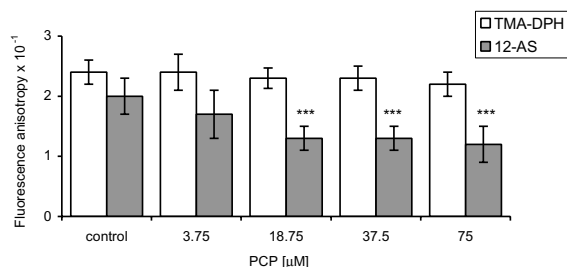
Fig. 2. The fluidity changes in the plasma membrane in digestive gland cells induced by PCP after 1 h of treatment. Experiments based on the TMA–DPH and 12-AS fluorescence anisotropy. Error bars denote SD; *** P < 0.001.
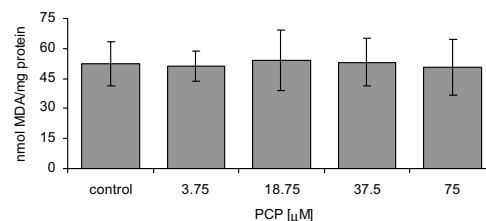
Fig. 3. MDA concentration in digestive gland cells induced by PCP after 1 h of treatment. The data were obtained from ten individual measurements.

peroxidation of polyunsaturated fatty acids. The results showed (Fig. 3) that peroxidation in samples incubated in PCP did not differ from controls.

Discussion

The genetic material (DNA) as well as plasma membranes affected by the activity of chemical compounds may pose a threat to the proper functioning of the cells in all organisms living in biosphere, including the aquatic biota (Livingstone, 1998; Arukwe *et al.*, 2000; Eufemia and Epel, 2000; Roche *et al.*, 2000; Schwaiger *et al.*, 2000). In this study genotoxic influence of egzogenic substance PCP has been assessed and changes in the lipid layer of plasmatic cell membranes in digestive glands of freshwater mussels (*Unio tumidus*) have been investigated *in vitro*. Mussels proved to be reliable indicators of rivers and lakes contamination with various chemical compounds and heavy metals that is why they are often used for monitoring the level of aquatic environment contamination (Richardson *et al.*, 2001; Piccardo *et al.*, 2001).

In vitro study revealed genotoxic damage in the cells of digestive gland of *Unio tumidus* as early as after one hour of incubation with PCP at the concentration of 3.75 μM. The rise in the concentration of this compound (18.75, 37.5 and 75 μM) was accompanied by the increase in DNA damage. However, *in vivo* study revealed genotoxic effects in haemocytes of zebra mussel (*Dreissena polymorpha* Pall.) as late as after 4 days of the exposure to PCP at a concentration of 37.5 or 56.25 μM (Pavlica *et al.*, 2001). It should be taken into consideration that in the aquatic environment the most frequent PCP concentrations are below 3.75 μM (1.125–1.462 μM), and this is a reason why genotoxic effects may appear after a longer exposure of the organisms to this compound (Brata-

nova *et al.*, 1998). As well, it should be taken into consideration that the model experiments performed on the cells are devoid of the influence of biochemical changes, which extend the time of the obtained results while being affected by the environmental factors.

The results show that at certain concentrations PCP induces single strand breaks in DNA. It is known that low doses of PCP increase the consumption of oxygen in the investigated organisms. It is caused by the loss of mitochondrial breathing as a result of the impairment of oxydative phosphorylation. High consumption of oxygen in those conditions can contribute to the process of excessive production of the reactive oxygen species (ROS), which could explain the mechanism of PCP toxicity in the case of DNA damage. On the other hand, the presence of other polyphenols such as polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), which are extremely toxic, can also contribute to DNA damage (Pavlica *et al.*, 2001). ROS generate different types of DNA damage, such as the damage of alkali, abasic sites as well as single and double strand breaks both in *in vivo* and *in vitro* systems (Dahlhaus and Appel, 1993). PCP penetrating into a cell may initiate not only the damage in the genetic material. The obtained results reveal that PCP causes the rise in the fluidity of the plasmatic membrane in the hydrophobic area wihout significant changes in its surface. Chlorofenol-derivatives are weak acids with a lypophil character. They distract the lipid phase of the cell membrane. In the natural environment chlorophenols appear in an ionized and non-ionized form. In the non-ionized form PCP penetrates the biological membranes more easily than in the ionized form which explains the influence of the pH on PCP toxicity. In consequence this xenobiotic contributes to the rise in the fluidity of the plasmatic membrane in its hydrophobic area without affecting its surface. It can be supposed that PCP or its derivatives which accumulate in the hydrophobic layer can initiate its damage directly or as a result of a disfunction in the enzymatic activity. In spite of the possibility of the formation of free radicals which induce oxydation processes in the cell (*e.g.* lipid peroxidation), this phenomenon was not observed in the isolated pancreas-liver cells exposed to the PCP activity. It can also be supposed that MDA or other lipid peroxidation products may be washed out during one of the stages of the *in vitro* experiment performed in a model system or the process of peroxidation at the used PCP concentrations and during 1h incubation may occur on a very low level. It does not change the fact that the cell walls rich in lipids, similarly to DNA, become important shields against the toxic compounds including the products of PCP metabolism or generated by them ROS (Dahlhaus *et al.*, 1995). In consequence, the problem of their harmful influence on the structure of the cell membranes requires further investigation.

Referring the results obtained through *in vitro* experiments to *in vivo* systems, it should be remembered that the analysed reactions take place only in similar biochemical conditions and during the use of investigation techniques the potential damage of DNA or lipids may take place.

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