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Increase in number and size of kidney concretions as a result of PCP exposure in the freshwater snail *Planorbarius corneus* (Gastropoda, Pulmonata)

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ABSTRACT: Molluscan kidneys are able to excrete solids in the urine in the form of concretions. It is thought that increased formation of these concretions occur under pollutant, environmentally or reproductive induced stress. This study examined the formation of concretions in the kidney of the freshwater snail *Planorbarius corneus* L. experimentally exposed to pentachlorophenol (PCP). Light microscopic histopathological analysis of the PCP-exposed *P. corneus* revealed significantly enhanced production of the kidney concretions when compared to the kidneys of control individuals. Measurements of the number of kidney concretions, the apparent area of the concretions, and the epithelial area filled with concretions indicated an increase in the number and size of concretions in all treated snails. Lipofuscin content of excretory cell concretions was detected.

KEY WORDS: Kidney concretions · Mollusca · *Planorbarius corneus* · Pentachlorophenol

INTRODUCTION

The role of the excretory system of molluscs in bioaccumulation and depuration has as yet been insufficiently investigated. Molluscan kidney consists of a tubule enclosing a luminal cavity derived from the coelom. The nephrocytes lining the luminal surface of the tubule are involved in the secretion of waste and the resorption of metabolites from the urinary fluid (Simkiss & Mason 1983). One of the peculiarities of molluscan kidneys is their ability to produce and excrete solids in the urine in the form of concretions (Potts 1968, Doyle et al. 1978, Andrews 1981, Angulo & Moya 1989, Marigómez et al. 1995). It is thought that increased formation of these concretions occurs under pollutant (Yevich 1980, Seiler & Morse 1988), environmentally (Krahelska 1910) or reproductive (Doyle et al. 1978) induced stress. Studies by Doyle et al. (1978) and others (Yevich 1980, Sunila 1987, Regoli et al. 1992) point out that the kidney concretions may possibly serve as biological monitors of pollution.

Molluscs are widely used in different biomonitoring projects. Their histopathological analysis provides information about the general health of the animals and contaminant-specific changes in the tissues. Although laboratory as well as field studies suggest that pollutants cause toxic effects to molluscs, the histopathological effects of chemical contaminants have not generally been measured (Sunila 1986, 1989, Hemelraad 1990, Marigómez 1990a). When measured, different qualitative, semi-quantitative, and quantitative approaches were used (Lowe 1988, Cajaraville 1990). Only recently has computer-assisted image analysis begun to be used in ecotoxicological research to provide information regarding cellular and tissue abnormalities (Etxeberria et al. 1994, Krishnakumar et al. 1994, Soto & Marigómez 1997). The use of computer-assisted image analysis improves the precision and objectivity of the measurements while being much less time-consuming.

Pentachlorophenol (PCP) has been chosen as a reference compound because its chemistry, toxicology and fate in the environment are fairly well understood (Rand & Petrocelli 1985, WHO 1987).

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The purpose of this investigation was to determinate whether appearance of concretions in the kidney of the freshwater snail *Planorbarius corneus* exposed to PCP is dose dependent.

MATERIALS AND METHODS

Adult specimens of the freshwater pulmonate snail *Planorbarius corneus* L. were collected from the Odra River (16° 08′ E, 45° 41′ W), Croatia. Acclimatization of snails to laboratory conditions in dechlorinated tap water lasted for 48 h. The specimens used for the study averaged 22 ± 3 mm in height. PCP (sodium salt) was obtained from BDH Chemicals Ltd, Poole, United Kingdom. Stock solutions of PCP (100 mg PCP l^{-1}) were prepared in distilled water. The experiment was carried out in aerated glass dishes, each containing 5 l of dechlorinated tap water. Based on the results of the preliminary mortality tests (the LC_{50} value for 96 h was 1420 µg PCP l^{-1}), 3 concentrations were chosen: 450, 600 and 800 µg PCP l^{-1}. The experiment was repeated 3 times, with parallel controls. One dish was used as the control, receiving dechlorinated tap water only. There were 20 snails per dish. The test solutions and control water were renewed daily. The experiment lasted for 7 d. The temperature and pH of the test solutions and control water were determined every 24 h by routine procedures (APHA 1985). The physicochemical characteristics of the test water were as follows: temperature 20 to 21°C; pH 7.6 to 8.0; dissolved oxygen 7.0 to 8.2; hardness 288 mg $CaCO_3$ l^{-1}. The snails were not fed throughout the assay because we wanted to prevent any possible changes in concretion production due to the digestion and excretion of food.

For histological analyses, live specimens were removed from each exposure concentration and control on Days 2, 4 and 7. Nine to ten individuals were analyzed per experimental group. Snails were placed in Bouin's fixative for 24 h. After fixation, the snails were embedded in paraffin and cut with a microtome into 6 to 8 µm thick slices. The sections were then stained with haematoxylin and eosin for histopathological analysis. Lipofuscin content of excretory cell concretions was detected by using the Schmorl reaction (Pearse 1968). From each snail, 2 sections that contained kidney tissue were examined. Five images of kidney saccular portion were randomly taken from each section. The image size was approximately 0.1224 mm^2. Tissue sections were analyzed using computer assisted image analysis. The image analysis system consists of a color CCD video camera (Sony 151 P) mounted on a light microscope and a PC where the images were examined utilizing SFORM image analysis system (made by VAMS d.o.o., Zagreb, Croatia). We measured the average number of concretions ($N_{ca} = N_c/A_e$), the average apparent area of concretion ($A_{ca} = A_c/N_c$), and the average epithelial area filled with concretions ($A_{ec} = A_e/A_c \times 100$), where N_c = number of concretions in the epithelium, A_e = area of epithelium, and A_c = area of the concretions found in the epithelium. Statistical analyses were measured by using non parametric Kruskal-Wallis and Mann-Whitney *U*-tests.

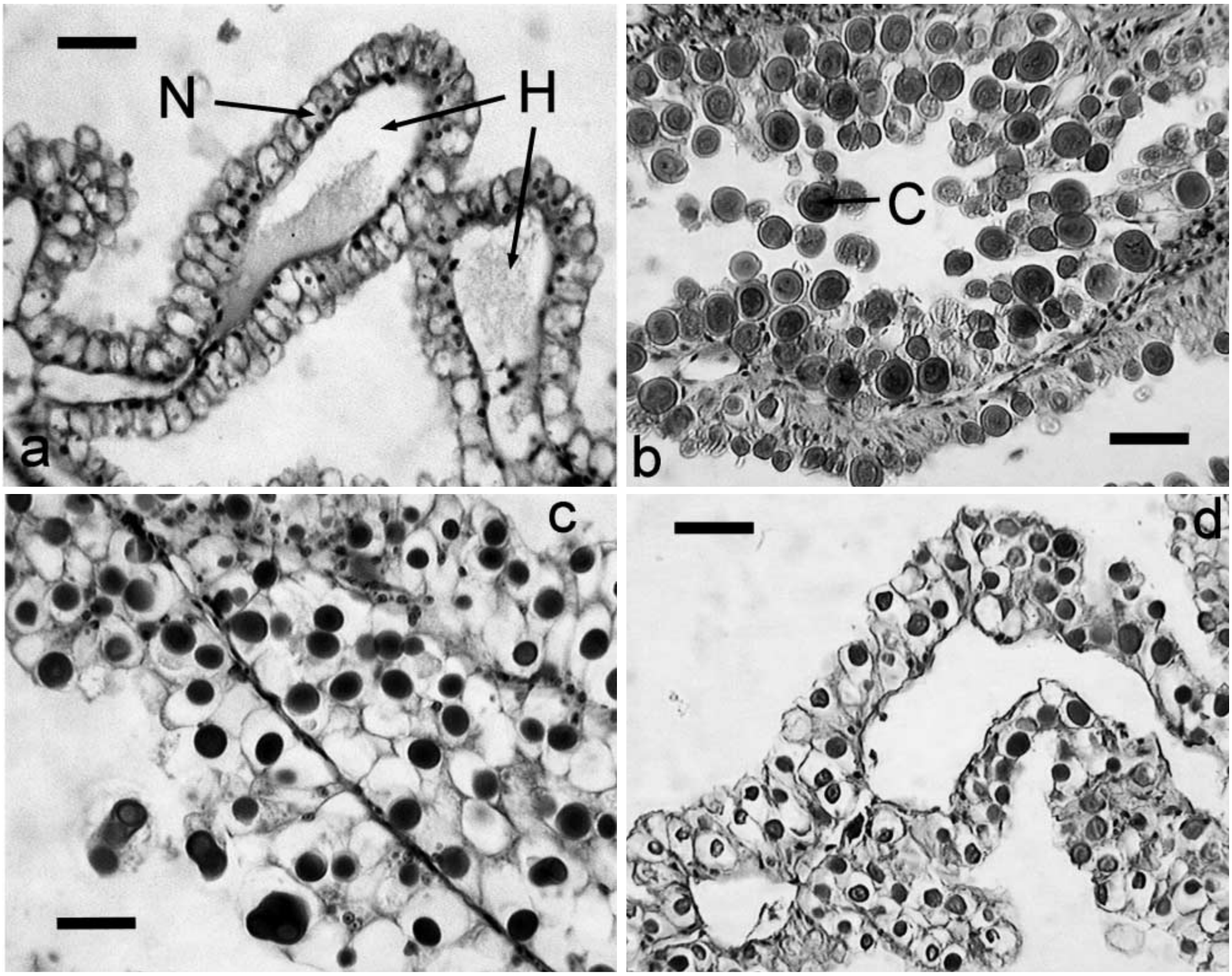
RESULTS

The kidney of *Planorbarius corneus* consists of a small saccular portion, an elongated tubular portion, and a short ureter that opens into the mantle cavity at the pneumostome. The internal surface of the nephridium is much increased by the formation of multiple folds. The epithelial cells of the wall of the saccular portion are columnar and are taller than those of the tubular portion but are arranged in the same manner. The folds are clothed with the excretory epithelium of nephrocytes (Fig. 1a). The apical portion of nephrocytes consists of a vacuole in which the excretory product accumulates, often in the form of large sphere concretions (Fig. 1b). The nuclei are round to oval and are basal in position. The folds consist of connective tissue containing blood vessels and sinuses (Fig. 1a). Another feature characterizing the transition of the saccular portion into the tubular portion is the absence of excretion concretions from the tubular portion epithelium. The crystalline concretions are lightly refractive, round bodies with a central core (Fig. 1b) and appear yellowish-brown in haematoxylin-eosin preparations. Generally, only 1 concretion is formed per cell. Eventually, the cells extrude the concretions into the lumen of the kidney from where they are excreted (Fig. 1b).

Histopathological measurements

On Day 2 of exposure only the highest concentration increased the number of concretions in the kidney epithelium. After 4 d of exposure, massive production of concretions was observed in nephrocytes of snails treated with 600 and 800 µg l^{-1} PCP (Fig. 2a). Lower concentration (450 µg l^{-1}) of PCP exerted significantly enhanced concretion formation on Day 7 of the exposure (Fig. 1c), which then matched the slightly depressed numbers of the 2 highest concentrations (Fig. 1d).

The average apparent area of the concretion found in the kidneys of the control group during the whole experiment ranged from 23 to 43 µm^2 (5.4 to 7.4 µm in diameter). On Day 4 of the experiment, a significant enlargement of concretions was observed in all treated



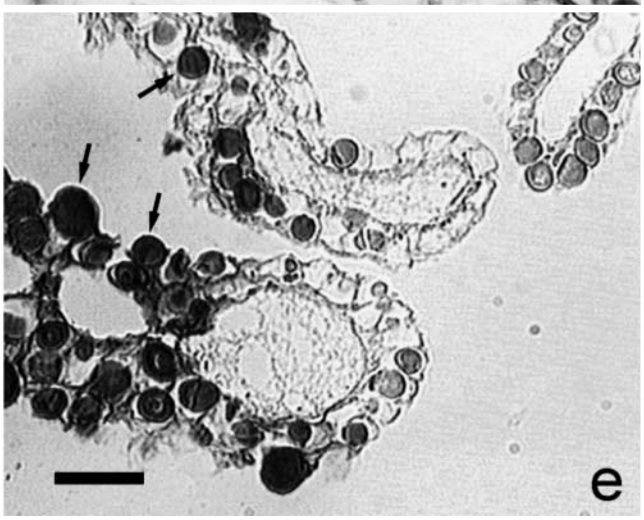


Fig. 1. Excretory organ of a *Planorbarius corneus*. Scale bar = 50 µm. (a) Excretory organ of an untreated snail on Day 4 of the experiment. The folds are clothed with the excretory epithelium of nephrocytes (N). Hemolymphatic spaces (H). (b) Snail treated with 600 µg l^{-1} pentachlorophenol (PCP) for 4 d. Massive production of concretions (C). (c) Snail treated with 450 µg l^{-1} PCP for 7 d. Increased number of concretions. (d) Snail treated with 800 µg l^{-1} PCP for 7 d. (e) Intensive staining of concretions with Schmorl reaction (arrows) in excretory organ of a snail treated with 450 µg l^{-1} PCP for 7 d

groups of snails with the highest response (252 µm^2, diameter: 19.7 µm) in 600 µg PCP l^{-1} (Fig. 2b). Surprisingly, on Day 7 of the experiment, the same concentration showed much lower values.

When analyzed for the average epithelial area filled with concretions, on Day 2 of exposure, only the kidney epithelium of the snails treated with the highest concentration (800 µg PCP l^{-1}) showed a small but

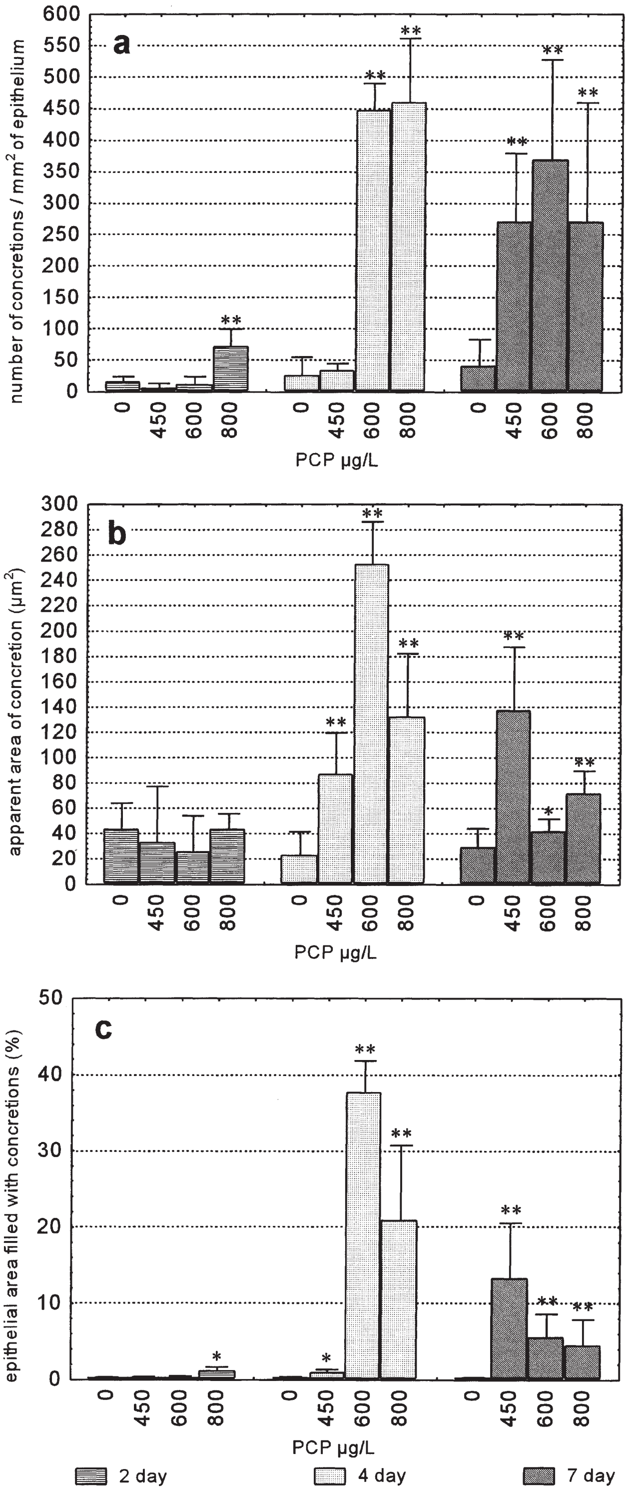


Fig. 2. Changes in the average size and number of concretions in the kidneys of the freshwater snail *Planorbarius corneus* exposed to 450, 600 and 800 µg l^{-1} PCP for 2, 4 and 7 d. Statistically significant values from respective control indicated by $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$. (a) Average number (±SD) of kidney concretions per mm^2 of epithelium. (b) Average apparent area (±SD) of the single concretion. (c) Average epithelial area (±SD) filled with concretions

significant increase compared to the control animals (Fig. 2c). On all the other observed days, the percentage was significantly larger in the treated snails than in the non-treated ones. On Day 4 of the experiment, the highest percentage (37.6%) was detected in 600 µg PCP l^{-1}. In these snails, the concretions occupied almost the entire interior of the nephrocytes, and consequent disruption of nephrocytes became apparent (Fig. 1b). It is evident from Fig. 2c that this value was followed with 21% epithelia, filled with concretions observed in snails treated with 800 µg PCP l^{-1} (Fig. 1d). The reaction of snails treated with the lowest concentration of PCP (450 µg l^{-1}) was slower and the highest percentage (13.11%) of concretion abundance was apparent on Day 7 of the experiment (Fig. 1c).

In our experiment, all of the yellowish-brown concretions (both control and treated snails) in nephrocytes of *Planorbarius corneus* were demonstrated to be Schmorl positive (Fig. 1e).

DISCUSSION

Light microscopical analysis of the PCP-exposed *Planorbarius corneus* revealed a multiple increase in the number and size of kidney concretions when compared to the kidneys of control snails. An increase is evident in all measurements used (N_{ca}, A_{ca}, A_{ec}). Up to now, this kind of event has been mostly associated with heavy metal exposure (Sunila 1986, 1989, Ballan-Dufrancais et al. 1990, Hemelraad et al. 1990, Hyne et al. 1992, Giamberini et al. 1996) although one can find work concerning toxic effects of non metals on molluscan kidney (Cajaraville et al. 1990).

A mutual characteristic of all analyses used (N_{ca}, A_{ca}, A_{ec}) is that the 2 highest concentrations elicited a quicker and stronger response than the lowest one. Also, snails exposed to the lowest concentration (450 µg PCP l^{-1}) exhibited greater concretion growth whilst in those exposed to higher concentrations the effect appeared to be an increase in the number of concretions. The decrease in number and size of concretions in snails treated with 600 and 800 µg PCP l^{-1} after Day 4 of exposure could be caused by the higher turnover rate in these concentrations.

Increase in the number of concretions has been observed in the kidneys of bivalves *Mercenaria mercenaria* and *Mytilus edulis* under heavy metal stress (Rheinberger et al. 1979, Sunila 1986) and in the kidneys of bivalve *Donacilla cornea* exposed to Cu and Cd (Regoli et al. 1992). Light microscopical observations of Marigómez et al. (1990a) on the excretory epithelium of *Littorina littorea* revealed that the amount of concretions increases at low Cd exposures. The kidneys of the same species showed increased occurrence of membrane-bound dark bodies which became enlarged after 96 h of exposure to naphthalene (Cajaraville et al. 1990).

Seiler & Morse (1988) reported that the kidney cells of *Mya arenaria* specimens collected from polluted sediments showed qualitatively higher numbers of granules when compared to those from a nonpolluted site. A comprehensive observation on this matter was made by Doyle et al. (1978), who noted that, sometimes, concretions are also detected in molluscs from apparently nonpolluted areas. Thus, pollution may be primarily related to concretion formation in that it serves as a stress to the organism. In molluscs collected from unpolluted environments, stress factors which may lead to concretion formation are abnormal temperature or salinity or proximity in the reproductive cycle to spawning. Indeed, a significant increase in the number of the concretions was observed in land pulmonates rendered inactive by cold or drying (Krahelska 1910). Cyclic changes in the reproductive system and digestive diverticula, as well as reactions to parasites and microorganisms, must be taken into account before an attempt is made to correlate histopathological changes with pollution (Sunila 1987). In between spawning, during the sexual repose, the nephrocytes are usually free of concretions (Yevich 1980). *Planorbarius corneus* does not spawn in autumn (Costil & Daguzan 1995), at the time when our experiments were performed. We found no egg capsules during our experiment; however, influences from reproductive activity could not be completely excluded.

The average apparent area of the single concretion in non-treated *Planorbarius corneus* ranged between 23 and 43.1 μm^2 (5.4 to 7.4 µm in diameter respectively), which is similar to the size of concretions (3 to 7 µm in diameter) observed in *Arion ater* (Angulo & Moya 1989) and excretion granules measuring 5 to 20 µm in nephrocytes of *Lymnaea stagnalis* (Wendelaar Bonga & Boer 1969). The maximum observed concretion diameter for *P. corneus* in our experiment was 19.7 µm (600 µg PCP l^{-1}). Yevich (1980) also observed that quahogs (*Mercenaria mercenaria*, *Arctica islandica*, *Mercenaria campehensis*) collected from polluted areas had more and larger-sized concretions in the kidneys than those found in molluscs collected from a non-polluted site.

The granules in the different invertebrate tissue can be classified into 3 types depending on cytochemical characteristics: iron rich granules, Cu-S containing granules and Mg/Ca concretions (Viarengo & Nott 1993). In gastropods, the mineralized Mg/Ca concretions exist as phosphate and carbonate types. In the nephrocytes of gastropods, as well as bivalves, concretions are essentially calcium phosphates (Doyle et al. 1978, Taylor & Andrews 1991, Regoli et al. 1992, Marigómez et al. 1995, Giamberini et al. 1996). In addition to calcium phosphate, lipofuscin and other lipid pigments (chromolipids, ceroid pigments) have been shown to accumulate in degenerative digestive and excretory cells in terrestrial (Angulo & Moya 1989, Vivar et al. 1990) and aquatic (Wendelaar Bonga & Boer 1969) pulmonate gastropods and to be present in the tertiary lysosomes of marine molluscs (Cajaraville et al. 1990, Marigómez et al. 1990b). In the Archaeogastropoda, lipofuscin is one of the most important constituents of the excretory granules (Andrews 1985). The presence of lipofuscin in the concretions of *Planorbarius corneus* has been confirmed in our research. Lipofuscins are mainly lipid peroxidation end products which are accumulated in the lysosomes as insoluble lipoprotein granules. PCP induced increased lipid peroxidation in the digestive gland of *P. corneus* exposed to 450 and 800 µg l^{-1} of PCP (Klobučar et al. 1997). Increased oxygen consumption that has been noted as an initial response of PCP-exposed aquatic animals (Weinbach 1954, Holmberg et al. 1972) could have caused increased lipid peroxidation (Roszell & Anderson 1996). We can assume that higher production of kidney concretions in PCP-exposed snails could also be partly due to PCP-induced increase in lipid peroxidation.

Our studies revealed that snails exposed to PCP had more and larger-sized concretions then non-treated individuals but no dose-dependent pattern was observed. Increased concretion formation in kidneys of *Planorbarius corneus* is a useful stress indicator, but its role as a possible biological monitor of pollution requires more research.

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