Impact of sewage effluents on osmoregulation in a freshwater teleost, *Anabas testudineus*

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Summary

This study investigated the impact of sewage effluents of the polluted river, Parvathyputhenar in Trivandrum city, Kerala, India, on the activities of osmoregulatory enzymes such as Na^+/K^+ and Ca^{2+} ATPases, the concentration of sodium and potassium ion content in the gill and on the chloride cells (CCs) and pavement cells (PCs) that regulate ions transport in the gill epithelium of a freshwater fish. The results indicate a significant ($P<0.05$) decrease in the activity of branchial Na^+/K^+ ATPase and Ca^{2+} ATPase in the fish exposed to sewage effluents for 7, 14 and 28 days and the fish caught from the polluted river. When the fish caught from the polluted river were kept in normal pond water in the laboratory for 30, 60 and 90 days, the enzyme's activities were gradually increased and almost restored to the control level. Scanning electron microscopic analysis of gill epithelium showed noticeable changes in the surface area morphology of CCs and PCs in the fish exposed to sewage effluents. Exposure to the sewage effluents drastically altered the size and characteristic "finger print" pattern of PCs and also reduced number of CCs in the gill epithelium. As the ATPases play an important role in maintenance of functional integrity of gill epithelium it is suggested that measurement of the activities of ATPases may be used as a biomarker of exposure to sewage effluents. This work is highly pertinent in the context of increased level and effect of endocrine disrupting chemicals present in the aquatic systems, which are increasing day by day.

Key words : Sewage effluents, Na^+/K^+ ATPase, Ca^{2+} ATPase, chloride cells, pavement cells

Introduction

Aquatic ecosystems are at great risk from pollutants since all chemicals, whether on land or in the atmosphere, will eventually reach the rivers and oceans and the fish as aquatic inhabitants are subjected to a multi-pollution state. In teleost fish, osmoregulation is largely the result of integrated transport activities of gill, gut and renal system (Evans et al., 2005). Teleosts are capable of maintaining ionic composition and osmolarity of their body fluids at levels significantly different from the external environment. Most of the osmoregulatory disturbance resulting from pollutants reported in the literature appear to be the result of their action on gills and inter-renal function (Hwang and Tsai, 1993; Vang et al., 2002; Ahmad et al., 2006; Wu et al., 2008).

Gill is a multifunctional organ involved in gaseous exchange, acid base balance and transport of Na^+, Ca^{2+}, Cl^- (Perry, 1997). Gill epithelium provides an extensive surface of contact with the environment to facilitate ion transport and gaseous exchange (Evans et al., 2005). The gill epithelium consists of pavement cells (PCs), mitochondria-rich chloride cells (CCs) and mucus cells (Laurent, 1984; Zhou et al.,2003; Sunny and Oommen, 2004; Evans et al., 2005). The CCs, more than any other cells in the gill, have ultrastructural characteristics suggestive of specialized transport activity. The involvement of CCs in Cl^- secretion in fish has been confirmed (Foskett and Scheffey, 1982). Ca^{2+} influx is facilitated by a Ca^{2+} channel in the apical membrane of the CCs (Verbost et al.,1989). Extrusion of Ca^{2+} from the cell across the basolateral membrane is mediated by a high affinity Ca^{2+} ATPase and possibly by a Na^+/Ca^{2+} exchanger in the gills of freshwater fish (Zanatta et al., 2001). Sodium, potassium adenosine 5'-triphosphatase is an integral membrane enzyme that actively transports K^+ and Na^+ ions against the respective cellular concentration differences. The gradient produced by this enzyme is coupled to physiological functions such as cell proliferation, volume regulation, maintenance of the electrogenic potential required for the function of excitable tissues, *i.e.*, muscle and nerves, and secondary active transport (Kaplan, 1978; Boldyrev, 1993; Vasilets and Schwartz, 1993; Basavappa et al., 1998). By regulating sodium and potassium ion concentrations, this enzyme also participates in the control of plasma membrane and mitochondrial Na^+/Ca^{2+} exchange, the endoplasmic reticulum and plasma membrane Ca^{2+} ATPase activity, and Ca^{2+} channel activity (Nikezic and Metlas, 1985).

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Endocrine disrupting chemicals appear to produce severe haemodynamic effects on the gill vasculature, which could certainly affect osmoregulation indirectly via alterations in perfusion of specific areas of the gill epithelium (Stagg and Shuttleworth, 1982; Grossel et al., 2005). Heavy metals also have the potential to affect osmoregulation in seawater fish. Stagg and Shuttleworth (1982) demonstrated that exposure of the marine flounder *Platichthys flesus* to 170 ppb Cu^{2+} for 42 days resulted in significant increase in blood Na^+ and Cl^- concentrations. In a subsequent study, Stagg and Shuttleworth (1983) showed that acute exposure of perfused flounder gills to Cu^{2+} resulted in a concentration-dependent reduction of the electrical potential across this tissue. Concomitant measurements of Na^+/ K^+ activated ATPase activities indicated substantial inhibition at Cu^{2+} or Zn^{2+} concentrations above 325 ppb (Crespo and Karnaky, 1983).

There are biological end points, whether biochemical or physiological, which can shed some light on the potential impacts of chemical entities on aquatic systems. As a result, role of biomarkers as a comprehensive indicator has been increasingly recognized (Parvez et al., 2005). It is suggested that the assessment of ATPase activity may, therefore, be used as an early warning signal of pollutant-induced damage to the osmoregulatory and acid base regulatory system in the gills and the sensitive biomarker used for assessment of membrane fragility of gills (Tkatcheva et al., 2004; Viagno et al., 2004).

A number of estrogenic chemicals present in the sewage effluents exert deleterious effect on fish by action at gills, intestine and kidney (Laura et al., 2005). In recent years, sewage effluents have been shown to be polluted with a wide range of anthropogenic substances (Kummerer, 2000, 2001). In this context, an attempt was made in the present study to investigate the impact of municipal sewage effluents of a polluted river, Parvathyputhenar, Trivandrum, on the osmoregulaory enzymes and ion contents, and its effect on the CCs and PCs that regulate ions transport in the gill epithelium of the fish.

Materials and methods

Study site

Parvathyputhenar, an artificial canal dating back to the Travancore era (Lat-$8^0$29' 21'' Long- 76^0 55'17''), was used for navigation, as an avenue of leisure and even the water was used for domestic cosumption. But, over years, it has become a major source of environmental pollution, posing a health hazard for the residents of the city. Domestic wastes from the Trivandrum city are brought to the sewage farm established five decades back. These wastes are drained to the nearby grassland and after this the effluents, without any treatment, is directly emptied into Parvathyputhenar, which is the study site.

Experimental Design

The fish selected for the study was the freshwater teleost *Anabas testudineus*. This is one of the few fish that survive in the study area. To study the direct effect of sewage effluents, adult healthy fish collected from the study site were brought to the laboratory, anesthetized immediately with tricaine methane sulfonate (MS-222), and the gills were excised immediately and frozen at -80°C until biochemical analysis. To analyze the indirect effect, sewage effluents from the polluted river, Parvathyputhenar, was collected in large polythene containers and brought to the laboratory. The adult healthy fish, already acclimatized in the laboratory, having uniform size and weight (50 ± 5 g) were divided into four groups of ten each and kept in separate aquarium tanks under a natural photoperiod and constant temperature of 24 ± 2^0 C. Fish in groups I, II and III were exposed to the sewage effluents for 7, 14 and 28 days, respectively. The fourth group of fish served as control and were kept in normal pond water. To study the restoration of enzyme activity, if any, fish collected from the study site were brought to the laboratory and kept in dechlorinated tap water for a period of 30, 60 and 90 days. The fish were fed everyday with commercial fish feed *ad libitum*. The gills were collected and frozen as mentioned earlier for enzyme assays. The gill arches were fixed in glutaraldehyde immediately for scanning electron microscopic study. Two gill lamellae from each fish were used for ion estimation.

ATPase assay

The homogenate prepared to measure ATPase activities was similar to that described by Zaugg (1982). All sample preparation steps were carried out at 4°C. The gills were removed separately, kept in SEI buffer (75mM sucrose, 20mM EDTA, 10mM imidazole) and immediately frozen for biochemical assays. Briefly, 50 mg of gill was homogenized in 2 ml SEI buffer, and centrifuged at 1000 rpm for 10 min. The supernatant collected was used to measure the specific activity of ATPase (Gurnsey and Edeleman, 1986). Experimental and control cocktail of Na^+ K^+ ATPase contained 30mM Tris, 10mM NaN_3, 1mM EDTA, 130mM NaCl, 10mM KCl and 3mM ATP. Ca^{2+} ATPase cocktail contained 100mM $CaCl_2$ instead

of NaCl and KCl. The control contained all of these reagents and 3 mM Ouabain. The pH of all the cocktails was adjusted to 7.4. The tubes were shaken and incubated at room temperature for 15 minutes, after which the reaction was terminated by adding 1 ml of 10% TCA. The microfuge tubes were kept on ice for 30 minutes and centrifuged at 2000 rpm for 10 minutes. The clear supernatant was collected for the determination of inorganic phosphate (Fiske and Subbarow, 1925). The absorbance was measured at 640 nm in UV-visible spectrophotometer (Perkin-Elmer, USA). ATPase activity was calculated as the inorganic phosphate liberated and was expressed as nanomoles/min/mg protein. The protein content of the same tissue homogenate was measured using the method of Bradford (1976).

Determination of ion content of gills

Sodium, calcium and potassium ions in the gill of the fish were determined by APHA method (Greenberg et al., 1992). Two gill lamellae from each experimental fish were excised and washed in double distilled water (DDW) and allowed to dry under shade. The tissue was then kept in hot air oven for 2 to 3 hr. After this, it was powdered with the help of mortor and pestle. One hundred milligrams of the powdered tissue was digested in 5 ml of con nitric acid and perchloric acid in 4:1 ratio for 3 to 5 hr on a hot plate with constant stirring at regular intervals. When the acid mixture was completely evaporated, the residue was cooled and dissolved in 20 ml of DDW. Na^+, K^+ and Ca^{2+} ion content were determined in a flame photometer (Systronics CL 360, India) using known standards and the value is expressed as µg/g wt.

Scanning electron microscopic study of gill

The excised gills were washed in phosphate-buffered saline (PBS) and fixed overnight in 2.5% gluteraldehyde in 0.1M PBS at room temperature. After dehydration in ethanol series (30 to 100%), the tissues were treated with iso-amyl acetate for 10 min. Drying was done with liquid CO_2 in a critical point drier (HCP-O_2-Hitachi) and sputter-coated with a gold-palladium complex in a gold ion-sputtering unit (E101, Hitachi). The coated specimens placed on a grid were examined in a scanning electron microscope (Hitachi-S-2400) at an accelerating voltage of 15KV.

Chemicals

Gluteraldehyde and MS222 were purchased from sigma chemicals, USA. All other chemicals used were of analytical grade and purchased from SRL, Bombay, India. All glassware was acid-washed prior to use.

Statistical analysis

Data were collected from six animals in each group. Statistical analysis was done by SPSS package. Data were analyzed by one-way analysis of variance, which helps to understand whether or not there were differences between groups of means. Groups that were not significantly different in Duncan's (1955) multiple range tests were considered homogeneous. All data were analysed for significant differences $P<0.05$.

Results

ATPases activity

Figure1 represents branchial Na^+/K^+ ATPase activity in the fish exposed to sewage effluents for 7, 14 and 28 days, fish caught from the study site and the fish kept in normal pond water in the laboratory for 30, 60 and 90 days . Activity of the enzymes decreased after 7, 14 and 28 days. The activity of branchial Na^+/K^+ ATPase after 28 days of sewage exposure was comparable with that in the fish caught from the study site. When the fish caught from the study site were kept in normal pond water in the laboratory for 30, 60 and 90 days, the enzyme activities gradually increased and were restored to the control level after 60 and 90 days of exposure.

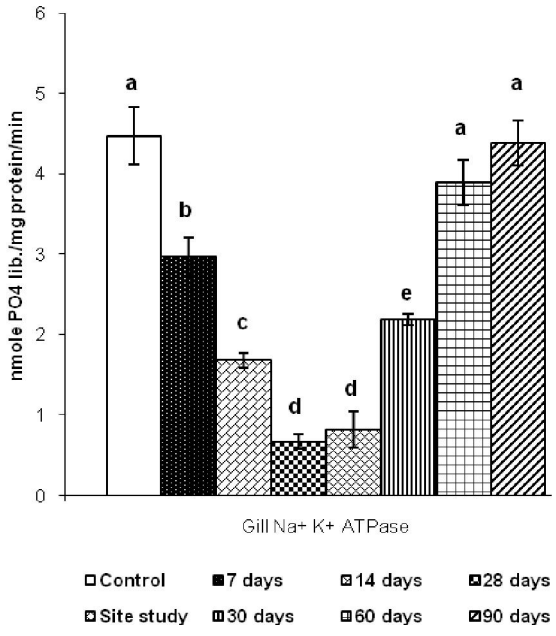

Fig.1 Effect of sewage effluents on branchial Na^+ ATPase activity
7, 14, 28 days – Exposure to sewage effluents
30, 60, 90 days – Exposure to control water
Each histogram represents mean ± SEM of 6 animals
Groups with different letter headings are significantly different ($P<0.05$)

Branchial Ca^{2+} ATPase activity is summarized in figure 2. Ca^{2+} ATPase activity was found to be significantly decreased in the fish exposed to the effluents for 7, 14 and 28 days. The maximum decrease in enzyme activity was found in fish caught from the study site. But the activity of the enzyme was significantly increased when the fish from the study site were kept in normal pond water in the laboratory for 30, 60 and 90 days.

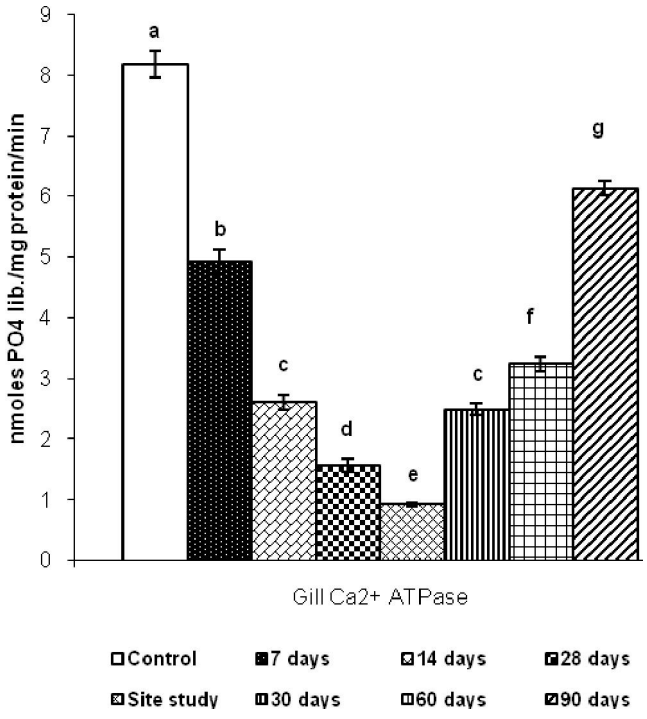

Fig. 2. Effect of sewage effluents on brancial Ca^{2+} ATPase activity. Other details, as in figure 1.

Ion content of gill

Figure 3 illustrates changes in the sodium, potassium and calcium ion content in the gill. Na^+ content decreased significantly after 7, 14 and 28 days compared to that of the control fish. After 28 days exposure, decrease in the Na^+ content was comparable with the branchial Na^+ content in the fish collected from the study site. When the fish caught from the study site were kept in normal pond water for 30, 60 and 90 days, Na^+ content was found to be significantly increased, and restored to normal control level after 60 and 90 days. Potassium ion content in the gill also showed corresponding significant decrease after 7, 14 and 28 days exposure and in the fish caught from the study site. When the fish caught from study site were kept in normal pond water for 30, 60 and 90 days there was a significant increase in branchial K^+ content.

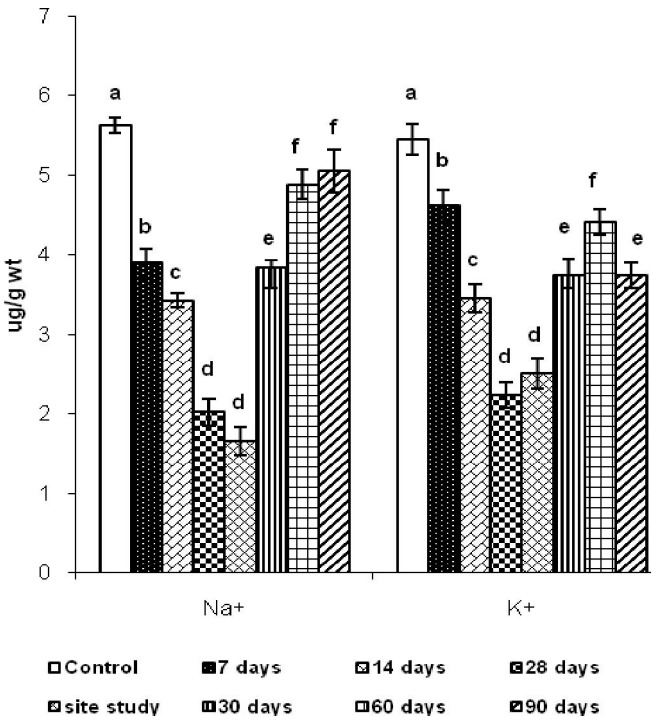

Fig.3 Effect of sewage effluents on the branchial ions content. Other details, as in figure 1.

Scanning electron microscopic study

Scanning electron micrographs of the gill epithelium revealed the surface morphology of the gill of control fish and fish exposed to sewage effluents. In gill epithelium of control fish, PCs were the most abundant cell type, when compared to CCs (Fig. 4 A, B). PCs showed characteristic concentrically arranged microridges on their surface, where as CCs possessed apical pits. The CCs were mainly located on the trailing edge of the filament epithelium and the bases of lamellae. In the control, the CCs displayed the characteristic apical surface morphology and were easily distinguishable from the neighboring PCs. Two types of CCs, namely shallow basin and wavy convex, were observed on the gill epithelium, and the shallow basin cells were in abundance. The apical membranes of wavy convex CCs gave a convex surface appearance with an aperture of larger size, shallow basin chloride cells having medium sized ovoid aperture and frequently decorated with short microvilli. The CCs were relatively less with characteristic apical area extrusions extending out between pavement cells. There were noticeable morphological changes in the surface area of CCs and PCs in the sewage-exposed fish in comparison to control. The morphology of CCs and PCs changed in all experiments (Fig. 4 C-H). The size and characteristic

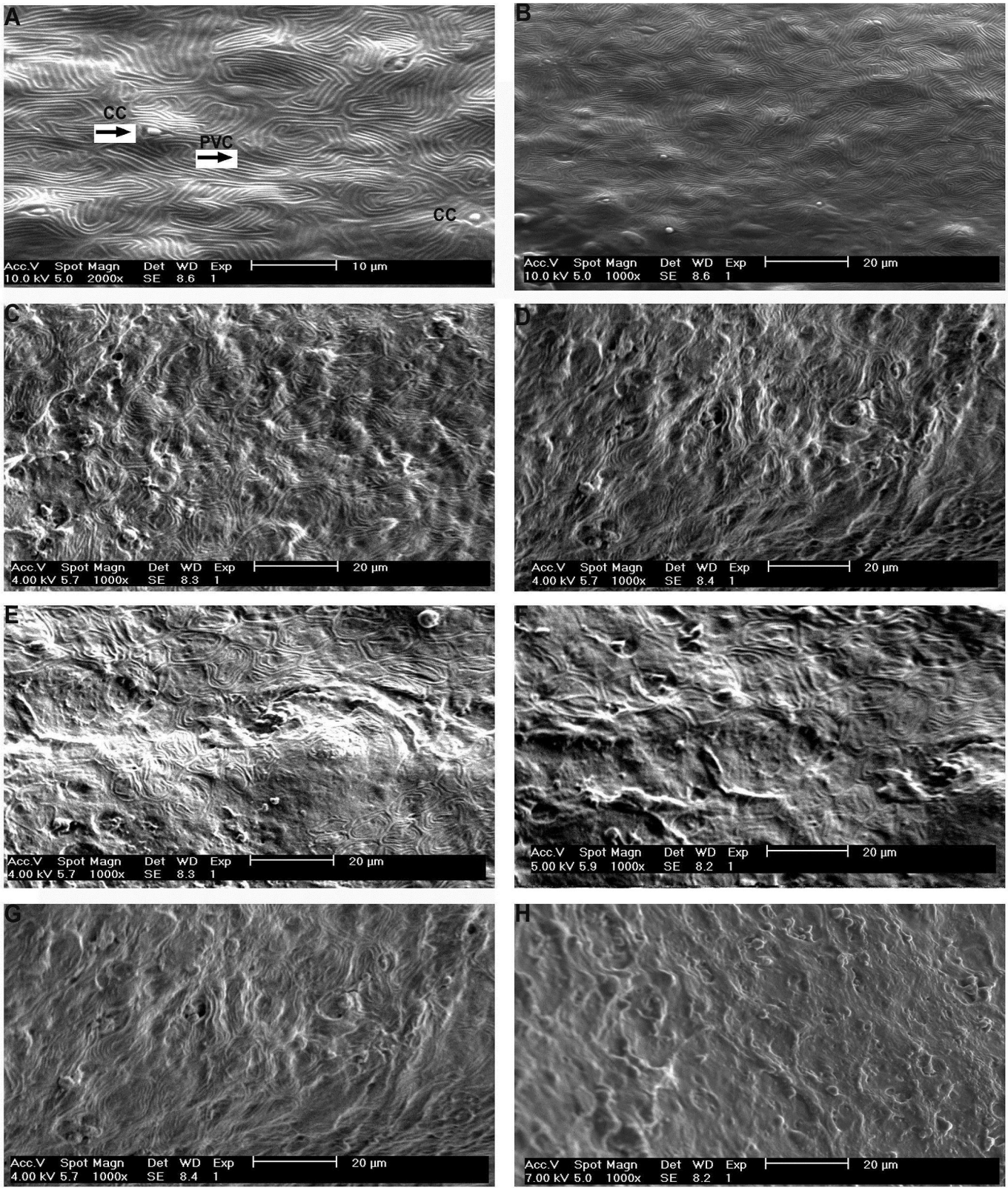

Fig. 4. Scanning electron micrographs of gill epithelium of fish exposed to sewage effluents. A, B gill epithelium of control fish; C, D, E, F - gill epithelium of fish exposed to sewage effluents for 7, 14 & 28 days in the laboratory; G, H – gill epithelium of fish collected from polluted river.

"finger print" pattern of PCs exhibited marked difference. Sewage effluents exposure caused severe damage to the gill epithelium by completely destroying the CCs and PCs. Exposure to sewage effluents apparently decreased the number and size of CCs.

The fish, after 7 days exposure, revealed reduction in the CC number and cell apical membrane area. The basic "finger print pattern" of PCs changed (Fig. 4C). After 14 day exposure the number of CCs significantly decreased and the concentric arrangement of PCs was alterred (Fig. 4D). The fish exposed to effluents for 28 days (Fig. 4 E, F) and those caught from the polluted river (Fig. 4 G, H) showed almost complete destruction of CCs and PCs.

Discussion

This study illustrates the hypo-osmoregulatory effect of the sewage effluents in the polluted river. Na^+, K^+ and Ca^{2+} ion concentrations showed a comparable decrease and activities of Na^+/ K^+ and Ca^{2+} ATPases were inhibited. Restoration of osmoregulatory capacity was observed in the fish caught from the study site when kept in normal pond water for stipulated periods.

Disruption of osmoregulation by pollutants is of paramount importance in several aquatic animals. Environmental organic pollutants usually affect the Na^+/ K^+ ATPase by decreasing its activity (Haya et al., 1983). Aquatic pollutants exert a biological effect on the ATPase system by partitioning the enzyme complex, which may cause an allosteric change that results in decreased ATPase activity (Reddy et al., 1992). The membrane-bound transport enzyme Na^+/K^+ ATPase is an integral part of active transport mechanisms for cations across the cell membrane (Das and Mukherjee, 2000). The branchial ion transport mechanisms provide useful focus for experiments to uncover the agents and mechanisms of differentiation and integration of transport function.

ATPases play a significant role in maintenance of functional integrity of plasma membrane and in several intracellular functions and are considered to be a sensitive indicator of toxicity (Plant et al., 2003). The apical channels are involved in the acid-base regulation, and the osmotic potential gradient is very important for the gill cells (Evans et al., 2005), which may lead to energy-related perturbations and, finally, affect basic metabolic and physiological activities (Parvez et al., 2005).

It has been reported that osmoregulation in fish is influenced by exogenous factors (Kumaraguru et al., 1982; Ewart and Klip, 1984; Bernardi, 1999; Evans et al., 2005). Among the exogenous ones, the exposure to aquatic pollutants should be highlighted. Inhibitory effects of Na^+/ K^+ ATPase in carp during acute exposure to pesticides were found in the laboratory studies (Segers et al., 1984). The same effect was also observed in juvenile specimens of *Cyprinus carpio* exposed to water from the Recoquista River (de la Torre et al., 1999). Paper mill effluents apparently altered the ionic profiles, Na^+ and K^+ in particular, thus indicating possible perturbations in the ATPase system along with disruption in the movement of ions across the ionic pumps. Similarly, lithium exposure significantly reduced Na^+ K^+ ATPase activity in gill of *Cyprinus carpio* (de la Torre et al., 1999). Desaiah and Koch (1975) reported that toxaphene inhibited ATPase activity in catfish tissues, with an identical pattern of inhibition in gill and brain. A recent study in a marine fish, *Sebastes schlegeli,* revealed decrease in the activity of Na^+/K^+ ATPase following perfluorooctanesulfonate (PFOS) exposure (Jeon et al., 2010). These findings strongly support our observations in the present study.

The reported action of toxicants on Na^+/K^+ ATPase activity in tissues may thus disturb the osmoregulatory capacity of fish (Delela et al*.,* 1978). In addition to this, branchial ATPases are intimately involved in osmoregulation, acid-base regulation, respiration, cellular volume, membrane permeability and osmotic pressure of fish (Parvez et al., 2005). Thus, it is evident that the decreased activity of Na^+/K^+ and Ca^{2+} ATPases observed in our study may lead to further perturbations in fish osmoregulatory physiology.

Scanning electron microscopic analysis of gill epithelium showed considerable changes in the fish exposed to sewage effluents and in the fish caught from the study site. The characteristic morphology of CCs and PCs changed. Size and characteristic "finger print" pattern

of PCs exhibited marked difference and caused severe damage to the gill epithelium by completely destroying the CCs and PCs. CCs, the ion-transporting cells in gills of fish, play an important role in the maintenance of ionic balance in these animals (Perry, 1997; Wenderlaar Bonga, 1997; Quadri and Ferrandi, 1998; Sunny and Oommen, 2004; Sreejith et al., 2007). Many evidences implicate CCs as the site of ion transport across the gill epithelium (Maetz and Bornancin, 1975; Pedersen et al., 2006).

The CCs, more than any other cells in the gill, have ultrastructural characteristics suggestive of specialized transport activity (Sunny and Oommen, 2004; Sreejith et al., 2007). These cells are characterized typically by the presence of large well developed mitochondria in close association with greatly amplified membraneous system and it is continuous with the baso-lateral membrane (Jagoe et al., 1996), which is so elaborate that it fills nearly the entire cell. In adult teleost fish gills, CCs are the major sites of ionic regulation. In embryos and larvae of several teleosts CCs are detected in the yolk membrane and body skin (Shiraishi et al., 2001). These extra branchial CCs are considered to be the sites of ionic regulation in early developmental stages without functional gills. In the present study, sewage effluents exposure caused severe damage to the gills, as the CCs were destroyed completely. Destruction of the CCs may be associated with impairment in the ion transport system in the gills as a high level of Na^+/K^+ ATPase activity has been specifically localized to the baso-lateral tubular membrane system of CCs in autoradiographic and cytochemical studies (Philipott, 1980).

Thus, the observations in this study reveal that sewage effluents influence the enzymes of osmoregulation and affect the ion contents and the chloride cell density in the gill epithelium. When the fish from the study site were exposed to normal pond water in the laboratory, the enzymes activity was almost restored to the normal control level. The hypo-osmoregulatory effect of sewage effluents is evidenced by the decreased activity of branchial ATPases, decreased Na^+ and K^+ ions content in the gill, reduction in size and density of CCs and the total damage to the CCs and PCs. Depletion of branchial Na^+/K^+ and Ca^{2+} ATPases and decrease in the branchial Na^+, K^+ and Ca^{2+} content after sewage effluents exposure and in the fish caught from the study site could be due to the inhibition of corresponding Na^+/K^+ pump or catabolic process of these pumps. Elevated branchial ATPase activity and branchial Na^+, K^+ and Ca^{2+} content in the fish kept in normal pond water show high turnover of chloride cells. Depleted Na^+/K^+ ATPase shows correlation to the loss of chloride cells. It is suggested that measurement of ATPase activities can be used as a surrogate biomarker of exposure to chemical pollutants. Na^+/K^+ ATPase may be considered as a marker enzyme to understand the physiological impairment of gills.

The male fish exposed to sewage effluents from the same river developed ovotestis and had increased levels of serum estrogen and vitellogenin (Binitha, 2007). This observation confirmed the presence of estrogenic chemicals in the river under consideration. Hence, it is assumed that the hypo-osmoregulatory effect in the present study my be due to the estrogenic action of sewage effluents of Parvathyputhenar.

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