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Mediterranean Marine Science

Vol 10, No 1 (2009)

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H.A. ABDEL-MOHSEN

doi: 10.12681/mms.118

To cite this article:

ABDEL-MOHSEN, H. (2009). Assessment of respiratory and ion transport potential of Penaeus japonicus gills in response to environmental pollution. *Mediterranean Marine Science*, *10*(1), 5–18. https://doi.org/10.12681/mms.118

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Mediterranean Marine Science
Volume 10/1, 2009, 05-18

Assessment of respiratory and ion transport potential of *Penaeus japonicus* gills in response to environmental pollution

H.A. ABDEL-MOHSEN

National Institute of Oceanography and Fisheries, Kayet-Bay, Al-Anfoushy, Alexandria, Egypt

e-mail: halamohsen@hotmail.com, sunsquare99@yahoo.com

Abstract

The present study aims to pinpoint the respiratory and ion transport potential of gills of Penaeus japonicus living in Abu-Qir Bay, East of Alexandria, Egypt. Our results revealed clear histological impairments in gill structure. These alterations were mainly represented by the presence of large vacuoles in gill axis and gill lamellae. In addition, narrow, disrupted gill lamellae with wavy cuticle and shrunk pillar cells were detected. Moreover, some cells clearly showed pyknosis. Gill ultrastructure also showed abnormal chromatin condensation inside the nucleus. Obvious alterations in the typical shape and structure of mitochondria were observed. Noticeably, the main characteristics of ion regulating gill epithelium were absent thus suggesting a low ion transport activity of P. japonicus gills. Statistically, this was further proved by the significantly higher activity levels of respiratory enzymes, namely, lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) compared to those of the ion transport enzymes, namely, adenosine triphosphatase (ATPase) and carbonic anhydrase (CA) in gills and haemolymph. SDH activity levels were higher than the corresponding levels of LDH in gills and its own level in haemolymph, indicating a contradictory effect of pollution on respiratory enzyme activity levels.

Keywords: *Penaeus japonicus* gills; Structure; Ultrastructure; Respiratory enzymes; Ion transport enzymes; Aquatic pollution.

Introduction

Numerous pollutants entering the aquatic ecosystem affect the normal functioning of its aquatic biota. These effects may be histopathological, biochemical, physiological or may be in the reproductive system affecting total yield of aquatic animals (VIJAYAVEL & BALASUBRAMANIAN, 2006). These pollutants pose an environmental hazard because of their great toxicity and persistence (MEADOR *et al.,* 1995). Aquatic organisms are exposed to pollutants in the environment across two possible routes: water and food. A persistent hydrophobic chemical like Poly Aromatic Hydrocarbon (PAH) accumulates in aquatic organisms through either direct uptake

from water by gills and by outer skin surface or via the consumption of contaminated food carried by haemolymph to various tissues. In both cases haemolymph functions as a vehicle for transporting nutrients as well as xenobiotics and its metabolites to different tissues (VAN DER OSST *et al.,* 2003; VIJAYAVEL & BALASUBRAMANIAN, 2006).

Crustacean gills are the first organ exposed to pollutants when ambient water is polluted (WU & CHEN, 2004). They are known to perform multiple physiological functions. In addition to being the organ of respiratory gas exchange (DEJOURS, 1975); they are also responsible for haemolymph acid-base regulation (TRUCHOT, 1978 & 1979) and nitrogen excretion (KORMANIK & CAMERON, 1981). On cellular bases, the epithelial cells of crustacean gills play a chief role in the iono-regulatory processes (PÉQUEUX, 1995; MASUI *et al.,* 2005). While thin epithelium predominates in anterior gills, posterior gills possess thick cells or ionocytes whose membrane has the dense apical enfoldings and basolateral invaginations involved in ion transport (COPELAND & FITZJARRELL, 1968; HENRY & WHEATLY, 1992; PÉQUEUX, 1995; TOWLE & KAYS, 1986).

The ultrastructure of the gill epithelium in *Penaeus japonicus* is simpler than that in most other crustacean groups (BOUARICHA *et al.,* 1994). The epithelium of the gills is formed of thin cells having, as their only apical differentiated structures, microvilli and mitochondria which are the principal cells of the gills of palaemonid and penaeid shrimps (TAYLOR & TAYLOR, 1992). The comparison with other species demonstrated that the gill epithelium is only slightly differentiated in *P. japonicus.* In other species such as *Gecarcinus lateralis* (COPELAND & FITZJARREL, 1968), *Carcinus maenas* (GOODMAN & CAVEY, 1988, 1990; COMPERE *et al.,* 1989) and *Procambarus clarkia* (BURGGREN *et al.,* 1974), two types of epithelia coexisted in the gill, a thin one supposedly implicated in gas exchange, and a differentiated epithelium involved in hydromineral exchanges. In adult *P. japonicus,* only one type of epithelium with apical microvilli but without basal enfoldings could be observed (BOUARICHA *et al.,* 1994). In addition, no morphological or ultrastructural differences between the anterior, median, and posterior gills of *P. japonicus* were observed. It was, thus, concluded that a single type of epithelium existed in *P. japonicus* which is implicated mainly in respiration and, less than in other species, probably in osmoregulation.

When an organism is exposed to xenobiotics, it undergoes inhibition or acceleration of the catalysed reaction rate of the enzyme systems. The mechanism includes either changing the enzyme activity, biochemical processes or directly affecting the enzyme molecule (HEATH, 1987). It is postulated that variations in the respiratory enzyme activities in particular serve as an early marker to assess the extent of pollution in the exposed aquatic animals (MCMAHON, 2001; CHINNI *et al.,* 2002).

In crustacean gills, mitochondrial respiratory enzymes such as lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) are involved in cellular respiration through glucose metabolism which yields the high energy compound adenosine triphosphate (ATP). On the other hand, Na^+/ K^+ adenosine triphosphatase (ATPase) and carbonic anhydrase (CA) play a central role in the process of osmoregulation by supporting the transport processes of all ions involved. While it is postulated that Na^+/ K^+ ATPase is involved in the transport of the

three Na^+ ions and two K^+ ions across the cell membrane at the expense of hydrolysis of a single ATP molecule (HORISBERGER, 2004; MASUI *et al.,* 2005), CA is known to catalyse the reversible hydration of CO_2 and water to H^+ and HCO_3^- ions.

Penaeus japonicus Bate, 1888 is an indo-west Pacific species that also lives in the south-eastern Mediterranean Sea. It originally migrated from the Red Sea through the Suez Canal (HOLTHIUS & GOTTLIEB, 1958; GELDIAY & KOCATAS, 1968; DOWIDAR & RAMADAN, 1972; SHIBER, 1976).

In Egypt, *Penaeus japonicus* caught from Mediterranean waters do not represent a high percentage of the landed catch; however, owing to the great adaptability of the species to the climatic conditions of the Mediterranean and other habitats, successful cultivation of this species and consequently an increase in its economic value have resulted (TAHA, 1994 & 2000). In spite of that, few studies have dealt so far with the respiration and ion transport physiology of *Penaeus japonicus.*

Therefore, the chief objective of this study was to assess the respiratory and ion transport activities of *Penaeus japonicus* gills in response to the polluted habitat in Abu-Qir Bay, one of the main fishery basins east of Alexandria City and one which is continuously facing serious pollution problems (ABOUL-NAGA *et al.,* 2002). The assessment was chiefly performed by examining the gill structure and ultra-structure of *P. japonicus.* In addition, activity levels of some respiratory enzymes such as lactate dehydrogenase and succinate dehydrogenase and ion transport enzymes, such as, Na^+/K^+ adenosine triphosphatase and carbonic anhydrase were measured in the gills and haemolymph of *P. japonicus.*

Materials and Methods

Study Area

Abu-Qir Bay is a shallow, semi-circular and semi-closed basin that lies 35 km east of Alexandria City. Its shoreline extends 50 km from the Rosetta branch of the River Nile east to the Abu-Qir peninsula (Fig.1). Abu-Qir Bay lies between 30° 4′ and 30° 21′ east and 31° 16′ and 31° 30′

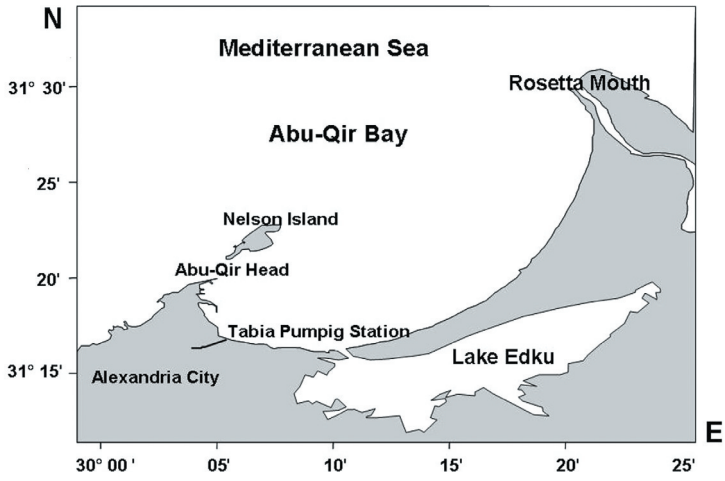

Fig. 1: Map of Abu-Qir Bay, Alexandria, Egypt.

north with a total area of about 560 km^2 and an average depth of about 12 m (NESSIM & EL-DEEK, 1993; ABOUL-NAGA *et al.,* 2002).

Abu-Qir Bay receives effluents of various waste categories discharged through three main openings, namely, the El-Tabia pumps, the outlet of Lake Edku and the Rosetta mouth of the River Nile (NESSIM *et al.,* 1994; MOHAMED, 2006). These discharges not only represent different industrial activities such as food processing, refineries, fertilizers and paper mills, but also represent domestic sewage discharge and agricultural waste as well (SAID *et al.,* 1995; ABOUL-NAGA *et al.,* 2002).

Study Species

Specimens of *Penaeus japonicus* were bought live from various fishermen at Abu-Qir Bay and brought to the laboratory in oxygen-packed polythene bags. As soon as the samples reached the laboratory, specimens were washed with distilled water to ensure contamination prevention during dissection and subsequent treatments. Adult individuals (19-22 cm) of both sexes were chosen for the present study; no discrimination between sexes was involved.

Structure and Ultrastructure of Gills

The gills of *P. japonicus* were cut free and the medial parts of the gill lamellae in particular were excised into small portions and were used to observe the osmoregulatory activity (if present) of the species. Some of these portions were fixed in Carnoy's Solution for 24 h, then transferred to 70% alcohol and processed using routine histological techniques. Paraffin sections of 4-5 µm were stained with Haemtoxylin and Eosin and examined microscopically (Olympus CX41).

Other portions were fixed in glutaraldhyde ($_4F_1G$) phosphate buffer solution (pH 7.2) at 4˚C for Transmission Electron Microscope (TEM) examination. The specimens were then post-fixed in 2% osmium tetroxide for 2h at 4˚C. Further, samples were dehydrated in graded ethanol baths and propylene oxide and were embedded in Epon 812. Thin sections of 70 nm were cut with a diamond knife, contrasted with uranyl acetate and lead citrate and then observed with Joel- 100 CX TEM. They were rinsed again in buffer, dehydrated in a graded ethanol series, and embedded in epon-araldite mixture. Ultrathin sections were cut and stained with uranyl acetate and lead citrate according to REYNOLDS (1963). The specimens were viewed in Jeol 100 CX TEM.

Biochemical Assay for Respiratory and Ion Transport Enzymes in Gills

One gram of gill tissue was first homogenized in 5-10 mL of cold buffer (50 mM potassium phosphate, 1 mM ethylene diamine tetraacetic acid, pH 7.5). Centrifugation at 10,000 xg for 15 min was then carried out at 4 ˚C. The supernatant was frozen at -80 ˚C for further analyses.

LDH Measurement

A UV optimized method for the determination of LDH activity was used (Alpha-Plus, Egypt). This method is based on the fact that LDH catalyses the reduction of pyruvate to lactate in the presence of reduced nicotinamide adenine dinucleotide (NADH) at pH 7.5. The reaction was monitored kinetically at 340 nm by the rate of decrease in absorbance resulting from the oxidation of NADH to NAD proportional to the activity of lactate dehydrogenase present in the sample. LDH activity was then expressed as follows:

U/mL = Δ A/minute x 8095 (37 ˚C),

where Δ A/minute is the average change in the absorbance/ minute.

SDH Measurement

SDH activity was determined by the method described by SLATER & BONNER (1952). SDH activity was expressed as nmoles of succinate oxidised/min/mg tissue.

Na^+/K^+ATPase Measurement

Activity of ATPase was measured according to the method mentioned by TAUSSKY & SHORR (1953) and BONTING *et al.* (1961) in which one unit of ATPase activity will liberate 1.0 micromole of inorganic phosphorus from ATP per minute at pH 7.8 at 37 ˚C in the presence of Na^+, K^+ and Mg^{++}. Na^+/K^+ ATPase activity was determined by comparing the inorganic phosphate between 2 media, with or without the addition of 1 mM ouabain (a specific inhibitor of Na^+/K^+ ATPase) and 20 mM $NH4^+$ and was calculated as follows:

Units/mg solid = units/mL enzyme divided by mg solid/mL enzyme.

CA Measurement

Activity of CA was measured according to the method of WILBUR & ANDERSON (1948) in which the time required (in seconds) for a saturated CO_2 solution to lower the pH of 0.012 M Tris HCl buffer from 8.3 to 6.3 at 0 ˚C is determined. The CA activity was then calculated as follows:

U/mg = $2(T_0\text{-}T)/Tx$ mg tissue in mixture, where T_0 is the recorded time without enzyme and T is the recorded time with enzyme.

Biochemical Assay for Respiratory and Ion Transport Enzymes in Haemolymph

A suitable amount of the haemolymph of *Penaeus japonicus* was collected from the pericardial sinus around the heart using a syringe and 21-gauge needle. The haemolymph was then centrifuged at 12,000 xg for 15 min at 4 ˚C. Respiratory and ion transport enzymes were detected in the haemolymph of the studied species following the above mentioned methods.

Statistical Analysis

Data are expressed as mean ± S.D. A Student t-test was used to compare between the mean levels of the studied enzymes in gills and haemolymph (*t* was considered significant at $p<0.05$).

Results

Structure and Ultrastructure of Gills

The present study aimed to clarify the respiratory and ion transport performance of *Penaeus japonicus* gills as a consequence of living in the highly polluted habitat of Abu-Qir Bay. The study mainly focused on the structure and ultrastructure of *P. japonicus* gills using light microscopy and electron microscopy techniques, respectively.

As gills are the first defense line of an organism against its surroundings, they should, therefore, be expected to be profoundly affected. Indeed, by studying sections taken from gills, a pronounced effect of pollution on gill structure was revealed. Large vacuoles were observed in different parts such as those shown in the central axis of gills (Fig. 2A). Most of the primary gill filaments were deformed (Fig. 2B). Secondary gill lamellae were shown to be narrow, disrupted and with little cellular organization. They also showed a wavy cuticle and epithelium and had shrunk-sized pillar cells (Fig. 2C). In addition, some cells in gill lamellae showed pyknosis (Fig. 2D).

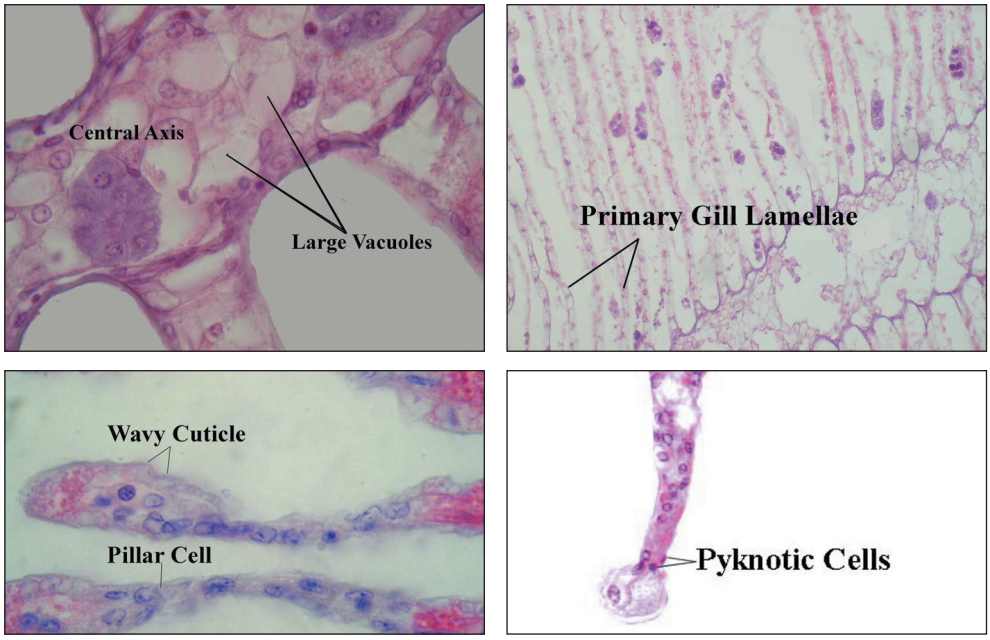

Fig. 2: *Penaeus japonicus.* Light micrographs of gills lamellae cross-sections. A. gill axis with large vacuoles (magnification X 100). B. deformed primary gill lamellae (magnification X 10). C. impaired secondary gill lamellae (magnification X 40). D. pyknotic cells (magnification X 40).

Figure 3 (A-D), shows TEM sections of *P. japonicus* gills in which more details were revealed of gill structure. Large vacuoles were detected inside gill lamellae with a general distinct disorganization of cytoplasm (Fig. 3A). Some nuclei showed abnormal structure; their nuclear envelope was shown to be wavy and not intact (Fig. 3B). In addition, chromatin patches were densely thickened and margined inside the nucleus (Fig. 3C). Mitochondria appeared to have lost their typical oval shape and became rounded or irregularly shaped with a disrupted membrane (Fig. 3D).

It is worth noting that only one type of epithelium in *P. japonicus* gills was observed. No structural characteristics that are associated with ion transport epithelium as described by SILVESTRE *et al.* (2005) could be detected.

Respiratory and Ion Transport Enzyme Activity

Figure 4, shows the levels of activities of respiratory and ion transport enzymes in the gills and haemolymph of *P. japonicus.* The detected levels of LDH activity in the gills and haemolymph of *P. japonicus* were 0.16 U/mg tissue and 71 U/L, respectively. On the other hand, SDH activity value in the gills was 39 U/mg tissue and that of haemolymph was 44.7 U/L. From a statistical viewpoint, SDH activity level in gills was significantly higher than that of LDH. The reverse was true for LDH value in haemolymph, which was significantly higher than that of SDH value.

In this study it was shown that there was a statistically significant difference between average values of respiratory enzymes over the corresponding values of ion transport

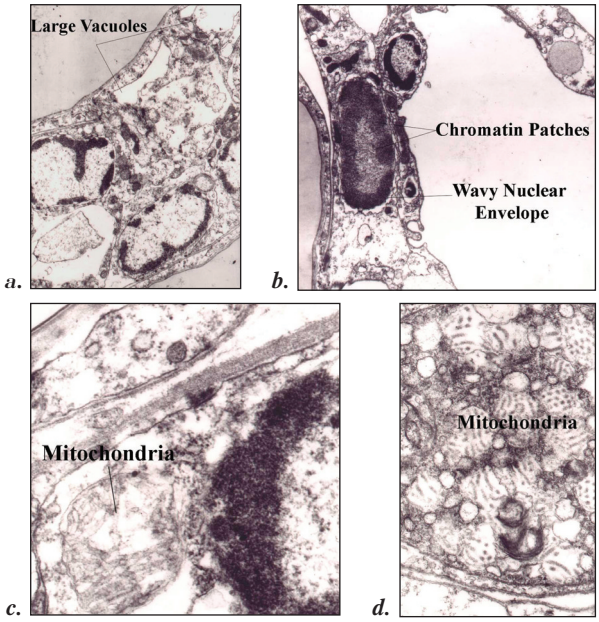

Fig. 3: *Penaeus japonicus.* Electron micrographs of gills lamellae cross-sections. A. gill lamellae cytoplasm with large vacuoles (magnification X 5000). B. nucleus with wavy nuclear envelope and chromatin patches (magnification X 5000). C. chromatin condensation and a single mitochondrion (magnification X 25000). D. bundles of mitochondria (magnification X 13000).

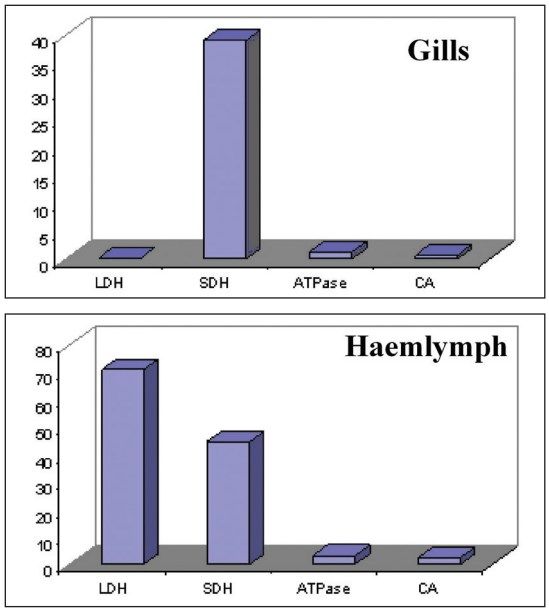

Fig. 4: *Penaeus japonicus.* Activity of respiratory enzymes; lactate dehydrogenase (LDH), succinate dehydrogenase (SDH) and ion transport enzymes; adenosine triphosphatase (ATPase) and carbonic anhydrase (CA) ± S.E. measured in gills (U/mg tissue) and haemolymph (U/L).

enzymes in gills and haemolymph. Na^+/K^+ ATPase average values in gills and haemolymph were 1.25 U/mg tissue and 3.2 U/L, respectively, whereas those of CA were 0.75 U/mg tissue and 2.63 U/L, respectively.

Discussion

Study Area

It is believed that pollutants affect aquatic animals mainly by causing respiratory and circulatory interference or by specific toxic action after absorption (RAND & PETROCELLI, 1985; HEATH, 1987). Hence, the respiratory potential of an animal is, in general, one of the most important physiological parameters in order to assess environmental stress on aquatic organisms (VIJAYAVEL & BALASUBRAMANIAN, 2006).

In this study we investigated the respiratory and ion transport performance of the gills of *Penaeus japonicus* living in Abu-Qir Bay, east of Alexandria. Abu-Qir Bay was chosen as our study area as it is one of the most polluted basins, receiving discharges of nearly all types of pollutants (NESSIM *et al.,* 1994; MOHAMED, 2006). ABOUL-NAGA *et al.* (2002) recorded considerable levels of lead, nickel and cadmium in different regions of the bay in the vicinity of points recognized as potential sources of pollution. Additionally, many unfavourable conditions for living organisms, such as increased levels of ammonium, nitrite, nitrate, reactive phosphate and silicate were highlighted by MOHAMED (2006) in his chemical study of the basin. These conditions were especially recorded near the overlying bottom of the basin where maximum concentrations of *P. japonicus* could be found (DOWIDAR & RAMADAN, 1972).

Structure and Ultrastructure of Gills

In crustaceans, gills are immediately exposed to the external environment and are thus the first organ exposed to pollutants when ambient water is polluted (WU & CHEN, 2004). Our study revealed that the structure and ultrastructure of the gills of *Penaeus japonicus* were highly affected by the surrounding pollutants. Large vacuoles were clearly seen in the gill axis and gill lamellae. Secondary gill lamellae were exceptionally narrow with shrunk sizes of pillar cells which could consequently lead to a reduction in the volume of circulating haemolymph (SILVESTRE *et al.,* 2005). Though a reduction in the efficiency of respiratory activity of *P. japonicus* –especially in that of oxygen consumption - could be theoretically expected, however, according to CHEN & LAI (1992) oxygen consumption and ammonia-N excretion of *Penaeus japonicus* adolescents increased with increased ambient ammonia-N. Increased ambient ammonia in Abu-Qir Bay was positively recorded by MOHAMED (2006). MCMAHON (2001) stated that chronic exposure to hypoxia may increase O_2 binding capacity and promote the synthesis of new high O_2 affinity carrier molecules.

Utlrastructure sections of *P. japonicus* gills indicated obvious alterations in the nucleus as shown in its wavy nuclear envelope. A general condensation and margination of chromatin was also observed. Utlrastructure sections especially revealed mitochondria to be profoundly affected. Alterations in mitochondrial structure could have serious consequences for the normal physiological functions of the organism and its respiratory process (BURCHAM & HARMAN, 1991).

Generally speaking, more drastic histological and cytological damage was previously recorded in other investigations

(SOEGIANTO *et al.,* 1999 & SILVESTRE *et al.,* 2005). Contrary to the present study in which specimens were taken directly from the field, those researches were experimentally executed where acute effects of pollutants could be recorded. In our case only chronic effects could detected. Nevertheless, the present changes in the tissue and cell structures of *Penaeus japonicus* gills may mark the beginning of more obvious and drastic changes.

It is worth noting that both structural and ultrastructural results revealed the presence of only one type of epithelium and the absence of the main characteristics of an osmoregulating epithelium recorded by SILVESTRE *et al.* (2005). These characteristics mainly include a thick epithelium with apical plasma membrane enfoldings and an enlarged subcuticular compartment and were clearly observed in the Chinese mitten crab *(Eriocheir sinensis)* which is a strong osmoregulatory species. BOUARICHA *et al.* (1994) recorded that there were no morphological or ultrastructural differences between the anterior, median and posterior gills of *P. japonicus* and thus concluded that a single type of epithelium existed in this species. The presence of a single epithelium type and the disappearance of ion osmoregulatory characteristics clearly indicated a low osmoregulatory activity exhibited by *P. japonicus* gills.

Respiratory and Ion Transport Enzymes Activity

It is believed that mitochondrial membrane damage leads to the inhibition of mitochondrial enzymes (EATON & GALLAGTER, 1994). This might be due to the fact that the inner and outer mitochondrial membranes contain unsaturated lipids and are hence more susceptible to attack by oxidants (BIRONAITE & OLLINGER, 1997).

The present study showed an acute decrease in the activity value of the gills mitochondrial LDH compared to its level in haemolymph. LDH is generally associated with cellular metabolic activity and acts as pivotal enzyme between the glycolytic pathways and tricarboxylic acid cycle. Therefore, it is believed that its inhibition leads to decreased respiration and partial uncoupling of oxidative phosphorylation (VIJAYAVEL & BALASUBRAMANIAN, 2006). Moreover, the higher value of LDH activity recorded in haemolymph than that in gills could be either due to anaerobic tissue metabolism in crustaceans (MCMAHON, 2001) or due to lysis or damage of gill cells. The latter reason is more plausible regarding the impairment in structure and ultrastrucutre of gills shown in this study. This is supported by the finding of WOLTERBEEK & VAN DEER MEER (2005) who stated that LDH is released to the surrounding medium upon cell damage or lysis and its activity, therefore, can be used as an indicator of cell membrane integrity and as a measurement of cytotoxicity.

It is worth noting that the response of respiratory enzymes to environmental pollution reported in the present study completely contradicted. On the one hand, the effect of pollution in Abu-Qir Bay on LDH activity in gills was an inhibitory one. On the other hand, higher levels of SDH activity in the gills and haemolymph of *P. japonicus* were detected. The considerable levels of SDH in gills and haemolymph agreed with the work of MIHELIC *et al.* (1999) who recorded high levels of SDH activity in polluted areas with heavy metals, pesticides and hydrocarbons.

Osmoregulation is a fundamental physiological measure that allows marine and

estuarine animals to live in the most dilute areas of aquatic habitat (FLORKIN & SCHOFFENIELS, 1969; HENRY 2001). It involves the active uptake of salts from the ambient medium by the gills (SKAGGS & HENRY, 2002). The capacity of transporting ions to cope with salinity variations is dependent largely on the up-regulation of transport-related proteins, Na+/K+ ATPase and CA (GENOVESE *et al.,* 2005).

Since in Abu-Qir Bay there was minor variation in salinity near the bottom where *P. japonicus* live (MOHAMED, 2006), little or low osmoregulatory activity in its gills would be expected. Indeed, the present study generally revealed a significant difference between activities of respiratory enzymes over the corresponding activity levels of ion transport enzymes (Na^+/K^+ ATPase and CA) in gills and haemolymph which coincided with our histological observations on gill epithelium. Our results specifically showed low levels of Na^+/K^+ ATPase activity in the gills and haemolymph of *P. japonicus* which agreed with COOPER & MORRIS (1997); COROTTO & HOLIDAY (1996) and LUCU & FLIK (1999) who reported that there was a clear link between the process of hyper-regulation and Na^+/K^+ ATPase activity. The latter was recorded to be increased several-fold when hyperregulating species were transferred to dilute media.

CA is known to play a role in a variety of physiological and biochemical processes. This involves CO_2 excretion and fixation, acid-base balance, ion transport and mineralization of calcified skeletal structures (HENRY, 1988a). It is also known to have multiple locations within the cells. While membrane associated CA functions to facilitate branchial CO_2 excretion through catalysed dehydration of haemolymph HCO_3, cytoplasmic CA functions in the hydration of CO_2 to H^+ and HCO_3, which are then used in ion transport (HENRY, 1988b). In the present work, lower levels of CA activity were observed in gills and in haemolymph compared to higher levels of LDH and SDH in the same organs, implying that the dominating species of CA in *P. japonicus* was the membrane associated one and fortifying the suggestion of low osmoregulatory activity in *P. japonicus* gills. Taken altogether, it can be said that the function of the gills of the *P. japonicus* living in Abu-Qir Bay could be mainly a respiratory one. Moreover, an interaction between pollution and osmoregulation in this species could hardly be established according to our results.

In conclusion, we can infer that pollution in Abu-Qir Bay had an apparent negative effect on the structure and function of respiratory enzymes activity of *Penaeus japonicus* gills. However, owing to the low activity levels of ion transport enzymes recorded in *P. japonicus* gills, the impact of pollution on this particular activity could not be clarified. Further bioassay studies on this relation are recommended.

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Submitted: April 2008
Accepted: February 2009
Published on line: June 2009