The effect of depuration on heavy metals, petroleum hydrocarbons, and microbial contamination levels in *Paphia undulata* (Bivalvia: Veneridae)

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ABSTRACT: The depuration of 8 heavy metals (Zn, Pb, Ni, Mn, Cu, Cr, Co and Cd), total petroleum hydrocarbons and pathogenic bacteria of *Paphia undulata* was tested and the survival of depurated clams was evaluated. Investigated samples were collected from Ismailia, Egypt. The initial metal concentrations were significantly higher in the whole soft tissues than in water and sediment except for Mn. After 24 h depuration, Zn, Pb, Ni, Mn, Cu, Cr and Cd were significantly reduced to 44, 23, 25, 17, 61, 41, and 75%, respectively. After three days of depuration the reduction was significant only for Cu, Cr, Co and Cd (27, 15, 23 and 52%, respectively). The total petroleum hydrocarbons were reduced significantly to 72% after three days of depuration, while after 24 h they were reduced to 90% compared to their initial concentrations. Four pathogenic bacteria were identified in the soft tissues of *P. undulata* (*Vibrio* sp., *Shigella* sp., *Escherichia coli* and *Salmonella* sp.). After one-day depuration the results evidenced the mean microbial reduction to 75, 31, 68, and 36%, respectively, compared to their initial counts. After three days of depuration the counts of *Vibrio* sp. and *Salmonella* sp. were reduced to 3% and 8%, respectively, while *Escherichia coli* was not detected on the third day. *Shigella* sp. was increased by 22% compared to the first day of depuration. The viability and mortality were not influenced by the depurative treatment.

Keywords: bivalve; depuration; heavy metals; pathogenic bacteria; petroleum hydrocarbons; survival

Shellfish are a popular and nutritious food source worldwide and their consumption has risen dramatically. Since bivalves are filter feeders, they concentrate contaminants to a much higher level than those of the surrounding sea (Cosson, 2000; Fang et al., 2003). These contaminants may cause diseases of humans, especially microbial contaminants, because shellfish are often eaten raw or lightly cooked (Rippey, 1994; Croci et al., 2002; Formiga-Cruz et al., 2003). To reduce or minimize the risk, the source of the shellfish should be investigated and better quality would be attained by appropriate treatment following the harvest. The best and the easiest strategy that has been developed for bivalve risk management is utilizing their capacity to eliminate pathogenic microorganisms and toxic substances when bivalves are kept in clean disinfected seawater tanks (Wong et al., 1997; Sobsey and Jaykus, 1999; El-Shenawy, 2004). The above method (depuration) helps bivalves to expel and separate contaminants from their gills and intestinal tract over a period of time and prevents their recontamination. Although Arnold (1991) restricted the role of this type of depuration to remove bacterial contamination, others emphasized and encouraged it for reducing the toxicity of heavy metals (Wilson, 1980; Hung et al., 2001; El-Shenawy, 2004; Katayon et al., 2004) and petroleum hydrocarbons (Lee et al., 1972; Stegman and Teal, 1973; Linden et al., 1979; Clement et al., 1980; Rainio et al., 1986; Rantamaki, 1997). Many factors influence the degree of depuration as follows: the system design, initial water quality, oxygenation and flow rates, salinity, temperature, shellfish-to-water ratios, removal and settlement

of faecal material, types of pollutants in seawater and the period of purification (Lee and Younger, 2002; Manfra and Accorneo, 2005).

Most studies concerning depuration exposed bivalves to pollutants in the laboratory and then transferred them to clean waters under laboratory or field conditions (Seymour and Nelson, 1971; Mason et al., 1976; Zaroogian, 1979; Wahi et al., 2009). A few studies used clams containing naturally high concentrations of heavy metals and followed their depuration at a relatively clean field (Okazaki and Panietz, 1981; El-Shenawy, 2004). No information was reported for *Paphia undulata* depuration or the impact of their depuration on survival. The aim of the present study was: (1) to evaluate the efficiency of depuration on the elimination of some pollutants such as heavy metals, hydrocarbons and pathogenic bacteria from *Paphia undulata* collected from a polluted area (Ismailia, Egypt); (2) to verify the survival of clams during depuration to help in the establishment of public health guidelines for commercial harvesting of clams.

MATERIAL AND METHODS

Sampling collection

Paphia undulata were collected in February 2009 from Ismailia near Buheirat-Marrat-el-Kubra, station No. 6, Egypt (Figure 1). The shell sizes of the detected samples were 2.6–5.8 cm in length and 1.2–3.2 cm in width. Dead or damaged specimens were eliminated and a standardized shell size 4.5 cm in length and 2.5 cm in width was used. Sediments and water samples were collected from a precise depth corresponding to the clam settlements to determine the initial level of heavy metals and total petroleum hydrocarbons (TPHs). Salinities were measured on a field salinometer during the sampling period and averaged about 40.18 ± 0.023‰. Temperatures averaged 21.13 ± 2.08°C, pH = 7.8 ± 0.06, turbidity = 0.5 NTU (Nephelometric Turbidity Units) and dissolved oxygen was 5.6 ± 0.32 mg/l during the sampling period.

Depuration experiment

The experiment was commenced within 3 h since the shellfish collection and studied for 24 and 72 h. For each period three replications of twenty clams of similar dimensions were placed in 5 l aquaria containing 3.5 l of autoclaved artificial sea water (Instant Ocean Aquarium System) to be free of chemical contaminants and microbial infection. The culture was kept under laboratory conditions (temperature 22 ± 2°C; salinity 40‰ and with continuous aeration). In three days depuration water was changed and the aquaria were cleaned every day to avoid refiltration of depurate contaminants (no food was added during the depuration).

Metal analysis

Heavy metal concentrations in samples of water, sediment and soft tissues of *P. undulata* were analysed according to the methods of Dybern (1983), Chevereuil et al. (1996) and Coughlan (2002) using an Atomic Absorption Spectrometer (Perkin-Elmer model 2380). The results of metal concentrations

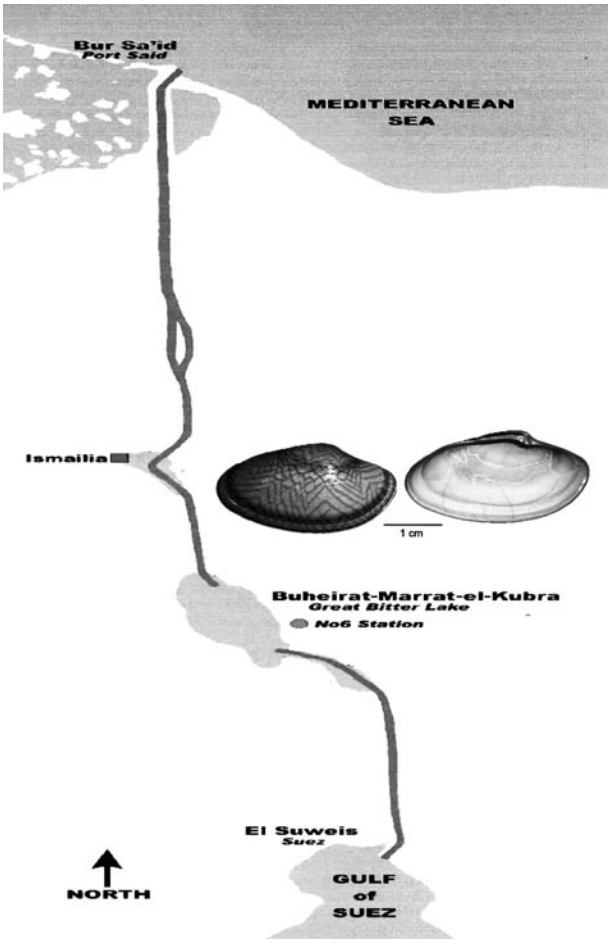


Figure 1. Map of the site of collection and collected *Paphia undulata*

are expressed as µg/ml (ppm) in water, µg/g of dry weight in soft tissues and in sediment.

Total petroleum hydrocarbons analysis (TPHs)

In water. Seawater samples were extracted three times with 60 ml of dichloromethane in a separating funnel. Sample extracts were combined and concentrated by rotary evaporation to 5 ml. Finally, the samples were concentrated under a gentle stream of pure nitrogen to a final volume of 1 ml, then measured using a UV-spectrofluorometer at 410 nm emission after excitation at 360 nm and chrysene as standard (Parsons et al., 1985).

Sediment. The sediment was freeze-dried, dry/wet ratios were determined and then it was sieved through a stainless steel mesh (250 mm). Each sediment sample (10 g) was Soxhlet extracted with 250 ml of hexane for 8 h and then re-extracted for 8 h into 250 ml of dichloromethane (Colombo et al., 1989). Then the extracts were combined and concentrated down using rotary evaporation at 30°C followed by concentration with a nitrogen gas stream down to a volume of 1 ml, then measured using a UV-spectrofluorometer at 410 nm emission after excitation at 360 nm and chrysene as standard.

Soft tissues. Accurately 5 g of dried *P. undulata* tissues was acidified to pH 2 with 0.1 ml concentrated HCl, then homogenized in 5 g anhydrous $MgSO_4$ for about 15–30 min at room temperature. The contents were Soxhlet extracted using chlorotrifluoroethylene (S-316) then dried through anhydrous magnesium sulphate. The solvent was evaporated to about 10 ml under vacuum and low temperature (not higher than 50°C). The residue (TPH) was cooled, dried in a desiccator for 2 h and weighed again. The residue was dissolved in chlorotrifluoroethylene and quantitatively transferred into a 25 ml volumetric flask and made up to the volume to be directly analysed using quantitative FT-IR (Fourier Transform Infrared).

Bacterial analysis

Bacteria were isolated from freshly harvested 10 clams in different selective media. MacConky, TCBS and Salmonella & Shigella (SS) media were used to isolate the most common Gram negative bacteria such as *Vibrio* spp., *Salmonella* and *Shigella*. Cells were stained with the Gram stain and examined under a phase contrast microscope. The isolated bacteria were applied to total count (APHA, 1989) and counted according to Hitchins et al. (1995), where CFU/ml = No. of colonies/amount plated × dilution. Determination was done according to Bergy's Manual of Determinative Bacteriology (Singhet and Prakash, 2008). These analyses were performed with freshly collected clams directly after harvesting (0 day), one day and three days after depuration. Ten clams were pooled for one analysis.

Mortality ratio

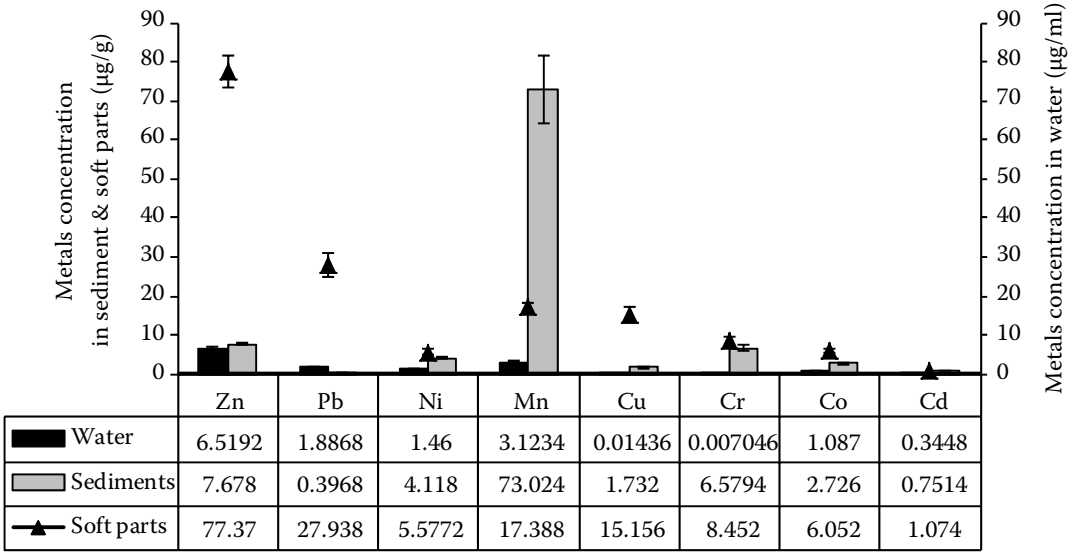
Mortality was determined by visual and tactile analysis of each specimen and was performed daily.

Statistical analysis

The results are presented as mean ± SD values. One-way analysis of variance (ANOVA) was used to test the significance of depuration in each metal concentration, TPHs and bacterial count. Post hoc test was used to analyse the multiple comparisons among water, sediment and soft parts, and between the zeroth, first and third day of depuration. All statistical analyses were performed using the SPSS 15.0 software (SPSS 2006).

RESULTS

The metal concentrations in water, sediment and clams' whole soft tissues at the beginning of the experiment are represented in Figure 2. One-way ANOVA (Table 1) shows significant differences ($P < 0.000$) between the three groups (water, sediment and soft parts). Baseline concentrations of the 8 metals were significantly higher in the whole soft tissues than in water and sediment ($P < 0.000$) except for Mn, which was significantly higher in sediment than in water and soft tissues ($P < 0.001$) as indicated by the post hoc test. The bioconcentration factor (BCF), which can be defined as the rate of accumulation of the metal in the organism relative to its environment, was higher in soft parts than in water especially for Cu and Cr (1083 and 1208 folds higher than in water, respectively). The



	Zn	Pb	Ni	Mn	Cu	Cr	Co	Cd
Water	6.5192	1.8868	1.46	3.1234	0.01436	0.007046	1.087	0.3448
Sediments	7.678	0.3968	4.118	73.024	1.732	6.5794	2.726	0.7514
Soft parts	77.37	27.938	5.5772	17.388	15.156	8.452	6.052	1.074

Figure 2. Initial concentrations of heavy metals in seawater (µg/ml), sediment and soft parts (µg/g dry weight) of *Paphia undulata* at the beginning of experiment

BCF values for the rest of the metals were 12, 15, 4, 6, 6, and 3 folds higher than in water for Zn, Pb, Ni, Mn, Co, and Cd, respectively.

On the first day of depuration, the levels of heavy metals tended to decrease in the order Mn > Pb > Ni > Cr > Zn > Cu > Cd > Co, while after three days the order was Co > Cu > Cr > Cd > Pb > Zn > Mn > Ni. The rates of heavy-metal reduction during depuration are presented in Figure 3. The concentrations of Zn, Pb, Ni, Mn, Cu, Cr, and Cd in the clams' tissues were significantly reduced to 44, 23, 25, 17, 61, 41, and 75%, respectively, after 24 h of depuration compared to their initial concentrations, while Co was reduced only to 93%. After three days of depuration the post hoc tests revealed a significant reduction for Cu, Cr, Cd, and Co (27, 15, 52, and 23%, respectively, compared to their initial concentrations.

The total petroleum hydrocarbons (TPHs) in water, sediment and soft parts at the zeroth, first and third day of depuration are illustrated in Figure 4. Statistical analysis (ANOVA) showed significant differences in TPHs between water, sediment and soft parts of *Paphia undulata*. They were significantly higher in sediments than in water and soft tissues ($P < 0.001$ and $P < 0.05$, respectively). No significant differences were noted between the zeroth and first day of depuration ($P > 0.05$), while a

Table 1. One way analysis of variance for heavy metals in water, sediment and clams' whole soft tissues at the beginning of the experiment

Metal	Zn	Pb	Ni	Mn	Cu	Cr	Co	Cd
F-ratio	1485.	424.2	100.5	268.1	246.1	123.2	343.1	35.0
P-value	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 2. Bacterial count (CFU/ml) in soft tissues of *Paphia undulate* during the depuration time

Day of depuration	Total counts	*Vibrio* sp.	*Shigella* sp.	*Escherichia coli*	*Salmonella* sp.
0 day	24.1×10^5	12.6×10^5	5.1×10^5	4.1×10^4	3.9×10^3
1st day	21×10^5	9.5×10^5	1.6×10^5	2.8×10^4	2.5×10^3
3rd day	2.8×10^5	4.3×10^4	2.7×10^5	free	3×10^2

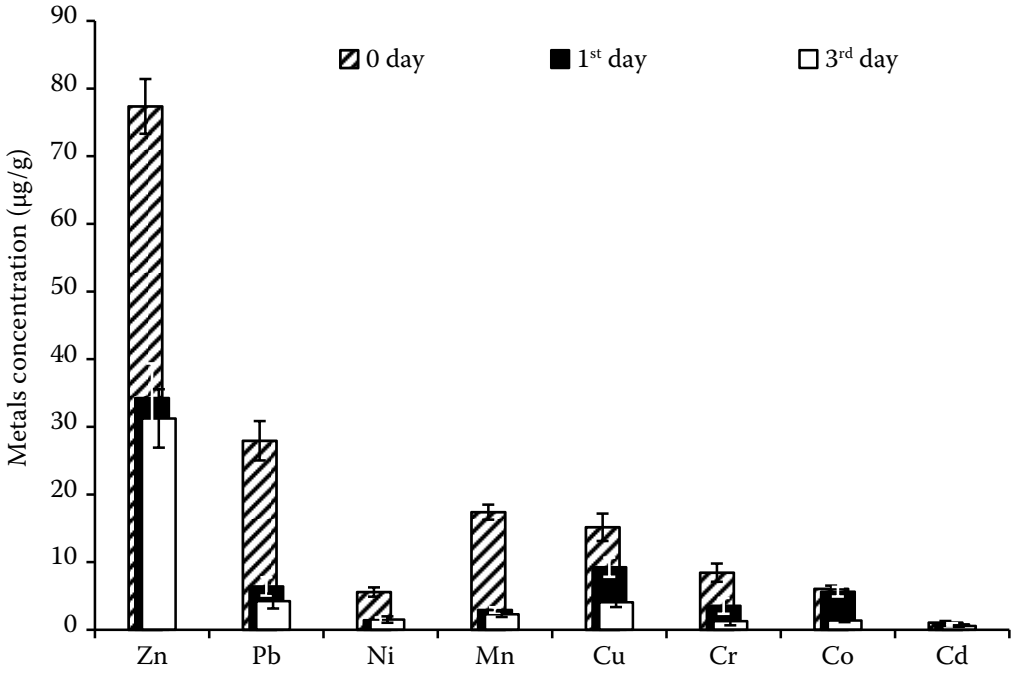


Figure 3. Metal concentrations in the soft parts of the *Paphia undulata* during the zeroth, first and third day of depuration. Values are means ± SD of three groups, each group contained five individuals

significant reduction was recorded on the third day. The concentrations of TPHs in clam's tissues were reduced to 90% after 24 h, while after three days of depuration they were reduced to 72% compared to their initial concentrations.

The initial bacterial count in the soft parts of *P. undulata* on the day of collection was 24.1×10^5 CFU/ml, while at the end of the depuration time it was reduced to 2.8×10^5 CFU/ml (12%) compared to its first concentration as shown in Table 2. Four pathogenic bacteria were isolated and identified (*Salmonella* sp., *Escherichia coli*, *Shigella* sp. and *Vibrio* sp.). The highest count was determined in *Vibrio* sp. (12.6×10^5 CFU/ml), followed by *Shigella* sp. (5.1×10^5 CFU/ml) and *Escherichia coli* (4.1×10^4 CFU/ml), while *Salmonella* sp. showed the lowest count (3.9×10^3 CFU/ml). The ability of *P. undulata* to depurate bacterial contamination after 24 and 72 h is presented in Figure 5. After one day of depuration the counts of *Vibrio* sp., *Shigella* sp., *Escherichia coli* and *Salmonella* sp. were reduced to 75, 31, 68, and 64%, respectively, from their initial count. After three days of depuration the counts of *Vibrio* sp. were significantly reduced to 3%, *Salmonella*

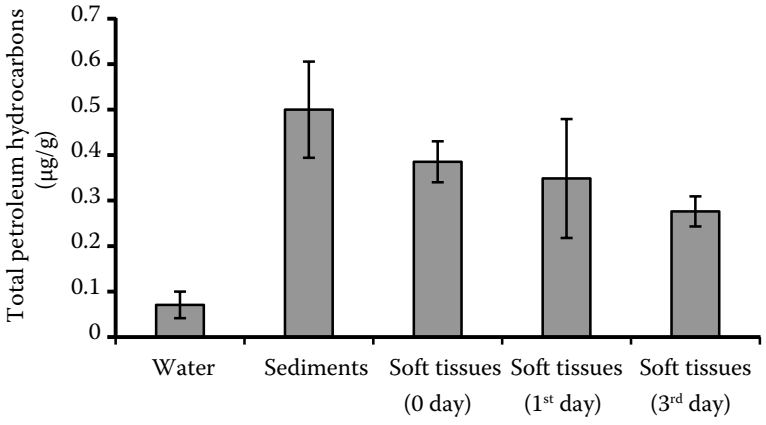


Figure 4. Total petroleum hydrocarbons in seawater, sediment and soft parts of *Paphia undulata* during the zeroth, first and third day of depuration

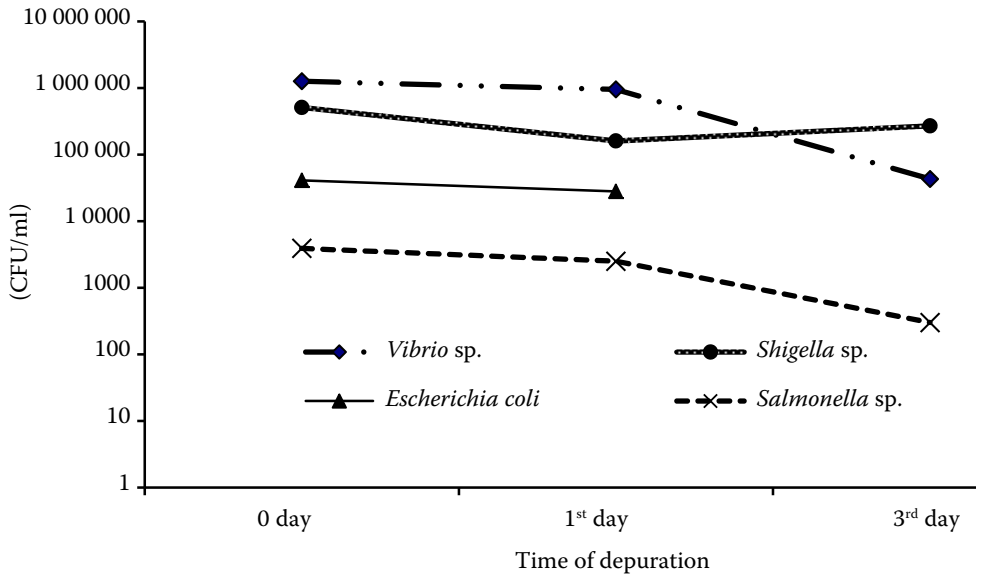


Figure 5. Counts of the patho-genic bacteria (log count/ml) in soft tissues of *Paphia undulata* during the depuration time

sp. to 8% and *Escherichia coli* was completely absent. The counts of *Shigella* sp. rose by 22% compared with the first day of depuration. So, the total number of pathogenic bacteria was reduced to 63% after one-day depuration, while after the third day it was reduced to 17% compared with its original concentration.

The mortality of clams is expressed as the percentage of clams that were lost on each day of depuration and compared with non-depurated ones. No significant changes were observed in the mortality ratio of *P. undulata* ($P > 0.05$) due to depuration. On the contrary, a decrease of mortality was observed on the first and third day of depurated clams as illustrated in Figure 6. The total mortality ratio that was observed at the end of the third day was 14% in depurated clams against 16% in non-depurated ones.

DISCUSSION

The area under investigation (Ismailia) has been exposed to municipal and different industrial wastes (Tarek and Ali, 2007). These wastes were accumulated by the *Paphia undulata* soft parts to levels higher than in water and sediments. This phenomenon was observed in other molluscs when compared to water and sediments, they exhibited greater spatial sensitivity, higher concentrations of heavy metals in their soft parts (Szefer, 1986; El-Gamal and Sharshar, 2004; Yap et al., 2009). Generally, the levels of the recorded metals did not exceed the maximum permissible levels except for Cu, Pb, and Cr as mentioned by WHO (1982). The herein species showed a good depuration for the tested metals that was time dependent. Barsyte-

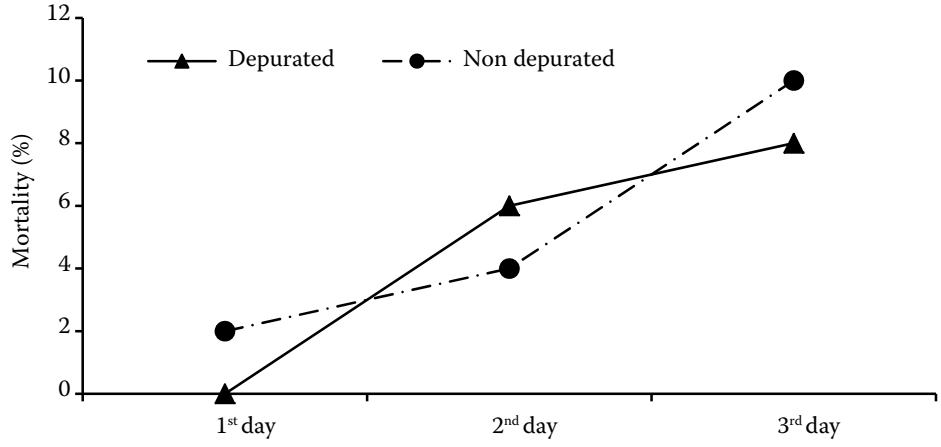


Figure 6. The mortality ratios of depurated and non-depurated *Paphia undulata*

Lovejoy (1999) and Anandraj et al. (2002) reported that molluscs had a depuration mechanism to reduce heavy metal toxicity in their body and this mechanism diminished the effectiveness of molluscs as biomonitoring organisms (Langston and Spence1995). After 24 h depuration, manganese was eliminated more quickly (83% of the initial concentration was depurated) than the other elements in spite of its high concentration in the sediment, while the lowest depurated one was cobalt (7% compared to its initial concentration). The high depuration of manganese reflects its weak binding within the *Paphia* tissues. Roesijadi (1980) related the high affinities of metals to bind with metallothioneins to their ability to fix themselves within the different tissues. On the contrary, low affinities reflect high depuration of metals from the tissues as explained by Ruddell (1971), who found out that metals that can be eliminated may be bound in amoebocytes and not fixed within the molluscan tissues. After three days of depuration cobalt, copper and cadmium were affected by the duration and eliminated faster than the other elements. In Geffard et al. (2002) study, cadmium was eliminated more quickly than copper or zinc when the kinetics of heavy metal elimination from *Crassostrea gigas* was recorded. Numerous studies were carried out concerning the accumulation and depuration of hydrocarbons especially fuel oils in bivalves (Rainio et al., 1986). The ratio of depurated TPHs (29%) was low compared with the depurated heavy metals in the herein *P. undulata*. The same was stated by DiSalvo et al. (1975) and Boehm and Quinn (1977): when they transferred molluscs chronically contaminated by hydrocarbons to clean water, they found out only a slight depuration. The slow and weak reduction of the TPHs in the present study might be related to the chronic pollution in Ismailia water (Tarek and Ali, 2007). In his experiment Rantamaki (1997) revealed that hydrocarbons which were slowly released to the water column were transferred to mussels, accumulated in them and at a longer exposure they were transported to the compartments, which slowly released them to water, even when it was again free of hydrocarbons. According to Stegeman and Teal (1973) it was important for an organism to retain biogenic hydrocarbons and the same mechanism may serve to prohibit the depuration of foreign hydrocarbons. A different conclusion was drawn by Lee et al. (1972) and Farrington et al. (1982) in acute oil spills, when molluscs were transferred to clean water, depuration could be rapid and might decline by several orders of magnitude. Concerning the duration of depurated TPHs, it had a slow beginning at the start of the experiment (10% depuration after 24 h) followed by a faster release after 72 h (29%) to be 72% compared to their initial concentration. On the other hand, Lee et al. (1972) supported the model of rapid initial elimination and delayed final depuration of polycyclic aromatic hydrocarbons after a short exposure. This contradiction may be due to the degree of pollution, time of exposure and the molluscan species.

Many studies reported the ability of bivalves to accumulate bacteria (Humphrey and Martin, 1993; El-Shenawy, 2004). At the same time, they can purge themselves of bacterial contaminants within 48 h, or they may be held up to 72 h in some cases (Dore and Lees, 1995). According to the results presented here, the counts of total bacteria were monitored to assess the efficiency of microbial depuration of *P. undulata*. The results of Van Slooten and Tarradella (1994) confirmed the present data; they found out that coliforms were reduced by 85% after 4 days of depuration, while pathogenic bacteria like *Vibrio* and faecal streptococci decreased to less than 50%. On the other hand, depuration was found to be effective against *Salmonella typhimurium* after 72 h and *Vibrio parahaemolyticus* after 36 h in *Mytilus galloprovincialis* (Barile et al, 2009). El-Shenawy (2004) correlated the depuration of bacteria to the degree of pollution, for example *Vibrio* was reduced by less than 50% when collected from a heavily polluted site. In *Paphia undulata* the counts of pathogenic bacteria were reduced by 83% after three days of depuration. Only *Shigella* sp. decreased after one day to 31% compared to its initial concentration, and then rose again by 22% after the third day of depuration (53% compared to the initial concentration). This might be influenced by the digestive process or bacterial ability to survive in the marine environment for days or weeks as mentioned by Le Guyader et al. (2000). Another explanation can be that the elimination by defecation is lower than the rate of reinfection.

The mortality of clams was not affected negatively by depuration. On the contrary, a decrease of mortality was observed on the first and third day of depuration. The same was observed by Maffei et al. (2009) in *Chamelea gallina*, where the viability and mortality were not influenced by the depurative treatment. The low mortality on the third day

may be correlated with the low bacterial and metal contamination.

Generally, the depuration of the studied pollutants was fast and brought about satisfactory results. Katayon et al. (2004) postulated that depuration under laboratory conditions was faster for reducing the metal contents of oysters compared to field depuration. Others (see for example El-Shenawy, 2004; Wahi et al., 2009) suggested that laboratory depuration needed a shorter period of depuration that ranged from 48 h to 32 days. On the contrary, depuration by transplanting the clams or oyster in another clean field needed a long period of depuration that ranged from 50 days to 6 months (Sericano et al., 1996, Rantamaki 1997; Gaber et al. 2008).

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