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Sublethal Effects of Zn^{++} and Cd^{++} on Respiration Rate, Ammonia Excretion, and O:N Ratio of *Donax trunculus* (Bivalvia; Donacidae)

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Anthropogenic activity along coastal regions may result in the introduction of metal ions into the marine environment. Shallow water organisms are subject to effluent water containing metal ions. Studies conducted on the shallow sandy beaches of Haifa Bay (Israel) - an area a with high level of industrial activity and input of urban and industrial effluents - showed no significant Cu, Cd, Pb or Zn contamination in the sediments or in benthic biota except at a single sampling site, opposite the polluted Kishón River estuary (Hornung et al 1989; Fishelson et al. 1996).

Donax is a cosmopolitan bivalve characteristic of sandy beaches. *Donax trunculus* is a common inhabitant of Mediterranean sandy beaches and it is common in Haifa Bay (Neuberger-Cywiak et al. 1990; Fishelson et al. 1996). Mussels and other bivalves have proved to be particularly sensitive to environmental levels of pollutants, and in polluted areas they may be exposed to xenobiotic material. The evaluation of the physiological status of "sentinel" organisms living in a monitored environment is the principle of biological monitoring. This can be done by determining the level of selected biological activities that are often reported as stress indices or biomarkers (Walker et al 2001)

Changes in respiration rate are one of the most common physiological responses to stress and are relatively easy to detect (Nordtug et al. 1991; Spicer and Weber 1991; Walker et al. 2001). Determination of nitrogenous excretory products, which are derived from catabolism of proteins, is also used as a measure of physiological responses to stressors (Stickle and Bayne 1982; Cockcroft 1990; Jiang et al. 2000). An important index used to observe stress response is the O:N ratio, which determines alterations in the balance between catabolism of carbohydrates, proteins, and lipids. This is a measure of the utilization of body reserves in the organism under a pollutant effect, and it provides an integrated physiological measurement of alteration in the balance between catabolic processes (Widdows 1985; Axiak and George 1987.)

The aim of the present study was to determine the effect of two metals ions(Zn an essential metal and Cd a non-essential metal) on *D. trunculus* physiological responses, their metabolic rate (measured as oxygen consumption and ammonia excretion), and on the O:N ratio as a stress index or biomarker of pollution.

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MATERIALS AND METHODS.

Donax trunculus was collected from unpolluted areas in Akko (Israel) and brought to the laboratory at Bar-Ilan University (Neuberger-Cywiak et al. 1990). The physiological responses were based on their metabolic rate (measured as oxygen consumption); and ammonia excretion (estimated after exposure of the animals to different concentrations of Zn and Cd ions). Mussels of 24-30 mm shell length were acclimated during 7 days to t=20°C ± 1°C; S= $40^0/_{00}$ ± $1^0/_{00}$ and 12D:12L photoperiod in the laboratory. Three-liter plastic containers were filled with 1,5 l of continuously aerated filtered sea water (0.45μm Millipore) and a 10 cm layer of local sand, enabling the animals to borrow inside the sediment. Under the experimental conditions, pollutants were added to each plastic container at concentration below (1 ppm and 0.1 ppm) or above (10 ppm) the LC_{50}, measurements began at 24h and 48h after exposure to the metals ions without water replacement (Neuberger-Cywiak et al. 2003). Zn^{++} was added as ZnCl, and Cd^{++} as $CdCl_2$, separately for each concentration and determination procedure. The plastic containers were sealed with a transparent plastic cover in order to reduce water loss by evaporation.

For oxygen determination, 15 mussels were placed in each of 14 plastic containers. After exposure to each of the metal ions, oxygen consumption was determined for a single mussel in a closed metabolic chamber (volume = 162 ml), using a Clark type electrode (YSI 5331). The metabolic chambers were kept at 20°C. The animals were introduced individually to the container containing sterilized sand and O_2 saturated filtered seawater stirred gently from the top. Before determination, the mussels were allowed to settle in the chamber for 15-20 minutes. Oxygen depletion was monitored for a period of 20 minutes, and used for calculating the rate of oxygen consumption. One control was used for both metals and consisted of empty containers filled with sand and filtered seawater. The rates were corrected toward the control. Only mussels that extended their siphons during the experiment were included in the analysis of results.

For ammonia determination, six to nine mussels were placed in each of 16 -250 ml beakers filled with 200 ml filtered sea water (0.45 μm). Metal ion concentrations and time were as above in the oxygen consumption experiments with control for each metal. Two samples of seawater of 10 ml each were taken at different times intervals, T_0=30 min; T_1= 2 h, T_3=3.5 h and T_4=5 h. The mean ammonium concentration determined at each metal concentration and time interval by the phenol-hydrochloride method (Solórzano, 1969; Parsons et al., 1984). The metal ions studied and air bubbling did not interfere in the ammonium determination method. At the end of the experiment, the organisms were dissected, removed from the shell, lyophilized and its dry weight was determined (0.1 mg).

Based on the rates of oxygen consumption and ammonia excretion, the O:N ratio in its atomic equivalents was calculated by the equation used by Axiak (1991).

Statistical analyses were carried out using the SPSS package. The Levene's test was performed for the homogeneity of group variance. The Univariate General Lineal Model, and the ANOVA were carried out and Dunnett's T3 Post Hoc multiple comparisons test was calculated in heteroscedastic cases, based on pair-wise comparisons between the treatment and with the control; and the Tukey's test was applied in homoscedastic data (Zar 1999; SPSS, 2003).

RESULTS AND DISCUSSION

The results obtained from the oxygen consumption experiments are summarized in Table 1. Oxygen consumption rate of *D. trunculus* decreased with increasing concentration of Zn^{++} and Cd^{++} ions. The differences were statistically higher in organisms exposed to the Cd^{++} than in those exposed to Zn^{++} at each concentrations and for both exposure periods (Table 2 a and Table 2 b). However, the effect of time at the same concentration was not significantly different in the animals exposed for 24 h period. Significant decreases were observed at 1 ppm and 10 ppm Cd^{++} exposure (Table 1 and 3). No significant differences in oxygen consumption between metals were observed for mussels exposed 24 h exposure period (Table 1 and 4). Significant differences were observed at 48 h exposure between the metals at 0.1, 1.0 and 10 ppm, with less oxygen consumption when exposed to Cd^{++} than when exposed to Zn^{++} (Table 1 and 4). Different authors also observed a decrease in oxygen consumption as result of toxicant concentration increases (Avolizi and Nuwayhid 1974; Spicer and Weber 1991; Mizrahi and Achituv 1994).

Connell et al. (1999) suggested that two possible strategies may be used by an organism to protect itself against a pollutant: get rid of the toxicant by excretion sequestering the toxicant in inactive tissues, or evading the area containing the toxicant and so reduction the exposure to the toxicant. The strategy of excreting the toxicant is involved in the synthesis of specific proteins such as metallothionein that detoxify metal ions by binding the ions and decreasing its bioavailability. This synthesis may require energy expenditure (metabolism, excretion, deposition of toxicant, physiological compensation, and avoidance behaviors) and eventually will be reflected in an increase in respiration rate. Reduction in the exposure to toxicants may be achieved by entering a dormant stage or by decreasing normal activities. Mussels may reduce feeding and close their valves to diminish uptake of toxicants; as a result, their normal physiological and metabolic functions are impaired and the respiration rate is decreased.

Our results showed a decrease in oxygen consumption when *D.trunculus* was exposed to higher toxicant concentrations. Widdows (1985) argued that behavioral responses must be measured simultaneously with oxygen consumption rate, since the decrease in respiration rate is mainly the result of suppression of activity and of partial or total closure of shells. When mussels are exposed to high concentrations of hydrocarbons, the apparent direct effect is enhanced oxygen consumption, also at elevated metal concentrations, bivalve molluscs keep their shells closed for long periods of time (Kramer et al. 1989). *Mytilus edulis* closed

Table 1. Rate of oxygen consumption (ml $O_2.h^{-1}.g^{-1}$tissue dry wt) of *Donax trunculus*, under sublethal concentrations of Zn^{++} and Cd^{++}, in organisms exposed to the metal for 24 h and 48 h.

	Treatment	Oxygen Consumption (ml $O_2.h^{-1}.g^{-1}$d.w.)	n		Treatment	Oxygen Consumption (ml $O_2.h^{-1}.g^{-1}$d.w.)	n
Exposure 24 h	**Control**	1.767 ± 0.557	15	Exposure 48 h	**Control**	1.988 ± 0.541	10
	Zn 0.1 ppm	1.191 ± 0.474	10		**Zn 0.1 ppm**	1.345 ± 0.848	12
	1 ppm	0.428 ± 0.482	9		**1 ppm**	0.650 ± 0.377	12
	10 ppm	0.235 ± 0.162	7		**10 ppm**	0.324 ± 0.200	7
	Cd 0.1 ppm	0.854 + 0.393	13		**Cd 0.1 ppm**	0.694 ± 0.357	10
	1 ppm	0.636 + 0.278	14		**1 ppm**	0.309 ± 0.188	10
	10 ppm	0.240 + 0.118	12		**10 ppm**	0.067 ± 0.059	11

(n= number of animals tested)

Table 2. Post Hoc Dunnett's T3 test for oxygen consumption of *Donax trunculus* exposed to different concentrations of Zn^{++} and Cd^{++} at 24h and 48h.

a) 24 h

24 h	Metal: Zn			Metal: Cd		
	Control	0.1ppm	1ppm	Control	0.1ppm	1ppm
0.1ppm	N.S.			***		
1 ppm	***	N.S.		***	N.S.	
10 ppm	***	*	N.S.	***	***	***

b) 48h

48h	Metal: Zn			Metal: Cd		
	Control	0.1ppm	1ppm	Control	0.1ppm	1ppm
0.1ppm	N.S.			***		
1 ppm	***	N.S.		***	N.S.	
10 ppm	***	**	N.S.	***	**	**

*** Significance level, α= 0.001; **Significance level, α= 0.01;
*Significance level α=0.05 N.S.= non-significant

Table 3. Post Hoc Dunnett's T3 test for oxygen consumption of *Donax trunculus* exposed to different concentrations of Zn^{++} and Cd^{++} at 24 h and 48 h

24h-48h	Control	0.1 ppm	1 ppm	10 ppm
Zn^{++}	N.S.	N.S.	N.S.	N.S.
Cd^{++}	N.S.	N.S.	*	*

(Differential effect of time on the physiological responses measured for each metal. Significance level, α=0.05.)

Table 4. Post Hoc Dunnett's T3 test for oxygen consumption of *Donax trunculus* exposed to different concentrations of Zn^{++} and Cd^{++} at 24 h and 48 h

Zn-Cd	0.1 ppm	1 ppm	10 ppm
24 hours	N.S.	N.S.	N.S.
48 hours	*	*	*

(Differential effect of metal concentration on the physiological responses measured at both times. Significance level, α=0. 05.)

their shells for considerable periods of time when exposed to different toxicants (Nordtug et al. 1991). In the present study, qualitative observations were made on siphon form and the closure of valve, these responses were related to previous results on burrowing behavior of *D. trunculus* (Neuberger-Cywiak et al. 2003). At high toxic level closure of shells was observed and the filtering siphons were not extended. However, at 10 ppm the valves remained open and no turgescence in the siphons was observed.

A decrease in oxygen consumption may also indicate induced pathological damage by heavy metal as a result of interference with a number of respiratory processes such as a decrease in ventilation, impeded gas exchange at respiratory surfaces, disrupted perfusion, impaired respiratory gas transport to/from tissues, or direct inhibition of cellular respiration (Spicer and Weber 1991). None of these are mutually exclusive and the effect can depend on the identity, as well as the ionic species or complex, of the metal (Spicer and Weber 1991). Pringle et al. (1968) stated that it is most probable that toxicity and decrease in metabolic activity is due to inhibition in enzyme systems. Mizrahi and Achituv (1994) studied the effect of Cd^{++}, Hg and Zn^{++} on the enzyme activities of cytochrome oxidase of the gastropod *Nassarius gibulosa*, they found a correlation between enzyme activity and oxygen consumption. Mizrahi and Achituv (1989) also showed inhibition of the cytocrome oxidase activity in *D. trunculus* exposed to 0.1 ppm, 1 ppm and 10 ppm of Cd^{++} and 10 ppm of Zn^{++}.

In mussels, valve closure act also as a defense mechanism to exposure to toxicant (Neuberger-Cywiak 2003). The animal may switch to anaerobic respiration; facultative anaerobic respiration was reported by Newell (1977) in intertidal invertebrates. Our results and the behavioral responses observed by Neuberger-Cywiak 2003, suggest that in order to obtain a realistic overview of the metabolism of bivalves that can switch to anaerobic metabolism, it is important to measure both aerobic and anaerobic metabolism.

Table 5 presents the ammonia excretion values. At 24 h there is difference between exposure to Zn^{++} and Cd^{++}. The differences between control and 10 ppm in the Zn^{++} experiments were significant (Table 5 and Table 6 a). In the Cd^{++} experiments the differences between control and 10 ppm, 0.1 ppm values-10 ppm, and between 1 ppm- 10 ppm were statistically significant (Table 5 and Table 6 a). Significant differences in ammonia excretion at different ion concentrations were

Table 5. Rate of ammonia excretion (μg NH_4-N. $hr^{-1}.0.1g^{-1}$ tissue dry wt) with the respective number of samples (n), under sublethal concentrations of Zn^{++} and Cd^{++}, in organisms of *Donax trunculus* exposed to the metal 24 h and 48 h.

	Treatment	Ammonia Excretion (μgNH_4-N.hr^{-1} .$0.1g^{-1}$ d.w.)	n		Treatment	Ammonia Excretion (μgNH_4-N.hr^{-1} .$0.1g^{-1}$ d.w.)	n
Exposure 24 h	**Control**	4.713 ± 1.412	7	Exposure 48 h	**Control**	7.573 ± 4.253	8
	Zn 0.1 ppm	6.708 ± 4.433	6		**Zn 0.1 ppm**	6.597 ± 1.652	6
	1 ppm	5.975 ± 3.567	7		**1 ppm**	9.708 ± 2.513	6
	10 ppm	1.209 ± 0.637	6		**10 ppm**	2.319 ± 0.519	9
	Control	2.819 ± 0.981	8		**Control**	6.435 ± 1.821	8
	Cd 0.1 ppm	3.044 ± 1.282	6		**Cd 0.1 ppm**	6.451 ± 2.479	7
	1 ppm	3.677 ± 0.903	9		**1 ppm**	5.299 ± 1.023	7
	10 ppm	0.609 ± 0.312	3		**10 ppm**	0.934 ± 0.458	9

Table 6. Post Hoc Dunnett's T3 test for ammonia excretion of *Donax trunculus* exposed to different concentrations of Zn^{++} and Cd^{++} at 24h and 48h.

a) 24 h

24 h	Metal: Zn			Metal: Cd		
	Control	0.1ppm	1ppm	Control	0.1ppm	1ppm
0.1ppm	N.S.			N.S.		
1 ppm	N.S.	N.S.	.	N.S.	N.S.	
10ppm	***	N.S.	N.S.	*	*	*

b) 48 h

48 h	Metal: Zn			Metal: Cd		
	Control	0.1ppm	1ppm	Control	0.1ppm	1ppm
0.1ppm	N.S.			N.S.		
1 ppm	N.S.	N.S.		N.S.	N.S.	
10ppm	*	**	**	***	**	***

**Significance level, α= 0.001; **Significance level, α= 0.01; *Significance level, α=0.05 N.S.= non-significant

Table 7. Post Hoc Dunnett's T3 test for ammonia excretion of *Donax trunculus* exposed to different concentrations of Zn^{++} and Cd^{++} at 24h and 48h.

24h-48h	Control	0.1 ppm	1 ppm	10 ppm
Zn^{++}	N.S.	N.S.	N.S.	N.S.
Cd^{++}	*	*	*	N.S.

(Differential effect of time on the physiological responses measured for each metal. Significance level, α=0.05.)

Table 8. Post Hoc Dunnett's T3 test for ammonia excretion of *Donax trunculus* exposed to different concentrations of Zn^{++} and Cd^{++} at 24h and 48h.

(Zn-Cd)	Control	0.1 ppm	1 ppm	10 ppm
24 h	*	N.S.	N.S.	N.S.
48 h	N.S.	N.S.	*	*

(Differential effect of metal concentration on the physiological responses measured at both times. Significance level, α=0.05.)

Table 9. Values of the equivalent O:N Ratio in atomic equivalent: oxygen consumed (metabolic rate) /ammonia excreted (metabolic waste) in *Donax trunculus*

	Treatment Zn	O:N	% decrease		Treatment Cd	O:N	% decrease
24 h	Control	46.85		24 h	Control	78.34	
	0.1 ppm	22.18	-52.66		0.1 ppm	31.00	-60.43
	1 ppm	8.96	-80.87		1 ppm	21.63	-72.39
	10 ppm	22.74	-51.46		10 ppm	49.30	-37.07
48 h	Control	32.67		48 h	Control	38.60	
	0.1 ppm	17.31	-47.01		0.1 ppm	13.44	-65.18
	1 ppm	8.37	-74.38		1 ppm	7.29	-81.11
	10 ppm	17.45	-46.58		10 ppm	8.99	-76.70

also found after 48 h exposure to Zn^{++} (Table 6 b). The effect of time upon exposure was significantly different for Cd^{++} but not for Zn^{++} (Table 7). Ammonia excretion in organisms exposed for 48 h to 1 ppm and 10 ppm Cd ions was significantly different (Table 8). The ammonia excretion response to Cd^{++} exposure was lower than that observed for Zn^{++} (Table 5).

Ammonia excretion in control of Cd experiment increased with time (Table 5). Ansell and Sivadas (1973), working on *D. vittatus* pointed out that due to protein catabolism during starvation NH_3 excretion increase. Bayne and Scullard (1977) indicated that can be a 10 fold increase of the excretion rate. A slight increase in ammonia excretion was observed at some concentrations of Zn ions as the concentration rose until reached highest concentrations of ion metals, when a decrease in the ammonia response was observed (Table 5). In the Cd^{++}exposure experiments ammonia excretion remained at the same levels as in the control in all ion concentration, but at the high concentration of 10 ppm a decrease on the response was observed (Table 5). Axiak (1991) obtained an increase in ammonia excretion in *Venus verrucosa* as a response to exposure to fractions of crude oil exposure. The amount of nitrogenous excretions resulted from the use of protein for energy and by the breakdown rate and turnover of constituent body cell catabolism (Widdows 1985; Griffiths and Griffiths 1987). Griffiths and Griffiths (1987) indicated that in most researches in bivalves, excretion rates are measured

as ammonia. Ammonia is regarded as an indicator of rate of protein catabolism, which varies with the nutritional and reproductive status of the animal (Widdows, 1985; Mathew and Menon, 1993). However, in *D. variabilis*, nitrogenous losses were 74% in nitrogenous compounds, 25% in amino acids and 1% in uric acid (Hammen 1968). Thus, Cockcroft (1990) found that in *D. serra* and *D. sordidus*, ammonia and amino acids were 70-78% and 21-30%, respectively, of total dissolved nitrogen excreted and the proportion of excreted nitrogen forms changed with stress. We detected a decrease in ammonia production (Table 5), exhibiting less ammonia production on organisms exposed to Cd^{++}. Ammonia production by bivalves has generally been assumed to be derived from aerobic amino acid and protein metabolism (Gabbott 1976); and carbohydrates or lipids are the predominant energy source at the outset of anaerobiosis (Sadok et al. 1999), while lactic acid and other end-products accumulate during this process (Newell, 1979). Valve closure and anaerobic mechanisms in the organism could be activated as part of the behavioral response to stressors.

It should be pointed out that the differences observed after exposure to Zn and Cd ions might be due to the way Zn and Cd ions affected the mussel metabolism. Zn^{++} is an essential component of at least 150 enzymes, on the other hand, Cd^{++} is a non-essential metal that in addition to being toxic above certain levels, may affect organisms by inducing deficiencies of essential elements through competition at active sites in biologically important molecules (Walker et al. 2001). Also, the differential behavioral response to the essential component (Zn^{++}) and non-essential metal (Cd^{++}), can also affect the metabolic pathway to be taken by the organism (Neuberger et al. 2003).

The use of the O:N ratio as a stress index has been discussed by Bayne and Widdows (1978), Mathew and Menon (1993). and Tedengren et al.(1999). We found a clear decrease between O:N ratio and increase in pollutant concentration (Table 9). Only at 24 h and 48 h of 10 ppm of Zn^{++} exposure and 10 ppm of Cd^{++}, the O:N ratio increased. At 10 ppm, Cd^{++} concentration is at the upper range of the LC_{50} value (Cd^{++} for 48 h LC50=7.6ppm, Neuberger et al. 2003) probably causing collapse of biochemical processes of the organism. This might explain why at 10 ppm, oxygen uptake and ammonia excretions are extremely low analyzed. The only metabolic source of energy measured by us is aerobic oxygen consumption; therefore, measuring the anaerobic processes is lacking and should be evaluated. In addition, ammonia excretion was the only form for excretion determined; other products that resulted from anaerobic metabolism were not identified and estimated. In all these cases, behavior was an important variable for avoiding the stressor and behavioral responses must be considered also as a stress biomarker (Neuberger et al. 2003; Wo et al. 1999). The importance of using this integrate value O:N ratio as a stress index must be considered when studying the effect of stressors at sub-lethal concentrations.

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