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Cellular energy allocation in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea) following tributyltin exposure

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Abstract

Recently, we described the cellular energy allocation (CEA) methodology to asses the effects of abiotic stress on the energy metabolism of the estuarine crustacean *Neomysis integer* (Crustacea: Mysidacea) [J. Exp. Mar. Biol. Ecol. 279 (2002) 61]. This short-term assay is based on the biochemical assessment of changes in the energy reserves (total carbohydrate, protein and lipid content) and the energy consumption (electron transport activity), and has been shown to be predictive of effects at the population level in daphnids [J. Aquat. Ecosyst. Stress Recovery 6 (1997) 43]. In the present study, the CEA methodology was evaluated using adult *N. integer* exposed for 96 h to the antifoulant tributyltinchloride (TBTCl). From a range-finding experiment with juvenile *N. integer*, a 96-h LC50 of 164 ng TBTCl/l was calculated. The energy metabolism of *N. integer*, as summarized by the CEA, was significantly altered by TBTCl exposure. Mysids exposed to 10, 100 and 1000 ng TBTCl/l consumed less energy and had lower respiration rates (in 10 and 1000 ng TBTCl/l treatments) than the control, resulting in a lower CEA. These changes at the cellular level occurred at environmentally relevant concentrations of the toxicant TBTCl which were an order of magnitude lower than reported effect concentrations for scope for growth in other marine invertebrates.

Keywords: Biomarker; Cellular energy allocation; Endocrine disruption; Energy metabolism; *Neomysis integer*; Tributyltin

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1. Introduction

Tributyltin (TBT) is a highly toxic and widespread contaminant in aquatic environments and has caused worldwide imposex (a pseudohermaphroditic condition, characterized by the development of a penis, vas deferens, and seminiferous tubules in females) in marine gastropods (deFur et al., 1999). The main inputs of TBT in the marine environment are from antifouling paints used on boats. In 1985, the world production of triorganotins with biocide properties was in the range of 8–10,000 tons annually (de Mora, 1996). Over recent decades, the use and the production of TBT-containing paints has been restricted, resulting in significant reductions in environmental concentrations (Alzieu, 1998). Despite these restrictive regulations, coastal TBT contamination can still reach up to 200 ng TBT/l (Michel et al., 2001).

In order to evaluate effects of pollutants, a number of assays have been developed linking effects at the (sub)organismal level with population level effects (Kooijman and Metz, 1984; Nisbet et al., 1989). The 'metabolic cost' hypothesis suggests that toxic stress induces metabolic changes which might lead to a depletion of its energy reserves resulting in adverse effects on growth and reproduction (Calow and Sibly, 1990). Probably the most successful application of this principle has been the 'scope for growth' concept, combining individual responses, such as respiration, excretion rate and assimilation efficiency into a single, integrated bioassay (Widdows and Salkeld, 1993). Scope for growth provides a measurement of the energy status of an organism, but remains labor-intensive, often results in a highly variable response (Bühringer and Danischewski, 2001) and is not easily employed in routine environmental impact assessments (De Coen and Janssen, 1997). Recently, we described an alternative methodology (cellular energy allocation, CEA) to assess the energy budget for the estuarine crustacean *Neomysis integer* (Verslycke and Janssen, 2002). The CEA methodology not only provides an integrated quantification of the organism's energy budget but also helps to elucidate different modes of action upon exposure to a varying abiotic environment and could potentially explain the different modes of action of toxicants.

In this paper, the CEA methodology was used to assess the effect of tributyltinchloride (TBTCl) on the energy allocation in the mysid *N. integer*. To evaluate acute toxicity of TBTCl to *N. integer*, an initial toxicity test was set up with juvenile mysids. In a subsequent exposure experiment, the effects of TBTCl on the energy metabolism of *N. integer* were evaluated.

Mysids are routinely used in laboratory toxicity tests and standard guides for conducting life-cycle toxicity tests with saltwater mysids have been developed using the subtropical American species *Americamysis* (*Mysidopsis*) *bahia* as a model (USEPA, 1995, 1997; ASTM, 1999). While specifically derived from work with *A. bahia*, these standard methods can be modified for testing with other mysid species (Roast et al., 1998a; Verslycke et al., accepted for publication). Since *A. bahia* has limited tolerance for lower salinities and is not a representative for European waters, *N. integer* has been proposed as a test species for pollution monitoring in European estuaries (Emson and Crane, 1994; Roast et al., 1998a,b, 1999; Verslycke et al., accepted for publication). *N. integer* is the dominant hyperbenthic mysid in European

estuaries (Mees et al., 1995; Mees and Jones, 1997) and is sensitive to many toxicants at environmental concentrations (Roast et al., 1999; Verslycke et al., accepted for publication; Wildgust and Jones, 1998). Furthermore, the extensive works of McKenney (1986), McKenny and Celestial (1996), McKenny and Matthews (1990) and McKenny et al. (1991) with *A. bahia* have resulted in several test procedures evaluating alterations in growth and reproductive responses upon toxicant exposure. In addition, transgenerational exposures are possible, gross morphological and histopathological changes may be observed, feeding and mating behavior and osmoregulatory capacities may be quantified, and cage experiments with mysids have been successfully performed in the field (deFur et al., 1999).

2. Materials and methods

2.1. Animal collection and maintenance

Initial *N. integer* populations were collected from the shore by hand net in the Galgenweel (a brackish water with a salinity of 3–5‰ near the river Scheldt, Antwerp, Belgium). After a 24-h acclimation period to the maintenance temperature, the organisms were transferred to 200-l glass aquaria. Culture medium was artificial seawater (Instant Ocean®, Aquarium Systems, France), diluted with aerated deionized tap water to a final salinity of 5‰. A 14-h light/10-h dark photoperiod was used during culturing and water temperature was maintained at 15 ± 1 °C. Cultures were fed daily with 24- to 48-h-old *Artemia* nauplii ad libidum to prevent adult mysids from cannibalizing their young. Hatching of the *Artemia* cysts was performed in 1-l conical vessels under vigorous aeration and continuous illumination at 25 °C.

2.2. Acute toxicity of TBTCl

Juvenile mysids were randomly distributed to 1.5-l glass beakers (10 per concentration, two replicates), each containing 1 l of the required TBTCl concentration (10, 100, 1000, 10,000 and 100,000 ng TBTCl/l) in water with a salinity of 5‰ (diluted from artificial sea water, Instant Ocean®). The tributyltin was delivered to the exposure solutions in absolute ethanol. The concentration of ethanol in the solvent control was 0.01%. Exposure temperature was 15 ± 1 °C and exposure solutions were renewed after 48 h. Animals were fed twice daily with 24- to 48-h-old *Artemia* nauplii (about 75 *Artemia*/mysid). Mortality was noted daily.

2.3. TBTCl analysis

The pH of the medium was adjusted to 5.3 with a sodium acetate/acetic acid buffer. After ethylation in an aqueous solution containing 1% of tetraethylborate, organotins were extracted with hexane, separated by gas chromatography (GC) and measured with inductively coupled plasma with mass spectrometry (ICP-MS). Tripro-

pyltin was used as internal standard. Details on the GC-ICP-MS operating conditions are given in De Smaele et al. (2001).

2.4. Exposure system

Duplicate groups of *N. integer* were exposed to 10, 100 and 1000 ng TBTCl/l and a control for 96 h. Animals were exposed in duplicate glass aquaria ($20 \times 15 \times 15$ cm) divided into six chambers by nylon screen (each chamber containing two individuals) resulting in 24 mysids per test concentration. Test organisms of about equal size (average weight of all animals used: 10.9 ± 3.3 mg) were collected from the cultures and either directly shock-frozen in liquid nitrogen and kept at -80 °C until analysis (day 0 animals) or randomly distributed into the test chambers. After 96 h, the mysids were removed from the exposure, shock-frozen in liquid nitrogen and kept at -80 °C until analysis (day 4 animals). It was decided not to feed the animals during the exposure period, to avoid variance due to individual differences in feeding. In case of partial mortality at the end of the 96-h exposure period, surviving animals were divided over the different biochemical analyses (sugar, lipid, protein and electron transport activity) in a way that at least three replicate measurements were performed for each parameter in each treatment.

All exposures were done in a temperature-controlled chamber (Liebher®, Laborimpex, Brussels, Belgium). Temperature was set at 15 °C (± 1 °C) and dissolved oxygen was maintained at saturation by aeration. The required salinity (5‰) was obtained by diluting artificial sea water (Instant Ocean®, Aquarium Systems) with carbon-filtered deionized tap water. Final salinity was confirmed with a portable refractometer (Digit 032, CETI, Belgium). Dissolved oxygen and temperature were measured at least twice a day (Oxi 191, WTW, Germany) and were within 5% of the desired value. The tributyltin was delivered to the exposure solutions in absolute ethanol and concentrations were checked with ICP-MS. The concentration of ethanol in the solvent control was 0.01%. Test concentrations were renewed after 48 h.

2.5. CEA measurement

The CEA was measured according to Verslycke and Janssen (2002). The different energy reserve fractions E_a (lipid, protein, sugar) were determined spectrophotometrically and transformed into energetic equivalents using their respective energy of combustion (39,500 mJ/mg lipid, 24,000 mJ/mg protein, 17,500 mJ/mg glycogen) (Gnaiger, 1983). The energy consumed (E_c) was estimated by measuring the electron transport activity (ETS) according to Owens and King (1975). The quantity of oxygen consumed per mysid, as derived from the ETS data, was transformed into energetic equivalents using the oxyenthalpic equivalents for an average lipid, protein and sugar mixture (484 kJ/mol O_2) (Gnaiger, 1983).

The E_a, E_c and CEA value were calculated as follows:

ΔE_a (difference in 'available energy, 'E_a) $= E_{a,day\ 0} - E_{a,day\ 4}$
4d-E_c (average 'energy consumption, 'E_c) $= 1/2 \times (E_{c,day\ 0} + E_{c,day\ 4})$
CEA (cellular energy allocation) $= \Delta E_a / E_c$.

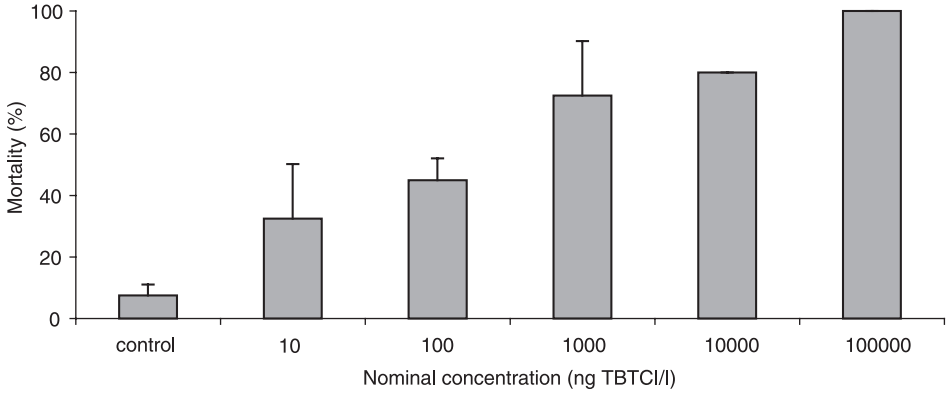

Fig. 1. Acute 96-h toxicity of tributyltinchloride (TBTCl) to the mysid *N. integer*. Error bars represent standard deviation on the mean of two replicates; $n = 10$ in each replicate treatment.

2.6. Statistical analysis

All data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's test, respectively, with an $\alpha = 0.05$. The effect of the treatment was tested for significance using a one-way analysis of variance (Dunnett's test, Statistica™). The 96-h LC50 values were calculated using the moving average method (Stephan, 1977).

3. Results

3.1. Acute toxicity of TBTCl to N. integer

N. integer were exposed to different concentrations of TBTCl in the water (10, 100, 1000, 10,000, 100,000 ng TBTCl/l) for 96 h and mortality was recorded (Fig. 1). Experimental TBTCl concentrations in freshly prepared solutions and after 48 h were determined with GC-ICP-MS (Table 1). The experimental TBTCl concentrations in the

Table 1
Aqueous tributyltinchloride (TBTCl) concentrations (ng TBTCl/l) in the 96-h toxicity experiment with *N. integer* as determined by GC-ICP-MS

Nominal TBTCl concentrations	Experimental TBTCl concentrations	
	Fresh solution	After 48 h
Control	8	9
10	32	28
100	150	86
1000	1050	1210
10,000	8540	2630
100,000	ND	ND

ND, not determined.

Table 2
Effects of 96-h exposure to tributyltinchloride (TBTCl) on the cellular energy allocation (CEA) of the mysid *N. integer* (data are shown as mean ± standard deviation)

Nominal concentration (ng TBTCl/l)	Energy allocation				
	ΔSugar reserve (mJ/mg ww)[a]	ΔProtein reserve (mJ/mg ww)[a]	ΔLipid reserve (mJ/mg ww)[a]	ΔE_a (mJ/mg ww)	4d-E_c (mJ/mg ww)
Control	66.6 ± 49.1	449.3 ± 364.3	1036.8 ± 423.3	1552.6 ± 687.7	5903.8 ± 1193.7
10	34.4 ± 87.9	21.1 ± 154.0	789.5 ± 350.5	845.0 ± 202.5	5334.7 ± 862.1
100	63.6 ± 38.4	73.9 ± 521.3	920.5 ± 467.0	1058.0 ± 345.1	6663 ± 1124.0
1000	35.3 ± 65.6	− 300 ± 638.5	975.3 ± 380.1	710.6 ± 290.4*	4990.8 ± 232.1

[a] Energy reserve day 0 − energy reserve day 4.
* Significantly different from control ($p < 0.05$).

freshly prepared solutions correlated well with the nominal values, except in the lower concentrations (10 and 100 ng TBTCl/l). From the mortality data, a 96-h LC50 of 114 ng TBTCl/l could be calculated (95% confidence limits: 33–436 ng TBTCl/l) based on the nominal concentrations. Based on the measured average concentrations (average between 0- and 48-h-old concentrations), the 96-h LC50 was slightly higher: 164 ng TBTCl/l (95% confidence limits: 52–373 ng TBTCl/l).

3.2. Cellular energy allocation

The effects of the 96-h exposure to tributyltinchloride on the different CEA parameters and the CEA of *N. integer* are shown in Table 2 and Fig. 2, respectively.

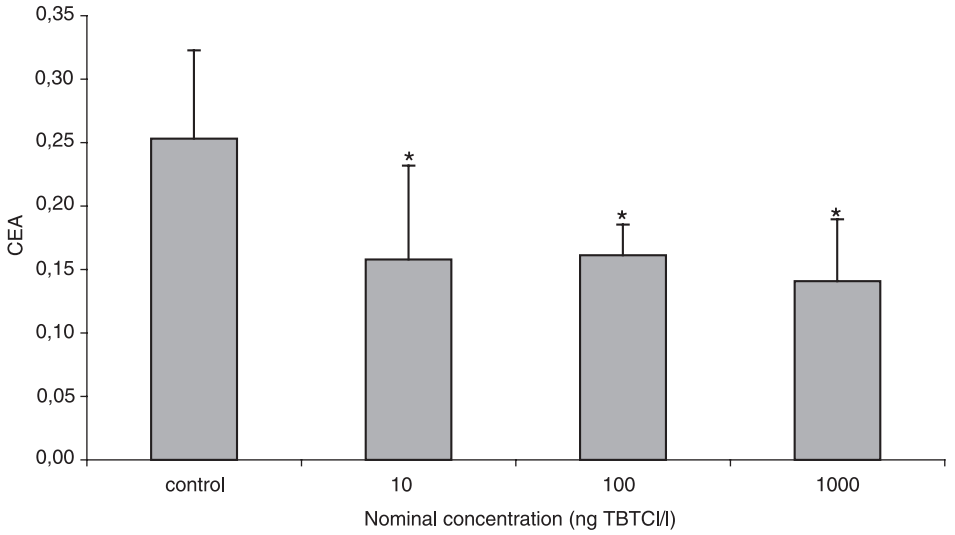

Fig. 2. Effect of 96-h exposure to tributyltinchloride (TBTCl) on the cellular energy allocation (CEA) in *N. integer*. Error bars represent standard deviation on the mean, with $n = 5$; *significantly different from control ($p < 0.05$).

Compared with mysids in the control, metabolic use of lipid, protein and sugar reserves was reduced (although not significantly) in all TBTCl-exposed mysids. Energy consumption after 96-h exposure to TBTCl ($E_{c,day\ 4}$), as derived from the electron transport activity, was not significantly affected by TBTCl exposure. A lower average energy consumption (4d-E_c) in mysids exposed to 10 and 1000 ng TBTCl/l was observed, but this reduction was not significant when compared with the control ($p = 0.4$ and $p = 0.2$, respectively). The ratio of the observed changes in energy reserves over the average energy consumption, defined as the cellular energy allocation (CEA), was significantly lower in all exposed mysids.

4. Discussion

Although the direct toxic effects and alterations in steroid metabolism leading to imposex following TBT exposure have been extensively described, relatively few data are available on the interaction of this toxicant with the processes that regulate energy metabolism (for review, see Fent, 1996). The imposex and intersex phenomena have demonstrated that it is important that toxicity and effects of environmental chemicals are studied on their basic modes of action at the subcellular level. Still, in the case of TBT-induced imposex, these underlying mechanisms have not been conclusively unraveled (deFur et al., 1999).

Organotins exert a number of important cellular, biochemical and molecular effects. For instance, elevation of intracellular calcium concentration appears to be responsible for the thymocyte killing and stimulation of apoptosis and thus cytotoxic action of organotins (Aw et al., 1990). In reference to the energy metabolism, organotins are inhibitors of oxygen uptake into tissues and mitochondria of cells, and are potent inhibitors of ATP synthesis (Matsuno and Hatefi, 1993). The ability of trisubstituted organotins to uncouple or directly inhibit oxidative phosphorylation in mitochondria has been attributed to three basic modes of action: direct inhibition of the ATPase complex (Aldridge and Street, 1964), inhibition of proton flow through the inner mitochondrial membrane (Selwyn, 1976), and gross swelling of mitochondria leading to the loss of most energetic functions (Aldridge and Rose, 1969).

Consequently, it can be hypothesized that changes in the energetic processes of *N. integer* following acute TBT exposure could be good indicators of the sublethal effects of this toxicant. In this perspective, we used the recently described cellular energy allocation technique to investigate the effects of TBT on the energy metabolism in the mysid *N. integer* (Verslycke and Janssen, 2002).

First, the acute toxicity of TBTCl to juvenile *N. integer* was assessed with a range-finding test. The acute LC50 for juvenile *N. integer* of 164 ng TBTCl/l (95% confidence limits: 52–373 ng TBTCl/l) found in our study is slightly lower than that reported by Goodman et al. (1988) for juvenile *A. bahia* (1100 ng TBTCl/l). Davidson et al. (1986) and Valkirs et al. (1987) found a similar acute effect level (300–420 ng TBT/l) for mysid shrimp (for review, see Fent, 1996). TBT compounds are thus highly toxic to mysid shrimp, especially juveniles. A greater sensitivity of the younger stages of shrimp was previously found by Cripe (1994) from a review of toxicity data for *A. bahia*. The 60% mortality in the 1000 ng TBTCl/l treatment with adult mysids for the CEA experiments

illustrates that adult mysids differ from juveniles in their sensitivity to TBTCl (96-h LC50 of TBTCl for adult *N. integer* in the μg/l range, data not shown). It can be speculated from our toxicity results that coastal TBT contamination, which can still reach up to 200 ng TBT/l despite restrictive regulations (Michel et al., 2001), is a potential threat to resident mysid populations.

Second, the effects of TBTCl exposure on the cellular energy allocation in adult mysids were investigated. The amount of protein, lipid and sugar allocated over the 96-h exposure period was lower in all treatments compared with the control (although not statistically significant), and this effect was strongest on the protein allocation in the 1000 ng TBTCl/l treatment ($p = 0.05$, compared with control). The change in available energy (ΔE_a) over the 96-h exposure period to TBTCl was lower in the 10 ($p = 0.07$), 100 ($p = 0.14$) and 1000 ng/l ($p = 0.04$) treatments compared with the control. These results indicate that TBT interferes with energy metabolism in *N. integer* by disrupting energy-producing processes of the catabolism. Since animals were not fed during the exposure, the intermediary energy-producing metabolism relies on the substrates available at day 0 (protein, sugar and lipid). Deleterious effects of organotins on the mitochondria, which are the sites of electron transport, oxidative phophorylation and fatty acid oxidation, have been described in literature (Fent, 1996). Alterations in the mitochondrial function would lead to a disruption of the catalytic processes and thus to a reduced breakdown of cellular energy reserves, as observed in the TBT-exposed mysids in this study. Obviously, these catalytic processes do not solely depend on mitochondrial integrity. The first steps not only in the oxidation of fatty acids but also in the protein degradation and glycolysis occur in the cytosol, independent of mitochondrial function. This could explain why significant breakdown of protein, sugar and lipid reserves still occurs in TBT-exposed mysids.

The differential effect on sugar, lipid and protein reserves is more difficult to explain. Protein allocation was more affected than lipid and sugar allocation. The biochemical composition of the estuarine mysid *N. integer* contrasts sharply with that of a typical mammal, i.e. the former has low carbohydrate reserves and fat depots. The potential use of protein as a metabolic reserve has previously been suggested by Bhat and Wagh (1992) for marine zooplankton and was also found in our previous studies with *N. integer* (Verslycke and Janssen, 2002). Still, we found that protein reserves exhibit high variability, which is probably partly related to ecdysis in these organisms. Further investigation and longer exposures should provide a better insight into the differential effect of TBT on protein, sugar and lipid metabolism.

The electron-transport system (ETS) is found in a cell's mitochondria and its microsomes, and consists of a complex chain of cytochromes, flavoproteins and metallic ions that transport electrons from catabolized foodstuffs (lipid, sugar, protein as fatty acids, glucose and amino acids) to oxygen (Packard, 1971). Owens and King (1975) demonstrated that the use of INT (*p*-iodonitrotetrazolium violet) as an artificial electron acceptor in the ETS with the suitable substrates provides a measure of planktonic potential oxygen consumption. We previously found a high correlation between potential whole organism respiration rates (as derived from the ETS assay) and real-time respiration measurements with a respirometer (Verslycke and Janssen, 2002). TBT has a selective and species-specific effect on different enzymes of the

microsomal ETS in freshwater fish, leading to destruction of native enzymes and inhibition of enzyme activity (Fent et al., 1998). It has been suggested that damage to mitochondria (and so decreasing respiration) is one of the most important mechanisms of TBT toxicity (Huang and Wang, 1995). We only observed a modest nonsignificant decrease in energy consumption in the 10 and 1000 ng TBTCl/l treatments and a nonsignificant increase in ETS activity in the intermediate exposure treatment. Either actual respiration rate was indeed affected, but these effects were masked because potential in vitro respiration rates were measured, or the possible differential effects of TBT on the mitochondrial or microsomal ETS were not detected by the ETS assay. Selective administration of NADH (electron donor for the mitochondrial ETS; Garret and Grisham, 1995) or NADPH (electron donor for the microsomal ETS; Lemberg and Barrett, 1973) could provide more information on potential selective effects of TBT on the microsomal and/or mitochondrial ETS. Actual mysid respiration rates should be monitored in future exposures to confirm if the respiration of TBT-exposed animals was actually reduced. Lower respiration rates would result in a lower metabolic fuel demand and so a lower CEA, as observed in our study. Based on the present study, it is difficult to assign the observed decline in CEA to a single effect of TBT on either a decrease in catabolic activity, a decrease in actual respiration rates, an inhibition of the ETS, or an effect on the mitochondrial integrity. Likely, a combination of the above led to the observed decrease in CEA, even at the lowest exposure concentration.

Generally, imposex in gastropod mollusks is believed to be the most dramatic effect of TBT exposure, occurring at levels of 2 ng TBT/l and above (Alzieu et al., 1986; Bryan et al., 1986; Curtis and Barse, 1990; Smith, 1980). The effects of TBT on gastropods are the most complete example of endocrine disruption in marine invertebrates. However, TBT has also been reported to exert some endocrine-disruptive effects in crustaceans. The following observations have been described in literature: effects on testosterone metabolism in daphnids (Leblanc and McLachlan, 2000; Oberdörster et al., 1998) and mysids (Verslycke et al., in press); effects on regenerative limb growth (Reddy et al., 1991; Weis et al., 1987); effects on molting (Reddy et al., 1992) and on calcium resorption from the exoskeleton (Nagabhushanam et al., 1990). These effects typically occur at levels in μg or mg/l range, levels which are acutely toxic to *N. integer*. We previously found significant interference of TBT with the testosterone phase I and II metabolism in *N. integer* (Verslycke et al., in press). Phase I reductase and hydroxylation activity and metabolic androgenization were induced in the 10 ng TBTCl/l treatment, whereas higher concentrations (100 and 1000 ng TBTCl/l) resulted in a reduction of phase II sulfate conjugation. In the present study, we found a significant alteration of the cellular energy allocation at the lowest test concentration (10 ng TBTCl/l). The general definition of an endocrine disruptor, i.e. an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function, would allow the observed effects on cellular energy allocation to be cataloged as endocrine-disruptive, since effects on CEA have been linked to effects on reproduction at the population level (De Coen and Janssen, 1997). On the other hand, it is easy to argue the contrary, since no direct disruption in endocrine function was observed, only the secondary effects. In conclusion, although a discussion on ‘actual’ endocrine

disruption is valid and a hot topic in current environmental science, the outcome of this discussion and so the actual underlying mechanism leading to individual and population effects is irrelevant for the organism in question. The observed effects on CEA and testosterone metabolism in *N. integer* occur at environmentally relevant concentrations of TBT, and research should be directed to the extrapolation of these data to field situations.

5. Conclusion

This study shows that the CEA assay can be used to detect sublethal interactions of environmental pollutants with the energetic processes of an organism. These changes at the cellular level occurred at environmentally relevant concentrations of the toxicant TBTCl and were an order of magnitude more sensitive than reported TBT effects on scope for growth in other marine invertebrates (Bühringer and Danischewski, 2001; Widdows and Page, 1993). In addition, the CEA assay allows an evaluation of specific interactions with subcellular mechanisms linked with the energy metabolism, such as electron transport activity and lipid, protein and sugar allocation.

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