ELSEVIER

Journal of Experimental Marine Biology and Ecology
306 (2004) 1–16

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

www.elsevier.com/locate/jembe

Cellular energy allocation and scope for growth in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following chlorpyrifos exposure: a method comparison

Tim Verslycke[a,*], Stephen D. Roast[b], John Widdows[c], Malcolm B. Jones[b], Colin R. Janssen[a]

[a] *Laboratory of Environmental Toxicology and Aquatic Ecology, Ghent University, J. Plateaustraat 22, B-9000 Ghent, Belgium*
[b] *Plymouth Environmental Research Centre, Department of Biological Sciences, University of Plymouth, Drake Circus, Plymouth, Devon PL4 8AA, UK*
[c] *Plymouth Marine Laboratory, Prospect Place, West Hoe, Plymouth, Devon PL1 3DH, UK*

Received 19 August 2003; received in revised form 18 October 2003; accepted 19 December 2003

Abstract

Mysids (Crustacea: Mysidacea) are used routinely in acute toxicity testing to evaluate the comparative toxicity of chemicals to aquatic organisms. The need for sublethal endpoints that provide comprehensive understanding of the potential impacts of toxicants to natural populations has resulted in examination of several physiological responses in mysid shrimp, including scope for growth (SFG) and cellular energy allocation (CEA). Both assays, based on the concept that energy in excess of that required for normal maintenance will be available for growth and reproduction, have been reported independently for the mysid *Neomysis integer*. The present study compares the responses of *N. integer* following exposure to environmentally realistic concentrations of the organophosphate pesticide chlorpyrifos using both assays. Oxygen consumption in the SFG assay was significantly correlated with cellular respiration rate in the CEA assay, and both were significantly increased by chlorpyrifos exposure. In addition, the protein, sugar, lipid and total energy content in the CEA assay and the egestion rate in the SFG assay were significantly different in chlorpyrifos-exposed mysids compared with control mysids. In contrast, absorption efficiency in the SFG assay was unaffected by pesticide exposure. Significant effects in the SFG and CEA assays were more pronounced following short (i.e. 48 h) compared with longer exposure periods (e.g. 168 h). SFG was significantly reduced at near-lethal concentrations (0.072 and 0.100 μg chlorpyrifos l^{-1}),

* Corresponding author. Tel.: +32-9-264-37-07; fax: +32-9-264-37-66.
E-mail address: Tim.Verslycke@UGent.be (T. Verslycke).

doi:10.1016/j.jembe.2003.12.022

whereas CEA was reduced in all chlorpyrifos-exposed mysids (0.038, 0.056, 0.072 and 0.100 μg chlorpyrifos l^{-1}) although there was no concentration response.

Keywords: *Neomysis integer*; Scope for growth; Cellular energy allocation; Biomarker; Energy metabolism

1. Introduction

To evaluate effects of pollutants on animal populations, communities and ecosystems, various methods have been developed ranging from the (sub)cellular to the ecosystem level of biological response. However, the predictive ability of measurements at higher levels of biological organization is limited because ecologically important effects (e.g. death or impaired organismal function) have already occurred before they can be detected at population and community levels. Over the last decades, biomarkers at suborganismal levels of organization (biochemical, physiological, and histological) have been considered to be viable measures of responses to stressors (Huggett et al., 1992). Some of the most successful types of biomarkers are those linked to metabolism and energetics. While typical and well-studied challenges to the endogenous crustacean energy metabolism include environmental hypoxia, functional (internal) hypoxia, changing energetic requirements, disturbance to water balance/ion-homeostasis and changes in temperature (for a review, refer to Morris and Airriess, 1998), exposure to toxicants will also result in an energetic challenge (i.e. McKenney, 1998).

Physiological energetics provide information on key processes in the organism's energy acquisition and expenditure, possibly also elucidating the mode of action of the toxicant. For example, the allocation of specific amounts of energy to basal metabolism, growth and reproduction will vary in response to changing environmental conditions, and theoretically exposure to a pollutant will disturb this allocation. In addition, changes in metabolic turnover and specific allocations will be linked to effects at higher levels of ecological organization (McKenney, 1998; De Coen, 1999). Based upon this concept, several single integrated bioassays, such as 'scope for growth' (SFG) and 'cellular energy allocation' (CEA), which both provide rapid, instantaneous measurements of the energy status of an organism, were developed (Widdows and Donkin, 1992; De Coen and Janssen, 1997). To date, there have been few attempts to compare different physiological responses of the same species to determine which is the most sensitive, or most appropriate, for environmental monitoring (e.g. Bamber and Depledge, 1997; Roast et al., 1999c).

Recently, the effects of the pesticide chlorpyrifos (*O*, *O*,-diethyl *O*-3,5,6-trichloro-2-pyridylphosphorothioate) on the respiration, feeding rate, absorption efficiency and SFG of the estuarine mysid *Neomysis integer* (Leach) were reported (Roast et al., 1999c). Also, the CEA assay has been used with *N. integer* to detect subtle effects of exposure to the antifoulant biocide tributyltinchloride (Verslycke et al., 2003). The present study set out to compare and evaluate both assays through an identical test set-up with the same species, *N. integer*, and the same toxicant, chlorpyrifos. Mysids are used routinely for marine and estuarine toxicity testing (USEPA, 1995, 1997; Roast et al., 1998a) and chlorpyrifos is one of the most widely used nonsystemic organophosphate insecticides (Whitehead, 1997).

Recommended application concentrations in the USA range from 0.28 to 0.56 kg chlorpyrifos ha^{-1} which results in concentrations of 9–18 μg chlorpyrifos l^{-1} (Marshall and Roberts, 1978). The potential of each individual CEA and SFG parameter as toxicity test endpoints are assessed, and the response of both CEA and SFG are compared.

2. Material and methods

2.1. Animal collection and maintenance

For the CEA assays, *N. integer* was taken from a laboratory culture in the Laboratory for Environmental Toxicology and Aquatic Ecology (Ghent University, Belgium); mysids were collected initially from the Galgenweel (a brackish-water inlet with a salinity of 4 ± 1 ‰ near the river Scheldt, Antwerp, Belgium) as described by Verslycke and Janssen (2002). 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 ad libitum with 24–48-h-old *Artemia* nauplii 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.

For the SFG experiments, *N. integer* was initially collected from the southern side of Terras Bridge, East Looe River estuary (Cornwall, UK) as described by Roast et al. (1998b). Animals were returned to the laboratory and placed in a shallow 15-l holding tank at a salinity of 10 ± 1 ‰ (made by combining filtered seawater and double-distilled, deionized water) in a constant-temperature room (15 ± 1 °C). Lighting was provided at ambient laboratory levels by overhead fluorescent lights; a time switch provided a 16-h light/8-h dark photoperiod. Mysids were fed ad libitum with 24–48-h-old *Artemia* nauplii hatched in the laboratory from cysts.

2.2. Chlorpyrifos exposure

In the SFG experiment, mysids were exposed to chlorpyrifos concentrations of 0.038, 0.056, 0.072 and 0.100 μg l^{-1} for three time periods (48, 96 and 168 h) as described by Roast et al. (1999c). In the CEA experiment, exposure concentrations and conditions were identical to those of the SFG experiment, except that only two time periods (48 and 168 h) were evaluated and animals were acclimated gradually for 72 h to the test salinity of 10 ‰. As oxygen consumption by *N. integer* is gender specific (Roast et al., 1999b), only males were used in these experiments. In the SFG experiment, 10 mysids of equal length (12 ± 1 mm from the anterior margin of the rostrum to the tip of the telson; average wet weight was 7.5 mg) were placed together in 2-l tall-form glass beakers containing 1500 ml of exposure water. In the CEA experiment, 50 mysids of equal length (visual selection of animals with a length of about 10 mm; average wet weight was 7.3 mg) were placed together in 10-l glass aquaria containing 5 l of exposure water. Two replicate aquaria were used for each concentration in the CEA experiment, and acetone only (100 μl acetone l^{-1}) controls were also set up to confirm that acetone did not affect any aspect of mysid

physiology. Due to the unstable nature of chlorpyrifos (ca. 55% loss within 24 h; Roast et al., 1999a), exposure solutions were replaced every 24 h with a freshly made solution (chlorpyrifos concentrations were not measured in the CEA experiment, but considered similar to those in the SFG experiment). At this time, dead or moribund mysids were removed from the test vessels (mortality was concentration-dependent and, in both test set-ups, more than 50% had died after 7 days exposure to 0.100 μg chlorpyrifos l^{-1}). Toxicant concentrations were prepared from an initial stock of 1 g chlorpyrifos l^{-1} acetone by dilution in 10‰ water. Mysids were fed twice daily with equal amounts of 36 ± 12-h-old *Artemia* nauplii (100 *Artemia* $mysid^{-1}$ day^{-1}), resulting in a continuous availability of food.

2.3. Scope for growth (SFG) assay

Oxygen consumption, feeding rate and absorption efficiency by *N. integer*, and subsequent calculation of SFG, were measured by Roast et al. (1999c). In summary, nine replicate respiration measurements were performed in closed respiration chambers (Strathkelvin Instruments) at 15 ± 1 °C and 10 ± 1 ‰, using freshly made exposure solutions of the same chlorpyrifos concentration to which the mysids had been exposed. Mysid egestion rate (measured by faecal production) correlates well with ingestion rate, and the former is a valid, indirect measurement of feeding rate (Murtaugh, 1984). Faecal production by *N. integer*, measured according to the method described by Roast et al. (2000), was measured from 10 replicates for each pesticide concentration and exposure period. Finally, mysid absorption efficiency was estimated by the ratio method (Conover, 1966), using pooled faecal material from several mysids. SFG was calculated by converting oxygen consumption and feeding rates into energy equivalents (J h^{-1}), and calculating the net energy gain/loss through physiological processes using the equation:

$$\mathrm{SFG} = A - (R + U) \quad (\mathrm{J\ mg^{-1}\ dry\ wt\ h^{-1}})$$

where: A = energy absorbed, R = energy respired and U = energy excreted (Widdows and Salkeld, 1993). The rate of ammonia excretion is usually closely related with respiration rate and, contributing usually < 5% of metabolic energy expenditure, is usually omitted from the SFG calculation (Widdows and Salkeld, 1993). Ammonia excretion was not measured in the present study, thus SFG was calculated from the equation $P = A - R$ (units as defined above) (Roast et al., 1999c).

2.4. Cellular energy allocation (CEA) assay

Mysids were shock-frozen in liquid nitrogen and kept at − 80 °C until analysis. CEA was measured according to Verslycke and Janssen (2002) with minor modifications. The different energy reserve fractions E_a (lipid, protein, sugar) were determined spectrophotometrically and transformed into energetic equivalents using their respective energy of combustion (39.5 J mg^{-1} lipid, 24 J mg^{-1} protein, 17.5 J mg^{-1} glycogen) (Gnaiger, 1983). Total lipids were extracted according to Bligh and Dyer (1959) and total lipid content was determined by measuring the absorbance at 370 nm with tripalmitin as a

standard. Total protein content was determined using Bradford's reagent (Bradford, 1976) by measuring the absorbance at 572 nm with bovine serum albumin as a standard. Total carbohydrate content was determined following the method of Roe and Dailey (1966) by measuring the absorbance at 492 nm with glucose as a standard.

The energy consumed (E_c) was estimated by measuring the electron transport system 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^{-1}) (Gnaiger, 1983). The E_a, E_c and CEA value were calculated as follows:

$$E_a \text{ (available energy } E_a) = \text{sugar} + \text{lipid} + \text{protein (mJ mg}^{-1} \text{ wet wt)}$$

$$E_c \text{ (energy consumption } E_c) = \text{ETS activity (mJ mg}^{-1} \text{ wet wt h}^{-1})$$

$$\text{CEA (cellular energy allocation)} = E_a/E_c$$

From this calculation, it can be deduced that a decline in CEA indicates either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction. Ten replicate measurements of lipid, sugar and protein content and ETS activity were performed for each pesticide concentration and exposure period, except in the highest test concentration after 7 days exposure ($n=7$) due to high mortality. All data are expressed as mJ per mg weight of the animal. For wet weight measurements, mysids were blotted dry and weighed with an analytical balance ($\pm$ 0.01 mg). To express results on a dry-weight base, a mysid dry wt/wet wt ratio of 20% can be used (Mauchline, 1980). The weight of the exposed animals was unaffected by chlorpyrifos exposure (ANOVA, $p>0.05$).

2.5. Statistical analysis of results

All data were checked for normality and homogeneity of variance using Kolmogorov–Smirnov and Levene's test, respectively, with an $\alpha=0.05$. The effects of exposure concentration and exposure duration were examined statistically by one- and two-way analysis of variance (ANOVA). SFG data are presented as means of the SFG calculated for the 48, 96 and 168-h exposure periods. Similarly, CEA data are presented as means of the CEA calculated for the 48- and 168-h exposure periods. Where significant F-ratios were calculated by ANOVA, Tukey's Honestly Significant Difference (HSD) test or Dunnett's Test were applied to identify which data sets were different.

3. Results

Mysids exposed to 100 μl acetone l^{-1} only (acetone controls) did not differ significantly from dilute seawater control mysids for any of the physiological parameters measured.

3.1. Oxygen consumption

Respiration by *N. integer*, measured in vivo, was affected by exposure concentration (ANOVA, F-ratio = 718, $df = 4$, $p < 0.001$) and duration (ANOVA, F-ratio = 109, $df = 4$, $p < 0.001$). All chlorpyrifos concentrations caused increased oxygen consumption with a clear concentration response ($p < 0.01$; Fig. 1). Mysids exposed to chlorpyrifos for 48 h showed a higher rate of oxygen consumption than those exposed for 96 or 168 h ($p < 0.01$). Although there was a significant interaction between pesticide concentration and exposure period (two-way ANOVA, F-ratio = 4.65, $df = 6$, $p < 0.01$), the effect was not obvious, and the individual effects of concentration and exposure are considered more important.

Cellular respiration was measured in mysids following chlorpyrifos exposure for 48 and 168 h via the in vitro electron-transport system (ETS) activity. ETS activities were significantly affected by exposure concentration (ANOVA, F-ratio = 5.90, $df = 4$, $p < 0.001$; Table 1), but not by exposure duration (ANOVA, F-ratio = 3.08, $df = 2$, $p > 0.05$). At the three highest exposure concentrations, mysids had higher respiration rates ($p < 0.05$) than control mysids after 48 and 168 h (not significant in the 0.072 μg chlorpyrifos l^{-1} treatment after 168 h). In addition, a significant correlation was found between the real-time respiration rates in mysids from the SFG experiment and ETS activities in mysids from the CEA experiment via linear regression analysis ($R^2 = 0.65$, $p < 0.01$; Fig. 2).

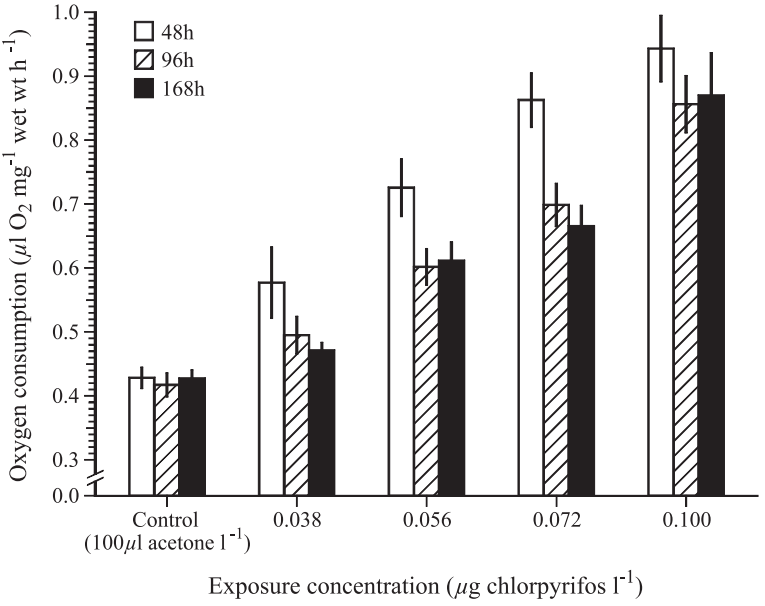

Fig. 1. Oxygen consumption by *N. integer* following exposure to chlorpyrifos. $n = 9$ for each exposure concentration/period. Error bars correspond to standard deviations of the mean (all chlorpyrifos-exposed mysids had a significantly higher oxygen consumption rate than control mysids, $p < 0.01$).

Table 1
Effects of chlorpyrifos exposure on the cellular energy allocation (CEA) in *N. integer* (data are shown as mean ± standard deviation)

Nominal concentration (μg chlorpyrifos l^{-1})	Exposure duration (h)	Energy allocation				
		Sugar reserve (mJ mg^{-1} wet wt)	Protein reserve (mJ mg^{-1} wet wt)	Lipid reserve (mJ mg^{-1} wet wt)	E_a [a] (mJ mg^{-1} wet wt)	E_c [b] (mJ mg^{-1} wet wt h^{-1})
	0	22.45 ± 11.45	508.09 ± 202.19	1283.59 ± 455.15	1814.14 ± 498.18	13.74 ± 2.94
Control ($n=10$)	48	32.71 ± 13.39	517.14 ± 87.80	1667.13 ± 458.42	2216.98 ± 466.96	14.55 ± 4.31
	168	16.75 ± 6.35	1186.25 ± 407.45	1197.30 ± 317.91	2389.29 ± 516.99	11.41 ± 2.96
0.038 ($n=10$)	48	36.97 ± 25.97	941.01 ± 358.01**	1752.08 ± 360.16	2730.06 ± 508.50	16.22 ± 3.53
	168	20.98 ± 9.33	898.50 ± 326.89	1364.81 ± 593.70	2284.28 ± 677.96	22.11 ± 9.60
0.056 ($n=10$)	48	39.49 ± 12.87	1053.66 ± 414.00***	1587.87 ± 755.85	2681.09 ± 862.01	31.04 ± 13.22***
	168	33.88 ± 12.44***	1079.98 ± 292.96	1533.52 ± 459.83	2647.37 ± 545.68	27.67 ± 18.62*
0.072 ($n=10$)	48	45.50 ± 15.72	1178.55 ± 176.17***	1728.23 ± 612.31	2952.29 ± 637.45*	28.93 ± 11.44***
	168	25.86 ± 10.65	796.11 ± 235.86*	1875.51 ± 804.63*	2697.94 ± 838.66	23.60 ± 12.97
0.100 ($n=7$)	48	32.36 ± 13.96	1080.20 ± 266.49***	2135.42 ± 962.47	3247.98 ± 998.86**	27.79 ± 12.81***
	168	16.19 ± 8.49	851.97 ± 330.12	2015.43 ± 1005.70*	2883.59 ± 885.18	29.09 ± 13.86*

Significantly different from control of the same exposure duration (ANOVA, Dunnett; *$p<0.05$;.**$p<0.01$; ***$p<0.001$).

[a] E_a = energy available (sum of sugar, protein and lipid).

[b] E_c = energy consumption (as derived from electron transport activity).

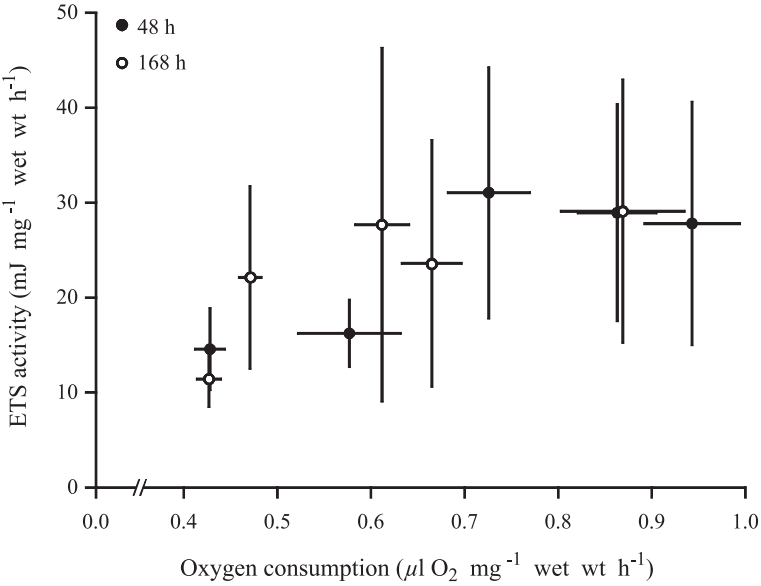

Fig. 2. Correlation between in vitro electron transport system (ETS) activities and whole organism respiration rates in *N. integer* exposed to chlorpyrifos. $n = 9$ for each exposure concentration/period. Error bars correspond to standard deviations of the mean.

3.2. Egestion rates and absorption efficiency

Egestion rates of *N. integer* were significantly affected by chlorpyrifos concentration (ANOVA, F-ratio = 27.59, $df = 4$, $p < 0.001$) but not by exposure duration (ANOVA, F-ratio = 0.72, $df = 2$, $p > 0.05$). Egestion rates were significantly different from control mysids following each of the three exposure periods at 0.100 μg chlorpyrifos l^{-1} ($p < 0.01$), and after 48 h at 0.072 μg chlorpyrifos l^{-1} ($p < 0.05$) (Fig. 3).

Irrespective of exposure concentration and duration, the absorption efficiency of *N. integer* was unaffected by chlorpyrifos. Absorption efficiencies were extremely consistent in all treatments, including controls, ranging between 0.342 and 0.362 (data presented by Roast et al., 1999c). The small variations recorded in absorption efficiency (absorption efficiencies of all pesticide-exposed mysids were within 1% of control mysid values) are assumed to be due to experimental variation and not to pesticide exposure.

3.3. Sugar, protein, lipid and total energy content

The effects of chlorpyrifos on the different CEA component values (sugar, lipid and protein) and the total energy content of *N. integer* (E_a) are shown in Table 1. Sugar content of *N. integer* was affected significantly by chlorpyrifos concentration (ANOVA, F-ratio = 2.84, $df = 4$, $p < 0.05$) and exposure duration (ANOVA, F-ratio = 19.31, $df = 2$, $p < 0.001$). No significant interaction effects between exposure concentration and duration on sugar content were observed (two-way ANOVA, F-ratio = 0.71, $df = 4$, $p > 0.05$). All

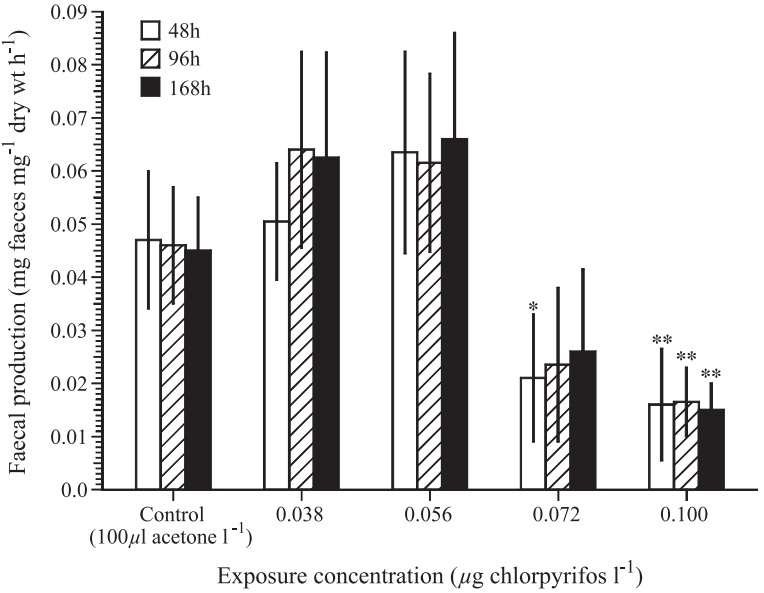

Fig. 3. Egestion by *N. integer* following exposure to chlorpyrifos. $n = 10$ for each exposure concentration/period. Error bars correspond to standard deviations of the mean. *Significantly different from control of the same exposure duration (*$p < 0.05$, **$p < 0.01$).

chlorpyrifos-exposed mysids had a higher sugar content than control mysids, except those in the highest exposure concentration. Sugar content was significantly higher in mysids exposed to 0.056 μg chlorpyrifos l^{-1} for 168 h than at other exposure concentrations (ANOVA, Dunnett, $p < 0.001$).

The mean protein content of *N. integer* exposed for 48 and 168 h was significantly affected by exposure duration (ANOVA, F-ratio = 35.07, $df = 2$, $p < 0.001$), but not by chlorpyrifos concentration (ANOVA, F-ratio = 1.04, $df = 4$, $p > 0.05$), although significant exposure effects were observed when considering 48-h and 168 h-exposed mysids separately. Significant interaction effects between exposure concentration and duration on protein content were observed (two-way ANOVA, F-ratio = 8.27, $df = 4$, $p < 0.001$). Following 48-h exposure to chlorpyrifos, all mysids had significantly higher protein contents than control mysids (ANOVA, Dunnett, $p < 0.01$). However, the opposite was observed in the 168 h-exposed mysids, where protein concentrations in chlorpyrifos-exposed mysids were lower than those of control mysids, although this was not statistically significant ($p > 0.05$).

Finally, lipid content of *N. integer* was significantly affected by exposure duration (ANOVA, F-ratio = 7.54, $df = 2$, $p < 0.001$) and concentration (ANOVA, F-ratio = 2.57, $df = 4$, $p < 0.05$), but no significant interaction effects between exposure concentration and duration were observed (two-way ANOVA, F-ratio = 0.74, $df = 4$, $p > 0.05$). All chlorpyrifos-exposed mysids had a higher lipid content than control mysids, but this was only significant in the two highest test concentrations following 168-h exposure (ANOVA, Dunnet, $p < 0.05$).

Total energy available (E_a) was calculated as the sum of the individual energy components sugar, lipid and protein. The E_a of *N. integer* was significantly affected by exposure duration (ANOVA, F-ratio = 26.62 $df = 2$, $p < 0.001$) and concentration (ANOVA, F-ratio = 3.194, $df = 4$, $p < 0.05$), but no significant interaction effects between exposure concentration and duration were observed (two-way ANOVA, F-ratio = 0.58, $df = 4$, $p > 0.05$). The total energy content of chlorpyrifos-exposed mysids was higher than the E_a in control mysids. This effect was significant in 48 h-exposed mysids in the two highest test concentrations (ANOVA, Dunnett, $p < 0.05$).

3.4. Scope for growth

Since different mysids were used to measure the individual components of the SFG calculations (i.e. oxygen consumption, feeding rate and absorption efficiency), SFG was estimated by using the mean component values. True replicates were, therefore, not possible and a single SFG estimate was calculated for pesticide concentration and exposure period. However, with the exception of mysids exposed for 48 h, there was no effect of exposure duration on the oxygen consumption or egestion rate of *N. integer*; therefore, mean SFG values at each pesticide concentration were calculated by averaging SFG values at 48, 96 and 168 h, allowing comparison of concentration effects. Exposure to chlorpyrifos had a significant effect on SFG of *N. integer* (ANOVA, F-ratio = 61, $df = 4$, $p < 0.01$) (Fig. 4). At the two higher pesticide concentrations, mysids had significantly reduced SFG compared with controls ($p < 0.01$). The mean SFG for mysids exposed to

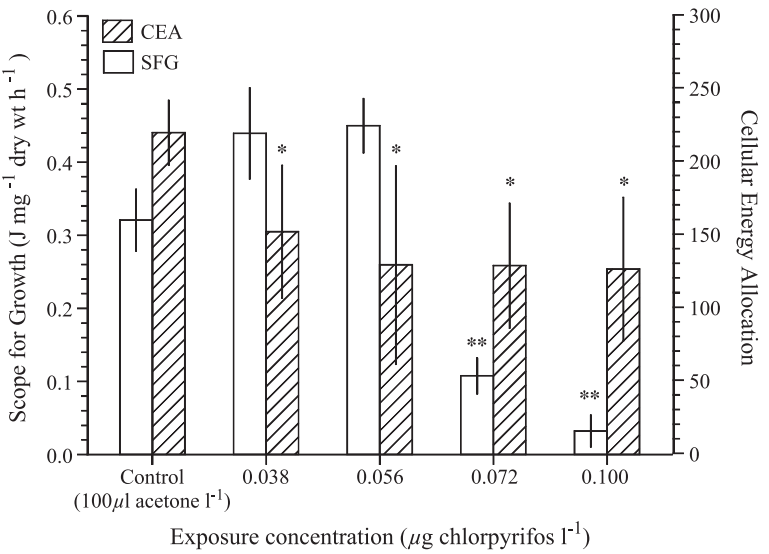

Fig. 4. Scope for growth (SFG) and cellular energy allocation (CEA) of *N. integer* following exposure to chlorpyrifos. Data pooled from SFG calculated after 48, 96 and 168 h and CEA calculated after 48 and 168 h. Error bars correspond to standard deviations of the mean. *Significantly different from control (*$p < 0.05$, **$p < 0.01$).

0.100 μg chlorpyrifos l^{-1} was close to zero, indicating some mysids may have been utilizing more energy than they were assimilating.

3.5. Cellular energy allocation

Cellular energy allocation was calculated as the ratio of the available energy E_a (sum of protein, sugar and lipid reserve) to the energy consumption E_c (as derived from the ETS activity) (Fig. 4). Thus, a decline in CEA indicates either a reduction in available energy or a higher energy expenditure, both resulting in a lower amount of energy available for growth or reproduction. Although the exposure period had an effect on all CEA components (except energy consumption), a single CEA estimate was calculated by averaging CEA values at 48 h and 168 h, allowing comparison of concentration effects and allowing a comparison with the SFG results. From this calculation, it was derived that all chlorpyrifos-exposed mysids had a reduced CEA compared with control mysids ($p < 0.05$).

4. Discussion

In the present study, chlorpyrifos was found to alter energy allocation and scope for growth in the mysid *N. integer*. Mysids exposed to the highest chlorpyrifos concentration had cellular (ETS) and whole animal respiration rates that were twice the rate of control mysids. In addition, a significant correlation ($R^2 = 0.65$, $p < 0.01$) was found between cellular respiration rate and whole animal respiration rate, despite measurements being carried out on different and separately exposed animals. Previously, we reported a high correlation ($R^2 = 0.94$; $p < 0.01$) between ETS activities in vitro and real-time in vivo animal respiration in *N. integer*, but these were measured on the same animals (Verslycke and Janssen, 2002). Thus, ETS activities appear to be a valid alternative to whole animal respiration measurements. Increased rates of oxygen consumption have been reported previously for mysids exposed to toxicants, including *Neomysis americana* exposed to naphthalene (Smith and Hargreaves, 1984) and *Americamysis bahia* exposed to the pesticides thiobencarb, endrin, fenthion and DEF (McKenney, 1998). In addition to the concentration response, there was also an exposure period effect on whole animal respiration, where mysids exposed to chlorpyrifos for 48 h consumed oxygen at a greater rate than mysids exposed for 96 or 168 h. However, this effect was not observed in the ETS activities. The ETS assay measures the maximum ETS activity under saturated substrate (NADH, NADPH) conditions and observed changes in activity must therefore be realized through changes in the amount of enzymes produced by the organism. Consequently, it is assumed that ETS activity is an overestimation of the ambient respiration and responds much slower to changes in the environment than the respiration rate (Båmstedt, 1980; Skjoldal et al., 1984; Mayzaud, 1986). Nevertheless, the concentration response in ETS activities was significant after 48 h of exposure and was not significantly different from the response after 7 days of chlorpyrifos exposure. It could, therefore, be hypothesized that mysids suffered from acute physiological stress following 48-h exposure, whereas at 168 h, although the cellular effects were still present, other mechanisms were operating, thus resulting in lower whole animal respiration rates.

Crustacean feeding rates are frequently suppressed following exposure to toxicants (e.g. Crane and Maltby, 1991; Crane et al., 1995; Guerin and Stickle, 1995). Although feeding rates have been studied in mysids (Nimmo et al., 1981; Jerling and Wooldridge, 1995; Roast et al., 2000; Engstrom et al., 2001; Viherluoto and Viitasalo, 2001a,b), few studies have investigated the effects of toxicants on mysid feeding rates. One study demonstrated a 50% decrease in cadmium-exposed mysid feeding rates, compared with control mysids (Gaudy et al., 1991). In the present study, egestion rate of *N. integer* (and, therefore, feeding rate) following exposure to high chlorpyrifos concentrations was significantly reduced compared with control mysids. On the other hand, absorption efficiencies were unaffected. In general, absorption efficiency is less sensitive to toxicant exposure than actual feeding rates and ingestion rate is considered the most important process in energy acquisition (e.g. Crane and Maltby, 1991; Roast et al., 2000). It may be anticipated that feeding rate will be closely related to the energy content of the mysids. Both E_a and egestion rates were affected most strongly at the two highest exposure concentrations (Fig. 2, Table 1); however, the observed effects were contradictory. Since mysid egestion rates are a good estimate of feeding rate (Murtaugh, 1984), lower egestion rates are expected to decrease the energy uptake and consequently the energy content of the organisms. Unexpectedly, while egestion rates decreased at the two highest exposure concentrations in the SFG study, mysids had a higher energy content (significant after 168 h) than controls at these concentrations in the CEA study. The reason behind this observation remains unclear. Perhaps, the correlation between feeding and egestion could be altered by toxicant exposure, although no chlorpyrifos-induced effects on absorption efficiency were apparent.

Clearly, present results indicate that chlorpyrifos exposure has significant effects on the energy expenditure/acquisition of *N. integer*. In the CEA assay, the increase in energy consumption in chlorpyrifos-exposed mysids was the determining factor for the observed decline in CEA. In the SFG assay, on the other hand, feeding rate was reflected most strongly in the mysid SFG value.

Both SFG and CEA incorporate the various components of an organism's energy budget by transformation of these components into energy equivalents (i.e. J h^{-1}), thus identifying the overall physiological effect of chlorpyrifos on *N. integer*. In this study, exposure to 0.072 and 0.100 μg chlorpyrifos l^{-1} led to a marked and concentration dependent reduction in SFG compared with control mysids (Fig. 4). Significant effects of chlorpyrifos on CEA were detected above 0.038 μg l^{-1}, but there was no evidence of a concentration–response relationship (Fig. 4). These effects on energy allocation would ultimately result in the mysid utilizing its energy reserves, and thus a decrease in E_a. Indeed, mysids exposed to 0.100 μg chlorpyrifos l^{-1} had a mean SFG close to zero and the lower standard deviation approaches zero, indicating that some individuals were potentially mobilizing their energy reserves. However, the E_a was not significantly lower in these animals in the present study and such an effect might only become apparent following longer exposures, which are likely to result in complete mortality, given the 168-h LC50 of 0.084 μg chlorpyrifos l^{-1} for N. integer (Roast et al., 1999a).

One final consideration is to assess which of the physiological responses measured in the present study offers the best potential for regulatory or environmental assessment purposes. Oxygen consumption was the most sensitive response and increased following exposure to the lowest pesticide concentration (0.038 μg chlorpyrifos l^{-1}) in the SFG

experiment. Although ETS activities in the CEA experiment were clearly increased in mysids exposed to the same concentration, this effect was only significant at 0.056 μg chlorpyrifos l^{-1}. ETS measurements had a greater variance than whole animal respiration rates in the present study (Fig. 2), nor did not show a linear concentration relationship, making them more difficult to interpret. In its favour, the ETS assay can mechanistically explain alterations in the energy consumption through specific interaction of the toxicant with the electron transport system (Spicer and Weber, 1991; Oberdörster et al., 1998). In general, changes in oxygen consumption are difficult to interpret as an isolated physiological response, especially since respiratory responses are also highly dependent on several abiotic and biotic factors (Roast et al., 1999b).

Scope for growth and cellular energy allocation are both appropriate responses to measure from a physiological point of view. However, SFG was only disrupted at concentrations approaching those causing lethal effects, especially for the longer exposure periods used in the present study. Egestion rates were significantly affected at 0.072 μg chlorpyrifos l^{-1} in the SFG experiment, which is just below the 168-h LC50 of 0.084 μg chlorpyrifos l^{-1} for *N. integer*. This narrow range between sublethal and lethal concentrations may be a feature of active (motile) crustaceans in contrast to sessile bivalves where SFG is affected at much lower toxicant concentrations than causing mortality (Widdows and Donkin, 1992). The cellular responses of the CEA were more sensitive (lowest observed effect concentration (LOEC) of 0.038 μg chlorpyrifos l^{-1}) than the organismal responses of the SFG (LOEC of 0.072 μg chlorpyrifos l^{-1}), which corroborates the theory of higher sensitivities of endpoints at a lower level of biological organization (e.g. biochemical vs. physiological). A disadvantage of the CEA response in the present study was the lack of a linear concentration-dependent response which limits its value because it did not distinguish between low sublethal and high sublethal effects of chlorpyrifos.

Although the interpretation of the individual components of the CEA (lipid, sugar and protein content) requires extensive background and control data, especially when used in field-collected organisms (Verslycke, 2003), the incorporation of energy consumption makes the CEA a relevant biomarker with a similar concept to SFG. In addition, CEA offers the possibility to assess (separately) the toxicant-induced effects on the major metabolic pathways, e.g. energy conversion towards growth (protein metabolism) might be separated from reserve depletion (lipids and sugar) to meet metabolic demands (De Coen, 1999). Finally, the biochemical responses of the CEA assay have been correlated significantly with population-level effects in toxicant-exposed daphnids, such as the intrinsic rate of natural increase and the mean total offspring per female (De Coen and Janssen, 1997). In our study, proteins were the fraction that was most strongly affected in chlorpyrifos-exposed mysids (Table 1). Several investigators have demonstrated that mysid metabolism is protein-based (Fergusson, 1973; Chin, 1974; Gaudy et al., 1980) and, therefore, protein metabolism might be an important endpoint to measure in toxicant-exposed mysids.

5. Conclusion

In summary, exposure to chlorpyrifos caused significant effects on the physiology of *N. integer* which were detected by both the SFG and CEA assays. While some of the

individual SFG and CEA components, such as oxygen consumption and protein content, were sensitive endpoints, it is unknown whether these would provide a consistent response to a wide range of chemicals. Furthermore, both SFG and CEA are integrative and provide more information concerning the effect of contaminants on the overall physiology of the animal, and potential effects at the population level. From the present study, CEA would appear to be a more sensitive biomarker than SFG [perhaps because it measures effects at a lower level of biological organization (cellular) than SFG (suborganismal)], but unlike SFG does not show a concentration-dependent response. However, this is only the first publication in which CEA and SFG responses are compared in a similar test set-up. Furthermore, a considerable amount of work has been conducted on the influence of extrinsic and intrinsic factors on SFG, and, of all stress indicators, SFG is probably the most developed and field-validated bioassay through several ecosystem monitoring programs (Huggett et al., 1992). The present study should, however, stimulate the use of CEA as an alternative or complementary approach to measure physiological aberrations in animals. The present study does not permit a conclusive statement as to whether one assay is better than the other, as both assays have their own strengths and weaknesses.

Acknowledgements

This research was supported by the Flemish Institute for the Promotion of Innovation by Science and Technology (IWT-V) and the Ghent University Research Fund (VEO-BOF). The authors wish to recognize especially K. Verheyden and B. Deryckere for their support in the practical work. The SFG study was funded by a Natural Environment Research Council grant [No. GT4/94/399/A (to S. Roast)], in collaboration with the Plymouth Marine Laboratory. **[SS]**

References

Bamber, S.D., Depledge, M.H., 1997. Evaluation of changes in the adaptive physiology of shore crabs (*Carcinus maenas*) as an indicator of pollution in estuarine environments. Mar. Biol. 129, 667–672.

Båmstedt, U., 1980. ETS activity as an estimator of respiratory rate of zooplankton populations. The significance of variations in environmental factors. J. Exp. Mar. Biol. Ecol. 42, 267–283.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. 37, 911–917.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 249–254.

Chin, P., 1974. Acclimation and metabolic attitude of *Neomysis awatschensis*. Publ. Mar. Lab., Busan Fish. Coll. 7, 1–20.

Conover, R.J., 1966. Assimilation of organic matter by zooplankton. Limnol. Oceanogr. 11, 338–345.

Crane, M., Maltby, L., 1991. The lethal and sublethal responses of *Gammarus pulex* to stress: sensitivity and sources of variation in an in situ bioassay. Environ. Toxicol. Chem. 10, 1331–1339.

Crane, M., Delaney, P., Watson, S., Parker, P., Walker, C., 1995. The effect of Malathion 60 on *Gammarus pulex* (L.) below water cress beds. Environ. Toxicol. Chem. 14, 1181–1189.

De Coen, W.M., 1999. Energy metabolism, DNA damage and population dynamics of the waterflea *Daphnia magna* Straus under toxic stress. PhD thesis, Ghent University, Belgium, p. 545.

De Coen, W.M., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing: IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed *Daphnia* populations. J. Aquat. Ecosyst. Stress Recovery 6, 43–55.

Engstrom, J., Viherluoto, M., Viitasalo, M., 2001. Effects of toxic and non-toxic cyanobacteria on grazing, zooplanktivory and survival of the mysid shrimp *Mysis mixta*. J. Exp. Mar. Biol. Ecol. 257, 269–280.

Fergusson, C.F., 1973. Aspects of nutrition and metabolism of *Neomysis integer* (Leach). PhD thesis, University of Southampton, Southampton.

Gaudy, R., Guérin, J.P., Pagano, M., 1980. Comparative ecophysiology of mysidacea *Hemimysis speluncola* (cave-dwelling) and *Leptomysis lingvura* GO Sars (non-cave dwelling)—respiration and excretion. J. Exp. Mar. Biol. Ecol. 44, 29–46.

Gaudy, R., Guérin, J.P., Kerambrun, P., 1991. Sublethal effects of cadmium on respiratory metabolism, nutrition, excretion and hydrolyse activity in *Leptomysis lingvura* (*Crustacea: Mysidacea*). Mar. Biol. 109, 493–501.

Gnaiger, E., 1983. Calculation of energetic and biochemical equivalents of respiratory oxygen consumption. In: Gnaiger, E., Forstner, H. (Eds.), Polarographic Oxygen Sensors. Aquatic and Physiologica Apllications. Springer Verlag, Berlin, pp. 337–345.

Guerin, J.L., Stickle, W.B., 1995. Effects of cadmium on survival, osmoregulatory ability and bioenergetics of juvenile blue crabs *Callinectes sapidus* at different salinities. Mar. Environ. Res. 40, 227–246.

Huggett, R.J., Kimerle, R.A., Mehrle Jr., P.M., Bergman, H.L., 1992. Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress. Lewis Publishers, Boca Raton, FL, p. 500.

Jerling, H.L., Wooldridge, T.H., 1995. Feeding of two mysid species on plankton in a temperate South African estuary. J. Exp. Mar. Biol. Ecol. 188, 243–259.

Marshall, W.K., Roberts, J.R., 1978. Ecotoxicology of chlorpyrifos. Publication NRCC, vol. 16079. Natural Research Council Canada, Ottowa, Canada, p. 314.

Mauchline, J., 1980. The biology of mysids. Adv. Mar. Biol. 18, 1–369.

Mayzaud, P., 1986. Enzymatic measurements of metabolic processes concerned with respiration and ammonium excretion. In: Corner, E.D.S., O'Hara, S.C.M. (Eds.), The Biological Chemistry of Marine Copepods. Oxford Univ. Press, Oxford, pp. 226–259.

McKenney Jr., C.L. 1998. Physiological dysfunction in estuarine mysids and larval decapods with chronic pesticide exposure. In: Wells, P.G., Blaise, C. (Eds.), Microscale Testing in Aquatic Toxicology: Advances, Techniques, and Practice. CRC Press, Boca Raton, FL, pp. 465–476.

Morris, S., Airriess, C.N., 1998. Integration of physiological responses of crustaceans to environmental challenge. S. Afr. J. Zool. 33, 87–106.

Murtaugh, P.A., 1984. Variable gut residence time: problems in inferring feeding rate from stomach fullness of a mysid crustacean. Can. J. Fish. Aquat. Sci. 41, 1287–1293.

Nimmo, D.R., Hamaker, T.L., Matthews, E., Moore, J.C., 1981. An overview of the acute and chronic effects of first and second generation pesticides on estuarine mysid. In: Vernberg, J., Thurberg, F., Calabrese, A., Vernberg, W. (Eds.), Biological Monitoring of Marine Pollutants. Academic Press, New York, NY, pp. 3–19.

Oberdörster, E., Rittschof, D., McClellan-Green, P., 1998. Induction of cytochrome *P*450 3A and heat shock protein by tributyltin in blue crab, *Callinectes sapidus*. Aquat. Toxicol. 41, 83–100.

Owens, T.G., King, F.D., 1975. The measurement of respiratory electron transport activity in marine zooplankton. Mar. Biol. 30, 27–36.

Roast, S.D., Thompson, R.S., Widdows, J., Jones, M.B., 1998a. Mysids and environmental monitoring: a case for their use in estuaries. Mar. Freshw. Res. 49, 827–832.

Roast, S.D., Widdows, J., Jones, M.B., 1998b. The position maintenance behaviour of *Neomysis integer* (*Peracarida: Mysidacea*) in response to current velocity, substratum and salinity. J. Exp. Mar. Biol. Ecol. 220, 25–45.

Roast, S.D., Thompson, R.S., Donkin, P., Widdows, J., Jones, M.B., 1999a. Toxicity of the organophosphate pesticides chlorpyrifos and dimethoate to *Neomysis integer* (*Crustacea, Mysidacea*). Water Res. 33, 319–326.

Roast, S.D., Widdows, J., Jones, M.B., 1999b. Respiratory responses of the estuarine mysid *Neomysis integer* (*Peracarida: Mysidacea*) in relation to a variable environment. Mar. Biol. 133, 643–649.

Roast, S.D., Widdows, J., Jones, M.B., 1999c. Scope for growth of the estuarine mysid *Neomysis integer* (*Peracarida: Mysidacea*): effects of the organophosphate pesticide chlorpyrifos. Mar. Ecol. Prog. Ser. 191, 233–241.

Roast, S.D., Widdows, J., Jones, M.B., 2000. Egestion rates of the estuarine mysid *Neomysis integer* (*Peracarrida: Mysidacea*) in relation to a variable environment. J. Exp. Mar. Biol. Ecol. 245, 69–81.

Roe, J.H., Dailey, R.E., 1966. Determination of glycogen with the anthrone reagent. Anal. Biochem. 15, 245–250.

Skjoldal, H.R., Båmstedt, U., Klinken, J., Lain, A., 1984. Changes with time after capture in the metabolic activity of the carnicorous copepod *Euchaeta norvegica* Boeck. J. Exp. Mar. Biol. Ecol. 83, 195–210.

Smith, R.L., Hargreaves, B.R., 1984. Oxygen consumption in *Neomysis americana* (*Crustacea: Mysidacea*) and the effects of naphthalene exposure. Mar. Biol. 79, 109–116.

Spicer, J.I., Weber, R.E., 1991. Respiratory impairment in crustaceans and molluscs due to exposure to heavy metals. Comp. Biochem. Physiol. 100C, 339–342.

USEPA, 1995. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Surface Waters to Marine and Estuarine Organisms, 3rd ed. U.S. Environmental Protection Agency, Cincinnati, OH, USA EPA-600/4-91-002.

USEPA, 1997. Office of pollution prevention and toxic substances (OPPTS) test guidelines. Series 850 on Ecological Effects. Environmental Protection Agency, Washington DC, USA.

Verslycke, T., 2003. Endocrine disruption in the estuarine invertebrate *Neomysis integer* (Crustacea: Mysidacea). PhD thesis. Ghent University, Belgium, p. 224.

Verslycke, T., Janssen, C.R., 2002. Effects of a changing abiotic environment on the abiotic environment on the energy metabolism in the estuarine mysid shrimp *Neomysis integer* (Crustacea: Mysidacea). J. Exp. Mar. Biol. Ecol. 279, 61–72.

Verslycke, T., Vercauteren, J., DeVos, C., Moens, L., Sandra, P., Janssen, C.R., 2003. Cellular energy allocation in the estuarine mysid *Neomysis integer* (Crustacea: Mysidacea) following TBTCl exposure. J. Exp. Mar. Biol. Ecol. 288, 167–179.

Viherluoto, M., Viitasalo, M., 2001a. Effect of light on the feeding rates of pelagic and littoral mysid shrimps: a trade-off between feeding success and predation avoidance. J. Exp. Mar. Biol. Ecol. 261, 237–244.

Viherluoto, M., Viitasalo, M., 2001b. Temporal variability in functional responses and prey selectivity of the pelagic mysid, *Mysis mixta*, in natural prey assemblages. Mar. Biol. 138, 575–583.

Whitehead, R., 1997. The UK Pesticide Guide 1997. C.A.B International and British Crop Protection Council Univ. Press, Cambridge, UK.

Widdows, J., Donkin, P., 1992. Mussels and environmental contaminants: bioaccumulation and physiological aspects. In: Gosling, E.M. (Ed.), The Mussel *Mytilus*. Elsevier, Amsterdam, pp. 383–424.

Widdows, J., Salkeld, P.N., 1993. Practical procedures for the measurement of scope for growth. MAP Tech. Rep. Ser. 71, 147–172.