Research Articles

Suitability of *Crangonyx pseudogracilis* (Crustacea: Amphipoda) as an Early Warning Indicator in the Multispecies Freshwater Biomonitor

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Abstract

Background. Biological monitors are increasingly important in 'Biological Early Warning Systems' (BEWS) for monitoring water quality. This study examines the freshwater amphipod *Crangonyx pseudogracilis* as a potential new indicator species when used in the Multispecies Freshwater Biomonitor (MFB). The MFB is an online continuous biomonitor which uses impedance conversion to record behavioural responses of vertebrates and invertebrates.

Methods. Four experiments were undertaken to establish: (1) if the electrical field generated by the MFB affected the organisms' behaviour, (2) if defined behaviours and their response to a gradient of ammonium chloride could be detected by the MFB, (3) if there was variation in the behaviour of *C. pseudogracilis* over a diel cycle, and (4) if behaviour changed significantly in response to a pulse of ammonium chloride.

Results and Discussion. Results showed no significant effect of the MFB's current on behaviour of *C. pseudogracilis*. Four behaviours; swimming, walking, grooming and inactivity, were observed and identified in the MFB. In the MFB, each behaviour changed significantly in response to an increasing gradient of ammonium chloride exposure. The MFB also detected increases in nocturnal activity by C. pseudogracilis. The MFB also detected a significant increase in activity after a pulse of ammonium chloride.

Conclusion. The range of behaviours exhibited by *Crangonyx pseudogracilis*, together with its ease of culture, suggest future potential of this species as an indicator species for the Multispecies Freshwater Biomonitor.

Recommendations and Outlook. Further testing is required over a range of toxicants and concentration gradients to establish threshold responses and the full compliment of behaviours that could be useful in online biomonitoring.

Keywords: Ammonia; Amphipoda; behaviour; *Crangonyx pseudogracilis*; impedance conversion; Multispecies Freshwater Biomonitor

Introduction

The need for Biological Early Warning Systems (BEWS) for the rapid toxicity assessment of water for drinking purposes and for the protection of the environment from toxic discharges has driven research into biological monitors (Kramer and Botterweg 1991, Gerhardt 1999). This is also important given the threat posed by bioterrorism. Biomonitors installed in remote locations, which use satellite techniques and modem connections, can alert operators based at central sites to problems. In several European countries online biomonitors have been installed along the large rivers, for example Rhine, Ruhr, Elbe (Bode and Nusch 1999). Biomonitors using light beam interruption, infra-red detection, impedance conversion and magnets have enabled the use of invertebrates as biological monitors and have eliminated the subjectivity of behavioural observations (Morgan et al. 1984, Gerhardt et al. 1994, Van Hoof et al. 1994, Borcherding and Jantz 1997, Gerhardt et al. 1998, Gerhardt 1999, 2000). ECOTOX using *Euglena gracilis* (Tahedl and Hader 1999, 2001) and the Dreissena-Monitor using *Dreissena polymorpha* (Kramer et al. 1989, Borcherding and Jantz 1997) use single species, however, the Multispecies Freshwater Biomonitor (MFB) can examine multiple species, both invertebrates and vertebrates, simultaneously (Gerhardt et al. 1994). The MFB is based on impedance conversion (Gerhardt, 1995) with organisms placed in chambers. On the chamber walls two pairs of electrodes are installed so that one pair generates a high frequency alternating current (100 kHz) and the other pair senses changes of impedance caused by the movements of the organism with different types of signals representing different types of behaviour (Gerhardt et al. 1998, Gerhardt 2000). The MFB measures quantitatively changes in the behavioural pattern of both aquatic vertebrates and invertebrates (Gerhardt et al. 1994).

Biomonitors often rely on rapid and sensitive behavioural responses of their indicator species. Preference-avoidance behaviour to various stimulants or toxicants has been studied mainly in fish (Beitinger 1990, Cherry and Cairns Jr 1982, McNicol and Scherer 1991) whilst behaviour of aquatic invertebrates is receiving increasing importance in eco(toxico)-logical research. *Gammarus* species, have become model biomonitor species due to their ease of culture and several readily measurable sub-lethal end-points including: changes in feeding activity (Taylor et al. 1993), precopula separation (Pascoe et al. 1994), locomotory activity and ventilation (Gerhardt 1995, 1999). Other end points may include stress response proteins, reproductive output and developmental abnormalities However, the use of *Gammarus* species in laboratory studies is not always useful as they are particularly sensitive to laboratory conditions, namely static tests where survival within controls is often poor (Martin and Holdich 1986).

In this study our aim was to examine the merits and suitability of *Crangonyx pseudogracilis* as a potential new early warning system species in conjunction with the MFB. The focus of this paper is not to examine the sensitivity of *C. pseudogracilis* to the toxicant. *Crangonyx pseudogracilis* (Bousfield 1958), is a common and widely distributed amphipod native to mid- and eastern North America (Holsinger 1977). Smaller than members of the genus *Gammarus* it differs in having an upright walking orientation and in the direction of sexual dimorphism, females being much larger (up to 11 mm) than males (3–4 mm) (Hynes 1955, Orme 1983, Martin 1986). *C. pseudogracilis* also has a much broader international distribution owing to a history of unintentional introductions to Europe. First introduced to England in 1930 it has expanded its range to become common throughout the British Isles, the Netherlands and Belgium (Holmes 1975, Pinkster et al. 1980, Martin 1986, Dick et al. 1999). This species has also spread throughout western Europe and now occurs from the Mosel and Rhine east to the German Danube, and north as far as Finland (Silfverberg 1999, Tittizer 2000, Bernerth and Stein 2003). *C. pseudogracilis* is amenable to laboratory culture and has a greater tolerance of static water conditions than *G. pulex* (Martin and Holdich 1986). The number of juveniles produced is also higher than for *G. pulex* (Dick 1996) and could be an important consideration when examining effects on juveniles or when following multiple life cycles. In terms of behavioural characteristics *C. pseudogracilis* also exhibits a wider range of behaviours including active brood care of the eggs (Dick et al. 1998) which *G. pulex* does not. Before commencing our studies with *C. pseudogracilis* we wanted to assess the MFB to be sure that *C. pseudogracilis* wasn't affected by the MFB itself, and that it responded in similar ways to *Gammarus* and was therefore comparable.

The aims of this study were; (1) to assess effects of the MFB current on *Crangonyx pseudogracilis* behaviour; (2) to determine whether a range of behaviours of *C. pseudogracilis*, and any change due to ammonium chloride, could be characterised by the MFB; (3) to use the MFB to examine for diel cycles in *C. pseudogracilis* activity, and (4) to examine behavioural changes of *C. pseudogracilis* in response to a pulse of ammonium chloride.

1 Materials and Methods

1.1 Test species

In this study, *Crangonyx pseudogracilis* were collected from September 2000 to May 2001 from Kiltonga Lake, Northern Ireland (Lat. 54° 34' 50 N, Long. 5° 43' 15 W) using a hand net (mesh 500µm). Females, larger and thus easier to observe, were placed into dishes (9 cm diameter) of standard hard water (pH 7.5 ASTM 1992, Rand 1995).

1.2 Multispecies Freshwater Biomonitor (MFB)

Test organisms were placed individually in a cylindrical flow-through clear acrylic plastic test chamber with nylon nets screwed at both ends. Chambers (2 cm diameter, 3 cm length) were attached to the MFB, and channel information, noise level (50 mV) and band frequencies (Band 1: 0.5–2.0 Hz for locomotory activity, Band 2: 2.0–8.0 Hz for ventilation while

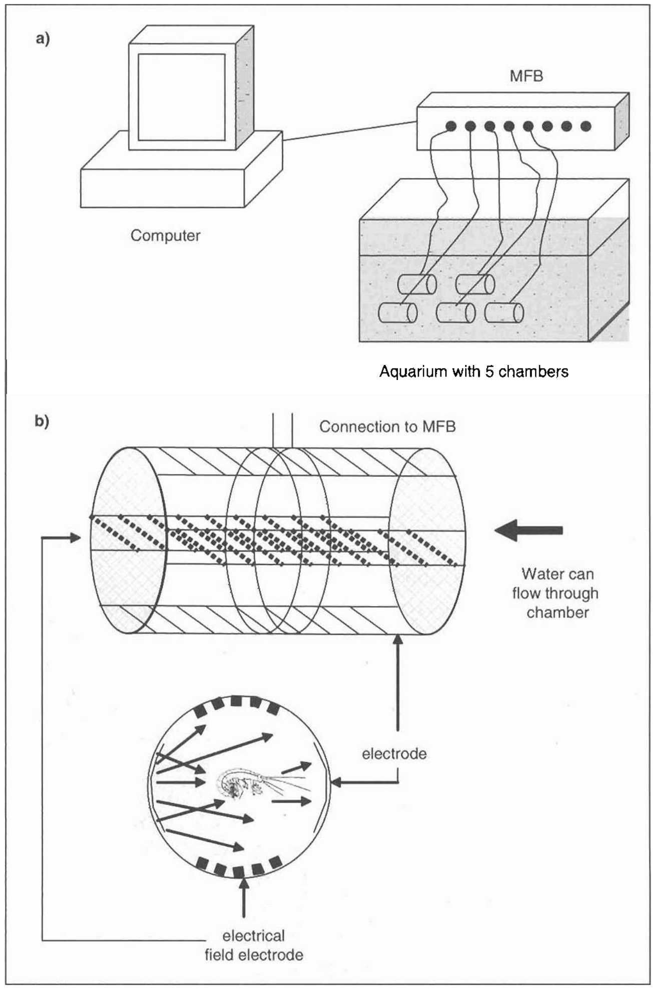

Fig. 1a,b: Schematic diagram of the Multispecies Freshwater Biomonitor (MFB): a) shows generalised layout of computer with MFB and chambers (in aquarium) attached; b) shows chamber with two electrode types, one pair of electrodes generate the current whilst the second pair detect the changes in the electrical field due to the resistance of the organism within that field (impedance)

inactive) programmed prior to recording. Recording occurred over 4 minute durations with 6 minute intervals between recording periods (**Fig. 1a,b**).

1.3 Toxicant

Ammonia was chosen as the toxicant in this behavioural assessment of *C. pseudogracilis* as it is present throughout aquatic ecosystems. It is environmentally hazardous both because of its toxicity and its ease of oxidation, enabling it to deplete dissolved oxygen rapidly (Russo 1985). Ammonia can exist in two chemical forms and both are highly soluble in water, with the non-toxic ammonium ions (NH_4^+) predominant in acidic (low pH) conditions and the highly toxic unionised ammonia (NH_3) in alkaline (high pH) conditions (Emerson et al. 1975). Added together, the two species are referred to as 'total ammonia'. In this study ammonium chloride (NH_4Cl) solutions were formulated using standard hard water. Average pH and temperature was used to calculate the proportion of un-ionised ammonia (mg NH_3-$N \cdot l^{-1}$) (Trussell 1972).

1.4 Experimental setup

1.4.1 Choice test using on/off chambers

For the MFB chamber on/off choice test two MFB chambers were attached to each other using rubber tubing. One end of each 'pair' was enclosed with mesh, the other with a microscope slide. Four such pairs were constructed and used simultaneously. These choice chambers were placed in a 1.5 l crystallising dish, which contained 1 l of solution (see below) at 19±2°C and pH 7.5. Both choice chambers of each pair were attached to the MFB, a female placed within and given 4 minutes to settle, then one chamber at random was switched on for 4 minutes. The location of each female in its choice chamber was noted at 1, 2, 3 and 4 minutes. There were two experimental groups: (1) standard hard water and (2) 0.1 mM ammonium chloride (=1.4 mg $N \cdot l^{-1}$ or 0.02 mg NH_3-$N \cdot l^{-1}$). Forty females were used in group 1 and, after 24 hours, the same females used in group 2.

1.4.2 Examining and comparing direct observation of individual behaviours with that recorded by the MFB during on and off periods

To observe individual behaviours in on/off chambers four MFB chambers were placed with their mesh end on the base of the aquarium and the top end replaced with a weighted microscope slide. This permitted visual observation of one female per chamber. After acclimatisation for 20 minutes, individuals were observed for a total of 24 minutes; 3 subsequent observations of 4 minutes when the MFB was on and similarly when the MFB was off. Observations were undertaken randomly over 2 hours for each female. Additionally behaviour was visually recorded using a Psion II hand-held computer (Psion PLC, London, England), programmed using the Observer (4.0) package (Noldus Information Technology, Wageningen, The Netherlands) for three subjectively pre-defined behavioural components, grooming, walking and swimming, obtained from qualitative observations (Kirkpatrick 2001). This direct observation of the individuals behaviour in real time with the MFB enabled the MFB trace to be characterised for each of the specific behaviours listed above. There were three experimental groups: (1) standard hard water; (2) 0.1 mM ammonium chloride (=1.4 mg $N \cdot l^{-1}$ or 0.02 mg NH_3-$N \cdot l^{-1}$) and (3) 1 mM ammonium chloride (=14 mg $N \cdot l^{-1}$ or 0.2 mg NH_3-$N \cdot l^{-1}$), all in a static water system. Twelve females were observed per group.

1.4.3 Testing for diel activity patterns

To test diel activity patterns of *Crangonyx pseudogracilis* eight MFB chambers were placed in 1 l of standard hard water in a static water system without renewal, temperature 15±1°C, photoperiod 14:10 hour light:dark and recordings taken over 48 hr. Sixteen females (one per chamber) were used in total.

1.4.4 Examining changes in activity due to a pulse of ammonium chloride

Females were placed in MFB chambers and acclimated for 12 h in standard hard water (pH 7.5; 21±1°C). Following this period a pulse of ammonium chloride was added which, when mixed in the aquarium, equated to 140 mg $N \cdot l^{-1}$ or 1.84 mg NH_3-$N \cdot l^{-1}$, close to the 96 hr LC_{50} derived for this species (1.1 mg NH_3-$N \cdot l^{-1}$; Kirkpatrick 2001). Sixteen females were monitored for 1hr pre- and 1hr post addition.

1.5 Statistical analyses

1.5.1 Choice test using on/off chambers

The distribution of females between on/off chambers in the choice test was assessed by χ^2. As there were four tests (i.e. 1, 2, 3, and 4 min) undertaken on each data set, we used the Bonferroni adjustment to avoid type 1 errors (erroneous rejection of a true null hypothesis), giving the level of significance as $P<0.0125$ (i.e. 0.05/4; see Sokal and Rohlf 1995).

1.5.2 Examining and comparing direct observation of individual behaviours with that recorded by the MFB during on and off periods

The examination of individual behaviours of the females in on/off chambers was analysed as follows: the total durations (s) for each behaviour were examined in a repeated measures MANOVA, the dependent variables being grooming, walking, and swimming, with factors MFB state (on/off; repeated measure) and ammonium chloride concentration. Further, with this data we examined MFB traces of behaviour that had simultaneous recordings made with the Observer, such that (1) the fidelity of the MFB could be assessed and (2) actual behaviour could be matched with the pre-defined MFB Bands: Band 1 (locomotion, including walking and swimming) and Band 2 (ventilation). For each MFB trace, we took the frequency and amplitude of the first example of each behaviour and calculated the range and mean. We then visually estimated, from the entire MFB trace for each female, the time spent in each behaviour and compared this with actual times as recorded by the Observer, answering (1) above. This was done with a repeated measures MANOVA, the dependent variables being grooming, walking, and swimming and the factors data source (MFB/Observer; repeated measure) and ammonium chloride concentration. Then, we compared the range and mean frequency and amplitude of each behaviour with the limits predefined for Bands 1 and 2, answering (2) above.

1.5.3 Testing for diel activity patterns

The data for the diel activity pattern test were analysed in a 2-Factor ANOVA where we examined percentage time (arcsine transformed; see Sokal and Rohlf 1995) with respect to activity band (Band 1/Band 2) and light regime (light/dark), both factors as repeated measures.

1.5.4 Examining changes in activity due to a pulse of ammonium chloride

We examined percentage time (arcsine transformed) with respect to toxicant status (pre-/post-addition) and activity type (Band 1/Band 2) in a 2-Factor ANOVA, both as repeated measures.

2 Results

2.1 Choice test using on/off chambers

There was no significant attraction to, or avoidance of, on/off test chambers at any time period or with either solution (**Fig. 2a,b; Table 1**).

2.2 Examining and comparing direct observation of individual behaviours with that recorded by the MFB during on and off periods

There was no significant difference in behaviour with the MFB on as compared to off ($PF_{3,31}$=0.97, NS; **Fig. 3a,b**). There was a marginally significant effect of ammonium chloride concentration on overall behaviour ($PF_{6,64}$=2.49, P<0.05), but no significant interaction effect ($PF_{6,64}$=2.02, NS). The latter effects appear due to a slight decrease in walking as concentration increased. There was a significant difference in behaviour recorded by the MFB as compared to the Observer ($PF_{3,22}$=3.37, P<0.05; **Fig. 4**), due to under-recording of nothing, i.e. inactivity, by the latter ($F_{1,24}$=6.23, P<0.05). The MFB overall appears to display high fidelity in discriminating actual behaviours (c.f. Figs. 3b and 4). There was no effect of ammonium chloride concentration on behaviour ($PF_{6,46}$=2, NS) and no significant interaction effect ($PF_{6,46}$=0.8, NS). However, this analysis views each concentration as an independent treatment and does not account for the gradation in ammonium chloride concentration. Thus, using log transformed data we regressed each of the four behaviours against ammonium chloride concentration. As concentration increased there was a significant change in each of the four behaviours (**Table 2**). Time spent walking and swimming decreased while that for grooming and doing nothing increased. Different types of behaviour showed distinct signal patterns, with mean frequencies for grooming, walking and swimming around 0.8 Hz (range 0.5–1 Hz) and with amplitudes of 0.2–1 V, 0.4–2.5 V and 1.3–2.5 V respectively (**Figs. 5a–d**). The frequencies correspond with the MFB pre-defined Band 1 or locomotory activity measure. When animals are ventilating, the frequency is higher (> 2 Hz) although the amplitude is very small (0.08–0.12 V). Ventilation dominates the signal, when the organism is inactive, however, it is also recorded when superimposed on locomotion signals. This frequency corresponds with the MFB pre-defined Band 2 or ventilation/inactivity.

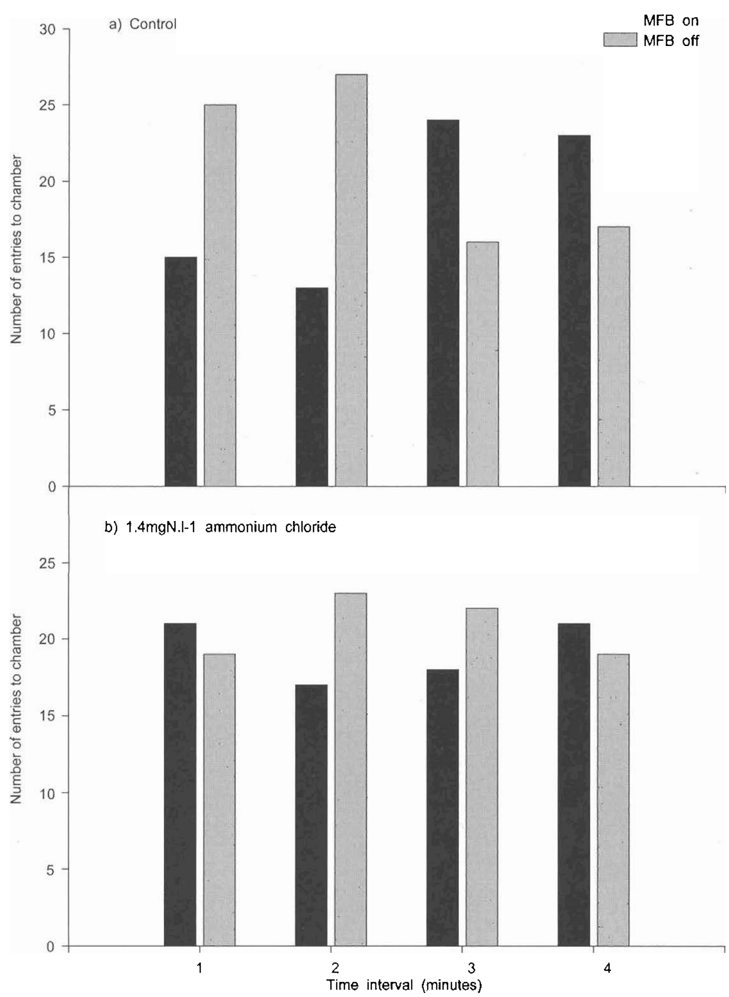

Fig. 2a,b: Number of times *Crangonyx pseudogracilis* was recorded as located, at one minute consecutive time intervals (n= 40) in a MFB chamber when the MFB was 'on' compared with when the MFB was 'off' in (a) 0 (control, standard hard water) and (b) 1.4 mgNl⁻¹ ammonium chloride

Table 1: χ^2values at one minute time intervals for *C. pseudogracilis* in choice chambers

Time (mins)	Control			1.4 mgN·l^{-1} ammonium chloride		
	χ^2	df	p-value	χ^2	df	p-value
1	2.5	1	NS	0.1	1	NS
2	4.9	1	NS	0.9	1	NS
3	1.6	1	NS	0.4	1	NS
4	0.9	1	NS	0.1	1	NS

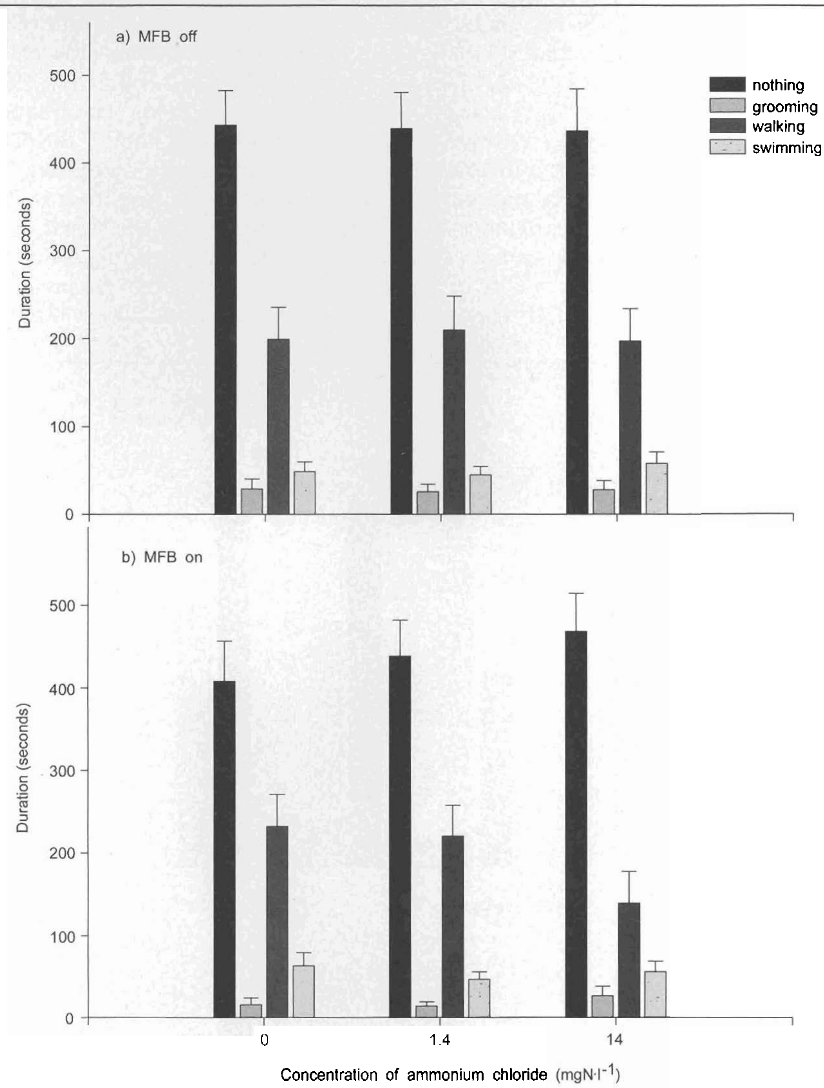

Fig. 3a,b: Duration spent per behaviour (as recorded by the Observer) in a standard hard water control and two concentrations of ammonium chloride when the MFB was switched (a) off and (b) on

Table 2: Results of regression analyses examining the relationship between increasing ammonium chloride concentration and duration spent in each of the four behavioural responses

Behaviour	R	t-value	P (df=25)
Nothing	0.313	37.18	<0.0001
Walking	–0.333	6.89	<0.0001
Swimming	–0.1	9.78	<0.0001
Grooming	0.456	4.69	<0.0001

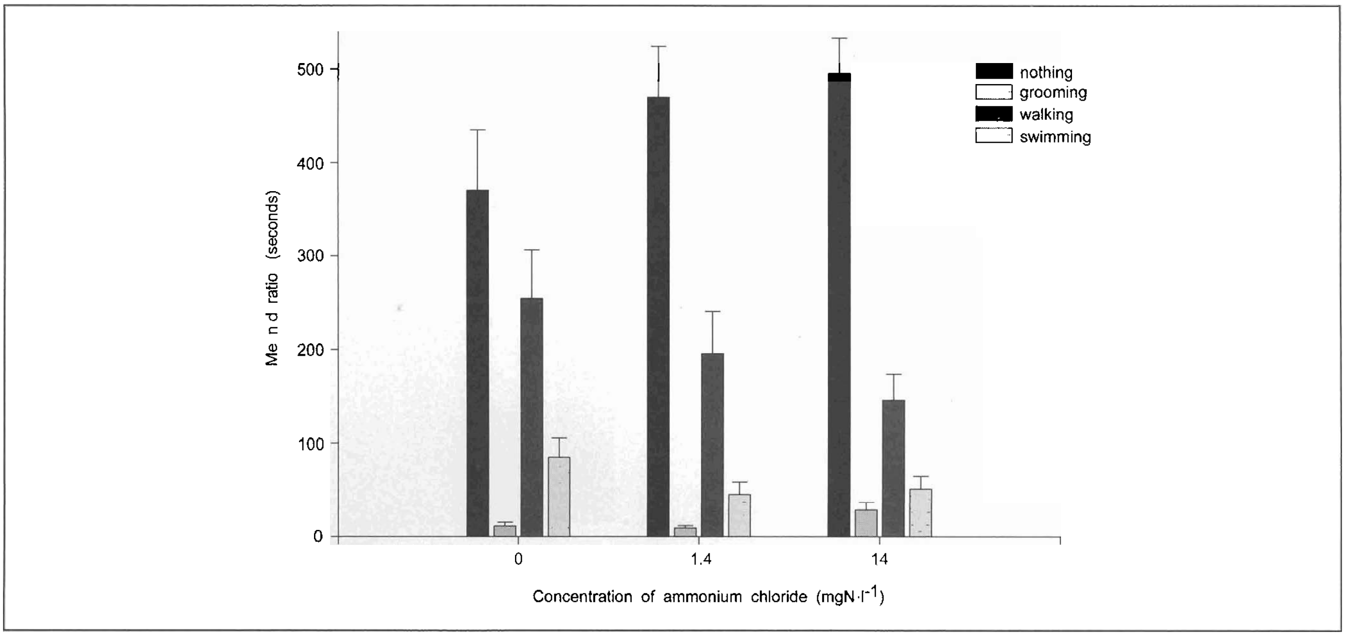

Fig. 4: Duration spent per behaviour (as recorded by the MFB) in a standard hard water control and two concentrations of ammonium chloride when the MFB was switched 'on'

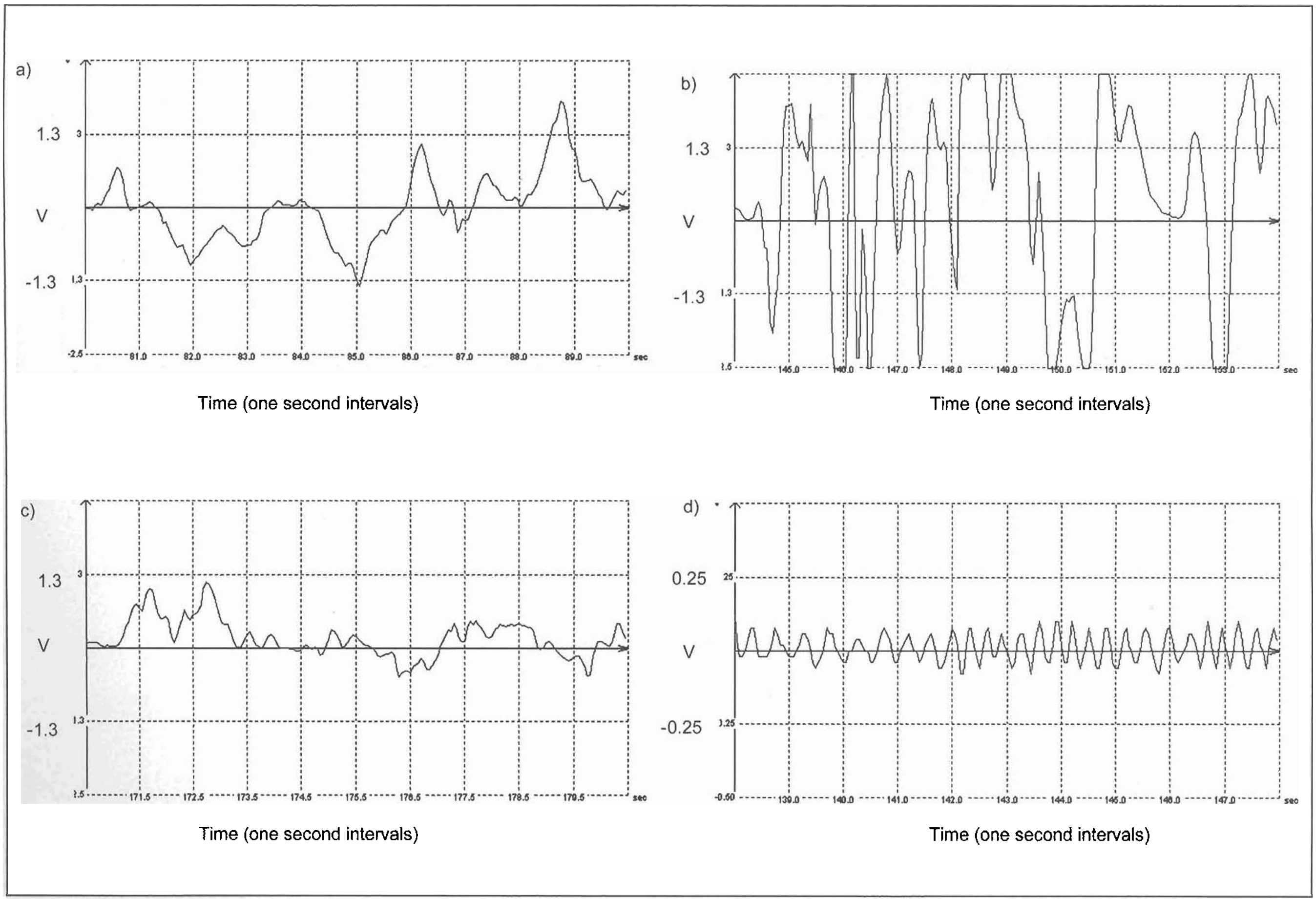

Fig. 5 a–d: MFB traces showing the characteristic waveforms for *Crangonyx pseudogracilis* behaviours observed (a) 'walking', (b) 'swimming', (c) 'grooming' and (d) 'ventilation while inactive'

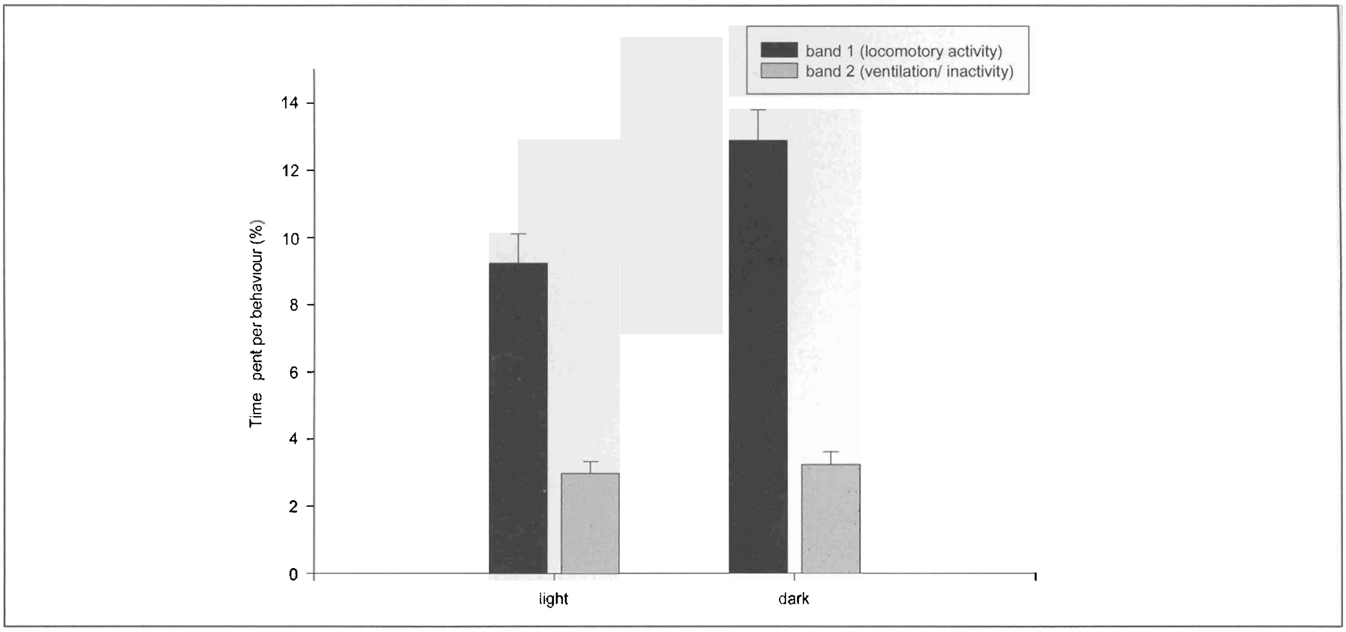

Fig. 6: Mean percentage proportion of time spent by *C. pseudogracilis* on band 1 (locomotory activity) and band 2 (ventilation/ inactivity) during light and dark periods in the MFB

2.3 Testing for diel activity patterns

The MFB recorded *C. pseudogracilis* as, overall, more locomotory active (i.e. Band 1) than ventilation while inactive (i.e. Band 2) ($F_{1,15}$=116.7, P<0.001) in both the light and dark (**Fig. 6**). Overlap of behavioural frequencies explains why time spent in Bands 1 and 2 are not strictly mutually exclusive. There was more active (i.e. Band 1) behaviour in the dark as compared to light as shown by the main effect of light regime and the activity band x light regime interaction ($F_{1,15}$=21.1 and 22.2 respectively, P<0.001; see Fig. 6).

2.4 Examining changes in activity due to a pulse of ammonium chloride

There was no significant effect of toxicant status (i.e. pre-exposure or post-exposure) on overall activity duration ($F_{1,15}$ = 21.05, p = 0.0654) but a significant activity type (Band 1 or Band 2) and toxicant status x activity type interaction effect (respectively, $F_{1,15}$=100.7 and 7.23; P<0.001 and <0.02), showed that animals spent a greater proportion of time in band 1 than band 2 behaviours, and greater activity during post-exposure than pre (**Fig. 7**).

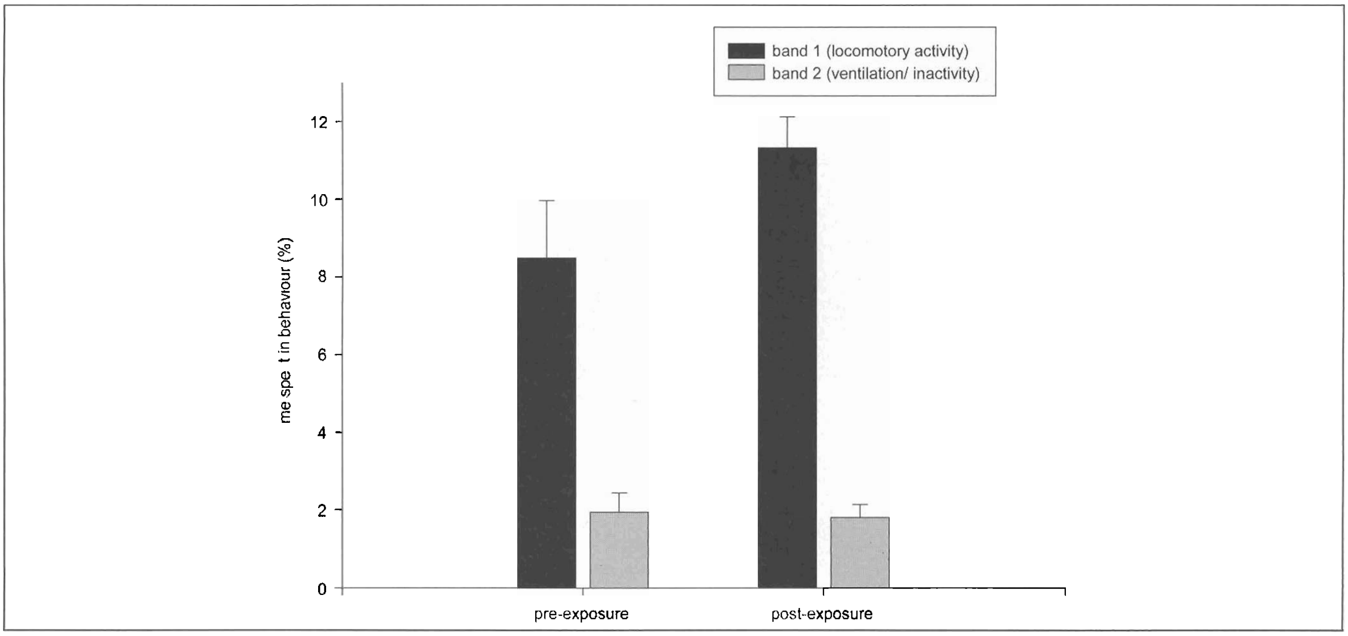

Fig. 7: Mean percentage proportion of time spent by *C. pseudogracilis* on band 1 (locomotory activity) and band 2 (ventilation/ inactivity) 1 h pre and 1 h post-exposure to a pulse of ammonium chloride in the MFB

3 Discussion

In this study, our aim was to determine the merits and suitability of *Crangonyx pseudogracilis*, as a new early warning system species rather than an explicit assessment of toxicant sensitivity. Our first experiment assessed if the monitoring system conditions imposed restrictions on normal behaviours since although the MFB was developed and tested for a range of species (see Gerhardt et al. 1994, Kirkpatrick et al. 2005) no study has assessed the influence of the electrical current on behaviour. Our first experiment showed no pattern in preference/avoidance of an on MFB chamber, both in control water and the low concentration ammonium chloride solution. This suggests that current and its alteration due to ammonium chloride, does not influence normal behaviour. From a wide range of behaviours (see Kirkpatrick 2001) we characterised and quantified four by direct observation which were also detected by the MFB. Walking and swimming behaviour produced similar traces as observed in the amphipods *G. pulex* (see Gerhardt 1996) and *Corophium volutator* (see Kirkpatrick et al. 2006).

The wider environmental tolerance of *Crangonyx* was suggested as an important advantage over *Gammarus* due to the latter species' low survival in laboratory conditions which compromises signal detection between treatment and control groups (Martin and Holdich 1986). However, this could also inhibit the use of *Crangonyx* due to its lower sensitivity to stressors (e.g. see MacNeil. et al. 2000). Nevertheless, our direct observations detected a significant change in behaviour with increasing toxicant concentration (0 to 1.4 mg/l to 14 mg/l). Although this was not significant among treatment levels in the MFB, regression analyses better account for the change in toxicant concentration, showing significant changes in all four behaviours as toxicant concentration increased. Locomotory activity decreased while animals spent more time grooming or doing nothing. The high concentration in our pulse experiment (140 mg N l^{-1}) also showed a significant affect on behaviour, but contrasted that of the gradient experiment by showing an increase in activity (Band 1) after exposure. This was probably due to changes in behaviour over time and the different time durations of each experiment. For example, the gradient experiment was carried out over a two hour period while the pulse experiment recorded behaviour over 1 hour. Indeed, in the pulse experiment, the initial increases in activity immediately after addition of the toxicant (not shown in Fig. 7) were followed by suppressed activity as time progressed. Such responses corroborate the 'Stepwise Stress Model' (see Gerhardt 1999) and that observed after toxicant exposure in other species. For example, Gerhardt and Quindt (2000) and Gerhardt et al. (1998) reported changes in locomotory and ventilatory activity in *G. pulex* after short-term exposure to lead and copper. Initial avoidance behaviour was followed by increased ventilatory and inactivity time as the sub-lethal effects became severe, eventually leading to death (Gerhardt 1999).

These experiments suggest that *C. pseudogracilis* exhibits behaviours with potential application in on-line biomonitoring. However, important considerations are the assessment of the species' diel activity rhythms to prevent false alarms, and the extent of inter-population or life-history variation in behavioural responses. For example, circadian or diel rhythms occur over a 24 h cycle, attributable to alternating light and darkness and predator avoidance (Green et al. 1990, Huhta et al. 2000). Consistent with this, the MFB recorded greater activity by *C. pseudogracilis* in darkness, a trait previously detected by the MFB in other invertebrates (Gerhardt 1996, Gerhardt and Quindt 2000, Gerhardt et al. 2004). As in these studies, the use of 'intelligent software programming' (Gerhardt 1996) would be a pre-requisite in online field biomonitoring for *C. pseudogracilis*. Differences in behaviour and among life-history stages or between populations could also influence the sensitivity response of the MFB and require further testing. For example, locally adapted populations may be less likely to trigger an alarm given small increases in toxicants as compared with non-acclimatised laboratory populations. Nonetheless, in ammonium chloride 96 hr LC50 tests Kirkpatrick (2001) found minimal difference between two regionally disjunct populations of *C. pseudogracilis*. In a separate test examining the effect of life-history stage on toxicity responses, ovigerous females were more tolerant than juveniles while both stages were more tolerant than non-ovigerous females (Kirkpatrick 2001). As a result, non-ovigerous females were used in the current MFB tests.

C. pseudogracilis may thus offer several advantages as a test species to the more commonly used *Gammarus* test group. *Gammarus* are largely restricted to unpolluted lotic habitats whereas the wider habitat distribution of *C. pseudogracilis* in ponds, lakes and rivers should provide easier collection (Martin and Holdich 1986, Gledhill et al. 1993, Dick et al. 1999). The higher reproductive potential of *Crangonyx* and its ease of culture in laboratory or outdoor mesocosms should also enhance its suitability as a test organism (Martin and Holdich 1986, Dick 1996) while a wider range of behaviours may provide greater sensitivity in sub-lethal assessments of environmental stress (Dick et al. 1998). The MFB has an advantage over other systems as it can record a range of behaviours in different environmental conditions, including sediment or when water conditions are turbid or coloured (Gerhardt et al. 1994). Given its wide range of behaviours (Kirkpatrick 2001), our preliminary work suggests that *C. pseudogracilis* has promise as a new biomonitoring species in the MFB. Future studies are needed to quantify threshold concentrations to a range of toxicants and the associated variation in the behavioural responses elicited.

4 Conclusions

We were able to identify distinct behaviours in *C. pseudogracilis* that were also detected by the MFB. Significant alterations in these behaviours were detected over a concentration gradient and due to a toxicant pulse suggesting that *C. pseudogracilis* could prove be an important species in future on-line biomonitoring. Future studies are required to assess this.

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