Hydrobiologia **341:** 159–167, 1996.

Evaluating decline parameters of rotifer *Brachionus plicatilis* populations as an interstitial water toxicity bioassay

T. A. DelValls[1], L. M. Lubián[2]*, M. González del Valle[2] & J. M. Forja[1]
[1]*Dpto. de Química Física, Facultad de Ciencias del Mar. Universidad de Cádiz, Apartado 40, 11510 Puerto Real, Cádiz, Spain*
[2]*Instituto de Ciencias Marinas de Anadalucía, C.S.I.C., Apartado Oficial, 11510, Puerto Real, Cádiz, Spain*
*(*Author for correspondence; Fax: 34-56-834701, e-mail: luis.lubian@uca.es)*

Received 2 May 1996; in revised form 24 September 1996; accepted 8 October 1996

Key words: ecotoxicology, interstitial water toxicity, rotifers, population decline parameters, particulate organic matter

Abstract

Population decline of the rotifer *Brachionus plicatilis* has been evaluated as a sensitive and reliable bioassay for assessing toxicity of marine sediment interstitial water. Three environmental conditions were examined using both interstitial and seawater cultures: (a) adverse effects from dissolved contaminant concentrations; (b) effects from particulate organic matter; and (c) increase or decrease of bacterial biomass from addition of mixtures of antibiotics. Three different parameters were measured to characterize decline: (i) time required for 50% of inoculated population to die (TL_{50}); (ii) curve of the decline rate of *Brachionus plicatilis* (μ_{BP}); and (iii) graphical area contained below plot of the egg:female ratio curve against time (A_{BP}). Results indicated that, for each of the different assays, the three parameters of the *Brachionus plicatilis* population decline test were sensitive to concentrations of contaminants dissolved in interstitial waters (principally: total ammonia, Cu, Cr and alkylbenzenesulphonates) but not to particulate organic matter. Nevertheless, the presence or absence of mixed antibiotics with the contaminants may influence the *Brachionus plicatilis* population decline test, principally by retarding the hatching of eggs. Based on these results, *Brachionus plicatilis* is confirmed as an appropriate organism for use as an indicator of interstitial water contamination, using either decline rate, TL_{50} or both parameters. The presence of particulate matter has no effect on these parameters, but the bacterial population may be an influence, although to a lesser extent than the toxicants.

Introduction

Determining the impact of contaminated sediments is a key element in environmental risk assessment and management of water resources. For this reason, the role of aquatic sediments as a repository for and source of significant amounts of contamination has led to the development of a wide variety of toxicity tests for the assessment of sediments. However, methods for measuring the potential toxicity introduced into the water column by contaminated sediment have not been extensively evaluated. Toxicity testing of interstitial waters is therefore important because dissolved forms of pollutants are more bioavailable to aquatic biota and are the primary cause of adverse impacts in an aquatic environment (Luoma & Ho, 1992; Chapman, 1988), especially in coastal ecosystems. In fact, most natural and anthropogenic chemical species which can affect physicochemical conditions throughout whole ecosystems are transported from the sediment to the water column by means of dissolution in the interstitial water. Solute presentation also has the advantage of being generally simpler to test than whole sediment, because solutions are less complex systems and easier to control. In addition, the potential distortion of results due to grain size or other physical properties of the sediments can be eliminated by removing contam-

inants from the sediments before testing (Carr et al., 1989; Giesy & Hoke, 1990; Long et al., 1990).

Examples of benthic macrofauna employed in interstitial water testing have been reviewed by Luoma & Ho (1992) and include: polychaete larvae (e.g. *Arenicola marina* – Walsh et al., 1986), sand dollar embryos (Meador et al., 1990), bivalve larvae (e.g. *Crassostrea virginica* – Cherr et al., 1990) and sea urchin larvae (*Strongylocentrotus purpuratus* - Long et al., 1990).

Several authors have pointed out that, among the zooplankton group, the Rotatoria are suitable organisms for use in tests to evaluate the condition of freshwater, estuarine and marine environments (Besch, 1982; Sladecék 1983; Fernández-Casalderrey et al., 1991a, b; Snell & Persoone, 1989; Weiss et al., 1992). However, rotifers have not been used before in toxicity bioassays of interstitial water from marine sediment samples. The rotifer *Brachionus plicatilis* is an ubiquitous species forming an important link in the marine and estuarine food chain and, in fact, is frequently used as live food for marine fish larvae. High densities of rotifers have been reported in enclosed areas, like estuaries and saltponds, with a relative frequency (Yufera et al., 1984) and sudden decreases in the population density could be originated by sediment disturbance. Rotifers reproduce by parthenogenesis and can be maintained as clones in laboratory cultures; they have a short generation cycle (e.g., 2 days at 25 °C), and experimental conditions are easily reproduced. Also, their small body size (0.18–0.25 mm) allows cultivation in small volumes with high densities of individuals.

The present study was carried out to determine three different environmental conditions using the decline of a *Brachionus plicatilis* population to assess interstitial water toxicity. The three samples assayed were: (1) an interstitial water of marine sediment, highly contaminated by urban effluent, for the main toxicity analysis; (2) the same interstitial water, both filtered (0.45 μ) and unfiltered, to evaluate the effect of the particulate organic matter present; and (3) the same interstitial water diluted with specific volumes of an antibiotic solution, to evaluate the potential effects of an increase or decrease in bacterial biomass on *Brachionus plicatilis* populations.

Material and methods

Sediment sample collection and interstitial water extraction

The sediment used in the test was collected using a Van Veen grab from the discharge point of highly contaminated urban effluent produced from San Fernando, an industrial town in SW Spain with approximately 100 000 inhabitants (DelValls, 1995). The samples were homogenized until textural and colour homogeneity was achieved, and then placed on ice and transported to the laboratory. Interstitial water was extracted from the sediment by centrifugation at $2600 \times \boldsymbol{g}$ for two hours at 2 °C (Ankley & Schubauer-Berigan, 1994); the resulting supernatant was placed in borosilicate erlenmeyer flasks of 500 ml and kept in darkness at 4 °C until used for assays, no more than 48 hours later. The aliquot for the chemical analyses was frozen until used. Oceanic water was collected (36°15′N 6 °20′W) using Niskin bottles placed on ice and transported to the laboratory, an then filtered (0.45 μm).

Chemical analysis of interstitial and sea waters

The pH (SWS) (Hansson, 1973) was measured with an ion analyzer (Radiometer, ION85) using combined glass electrodes (GK 2401 G). Nutrient analyses (ammonia, phosphate, nitrate and nitrite, silicate and dissolved organic carbon) were carried out in a TRACCS 800 Technicon autoanalyzer. The concentration of metals in the interstitial and sea water samples were determined using differential pulse anodic stripping voltametry (DPASV); measurements were taken with a static drop mercury electrode (SMDE), using the Metrohm 693 processor. The concentration of surfactants, linear alkylbenzenesulphonates (LAS), was measured following a procedure for the isolation and preconcentration of the LAS based on solid phase extraction using an octadecyl reversed phase silica column (C_{18}) and a strong anion exchange column (SAX). The resulting LAS was then analyzed in a Waters high-performance liquid chromatography equipped with a fluorescence detector (González-Mazo, 1995).

Organism selection and assay conditions

Toxicity tests were conducted with the estuarine rotifer *Brachionus plicatilis*, S-1 clone (Pozuelo & Lubián, 1993), isolated from saltmarshes near Cádiz and maintained in our laboratory under controlled conditions.

The S-1 clone is characterized by a low level of mictic female and male production. The rotifer inocula came from cultures in exponential growth phase, fed on the microalga *Nannochloropsis gaditana* (Eustigmatophyceae), and exposed to a constant temperature of 25 °C and continuous light (2000 lux), as the experimental conditions. To initiate tests, rotifers were sieved through a mesh of 63 μm pore size and poured back into different beakers with each assay medium in a total volume of 50 ml, as follows:

(A) Two control assays: the first one consisting of filtered seawater (C) and the second one of filtered seawater containing a mixed antibiotic solution (neomycin, amoxicillin and chloramphenicol in ratio 1:1:1 v/v), with a final mixture concentration of 21 mg l^{-1} (C^*).
(B) Two raw interstitial water assays: the first one consisting of interstitial water recently obtained from sediment (IW) and the second one of the same water containing the mixed antibiotic solution as specified above (IW*).
(C) Two filtered interstitial water assays: the first one consisting of interstitial water previously filtered (0.45 μm) (FIW), and the second one of the same water containing the mixed antibiotic solution as specified above (FIW*).

All assays were performed in duplicate. Initial environmental conditions for the toxicity bioassays were: salinities 28 ± 1 and 33 ± 1; pH: 7.9 ± 0.1 and 8.0 ± 0.1, and dissolved oxygen 5.3 ± 0.7 and 6.2 ± 1.0 mgO_2 ml^{-1}, for the interstitial water and for seawater respectively. In all the assays, the rotifers were unfed and the medium was not renewed during the six days of the exposure time to the different mediums described above. The initial rotifer concentration was about 270 individuals per ml for each duplicate of test mediums (50 ml), which allows a significant and feasible response in the decline population assay. The length of rotifers ranged from 180–200 μm, and the eggs:female ratio was 0.38.

The time between preparation of the medium solutions, culture treatment, and introduction of the test rotifers was kept to less than one hour so that changes in the toxicity of the interstitial water after sample collection were minimized.

Data calculation and statistical analysis

Subsamples (0.5 ml) of each rotifer culture (50 ml) were taken daily (at least twice per day) for 6 days and counted by stereoscopic microscopy (50 ×). This is the minimum sufficient time required to evaluate the intrinsic responses (e.g. mortality, reproduction) of the whole population to each of three treatments. A first count was done of dead rotifers (individuals showing no internal or external movement for 10 seconds); a second count was done of the total number of rotifers and amictic eggs, which had previously been fixed with 3% buffered formalin. Then diluted samples of the culture (10 μl each) were taken and placed on Marine Agar 2216 (Difco) and incubated for 48 hours at 35 °C, followed by a count of total heterotrophic bacteria. The number of bacteria was determined by the total viable cells (CFU) enumeration method (Buck, 1979; Ray, 1979; APHA, 1985). All counts were performed in duplicate.

From plots of the decline curves, three parameters were calculated: (a) time required for 50% of the population to die (TL_{50}); (b) the decline rate of *B. plicatilis* population (μ_{BP}); and (c) the area under the egg:female ratio curve against time (A_{BP}), as an index of viability of the rotifer population.

The parameter TL_{50} was calculated by linear regression of log toxicant time on decline probit values, using a probit analysis modified from the classic methodology to obtain the common toxicological parameter: EC_{50} or LC_{50} (Bliss, 1934; Rand et al., 1995). The modification uses the percent mortality from the initial population versus time. Only those regressions yielding significant χ^2 values were used to calculate the TL_{50}. The decline rate parameter (μ_{BP}) was calculated as the slope of the straight line resulting from exponential regression of the rotifer density against time (hours). The area under each set of egg:female ratio curves (A_{BP}), was calculated by Simpson's rule (Shenk, 1984).

It should be noted that the rotifer decline toxicity test is considered valid only if the mortality rate in the negative control (C) differs by less than 10% from the reference data for this species when cultivated in unfed conditions (Pozuelo, 1975).

The resulting parameters (TL_{50}, μ_{BP}, A_{BP}) from the duplicates of the six assays (including controls) were compared using MANOVA and Scheffe's F tests to identify significant differences in sensitivity between assays ($P<0.01$).

Table 1. Characterization of seawater (C) and interstitial water (IW) used to develop the six assay treatments evaluated in this study. Concentrations of dissolved carbon (DOC), and of the five nutrients (phosphate, silicate, total ammonia, nitrite and nitrate) are expressed in mg l^{-1}; the linear alkylbenzensulphonates (LAS) and the seven heavy metals (Fe, Mn, Zn, Cu, Pb, Cd and Cr) are expressed in $\mu g\ l^{-1}$.

Parameters	IW	C	Parameters	IW	C
DOC	111.15	3.25	Fe	459.9	12.1
phosphate	255.44	-*	Mn	1244.3	11.7
silicate	138.47	30.28	Zn	99.7	78.7
total ammonia	2801.49	15.21	Cu	450.7	103.1
nitrite	1.28	0.71	Pb	135.2	35.1
nitrate	12.5	3.61	Cd	1.39	0.9
LAS	2895.6	33.2	Cr	4.8	1.7

* not detected

Table 2. Results of the analysis of the three parameters (each n=2) used to characterize the decline process in *Brachionus plicatilis*, as described in text: μ_{BP} (units: k h^{-1}) $\times$ 10^{-3}; TL_{50} (units: hours), ABP [units: ratio of number of rotifer eggs and number of rotifers ml^{-1} h^{-1} $\times$ 10^3). The decline rates are shown with the correlation coefficient (r) from the adjusted start line.

Treatment	μ_{BP} (k h^{-1})	TL_{50} (h)	A_{BP} [egg female^{-1} ml^{-1} h^{-1}]
C	5.9 ± 0.3	123.4 ± 12.4	0.100 ± 0.001
	$r=-0.8 \pm 0.1$		
C*	8.21 ± 0.9	114.9 ± 3.7	0.131 ± 0.025
	$r=-0.8 \pm 0.1$		
IW	14.3 ± 1.6	34.6 ± 1.5	0.164 ± 0.001
	$r=-0.9 \pm 0.0$		
IW*	32.9 ± 3.3	20.3 ± 0.7	0.174 ± 0.020
	$r=-0.9 \pm 0.0$		
FIW	20.3 ± 0.4	35.5 ± 1.7	0.231 ± 0.002
	$r=-0.9 \pm 0.0$		
FIW*	24.2 ± 0.4	24.3 ± 3.6	0.184 ± 0.001
	$r=-0.9 \pm 0.0$		

Results

The principal characteristics of the sediment from which the interstitial water was extracted were: particle size (0.9% >20 μm; 22.7% 2–20 μm and 76.4% $\leq$2 μm); specific surface (7.35 m^2 m^{-3}); organic carbon (12.28%); total carbon (12.51 mg kg^{-1} dry sediment); total nitrogen (0.76 mg kg^{-1} dry sediment). Detailed analyses procedures are reported by DelValls (1995). Selected results from the analysis of the control seawater (C) and contaminated interstitial water (IW) are presented in Table 1. Measured levels of dissolved organic carbon (DOC), nutrients, LAS, and the heavy metals Fe, Mn, Pb, Cu, and Cr were higher in interstitial than in sea water. However, the other two metals analyzed (Zn and Cd) showed fairly similar concentrations in both interstitial and seawater.

The rotifer number and egg density curves for each toxicity test and control assay are shown in Figure 1. Neither males nor resting eggs were observed in any of the counts. Despite absence of additional nutrients, the rotifer densities increased during the first two days of all tests, as a result of the high initial egg:female ratio. After this time, there was an exponential decrease of population density that was significantly more pronounced in rotifers exposed to interstitial water. The three decline parameters calculated are presented in Table 2 and they all confirm a significant contamination effect on decline curves from interstitial water compared with control seawater. For a full discrimination between the effects of bacterial biomass and particulate organic matter, a three-way MANOVA test was performed on the results of the three parameters obtained from the curves (Table 3). For all three parameters, the differences observed between contaminated interstitial water and control seawater assays were significant ($P<0.05$), whereas presence of particulate organic matter showed no significant effect ($P<0.05$). Finally, antibiotic treatments produced different responses in the different parameters measured in the analysis. The effect of antibiotics on the bacterial population is represented in Figure 2. In the absence of antibiotics, the number of bacteria increased with time, whereas in the presence of antibiotics, the number of bacteria was maintained below six orders of magnitude during the first eighty hours.

The parameters μ_{BP} and TL_{50} showed significant differences between all the assays ($P=0.0086$) but the differences were more evident in the assays of interstitial waters ($P=0.0024$); the parameter A_{BP} was significantly homogeneous between all the assays (Table 3).

Discussion

The degree of contamination of a sediment sample is usually based on the chemical analysis of the bulk sample. Different contaminants, however, will be more or less bioavailable depending on the various chemical and physical characteristics of the sediment (e.g.

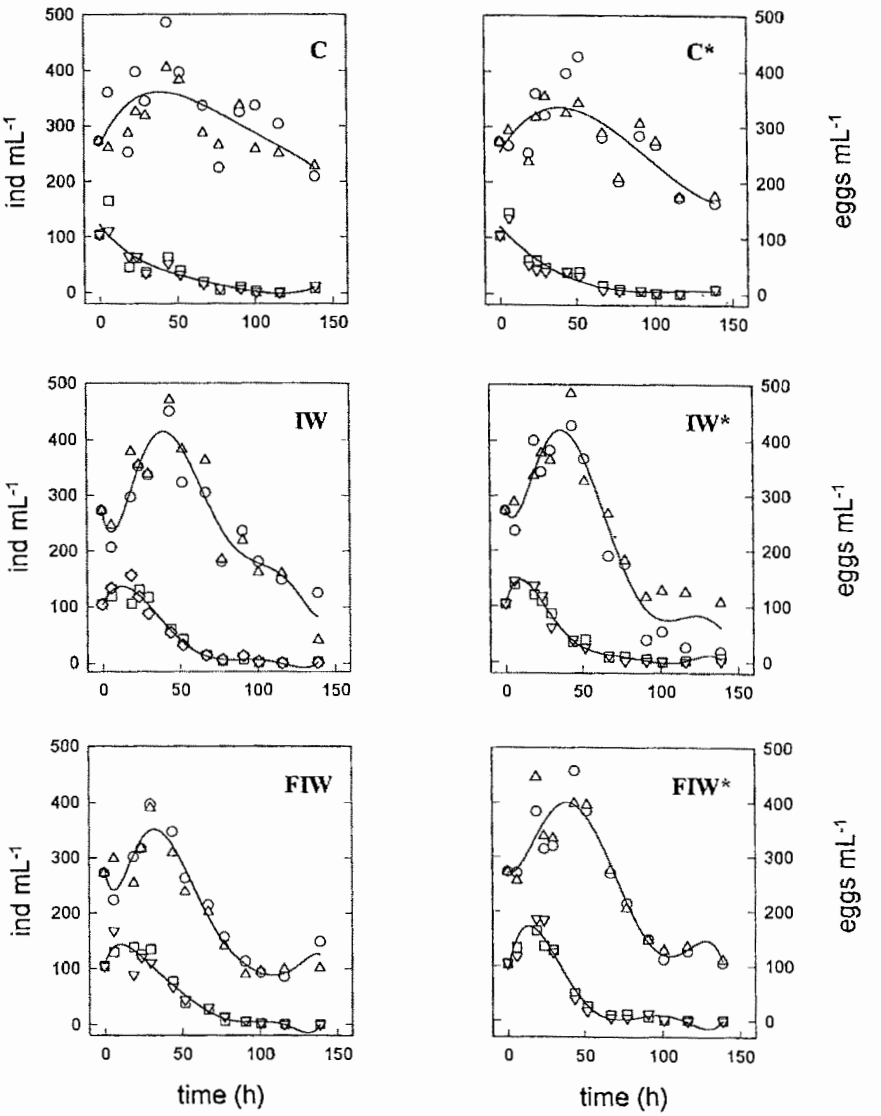

Figure 1. *Brachionus plicatilis* abundance (number of females per millilitre) and egg abundance (number of eggs per millilitre) during the time of the bioassay (hours) in the three different assays: (a) two seawater control assays (*C* and *C**); (b) two raw interstitial water assays (IW and IW*); and (c) two filtered interstitial water assays (FIW and FIW*). The treatments represented by (*) were associated with the use of antibiotic mixture to control the bacterial biomass. The two different symbols (triangle and circle to describe rotifer density duplicates and squares and inverse triangle to represent egg density duplicates) represent the two measurements for each duplicated assay and the line shows the fitted trend.

total organic carbon content, pH of interstitial water, grain size or specific surface). More specifically, recent studies have demonstrated that for certain nonpolar organic contaminants (Suedel et al., 1993) and for inorganic contaminants (Bryan & Langston, 1992), the key exposure route is from the interstitial (pore) water. Likewise, the toxicity of any contaminant can be expected to affect benthic invertebrates only if the chemical concentration in the sediments is high enough to result in the equilibrium interstitial water concentration produced by desorption being equal to or higher than the concentration demonstrated to cause an effect in a water exposure toxicity test (Campbell et al., 1988; Carr et al., 1989; Landrum et al., 1991). However, the resulting chemical data provide little or no evidence of biological consequences of contamination measured

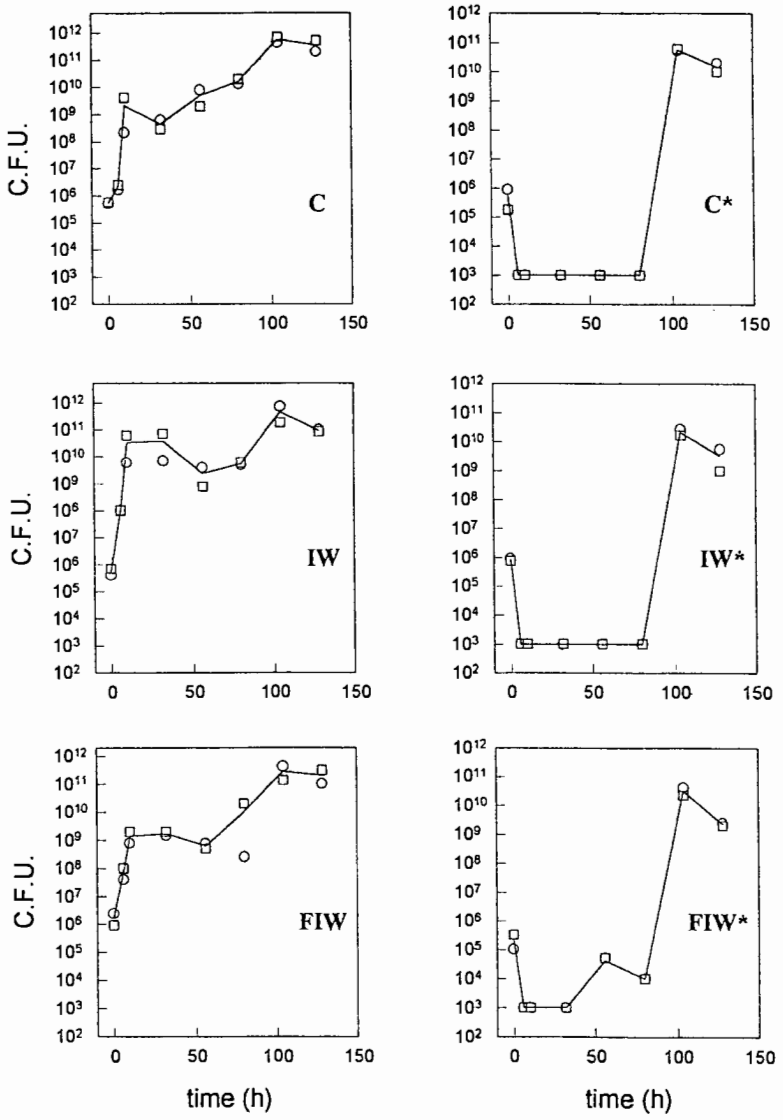

Figure 2. Total heterotrophic bacteria (by the CFU total viable cells enumeration method) during the time of the decline bioassay, using *Brachionus plicatilis* in the three different assays: (a) two seawater control assays (*C* and *C**); (b) two raw interstitial water assays (IW and IW*); and (c) two filtered interstitial water assays (FIW and FIW*). The treatments represented by (*) were associated with the use of antibiotic mixture to control the bacterial biomass. The two different symbols (circles and squares) represent the two measurements for each duplicated assay and the line shows the fitted trend.

in interstitial water which can only be demonstrated by toxicity tests. By conducting toxicity tests with pore water samples directly, all the key factors controlling bioavailability, and hence toxicity, can be evaluated without potential interference from non-related effects of other sediment characteristics (e.g. grain size). The principal disadvantage of the pore water sample approach to sediment toxicity testing is that the samples may be modified from their natural state in order to make them testable: salinity may be altered, dissolved oxygen and pH may be increased. However, since the rotifer *Brachionus plicatilis* was easily cultured, none of these variables had to be changed from their original states. As a result, the observed toxicity of the contaminants did not decrease (e.g. heavy metals).

Since the rotifers were unfed during the bioassay, any possible interaction between the effects of con-

Table 3. Results of the three-way (MANOVA) analysis on three different interstitial water assays: toxicity, bacterial biomass (antibiotic effects), and particulate organic matter (filtration effects), from the three parameters used to characterize the decline process in *Brachionus plicatilis*, as described in text: μ_{BP}; TL_{50} and A_{BP}. The signs (+) and (-) show whether significant differences ($P<0.05$) between assays were or were not found.

Parameter	Assay treatments		
	Toxicity	Antibiotics	Filtration
μ_{BP} (h^{-1})	+	+	–
TL_{50} (h)	+	+	–
A_{BP} [e f^{-1} ml^{-1} h^{-1}]	+	–	–

tamination on the rotifers and on the microalgae that serve as their food was eliminated. Only in treatments without antibiotic mixture, could a source of food, 'bacteria' be available, but the results (Figures 1 and 2) show no or minimum effect of this kind of food and in this environmental densities. In our experiments, the high eggs:female ratio resulted in an increase of the rotifer population in the first 48 hours, after which the rotifer densities decreased significantly faster in all the interstitial water assays than in seawater. This behaviour has been described by other workers testing the adverse effect of antrazine and bifenthrin in several freshwater rotifer species (Hoagland et al., 1993).

From chemical analysis of the interstitial water, only ammonia, LAS, and the metals Cu and Cr could be considered to reach toxic concentrations (Mance, 1987; Giesy & Hoke, 1990; Hoke et al., 1992). Deionized ammonia is considered to be one of the pollutants responsible for limiting the population growth of aquatic animals including rotifers (Hirayama, 1987). For *Brachionus rubens*, Schlüter & Groeneweg (1985) considered that, in the range of 3–5 mg NH_3-N l^{-1}, the reproduction rate is reduced, although no animals are killed, but at concentrations over 5 mg NH_3-N l^{-1} the rotifers die within 2 days. For *B. plicatilis*, Snell et al. (1991) establish 24 h LC_{50} value for free ammonia of 38 mg l^{-1}. Bearing in mind that, in our experimental conditions, the amount of free ammonia is significantly higher ($\sim$ 129 mg l^{-1}), it is reasonable to conclude that free ammonia in the interstitial water analysed is one of the principal contaminants responsible for the significant response. Free ammonia concentration has been calculated using the pH data for interstitial water using the values for the equation of the ionic equilibrium constant for the ammonia (K_i) and the ionic equilibrium constant of water, (K_w) reported by Trussell (1972). This calculation offers a approximate value based in the approximation of the values reported by Trussel (1972) for the expressions of the ionic constants, in which are not included effects like ionic strength (see Whitfield, 1974, 1993; Johanson & Wedborg, 1980). The copper concentration in our assays is higher than those reported for LC_{50} values in *B. plicatilis* by Snell & Persoone (1989) and Snell et al. (1991). For the contaminants LAS and Cr, there is no available toxicity data for *Brachionus plicatilis*, but dissolved concentrations in our samples are higher than those associated with adverse effects for other organisms in tests developed using solute exposure media (Mance, 1987; Saquid, 1992). The concentrations of the other metals identified are below those reported for NOEC ('no observed effect' concentration) in *B. plicatilis* (Snell & Persoone, 1989; Snell et al., 1991).

The presence of particulate matter in interstitial water did not influence the three parameters used to characterize the decline process, whereas the antibiotic treatment did have a significant effect on both decline rate and TL_{50} parameters. Although the possibility that the antibiotic mixture directly affected the rotifers cannot be ruled out, Arndt (1993) makes it clear that *B. plicatilis* has a low-to-medium ability to feed on bacteria in natural ecosystems but that, at high population densities, such as in live feed cultures, rotifers may be efficient at removing bacteria from the culture. This process could have an influence which contributes to the lower of decline curves observed in rotifer populations without antibiotics, whereas the area below the egg:female ratio curve could not have been affected. However, this latter parameter is significantly lower in control seawater than in interstitial water assays.

Nevertheless, it is interesting to note that the number of eggs increases slightly in the early period in all cases and later decreases as the rotifers hatch. This decrease is more rapid in seawater than contaminated water because egg numbers are kept higher for a longer period in the contaminated water (see Figure 1), whereas the assays with antibiotics have no influence on this process. These results suggest the possibility that contamination in the water can affect the rotifers by retarding the time taken for eggs to hatch, although further, more specific experiments are necessary to confirm and quantify this effect.

Other standarized toxicity tests (Snell & Moffat, 1992) are also based on rotifer population life cycle but these use freshwater species. Since the focus of this study is marine interstitial water, the more appropriate seawater species, *Brachionus plicatilis* was used in these tests. The criteria for the tests, e.g. a popu-

lation parameter, were not changed. Previous studies using *B. plicatilis* analyzed individual responses (Snell & Janssen, 1995) and specific responses of the population (ingestion rates of populations in exposures times of 1 h, (Juchelka & Snell, 1995)), whereas in the present paper we evaluated the responses of the whole population in a exposure time of 6 days, until the death of almost all the individuals. To the author's knowledge *Brachionus plicatilis*, has not been used previously to evaluate in situ interstitial water toxicity. A range of the relevant environmental variables must still be investigated, but the use of *Brachionus plicatilis* has already showed promising sensitivity relative to conventional tests, e.g. Microtox® test (DelValls, 1995).

In conclusion, *B. plicatilis* is a suitably sensitive organism for use as a measure of the contamination of interstitial water, by means of either the decline rate or TL_{50} parameter, or both. Presence of particulate matter has no effect on these parameters, but the bacterial population can have some influence, although less than the toxicants.

Acknowledgments

The authors would like to thank Dr Terry Snell for his comments on the manuscript and two anonymous referees for their critically reading. The described work was supported as a part of project AMB94-0291, financed by a grant from the Spanish Science and Technology Interministerial Commission (CICYT).

References

APHA, 1985. Standard methods for the examination of water and wastewater. 16th edn. American Public Health Association, Inc., Washingthon, DC, 126 pp.

Ankley, G. T. & M. K. Schubauer-Berigan, 1994. Comparison of techniques for the isolation of sediment pore water for toxicity testing. Arch. envir. Contam. Toxicol. 27: 507–512

Arndt, H., 1993. Rotifers as predators on components of the microbial web (bacteria, heterotrophic flagellates, ciliates) – a review. Hydrobiologia 255/256: 231–246.

Besch, W. K., 1982. Kritische Bewertung der Rolle von toxikologischen Test bei der Gewässerüberwachung. Decheniana-Beihefte 26: 67–77.

Bliss, C. I., 1934. The method of probits. Science 79: 38–39.

Bryan, G. W. & W. J. Langston, 1992. Bioavailability, accumulation and effects of heavy metals in sediments with special reference to United Kingdom estuaries: a review. Envir. Pollut. 76: 89–131.

Buck, J. D., 1979. The plate count in aquatic microbiology. In S. W. Costerton & R. R. Colwell (eds), Native aquatic bacteria: enumeration, activity and ecology. American Society for Testing and Materials, Philadelphia: 19–28.

Campbell, P. G. C., A. G. Lewis, P. M. Chapman, A. A. Crowder, W. K. Fletcher, B. Imber, S. N. Luoma, P. M. Stokes & M. Winfrey, 1988. Biologically available metals in sediments. NCRR/CNRC (27694), Otawwa, 298 pp.

Carr, R. S., J. W. Williams & C. T. B. Fragata, 1989. Development and Evaluation of a Novel Marine Sediment Pore Water Toxicity Test with the Polychaete *Dinophilus gyrociliatus*. Envir. Toxicol. Chem. 8: 533–543.

Chapman, P. M., 1988. Marine sediment toxicity tests. In J. J. Lichtenberg, F. A. Winter, C. T. Weber & L. Fradkin (eds), Chemical and Biological Characterization of Sludges, Sediments, Dredge Spoils and Drilling fluids (STP 976). American Society for Testing and Materials, Philadelphia: 391–402.

Cherr, G. N., J. Shoeffner-McGee & J. M. Shenker, 1990. Methods for assessing fertilization and embryonic/larval development in toxicity test using the California mussel *Mytilus californianus*. Envir. Toxicol. Chem. 9: 1137–1145.

DelValls, T. A., 1995. Aplicación de un método integrado para la medida de la calidad ambiental en ecosistemas litorales del golfo de Cádiz. Tesis Doctoral. Universidad de Cádiz, 389 pp.

Fernández-Casalderrey, A., M. D. Ferrando, M. Gamon & A. Andreu-Moliner, 1991a. Acute toxicity and bioaccumulation of ensulfan in rotifer (*Brachionus calyciflorus*). Comp. Biochem. Physiol. 1/2: 61–63.

Fernández-Casalderrey, A., M. D. Ferrando & E. Andreu-Moliner, 1991b. Demographic parameters of *Brachionus plicatilis* Pallas (Rotifera) exposed to sublethal endosulfan concentrations. Hydrobiologia 226: 103–110.

Giesy, J. P. & R. A. Hoke, 1990. Freshwater sediment quality criteria: toxicity bioassessment. In R. Baudo, J. P. Giesy & H. Muntau (eds), Sediments: Chemistry and Toxicity of In-Place Pollutants Ann Arbor, Boca Raton, Boston, Lewis Publishers, Inc. 9: 265–348.

González-Mazo, E., 1995. Comportamiento de Alquilbenceno Lineal Sulfonato (LAS) en sistemas litorales del golfo de Cádiz. Tesis Doctoral, Universidad de Cádiz, 254 pp.

Hansson, I., 1973. A new set of pH-scales and standard buffers for seawater. Deep-Sea Res. 20: 479–491.

Hirayama, K., 1987. A consideration of why mass culture of the rotifer *Brachionus plicatilis* with baker's yeast is unstable. Hydrobiologia 147: 269–270.

Hoagland, K. D., R. W. Drenner, J. D. Smith & D. R. Cross, 1993. Freshwater community responses to mixtures of agricultural pesticides: Effects of atrazine and bifenthrin. Envir. Toxicol. Chem. 4: 627–637.

Hoke, R. A., J. P. Giesy & G. Jr. Kreis, 1992. Sediment pore water toxicity identification in the Lower Fox River and Green Bay, Wisconsin, using the Microtox assay. Ecotox. envir. Saf. 23: 343–354.

Johanson, O. & M. Wedborg, 1980. The ammonia-ammonium equilibrium in seawater at temperatures between 5 and 25 °C. J. solution chem. 9: 37–44.

Juchelka, C. M. & T. W. Snell, 1995. Rapid toxicity assessment using ingestion rate of cladocerans and ciliates. Arch. envir. Contam. Toxicol. 28: 508–512.

Landrum, P. F., J. E. Brian & W. R. Faust, 1991. Toxicokinetics and toxicity of a mixture of sediment associated polycyclic aromatic hydrocarbons to the amphipod *Diporeia* sp. Envir. Toxicol. Chem. 10: 35–46.

Long, E. R., M. F. Buchman, S. M. Bay, D. J. Breteler, P. M. Chapman, J. E. Hose, A. L. Lissner, J. Scott & D. A. Wolfe, 1990. Comparative evaluation of five toxicity tests with sediments from S. Francisco Bay and Tomales Bay, California. Envir. Toxicol. Chem. 9: 1193–1214.

Luoma, S. N. & K. T. Ho, 1992. The appropriate uses of marine and estuarine sediment bioassays. In: P. Calow (ed.), The Handbook of Ecotoxicology. Blackwell Scientific Publications, London: 193–226.

Mance, G., 1987. Pollution threat of heavy metals in aquatic environments. Elsevier Applied Science Publishers Ltd. New York, 372 pp.

Meador, J. P., B. D. Ross, P. A. Dinnel & S. J. Picquelle, 1990. An analysis of the relationship between a sand-dollar embryo elutriate assay and sediment contaminants from stations in an urban embayment of Puget Sound, Washington. Mar. envir. Res. 19: 13–37.

Pozuelo, M., 1975. Cultivo del rotífero *Brachionus plicatilis* obtención de un zooplancton artificial. Publ. Tec. Dccon. Gral. Pesca Marítima. Ministerio de Comercio 11: 401–416.

Pozuelo, M. & L. Lubián, 1993. Asexual and sexual reproduction in the rotifer *Brachionus plicatilis* cultured at different salinities. Hydrobiologia 255/256: 139–143.

Rand, G. M., P. G. Wells & L. S. McCarty, 1995. Introduction to aquatic toxicology. In G. M. Rand (ed.), Fundamentals of Aquatic Toxicology: Effects, Environmental fate and Risk assessment. 2^{nd} ed. Taylor & Francis, Washington, DC: 3–66.

Ray, B., 1979. Methods to detect stressed microorganisms. J. Food. Prot. 42: 346–355.

Saquid, M., 1992. Toxic metal chemistry in marine environments. arcel Dekker, Inc. New York, 390 pp.

Schlüter, M. & J. Groeneweg, 1985. The inhibition by ammonia of population growth of the rotifer *Brachionus plicatilis* in continuous culture. Aquaculture 46: 215–220.

Shenk, A., 1984. Calculus and analytic geometry. 3rd edn., Scott, Foresman and Company. Palo Alto, CA, 1131 pp.

SladeCék, V., 1983. Rotifer as indicators of water quality. Hydrobiologia 100: 169–201.

Snell, T. W. & G. Persoone, 1989. Acute toxicity bioassays using rotifers. I. A test for brackish and marine environments with *Brachionus plicatilis*. Aquat. Toxicol. 14: 65–80.

Snell, T. W., B. D. Moffat, C. Jensen & G. Persoone, 1991. Acute toxicity tests using rotifers. III. Effects of temperature, strain and exposure time on the sensitivity of *Brachionus plicatilis*. Envir. Toxicol. Wat. Qual. 6: 63–75.

Snell, T. W. & B. D. Moffat, 1992. A two-day life-cycle test with *Brachionus calyciflorus*. Envir. Toxicol. Chem. 11: 1249–1257.

Snell, T. W. & C. R. Janssen, 1995. Rotifers in Ecotoxicology: a review. Hydrobiologia. 313/314: 231–147.

Suedel, B. C., J. H. Jr. Rodgers & P. A. Clifford, 1993. Bioavailability of fluoranthene in freshwater sediment toxicity tests. Envir. Toxicol. Chem. 12: 155–165.

Trussell, R. P., 1972. The percent un-ionized ammonia in aqueous ammonia solutions at different pH levels and temperatures. J. Fish. Res. Bd Canada 29: 1505–1507.

Walsh, G. E., M. K. Louie, L. L. McLaughlin & E. M. Lores, 1986. Lugworm *Arenicola cristata* larvae in toxicity tests: survival and development when exposed to organotins. Envir. Toxicol. Chem. 5: 749–754.

Weiss, K., J. P. Lay, A. Goeppel & R. Lang, 1992. The rotifer toxicity test. Chemosphere 25: 517–524.

Whitfield, M., 1974. The hydrolysis of ammonium ions in sea water – a theoretical study. J. Mar. Biol. Ass., UK 54: 565–580.

Whitfield, M., 1993. Establishment of practical pH scales and ammonia ionisation in estuaries. Interim Report: Validated ammonia model edited by National Rivers Authority. Plymouth Marine Laboratory, UK, 50 pp.

Yufera, M., L. M. Lubián & E. Pascual, 1984. Estudio preliminar del Zooplancton de las salinas de Cádiz. Limnetica I: 62–69.