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The effects of fluid motion on toxicant sensitivity of the rotifer *Brachionus calyciflorus*

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Abstract

Standardized methods for estimating the toxicity of anthropogenic compounds to aquatic organisms frequently fail to consider key elements of the test organisms' environment. Aquatic organisms exist in a fluid environment, and fluid dynamics may have an important influence on the response to toxicants. Rotifers are one of the three major groups of zooplankton and have been increasingly utilized in standardized toxicity testing. However, like other toxicity tests, assays with the species *Brachionus calyciflorus* are performed under static conditions in the absence of fluid motion. We investigated how fluid motion modifies pentachlorophenol (PCP) toxicity to *B. calyciflorus* using 24 h acute and 48 h reproductive toxicity tests. Estimates of PCP LC50s and reproduction EC50s in static conditions decreased from 738 and 1082 μg l^{-1}, respectively, to as low as 262 and 136 μg l^{-1}, respectively, in fluid motion. Flow analysis indicated that increased toxicant sensitivity can occur at ecologically relevant levels of fluid motion. Mechanistic studies indicated that fluid motion/toxicant interactions may result from the ability of fluid motion to cause shear stress, alter toxicant uptake, and/or alter the bioavailability of food. As fluid motion may have an important effect on the life histories of a wide variety of aquatic organisms, fluid motion/toxicant interactions may be an important consideration in other standard test organisms. These results raise questions about the accuracy of ecological risk assessments based on toxicity data from static conditions.

Keywords: Toxicity; Pentachlorophenol; Rotifer; Fluid motion; Shear turbulence

1. Introduction

Toxicity tests conducted in the laboratory frequently fail to consider key elements in the ecology of test organisms. This is despite the fact that spatial and temporal heterogeneity in environmental conditions and the presence of natural stressors may have significant effects on organism fitness (Snell, 1980; Kirk, 1997; Sprague, 1995). Natural zooplankton populations, for example, are strongly influenced by food availability and quality (Hirayama et al., 1989; Korstad et al.,

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1989; Radwan and Popiolek, 1989), water temperature (Mikschi, 1989; Walz et al., 1989), competition (MacIsaac and Gilbert, 1989; Rothhaupt, 1990; Pace and Vaque, 1994; Sarma et al., 1996), and predation (Kerfoot and Sih, 1987; Williamson et al., 1989). Furthermore, a number of studies have demonstrated that anthropogenic toxicants interact with a variety of ecological factors affecting zooplankton abundance (Kluttgen and Ratte, 1994; Faber et al., 1998; Sierszen and Lozano, 1998; Monson and Brezonik, 1999; Preston et al., 1999a,b,c). Ecologically relevant assessments of toxicity and subsequent predictions of ecosystem level effects, necessitate considering the ecology of test organisms and incorporating critical ecological factors into toxicity tests.

Fluid motion is an important physical factor affecting the ecology of planktonic organisms (Reynolds, 1992). Fluid motion contributes to the spatial distribution of plankton in aquatic systems, which in turn contributes to ecosystem structure and function (Peterman and Bradford, 1987; MacIntyre, 1993; Ruiz et al., 1996; Abraham, 1998). Fluid motion influences the uptake of nutrients across cellular membranes in phytoplankton (Munk and Riley, 1952; Lewis et al., 1984; Lazier and Mann, 1989; Reynolds, 1992; Karp-Boss et al., 1996) and oxygen across ventilatory surfaces in aquatic animals (Philipson, 1954; Kovalak, 1978). Fluid motion also affects animal energetics, metabolism, and behavior and influences intra- and interspecific interactions, such as mate location and predator–prey interactions (Rothschild and Osborn, 1988; Marrase et al., 1990; Costello et al., 1990; Granata and Dickey, 1991; Alcaraz et al., 1994; Kiorboe and Saiz, 1995; Saiz and Kiorboe, 1995; Shimeta et al., 1995; Osborn, 1996).

Considering the fundamental importance of fluid motion in the ecology of aquatic organisms, it is surprising that this factor has not been investigated by aquatic toxicologists. The small-scale motion of water may be either laminar (ordered) or turbulent (chaotic). The boundary between these two flow regimes may be estimated by the Kolmogorov turbulence microscale, defined as the size at which turbulent eddies are dampened by molecular viscosity to laminar fluid shear (Gill, 1982). The Kolmogorov scale for the upper mixed layer of freshwater systems is on the order of 10^{-3} m, but varies with the energy dissipation rate, a measure of the amount of turbulent energy in a fluid system (Lazier and Mann, 1989). Thus, organisms smaller than the Kolmogorov scale experience small scale fluid motion as laminar shear. Both of these flow regimes have implications for the life histories of plankton. Small-scale fluid motion creates relative motion between an organism and the fluid environment that can affect rate-limited processes such as the uptake of nutrients or toxicants, elimination of wastes, or contact rates with food sources (Rothschild and Osborn, 1988; Lazier and Mann, 1989; Marrase et al., 1990; Reynolds, 1992; Shimeta et al., 1995; Karp-Boss et al., 1996). However, for motile plankton, there is a lower size limit (~ 100 μm) below which increasing relative motion has negligible effects on diffusion-limited processes relative to the effects of swimming (Lazier and Mann, 1989; Karp-Boss et al., 1996). In addition, velocity gradients within the fluid can cause mechanical shear stress on plankton (Berdalet, 1992; Hondzo et al., 1998; Hondzo and Lyn, 1999). Thus, small-scale fluid motion may have important implications for organism fitness.

Rotifers are one of the three major groups of zooplankton and are common inhabitants in the surface mixed layer of aquatic systems, where fluid dynamics may be most intense. In addition, rotifers have been increasingly used for toxicity assessments, and standardized methods exist for estimating acute and chronic toxicity of chemicals to rotifers (Snell and Janssen, 1995; ASTM, 1998a; APHA, 1998a). Free-swimming rotifers typically range in size from 100 to 1000 μm in length and vary in swimming speeds from 100 to 1000 μm s^{-1} (Nogrady et al., 1993). As such, they are characterized by low Reynolds' numbers (0.01–1.0) (the ratio of inertial to viscous forces), and their physical environment is dominated by viscous forces. Rotifers are generally smaller than the Kolmogorov scale and experience laminar fluid motion. However, given their size and swimming speed, fluid motion is predicted to have a greater influence on rate-limited processes than swimming (Karp-Boss et al., 1996).

Standardized toxicity tests with the rotifer *Brachionus calyciflorus*, as well as with the cladocerans *Daphnia* and *Ceriodaphnia*, are performed under static or static-renewal conditions (APHA, 1998a; ASTM, 1998a,b,c). Such test conditions ignore the fluid dynamics of natural aquatic systems and give little consideration to the effects of fluid motion on the toxicant sensitivity of aquatic organisms. Flow-through toxicity tests and toxicity testing with artificial streams are designed to maintain consistent levels of toxicants, oxygen, and metabolic wastes (Muirhead-Thompson, 1978; Shriner and Gregory, 1984; Kosinski, 1989), not to reproduce ecologically relevant fluid dynamics. Because fluid motion is specifically excluded from standardized zooplankton toxicity tests, the ecological relevance of the data generated by them is currently unknown.

In this study, we investigated the effects of pentachlorophenol (PCP) on reproduction and mortality of the freshwater rotifer *B. calyciflorus* in the presence and absence of artificially generated fluid motion to determine the effects of fluid motion on rotifer sensitivity to PCP. Results from this study were compared to previous reports of PCP toxicity in this species. In addition, plausible mechanisms that account for our results also were examined, including effects of fluid motion on food availability, physical stress and energetics, and the uptake of toxicants.

2. Materials and methods

2.1. Test animals

B. calyciflorus (Pallas) test animals consisted of neonate females (4–6 h old) hatched from cysts as previously described by Gomez et al. (1997). The *B. calyciflorus* strain used was originally collected in 1983 in Gainesville, FL (Snell et al., 1991). Since then, this strain has been cultured continuously with periodic collection and storage of cysts.

2.2. Generation and analysis of fluid motion

Fluid motion was generated artificially by using a culture rocker manufactured by Reliable Scientific (Model # 55). The rocker consisted of a 40×30 cm platform that rocked back and forth (5° inclination/declination) on a single axis at a variable frequency. The small test volumes and the irregular fluid motion produced by the experimental design prevented direct quantification of fluid motion. However, the response of the freshwater green alga *Scendesmus quadricauda* in response to quantified fluid motion has been reported (Hondzo et al., 1998; Hondzo and Lyn, 1999). In these studies, the growth rates of *S. quadricauda* were calculated by measuring changes in chlorophyll a concentrations in response to quantified fluid motion generated via a rotating cylinder apparatus (Hondzo et al., 1998) and an oscillating grid apparatus (Hondzo and Lyn, 1999). Growth was measured in response to a broad range of energy dissipation rates (ε), including ecologically relevant values (Hondzo and Lyn, 1999). Therefore, the reported response of *Scenedesmus quadricauda* to quantified fluid motion was used as a bioassay for the intensity of turbulent fluid motion in our experiments.Growth of *S. quadricauda* in response to the fluid motion produced in the current study was measured using identical culture conditions as Hondzo et al. (1998), Hondzo and Lyn (1999). Suspensions of *S. quadricauda* were prepared by diluting a stock culture in sterile Bristol's media (Starr and Zeijus, 1993) to yield an initial (t_0) chlorophyll a concentration of 0.07–0.1 mg l^{-1}. Multiple 12 ml aliquots of this suspension were placed in 30 ml polystyrene cell culture flasks (10 cm l^{-1}, 4 cm W, 2 cm H). Flasks were placed horizontally on the culture rocker perpendicular to the axis of rotation and incubated at 25°C under a constant photoperiod of (16:8 h light:dark). Rocker frequencies were 0, 1, 5, 10, 20, 30, and 40 oscillations/min, and chlorophyll a concentration was quantified at 12, 24, 36, 48, 60, 72, and 96 h. At each sampling time, 4–6 replicate flasks were sacrificed and a 5 ml sample from each flask was filtered on 0.45 μm filter paper to collect the algae. Filters were placed in 10 ml centrifuge tubes containing 5 ml of 90% acetone, and homogenized to facilitate transfer of chlorophyll into the solvent phase. Tubes were allowed to steep for 24 h, after which the tubes were centrifuged at 8000

rpm for 15 min. The absorbance of the liquid phase was measured at 664, 647, and 630 nm using a Shimadzu spectrophotometer. Chlorophyll a concentrations in mg l^{-1} and intrinsic rates of increase (r) were calculated from these measurements using the equations of APHA (1998b), Hondzo et al. (1998), respectively.

Tests were accepted if the growth constant for *S. quadricauda* in the controls (static conditions) fell within 10% of the observed control growth rate for Hondzo and Lyn (1999). Growth under different fluid motion treatments (r_t) was compared to growth in static controls (r_c) using one-way analysis of variance (ANOVA). Data on inhibition of growth in *S. quadricauda* in response to increasing ε from Hondzo et al. (1998), Hondzo and Lyn (1999) were modeled using linear, exponential, and third order polynomial functions, the latter of which had the best fit. Observed inhibition in *S. quadricauda* growth (r_t/r_c)under the flow regimes used in the current study were compared to the polynomial model to estimate ε. Shear rates (γ) were subsequently estimated from ε values using the equation (Hondzo and Lyn, 1999): $\gamma = 1/(15)^{1/2}][\varepsilon/\upsilon]^{1/2}$ where, υ = the kinematic viscosity of the fluid (for freshwater at 20°C, $\upsilon = 1.004 \times 10^{-6}$ m^2 s^{-1} (Vogel, 1996)) and ε = energy dissipation rate (m^2 s^{-3}). The Kolmogorov scale (L_v) for each fluid motion treatment was as also estimated using the equation (Gill, 1982): $L_v = (\upsilon^3/\varepsilon)^{1/4}$.

2.3. Toxicant preparation

Pentachlorophenate sodium salt (PCP), was obtained from Aldrich Chemical Company (Milwaukee, WI). Test solutions were prepared by diluting specific volumes of a PCP stock solution in moderately hard synthetic freshwater. Dilution water consisted of 96 mg $NaHCO_3$, 60 mg $CaSO_4 \cdot H_2O$, 60 mg $MgSO_4$, and 4 mg KCl in 1 l deionized water, adjusted to pH 7.5 with 10 M NaOH or HCl (EPA, 1985). Preparation of PCP test solutions according to this method has previously been demonstrated to yield concentrations within 10% of nominal values (Snell and Moffat, 1992). All experiments were conducted at nominal PCP concentrations.

2.4. Reproduction tests

Interactions between fluid motion and sublethal PCP exposure were assessed using a modified 2 day *B. calyciflorus* reproduction test as described by Snell and Moffat (1992). Tests consisted of six PCP exposure treatments (0, 60, 110, 190, 330, or 450 μg l^{-1}) with six fluid motion treatments per PCP exposure (0, 1, 10, 20, 30, and 40 oscillations/min). Six female neonate rotifers were placed in a 30 ml polystyrene cell culture flask containing 12 ml of a 2.0×10^6 cells/ml suspension of *Nannochloris oculata*. Four replicate flasks were used per PCP exposure and fluid motion treatment. After the addition of animals, tubes were placed horizontally on the culture rocker and incubated for 48 h in darkness at 25°C. After 48 h, tubes were emptied into a glass petri dish and the number of animals per flask were counted. Rotifer reproduction was exclusively parthenogenetic during the test. Two-way analysis of variance and Dunnett's test was used to assess the individual effects of fluid motion or PCP exposure on reproduction. Linear regression was used to calculate reproduction EC50s for PCP for each fluid motion treatment. Regression lines were calculated from reproduction data expressed as a percentage of control and transformed to a probit scale plotted against log PCP concentrations.

2.5. Mortality tests

B. calyciflorus acute tests consisted of six PCP exposure treatments (0, 190, 330, 450, 600, and 750 μg l^{-1}) with seven fluid motion treatments per PCP exposure (0, 1, 5, 10, 20, 30, and 40 oscillations/min). A total of 25 animals were placed in a 30 ml polystyrene cell culture flask containing 12 ml of PCP test solutions. Flasks then were placed horizontally on the culture rocker and incubated for 24 h in darkness at 25°C. No food was provided in the acute tests. Four replicate flasks were used per PCP exposure and fluid motion treatment. After 24 h, the

number of dead and live animals were enumerated using a dissecting microscope at 10 × magnification. Death was judged by the absence of ciliary and internal movement. Arcsine transformations were performed on mortality frequencies and two-way analysis of variance and Dunnett's test were used to assess the effects of fluid motion or PCP exposure on mortality. Linear regression was used to estimate the LC50s for PCP in controls and fluid motion treatments. Regression lines were calculated from mortality frequencies transformed to a probit scale and plotted against log PCP concentrations.

2.6. Rotifer ingestion tests

The effects of prior exposure to fluid motion on rotifer ingestion was assessed using a simplified version of a procedure described by Juchelka and Snell (1994) as an additional assay for stress effects resulting from fluid motion. Experiments consisted of seven fluid motion treatments (1, 5, 10, 20, 30, and 40 oscillations/min). A total of 25 animals were placed in a 30 ml polystyrene cell culture flask containing 12 ml of synthetic freshwater. Five replicate flasks were used for each fluid motion treatment. After the addition of animals, flasks were placed horizontally on the culture rocker and incubated for 24 h in darkness at 25°C. After 24 h, flasks were removed from the rocker, and 10 μl of a solution of 5 μm diameter red polystyrene microspheres was placed in each flask, yielding a final microsphere concentration of $\sim 25\,000\ \mathrm{ml}^{-1}$ in each flask. Flasks were incubated in darkness at 25°C for 15 min under static conditions, then emptied into a glass petri dish where animals were killed and fixed by adding 120 μl of a 10% formalin solution. Animals were then transferred via transfer pipette to a glass microscope slide, and the gut contents of each rotifer were examined at 30 × magnification using a dissecting microscope. The number of animals feeding in each replicate was enumerated. Arcsine transformations were performed on ingestion frequencies, and one-way analysis of variance was used to assess the effect of prior exposure to fluid motion on *B. calyciflorus* ingestion.

2.7. Algae sedimentation tests

The effect of fluid motion on algae sedimentation was assessed by direct sampling of algae cell density in test flasks after 24 h to determine if fluid motion had a significant effect on food availability. Thirty ml cell culture flasks containing 12 ml of a 2.0×10^6 cells/ml suspension of *N. oculata* were placed on the culture rocker and incubated for 24 h in darkness at 25°C. Ten replicate flasks were used per fluid motion treatment (0, 1, 5, 10, 20, 30, and 40 oscillations/min). After 24 h, the rocker was stopped, and 100 μl samples of the algal suspension were withdrawn from each flask, and algal concentration was quantified using a hemacytometer. One-way analysis of variance was used to assess the effects of fluid motion on *N. oculata* sedimentation.

2.8. Substrate uptake tests

The effect of fluid motion on the uptake of chemical substrates was assessed using two fluorescent dyes of different molecular weights, fluorescein (MW = 376.3) or rhodamine-B (MW = 479.0). Experiments were conducted in two phases. First, time series tests were conducted to determine the duration of exposure necessary to ensure uptake of the dyes had reached a steady-state equilibria. Second, a definitive test of a fixed duration was conducted. Stock solutions of fluorescein and rhodamine were prepared by dissolving the dyes in 100% acetone. Test solutions of the dyes were prepared by diluting the stock solutions to 12 ml in synthetic freshwater. The test concentrations of fluorescein and rhodamine were 10 and 5 μmol, respectively, and the concentration of acetone in test solutions was $< 1\%$. Time series tests consisted of seven fluid motion treatments (0, 1, 5, 10, 20, 30, and 40 oscillations/min). A total of 25 animals were placed in a 30 ml polystyrene cell culture flask containing 12 ml of a fluorescein or rhodamine solution. After the addition of animals, flasks were placed horizontally on the culture rocker and incubated in darkness at 25°C without food for 0, 10, 20, 40, 80, 140, or 240 min. One replicate flask was used per fluid motion treat-

ment and incubation time. After incubation, flasks were emptied into a glass petri dish, and the animals were fixed with the addition of 100 μl of a 10% formalin solution. A total of 15 animals were selected at random and placed on a glass microscope slide and covered with a glass cover slip. Animals were examined at 100 × magnification using epifluorescence microscopy. Images were captured using a digital CCD camera connected to a computer using National Institutes of Health Image 1.61 (US National Institutes of Health; available on the Internet at http://rsb.info.nih.gov/nih-image). Net animal fluorescence was calculated by quantifying fluorescence of the animals (F) and subtracting background fluorescence of a space adjacent to the animal of the same area. The relative effect of fluid motion on net fluorescence of *B. calyciflorus* was quantified as the ratio of fluorescence in fluid motion treatments (F_t) to stagnant controls (F_c). The effects of exposure duration and flow treatment on net fluorescence of *B. calyciflorus* was assessed using one-way ANOVA and Dunnett's test. Definitive tests were subsequently performed in an identical fashion except that two replicate flasks were used per flow treatment and fluorescence was quantified after a single exposure time (4 h) based on the results of the time series tests. In addition, the effect of a 4 h exposure to fluorescein and rhodamine on *B. calyciflorus* ingestion was tested in a similar fashion to the ingestion tests described above to ensure that exposure to the fluorescent dyes did not adversely effect the feeding of *B. calyciflorus*. This verified that the observed effects were the result of fluid motion rather than changes in feeding behavior caused by exposure to the dyes.

Table 1
Analysis of the flow regimes used in the current study

Flow	r_c/r_t[a]	ε[b]	γ[c]	L_v[c]
0	1.00	1.0×10^{-6}	0.03	1.000
1	1.01	–	–	–
5	1.02	–	–	–
10	0.93	1.9×10^{-5}	1.13	0.480
20	0.81	8.9×10^{-5}	2.43	0.330
30	0.73	2.1×10^{-4}	3.72	0.260
40	0.61[d]	6.9×10^{-4}	6.77	0.200

[a] r_c/r_t represents the change in S. *quadticauda* growth rates in fluid motion treatments (r_t) relative to controls (r_c).

[b] ε, energy dissipation rate ($m^2\ s^{-3}$) estimated by comparison of r_t/r_c values to the data from Hondzo and Lyn (1999).

[c] γ and L_v represent the shear rate (s^{-1}) and Kolmogorov scale (mm) predicted by the ε values.

[d] Indication of significant difference from control ($P<0.05$) by ANOVA.

3. Results

3.1. Flow analysis

The mean initial intrinsic rate of increase (r) of *S. quadricauda* controls (static conditions) in the current study was 0.880. This value differs by $<10\%$ from the *S. quadricauda* initial growth rate under stagnant conditions of 0.799 reported by Hondzo and Lyn (1999), indicating successful reproduction of Hondzo and Lyn's (1999) culture conditions and the general comparability of results between the two studies. The oscillation rate of the rocker used to generate fluid motion in the current study significantly reduced r over the initial growth phase as well as at the termination of the experiment (96 h) ($df=1$, $F=4.5$, $P<0.039$ and $df=1$, $F=6.0$, $P=0.015$, respectively). In addition, initial growth of *S. quadricauda* was significantly lower than controls at 40 oscillations/min (Table 1).

The reduction (r_t/r_c) in r over the initial exponential growth phase in response to fluid motion treatments was compared to the growth curve generated from Hondzo et al. (1998), Hondzo and Lyn (1999) to estimate energy dissipation rates (ε) and shear (γ) at each of the fluid motion treatments tested in the current study (Fig. 1, Table 1). The Kolmogorov scales (L_v) predicted by ε values were also calculated (Table 1). Growth at 1 and 5 oscillations/min differed by $<3\%$ from controls and thus, these levels of fluid motion were excluded from the analysis, as they could not be experimentally distinguished from controls. Estimated energy dissipation rates (ε) spanned three orders of magnitude from 10^{-6} to 10^{-4} $m^2\ s^{-3}$, while shear rates (γ) spanned two orders of magnitude from 0.03 to 6.77 s^{-1}. Kolmogorov scales

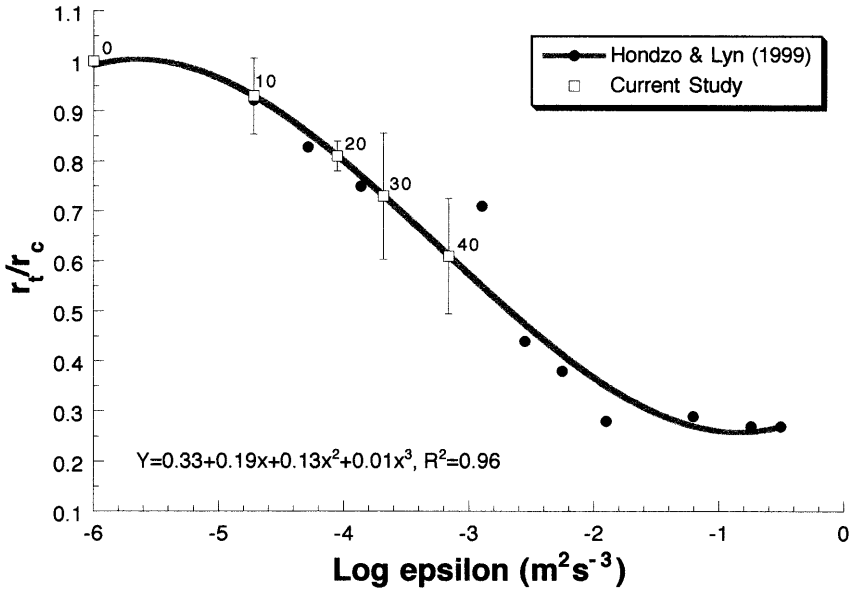

Fig. 1. Estimation of energy dissipation rates (ε) for flow treatments. Data on the inhibition of *S. quadricauda* growth rates in response to quantified fluid motion was taken from Hondzo et al. (1998), Hondzo and Lyn (1999) (black circles). Subsequently, these data were used to model changes in *S. quadricauda r* over a range of ε values, using a best-fit third-order polynomial function (grey curve). The equation for this model was then used to estimate ε values for the flow regimes used in the current study from the inhibition of *S. quadricauda r* observed in each flow regime (white squares). Vertical bars represent the standard error of the mean. r_t/r_c was assumed to be 1.0 under static conditions (0 oscillations/min), where t is treatment and c is the control *r* value.

(L_v) ranged from 1.0 mm at zero oscillations/min to 0.2 mm at 40 oscillations/min. These length scales were compared to the mean *B. calyciflorus* neonate female body length of 285 μm (Preston et al., 1999b), to determine the nature of the flow (i.e. turbulent versus laminar) experienced by *B. calyciflorus* in each fluid motion treatment.

3.2. Reproduction tests

In the absence of PCP, fluid motion had a significant effect on *B. calyciflorus* reproduction ($df = 5$, $F = 5.9$, $P = 0.002$). The mean number of animals after 2 days increased slightly as fluid motion increased up to 20 oscillations/min, but this increase was not statistically significant. An insignificant decrease in the mean number of animals after 2 days was observed at 30 oscillations/min relative to control and reproduction was significantly inhibited at 40 oscillations/min ($df = 1$, $F = 169.0$, $P < 0.0001$). Although PCP concentrations < 450 μg l^{-1} did not have a significant effect on reproduction under static conditions, effects of PCP at these lower concentrations became significant after exposure to fluid motion between 1 and 20 oscillations/min (Fig. 2). Beyond 20 oscillations/min, the effects of fluid motion were large enough to inhibit reproduction independent of PCP concentration. A significant interaction between PCP and fluid motion on reproduction was observed by two way ANOVA ($df = 25$, $F = 2.8$, $P = 0.0001$). The PCP reproduction no observed effect concentrations (NOECs) decreased from 450 μg l^{-1} in the absence of fluid motion to as low as 110 μg l^{-1} at 10 oscillations/min (Table 2). Increased fluid motion decreased PCP reproduction EC50s by up to eight-fold at ten oscillations/min (Table 2). Even at the lowest flow intensity (1 oscillation/min) the PCP EC50 decreased by approximately four-fold (Table 2). However, the high upper confidence limit around the stagnant PCP EC50 makes this estimate (1082 μg l^{-1}) questionable. If comparisons are made to the lower confidence limit for the stagnant PCP

EC50 (460 μg l^{-1}), fluid motion still decreases PCP EC50s by ~2–3 fold. Reproduction EC50s could not be calculated for fluid motion treatments of 30 and 40 oscillations/min, as the slope of regression lines did not differ significantly from zero.

3.3. Survival tests

In the absence of PCP, fluid motion had no significant effect on *B. calyciflorus* mortality. However, PCP alone had a significant effect on mortality (df = 5, $F = 43.4$, $P < 0.0001$), and PCP

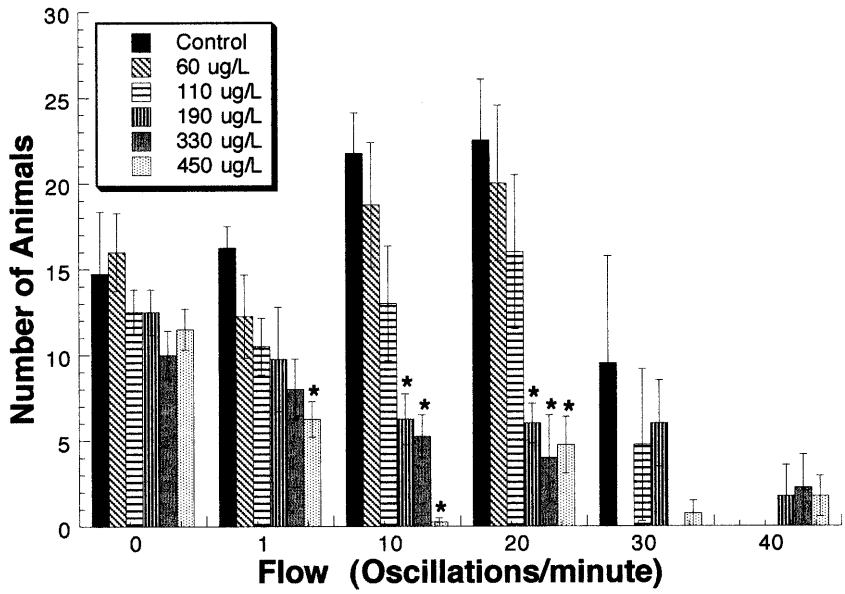

Fig. 2. Effects of sublethal pentachlorophenol (PCP) exposure and fluid motion on *B. calyciflorus* reproduction. Columns represent the mean number of animals appearing in tubes after 48 h (starting number = 6). Vertical bars represent the standard error of the mean. * indicates significant difference ($P < 0.05$) from PCP controls by analysis of variance (ANOVA) and Dunnett's test.

Table 2
Pentachlorophenol NOECs, LOECs, LC50/EC50 for mortality and reproduction tests with *B. calyciflorus* in response to different flow regimes[a]

Mortality				Reproduction		
Flow	NOEC	LOEC	LC50	NOEC	LOEC	EC50
0	450	600	738 (630, 1110)	450	N/A	1082 (460, 1.4×10^9)
1	450	600	450(373, 513)	330	450	287(221, 421)
5	450	600	714(655, 839)	N/T	N/T	N/T
10	190	330	262(191, 315)	110	190	136 (90, 187)
20	330	450	362(264, 432)	190	330	166 (71, 322)
30	450	600	567(448, 743)	N/A	N/A	N/A
40	330	450	378 (278, 486)	N/A	N/A	N/A

[a] NOECs, LOECs, LC50/EC50s are in μg PCP/L. LC50/EC50s with upper and lower 95% confidence limits (parentheses) were calculated using linear regression and probit analysis. N/T indicates flow intensity level was not tested. N/A indicates value could not be calculated due to limited data.

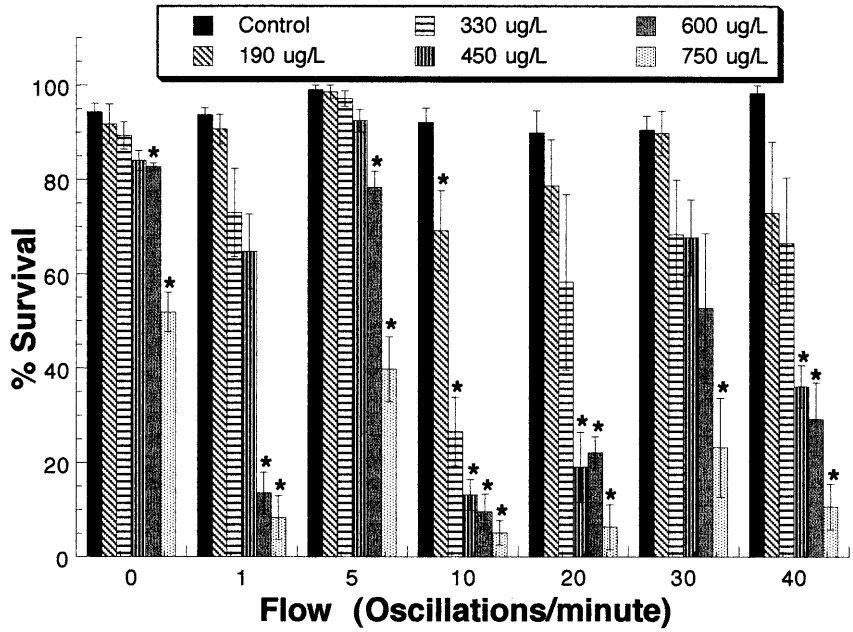

Fig. 3. Effects of acute PCP exposure and fluid motion on *B. calyciflorus* survival. Columns represent the mean percent survival in tubes after 24 h. Vertical bars represent the standard error of the mean. * indicates significant difference ($P < 0.05$) from PCP controls by ANOVA and Dunnett's test.

effects were observed in all fluid motion treatments (Fig. 3). A significant interaction between fluid motion and PCP was observed by two way ANOVA ($df = 30$, $F = 3.5$, $P < 0.0001$). PCP NOECs decreased from 450 μg l^{-1} in the absence of fluid motion to as low as 190 μg l^{-1} at 10 oscillations/min. Fluid motion decreased PCP LC50s by up to three-fold at 10 oscillations/min (Table 2).

3.4. Algae sedimentation tests

Fluid motion had a significant effect on the sedimentation of the alga *N. oculata* within the test flasks ($df = 6$, $F = 98.3$, $P < 0.0001$; Fig. 4). In the absence of fluid motion, sedimentation caused the alga concentration in test flasks to decrease by approximately one order of magnitude from its initial concentration of 2×10^6 cells/ml after 24 h. However, flow intensities at 1–5 oscillations/min and 10–20 oscillations/min increased alga concentrations by ~ 2 and 6-fold, respectively. Fluid motion intensities at 30 and 40 oscillations/min were nearly sufficient to eliminate the sedimentation of *N. oculata*.

3.5. Ingestion tests

Prior exposure to fluid motion had a significant effect on the feeding response of *B. calyciflorus* ($df = 6$, $F = 13.83$; $P < 0.0001$; Fig. 5). Ingestion decreased significantly by $\sim 20\%$ at 1 oscillation/min. Further reductions of $\sim 40\%$ were observed for fluid motion intensities at 30 and 40 oscillations/min.

3.6. Fluorescein and rhodamine uptake tests

Time series analysis indicated that the fluorescence of *B. calyciflorus* individuals increased significantly over time after exposure to fluorescein or rhodamine by two-way ANOVA ($df = 6$, $F = 371.2$, $P < 0.0001$ and $df = 6$, $F = 145.5$, $P < 0.0001$, respectively) (data not shown). Fluorescence of *B. calyciflorus* individuals exposed to fluorescein or rhodamine also increased

significantly with increasing fluid motion ($df = 5$; $F = 14.3$, $P < 0.0001$ and $df = 5$; $F = 16.1$, $P < 0.0001$, respectively). A significant interaction was observed between exposure time and fluid motion on fluorescence of *B. calyciflorus* individuals exposed to fluorescein or rhodamine ($df = 30$, $F = 8.8$, $P < 0.0001$ and $df = 30$, $F = 4.4$, $P < 0.0001$). For fluorescein, exposure times beyond 140 min did not significantly increase fluorescence of *B. calyciflorus* individuals, suggesting that uptake of fluorescein had reached a steady-state. For rhodamine, exposure times beyond 80 min did not

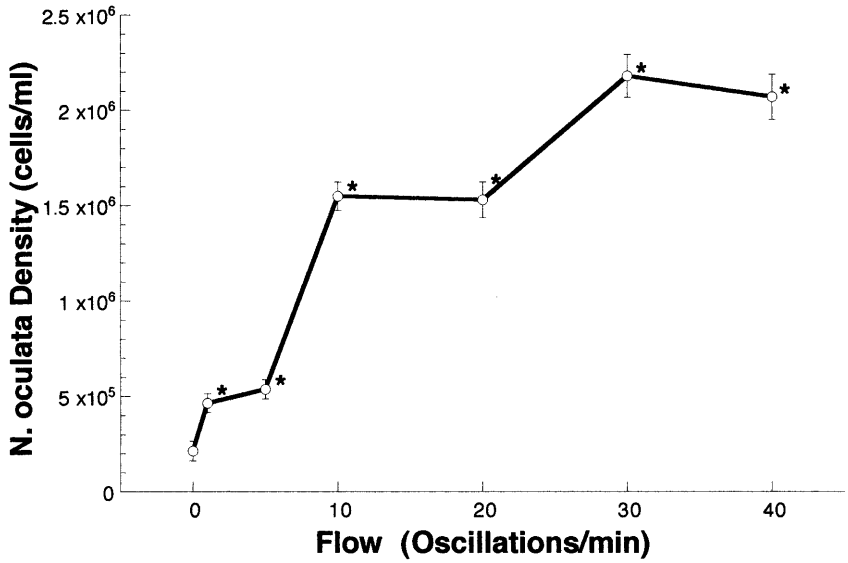

Fig. 4. Effects of fluid motion on sedimentation of the alga *N. oculata*. Vertical bars represent the standard error of the mean. * indicates significant difference ($P < 0.05$) from controls by ANOVA and Dunnett's test.

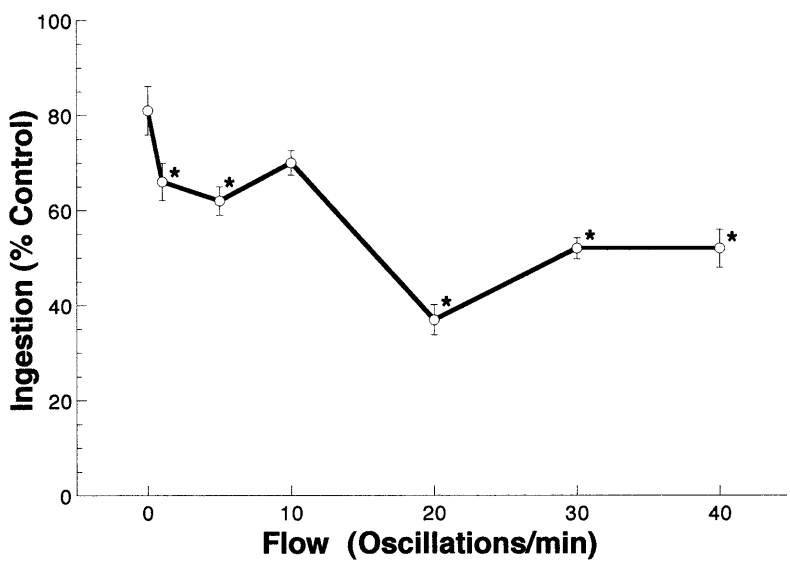

Fig. 5. Effects of fluid motion on post-exposure feeding behavior of *B. calyciflorus*. Vertical bars represent the standard error of the mean. * indicates significant difference ($P < 0.05$) from controls by ANOVA and Dunnett's test.

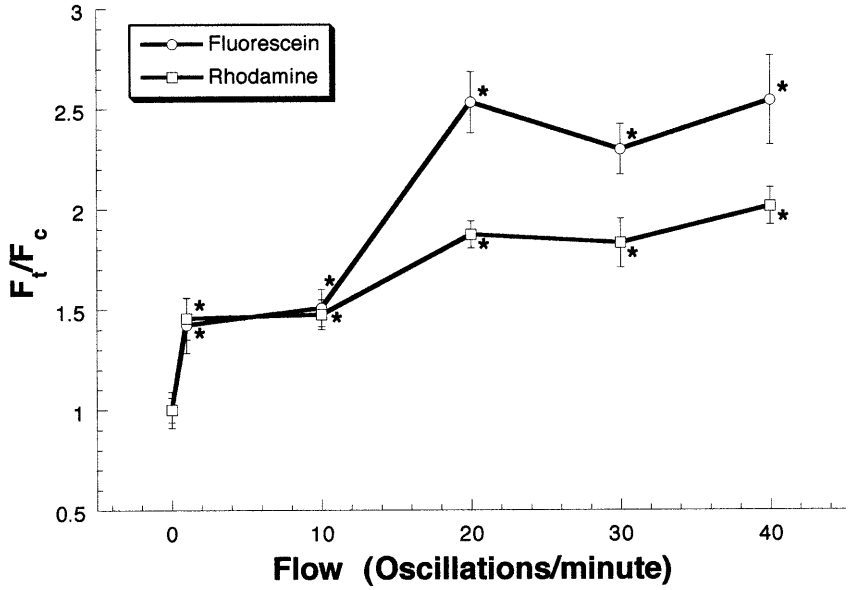

Fig. 6. Effects of fluid motion on net fluorescence relative to static controls of *B. calyciflorus* exposed to fluorescein (circles) or rhodamine-B (squares). Vertical bars represent the standard error of the mean. * indicates significant difference ($P < 0.05$) from controls by ANOVA and Dunnett's test.

cause a significant additional increase in fluorescence.

Based upon the results from the time series analysis (see above and Section 2), definitive tests of the effects of fluid motion on uptake of fluorescent dyes by *B. calyciflorus* were performed after animals were exposed to fluorescein or rhodamine for 240 min (4 h). This duration was sufficiently long to ensure steady-state uptake/loss of the dyes, but sufficiently short to prevent dye-induced toxicity. As in time series tests, increasing fluid motion significantly increased the fluorescence (F_t/F_c) of *B. calyciflorus* exposed to fluorescein or rhodamine ($df = 5$, $F = 21.0$, $P < 0.0001$ and $df = 5$, $F = 17.4$, $P < 0.0001$, respectively). Although the relative increase in fluorescence in response to fluid motion was similar between the two compounds, a greater relative increase in fluorescence was observed with the lower molecular weight fluorescein. For fluorescein, fluorescence of *B. calyciflorus* increased significantly between 1 and 20 oscillations/min, beyond which further increases in fluid motion did not further increase fluorescence (Fig. 6). For rhodamine, fluorescence of *B. calyciflorus* individuals increased significantly at 20 oscillations/min, beyond which further increases in fluid motion did not further increase fluorescence (Fig. 6). Exposure to fluorescein or rhodamine had no significant effects on *B. calyciflorus* ingestion.

4. Discussion

Small-scale fluid motion has significant effects on the toxicant sensitivity of *B. calyciflorus*. Large reductions in both the LC50 and the reproduction EC50 for *B. calyciflorus* were observed in the presence of fluid motion compared to static conditions (Table 2). Reductions in these endpoints were observed at the lowest level of fluid motion intensity tested (1 oscillation/min), indicating that even small changes in fluid dynamics can increase toxicant sensitivity. However, there did not appear to be a linear dose-response to increasing intensities of fluid motion. Snell and Moffat (1992) reported a 24 h PCP LC50 for the same strain of *B. calyciflorus* of 1200 $\mu g\ l^{-1}$ versus the 738 $\mu g\ l^{-1}$ reported here under static conditions. Although these values differ by $\sim 40\%$, it falls

within the reported among-test variability for the *B. calyciflorus* 24 h acute test of ~50% (Persoone et al., 1993). The *B. calyciflorus* reproduction EC50 reported here under stagnant conditions of 460–1082 μg l^{-1} is higher than the value of 270 μg l^{-1} reported by Snell and Moffat (1992). The latter value was obtained in the presence of unquantified fluid motion generated by a culture rotator to reduce algal food sedimentation, which cannot be compared to the fluid motion in the current study. The 270 μg l^{-1} reproduction EC50 of Snell and Moffat (1992) is more comparable to the values obtained in this study (166–287 μg l^{-1}).

Estimated Kolmogorov scales for the tested flow regimes were larger, or comparable, in size to *B. calyciflorus*, suggesting that the fluid motion experienced by *B. calyciflorus* was predominantly laminar shear. As a result, the observed interaction between fluid motion and PCP exposure may be attributed to the combined effects of physical shear stress and toxicity and/or fluid motion effects on rate-limited phenomena such as toxicant uptake or food availability. As a result, we propose three hypotheses to account for our observations. First, both fluid motion (Oviatt, 1981; Harris and Malej, 1986; Alcaraz et al., 1988, 1994) and PCP (Janssen et al., 1994; Penttinen and Kukkonen, 1998) have been demonstrated to act as physiological stressors to zooplankton by increasing metabolic rates and altering energetics. The cost of locomotion for *Brachionus* has been estimated at 62% of total metabolism (Epp and Lewis, 1984), indicating its sensitivity to factors such as fluid motion that may affect metabolic efficiency. It is therefore plausible that these two stressors may act additively or synergistically. Second, fluid motion has been demonstrated to inhibit rotifer feeding behavior (Miquelis et al., 1998), and food limitation has been demonstrated to increase toxicant sensitivity in zooplankton, due to reductions in the energy available for detoxification (Kluttgen and Ratte, 1994; Gomez et al., 1997; Monson and Brezonik, 1999). Thus, increased toxicant sensitivity in the presence of fluid motion may be the result of fluid motion-induced inhibition of feeding behavior (Miquelis et al., 1998). Third, previous studies have demonstrated the ability of fluid motion to increase nutrient uptake in plankton of 10–100 μm in size, although the critical size range necessary for fluid shear to significantly enhance uptake remains controversial (Lazier and Mann, 1989; Karp-Boss et al., 1996). Thus, fluid motion may increase the uptake of toxicants across membranes in a similar fashion to nutrients. These mechanisms are not mutually exclusive, and combinations of these effects may account for our observations.

The hypothesis that fluid motion causes physical stress in *B. calyciflorus* is supported by data from the current study. Although in the absence of PCP, *B. calyciflorus* survival was unaffected by increasing fluid motion, *B. calyciflorus* reproduction was inhibited at rocker frequencies of 30 and 40 oscillations/min. Furthermore, prior exposure to fluid motion caused moderate reductions in the feeding of *B. calyciflorus* at all rocker frequencies compared to controls. Together these data suggest that the physical stress of fluid shear can cause adverse effects *in B. calyciflorus* that may interact with PCP toxicity. For example, increased demands to maintain homeostasis and increased metabolic activity in the presence of fluid motion may force trade-offs in the allocation of energy between detoxification and other metabolic processes. Prolonged exposure to high intensities of fluid motion may be a sufficient stressor to cause severe physiological stress or mortality in *B. calyciflorus* independent of other stressors such as toxicants. Adverse effects on *S. quadricauda* were observed at γ values of ~2 s^{-1} and higher, corresponding to rocker frequencies between 20 and 40 oscillations/min. This suggests that fluid motion alone may not be a significant physical stressor to *B. calyciflorus* at shear rates lower than 2 s^{-1}.

The hypothesis that laminar shear interacts with PCP toxicity through its effects on rate-limited processes also is supported by data from the current study. Experiments with fluorescein and rhodamine indicated that toxicant uptake may increase significantly in response to increases in relative motion between *B. calyciflorus* and its fluid environment. It is interesting to note that the 2–3 fold increase in fluorescein and rhodamine uptake by *B. calyciflorus* in response to fluid motion corresponds to the 2–3 fold increase in acute and reproductive PCP toxicity. The influ-

ence of fluid motion on food acquisition by *B. calyciflorus* may be an important consideration as well. Although ingestion tests indicated that fluid motion had a moderate adverse effect on *B. calyciflorus* ingestion, increasing fluid motion had a large effect on the sedimentation of *N. oculata*. In addition, an insignificant trend of increasing reproduction with increasing fluid motion between 0 and 20 oscillations/min was observed, suggesting that at these intensities, the benefits of fluid motion on food availability outweigh the stress incurred by *B calyciflorus*. However, it should be noted that toxicant sensitivity increased with fluid motion in mortality tests where food was not provided. Thus, low levels of fluid motion ($\varepsilon < 10^{-4}$ m^2 s^{-3}) may interact with toxicants by increasing rates of uptake or altering the efficiency of food acquisition, while at higher levels, toxicant interactions become less important because the physical effects of shear stress dominate, causing adverse effects even in the absence of other stressors.

Although the oscillating motion used to generate fluid motion in the current study differs from the forces driving fluid turbulence in natural aquatic systems, over size scales relevant to low Reynolds' number organisms (e.g. algae), fluid motion is isotropic and homogenous regardless of the manner in which it is generated (Jiminez, 1997). Thus, simple, artificial methods for generating fluid motion may create fluid dynamics that are ecologically relevant to aquatic organisms. Energy dissipation rates in the surface mixed layers of natural freshwater ponds and lakes span several orders of magnitude, from $\sim 10^{-8}$ to 10^{-5} m^2 s^{-3} under intense conditions (Reynolds, 1992; Imberger, 1991; Hondzo and Lyn, 1999). Bioassays with the green algae *S. quadricauda* indicate that the method for generating fluid motion in the current study has effects on *S. quadricauda* growth rate similar to ε values of $\sim 10^{-6}$–10^{-4} m^2 s^{-3}, which are comparable to naturally occurring turbulence levels. *S. quadricauda* growth rates at 1 and 5 oscillations/min were indistinguishable from stagnant controls, suggesting these levels of fluid motion resemble near stagnant conditions. However, increases in PCP sensitivity were observed at all intensities of fluid motion used in the current study. Thus, these data suggest that the interaction between fluid motion and PCP toxicity observed in the current study are likely generalizable to natural ecosystems.

This interesting interaction between fluid motion and toxicant sensitivity merits further study. Our results have implications for a wide range of low Reynolds' number aquatic organisms, from phytoplankton to larger zooplankton species such as *Daphnia*. In addition, turbulent fluid motion may have effects on toxicant sensitivity of larger animals as well by affecting toxicant uptake across respiratory surfaces such as fish gills. However, the organisms to which these results can be generalized and the mechanism(s) responsible for fluid motion-induced alterations in toxicant sensitivity needs further clarification. A number of experimental approaches could be productive, including correlating metabolic rates with turbulence levels and toxicant effects, measuring tissue concentrations of toxicants under different flow regimes, or identifying differential toxicant effects at different food concentrations and flow regimes. In addition, methods are available that allow direct quantification of fluid motion (Peters and Redondo, 1997; Weissburg, 2000). Clarification of how fluid motion modifies the toxicant sensitivity of aquatic organisms is necessary given the importance of standardized toxicity tests in performing ecological risk assessments and determining safe environmental concentrations of toxicants. The failure to use ecologically relevant toxicity data may impede attempts to preserve the health and sustainability of aquatic ecosystems.

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