The early warning of aquatic organophosphorus pesticide contamination by on-line monitoring behavioral changes of *Daphnia magna*

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Abstract In this paper, the movement behavior of *Daphnia magna* was studied as a bio-indicator of organophosphorous pesticide (OP) contamination, using an on-line bio-monitoring method, the Multi-species Freshwater Biomonitor®. A static test of acute toxicity test revealed the 24-h and 48-h LC_{50} values (95% confidence limit) for *Daphnia magna* to be respectively 0.45 μg/l and 0.21 μg/l for dipterex 3.80 μg/l and 0.90 μg/l for malathion, and 1.25 μg/l and 0.38 μg/l for parathion. The behavior strength of *Daphnia magna* was a sensitive indicator of sublethal OP stress and resulted in significant concentration–response relationships for the three OPs. Increasing OP concentration will result in more intensive behavioral responses of *Daphnia magna* and shorter response time, which could be described by the Stepwise Stress Model (SSM). Therefore movement behavior can be effectively applied in early warning of environment quality by on-line bio-monitoring. The intensive changes in behavior strength of *Daphnia magna* over a short time follow the SSM concept and can be used as an indicator of early stress response to OP accidental contamination.

Keywords Organophosphorous pesticide ·
Daphnia magna · On-line bio-monitoring ·
Behavior strength · Stepwise Stress Model ·
Aquatic environmental accidents

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Introduction

Organophosphorous pesticides (OPs) are among the most potent insecticides known and have been used throughout the world to control pests in agricultural crops, forests, and wetlands for more than four decades (Smith 1987). Their wide use is due to high toxicity and rapid environmental degradation (Eto 1974). Unfortunately, OPs lack target specificity and can cause severe and persistent population effects on aquatic non-target species, particularly invertebrates (Fulton and Key 2001; Schulz and Liess 1999). Extensive use of OPs and the release into aquatic environments may alter the balance of biological communities.

OPs can be taken up by organisms through the skin, the respiratory system and the digestive tract. They then react with acetyl cholinesterase (AChE) to inhibit the activity of the enzyme, which may induce

the loss of the nerve conduction ability (Christopher et al. 2001). In invertebrates, AChE inhibitors are known to exert their toxicity on the central nervous system (Eldefrawi 1985). AChE inhibition may cause hyperactivity, loss of coordination, convulsions, paralysis and other kinds of behavioral changes (Kuhr and Dorough 1976). Hence, it may be appropriate to assess OP toxicological impact by detecting the behavioral changes in a non-target invertebrate species.

Daphnia magna, a small planktonic invertebrate crustacean (0.5–5.0 mm) with a short life cycle, is very sensitive to changes of the chemical composition in aquatic environments. *Daphnia magna* is a standard organism for toxicity tests, and much literature exists on its response to different types of toxins (Gerhardt et al. 2005; Knops et al. 2001; Peters and De Bernardi 1987). The species has often been used in bioassays and environmental monitoring of aquatic systems due to the ease and economy of maintaining cultures (Tomasik and Warren 1996). Some studies showed that *Daphnia magna* was very sensitive to ethyl parathion (Sturm and Hansen 1999), as it often inhabits small water bodies in and around agricultural fields receiving OP treatments (Peters and De Bernardi 1987). In addition, the movement patterns of *Daphnia magna* have many different components.

Stress is not something to be avoided, but the nonspecific response of an organism to any demand made upon it. A lower limit or "stress threshold" may be defined. Stress, including stress due to toxicants, will initially evoke an "alarm reaction" but no organism can maintain a chronic state of alarm and it will eventually enter a phase of adaptation or resistance (Selye 1973). The external manifestation of stress typically involves physiological responses mediated by chemical messengers and the nervous system. An organism may respond to a stressor in many ways, including changes in movement patterns.

Motility is a characteristic feature of many organisms that cannot be neglected as an important physiological factor in survival. Changes in the movement behavior of organisms can therefore be used as a suitable indicator in ecotoxicological risk assessment (Harald and Donat-P. 2001). Behavioral change in organisms associated with stress and toxicant exposure provides novel information that cannot be gained from traditional toxicological methods, which include short-term and median lethal exposure effects, and the potential for mortality (Andrew et al. 2004). From the

1980s forward, a great deal of research on fish movements has been done to evaluate environmental stress (Park et al. 2005; Steinberg et al. 1995; Yachida et al. 1981). There are also some studies on the consequences of perinatal exposure to OPs on invertebrate behavior (Arthur et al. 1983; Roast et al. 2000). The progress of behavioral toxicology as a consensus-based discipline has been hindered by a lack of test standards, homogeneity of samples, variation in measured endpoints, and small sample sizes. Therefore, no generally accepted model for movement based on behavioral change under OP stress has yet been produced.

Though behavioral responses of *Daphnia magna* and other aquatic organisms to some environmental contaminants have already been investigated (Andrew et al. 2004; Harald and Donap-P. 1999; Hubert et al. 2003), hardly any literature is currently available which deals with behavior strength changes of aquatic invertebrates under contaminant stress.

In the present studies, bioassays were carried out to assess the acute effects of three model OPs: Dipterex ($C_4H_8Cl_3O_4P$), malathion ($C_{10}H_{19}O_6PS_2$) and parathion ($C_{10}H_{14}O_5NSP$) on behavior strength of *Daphnia magna*. The effects of the OPs on the movement behavior of *Daphnia magna* were observed using the movement signal detected by a Multispecies Freshwater Biomonitor® (MFB, Limico International, Germany). Finally, a behavioral change model for *Daphnia magna* was constructed as an early warning system of aquatic OP contamination.

Materials and methods

Daphnia magna culture

The experimental *Daphnia magna* (24-h young) were obtained from the Chinese Environmental Science Academy and cultured in our laboratory for more than three generations.

The culture was maintained at $20 \pm 2°C$ under a 16-h light: 8-h dark photoperiod (illumination ranged between 3,000 and 4500 lx). Culture medium was prepared according to the components of the Standard Reference Water (SRW) (ISO 1996a) and *Daphnia magna* were fed *Scenedesmus obliquus*.

Before the exposure experiments, the gravid female *Daphnia magna* were taken out and cultured individually in 50-ml glass beakers of SRW until they

oviposited. As *Daphnia magna* neonates from the first brood are usually the smallest (Lampert 1993) and much more sensitive to different growth media, healthy neonates (about 24 h) were always taken from the second and following clutches as recommended (APHA 2001). During the individual culture of female *Daphnia magna* with eggs, the growth medium was renewed regularly and *Daphnia magna* were fed *Scenedesmus obliquus* algae three times a week. Before feeding *Daphnia magna*, the culture medium of *Scenedesmus obliquus* was filtered and then diluted by SRW until the concentration reached 1×10^5 cells/ml. The quantity of the algae was about 1% beaker volume.

Sample preparation

Dipterex (GSGB23022-92, 100 mg/ml × 1 ml), malathion (GSGB23018-92, 100 mg/ml × 1 ml) and parathion (GSGB23009-92, 100 mg/ml × 1 ml) were purchased from the Standard Sample Center of People's Republic of China.

Dimethyl sulfoxide (DMSO, analytical pure) was compatible with earlier ecotoxicity assessments (De la Torre et al. 1995), so the solvents of the three samples were added to 1 ml DMSO. The samples were stored at 4°C until use.

The control concentration of DMSO was less than 0.5% in the tests. Studies showed that DMSO of such concentration would neither lead to acute toxicity to *Daphnia magna* nor affect the mobility of *Daphnia magna* (Sandbacka et al. 2000).

Acute toxicity tests

Preliminary acute toxicity tests were conducted in order to evaluate the effects of different OPs on the movement behavior of *Daphnia magna*. The acute test was performed in accordance with the standard protocol for *Daphnia magna* acute test (ISO 1996b). 24-h and 48-h static exposure experiments were performed to assess the environmental risk of the three model OPs. During the studies, laboratory conditions were kept the same as in the culturing room. All determinations were repeated three times. The evaluation of median lethal concentrations (LC_{50-24} and LC_{50-48}) of dipterex, malathion and parathion to *Daphnia magna* was regarded as an elementary step before studying the median lethal and sublethal behavioral changes under OP stress. During the exposure experiments, no food was added. Death of *Daphnia magna* was defined according to Villegas-Navarro et al. (1999) as the inability to swim for more than a few strokes within 15 s after gentle agitation of the test vessel.
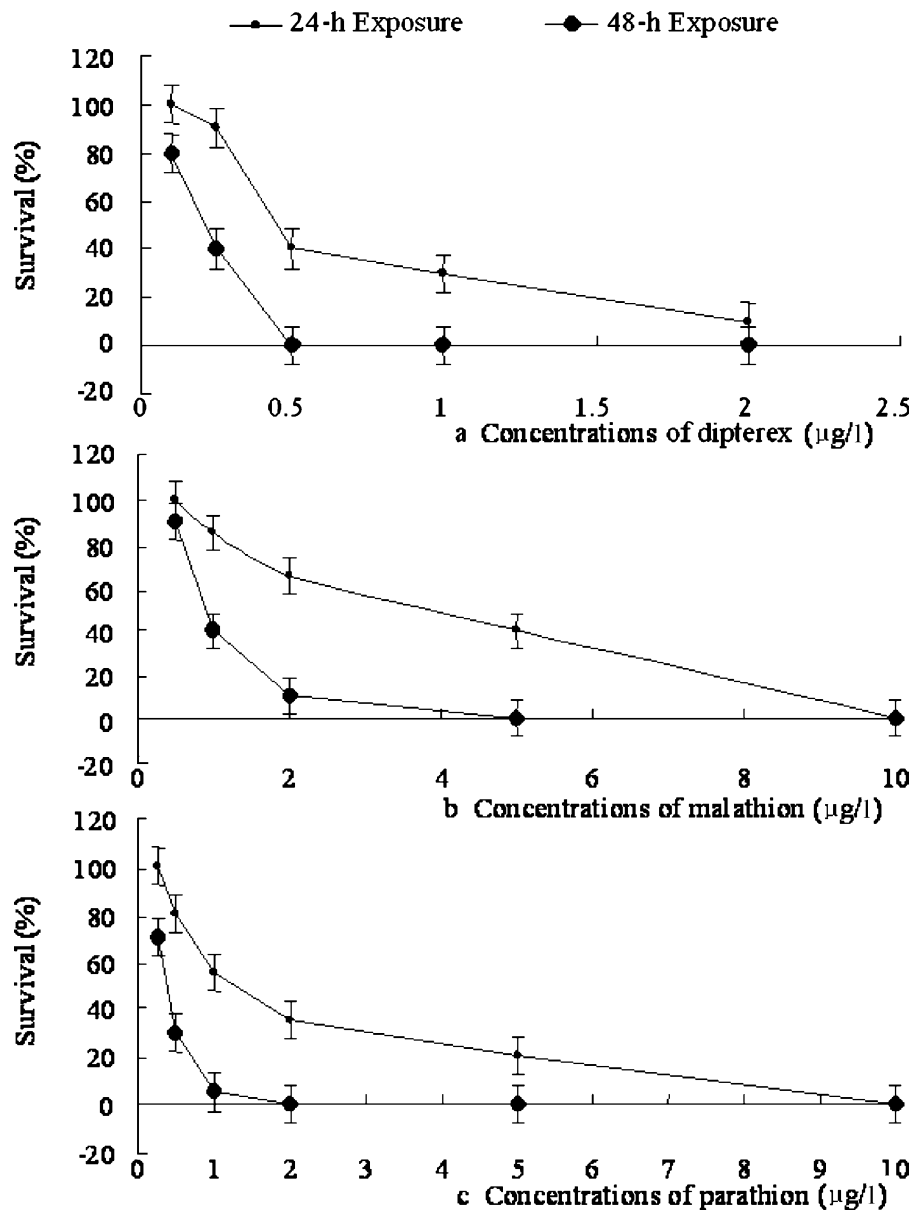
Behavioral change analysis

Multispecies Freshwater Biomonitor® (MFB) is an online instrument for continuous water control. The MFB can be used as an early warning system by detecting changes in behavior of selected bioindicators to demonstrate degradation of the water quality. The measurement is based on an easy and robust measurement technique, allowing for the use of selected animal species up to 96 measurement channels (Gerhardt et al. 2002). The biosensor of MFB is located in the test chamber. There are two pairs of electrodes at the walls of each test chamber. One pair sends a high frequency signal of alternating current, which is then received by the second pair of non current-carrying electrodes. The sending electrodes work in synchrony and hence avoid interferences between the channels. Movements of the test animals cause changes in the amplitude of the generated sinus signal, dependent on both the degree of activity of the animal and the size of the animal relative to the size of the chamber. Translated by discrete Fast Fourier Transform (FFT) designed in MFB, movement frequency signal was changed into time series data simultaneously. It provided a histrogramme of different frequencies per signal, where the height of the bars indicated how much time the animal spent on each frequency.

In situ long-term operation of MFB was carried out to obtain the behavioral change data. The monitoring data were analyzed using functions of "alarm generation", which is based on the alarm algorithm in an ARIMA-model, of MFB software package. The average values of every six long-term monitoring data records for 48 h exposure and every three data records for 24 h exposure experiments were obtained automatically to calculate the motility of tested organisms, and the data were recorded as continuous number signal transformed by FFT.

Groups of experimental *Daphnia magna*, each consisting of 10 individuals, were selected at random and placed in the smaller test chamber (Biosensor,

3 cm long, 2 cm in diameter) of the MFB, which was closed off on both sides with nylon nets (250 μm). The behavioral change studies were carried out in an airtight aquarium in order to decrease the effects of in vitro environmental factors on the movement behavior of *Daphnia magna*. No food was added during the experiments.

A 48-h exposure experiment was carried out to evaluate the effects of different OPs on the movement behavior of *Daphnia magna* detected by the MFB. The concentration settings of OPs were based on the results of acute toxicity tests.

In this study, behavior strength, as the main behavior parameter of *Daphnia magna*, was determined by changes in different frequency movements during a 48-h exposure period.

Behavior strength is one of the behavior parameters of organisms with motility. It is not a specific aspect, but an integrative reflection of all instantaneous motions, e.g. swimming velocity, behavioral frequency, and movement extent and so on. In this study, full behavior strength was regarded as 1 unit. Using the MFB, one can obtain the change of behavior strength over time, and from the change, the early warning of

Table 1 *Daphnia magna* acute test: LC$_{50}$ values (µg/l) for each organophosphorous pesticides

Organophosphorous pesticides	Concentration levels	LC$_{50-24}$*	LC$_{50-48}$*
Dipterex	0.10–5.00	0.45	0.21
Malathion	0.50–10.00	3.80	0.90
Parathion	0.25–10.00	1.25	0.38

*mean value of LC$_{50}$ and with 95% confidence limit

environmental contaminants could be made. All the studies on behavioral changes were repeated three times.

In order to further demonstrate the relationship between behavior strength change and the OP concentration in water bodies, additional experiments were conducted using OP concentrations based on the existing environmental quality standard for surface water in People's Republic of China (GB3838-2002 of China P. R. 2002).

Statistical analysis

For the purpose of this study, all of the statistical data was treated by SPSS 13.0 using a statistical criterion of $p<0.05$. In this study, the average values are presented. Probit analyses using SPSS were performed to determine the acute toxicity of dipterex, malathion and parathion to *Daphnia magna* (LC50 for each OP). One-way analysis of variance (ANOVA) was performed to compare the effects of median lethal OP stress on the behavior strength of *Daphnia magna* with the controls.

Results

Acute toxicity tests

In 24-h and 48-h acute toxicity tests with neonates, the mortalities of *Daphnia magna* in different water bodies were first measured to evaluate LC$_{50}$. Control groups were tested in a solvent solution (SRW99.5%+ 0.5% DMSO). The measured concentration levels and the survival of *Daphnia magna* are shown in Fig. 1, and the calculated LC$_{50}$-24 h and LC$_{50}$-48 h values with linear interpolation are shown in Table 1. The slopes of the probit regression lines were uniform, suggesting that the concentration–response relationship was similar for different OPs.

Table 2 The effects of median lethal organophosphorous pesticide stress on the behavior strength of *Daphnia magna*

Organophosphorous pesticides	Concentrations (µg/l)	Mean values of behavior strength (M±S.D.)						
		0.5 Hz*	1.0 Hz*	1.5 Hz*	2.0 Hz*	2.5 Hz*	3.0 Hz	Above 3.0 Hz
Dipterex	0.21	0.38±0.24	0.28±0.19	0.13±0.13	0.05±0.05	0.02±0.03	0.01±0.01	0.00±0.00
	0.45	0.10±0.21	0.06±0.16	0.02±0.07	0.01±0.02	0.00±0.01	0.00±0.00	0.00±0.00
Malathion	0.90	0.57±0.17	0.51±0.18	0.29±0.14	0.11±0.08	0.03±0.04	0.00±0.01	0.00±0.00
	3.80	0.21±0.27	0.17±0.23	0.08±0.13	0.03±0.06	0.01±0.03	0.00±0.01	0.00±0.00
Parathion	0.38	0.56±0.31	0.43±0.23	0.25±0.17	0.16±0.16	0.07±0.10	0.03±0.05	0.00±0.00
	1.25	0.45±0.29	0.35±0.23	0.17±0.10	0.07±0.06	0.02±0.03	0.01±0.01	0.00±0.00

*showed the differences between the same OPs of different concentrations and $p<0.05$

Fig. 2 The effects of suble-thal dipterex, malathion and parathion on the behavioral changes of *Daphnia magna* *: $p<0.05$
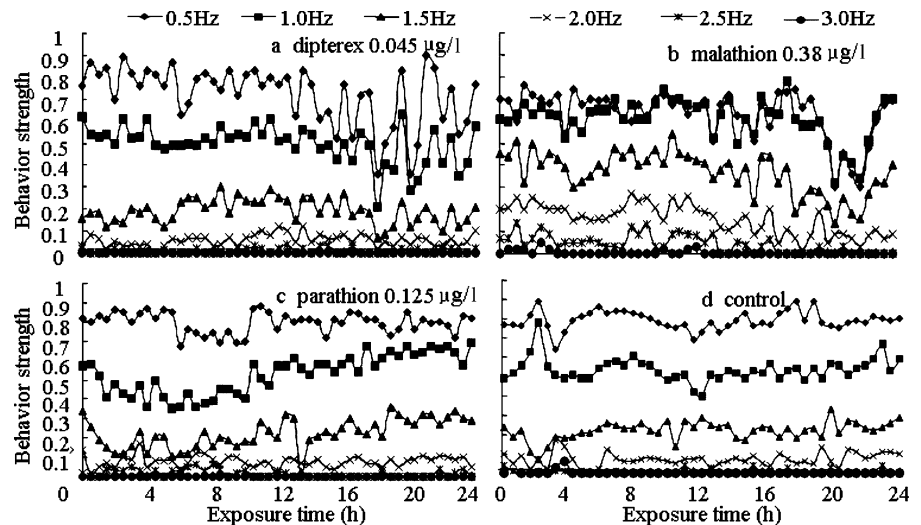
Behavioral change analysis

Sublethal concentrations may be regarded as 10% LC_{50} or less (Kong et al. 2000). For the behavioral experiments, therefore, the 24-h sublethal concentrations were defined as the lower 95% confidence limit of the 24-h LC_{50}. The sublethal exposure concentrations were 0.045 μg/l for dipterex, 0.38 μg/l for malathion and 0.125 μg/l for parathion. Within the lethal exposure range, concentrations of the three OPs (Dipterex: 0.45 μg/l, 0.21 μg/l; Malathion: 3.80 μg/l, 0.90 μg/l; Parathion: 1.25 μg/l, 0.38 μg/l) were selected to carry out the behavioral change studies on *Daphnia magna* under median lethal stress. SRW99.5%+0.5% DMSO was used as the control.

Analyzed by FFT, the movement frequencies were most often measured at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 Hz, with less than 0.1% of movement behavior exceeding 3.0 Hz (Table 2). Therefore, movement behavior between 0.5 Hz and 3.0 Hz was chosen to indicate behavior strength changes under OP stress.

In this study, movement behavior data were taken once an hour for 48 h exposure and twice an hour for 24 h exposure. The continuous number signal translated by FFT in MFB could be exported in the form of Excel worksheet. In movement behavior data processing, the average values are discussed. At first, the synchronous monitoring data of the same movement frequency of three replications for each treatment were averaged, and then the averages of different movement frequencies were used to evaluate the effects of different concentrations OPs on the movement behavior of *Daphnia magna*.

The behavioral responses of *Daphnia magna* to sublethal and median lethal OP stress with $p<0.05$ are shown in Figs. 2 and 3. Within the control group, no significant changes in behavior were detected. In the case of OP exposures, the results demonstrate that the changes of movement behavior coincided at the different frequencies for the different OPs; that is, movements of the same frequency changed to the same extent. The changes of behavior strength depended mainly on the lower frequency movements (0.5 Hz and 1.0 Hz). And, in general, the relationship between behavior strength and movement frequency was inverse: the higher the movement frequency, the lower the behavior strength. Behavioral changes showed an evident concentration–response relationship with median lethal OP stress (Table 2). The movement behavior of different frequencies changed simultaneously, and higher concentrations of dipterex, malathion and parathion resulted in a greater degree of the behavioral changes, as well as a shorter response time for behavioral changes.

The changing trends of behavior strength to median lethal OP stress were the same as in higher concentrations designed according to environmental quality standard for surface water in People's Republic of China (GB3838-2002 of China P. R. 2002) (Fig. 4), with the exception of a shorter response time. Even under the sublethal stress, the behavioral strength changes of *Daphnia magna* were evident (Fig. 2),

Fig. 3 The effects of median
lethal dipterex, malathion and
parathion on the behavioral
changes of *Daphnia magna*
*: *p*<0.05
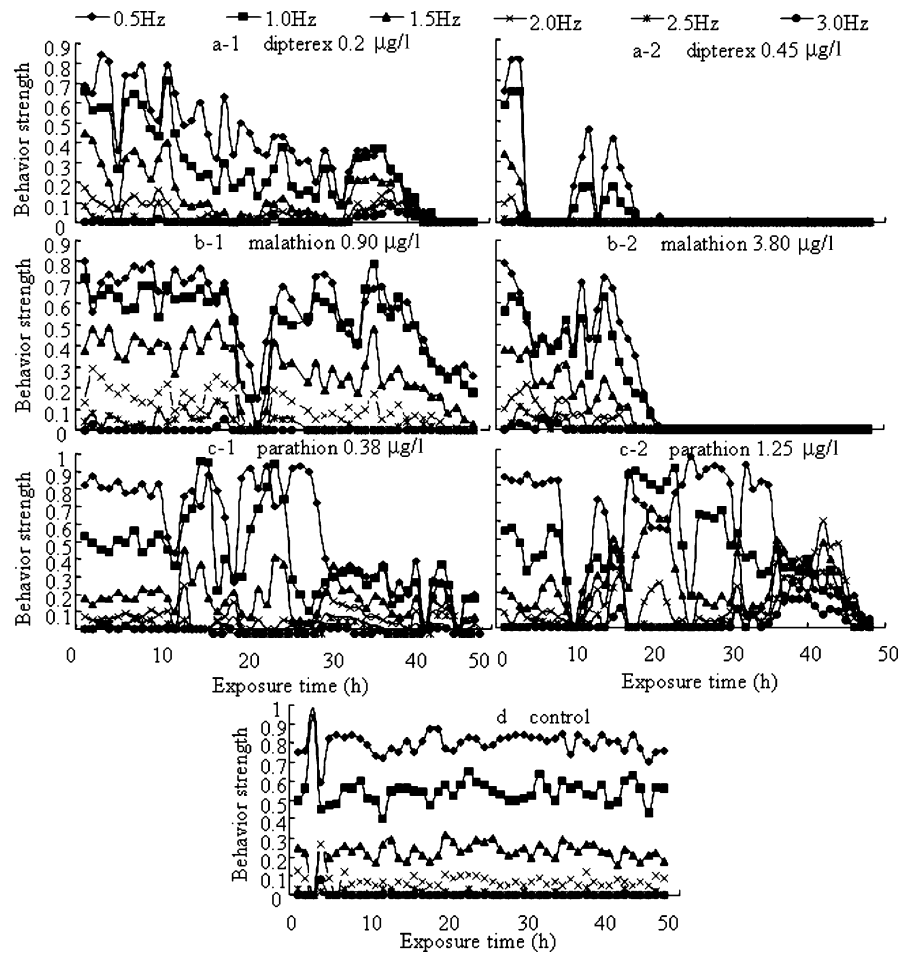
which showed that the behavior strength was more
sensitive in monitoring aquatic environment quality
changes than the other parameters in acute toxicity.

## Discussion

Acute toxicity tests

The toxic action of OPs is similar (Christopher et al.
2001; Eldefrawi 1985; Kuhr and Dorough 1976) in
that they inhibit AChE leading to the loss of nerve
conduction ability (Eldefrawi 1985). Tables 1 and 2
demonstrate that the three OPs were highly toxic to
Daphnia magna and that the acute toxicity sequence
was dipterex > parathion > malathion. Other inverte-
brates and some fish also demonstrated the same
order of toxicity among the three OPs, including fresh
water teleost *Saccobranchus fossilis* (Verma et al.

1982), midges (Belden and Lydy 2001), grass shrimp
Palaemonetes pugio (Peter et al. 1998) and goldfish
Carassius auratus (Ana et al. 2004). This suggested
that the acute relative toxicity of the OPs might be
analogous across these species.

Behavioral change analysis

The results of acute toxicity and the behavioral
studies showed that behavioral changes were time-
dependent. There were also obvious concentration–
response relationships between the three model OPs
and the behavioral response of *Daphnia magna*.
Behavioral response of *Daphnia magna* to sublethal
and median lethal OP stress (Figs. 2 and 3) suggested
that whether exposed in high or low concentrations
OPs, the movements of *Daphnia magna* changed
similarly. Additionally, the strength of movement
behavior detected at different frequencies showed a

Fig. 4 The effects of higher concentrations dipterex, malathion and parathion on the behavioral changes of *Daphnia magna* *: *p*<0.05
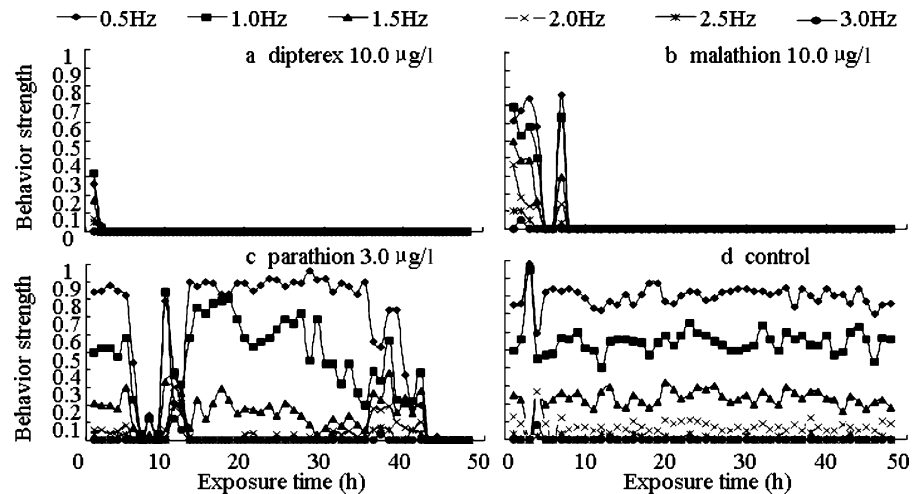
sudden decrease after a certain length of exposure, although after a period of low strength time, motility resumed. At the same time, the readjusted behavior strength showed some differences:

(1) In dipterex exposure, the readjusted behavior strength of different frequencies changed simultaneously. It was obviously lower than the first exposure period and a gradual decreasing trend occurred, e.g. a-1 and a-2 in Fig. 3;

(2) In parathion exposure, the discrepancy between the adjusted behavior strength and start motility was not clear, but after a period of maintenance, there was a sudden decrease to immobility, e.g. b-1 and b-2 in Fig. 3;

(3) In malathion exposure, after motility resumed, the behavioral changes showed greater diversity among different frequency movements. Different frequency movements were displayed separately. Behavioral strength sometimes decreased or increased suddenly. After a period of maintenance, a second depression occurred, and occasionally, motility resumed afterward, e.g. c-1 and c-2 in Fig. 3. These changes were different from the regular ones in other exposures (a-1, a-2, b-1 and b-2 in Fig. 3).

(4) The behavioral response trends of *Daphnia magna* to sublehtal OP stress (Fig. 2) were similar at median lethal concentrations and the degree of change showed significant concentration–response relationships.

The movements of *Daphnia magna* changed more significantly at higher concentrations than under median lethal OP stress (Fig. 4). This result suggested

that the movement behavior of *Daphnia magna* could be feasible as an early warning system for aquatic environmental quality by on-line monitoring behavior strength changes, according to the requirements of the environmental quality standard for surface water of People's Republic of China.

Stepwise Stress Model (SSM) under OP stress

The Stepwise Stress Model (SSM), described by Gerhardt (Gerhardt 1999, 2001; Gerhardt et al. 2005, anticipates that with either increasing toxicant concentration or exposure time, a cascade of regulatory behavioral stress responses are activated and performed by the organisms. SSM postulates that an organism displays a time-dependent sequence of different regulatory or compensatory behavioral stress responses during exposure to pollutants above their respective thresholds of resistance. An increasing stress stimulus provokes regulatory responses (loading stress). Above a certain stimulus level, however, several reactions are possible: (1) the homeostasis cannot be maintained and a toxic effect in
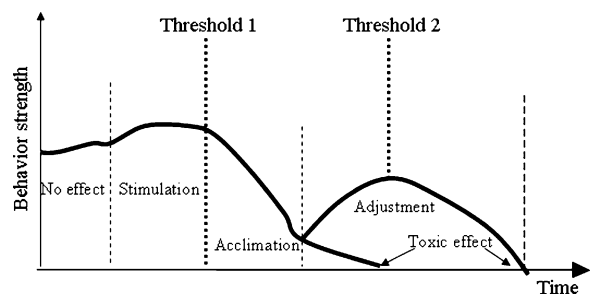
Fig. 5 Stepwise Stress Model (SSM) under OP stress

the organism occurs (limiting stress), (2) the organism can acclimate to the increased stress level and (3) the organism decreases the performance of the response and increases the performance of another response to the stimulus. If the first stress response decreases to less than the original level, a toxic effect occurs.

According to the relationship between the exposure time and the movement behavioral changes of *Daphnia magna* in each kind of OP, along with the behavioral change trends shown in Figs. 2, 3 and 4, we found that the behavioral change trends of *Daphnia magna* were consistent with SSM and proved SSM for the first time for OPs in *Daphnia magna*, by showing changes in behavioral strength (movements characterized by different signal frequencies). Based on the reports of Gerhardt in 1999 and 2001, the theoretical reactions of *Daphnia magna* in a certain behavior under OP stress that included the categories No effect, Stimulation, Acclimation, Adjustment, and Toxic effect are shown in Fig. 5.

In theory, the first behavior modulation of *Daphnia magna* was to increase the strength of all frequency movements presumably to try to escape from the polluted aquatic environment (Avoidance behavior), which explains the behavioral changes observed in Stimulation after No effect. Presumably, certain OP concentrations would be too high for *Daphnia magna* to start Avoidance behavior because this type of behavior was absent or of short duration at high OP concentrations (Fig. 4). In these cases, behavior strength tended to decrease gradually until the ability for movement was lost. In other cases, *Daphnia magna* were apparently able to cope with the neurotoxic stressors and increased behavioral strength. Associated with this response, the length of Stimulation was close to the expected value, about 1–3 h (Fig. 3) and it was in inverse ratio to the OP concentrations.

Stimulation was usually followed by Acclimation which was a continuous weakening of behavior strength. The main reason for the fall of movement behavior in Acclimation was that the behavior adjustment reached extreme of "Alarm reaction" (Threshold 1). As time passed, stress gradually decreased the motility of *Daphnia magna*.

After Acclimation, movement behavior split in two directions: First, if *Daphnia magna* could not overcome Threshold 1, Toxic effect would occur. Secondly, if *Daphnia magna* lived through Threshold 1, the movement behavior strength resumed in a short time as shown in Adjustment and there would be a second extreme of "Alarm reaction" (Threshold 2) until Toxic effect occurred.

Based on these results, "avoidance behavior" is quite appropriate to be used in early warning pollution stress responses. Almost all aquatic animals have the ability of actively escaping from a polluted environment to an unpolluted area (Robin and Don 1971). A trend for behavior modulation to maintain a stable internal environment and diminish dependence on an external environment was also shown (Putman and Wratten 1984).

Conclusions

This study proved behavioral responses of the freshwater invertebrate cladoceran *Daphnia magna* to be fast and sensitive. Median lethal and sublethal bio-indicators to OP stress and the movement behavior under OP stress showed regular changes. Movement behavior can be effectively applied in early warning of environment quality by on-line bio-monitoring. The intensive changes in "behavior strength" of *Daphnia magna* over a short time follow the SSM concept and can be used as an indicator of early stress response to OP contamination. This knowledge could be applied to monitor aquatic environmental accidents. If in the future, relationships between median lethal contaminant stress and behavior responses could be described by a mathematical formula, early warning systems would be greatly improved. To this aim, more studies regarding the behavioral changes of invertebrates under different types of contaminant stress are needed to further prove the SSM.

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