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The Stepwise Behavioral Responses of Medaka (*Oryzias latipes*) to Organophosphorus Pesticides in an Online Monitoring System

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Abstract

The stepwise behavioral responses (SBR) of medaka (*Oryzias latipes*) were continuously monitored by an online monitoring system (OMS) after exposure at low concentrations of Trichlorfon (T), Parathion (P) and Malathion (M). The Self-organizing map (SOM) was used to train behavior strength (BS) measured by OMS according to different chemicals and concentrations. Behavioral difference was observed according to different levels of concentrations, and some differences were observed according to chemicals and concentrations. Differences in photo- and scotophases were also observed in control and treatments with lower concentrations and short exposure at high concentrations. Meanwhile, the changes of BS demonstrated SBR model correctly. SBR of medaka would be useful for presenting behavioral states in responses to treatments. The SBR model supported and developed the Stepwise Stress Model (SSM), which included No effect, Stimulation, Acclimation, Adjustment (Readjustment) and Toxic effect.

Keywords: Stepwise Behavioral Responses; Medaka (*Oryzias latipes*); Organophosphorus Pesticide; Behavior Strength.

1. Introduction

Numerous pesticides pose incidental and potential threats to non-target organisms as negative consequences consulting environmental pollution and damaging people's health. As one of the most efficient and important agents, organophosphorous pesticides (OPs) have been used throughout the world

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to control pests in agricultural crops, forests, and wetlands for more than four decades [1]. The widely used OPs can exert their toxicity by inhibiting cholinesterases (ChE) of organisms [2-5]. Extensive use and discharges of OPs in environment, however, may impair biological communities. Accidental release into source water may also cause unpredictable toxicity to human and numerous biological organisms. Thus, it is necessary to have a clear understanding of effects of these substances in the aquatic environment, and to monitor accurately pesticides released in aquatic ecosystems.

Among all of the test endpoints, behavioral responses can be used as a suitable indicator in OP toxicological impact assessment. Due to ChE inhibition, OPs may cause hyperactivity, loss of coordination, convulsions, paralysis and other behavioral changes [6]. According to numerous accounts of research [7-14], behavioral responses were reported to be an adaptive behavior to reduce exposure to harmful conditions. Failure to avoid exposure, however, may cause reduction in fitness and survival.

Medaka (*Oryzias latipes*) was used as indicator in this study and has been reported as a suitable indicator in risk assessment regarding availability of biological information, physiological/behavioral regulation and vulnerability to chemical stress [15-17]. Medaka is listed as a standard test species in the OECD guidelines [18]. Medaka has been used in continuous monitoring with computational methods [19-22]. The progress of behavioral toxicology, however, was hindered by a lack of test standards as a consensus-based discipline, due to homogeneity of samples, and variation in measured endpoints. It is essential to provide an objective and comprehensive ground to evaluate behavioral responses. The stepwise behavioral response (SBR) could be a candidate for assessing toxic effects on test animals through continuous changes of behavior responses detected by an online monitoring system (OMS).

In this study, medaka was continuously exposed in three chemicals, Trichlorfon (T), Parathion (P) and Malathion (M) in an OMS. Subsquently behavioral data were trained by Self-Organizing Map (SOM) to differentiate behavioral responses according to different chemicals and concentrations. Then the SBR model was identified with differences in behavior strength. Experiments with continuous treatment along with computational methods were illustrated in objectively characterizing complex behavioral data in response to different chemicals and concentrations.

2. Materials and Methods

2.1. Equipment

Behavioral responses of medaka was monitored by an online monitoring system (OMS) built in Chinese Academy of Sciences [23]. Test organisms were placed in a flow-through test chamber (7cm long, 5cm in diameter), which was closed off with nylon nets (250 μm) on both sides. One pair of electrodes located at the walls of the test chambers, and sent a high frequency signal of altering current, which was received by a second pair of non current-carrying electrodes [24]. Overall response activity of test organisms is transformed by the A/D transformer and was analyzed by software installed in the equipment (Fig 1.). Deatiled mechanism in OMS can be referred to Ren and Wang [25]. We define the data recorded by OMS as behavior strength (BS) in the chamber. BS was automatically sampled by OMS in each second, and average values in every 6 minutes were produced as output and were used to present behavioral changes. BS ranged from 0 (Loss of motility) to 1 (Full behavior express). The judgment of significant decrease of BS (SD-BS) was made when difference of BS (30 min) mean values were greater than or equal to 20% [26].

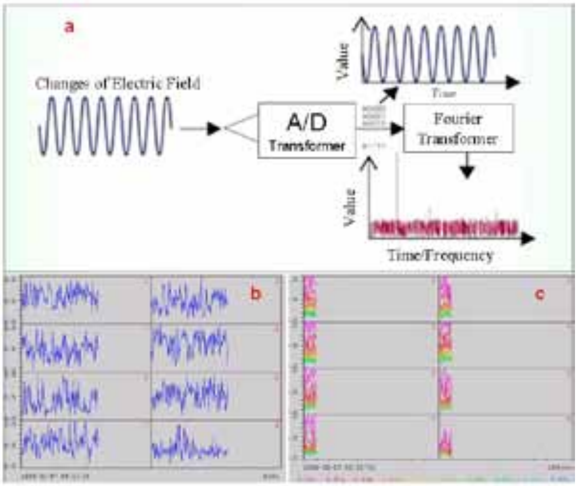

Fig. 1. Aignal acquisition and transmission in OMS. (a) The signal acquisition of BEWS, (b) The normal signal analysis (BS) and (c) The signal analysis after Fast Fourier Transform

2.2. Test species

The individuals of medaka fish were kingdly provided by the Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences (Beijing, China). The brood stock was raised in flow-through system with dechlorinated tap water (using active carbon) at a constant temperature of 20±2°C, and has been maintained in our laboratory for more than three years. Control the culturing photoperiod of 16 h light (04:00-20:00, with 4000 lux light intensity) and 8 h dark (20:00-04:00, no light given). The brood stock was fed with newly hatched brine shrimp in the morning and flake food (Trea®, Germany) in the afternoon. By 15 days later after hatching, medaka was fed 2 times every day. Medaka individuals (2 months) about 2.5~3.0 cm long were selected as the test organisms.

2.3. Test chemicals

Trichlorfon (T), Parathion (P) and Malathion (M) were purchased from J&K Chemical Ltd. All compounds were technical grade (>95% purity). Stock solutions (stored at 4°C until use) with proper concentrations were prepared with dimethyl sulfoxide (DMSO) as a solvent, and were accordingly diluted with appropriate aliquots. All solvents were of analytical grade. The concentration of DMSO used for test was less than 0.5% in all experiments, which would neither lead to acute toxicity to medaka nor affect the mobility [27].

2.4. Experimental setup

BR monitoring was carried out under flow-through conditions. Three healthy medaka were selected at random for each test chamber and three test chambers were used for each treatment. The flow rate of each test chamber was controlled about 2 liters per hour, which was proved to have no effect on the motility of test organisms [28]. During observation period, no food was provided to test medaka. Observation started in 16:00, and finished in 16:00 of the third day for all chambers. Light and temperature conditions were the same as given in rearing stock populations.

Exposure of T, P and M was conducted for 48 hours to investigate medaka SBR under flow-through conditions. Chemical Toxic Unit (TU) to test organisms was used for comparison of toxic effects and the 48-hour median Lethal Concentration (LC50-48), for instance, was taken as one unit (1 TU) [31]. The calculation of TU value is shown as follows:

$$TU = \sum \frac{C_i}{LC_{50}^i} \quad (1)$$

where Ci was the total concentration of chemicals, LC50i was the 48-hour median lethal concentration. Sublethal concentration was regarded as 10% LC50 or less dose [29]. Four concentrations of 0.1TU, 1 TU, 5 TU, and 10 TU were tested respectively with each chemical.

Based on the previous reports [30, 31], the LC50-48 of T, P and M, which was regarded as 1TU in this study, was 10 mg/L, 2.9 mg/L, and 0.75 mg/L respectively.

2.5. Self-organizing map (SOM)

Output data from OMS were analyzed with SOM to provide overall patterns of behavior in response to chemicals.

×10 nodes was used in this study.

The Euclidian distance (dj (t)) at the j-th node on the SOM between weight at iteration time t and the input vector was calculated through learning processes:

$$d_j(t) = \sum_{i=0}^{p-1} [x_i - w_{ij}(t)]^2 \quad (2)$$

where xi is the value of the i-th parameter, wij(t) is the weight between i-th parameter and the j-th node on the SOM and P is the number of the parameter.

The best matching neuron, which has the minimum distance, was chosen as the winner. For the best matching neuron and its neighborhood neurons, the new weight vectors are updated as:

$$w_{ij}(t+1) = w_{ij}(t) + a(t)[x(t) - w_{ij}(t)] \quad (3)$$

where t is the iteration time and α(t) is the learning rate. The learning process of the SOM was conducted using the SOM Toolbox developed by the Laboratory of Information and Computer Science, Helsinki University of Technology in Matlab environments [33]. The initialization and training processes followed suggestions by the SOM Toolbox by allowing optimization in algorithm. Detailed description regarding application of the SOM to behavioral data can be referred to other relative reports [34, 35].

As the input data were provided to the SOM for training (equation 3), the weights of the best matching unit and computation nodes close to it were adjusted towards the input vector through interactive calculation. In order to reveal the degree of association between the SOM units, the Ward's linkage method was used to cluster the movement data based on the dendrogram according to the Euclidean distance [36, 37]. The linkage distances were rescaled in 0%-100%.

Based on the solutions presented in Rabiner [38], the process was conducted with the programs provided in the HMM toolbox (MATLAB7.8, The Math Works, R2009).

3. Results and Discussion

3.1. The effects of OPs on medaka BR

After SOM, medaka BR was shown according to different OPs (Fig 2.). According to cluster analysis, four groups were identified based on the mean values of BS (Fig 2. (a, b)). Cluster 4 at the bottom left corner of SOM presented initial period of OP treatment (Fig 2. (a), from 16:00 to 20:00). Cluster 3 and 2 reflected subsequent exposure time from 20:00 to the next day 16:00. Clear time differentiation between cluster 3 and 2 was not observed. But a loop was observed according to time flow (Fig 2. (b)). Cluster 1 matched to the final period from the second 16:00 to the third day (16:00). During the exposure time series cluster order was 4, 3, 2 and 1. Exposure time was also in accordance with photoperiod (Fig 2. (c)). The cluster distances according to the Ward's linkage method indirectly suggested closeness between clustered groups. It was notable that the patterns were mostly variable along with diagonal line matching to intermediate period after exposure and that some patterns were in the farthest position at upper-left corner from the cluster of initial period at bottom-right corner.

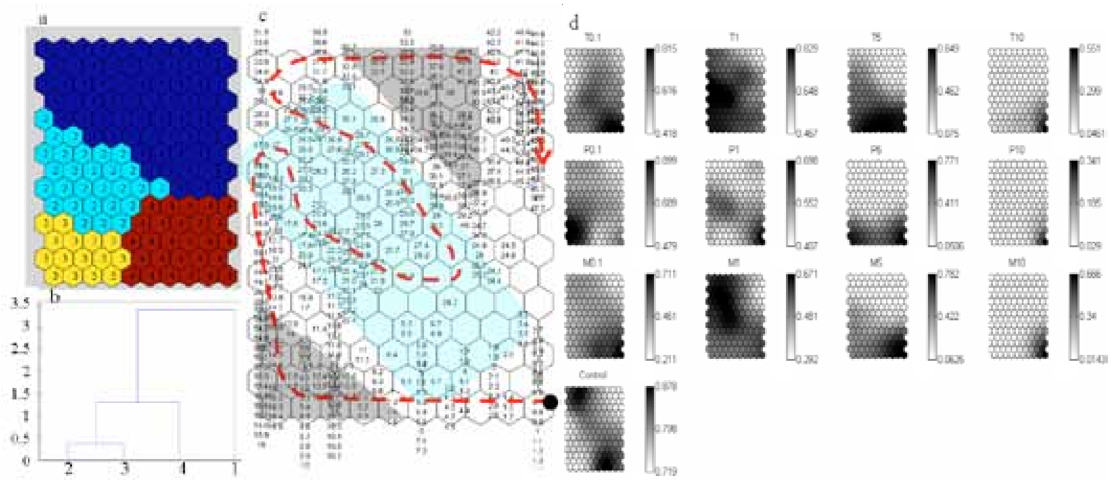

Fig. 2. Clustering of BS on SOM in different OPs and different concentrations. The SOM showed different clusters based on BS in different OPs and different concentrations: (a) Four clusters classified by the SOM; (b) Cluster distances according to the Ward's linkage method; (c) Cluster along with time series (Black dot indicating the starting position). The black shadow means the scotophase, while the gray shadow indicates the photophase and (d) Profiles of BS values visualized on the SOM (a) in different OPs. T0.1 means the exposure in 0.1TU Trichlorfon, and the values in the vertical bar indicate range of the mean values of BS.

Fig 2. (d) showed the profiles of BS values visualized on the SOM (Fig 2. (a)) in different OPs. The values in the vertical bar indicate the range of BS mean values according to different concentrations and different time period. In the control group, BS ranged between 0.719 and 0.878, and higher values were observed at two corners of upper-left and bottom-right areas of SOM (Fig 2. (d)). Degree of changes was relatively lower than that by treatments. By matching with Fig 2. (c), BS changes of medaka showed an evident rhythm: higher BS value matched to the light photoperiod along with diagonal line from upper-left to bottom-right, while the lower values were in accordance with area of SOM in the dark period. At the end of the exposure at upper-right corner, though it was in photophase, BS values was lower than the area long with diagonal line due to no food supplied in 48 hours.

While BS showed some differences among different OPs gradient was observed in different concentrations. The profiles of BS on SOM were variable depending upon levels of concentration. At low levels, profiles were different according to different OPs, but the values were lower, which suggested that the circadian rhythms could affect medaka BS in lower concentration exposure, and even sublethal concentration could induce behavior toxic effects. Significant BS decrease happened in cluster 1 and 2 in 5 TU exposure, and there were no evident circadian rhythms. Meanwhile, medaka BS dropped to less than 0.1 soon after the start of 10 TU exposure and no circadian rhythms was showed.

Fig 3. showed the exposure time of medaka in different OPs before the first SD-BS. The results suggested that the exposure time was strongly depended on exposure concentrations. The first SD-BS kept similar at the same concentrations in different OPs.

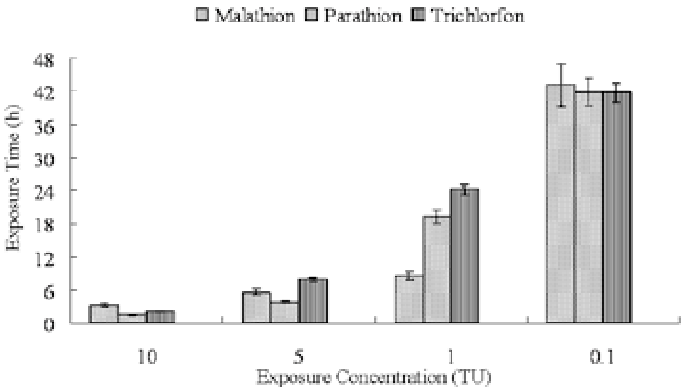

Fig. 3. the exposure time of medaka in different concentration OPs before the first SD-BS

After the first SD-BS, BR of medaka were different in different concentrations: in 10TU, BS dropped suddenly to less than 0.1, which suggested that test medaka might lose movement; in 5TU, BR showed apparent stepwise modulation, which included Adjustment and Readjustment till the disappearance of behavior movement; in 1TU and 0.1TU, BR showed more and more stepwise process. It was concluded that SBR was very important to help medaka adapt to the environmental stress, especially in lower concentrations.

3.2. Medaka SBR

The effects of T, P and M on medaka BR in different exposure time were showed in Fig 4. Medaka BS in the control kept about 0.8, which was similar to the start period of every exposure (the first 6 min). In the same chemical treatments, different concentration and different exposure time induced different BS. Meanwhile, the results suggested that the tendency of medaka BR in the exposure of different OPs was similar. In the same treatment, medaka BS decreased with exposure time and in the same exposure time, it decreased with the increase of OP concentration. The results of continuous exposure in OPs were that medak BS went to less than 0.1, and during this proceed, BR of medaka did not show a smooth change way in gradually decrease, but with several adjustments/readjustment, which illustrated that BR was stepwise modulation during OPs exposure in different exposure time.

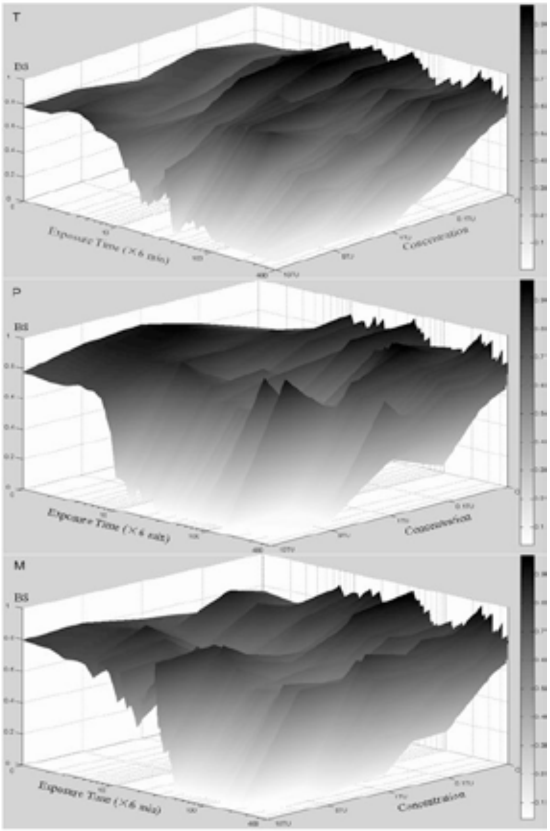

Fig. 4. the effects of different treatments on medaka BR in different time. Exposure time was showed as log value, and BS values were used to statistic the tendency of medaka BR in different OPs. T, P and M meant the effects of Trichlorfon, Parathion, and Malathion separately on medaka BR.

These results suggested that different environmental stress in different exposure period dicided medaka BR. In lower concentration treatments, e.g. 0.1 TU and 1TU, medaka BS SBR was more significant, which mainly contained Stimulation, Acclimation, and Adjustment (Readjustment), without Toxic effect. In 5 TU OP treatments during the exposure period, medaka BR met a great many changes, which mainly included Stimulation, Acclimation, Adjustment (Readjustment) and Toxic effect [39]. In the highest concentration exposure, it was hard for medaka to have the capability of adjustment.

Therefore, it was concluded that medaka SBR was affected by both exposure time and exposure concentrations, and it was mainly made up of No effect, Stimulation, Acclimation, Adjustment (Readjustment) and/or Toxic effect.

3.3. SBR model of medaka

The results of medaka SBR suggested that the behavior movements of medaka in different treatments went through No effect, Stimulation, Acclimation, Adjustment (Readjustment) and/or Toxic effect (Fig 5.). These results were accordance with previous studies on *Daphnia magna* [39, 40], which advised that either increasing toxicant concentration or exposure time, a cascade of regulatory behavioral stress responses were activated and performed by the organisms. Medaka SBR postulated that an organism displayed 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 provoked regulatory responses (loading stress). Above a certain stimulus level, however, several reactions were possible: 1) the homeostasis could not be maintained and a toxic effect in the organism occurs (limiting stress), 2) the organism could acclimate to the increased stress level and 3) the organism decreased the performance of the response and increased the performance of another response to the stimulus. If the first stress response decreased to less than the original level, a toxic effect occurs.

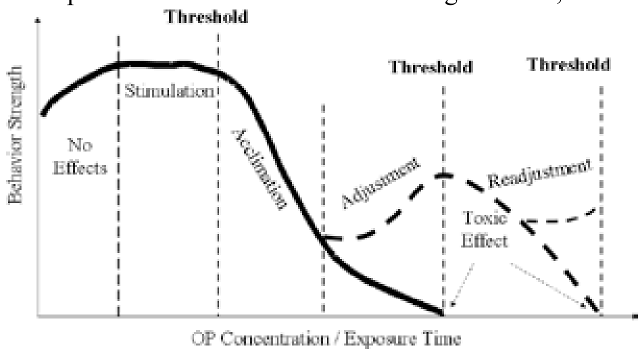

Fig. 5. SBR model of medaka in OPs exposure

In theory, the first behavior modulation of medaka was to increase the strength of all frequency movements presumably to try to escape from the polluted aquatic environment (Avoidance behavior) [40], which explained the BR observed in Stimulation after No effect. Presumably, stress of certain OP concentrations would be too high for medaka to start Avoidance behavior because this type of behavior was absent or of short duration at high OP concentrations (Fig 4.). In these cases, BS tended to decrease gradually until the ability for movement was lost. In other cases, medaka was apparently able to cope with the neurotoxin stressors and increased BS. Associated with this BR, the length of Stimulation was close to the expected value, about 1 to 3 hours (Fig 3.) and it was in inverse ratio to the OP concentrations.

Stimulation was usually followed by Acclimation which was a continuous weakening of BS. 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 test organisms.

After Acclimation, BR split in two directions: First, if medaka could not overcome Threshold 1, Toxic effect would occur. Secondly, if medaka lived through Threshold 1, BS 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” was quite appropriate to demonstrate SBR as reported in previous research [40]. The reason for these might be that almost all aquatic animals had the ability of actively escaping from a polluted environment to an unpolluted area [41]. According to medaka SBR, a trend for behavior modulation to maintain a stable internal environment and diminish dependence on an external environment was also shown as reported by Putman and Wratten [42].

The SBR model of medaka during the exposure supported and developed the Stepwise Stress Model (SSM) according to previous researches [39, 43], which included No effects, Stimulation, Acclimation, Adjustment (Readjustment), and/or Toxic effect. In this study, SBR model was affected by both the OP concentrations and exposure time, but in SSM, it only suggested the effects of exposure time on BS changes in certain concentration. Meanwhile, the results advised that it was “Threshold” that decided the effects of environmental stress on BR of medaka and the tendency of BR.

4. Conclusions

As the inhibitor of ChE, the toxicity characteristics of all OPs on the behavior movement of organisms were the same. The activity restrain of ChE could induce the loss of the nerve conduction ability, and then cause hyperactivity, loss of coordination, convulsions, paralysis and other kinds of behavioral changes. All of these behavior disorders could bring on SBR of organisms. Therefore, the SBR, which could be used as a suitable indicator in eco-toxicological risk assessment of OPs, would be affected by both OP concentrations and the exposure time as illustrated in Fig 5. Meanwhile, behavioral states obtained by using the SOM were accordingly classified in a heuristic manner, and subsequently, the movement strength data were verified with the SBR processes under stressful conditions. Data mining by SOM and SBR processes in combination could be efficiently used to illustrate the behavioral processes and to monitor toxic chemicals in the environment.

Since it might be the activity restrain of ChE that resulted in SBR of medaka, further investigation should focus on the relationship between the restrain degree of ChE and the behavioral responses by in vivo test to discuss the intrinsic response mechanism of the stepwise model. Meanwhile, as reported in this study, photoperiod worked on the nerve system of medaka could cause evident behavior rhythms, and the reason for this might due to the internal rhythm caused by biological clock [44]. According to this result, in future research on the behavior movement in lower concentration chemicals, the effects of circadian rhythms on BR should be paid more attention.

Acknowledgments

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