Quantitative characterization of response behaviors and individual variation in *Chironomus riparius* after treatments of diazinon

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Abstract

Response behaviors of indicator species in aquatic conditions were characterized by multivariate statistical methods after an insecticide, diazinon, was treated at low concentration (1 *ppb*). Seventeen larvae of *Chironomus riparius* were individually monitored using a CCD camera before (8 hours) and after (8 hours) the treatments. The variables presenting inactivity such as proportion of stops (p.stop) and mean weighting time for movement (m.wt) were increased, while mean speed (m.speed) and mean acceleration (m.acc) decreased after the treatments. A principal component analysis (PCA) based on the normed differences was conducted to elucidate toxic effects on the variables in an integrative manner after the treatments of the insecticide. The variables related to activity were accordingly grouped on the principal axes: the group of variables presenting inactivity (e.g., p.stop, m.wt) on the positive side and the group of variables associated with activity (e.g., m.speed, m.acc) on the negative side. The normed PCA could also illustrate degree of individual variation in behavioral changes in response to the treatments.

Keywords: response behavior, chironomids, diazinon, PCA, normed difference, behavioral monitoring.

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1 Introduction

Over use of pesticides has been a major concern regarding deterioration of ecosystem functioning including pest resistance to pesticides, change in pest status, high cost of control practices, residue problems in food and environment, etc [1]. All ecological compartments in aquatic ecosystems can be chronically affected given accumulation and persistence of pesticides in association with land use and surface run-off [2]. Recently, behavioral monitoring of indicator specimens [3–8] has garnered attention as one of the efficient assessment methods filling the gaps between large (i.e., community estimation) and small (i.e., molecular assessment) scales. Behaviors are collective projection of the small-scale molecular (and toxicological) responses, while behavioral measurements could be also projected upward to the large-scale in population (e.g., population growth) and community (e.g., biodiversity) responses. With establishment of behavioral measurements, the integrative risk assessment could be achieved on a full scale across different sizes in biological systems.

Behavioral detection of indicator species has been regarded as an efficient monitoring tool in aquatic ecosystems [3, 4]. Analyses of motility difference were conducted on fish species with various measurement techniques including videotaping [9], infrared-transmitters [10], and impedance conversion [5, 11] for monitoring presence of toxic chemicals [12–17] and hormonal effects [18]. Behavioral monitoring in response to toxic chemicals has been further extended to various taxa, covering crustaceans [19–21], parasite [22], snails [23], and insects [11, 24, 25].

Among insects, chironomids have been regarded as an efficient indicating group for monitoring biological water quality [26, 27]. A species in *Chironomus* has been reported to be more sensitive than fishes in response to organophosphates insecticides by affecting neuro-transmitter substance [28], while inactivation of chironomid larvae was reported after the treatments of chlorine dioxide [29]. Kim et al [24] extracted information from the locomotive data of chironomid larvae, *Chironomus flaviplumus*, by using wavelets and artificial neural networks to detect toxic responses after the treatments of carbofuran. Recently movement behaviors of *Chironomus flaviplumus* larvae have been characterized by decrease in fractal dimension [25].

Along with development of measurement techniques, theoretical research has been conducted to cope with the problem of complexity residing in behavioral data regarding auto-correlation [30], dynamics and statistical discrimination [31, 32], and fractal dimension [25, 33, 34]. Recently artificial neural networks have been used for recognizing response behaviors by utilizing Multi-Layer Perceptron (MLP) based on supervised learning [6, 35] and the Self-Organizing Map (SOM) based on unsupervised learning [8, 25].

The previous methods, however, have been mostly concentrated on variation in the variables in characterizing the movement data. There has been no extensive study on discussing variation in individual behaviors. While the methods of parameter extraction (e.g., fractal dimension) are concise and effective in revealing data structure, the parameters (e.g., fractal dimension) are highly compressed and usually express behavioral states of the specimens in overall terms only. Artificial neural networks are feasible in the sense that the models could extract information locally and are flexible to be applicable to non-linear data. The neural networks, however, could not directly provide quantitative information on the obtained results (i.e., no eigen values) since the models are mostly based on heuristic algorithms. In this study, a multi-variate analysis based on the normed difference was used to parametrically characterize the toxic effects on the variables and to elucidate individual variability in presenting response behaviors after the treatments.

2 Materials and methods

A strain of *Chironomus riparius* received from the Korea Research Institute of Chemical Technology (KRICT; Taejeon, South Korea) was used for test. The stock population was reared with an artificial dry diet (Tetramin) under the light regime of L10:D14 photoperiod (light phase from 8:00 a.m. to 6:00 p.m.) at a temperature of 22 ± 0.5°C. The moving patterns of 17 larvae were observed individually in an acrylic cage (5.5×5.5×1.6 (height) cm^3) from top view at every 0.25 second before (8 hours) and after (8 hours) the treatments of diazinon (1 ppb). The analog data captured by the CCTV camera were digitized by using a video overlay board (Dooin Electronics Co., LTD.; OSCARIII®) for image recognition (developed by the Artificial Intelligence Laboratory, Department of Electronics Engineering, Pusan National University). The middle point of the body was recorded as the *x, y* location of the specimens in the observation cage. In case the specimens moved, the center point of the moving portion of the body was recorded as the position of the specimens. For image processing, stable conditions were maintained for the test specimens in the monitoring system. Disturbances in observation cages were minimized; oxygen and food were not provided to the test specimens during the observation period.

The movement segments were defined as a vector (speed and direction) measured in every 0.25 second. Based on preliminary observations and previous reports [24, 25], we selected 13 variables to characterize the movement patterns accounting for speed, angular speed, stops, positions, and changes in direction (listed below). The movement segments being remained within the range of 0.2 mm from the previous segments was defined as the segment for 'Stop'. Regarding directional changes, three types were defined by comparing the axes of direction between two sequential movement segments in radian: positive (clockwise), negative (counter clockwise) and no (null) directions.

1) Mean (*mm/sec*; m.speed) and variation (mm/sec^2; cv.speed) of speed: The total distance of the movement divided by the cumulated time duration and coefficient of variation (CV; standard deviation divided by mean) of the measured distances, respectively.
2) Mean (*rad/sec*; m.angle) and variation (rad/sec^2; cv.angle) of angular speed: Mean of angle changes in absolute values in radian divided by

the cumulated time duration of movement and CV in the measured angular speeds, respectively.

3) Proportion of positive (p.po) and no (po.null) changes in direction: Proportion of the segments indicating positive and no movements out of the total movement segments, respectively.
4) Proportion of stops (p.stop): Proportion of 'stops' out of the total movement segments.
5) Distance (*mm*; m.dis) and variation in distance (cv.dis): Mean of measured distances from the center of observation cage and CV in the measured distances, respectively.
6) Mean waiting time (*sec*; m.wt) and variation (cv.wt): Mean time between two moving events and CV in the measured waiting times.

Effects of diazinon were tested for the selected variables using the paired *t*-test [36]. In order to explore the integrated effects of the treatments on the variables, a Principal Component Analysis (PCA) based on normed differences [37] was utilized to realize the ordination of the paired values on the component plan before and after the treatments. After obtaining the two fully matching data for individual-variable matrix, the normed differences (Z_{ij}) between two sets of data (before and after the treatments) were obtained:

$$Z_{ij} = \frac{y_{ij} - x_{ij}}{\sqrt{\partial}}$$

$$\partial(j) = \frac{1}{n} \sum_{i=1,n} (y_{ij} - x_{ij})^2$$

where n is the number of individuals, and x_{ij} and y_{ij} present the values of variable j (column) for individual i (row) before and after the treatments respectively. Subsequently diagonalization of matrix was carried out to extract information residing in the variable-individual relationships. Computations and associated graphical representations were produced according to 'ade4' library available in the *R* freeware [38]. Detailed method for analysis could be referred to [37–39].

3 Results

3.1 Testing variables

When the selected variables were compared before and after the treatments, the toxic effect was significantly observed ($p \leq 0.05$) in 8 variables (out of the 13 variables; n=17): mean speed (m.speed), mean angular speed (m.angle), variation in angular speed (cv.angle), proportion of null angles (p.na), proportion of stops (p.stops), mean acceleration (m.acc), variation of acceleration (cv.acc) and mean waiting time (m.wt) (Table 1). Mean waiting time increased in the maximal range, and proportion of stops also accordingly increased after the treatments. Speed and CV in speed correspondingly decreased after the treatments. The results suggested that activities of the specimens substantially decreased in intoxication.

Acceleration and CV in acceleration also increased after the treatments, indicating higher occurrence of abrupt movements that could be originated by differences in speed. Although speed of intoxicated specimens was low, the specimens' movements would be frequently initiated from stops or from the states of activity in the minimal range in intoxication and consequently this type of abrupt movements would produce a higher degree of velocity differences (i.e., high acceleration). The intoxicated movements were also characterized with increase in mean angular speed and decrease in variation of angular speed (Table 1).

Table 1: Difference in the variables in movement data of *Chironomus riparius* larvae in response to the diazinon treatments.

Variable	*Difference*	*Mean of the differences (% of initial mean[1])*	*p-value*	
m.speed	-	*10.4*	*0.0041*	**
cv.speed	-	*6.3*	*0.4407*	
m.angle	+	*8.4*	*0.0096*	**
cv.angle	-	*6.6*	*0.0011*	**
p.pa	<	*0.2*	*0.8454*	
p.na	+	*24.7*	*0.0308*	*
p.stops	+	*30.8*	*0.0004*	**
m.dis	+	*6.3*	*0.0526*	
cv.dis	-	*7.1*	*0.2175*	
m.acc	+	*31.4*	*0.0013*	**
cv.acc	+	*19.8*	*0.0229*	*
m.wt	+	*81*	*0.0009*	**
cv.wt	+	*53.7*	*0.0567*	

[1]"Initial mean" refers to the mean values before the treatments. **; highly significant ($\alpha = 0.01$), *; significant ($\alpha = 0.05$). Units for the variables are listed in the text. ($n = 17$).

3.2 PCA on the normed differences

The PCA on the normed differences before and after the treatments revealed a two dimensional pattern on the component plane (Fig. 1). Two axes respectively accounted for 44% and 27% of the total inertia on the coordinate 1 (x) and 2 (y) (Fig. 1A). The group of the variables indicating no activity (i.e., p.stop, m.wt) was placed on the positive position on the coordinate 1, while the group of variables indicating activity (i.e., m.speed and m.acc) was located on the negative side on the coordinate 1.

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Two variables (m.dis and cv.dis) that were not significantly different before and after the treatments (Table 1) were also associated with the groups of the variables placed along with the coordinate 1. The variable m.dis was placed closely to the group of low activity in the positive side, while cv.dis was associated with the group of high activity in the negative side. This indicated that, although the variables were not significantly different as single variables (Table 1), the variables were associated with other variables in presenting response behaviors. Association with m.dis with the group of inactivity in the positive side of coordinate 1 implied that the specimens did not remain close to the centre area in intoxication. The variable cv.dis was associated with the group of high activity in the negative side of coordinate 1. This is understandable by considering the fact that the specimens would move around in a higher degree in the cage when they were active.

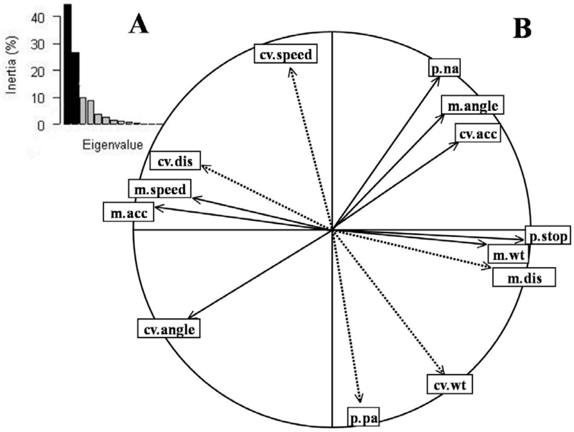

Figure 1: Diagram of eigenvalues of the PCA on normed differences (A) and projections of the variables on the correlations circle in two axes (B); dashed lines represent variables for which the treatment effect is not significant according to the paired-*t* test (Table 1).

In combination of coordinates 1 and 2, the variables of p.na, m.angle and cv.acc were grouped on the positive sides at the top left area of Figure 1B. Association of p.na and m.angle indicated that the intoxicated specimens tended to keep the same direction (i.e., high level in p.na) but the specimens turned in a wide angle once the specimens moved (i.e., high level in m.angle). The significant increase in cv.acc (Table 1) reflected that the specimens showed higher levels of variation in acceleration in this group in intoxication. In the opposite side for this group, cv.angle was placed and was not closely related with other variables or groups of variables. In the coordinate 2, cv.speed (positive side) and the group p.pa and cv.wt (negative side) were placed in the opposite direction and tended to show negative association on the component plane

Figure 2: Projections of the 17 individuals on the first factorial plan. Arrows length reflects the importance of change between before treatment (origin of the axes) and after treatment (end of arrows).

although the variables were not significant before and after the treatments in single variables (Table 1).

The PCA on the normed difference could also show variation of individual specimens on the component plane (Fig. 3). The tested specimens (17 individuals) could be individually located in association with ordination of the variables. The majority of individual variations were observed in the positive direction from left to right on the component plane along with coordinate 1, indicating that test specimens were mostly associated with the variables indicating inactivity. Variation among individuals was also accordingly presented in Figure 2 on the individual base. The specimens with most sensitive responses with long arrows could be identified along with the coordinate 1 (e.g., 2, 3, 14). In addition, a few specimens were observed to be located away from the majority of individuals diagonally to the positive (i.e., 5) and negative (i.e., 10) sides of the coordinate 2, while a few individuals (e.g., 8, 11, 16, 15) even remained near the origin, indicating minimal responses to the treatments (Fig. 2).

The normed test was further able to present the “degree” of behavioral change for each test specimen before and after treatments (Fig. 3). While Figure 2 presented association of the individuals with the variables occupying the same origins on the component plane, Figure 3 illustrated the degree of changes in response behaviors of individuals with the shape of arrows from “before treatment (origin of the arrows)” to “after treatment (end of the arrows)”. The changes in variation after the treatments were observed in the majority of individuals in the positive direction along with the coordinate 1. Some individuals showed the maximal changes after the treatments (e.g., 2, 14) on the

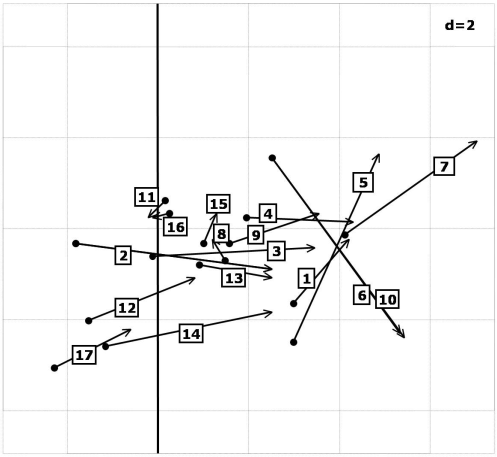

Figure 3: Simultaneous projections of the normed difference values for individuals before and after the treatments (respectively $x_{ij} / \sqrt{\partial}$ and $y_{ij} / \sqrt{\partial}$) on the first factorial plan. Arrows account for individual variations in response behaviors.

first factorial plan, while other individuals showed minimal changes (e.g., 8, 16 and 11). The positions of the specimens with minimal changes were in accord with the positions in Figure 2 (i.e., close to centre).

4 Discussion and conclusions

The pattern of locomotory modifications of *Chironomus riparius* exposed to diazinon revealed an acute sensitivity of the intoxicated organisms. The PCA on the normed difference efficiently presented the effects of toxic chemicals on response behaviors, identifying associations in the related variables (Fig. 1). Variability in individuals was further elucidated on the component plane by the PCA method (Figures 2 and 3). Although a numerous accounts of research on computational analysis of response behaviors have been reported as stated above, individual variation has not been extensively presented. Considering that individual variations, however, are frequently observed and problematic in observation of the movement data [6, 35], the method illustrating individual variation could contribute to development of sensitivity tests and for selecting appropriate individuals in different classes of response behaviors for monitoring.

The results accordingly presented differences in behaviors in the specimens treated with the insecticide. Diazinon, as an organophosphate, interferes with the membrane transport of sodium, potassium or other ions, inhibits selective enzyme activities (notably cholinesterase), and contributes to the release and/or the persistence of chemical transmitters at nerve endings. Diazinon exposure engendered a noticeable increase in the mean number of stops and mean waiting

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time between two moving events in this study (Table 1, Figure 1). The inactivity observed in response behaviors may be consequences of the diazinon effects and could be interpreted as a physiological impairment in the muscular activity [40]. However, further study is required in confirming the causative relationships between toxicological effects and response behaviors.

Proportion of no direction change (p.na) was statistically significant (Table 1), and was associated with the group of m.angle and cv.acc at the left corner of Figure 2. Although the variable was significantly different, however, the changes in p.na were in the small range: before the treatments the proportion of p.na occupied 2 ± 1% and 3 ± 1% after the treatments. Proportion of changes in the positive direction (p.pa) was placed on the negative side of the coordinate 2, but the variable was not associated with other variables. The positive (and negative) changes in direction before and after the treatments were negligible in this study (Table 1): the positive direction occupied 49 ± 1% before treatment and 49 ± 2% after treatment.

In conclusion, a multivariate statistical analysis based on the normed differences in the variables was feasible in illustrating the toxic effects on movement behaviors with parametric characterization. The PCA method could also elucidate individual differences in showing response behaviours after the treatments. This type of tracing individual variation in response behaviours would be useful for selecting appropriate individuals for checking sensitivity to the toxic chemicals and behavioural monitoring of water quality in aquatic ecosystems.

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