Inferring variance and invariance in multi-individual behavior of zebrafish (Danio rerio) responding to chemical stress

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Published online: 28 Aug 2015.

To cite this article: Kha Quang Quach, Hungsoo Kim, Tuyen Van Nguyen, Chunlei Xia, Woon-Seok Cho & Tae-Soo Chon (2015) Inferring variance and invariance in multi-individual behavior of zebrafish (Danio rerio) responding to chemical stress, Animal Cells and Systems, 19:4, 252-261, DOI: 10.1080/19768354.2015.1054308

To link to this article: http://dx.doi.org/10.1080/19768354.2015.1054308
Inferring variance and invariance in multi-individual behavior of zebrafish (Danio rerio) responding to chemical stress

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(Rceived 16 February 2015; received in revised from 30 April 2015; accepted 12 May 2015)

Variance and invariance residing in multi-individual movement of zebrafish under chemical stress were addressed concurrently before and after treatment with formaldehyde. Variance was observed in movement parameters. Without treatment, linear speed was highest in groups with two-individuals, followed by those with one- and four-individuals. Following treatment, linear speed decreased significantly in groups with one- and two-individuals, but not in those with four-individuals. Inter-distances between two-individuals in groups with two-individuals decreased markedly after treatment, whereas inter-distances between two randomly selected individuals in the groups with four-individuals were not affected. The zero- and peak-values in the time-lag of autocorrelation of inter-distances decreased further after treatment in groups of two individuals relative to those in groups of four individuals. However, the empirical transition probability matrix between predefined behavioral patterns remained invariant among groups with different numbers of individuals as well as before and after treatment. Variance and invariance in multi-individual behaviors are suitable in expressing complex behaviors under chemical stress and could be a reference for detecting contaminants in the environment using indicator species.

Keywords: Autocorrelation; behavior monitoring; group behavior; movement pattern; transition probability matrix

1. Introduction

Motion in groups is a typical expression of animal populations for survival during adaptation to external environmental stimuli. The grouping mechanisms of animals, including flock size, direction, and self-organization, have been actively investigated from both experimental and biological aspects since the 1950s (Emlen 1952; Breder 1954; Jones 1957). Complexity in group behavior garnered attention from model developers regarding simple and objective expression of movement dynamics in association with self-propelled particle systems (Vicsek et al. 1995; Cucker & Smale 2007), correlation function (Toner & Tu 1998), hierarchical group dynamics (Nagy et al. 2010), and information sharing (Couzin 2007). Group behavior models were also analyzed during evaluation of experimental data regarding drag coefficient and external forces acting on fishes (Suzuki et al. 2003).

Individual movement patterns have been separately investigated with regards to monitoring of stressors in the environment since the 1980s (Lemly & Smith 1986; Lawrence & Smith 1989; Baganz et al. 1998; Chon et al. 2005) including correlation analysis (Dray et al. 2010), parameterization (Weis & Weis 1974), data transform (e.g. Fourier transform, wavelets) (Park et al. 2005; Kim et al. 2006), informatics (e.g. self-organizing map) (Chon 2011), and hidden Markov model (Liu et al. 2011; Nguyen et al. 2011).

However, no study has been specifically focused on variability of movement behaviors observed in the observation arena per se. In this study, we focus on characterizing variance and invariance concurrently observed in complex response behaviors of an indicator species before and after chemical treatment. This study is also aimed to present behaviors in groups. Although there have been numerous accounts of group behavior studies as stated above, little research has been conducted to investigate group response behaviors under chemical stress. Recently, Quach et al. (2013) reported that the intermittency of the shadowing times (i.e. period over a predetermined threshold) on both forces and inter-distances for one- and two-individual groups were different before and after treatment with formaldehyde at a low concentration. In this study, we demonstrate variance and invariance of the movement behaviors of zebrafish in small groups as a suitable reference to detect toxic chemicals.

2. Materials and methods

2.1. Test organisms

For convenience of expression, we defined experimental conditions and observations according to the number of individuals separately. We had the groups of one-, two-, and four-individuals of zebrafish for experimental conditions and named as G1, G2, and G4, respectively. In
G1, G2, and G4, behaviors of one and two individuals were observed before and after the chemical treatment. We defined these as one-individual and two-individual observations. In G1 only one-individual observation was carried out, whereas one-individual and two-individual observations were separately conducted in G2 and G4. It is known that zebrafish is one of the most suitable vertebrate model species for various biological research (Blaser & Gerlai 2006; Levin et al. 2007). Due to vulnerability to chemical stress, the species has a strong potential being used as an indicator of the risk assessment studies (Liu et al. 2011). Stock population (approximately 300 individuals) of wild type zebrafish were obtained from a local fish dealer and were reared for two weeks before the observations (Blaser & Gerlai 2006) at a temperature of 25 ± 1°C and pH of 7.1 ± 0.3 under a light/dark cycle of 14/10 h (on at 7:00 and off at 20:00). The fluorescent lights (26 W) were placed 50 cm above the rearing container.

Test organisms (age: 5–6 months; body length: 30–40 mm) were randomly selected from the stock population and placed individually in a glass aquarium (30 × 30 × 30 cm³; water height 2 cm) with small groups (G1, G2, and G4). Before observation, organisms were acclimated to the observation system for 30 minutes (Gerlai et al. 2006). Formaldehyde (HCHO, 37 wt.% solution in water, ACS reagent, Gamma-Aldrich®) was used to treat the test organisms after dilution. The chemical was directly added to the water in the observation aquarium at a concentration of 0.1 mg l⁻¹.

2.2. Recording and quantifying behavioral data

The recording system consisted of an observation aquarium, a camera (Logitech® Vid™ HD), and a PC. Software (‘Multi-tracks’) for tracking the motion of multiple individuals was developed at the Laboratory of Ecology and Behavior Systems at Pusan National University based on the global nearest neighborhood algorithm and stereo vision by triangulation measurement (Xia et al. 2009). Movement of zebrafish was continuously observed before and after exposure to formaldehyde. The x–y position of each individual was recorded from top view in two dimensions before (30 minutes) and after (30 minutes) treatment at 30 fps. Five-minute segments were selected for analysis according to Suzuki et al. (2003) and Herbert-Read et al. (2011), and were sufficient to present response behaviors of zebrafish.

Based on a preliminary research (Liu et al. 2011), a time segment of 0.1 s was selected for recording. Since we aimed to observe overall changes in movement of fish specimens in response to the chemical treatment, the 0.2s segment was sufficiently short for presenting displacement of the organism location. Each movement segment was determined based on three points with two consecutive 0.1s segments (0.2 s in total). The observation was repeated 20 times each for G1, G2, and G4. Mean values of linear speed and turning angular were obtained from movement segments for each individual during the observation period. To compare parameters with equal numbers of observations in different tested groups, one individual (i.e. measuring linear speed and turning angular in G2 and G4) and a pair of individuals (i.e. measuring inter-distance in G4) were randomly selected from each replication in G4.

2.3. Computational methods

2.3.1. Autocorrelation. We applied autocorrelation (AC) to reveal time dependence in inter-distances between individuals (Dray et al. 2010). An AC without normalization, \( R_{xx}(m) \), for a time-series data set, \( x_n \), is defined as (Orfanidis 1985)

\[
R_{xx}(m) = \sum_{n=1}^{N-m+1} x(n)x(n + m - 1),
\]

where \( m = 1, 2, \ldots, M + 1, M \) is the number of time-lags, and \( N \) is the data length.

2.3.2. Behavioral patterns and transition probability matrix. As shown in Figure 1, eight behavioral patterns were identified based on the movement direction (e.g. move forward, turn to the right, etc.) following Liu et al. (2011). Direct backward movement was not included since straight backward movement was not observed under the experimental conditions. The angle (\( \theta \)) of directional change in fish was expressed in \( 0 \) to \( \pi \) rad. This value was positive when fish moved counter-clockwise and negative when fish moved clockwise. To determine the criterion of the same direction of movement, sigma (\( \sigma = 0.12 \) rad) was used based on the body size of a single fish following \( \sigma = \arctan(h/l) \), where \( h \) is the average body width of fish (\( h = 4.05 \pm 0.67 \text{mm} \)) and \( l \) is average body length of fish (\( l = 35.67 \pm 3.47 \text{mm} \)). According to the criteria described in Figure 1, the

\[
\begin{align*}
(a) & \quad (b) & \quad (c) & \quad (d) \\
& \quad \bullet & \quad \rightarrow & \quad \rightarrow \\
(e) & \quad (f) & \quad (g) & \quad (h) \\
& \quad \rightarrow & \quad \rightarrow & \quad \rightarrow
\end{align*}
\]

Figure 1. Definition of eight movement patterns of zebrafish: (a) stop (P1), (b) move-and-stop (P2), (c) stop-and-move (P3), (d) move straight (P4), (e) turn leftward with \( \sigma < \theta < \pi/2 \), (f) turn rightward with \( -\pi/2 < \theta < -\pi/2 \), (g) turn backward to the left with \( \pi/2 < \theta < 0 \), and (h) turn backward to the right with \( -\pi/2 < \theta < -\pi + \sigma \) (P8).
movement patterns were determined continuously in a 0.2s segment throughout the whole observation period (5 minutes) before and after treatment. An empirical transition probability matrix (TPM) was obtained by averaging transition probabilities between two consecutive patterns in the observed segments as time progressed for G1, G2, and G4. Initially, mean value of transition probabilities for each individual was obtained for each pattern change through the observation periods (5 minutes). Subsequently, the mean values were averaged for all individuals for each group to provide experimental TPMs used in this study.

3. Results

3.1. Movement patterns

Overall, the movement tracks of zebrafish appeared to be smoother and more linear before treatment (Figure 2(a–c)). After treatment, movement tracks showed more short turns and shaking, as well as higher frequency of swimming behavior in the center area (Figure 2(d–f)). Before treatment, the histogram of average linear speed varied according to the number of observed individuals in G1, G2, and G4 (Figure 3(a–c)). While the peak was observed at 24–32 mm/s for G1 (Figure 3(a)), the peak for G2 increased to 32–40 mm/s (Figure 3(b)). However, in G4, the peak markedly decreased to 16–24 mm/s (Figure 3(c)). The average linear speed was highest in G2 (39.21 ± 0.45 mm/s), followed by G1 (28.57 ± 0.53 mm/s) and G4 (27.08 ± 0.64 mm/s) before treatment (Figure 3(d)), and these values differed significantly among the groups (Tukey’s test, p < 0.05; n = 20).

After treatment, the peaks generally decreased to low levels, with values of 16–24, 16–32, and 8–16 mm/s being observed for G1, G2 and G4, respectively (Figure 3(a–c)). The average linear speeds were 32.16 ± 0.70 mm/s for G2, 25.8 ± 0.34 mm/s for G4 and 18.44 ± 1.10 mm/s for G1, which differed significantly (Tukey’s test, p < 0.05) (Figure 3(d)). These findings were similar to those observed before treatment. The changes in linear speed before and after treatment differed with significance in G1 and G2 (p < 0.01**) according to t-test (Figure 3(d)), but not in G4 (t-test, p > 0.15).

The turning angular velocities, defined as the rate of change of angular displacement in different directions relative to the position of fish at each moment, were also obtained for the movement segments. When compared with the linear

Figure 2. Movement of zebrafish in group in 20 seconds before (a–c) and after (d–f) the treatment. Different individuals are marked by different colors with starting arrow and ending circle.
speeds, the peak values of angular velocity did not change among groups, with peaks of 0–6 rad/s being observed (Figure 4(a–c)). The average turning angular velocities were similar between G1 (2.02 ± 0.24 rad/s) and G4 (2.08 ± 0.3 rad/s), but were significantly higher when compared with G2 (1.58 ± 0.22 rad/s) (Tukey’s test, p < 0.05) before treatment (Figure 4(d)). After treatment, turning angular velocities tended to increase slightly in G1 (3.12 ± 0.32 rad/s), G4 (2.27 ± 0.31 rad/s) and G2 (2.11 ± 0.3 rad/s). G1 differed significantly from the rest of the groups after treatment (Tukey’s test, p < 0.05) (Figure 4(d)). Angular velocity differed with high significance before and after treatment in G1 and G2 (t-test, p < 0.01 (**) (Figure 4(d)), but not in G4 (t-test, p > 0.07). The overall results demonstrated that the behaviors were different according to groups with different numbers of individuals before and after treatment.

Figure 5 shows the time-series data of inter-distances before and after treatment in G2 (Figure 5(a)), two individuals randomly selected from G4 (Figure 5(b)) and the averages of the inter-distances of two individuals selected among four individuals (Figure 5(c)) during the observation period. Before treatment, strong variations were observed in the inter-distance in G2. However, inter-distances were less variable among randomly selected individuals (Figure 5(b)) and appeared to be smooth on average (Figure 5(c)) in G4. Inter-distances were similar among different individual groups before treatment (129.6 – 141.23 mm). The inter-distances, however, were markedly different before and after treatment in G2 with high significance (t-test, p < 0.01 (**)). The distances were consistently lower during the course of observation after treatment (64.36 ± 6.78 mm) in G2, showing significantly lower levels than before treatment (141.23 ± 8.51 mm) on average (Figure 5(a) and Figure 6(a)). In contrast to G2, inter-distances for two individuals based on random selection (135.36 ± 9.82 mm) and the average (129.67 ± 5.95 mm) did not differ after treatment in G4 (Figure 6(a)). These findings demonstrated that the inter-distance in G2 was highly sensitive to the existence of stressors and could be suitable for detecting contamination.

3.2. Autocorrelation

Time-lag in AC was further observed in data for inter-distances in G2 and G4. In G2, strong negative and positive peaks were observed before treatment (Figure 6(b)). This time-lag effect was markedly diminished when the distance was observed from two individuals randomly selected in G4 (Figure 6(c)). It should be noted that a negative time lag reappeared when the averages of all inter-distances (i.e. from six combinations of individuals) were obtained in G4 (Figure 6(d)).

Following treatment, the AC curves changed substantially. Specifically, the long time-lag that was strongly observed in G2 before treatment disappeared. A trace of
the short time-lag was weakly observed in this case, appearing as numerous teeth-like indentations on the AC curve (Figure 6(b)). The patterns of AC presenting inter-distances in G4 also appeared to differ. In the case of random selection from two individuals (Figure 6(c)), time-lag effects were generally not strong. However, in G4, there were time-lags, although the periods were somewhat shorter than before treatment (Figure 6(d)). To compare the patterns of AC curves, the peak-value and zero-value time-lags, at which the value of AC crosses

Figure 4. The probability distributions of angular velocity before and after treatment in groups (a) G1, (b) G2, and (c) G4 and the averages of the absolute value of angular velocity (d) \( n = 20 \). The alphabets and symbol are the same as indicated in Figure 3.

Figure 5. The inter-distances between two individuals in G2 (a), randomly selected from G4 (b), and average of all combinations in G4 (c) before and after treatment.
zero values in the $y$-coordinate, were obtained as shown in Figure 7. In G2, the differences were outstanding in time-lags for both zero-values (Figure 7(a)) and peak-values (Figure 7(d)) before and after treatment. However, no differences were observed in the case of G4 for both random selection (Figure 7(b) and (e)) and the averages (Figure 7(c) and (f)). These findings demonstrated that time-lags observed during the course of interaction in G2 are variable in different number of individuals and sensitive to chemical stress.

3.3. Transition probability matrix

Although the parameters and inter-distances were variable, overall TPMs of movement patterns were markedly similar (Figure 8). Among TPMs, no statistical difference was found between the different number of individuals as well as before and after treatment based on the Kolmogorov–Smirnov tests (Massey 1951) ($p > 0.05$). This invariance in TPMs demonstrated structural properties residing in behavioral patterns in zebrafish. Some specific pattern changes within TPMs, however, were outstanding. The pre-pattern (listed in the left-most column in Figure 8) of stopping movement P1 (stop) and P2 (move and stop) (Figure 1 for the patterns) ended up with advancing pattern P3 (stop and move) as the post-pattern (listed in the top row in Figure 8). It should also be noted that the pre-patterns having ‘move’ as the last segment (P3–P8) changed to diverse patterns when compared with the stopping movement (P1 and P2) (Figure 8). Pattern P5 (turn leftward) remained the same with high probabilities in most cases. This indicated that although the parameters were different either before or after treatment or in different number of individuals, the transitions between the predefined movement patterns are stable.

Although the overall probabilities were in a similar pattern, some component probabilities in TPMs were locally variable and showed statistical significance among different numbers of individual groups according to multiple comparison tests (Figure 8). The numbers presented on each checkerboard plot in Figure 8 indicate statistical differences. For instance, the numbers (2, 4) in the checkerboard plot (P1, P1) in Figure 8(a) present that the transition probability from P1 to P1 in G1 is significantly different from G2 and G4 before treatment. Pre-patterns of P1 and P2 that changed to post-patterns, either P1 or P3, mostly showed statistical significance in different numbers of individuals (Tukey’s test, $p < 0.05$). These findings indicated that the stopping movements (i.e. ended with ‘stop’) changed to ‘stop’ and ‘stop and move’ variably among groups with different numbers of individuals. Further, probabilities P3–P8 tended to be variable across the different numbers of individuals when compared with P1 and P2. Variability in transition probabilities was also similarly observed after treatment (Figure 8). When specific transition probabilities were compared between ‘before’ and ‘after’ treatment, additional differences were observed. The underbars shown within checkerboard...
Figure 7. Zero-value time-lag (a–c) and peak-value time-lag (d–f) in G2, G4 with random selection and average of all combinations before and after treatment.

Figure 8. TPMs of the movement patterns before ((a) G1, (b) G2 and (c) G3) and after ((d) G1, (e) G2, and (f) G3) treatment. The color bar in the bottom right corner represents probability values. The numbers on checkerboard show statistical differences before and after treatment. The underlines of numbers in (d)–(f) indicate statistical differences between before and after treatment according to the paired sample t-test ($p < 0.05$).
plots (Figure 8(d)–(f)) represent statistical differences before and after treatment. Significant differences tended to be more frequently observed before and after treatment for the movement patterns of ‘stop and move’ (P3), continuous straight movement (P4), and forward movement with directional changes (P5 and P6).

4. Discussion

While numerous studies have been conducted to investigate group behaviors of animals based on field observation and modeling (Breder 1954; Jones 1957; Niwa 1994; Vicsek et al. 1995), collective motion has not been extensively reported, especially under chemically stressful conditions. In previous studies, overall group behavior was primarily discussed at the population level after treatment (Lee et al. 2009) based on group size (Griffiths & Magurran 1997; Hoare et al. 2004). Information directly addressing interactions with the nearest neighborhood, such as speeds and inter-distances under stressful conditions in groups, have not been specifically addressed as presented in this study. Here, we demonstrated variance and invariance coexisting in the movement data among group behaviors responding to chemical exposure.

Without treatment, behaviors were already different among groups with different numbers of individuals. The linear speeds, turning average velocities, and inter-distances (Figures 3–5 and Figure 6(a)) and their time-lags differed remarkably in G2 when compared with G1 and G4 (Figure 6(b–d) and Figure 7). These findings support the existence of close relationships between the two nearest individuals and unique characters in pairwise relationships in group formation. Recently, Herbert-Read et al. (2011) demonstrated the importance of repulsion and response to the single nearest neighbor in fish group behavior dynamics. Pairwise interactions are also regarded as important for qualitatively capturing the correct spatial interactions in small groups of fish when compared with observed data (Katz et al. 2011). Based on continuous tracking analysis, our study confirmed the significance of the two individual interactions in group formation.

After treatment, parameters such as linear speed and angular velocity tended to show a higher degree of change in G1 than in G2, although the significance level was generally in the same range (Figure 3(d) and Figure 4(d)). It is worth noting that differences in inter-distances and time-lag effects were substantial in G2 before and after treatment (Figure 6(a) and Figure 7). The decrease in inter-distance after treatment (Figure 5(a) and Figure 6(a)) supported the results of previous studies that showed fish were alert in the presence of low concentrations and tended to be close to neighboring individuals (Griffiths & Magurran 1997; Miller & Gerlai 2008; Quach et al. 2013). In this study, we further observed that organisms tended to stay in the central area rather than in boundary or corner areas. Consequently, inter-distances between two individuals were shortened after treatment. However, the inter-distances in the four-individual groups did not decrease substantially. A subgroup consisting of two or three individuals among four individuals was frequently observed with short inter-distances during the observation period. However, in such cases, outliers not belonging to the subgroup were also frequently found either in the boundary area or far away from the subgroup before or after treatment. Thus, the overall inter-distance in the four individuals group did not decrease substantially.

The results based on AC further indicated that pairwise interactions were more sensitive in G2 than G4 (Figure 6(b)), showing positive and negative correlations of inter-distances between two individuals. This type of time-lag effects was not observed for inter-distances in G4 when the inter-distance was randomly selected from each replication (Figure 6(c)). However, negative time-lag effects were observed when the averages were analyzed by AC (Figure 6(d)). These findings suggested that time-lag effects may emerge when overall interactions were accounted for among all individuals at the population level. However, it is currently not clear if the cause of changes in inter-distances is due to interaction force formed between individuals, disruptive response to the boundary, or some other physical reasons. Further studies are needed to investigate the collective behavior in response to stressors from both computational and experimental aspects.

Although parameters describing the movement of individuals were variable, the empirical transition probabilities between different movement patterns were invariant (Figure 8). Regarding single individual behavior, our study confirms the results of a previous study in which the overall TPMs were similar with partial difference in component probabilities (Liu et al. 2011). These findings imply that there is a stereotypic inner state that maintains basic behaviors in animals (Liu et al. 2011). It is remarkable that transition probabilities were similar in single or groups. In this study, we firstly demonstrated that TPMs are also invariant among groups containing different numbers of individuals, even though the parameters extracted from movement behaviors were highly variable (Figures 3, 4, and 7). These findings further indicate that structure property exist in behavior patterns in groups. Although strong variability hinders achieving objectivity in behavior monitoring, structural properties residing in the TPM could serve as an efficient reference system for evaluating complex response behaviors. While the invariant TPM could be a reference system, the partial difference in transition probabilities could serve further as a local detection criterion, specifying which pattern specifically changes before and after treatment. To the best of our knowledge, this is the first report observing both variance and invariance in movement data in groups under chemical stress. In order to infer behavior states, Markov processes such as hidden Markov model...
(HMM) (Rabiner 1989; Liu et al. 2011) could be utilized for parameter estimation by TPM. In the similar experimental situations where zebrafish individuals were exposed to formaldehyde at the same concentration, empirical TPMs were basically in the similar range to estimated TPMs based on HMM according to Liu et al. (2011). In the future, however, more studies are warranted to further evaluate transition and emission probabilities according to indicator species, different chemicals, concentrations, and experimental conditions.

In this study, we observed only the immediate olfactory response of zebrafish during the initial 5 minutes after exposure to the chemical. Since formaldehyde is a carcinogenic agent, and toxic response behavior will also be produced through physiological networks (Gerlai et al. 2006), a longer observation period would be required to analyze movement behaviors responding to chronic toxic effects.

Sex was not considered to affect the general behavior of the zebrafish population in this study. However, it should be noted that males and females used in the experiment were not influenced by any physiological stress (e.g. reproduction, mating) during the observation period. In the future, sex and age should also be considered when determining the effects of pairwise responses in association with different life stages.

In this study, one concentration of the chemical was tested. Behavior monitoring would be useful for providing early warnings under field conditions (Lemly & Smith 1986), whereas the method is less useful to reveal close quantitative relationships in response to varying degrees of stimuli when compared with other toxicological and molecular analyses. Nevertheless, any abnormal response behaviors observed in the test organisms would provide managers with a prompt signal that some aspect of the environment had changed, providing an opportunity to conduct further in-depth investigations. Consequently, both behavioral and physiological methods should be applied in combination. Demonstration of variability in response to different intensities of stressors would be still useful in differentiation and classification of behavioral states under stressful conditions; therefore, further study of this aspect is warranted.

5. Conclusions

The movement data were analyzed for small groups of zebrafish by continuously tracking individuals’ positions, and variance and invariance were addressed according to groups containing different numbers of individuals, as well as ‘before’ and ‘after’ treatment. Parameters of movement behaviors of groups of one-, two-, and four-individuals were markedly different before treatment, confirming the importance of neighbor individuals in group formation. Under chemical stress, parameters such as linear speeds and angular turning velocities changed significantly in groups of one- and two-individuals, but not strongly in groups of four-individuals. The inter-distance between two individuals in groups of two decreased markedly when compared with groups of four. The decrease in peak and zero-value time-lags was greater in groups of two-individuals. Invariance was further observed in TPM between different movement patterns in groups as well as before and after treatment. The structural property in TPM confirmed the possibility of a stereotypic inner state that maintains basic behavior changes in test organisms. In addition, specific transition probabilities in TPM occurred before and after treatment could be used as criteria for behavior changes. The variance in parameters (i.e. speed, inter-distance, time-lag) and invariance (i.e. TPM) observed in small groups of indicator organisms may be useful to detect toxic chemical in the environment, and could be suitable as a means of early warning signal of pollution.

Disclosure

No potential conflict of interest was reported by the authors.

Funding

This research was supported by Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (2011-0012960).

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