Effect of Sodium Hypochlorite on Zebrafish Swimming Behavior Estimated by Fractal Dimension Analysis

Kittiwann Nimkerdphol* and Masahiro Nakagawa

Department of Electrical Engineering, Faculty of Engineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan

Received 1 October 2007/Accepted 4 February 2008

The behavioral responses of zebrafish (Danio rerio) to sublethal concentrations of sodium hypochlorite (NaClO)-based household bleach were quantified in order to index toxicity of the solution. The swimming behavior of zebrafish was captured using nonplanar 3D stereocameras in combination with 3D coordinate computation with perspective correction (3DCCPC) to compute for actual 3D coordinates. Swimming trajectory and velocity were quantified by fractal dimension analysis. The results showed that, under incremental concentrations of NaClO-based aqueous solutions with the maximum of 0.005% v/v, the fractal dimension of swimming velocity trended to increase with the concentration. The fractal dimension of swimming trajectories trended to increase with pH. Hence, the results have proven that the system is a useful tool to indicate behavioral changes, which may be implemented in biomonitoring systems for acute toxicity bioassay.

[Key words: zebrafish, sodium hypochlorite, nonplanar stereoscopic camera, fractal dimension analysis, critical exponent method]

Sodium hypochlorite (NaClO, CAS no. 7681-52-9) has been used since the 17th century. Nowadays, NaClO is one of the most widely used chemical, i.e., for water disinfection (drinking water, swimming pool, and wastewater) and as a bleaching agent for paper and clothes. NaClO can be absorbed into the human body by inhalation of its aerosol and ingestion. It is not harmful to humans when used at low concentrations (<10%). However, at higher concentrations (>10%), it can cause respiratory disorder, skin irritation (short-term exposure), or skin sensitization (long-term exposure), abdominal pain, burning sensation, cough, sore throat, diarrhea, and vomiting (ICSC4082, ICSC1119). Moreover, the substance is very toxic to aquatic organisms (EINECS/ELINCS 231-668-3).

Household bleaches for laundering of clothes usually contain about 5% NaClO (about pH 11) at the time of manufacture, which slowly decreases when NaClO is heated or after a long storage (ICSC0482) (1). The recommended usage amount of bleach for a standard-size washer is about 50–250 ml per load. Hot water will increase the activity of the bleach, due to the thermal decomposition of hypochlorite, which ultimately generates environmentally undesirable corrosive gases including chlorine. NaClO is released into the environment accidentally and intentionally. Hence, it is worthwhile to measure the effect of NaClO to aquatic organisms.

Among test organisms, zebrafish has been used effectively in many different research areas, including biological and toxicological studies (2, 3). Guidelines on the proper care and use of zebrafish in laboratory have been extensively reported (4). Various biological activities of a fish can be used as measuring indicators (5). However, biological activities are very complex and inconsistent; therefore, conventional methods such as Euclidian geometry are insufficient to describe them (6, 7). Hence, complex geometry such as fractal geometry is required.

Fractal geometry was introduced by Mandelbrot (8) to describe irregular and complex structures such as those in nature. There are various methods to evaluate fractal geometry such as box-counting dimension analysis, capacity dimension analysis, correlation dimension analysis, information dimension analysis, Hausdorff dimension analysis, Hurst exponent analysis, Lyapunov dimension analysis (9, 10) and q-dimension analysis (11). Fractal information may have non-uniform scaling characteristics that are difficult to determine a unique scaling index. Therefore, Nakagawa and colleague (12, 13) proposed a critical exponent method (CEM) to estimate the fractal dimension D of self-affine time series information by determining the critical exponent of the moment of power spectral density to overcome such a problem.

Many researchers had tried to measure biological activities of various types of test animal (14–19). Hence, several types of camera and computer vision systems were used. In the case of a fish swimming behavioral study, in most of the investigations, a computer vision system was used to analyze fish movement in 2D, in which the water was shallow to prevent fish from swimming out of the camera’s focal plane (16, 17). In contrast, in the case of allowing the fish to swim freely in 3D (18), the swimming pattern will contain a higher degree of confidence and provide better results than 2D studies.
In many cases, to obtain a 3D coordinate of an object, a special 3D camera, such as a stereoscopic camera or a single optical sensor camera with special light modulation, is used. In this study, a new approach, 3D coordinate computation with perspective correction (3DCCPC), was carried out using two regular digital video camcorders placed nonplanar to an object, is proposed. Hence, a researcher only needs regular digital video camcorders to efficiently obtain 3D biological activities of a test animal or substance and then quantify the activities by fractal dimension analysis.

**MATERIALS AND METHODS**

**Fish maintenance** Zebrafish (*Danio rerio*) with the body lengths from 2.5 to 3 cm were bought from a local pet shop and maintained in a 20 l aquarium. They were kept for a week prior to the experiment to acclimatize them. The aquarium was filled and refilled with dechlorinated tap water (pH range from 6.5 to 7.2, and oxygen level range from 6.5 to 7.5 mg/l). The water temperature was controlled at 26±1°C using an aquarium heater (Kotobuki Safety Auto IC 55W; Kotobuki-Kogei, Osaka). A power filter (GEX Easy Filter–M; GEX, Osaka) and a biological aquarium supplement (Tetra EasyBalance; Tetra Japan, Tokyo and Nutrafin Cycle; Hagen Japan, Osaka) were used to maintain water quality. Fish care was in accordance with the guidelines for laboratory use of fish. The fish were fed once a day with commercial flakes (Tetra Killimin; Tetra Japan). The light cycle was controlled by programmable timer for 14 h light and 10 h dark (LD 14:10).

**Experimental procedures** Prior to the experiment, a 14 l (32 × 17 × 25 cm³) experimental glass chamber was filled with 10 l of dechlorinated tap water (19 cm water depth). An aquarium heater (Kotobuki AT-10X; Kotobuki-Kogei) was used to control water temperature at 26±1°C. Two video camcorders (DCR-PC300K; Sony, Tokyo) were placed nonplanar to the chamber (in front and beside the chamber). The devices and their positions are shown in Fig. 1.

A bottle of 600 ml NaClO-based household bleach for laundry (pH 13.60) was obtained from a local grocery store. The bleach was used as the main chemical in all the experiments. Swimming responses to acute toxicity in the experiment were measured using an incremental approach with a step of 0.001% concentration (v/v); 0.1 ml of the bleach was added to the experimental chamber in each experiment. The solution was stirred using a small aquarium pump (Rio 50; Kamihata fish industries, Hyogo) for 10 min. The room temperature was elevated to 27°C because the aquarium heater had to be removed during the experiment. Then, a male zebrafish was introduced to the experimental chamber and allowed to acclimate for 30 min before a trial; this will eliminate the effect of swimming behavioral changes due to changes in the size of fish and chemical composition in the water. After the acclimation, the devices in the chamber were removed and the swimming behavior were recorded for 30 min from both side of the chamber. After the recording, the fish was immediately returned to the raising aquarium.

A series of 6 trials (6 d) using the same fish for each trial, were performed to determine the relationship between solution properties and changes swimming behavior. The maximum accumulated concentration of NaClO was 0.005% v/v.

**3D image analysis** The video frames from video camcorders were digitized in the MPEG format with a frame size of 720 × 480 pixels and a frame rate of about 30 fps using Windows Movie Maker via IEEE1394 connection. MPEG movies were fed into the Template matching program to extract XZ and YZ coordinates of the fish. A template image was manually obtained from the beginning of each video file itself to overcome the problem of image registration in the template matching process. The extracted XZ and YZ coordinates were then used to construct the actual XYZ coordinates using the proposed non-planar scopic camera system, with which one can compute the actual coordinates and correct perspective problem from video frames. Figure 2 shows the parameters used in the 3DCCPC equations. The equations for computing 3DCCPC are as follows:

\[
\begin{align*}
\Delta x &= x' + \Delta x_c \\
\Delta y &= y' + \Delta y_c \\
\Delta z &= z' + \Delta z_c
\end{align*}
\]

where \(x', y', z'\) are the fish locations on the x-, y-, and z-axes obtained from the template matching of video frames taken from the long side of the experimental chamber; \(Y, Y, Z\) is the fish location on the y-axis obtained from the template matching of video frames taken from the short side of the experimental chamber; \(x, y, z\) are the computed actual coordinates in the x-, y-, and z-axes respectively; \(\Delta x, \Delta y, \Delta z\) are the computed offsets of the x-, y-, and z-axes respectively; \(x_c, y_c, z_c\) are the center positions of the x-, y-, and z-axes respectively. \(x_\text{outer}, y_\text{outer}, z_\text{outer}\) and \(x_\text{inner}, y_\text{inner}, z_\text{inner}\) are the widths of the inner wall and inner width, which are away from the short side of the experimental chamber.

FIG. 1. Experimental setup, two digital video camcorders (DCR-PC300K; Sony, Tokyo) were placed in front (XZ) and beside (YZ) the glass experimental chamber. The distance from the chamber to the camera was ~50 cm. The chamber size was about 14 l (37 × 32 × 25 cm³).

FIG. 2. Parameters used in the 3DCCPC equations. The equations for computing 3DCCPC are as follows:

\[
\begin{align*}
\Delta x &= x' + \Delta x_c \\
\Delta y &= y' + \Delta y_c \\
\Delta z &= z' + \Delta z_c
\end{align*}
\]
Vector velocity computation

\[ v_t = |r(t+h) - r(t)| / h \]  

(4)

where \( r(t) \) is the vector displacement, \( r(t) = [x(t), y(t), z(t)] \), \( h \) is a delay of approximately 33.36 ms (29.97 fps).

Fractal dimension analysis estimated by critical exponent method

The fractional Brownian motion (fBm) can be used to model random processes as can be found in nature, such as voice, cloud, and substance movement (20–22). Thus, to uniquely estimate scaling characteristics related to the fractal dimension \( D \) of those phenomena on the basis of power spectrum analysis, a critical exponent method (CEM) was suggested by Nakagawa (12).

Denoting the self-affine series fBm with the Hurst function as \( B_\beta(t) \) and the Gaussian white noise as \( w(t) \), the relationship between \( B_\beta(t) \) and \( w(t) \) can be represented by fractional differential equation as follows:

\[ \frac{d^{|\beta|} B_\beta(t)}{dt^{|\beta|}} = w(t) \]  

(5)

where \( H \) is the Hurst exponent (22) and \( t \) denotes the time. With Grunwald–Letnikov differintegral, the \( p \)-order fractional derivative \( D^p f(t) \) of the time series in Eq. 5 can be represented as follows:

\[ \frac{d^p f(t)}{dt^p} = \lim_{h \to 0} \left( 1 - \frac{e^{-\lambda t}}{h} \right)^p f(t) \]  

(6)

\[ = \lim_{h \to 0} h^p \sum_{n=0}^{\infty} \left( \frac{-\lambda}{n} \right)^p \left( \frac{p}{m+1} \right) f(t - mh) \]  

(7)

where \( f(t) = B_\beta(t) \), \( D \) is the differential operator, \( e^{\lambda t} \) is a shift operator, \( e^{-\lambda t} f(t) = f(t-h) \), and \( p \) is a scaling characteristic.

In the frequency domain, a scaling characteristic of fBm can be estimated by determining the critical moment exponent of its power spectral density (PSD). The PSD of fBm, \( P(\nu) \), can be defined as follows:

\[ P(\nu) \sim \nu^{(2H+\beta)} = \nu^p \]  

(8)

where \( \nu \) is the frequency, \( H \) is the Hurst exponent, and \( \beta \) is a scaling characteristic. Then, the fractal dimension determined on the basis of fBm can be given by

\[ D = 2 - H(5 - \beta)/2 \quad (0 < H < 1) \]  

(9)

To estimate the scaling index \( \beta \), Nakagawa (12) suggested CEM to estimate the moment exponent of PSD in the frequency domain.

The th moment, \( I_\alpha \), of PSD can be defined as

\[ I_\alpha \sim \frac{\nu^{\alpha}}{\Gamma(\alpha + 1)} \]  

(10)

where \( U \) is the upper limit of the integration and \( \nu \) is the normalized frequency whose lower cut-off corresponds to 1. By substitute Eq. 8 in Eq. 10,

\[ I_\alpha \sim \frac{\nu^{\alpha}}{\nu^{(2H+\beta)}} \]  

(11)

Given \( X = \alpha - \beta + 1 \) and \( U = \log(U) \),

\[ I_\alpha \sim \frac{\nu^{\alpha}}{\nu^{(2H+\beta)}} \]  

(12)

By computing the 3rd order derivative of the logarithm of moment, \( I_\alpha \), the critical value \( \alpha_c \) can be obtained at the zero-crossing point where \( X = \alpha - \beta + 1 = 0 \).

\[ \frac{d^3 \log I_\alpha}{d\nu^3} = -2 \frac{1}{X^3} + 2 \frac{1}{X^2} \nu \cosh \left( \frac{\nu X}{2} \right) \cosh \left( \frac{\nu X}{2} \right) = 0 \]  

(13)

Then, at the critical value \( \alpha = \alpha_c \), the exponent \( \beta = \alpha_c + 1 = 2H + 1 \).

Finally, the fractal dimension \( D \) can be derived from

\[ D = 2 - H(5 - \beta)/2 \]  

(14)

RESULTS

The swimming trajectories of a zebrafish in 0–0.005% v/v of NaClO based aqueous solution were recorded for 30 min using conventional digital video camcorders, placed nonplanar to the experimental chamber. At a frame rate of 29.97 fps, approximately 54,000 XZ and YZ coordinates of NaClO based aqueous solution were recorded for 30 min using conventional digital video camcorders, placed nonplanar to the experimental chamber. At a frame rate of 29.97 fps, approximately 54,000 XZ and YZ coordinates were extracted by template matching. Also, actual 3D coordinates were constructed by the 3DCCPC. Figure 3A and 3B shows the sample plots of the actual 3D coordinate swimming trajectories under 0.002% concentration of the time series plot of swimming coordinates of the XZ-axis (the swimming responses observed from the long side of the chamber) and the YZ-axis (the swimming responses observed from the short side of the chamber), respectively. Figure 3C shows the complete 3D plot of overall swimming responses. The plots show that the swimming trajectories were very complex and irregular. There was a higher density of trajectories at the water surface and along the wall owing to swimming behavior responses of a particular fish.

The power spectral density (PSD) \( P(\nu) \) of swimming tra-
The actual 3D coordinates determined by 3DCCPC were also used to compute for vector displacement, which was later used to compute swimming velocity (Eq. 4). As a result, Fig. 6A shows a sample plot of the vector displacement of swimming velocities at 0.002% concentration at frame numbers 20,000–39,000 and Fig. 6B shows the sample plot of computed velocities versus frame number at frame numbers 20,000–20,999. Then, swimming velocity was determined by fractal dimension analysis estimated by CEM (Eq. 5–14). Figure 7A shows the plot of log($P(\nu)$) versus log($\nu$). Figure 7B shows the plot $I_\alpha$ versus exponent $\alpha$, which was computed using the 3rd order derivation of log($I_\alpha$). The critical exponent $\alpha_c$ can be obtained at the zero-crossing point and D can be directly computed using Eq. 14.

pH was measured immediately after each experiment. A 100 ml sample of each solution was taken from the experimental chamber to measure pH using a handheld pH/DO meter (D-55; Horiba, Kyoto). The meter was connected to a computer to record the changes in pH then the steady pH was chosen. As a result, the pH at each percent concentration and the fractal dimension values of swimming trajectories and swimming velocities are shown in Table 1. Hence, the
The results shown in the table were used to generate visual results. The data were sorted according to relevant results; Fig. 8A shows the plot of D of swimming velocities, which were sorted by the concentration of the solution and Fig. 8B shows the plot of D of swimming trajectories, which were sorted by the pH of the solution. The trends are shown as lines in Fig. 8A and 8B obtained using linear regression analysis.

<table>
<thead>
<tr>
<th>Concentration (v/v)</th>
<th>pH</th>
<th>D of swimming trajectories</th>
<th>D of swimming velocities</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000%</td>
<td>7.60</td>
<td>2.1367</td>
<td>2.2200</td>
</tr>
<tr>
<td>0.001%</td>
<td>7.62</td>
<td>2.1433</td>
<td>2.2200</td>
</tr>
<tr>
<td>0.002%</td>
<td>7.59</td>
<td>2.1100</td>
<td>2.2350</td>
</tr>
<tr>
<td>0.003%</td>
<td>7.61</td>
<td>2.1433</td>
<td>2.2300</td>
</tr>
<tr>
<td>0.004%</td>
<td>7.60</td>
<td>2.1183</td>
<td>2.2800</td>
</tr>
<tr>
<td>0.005%</td>
<td>7.64</td>
<td>2.1317</td>
<td>2.2600</td>
</tr>
</tbody>
</table>

DISCUSSION

The results proved that the proposed scheme could effectively obtain and quantify 3D biological activities of test animals or substances using conventional digital video camcorders. Also, fractal dimension analysis was a very effective method to quantify information. Moreover, CEM serves as an excellent fractal estimator to uniquely determine the Hurst exponent and fractal dimension. Beside, the results show that the biological activity of zebrafish has a fractal structure and the change in fractal dimension of swimming trajectories correlated to pH, whereas the fractal dimension of swimming velocities correlated to the concentration of the solution.

Regarding pH as opposed to NaClO concentration, the pH value did always increase as NaClO was added. Although the experiments were repeated under controlled procedures and environment, the pH fluctuation still occurred owing to many other free factors that might affect the pH of the solution. Similar results were found previously, that is, pH and behavioral responses do not necessary correlate to the concentration of the solution (23, 24). Figure 8A shows that swimming velocity correlated to the concentration of the solution, whereas Fig. 8B shows that swimming trajectory correlated to the pH of the solution.

In this study, the method of using conventional video camcorders in combination with 3DCCPCP was proposed. Hence, one can use video camcorders to capture bioactivities of a substance or a test organism in a transparent experimental chamber (Fig. 1). Template matching was adopted to extract the swimming loci of the fish. However, the fish may cause noise in the template matching process when they swim along the water surface producing ripples, and their reflection when they swim along the wall of the chamber. To avoid such a problem, correct swimming coordinates can be obtained by selecting the position closest to the center of the chamber. Then, 3D coordination can be constructed precisely by 3DCCPC (Eqs. 1–3).

Although there are various methods to evaluate fractal geometry dimension (9–11), none can provide a unique scaling index to compute for a fractal dimension. Thus, Nakagawa (12) proposed an innovative method to estimate fractal dimension by CEM. By determining the critical point of the power spectrum density of long-tail self-affine time series information, the scaling index was uniquely computed and the fractal dimension.
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