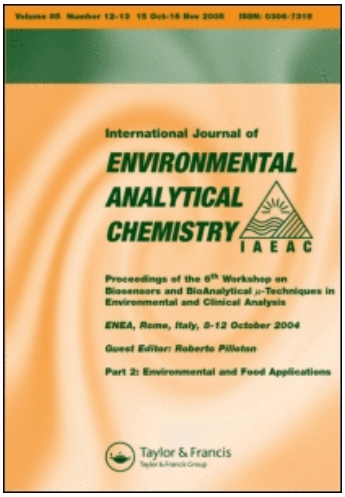

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Flow injection analysis to measure the production ability of superoxide with chemiluminescence detection in natural waters

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Flow injection analysis to measure the production ability of superoxide with chemiluminescence detection in natural waters

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In connection with the photo-chemical processes in the aquatic environment, the evaluation system for the production ability of superoxide anion was constructed. The technique was based on the flow-injection method with luminol chemiluminescence detection. The injected sample was first irradiated by a solar simulator in a vortex quartz cell (cell volume = 0.167 mL), whose vortex face was exposed to light after passing through two air-mass filters. The carrier was the aqueous solution at pH 11 adjusted by NaOH. After irradiation was finished, the carrier (with sample) flow was merged with 1.52 mM of the luminol solution and was introduced into a chemiluminescence detector. The results of the laboratory experiment show that the production of superoxide is linearly related to the concentration of humic acid up to 50 ppm, and also to that of dissolved oxygen. In addition, the chemiluminescence intensity (superoxide production) was proportionally related with the irradiation intensity of the solar simulator. By means of changing the flow rate of the carrier, the half-life of superoxide at pH 11 aqueous solution was estimated to be 15 s. ESR was measured for the sample containing 0.5% humic acid, 0.5% NaOH, and 20 μL DMPO (spin trap agent). ESR spectra were obtained after 5 min of irradiation of the solar simulator. In addition to the four sharp peaks due to OH radicals, a broad peak appeared at the middle of the OH signal. The obtained signal cannot conclusively be ascribed to superoxide, but the peak that appeared may be due to the radical produced in the humic acid molecule. The river water was collected at 18 points of the Tamagawa River located in Tokyo. Upstream, the production ability of superoxide was observed, but not downstream or in the estuarine district. Although the concentration of humic acid is much higher in the estuarine sample, some quenching mechanisms work for superoxide production.

Keywords: Superoxide anion; Flow-injection analysis; Humic acid; Life time; River water

1 Introduction

In natural waters, the superoxide anion is an active oxygen species generated via the photochemical process where the dissolved organic matter (DOM) plays an important role in their generation. In the initial stage of the solar-induced

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photochemical processes, the hydrated electron produced by the photolysis of DOM is proposed as the source of the superoxide anion. This process was examined by Zika [1] and Zafariou [2] in the early 1980s. The proposed reaction pathway for superoxide generation and quenching was as follows:

$$\begin{aligned} \text{Org.} + h\nu &\rightarrow \text{Org.}^* \\ \text{Org.}^* &\rightarrow \text{Org.}^+ + e^-_{aq} \\ e^-_{aq} + O_2 &\rightarrow O_2^- \\ 2O_2^- + 2H^+ &\rightarrow H_2O_2 + O_2, \end{aligned}$$

where Org. is the dissolved organic matter like humic acid, $h\nu$ is the solar radiation, Org.* is the excited dissolved organic matter, e^-_{aq} is the hydrated electron, O_2 is the dissolved oxygen, O_2^- is the superoxide anion, and H_2O_2 is the hydrogen peroxide. Since their studies, Millero estimated the lifetime of superoxide in natural waters [3]. Kochany *et al.* suggest that indoles, including those in aqueous coal oil, show a high quantum yield to give superoxide anions via hydrated electrons, which participates in their autocatalysed photo-oxidation [4]. In this case, the active oxygen species are utilized to remove organic substrates including polyaromatic hydrocarbons, coloured substances, and COD [5]. In general, humic acid has been focused on as an mediator of superoxide production [6–9]. Furthermore, it causes cellular injury, i.e. Liang *et al.* [10] found that the commercial humic acid reduces the viability of rabbit articular chondrocyte cells. The poisonous effect of the humic acid is ascribed to the generation of superoxide anions via lactate dehydrogenase released in this biological system.

There are several methods of detecting the superoxide anion; fluorometry [11], absorption spectrophotometry [12, 13], electrochemical technique [14], and chemiluminescence emission spectrometry [15–17]. These techniques are able to detect 1 μmol to 10 nmol/L of superoxide anion. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is commonly used as the reagent of chemiluminescence, i.e. it has been concluded that the superoxide anion reacts rapidly with luminol and primarily gives the chemiluminescence [18–20]. Since the occurrence of superoxide is of major interest in the biological tissue, luminol chemiluminescence is mainly applied in living systems [21–25]. However, other fields also use luminol chemiluminescence. Nosaka *et al.* used this method to monitor superoxide radicals produced in TIO_2 photocatalysis [26], where they found an immediate reaction between luminol and superoxide, but a slow reaction between luminol and H_2O_2.

In this paper, a flow-injection system with luminol chemiluminescence detection has been constructed for the sake of elucidating the photochemical production ability of superoxide anions in the respective natural water samples, i.e. the high capability of the flow injection system has been pointed out for the detection of superoxide not only for biological systems [27–32] but also in natural water [33].

2. Experimental

2.1 *Materials*

Analytical-grade sodium hydroxide and luminol were purchased from Wako Chem. Co. (Osaka, Japan). Humic acid (AHA) was purchased from Aldrich

Chem. Co. (St. Louis, MO, USA). These chemicals were used without further purification. In the case of humic acid, 1 (w/v)% AHA was dissolved in 0.01 M (=mol/L) NaOH and filtered with a glass-fibre filter with a 1-μm-diameter pore. This solution was diluted with Milli-Q water and used in the experiment. All the solutions (carrier and luminol in the flow-injection analysis) were saturated with oxygen (18 mg/L) by bubbling oxygen (DO) for about 15 min. The amount of dissolved oxygen in the solution was measured by a DO meter (Iijima Electronics Co., model B-505, Tokyo, Japan). In the ESR measurements, humic acid was dissolved in the NaOH solution at a concentration of 0.5%, and the solution was filtered using a membrane filter with a 0.7-μm-diameter pore. Then, 20 μL of DMPO (5,5-dimethyl-1-pyrrolin-*N*-oxide, purchased from Labotech Ltd, Tokyo, Japan) was added to 140 μL of the aforementioned humic acid solution.

2.2 *Instruments*

The system used in the present experiment is illustrated in figure 1. The flow lines were constructed with a Teflon tube of i.d. 0.5 mm and o.d. 1.5 mm. About 0.01 M NaOH aqueous solution (adjusted pH = 11) was sent as a carrier to the system by a pump (JASCO: model PU-980, Tokyo, Japan) at a flow rate of 5 mL/min. Without the sample injection, the carrier travelled to the flat vortex cell, which was made of a quartz tube (i.d. = 1 mm, o.d. = 2 mm). The volume of this cell was 0.167 mL. The vortex cell and two air mass filters (AM 0 (81090) and AM 1.5D (81092) purchased from Oriel Co., Stratford CO, USA) were set in the cylinder, which was placed in front of a 150 W solar-simulator lamp (Oriel Co. model 68905). The irradiated energy collected on the surface of the vortex cell was about 0.85 W in its radiation intensity, as measured by a power meter (Ophir Japan Ltd; type 89018, Tokyo, Japan). The sample was injected into a six-way injection port (Rheodyne Co. model 9725, ERC Gimble, Rimerling, Germany), from which to the exit of the vortex cell. The volume of the line from the injection port was 260 μL. Since the injection volume of the sample was 300 μL, the sample solution already reached the irradiation coil and was, therefore, immediately

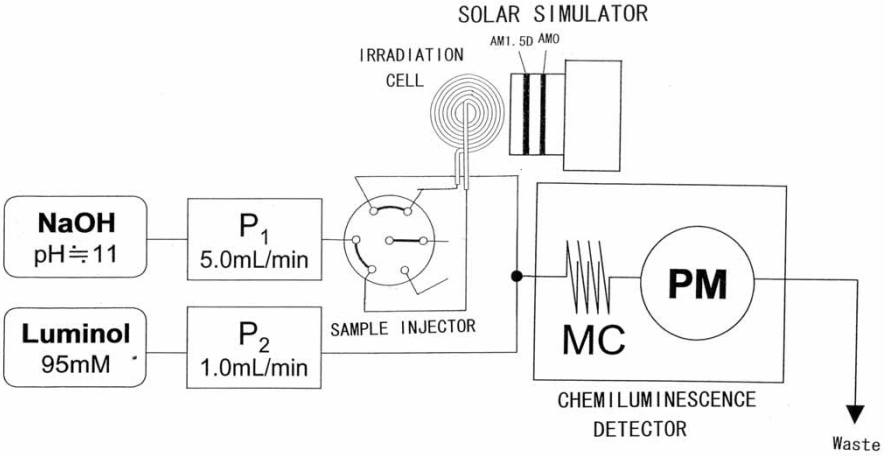

Figure 1. Flow-injection system for superoxide production. P_1, P_2: pump; AM1.5D, AM0: air-mass filter; MC: mixing coil; PM: photomultiplier; sample injector: six-way valve (Rheodyne 9275).

irradiated by the solar simulator after injection. The carrier flow started exactly 4 s after the sample injection. The solution flowed in a tube at the length of 10 cm and was merged with the flow line of luminol, which was dissolved in 0.01 M NaOH solution at a concentration of 95 mM. The flow rate of the luminol solution was 1 mL/min. Both the carrier and luminol solutions were saturated by oxygen. The sample solution mixed with luminol was sent to the light emission detector (JASCO, model 825-CL, Tokyo, Japan), in which the detection was performed in the Teflon coil (i.d.: 0.5 mm; length: 80 cm, cell volume = 0.16 mL) placed on the photomultiplier surface.

ESR (JEOL: JES-FR 30 Free Radical Monitor, Tokyo, Japan) was used for the measurement. The sample containing 0.5% humic acid, 0.5% NaOH, and 20 μL of DMPO (spin-trap agent) was irradiated by the same solar simulator illustrated in figure 1, for 4 min, and was measured in a 130 μL flat cell (LLC-048 made by LABOTEC Co. Ltd, Tokyo, Japan).

2.3 *Sampling of river water*

The river water was sampled in the Asakawa River, which is one of the branch streams of the Tamagawa River in Tokyo. Several points at the estuary of the Tamagawa River were also chosen for the sampling. These sampling points are indicated in figure 2. The river water was collected midstream and stored in a plastic bottle. After aeration, the sampled water filtrated with a sintered glass-fibre filter with 0.7-μm-diameter pores was measured according to the above-mentioned system. As an index of humic acid, the fluorescence of the river water samples was measured using a fluorescence spectrophotometer (JASCO, model FP-777), where the fluorescence intensity was taken against the variations of the wavelengths of emission and excitation.

3. Results and discussion

Figure 3 shows the chart track of flow injection when introducing various amounts of AHA through the injector shown in figure 1. In the figure, the chemiluminescence peak

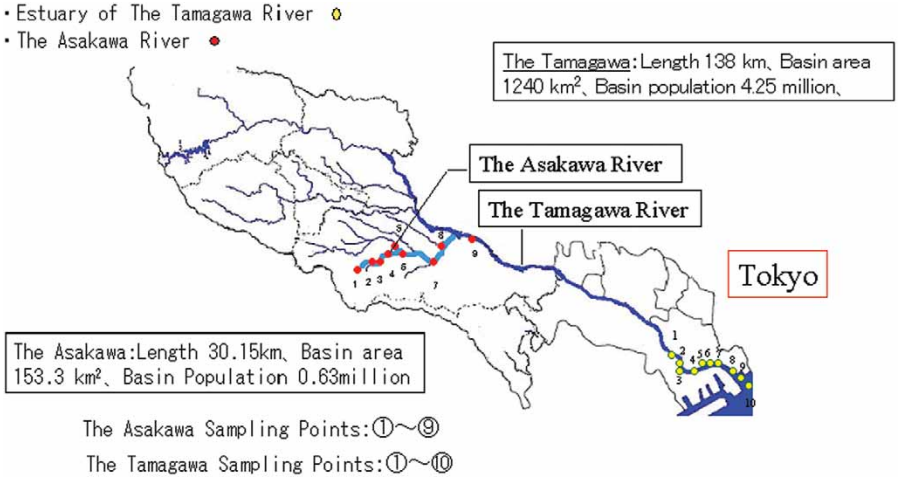

Figure 2. Sampling points in river water.

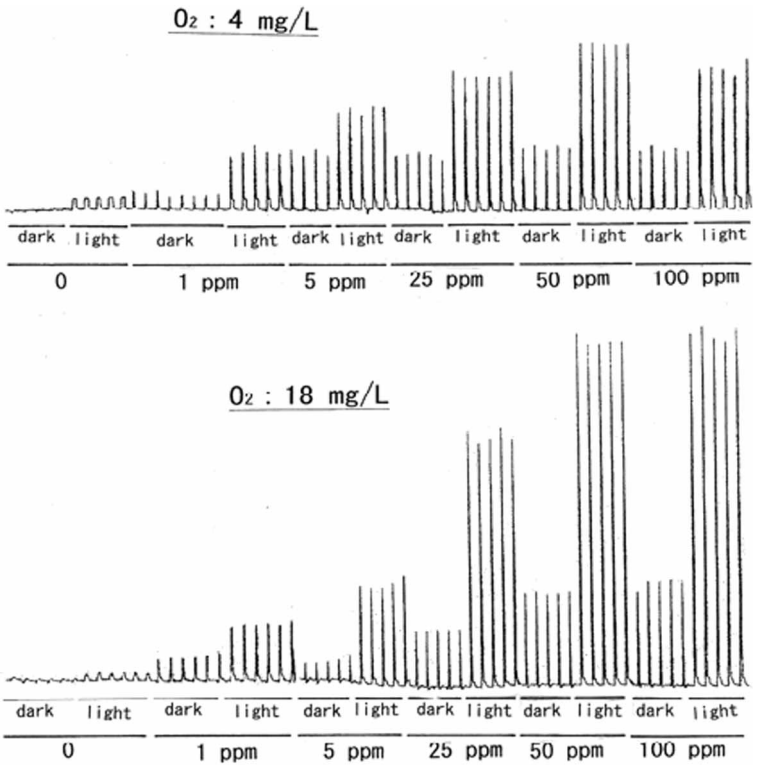

Figure 3. Flow-injection signals for humic acid solution of different concentrations. The concentration of O_2 (dissolved oxygen) was changed by bubbling oxygen through the solution, which was measured by a DO meter. The concentration of AHA was 1 (w/v)%. The concentration of NaOH was 0.01 M (=mol/L), and the pH of the solution after mixing NaOH was pH 11. The concentration of luminol was 1.52 mM. The irradiation time of the solar simulator was 4 s.

increases along with the increase in AHA up to 50 mg/L, which means that AHA quantitatively contributes to the superoxide generation under light radiation. Some signals appeared without irradiation from the solar simulator. Preparation of the solutions was carefully performed under the control of light. However, elimination of the signal due to the non-irradiated sample was difficult, i.e. the superoxide was not generated under complete darkness only. In figure 3, the flow-injection signals were taken for the solutions containing different DO concentrations. The higher-DO solution (18 mg/L) gave a higher chemiluminescence signal than the lower-DO solution (4 mg/L). The DO concentration in the injected solution was changed by the bubbling period of oxygen; the concentration of DO increased from about 3 to 4 mg/L and became constant at 14–20 mg/L by changing the bubbling period from 0 to 20 min.

The chemiluminescence emission intensity was also dependent on the irradiation intensity of the solar simulator onto the vortex flow cell, in which a linear relationship was found as shown in figure 4. The intensity of the solar simulator radiation intensity was varied by using neutral-density filters. It is evident that the production of superoxide anions (or the substance causing the luminescence of luminol) is due to the photoreaction under the existence of humic acid. Figure 5 shows the relationship

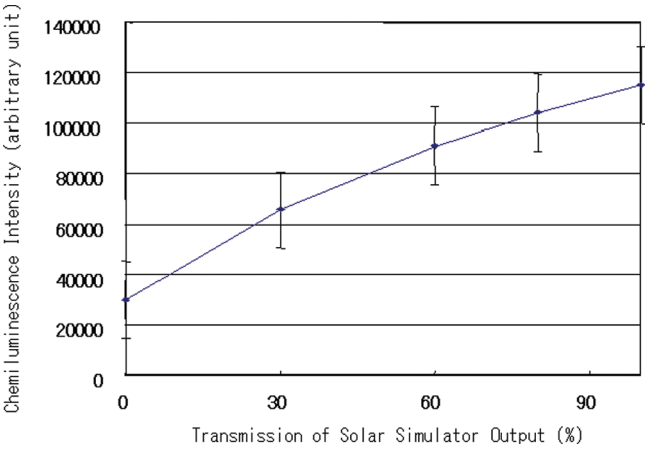

Figure 4. Dependence of chemiluminescence intensity on irradiation intensity of solar simulator.

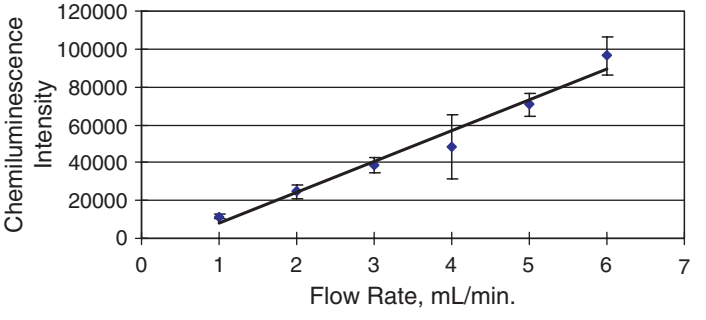

Figure 5. Dependence of chemiluminescence intensity (arbitrary unit) on the flow rate of carrier solution.

between the flow rate of the carrier solution and the chemiluminescence intensity, where the humic acid solution (50 mg/L) was injected. The injection volume was 300 μL, which was sufficient to fill the humic acid solution from the injector to the whole irradiation cell. The irradiation time was 4 s, which was a sufficient period to give the saturated signal for the chemiluminescence signal. The chemiluminescence signal detected (Iv) at the carrier flow rate of v is given as:

$$Iv = [I_b + I_p]e^{-\lambda(VL/v)} - I_b e^{-\lambda(VL)/v}, \quad (1)$$

where I_b and I_p are the chemiluminescence signal without the irradiation of the solar simulator and the chemiluminescence signal assumed at the exit of the irradiation cell, respectively. λ and VL are the attenuation constant and the volume from the exit of the irradiation cell to the chemiluminescence detector, respectively. It is difficult to estimate I_p. However, I_p is related to the difference between the chemiluminescence

signals at 100% (I_{100}) and 0% (I_0) irradiations in figure 3. In this case, equation (1) can be rewritten as

$$Iv = (I_{100} - I_0)e^{\lambda(VL)/v^*} \cdot e^{-\lambda(VL)/v}, \tag{2}$$

where v^* is the flow rate of the carrier obtaining I_{100} and I_0 (in the present case, the flow rate was 5 mL/min ($1/v^* = 12$ s/mL). Therefore, from equation (2), λ is given by

$$\lambda = \frac{1}{VL((1/v) - (1/v^*))} \ln \frac{I_{100} - I_0}{Iv}. \tag{3}$$

In the present flow-injection system, VL was 1.88 mL and $I_{100} - I_0$ was about 70 500. When these values were used, λ was given as 0.031–0.083/s. The half-life was

$$t_{1/2} = \frac{\ln 2}{\lambda}. \tag{4}$$

Since the average λ is about 0.064, a half-life of the superoxide anion in the pH 11 aqueous solution can be estimated to be about 15 s (range: 22.4–8.4 s). One should not ignore the possibility that some reactive oxygen species (except for $^{\bullet}OH$) interfere with chemiluminescence and cause a change in life time for different carrier flow rates. When the tube length from the radiation coil to the merge of luminol was changed from 50 cm (life time: 24 s) to 400 cm (life time: 11.5 s), the calculated half-life varied twice. This variation cannot be explained by the decay mechanism of superoxide in the present flow-injection system. This half-life is rather short compared with the value (100 s) given by Millero [3]; he mathematically calculated the half-life as the disproportionation of HO_2 based on the concentration of H_2O_2. His calculation was based on the two-body reaction of HO_2. Although Millero's result was given for seawater, the order of the calculation and the observed values are similar to each other. The linearity of flow rate and chemiluminescence was always given in the present samples, and seems to be negligible in the exponential component. As for the half-life of superoxide, 10 min was given in aprotic conditions, where ESR was employed for the measurement [34]. In addition, 5.7 min was obtained at pH 7.4 by the spin-trap ESR measurement [35]. The simple aqueous solution including seawater reduces the half-life of the superoxide anion compared with complex biological cells. Regarding the substances affecting the life time of superoxide, several metal ions existing in river water can be considered. We inspected the sulphates of Fe(II) and Cu(II). These ions reduce the flow injection peak, but the life time of superoxide was not influenced by their addition up to 100 μM (mol/L). The main extinguish mechanism of superoxide is in the reaction that participates with superoxide dismutase in the biological system. However, the hydrogen ion is a major species in river water as shown in the reaction scheme. Based on previous data [36], generation of hydrated electron was reduced by nitrate and nitrite ion. These ions are also possible interferents.

Figure 6 shows the ESR spectra, where DMPO was used as a spin-trap reagent. When DMPO was added after the irradiation, a very small signal was obtained. As shown in the figure, four peaks (which seem to be due to OH radicals) appeared irrespective of being with or without humic acid. Under the irradiation acid solution,

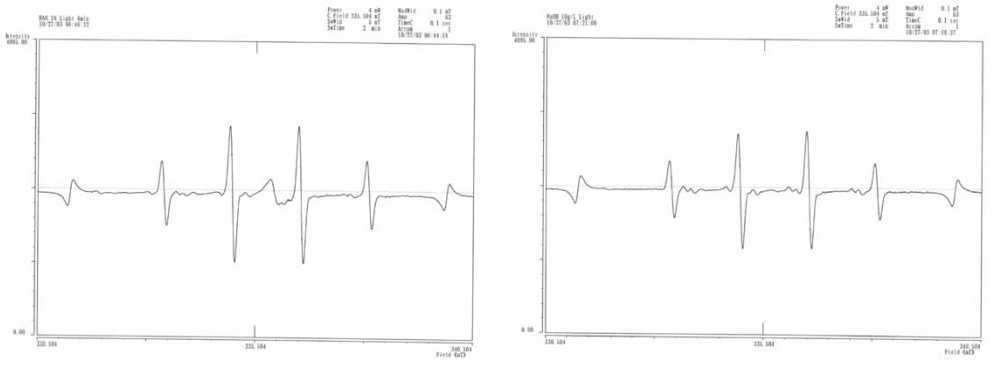

Figure 6. ESR spectra for the solutions with (left) and without (right) humic acid. On the left, ESR was measured for the sample containing 0.5% humic acid, 0.5% NaOH, and 20 μL DMPO (spin trap agent). ESR spectra were obtained after just 5 min of irradiation of the solar simulator. The right spectrum is the same without humic acid.

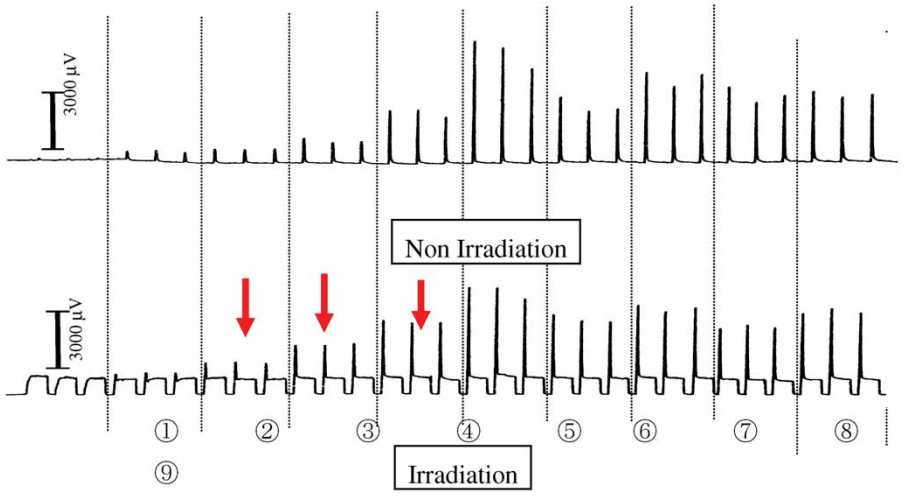

Figure 7. Flow-injection signals of the sample collected in the Asakawa River. The flow-injection signals with and without irradiation of the solar simulator were given. The number shown in the figure denotes the sampling points shown in figure 2.

a small peak appeared at the centre of the ESR chart in the humic acid solution. The data given [37] are insufficient to ascribe this signal to superoxide, i.e. superoxide shows various branch signals close to four peaks of the OH radical. The obtained signal that appeared in the centre of the ESR chart seems to correspond with the radicals occurring in the molecules of humic acid, which has numerous aromatic rings. The radicals may be generated in the humic acid molecule after solar irradiation.

The present flow-injection system was used for river water. The flow-injection signals are shown in figure 7, where the river water was taken from the Asakawa River, upstream of the Tamagawa River in the Tokyo district, Japan (the sampling points are shown in figure 2). In figure 7, one can see that the river water sampled upstream of the Asakawa River (sampling points: 2 ~ 4 in figure 2) provided a slight increase

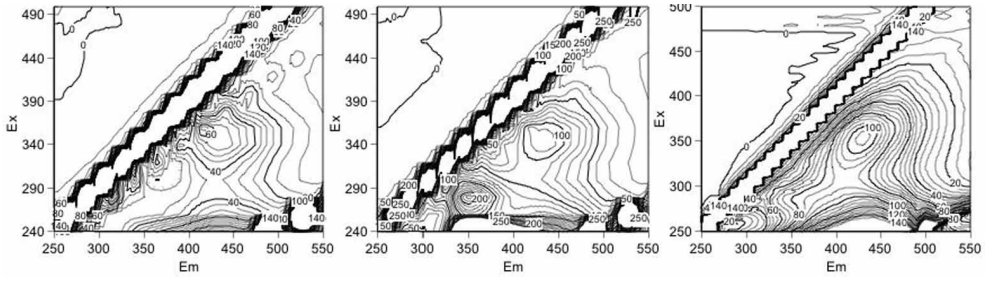

Figure 8. Three-dimensional measurement of excitation and emission (3DEEM) for river fluorescence.

in intensity of chemiluminescence by irradiation with the solar simulator. The water downstream gave a significant chemiluminescence signal either with irradiation or without irradiation from the solar simulator. This situation is the same in water sampled from the estuary of the Tamagawa River. The results obtained cannot be explained by means of the photo-production of superoxide in the river water except upstream of the Asakawa River, but some reactive components that originally exist in the river water made emission with luminol. Reports in the literature show that luminol reacts with H_2O_2, also other than superoxide. However, the reaction between luminol and H_2O_2 is slow, and the amount of H_2O_2 in river water is negligible for the chemiluminescence reaction. The several ingredients gave the luminol chemiluminescence either with or without the solar irradiation. As a result, the half-life can be determined only from river water sampled around the upper stream of the Asakawa river (2.5–3.2 s) by the addition of 50 ppm AHA. The river water sampled at the estuary of the Tamagawa river (sampling points 1–10) yielded chemiluminescence either with or without irradiation from the solar simulator. Figure 8 shows examples of the three-dimensional measurement of excitation and emission for river-water fluorescence. In this figure, emission observed at 450 nm corresponds to humic acid. The result in figure 8 shows that humic acid does not contribute to an increase (by photo-induction) in intrinsic chemiluminescence in river water.

In conclusion, the present flow-injection system is applicable for observing the photo-production of superoxide mediated by humic acid. However, because luminol yields chemiluminescence with substances in natural river water, the photo-production of superoxide was conducted on a limited natural sample. In spite of the limited cases, it is important that the flow-injection technique has the potential to measure the life time of some unstable chemical species as shown in the case of superoxide anions in the aquatic system.

Acknowledgements

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