Comparison of the Conventional Algal Growth Inhibition Tests Using Cell Counting and Algal Bioassay Using Delayed Fluorescence: Application to Industrial Effluents

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ABSTRACT

We propose that rapid algal bioassay using delayed fluorescence (DF) may be useful to apply to the whole effluent toxicity test. In this report, we compared the conventional algal growth inhibition test and the rapid algal bioassay using DF to detect the toxicity of industrial effluents. We used the industrial effluents from eight factories in Toyama Prefecture. Delayed fluorescence is a special type of luminescence; it detects the growth of only those cells that have photosynthetic capability. Therefore, DF can detect algal growth inhibition in a shorter time than the 72 h conventional test, as cells that have lost photosynthetic capability can be detected. The DF test in 6 h and 24 h exposure found algal inhibition from six out of eight effluent samples, and found chronic toxicity unit over 10 from four out of eight effluent samples, the same as the conventional 72 h growth test. These observations suggest that the rapid bioassay using DF is potentially applicable to the selected industrial effluents.

Keywords: algal growth inhibition test, delayed fluorescence, rapid bioassay, whole effluent toxicity

INTRODUCTION

Whole effluent toxicity (WET) testing has been introduced to the US, Canada, European countries and South Korea to compensate the shortcomings of conventional effluent regulations based on the chemical-specific approach (USEPA, 1991; Kusui, 2000; Tatarazako, 2006). Whole effluent toxicity testing evaluates the acute and chronic toxicity of effluent directly using bioassays of alga, daphnia and fish. Among these test species, alga is only the primary producer and is highly sensitive to chemical substances that inhibit photosynthesis and some antimicrobials (Tatarazako and Iguchi, 2012). Since these bioassays assess growth and reproduction of the test organisms, the conventional algal growth inhibition test requires several days to measure the response (e.g. 6 to 9 days, including preparation of the organisms). Regulations generally use the published test method (e.g. USEPA, 1991). On the other hand, there is motivation to develop a high throughput method for self-management tests or toxicity screenings for internal use by each institute (e.g. factory). For example, algal growth test using a microplate is proposed (Eisentraeger *et al*., 2003). Such a method may be a useful adjunct test to the whole effluent toxicity test.

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We propose rapid algal bioassay using delayed fluorescence (DF), which is a characteristic phenomenon of algae and other photosynthetic organisms. Delayed fluorescence is a prolonged ultra-weak fluorescence that can be detected in the dark. Delayed fluorescence decays for more than one minute after a short light excitation (Strehler and Arnold, 1951). Since DF originates from chlorophyll in reaction center of photosystem chemically excited by charge recombination in a back reaction in the photosynthetic electron transport chain, DF can be an intrinsic probe of the photosynthetic activity. Therefore, DF shows two interesting characteristics that may be useful for toxicity evaluation when algae are exposed to toxic chemical substances. First, the emission of DF is inhibited following exposure to certain chemical substances approximately 0.5 s after the excitation. In addition, the inhibition of DF can be used to estimate the 50% effective concentration (EC_{50}) that is obtained in the 72 h conventional growth inhibition test for typical herbicides, photosynthetic inhibitors and heavy metals even for exposure times less than 24 h (Katsumata *et al.*, 2006, 2009; Berden-Zrimec *et al.*, 2007). Second, it was also reported that the time profile of DF intensity (decay curve) shows characteristic behaviors that depend upon the substance (Schmidt and Senger, 1987; Bürger and Schmidt, 1988; Katsumata *et al.*, 2006). Although the analytical method of the characteristics of decay curve requires further research, the second characteristic may provide additional information that can be used for the toxicity identification evaluation in effluent that contains various unknown toxicants.

In this report, we apply the DF test to evaluate the toxicity of industrial effluents (mixtures), rather than specific chemical compounds. We compare the results obtained from conventional algal growth inhibition test and rapid algal bioassay using delayed fluorescence on the whole effluent toxicity test.

MATERIALS AND METHODS

Effluent sample

Between October, 2012 and January, 2013, the industrial effluents from eight factories were sampled in Toyama Prefecture, Japan (Kusui *et al.*, 2014). Effluents #2 and #5 were collected by composite sampling, that is, they were mixtures of effluents collected from different sites. Effluent #2 was from two different sites, #5 was from four sites. Other effluents were collected by grab sampling at a single time and place. In preparation for chemical analyses and bioassays, samples were filtered through a glass fiber filter (GA-55, pore size 0.6 μm, Advantec, Tokyo, Japan), then stored at 4°C in glass bottles. In principle, the biological testing was conducted within 36 h after sampling.

Chemical analyses of the effluents

The potential of hydrogen (pH) was measured by a pH meter (HM-30R, TOADKK, Tokyo, Japan). Dissolved oxygen (DO) was measured by a DO meter (Pro ODO, YSI Nanotech, Kanagawa, Japan). Electric conductivity (EC) was measured by a pH/EC meter (D-54, HORIBA, Kyoto, Japan). Hardness was measured in accordance with the standard methods for the examination of water (Japan Water Works Association, 2011). Since residual chlorine has strong acute toxicity to test alga, free chlorine and total chlorine were determined. Both free and total chlorine were measured by a spectrometer

(DR2800, HACH, Loveland, Colorado, USA) with N,N-diethyl-p-phenylenediamine (DPD) free chlorine reagent (#1407799, HACH) and DPD total chlorine reagent (#1407699, HACH). Since the chlorine concentration in Effluent #6 was relatively high, the water sample was dechlorinated by sodium thiosulfate (11.4 mg/L) before both algal toxicity tests. The concentration of sodium thiosulfate had no effect on algal growth in 24 h (data not shown).

Metal concentrations in the effluents were determined by the following procedure: 50 mL of filtered effluent was mixed with 5 mL nitric acid (EL grade, Kanto Chemical Co., Inc., Tokyo, Japan) in a metal-free PP tube (Digitube, SCP Science, Baie-D'Urfe, Quebec, Canada). The mixture was treated by the wet digestion method; heated to 95°C for 30 min then let to stand for 120 min at 95°C. The depredated mixture was refilled to 50 mL by adding ultrapure water (milli-Q), and then analyzed by ICP-MS (Agilent 7700e, Agilent Technologies, Santa Clara, California, USA). The calibration curve was constructed using a multi-elemental standard solution (XSTC-622, SPEX, Metuchen, New Jersey, USA).

Algal growth inhibition test, OECD test guideline 201, 1984

Algal growth inhibition tests were conducted in accordance with OECD test guideline 201 (TG201; OECD, 2011), using a green alga *Pseudokirchneriella subcapitata*, strain NIES-35. Algal suspensions, inoculated at 0.5×10^4 cells/mL in 60 mL of AAP medium in an Erlenmeyer flask (200 mL capacity), were exposed to a range of effluent concentrations (0, 5, 10, 20, 40, and 80%, diluent was AAP medium). These samples were prepared in triplicate for each effluent concentration. The samples were incubated under continuous illumination from fluorescent lamps (60 μmol/m^2/s) at a temperature of 23 ± 2°C in an orbital shaking culture. Algal cell density was determined with a particle counter (detection range between 3 μm and 12 μm, CDA-500, Sysmex, Japan) every 24 h in the 72 h growth test. The 50% effective concentration (EC_{50}) and the no observed effect concentration (NOEC) were determined by analyzing the cell density data using the default setting by an ecotoxicity statistical analysis package (Ecotox-Statics Ver. 2.6, The Japanese Society of Environmental Toxicology, Japan).

Rapid algal bioassay using delayed fluorescence

Rapid algal bioassay using delayed fluorescence is reported by Katsumata *et al.* (2011) and Katsumata (2012). The green alga *Pseudokirchneriella subcapitata*, strain NIES-35, was used for the rapid test. The test alga was provided as frozen algal kit preserved at –80°C (internal preparation, Hamamatsu Photonics, Japan). The frozen alga recovered to the exponential growth phase through a 1 h pre-incubation after thawing. One milliliter of the prepared algal suspension (20×10^5 cells/mL) was mixed with 9 mL of test sample solution prepared in a range of effluent concentration (0, 5, 10, 20, 40, and 80%; diluent was OECD medium) in round bottom glass culture tubes (ø25 mm × 80 mm). These samples were prepared in triplicate for each effluent concentration. The samples were incubated under continuous illumination from fluorescent lamps (60 μmol/m^2/s) at a temperature of 24 ± 1°C in an orbital shaking culture. The DF from algae in the tube was directly measured at 0 h (initial value), 1 h and 6 h after exposure by the high sensitivity luminometer (TYPE-7100 prototype, Hamamatsu Photonics, Japan). This luminometer was basically the same as the one that had used in our previous report (Katsumata *et al.*, 2009). Samples were left to stand in the dark for 60 s

and were then illuminated with excitation light condition for 30 s with a white LED light (500 μmol/m^2/s), and let to stand in the dark for 5 s, followed by a 1 s exposure by a 700 nm LED light (20 μmol/m^2/s). The DF intensity after the excitation was detected at 0.1 s intervals from 1 to 60 s. The DF signals were recorded as numerical data in a personal computer. The relative DF intensity was expressed as a count. The DF intensities in the 1 to 60 s period were integrated as an integrated delayed fluorescence (DFI). Since the DFI is inhibited through the influence of typical herbicides and photosynthetic inhibitors (Katsumata *et al.*, 2009), DFI can be used in the same manner as cell density in the conventional evaluation method to calculate the EC_{50} and NOEC. The Ecotox-Statics analysis software was used to determine the EC_{50} and NOEC using the DFI data as was used for the conventional algal growth inhibition test.

Generally, to evaluate chronic toxicity, the exposure time should be long enough to evaluate the effect on generations. In the present study, we chose 6 h as the exposure time because we are also considering its practical utility as a rapid test. A 6 h exposure time enables obtaining preliminary results in the same day when the test has started at the morning. For reference, under the experimental condition of 6 h-DFI in this report, the number of algal cells in 6 h becomes approximately 1.5 times as many as that in 0 h in the control medium.

RESULTS AND DISCUSSION

Figure 1 shows the growth curve of the conventional algal growth inhibition test in 72 h (72 h-TG201) for each effluent sample at concentration ratios of 0%, 5%, 10%, 20%, 40% and 80%. The results of eight effluents are displayed in the order of the effect of growth inhibition. Using the 72 h-TG201, growth inhibition was observed in Effluents #4, #5, #6, #7, and #8. Figure 2 shows the growth curve of the rapid test using DFI at 6 h (6 h-DFI). The figure displayed the same order as in Fig. 1. In the result of 6 h-DFI, a typical DF inhibition was observed in Effluents #5, #6, #7, and #8 at the same series of concentration ratios. Interestingly, the inhibition of Effluent #8 was much stronger in the 6 h-DFI than 72 h-TG201. For Effluent #4, significant growth inhibition was detected in higher concentrations (40% and 80%) using the 72 h-TG201 while no inhibition was found within the range of test concentrations using the 6 h-DFI.

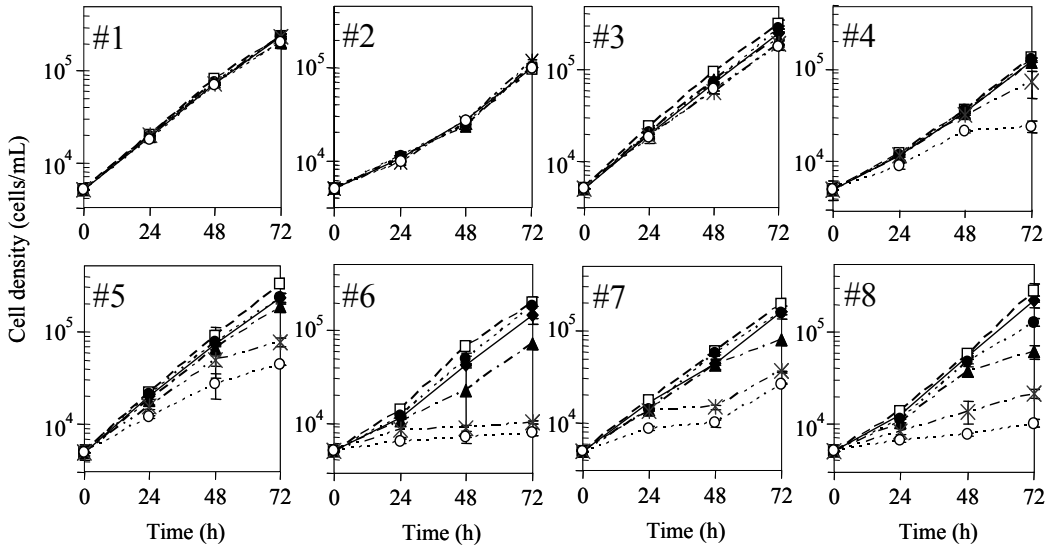

Fig. 1 - Growth curve of the conventional algal growth inhibition test.

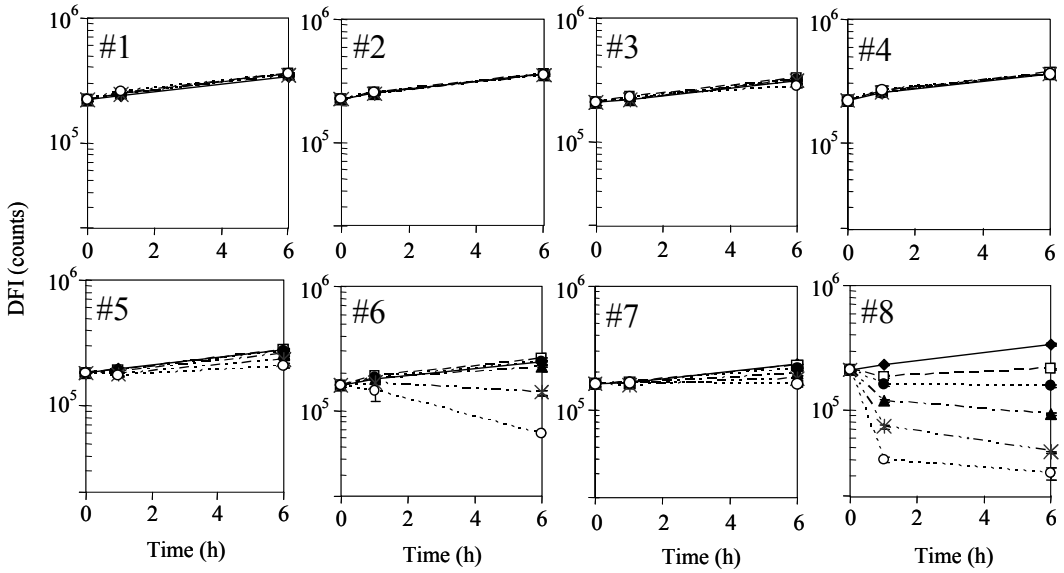

Fig. 2 - Growth curve of the rapid test using DFI.

Figure 3 shows the dose-response of growth rates of the eight effluents using the 72 h conventional test (72 h-TG201). The lowest observed effect concentrations (LOEC) were 80%, 40%, 20%, 20%, 20%, and 10% for Effluents #3, #4, #5, #6, #7, and #8, respectively. Since LOEC was not determined from all ranges of concentration for Effluents #1 and #2, NOEC was larger than the highest concentration such as > 80%. Figure 4 shows dose-response of growth rates using DF in the 6 h (6 h-DFI) rapid test. The lowest observed effect concentrations were 80%, 20%, 20%, 10%, and 5% for Effluents #3, #5, #6, #7, and #8, respectively. The lowest observed effect concentrations were not determined for Effluents #1, #2, and #4 (NOEC was > 80% concentration). Comparing the dose-response of 72 h-TG201 and 6 h-DFI (Figs. 3 and 4), Effluent #8 indicated much stronger inhibition in all concentrations in the 6 h-DFI than in the 72

h-TG201. For Effluent #4, LOEC was 40% in 72 h-TG201, while LOEC was not determined from all the samples in 6 h-DFI. We additionally observed a dose-response of DFI of Effluent #4 with extended exposure time to 24 h. The LOEC was 80% in 24 h-DFI (Fig. 4). The observation suggests that the toxicity of Effluent #4 requires a much longer exposure time than 6 h (i.e. at least 24 h) to be able to detect its influence by the DF test, which possibly indicates that the longer exposure time is required for significant toxicity.

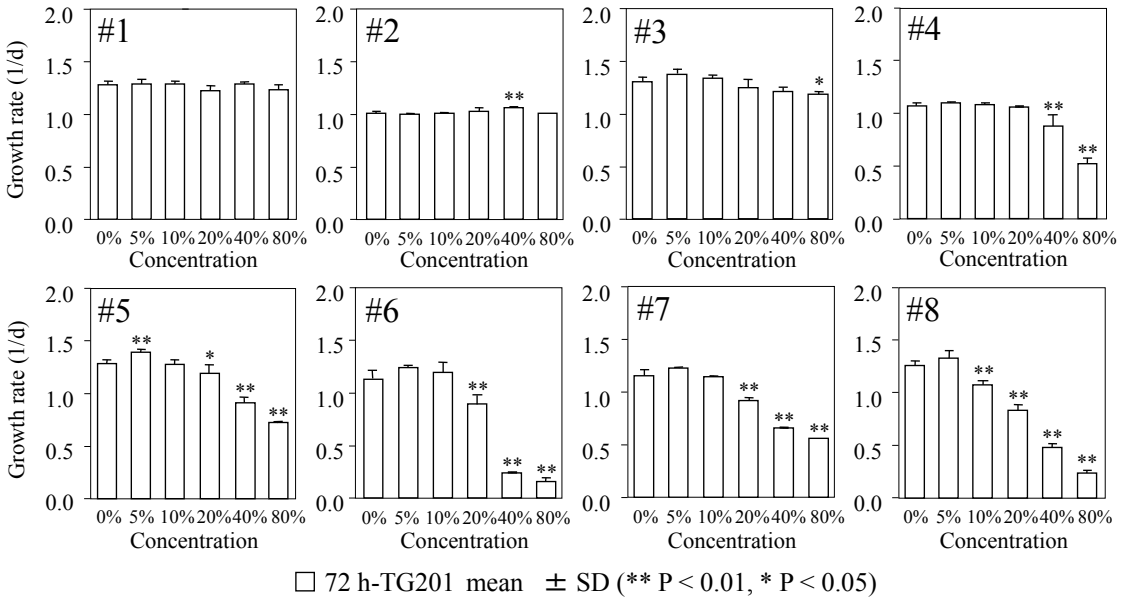

Fig. 3 - Dose-response of the conventional test (72 h-TG201).

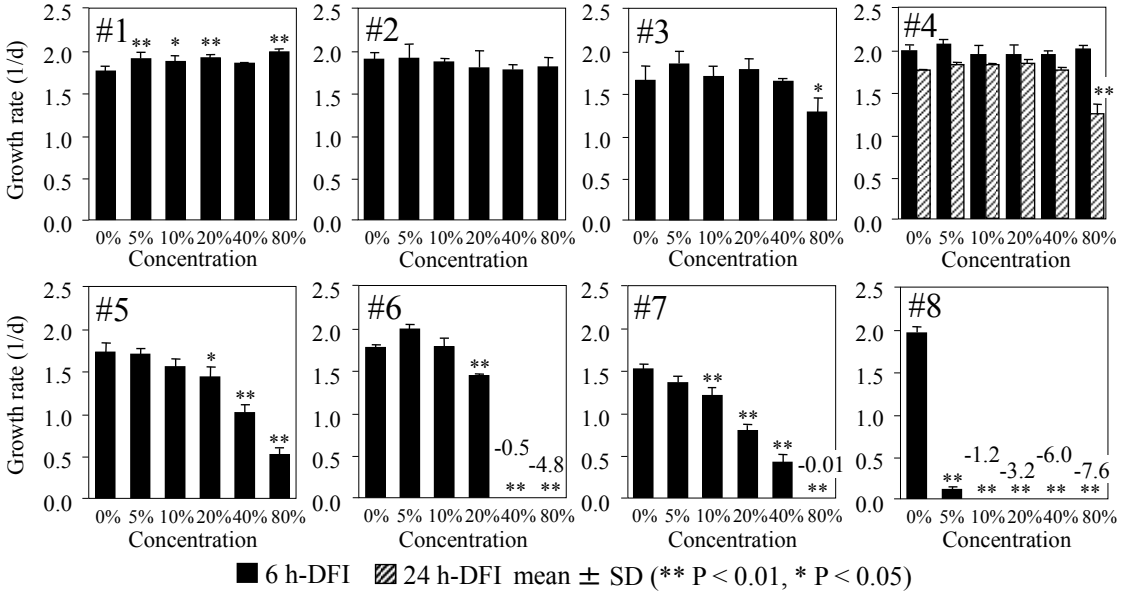

Fig. 4 - Dose-response of the rapid test using DFI (6 h-DFI and 24 h-DFI).

Figure 5 shows the comparison of 72 h-TG201 and 6 h-DFI for chronic toxicity unit (TUc) of the eight effluents. Chronic toxicity unit is defined as 100/NOEC (%). Chronic toxicity unit is considered as an ecotoxicity indicator value. That is, the higher the TUc value, the stronger the toxicity. The Effluents #1, #2, #3, #5 and #6 indicated identical TUc in both tests. On the other hand, Effluent #4 indicated strong TUc by the 72 h-TG201 than 6 h-DFI (and 24 h-DFI). Effluents #7 and #8 indicated higher TUc by the 6 h-DFI than the 72 h-TG201.

Table 1 shows the results of chemical analyses of the effluents. Referring to Table 1, the results of the toxicity observations in Fig. 5 can be discussed as follows: LOEC was not found for Effluents #1 and #2 in both the 72 h-TG201 and 6 h-DFI tests (NOEC > 80%), and TUcs of the effluents were < 1.25 (i.e. lowest value). Among the eight effluents, Effluent #2 showed slightly high Zn concentration (59 µg/L). This suggests that Zn at this level does not cause inhibition in the test. The NOEC of Zn for the alga (*P. subcapitata*, 96 h growth inhibition test) was reported as 20 µg/L (Chao and Chen,

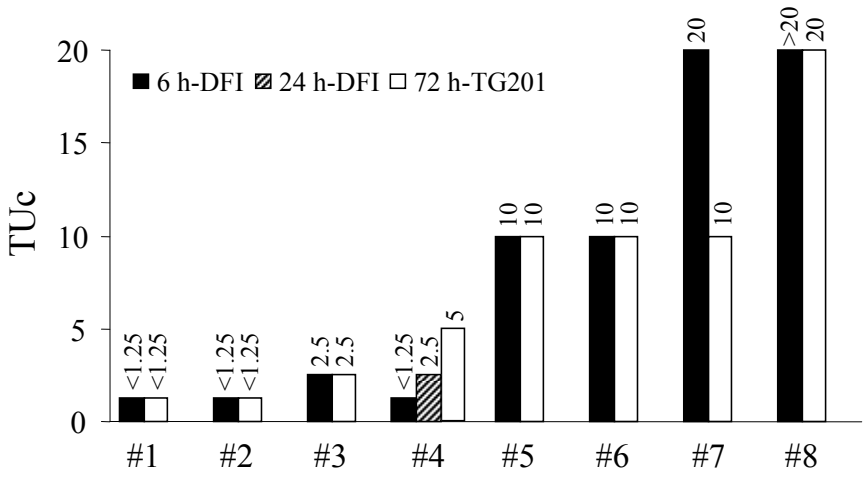

Fig. 5 - Comparison of toxicity units (TUc) in each test.

Table 1 - Results of water analysis.

	#1	#2	#3	#4	#5	#6	#7	#8
pH*	7.66	7.27	6.51	7.98	7.27-7.46	7.56	7.34	7.89
DO*(mg/L)	9.15	9.87-11.42	6.36	8.44	8.08-9.89	5.70	12.90	7.45
EC（S/m)	0.455	0.294	0.203	0.349	0.892	0.867	0.342	5.23
total-chlorine (mg/L)	0.01	0.01	0.07	0.06	0.04	1.00	0.01	0.00
free-chlorine (mg/L)	0.01	0.01	0.01	0.02	0.00	0.02	0.00	0.00
hardness (mg/L)	52	51	240	79	51	80	35	50
Metals (mg/L)								
Cd	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Pb	0.002	0.001	<0.001	0.011	0.001	<0.001	<0.001	<0.001
As	0.001	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	0.003
Se	<0.001	<0.001	<0.001	<0.001	0.010	<0.001	<0.001	0.007
Cu	0.008	0.005	0.003	0.036	0.012	0.009	0.002	0.005
Zn	0.008	0.059	0.017	0.078	0.042	0.042	0.011	0.017
Cr	0.002	0.002	0.001	0.001	0.002	0.004	0.002	0.003
Ni	0.008	0.003	0.086	0.003	0.190	0.002	0.576	0.003

* measured on site

2000). No detection of algal inhibition at 80% of Effluent #2 (Zn of approx. 47 μg/L) might be due to the attenuation by some unknown compounds and/or difference in speciation of Zn. Effluent #3 had TUc of 2.5 in both the 72 h-TG201 and 6 h-DFI. Effluent #3 indicated relatively high hardness (240 mg/L) and Ni concentration (86 μg/L). Since the NOEC of Ni for the alga was reported as 10 μg/L (Chao and Chen, 2000), Ni can be considered likely to cause toxicity. Biotic ligand model might give explanation to the attenuation of nickel toxicity by the relatively higher hardness in Effluent #3 (Lock *et al.*, 2007). The toxicity of a heavy metal (Cd) on *P. subcapitata* was reported to be reduced by high hardness (Källqvist, 2009). However, there is no reliable information how the hardness contributes in the case of Ni. Analysis of the data for Effluent #4 is instructive. The TUc was 5 for Effluent #4 in 72 h-TG201, but was < 1.25 (lowest) in 6 h-DFI. When measuring DFI at 24 h, the TUc was increased to 2.5. Effluent #4 indicated relatively high concentration for Pb, Cu, and Zn (11, 36, and 78 μg/L, respectively). The concentrations of Cu and Zn were much higher than NOECs (10, and 20 μg/L, respectively; Chao and Chen, 2000). The concentration of Pb was lower than NOEC (60 μg/L, Chao and Chen, 2000). Furthermore, hardness was slightly high (79 mg/L). The data for Effluent #4 suggests that there are effluents (e.g. a few metals at low concentrations and slightly high hardness) that require extended exposure times of at least 24 h to detect the influence of the effluent by the rapid algal bioassay using DF. This might also be due to the difference in speciation of the metals. The measured TUc was 10 for both Effluents #5 and #6 in both the 72 h-TG201 and 6 h-DFI. The two effluent samples contained relatively high EC and Zn concentration (#5: EC = 0.892 S/m, Zn = 42 μg/L; #6: EC = 0.867 S/m, Zn = 42 μg/L). Interestingly, Effluent #5 showed notably high Ni concentration (190 μg/L) than Effluent #6. Since TUcs of the two effluents were the same, the high Ni concentration in Effluent #5 might not influence its toxicity. This observation suggests that the toxicity of Ni and other heavy metals could be changed by speciation, EC, hardness, organic matter, and other physical/chemical properties. Specifically, it is difficult to estimate the toxicity from the concentrations of heavy metals in the effluent. Effluent #6 showed strong inhibition at 40% and 80% concentrations (Figs. 3 and 4). As described above, since Effluent #6 contained high chlorine (1.00 mg/L, 14 μM), the effluent was dechlorinated by sodium thiosulfate at excess concentration (11.4 mg/L, 72 μM) before the tests. However, there is still the possibility that the remaining chlorine might affect the inhibition. One possibility is the production of toxic by-products such as organo-chlorine compounds by the reaction of residual chlorine with organic matter contained in the effluent. The calculated TUc of Effluent #7 was 10 in the 72 h-TG201 and 20 in the 6 h-DFI. In other words, the TUc of Effluent #7 in 6 h-DFI was twice as large as in 72 h-TG201. Effluent #7 contained notably high Ni concentration (576 μg/L), but there was no other notable point on other chemical analyses. Therefore, Ni is a possible cause of inhibition. In addition, we should consider speciation of Ni and other metals. However, we do not have adequate data from these experiments. Currently, effluent limit of Ni is unavailable in the Japanese standards. However, bioassay may detect the toxicity of effluents, which include such unregulated/unknown chemicals. This is an advantage of the WET system. Effluent #8 was the most toxic, with TUc = 20 in the 72 h-TG201 and TUc > 20 in the 6 h-DFI. The chemical analysis of Effluent #8 showed notably high EC (5.23 S/m). Since the signal on the ICP-MS for Na in Effluent #8 was over the upper value of its calibration curve (0.1 mg/L), we have only preliminary data of Na concentration in Effluent #8. The preliminary data indicated 2 to 3 g/L of Na in Effluent #8. The 50%

effective concentration of sodium chloride is reported as 28.85 mg/L (Dvořáková *et al.*, 1999). This suggests that a part of toxicity in Effluent #8 was caused by Na. However, there is a possibility of influence from unknown physical/chemical properties. In addition, several metals were detected at relatively low concentrations (As, Se, Cu, Zn, Cr and Ni). This suggests that Effluent #8 contains a relatively high quantity of electrolytes probably including several metal ions. Since Effluent #8 showed definitively stronger inhibition using the 6 h-DFI than the 72 h-TG201 on the dose-response (Figs. 3 and 4), there is a possibility that mixtures containing several metals at relatively low concentrations can show strong inhibition in the 6 h-DFI. This result also suggests that it is difficult to estimate the toxicity of effluent from limited information of physical/chemical properties.

In this study, we only performed a limited analysis of the water quality. To identify the cause(s) of inhibition, toxicity identification evaluation (TIE) is necessary. A high throughput test method that we proposed in this report would potentially be useful for TIE that evaluates many types of effluents by characterizing their toxicity and assisting in the development of effective treatment methods by measuring the benefits (or lack thereof) of treatment, for example by chelation, filtration with activated charcoal, aeration and/or other methods.

CONCLUSIONS

The proposed rapid bioassay using DF estimates the chronic toxicity of effluents in a shorter exposure time than the standard TG201 growth inhibition test, and has the additional benefits of being a simple and rapid procedure. The DF test in 6 h and 24 h exposures found algal inhibition from six out of eight effluent samples, and found TUc over 10 from four out of eight effluent samples, the same as the conventional 72 h growth test. The estimation in 6 h exposure by the DF test indicated similar or higher toxicity values compared to the 72 h conventional growth test in seven of eight effluents in this report. For the single effluent sample that showed no toxicity in 6 h exposure by the DF test, these results suggest that some types of effluents require extended (> 24 h) exposure times for the toxicity effects to develop. Here, we conclude that the rapid bioassay using DF is potentially applicable for the rapid evaluation of the toxicity of industrial effluents, including unknown mixtures.

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