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공학석사 학위논문

PROPOSED METHOD FOR
CONTROLLING TURBID
PARTICLES IN SOLID-PHASE
BIOLUMINESCENT TOXICITY
MEASUREMENT

고형 시료의 탁도 제어에 따른 생태독성학적
평가방법의 산정법 제안

2015년 7월

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이 논문을 공학석사 학위논문으로 제출함

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여슬기 석사 학위논문을 인준함

2015년 7월

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Abstract

PROPOSED METHOD FOR CONTROLLING TURBID PARTICLES IN SOLID-PHASE BIOLUMINESCENT TOXICITY MEASUREMENT

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In the recent half century, numerous methods have been developed to assess ecological toxicity. However, the presence of solid-particle turbidity sometimes causes such tests to end with questionable results. Many researchers focused on controlling this arbitrary turbidity effect when using the Microtox® Solid-Phase toxicity system, but there is not yet a standard method. In this study, I examined four solid-phase sample test methods recommended in the Microtox® manual, or proposed from the literature, and compared the existing methods with the proposed method (c-BSPT, centrifuged-BSPT). Four existing methods use the following strategies to control turbid particles: complete separation of liquid and solid using 0.45- μm filtration before contacting solid samples and bacteria, natural settlement, moderate separation of large particles using coarser-pore-size filtration, and exclusion of light loss in the toxicity calculation caused by turbidity after full disturbance of samples. The proposed method uses moderate centrifugation to separate out the heavier soil particles from the lighter bacteria after direct

contact between them. Among the solid-phase methods tested, in which the bacteria and solid particles were in direct contact (i.e., three existing methods and the newly proposed one, c-BSPT), no single method could be recommended as optimal for samples over a range of turbidity. Instead, a simple screening strategy for selecting a sample-dependent solid-phase test method was suggested, depending on the turbidity of the solid suspension. The results of this study highlight the importance of considering solid particles, and the necessity for optimal selection of test method to reduce errors in the measurement of solid-phase toxicity.

Keywords: Toxicity, Bioassay, Turbidity, Microtox®, Centrifuging

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Chapter 1 Introduction

1.1 Background

Toxicity measurement of waste water and sediments takes a significant portion of environmental pollution monitoring. Since toxicants are ultimately adsorbed onto soil particles and the sediment is usually immobile compared to aqueous phase stream, evaluation of toxicity of solid phase sediment is desirable. The measurement of toxicity using bioluminescent bacteria is growing in popularity owing to its simplicity, short response time, and ease of maintenance. Among the various test methods using bioluminescent bacteria, Microtox[®] Bioassay is a commercial kit using *Aliivibrio fischeri* (*A. fischeri*, NRRL number B-11177, also formally known as *Vibrio fischeri*) as a toxic receptor. The bioluminescence enzyme is created by the set of genes named as lux, this operon is consisted of eight different genes (lux RICDABEG). Luciferase (lux AB) is functionalized as dioxygenase to oxidize the long-chain aldehyde to fatty acid by using substrate. Also it oxidizes FMNH₂ to FMN as well. During this process, the blue green light (490nm) comes out from the electronically excited flavin (Meighen, 1994). The reaction mechanism is as follows (Davis and Aubrey, 2007) and Figure 1. shows the light emission of *A. fischeri*.

Figure 1. *Aliivibrio fischeri* (left), the light emission of *A. fischeri* (right)

The freeze-dried bacteria are rehydrated with reconstitution solution to provide a ready-to-use suspension of organisms. Since *A. fischeri* is bioluminescent marine bacteria, it should be treated carefully following the instructions.

- It is commercially manufactured as frozen and should be kept in the refrigerator.
- It can be used without the sensitivity variance until 1-2 hours after reconstitution.
- 2% NaCl is used for sample dilution to adjust similar marine environment for bacteria.
- The reconstituted bacteria should be in place of constant temperature (15 °C) for its optimum condition.

It has been reported that the *A. fischeri* bioluminescence-assay is one of the most sensitive methods for a wide range of chemicals, compared to other bioassays (e.g., nitrification inhibition, respirometry, ATP luminescence, and enzyme inhibition) (Vanhala et al. 1994; Dalzell et al. 2002; Parvez et al. 2006). Compared to the test using *Daphnia magna*, Microtox[®] has advantages of short time for the test preparation (cultivation and reconstitution of bacteria) and relatively easier test method. Thus Microtox[®] has been used as a standard government eco-toxicological test method in a number of countries (i.e., Canada, The Netherlands, France, Germany, Sweden, and Spain) (Keddy et al. 1995; Park et al. 2001).

Microtox[®] Bioassay is a method originally designed for aqueous samples, so it has been reported to have relatively high accuracy and reliability for liquid samples test. However, some questionable results have been reported when it is used to test solid samples, mainly due to the blocking of light by turbid particles (Parvez et al. 2006; Lappalainen et al. 1999, 2001; Campisi et al. 2005; Ashworth et al. 2010, Kovats et al. 2012; Burga Perez et al. 2013, Xiaoyan et al. 2014). Volpi-Ghirardini et al. (2009) stated that previous research had proven that physical interference of particles with bioluminescence could be a confounding factor in solid-phase sample test (Brouwer et al. 1990; Tung et al. 1990; Bulich et al. 1992; Svenson et al. 1994;

Benton et al. 1995; Brouwer et al. 1995; Ringwood et al. 1997; Onorati et al. 1998; Volpi-Ghirardini et al. 1999).

To correct this negative influence of turbidity, researchers have suggested several methods for controlling turbid particles. Compared to the color effect that is easier to correct, the turbidity effect demands more sophisticated control (e.g., removing turbidity particles while keeping bacteria in suspension, or assuming suspended particles act in the same way as red-brown colored dye) (Ashworth et al. 2010). The Microtox[®] user manual provides two methods for testing samples that include solid particles. The first method is the basic solid phase test (BSPT) and the other is the solid phase test (SPT). The BSPT is essentially identical to the regular basic test (BT) method (the default method for liquid samples) except that the sample filtration step is omitted. In BSPT, the solid particles are partly removed by natural settling during 15–30 min of reaction time of the toxic acceptor. This method is regarded as acceptable when the light-blocking effect of the turbid particles is negligible. On the other hand, the SPT method uses a specially designed filter with 15–45 µm pore size. The test sample is filtered just before the light measurement, after direct contact of the bacteria and solid suspension. This method was conceptually designed to separate the solid particles and bacteria using the SPT filter, but the efficiency of separation is limited samples with coarse solid particles.

Campisi et al. (2005) suggested an alternative test method (mBSPT: modified Basic Solid Phase Test), which considered the inhibition effect of turbidity particles. The modified BSPT is similar to the conventional BSPT, but has the additional step of disturbing the solid suspension before reading the initial light emission (right after bacterial contact with the sample) and the final light emission (after bacterial contact with the sample for a designated time). The light reduction observed in the initial step is assumed to indicate the reduction of luminescence caused only by the physical blocking of light by turbidity particles. Burga-Perez et al. (2012) investigated different bioassays (Microtox[®] Solid Phase Assay or MSPA, and Microtox[®] Leachate Phase Assay or MLPA) based on different intervals and methods of filtration to get rid of the turbidity effect (Burga Perez et al. 2012, 2013). Owing to

these shortcomings of solid-phase toxicity test methods, many researchers have tested the solid particles regardless of their toxic effects, after completely separating the solid particles from the sample using centrifugation or filtration (Dombroski et al. 1996; Ringwood et al. 1997; Romkens et al. 1999; Harkey et al. 2000; Park et al. 2001; Tiensing et al. 2001; Jung et al. 2001; Loureiro et al. 2005; Parvez et al. 2006). However, removal of the particles by those methods (leachate-basic test, L-BT) may cause an underestimation of total toxicity, if the particles removed have a toxic load.

In this study, I developed a new method of controlling turbid particles (the c-BSPT: centrifuged-Basic Solid Phase Test) by adding a centrifugation step at moderate rpm and time, and compared results obtained with those from four existing test methods. Based on the test results and literature review, the strengths and shortcomings of each method were examined. Furthermore, the optimal solid-phase test method was proposed in a sample-specific manner to minimize possible errors and false toxicity readings when using the Microtox[®] system for the toxicity testing of solid phase samples.

Chapter 2 Materials and Methods

2.1 Sample preparation

The solid samples used for toxicity measurements were stream sediments obtained from two different abandoned mining sites in Busan, South Korea (Figure 2.).

Figure 2. Sampling points near by the abandoned mines in Busan, South Korea

Samples were collected from downstream of abandoned adits and were wet-sieved (#100 standard) to collect sediments passing through the 150 μm pores. Fifteen samples were selected for experiments and classified into four categories depending on their toxicity and turbidity. First letter stands for toxicity and the second letter stands for turbidity (i.e., HH: High Toxicity and High Turbidity, HL=High Toxicity and Low Turbidity, LH: Low Toxicity and High Turbidity, LL=Low Toxicity and Low Turbidity). Figure 3. indicates the perceptible way to understand the sample categorization based on toxicity and turbidity.

Figure 3. Sample categorization

To measure the turbidity of a sample, 0.05 g of solids were mixed with 10 mL distilled water and turbidity was measured using a HACH 2100Q (HACH, USA). A higher dilution factor was applied when the turbidity reading was unavailable at solid/solution ratios greater than 1:200 in this case. Figure 4. shows the variation of turbidity (NTU) depending on dilution factor. ‘Initial’ in x-axis means no dilution at all (1:200) and X2, X4, and X8 dilutions means 1:400, 1:800, and 1:1600 dilution of initial state, respectively.

The chemical-composition results obtained from acid digestion of the samples, followed by analytical measurement with an inductively coupled plasma-optical emission spectrometer (Perkin Elmer, USA), and are presented in Table 1.

Figure 4. Turbidity variation on sample dilution factor

Table 1 Chemical properties and turbidity of each sample

	Initial turbidity (NTU)	15-min turbidity ^a (NTU)	pH	Fe	Al	Mn	S	Cu	Pb	Zn	As	Cr
				(%)				(mg/kg)				
HH#1	1800	684	3.35	10.4	7.71	0.14	0.694	37	83	110	87	38
HH#2	2180	568	5.36	5.49	6.9	0.2	0.04	35	98	280	12	23
HH#3	1020	342	3.5	8.75	8	0.27	0.353	38	110	150	56	33
HL#1	436	121	2.81	3.6	4.9	-	-	80	48	78	920	12
HL#2	630	153	3.18	4.97	5.76	-	-	180	73	130	210	90
HL#3	430	104	3.1	2.82	7.16	0.0098	0.08	14	210	64	63	8
LH#1	748	173	6.54	3.88	8.25	0.12	0.039	29	87	220	5	21
LH#2	971	187	5.63	6.14	7.46	0.28	0.044	24	110	230	12	39
LH#3	765	194	5.8	5.89	7.63	0.37	0.014	25	130	230	99	18
LH#4	1608	310	5.76	6.01	7.96	0.28	0.014	28	110	310	96	140
LL#1	304	27.2	5.3	3.42	5.9	0.06	0.026	17	67	99	11	18
LL#2	553	40.4	5.63	5.36	6.92	0.096	0.021	6	52	76	< 2	15
LL#3	425	39.9	5.71	3.55	6.08	0.14	0.013	10	55	85	5	11
LL#4	678	72	6.33	3.01	7.12	0.12	0.025	26	77	180	< 2	21
LL#5	184	20.2	6.05	2.73	5.15	-	-	210	95	210	53	27

2.2 Toxicity tests suggested in Microtox[®] system (*L-BT*, *BSPT* and *SPT*)

To find the optimum test procedure among existing solid-phase test methods, four existing test methods were applied. The general concept of Microtox[®] is depicted in Figure 5.

Figure 5. General procedure for measuring light intensity in Microtox[®]

A diluent is transferred into a cuvette, which is placed in an M500 analyzer. Next, reconstituted bacteria are added to the cuvette. For the normal reaction between diluent and bacteria, a typical wait would be 15 min. Then the light intensity is measured and this is the control (initial light intensity: I_0). A proper amount of sample is pipetted into the cuvette measured I_0 and this starts another wait of 15 min for reaction between sample and bacteria. In this study, I used an experimental design based on 15-min reactions, but the time is adjustable by the tester (normally 5, 15, or 30 minutes). After the second 15 min wait, the light intensity measured is the final light intensity (I_t). Using I_0 and I_t , the effective concentration (EC_{50}) and toxicity unit (TU) can be calculated. EC_{50} is the half maximal effective concentration that refers to the concentration of toxicant inducing a response halfway between the initial

point and the maximum point after a specified exposure time. Every detail of each test should be clarified before the test starts. The conceptual figures and step-by-step test procedure of each method are presented in Table 2.

Table 2 Conceptual figures of each test method and step-by-step procedure

SPT	BSPT	L-BT	mBSPT	c-BSPT
<p>I_t</p>	<p>I_0 I_t</p>	<p>I_0 I_t</p>	<p>I_{mod} I_t</p>	<p>I_0 I_{ct}</p>
<p>Sediment + 2% NaCl</p> <p>↓</p> <p>Addition of <i>A. fischeri</i></p> <p>↓</p> <p>Reaction time (15 min)^b</p> <p>↓</p> <p>Filtration (SPT filter)</p> <p>↓</p> <p>Light intensity (I_t)</p>	<p>2% NaCl</p> <p>↓</p> <p>Addition of <i>A. fischeri</i></p> <p>↓</p> <p>Reaction time (15 min)^a</p> <p>↓</p> <p>Initial light intensity (I_0)</p> <p>↓</p> <p>Addition of sample</p> <p>↓</p> <p>Reaction time (15 min)^b</p> <p>↓</p> <p>Light intensity (I_t)</p>	<p>Sediment + 2% NaCl</p> <p>↓</p> <p>Filtration (0.45 µm filter)</p> <p>↓</p> <p>Addition of <i>A. fischeri</i></p> <p>↓</p> <p>Reaction time (15 min)^a</p> <p>↓</p> <p>Initial light intensity (I_0)</p> <p>↓</p> <p>Addition of filtered sample</p> <p>↓</p> <p>Reaction time (15 min)^b</p> <p>↓</p> <p>Light intensity (I_t)</p>	<p>2% NaCl</p> <p>↓</p> <p>Addition of <i>A. fischeri</i></p> <p>↓</p> <p>Reaction time (15 min)^a</p> <p>↓</p> <p>*Initial light intensity (I_0)</p> <p>↓</p> <p>Addition of sample</p> <p>↓</p> <p>Reaction time (15 min)^b</p> <p>↓</p> <p>Light intensity (I_t)</p> <p>↓</p> <p>Centrifuging 500 rpm (1 min)</p> <p>↓</p> <p>Light intensity (I_{ct})</p>	<p>2% NaCl</p> <p>↓</p> <p>Addition of <i>A. fischeri</i></p> <p>↓</p> <p>Reaction time (15 min)^a</p> <p>↓</p> <p>*Initial light intensity (I_0)</p> <p>↓</p> <p>Addition of sample</p> <p>↓</p> <p>Reaction time (15 min)^b</p> <p>↓</p> <p>Light intensity (I_t)</p> <p>↓</p> <p>Centrifuging 500 rpm (1 min)</p> <p>↓</p> <p>Light intensity (I_{ct})</p>

^a Reaction between diluent and microbes

^b Reaction between sample and microbes

* Measured only for comparison. It was not used for EC₅₀ calculation

With the L-BT method, the sediment suspension was filtered with a 0.45 µm syringe filter and only the supernatant was used to measure the toxicity of the clear solution. Thus, there was no direct contact of the solid particles with the bacteria. The details of the test procedure can be found in the Microtox® user manual (Azur Environmental, 1998).

The second and third methods used were the Solid Phase Test (SPT) and Basic Solid Phase Test (BSPT). The SPT uses an SPT filter (Evergreen Scientific, USA) to separate coarse particles from a solid suspension mixed with bacteria. Unlike the SPT, the BSPT does not require extra equipment (e.g., incubator block or filters). This method involves direct contact of solid particles with the bacteria. No other process is used to separate solid particle except the natural settling that occurs during the reaction time. Therefore, in principle, the effect on light intensity of suspended solids left in the suspension after the natural settling with BSPT should be negligible, but this is not always true.

The fourth method was adopted from Campisi et al. (2005). The mBSPT (modified Basic Solid Phase Test) method includes the light output at the highest level of turbidity by disturbing the sample just before measurement of the light (i.e shaking enough) and takes it as the initial light intensity (I_{mod}). With the mBSPT, the light intensity of a sample is read three times (Table 2). First, before sample injection, the initial light output (I_o) is read. It is read again just after putting the sample into the cuvette (modified initial light output: I_{mod}) and finally read a third time after bacteria are added to the sample (I_t). With the mBSPT the toxicity is calculated using I_{mod} and I_t .

In determining EC_{50} , the software provided by Azur Environmental automatically calculates the result as EC_{50} using the bioluminescence inhibition (%) parameter, defined as:

$$KF = \text{Correction Factor} = \left(\frac{I_t}{I_o} \right)_{control} \quad (2.1)$$

$$\text{Bioluminescence Inhibition (\%)} = 100 - \left[\frac{I_t}{I_o * KF} * 100 \right]_{sample} \quad (2.2)$$

The sample toxicity unit (TU) is calculated using the obtained EC_{50} in Eq. (2.3).

$$TU = 100/EC_{50} \quad (2.3)$$

2.3 c-BSPT (centrifuged-Basic Solid Phase Test)

In this research, I also tested a new alternative, named the c-BSPT. This proposed test method controls sample turbidity using centrifugation (*HITACHI CT 15E*, Max. speed = 15,000 rpm, Max. RCF = 21,500 x g). The c-BSPT is similar to the BSPT except that the added centrifugation step forces the turbidity particles to settle, while the bacteria remain suspended in the solution. High-speed centrifugation is a typical microbiological method used to isolate bacteria from a diluted suspension. In this study, a slower centrifugation speed (500 rpm) was used to settle soil or sediment particles, while allowing bacteria to remain suspended. In the c-BSPT, a 2-mL plastic micro-centrifuging tube was used as a sample cuvette instead of a commercial Microtox[®] sample cuvette. This way, the sample does not need to be transferred to a separate container for the centrifugation step. In the c-BSPT, the bioluminescence inhibition is defined in way similar to that explained earlier, except that I_{ct} (the light emission after centrifuging, followed by reaction of sample with bacterial reagent for 15 minute) is used instead of I_t in Eq.(2.1) and (2.2). The equation used for toxicity calculation in the c-BSPT is:

$$KF = \text{Correction Factor} = \left(\frac{I_{ct}}{I_0} \right)_{control} \quad (2.4)$$

$$\text{Bioluminescence Inhibition (\%)}_{c-BSPT} = 100 - \left[\frac{I_{ct}}{I_0 * KF} * 100 \right]_{sample} \quad (2.5)$$

Chapter 3 Results

3.1 Determination of c-BSPT method

To establish a proper centrifugation speed (in rpm, *revolutions per minute*), it was necessary to clarify whether centrifugation influenced light emission from the bacteria. I examined the change in light intensity within a range of 300 rpm to 10,000 rpm. Each test was replicated five times for consistency. From the results, it was observed that the light intensity increased after centrifuging due to bacterial activation. The change in light output was maximal when the centrifuge speed was 500 to 1,000 rpm, and declined with increasing centrifuge speed. The reduction of the light output change is attributable to the settlement of bacteria when the rpm is high. To define the centrifuge effect on bacterial viability, the change in light intensity was determined in relation to a range of centrifuge speeds (300–10,000 rpm) and Figure 6. shows the result. As centrifuge speed increased, light intensity decreased. This means that the viability of *A. fischeri* appears to be impacted by higher rpm. However, the rpm appropriate for c-BSPT, was one able to settle the turbidity particles, but allow the bacteria to remain in suspension. For this reason, I selected 500 rpm for the centrifuge speed in the experimental design, and a run time of 1 min. Regarding the settling of bacteria, the optimal centrifugation speed for the c-BSPT procedure was determined to be 500 rpm. The change in light output induced by centrifugation can be adjusted in the EC_{50} calculation using Eq. (2.4) and (2.5).

Figure 6. Change in light intensity at different centrifuge rpm

Figure 7. Correlation between BSPT and c-BSPT toxicity values (EC₅₀) tested with solid extracts (without solid particles)

To verify the effectiveness of the newly proposed c-BSPT method, solid extracts without particulate matter were tested using c-BSPT and BSPT. If the c-BSPT results were similar to those of BSPT (same as L-BT when aqueous samples are tested), within the acceptable range of the tolerance level of the general Microtox[®] test, then the new c-BSPT method should be systematically acceptable. Figure 7. shows the overall linear regression of EC₅₀ in BSPT versus c-BSPT testing of solid extracts. It indicates a relatively significant correlation ($r^2 = 0.9309$, linear regression = 0.9479) and the deviation was within the boundary of normal systemic deviation caused by Microtox[®]. This indicates that the centrifugation effect itself can be correctable in calculations. Thus, I selected 500 rpm with a run time of 1 min as optimal for settling the turbidity particles without precipitating the bacteria. It should be noted that a test-dependent (i.e., sample quality dependent) approach to selecting the proper rpm and run time could be applied, with a comprehensive understanding of the entire process.

3.2 Comparison of toxicity results tested using five different methods

The toxicity of fifteen samples was evaluated using the five different test methods (L-BT, SPT, BSPT, mBSPT, and c-BSPT) and the results were presented in Table 3 in EC_{50} units along with the results of turbidity measurement. These results were categorized in the four groups based upon the cross-compared trends of EC_{50} values obtained from each method. It was found that the 15-min turbidity value was an effective parameter among the categorizing criteria. The categorized results of EC_{50} depending on the 15-min turbidity of samples are presented in Figure 8. as pentagonal graphs. The EC_{50} of the L-BT was higher in most samples than was the EC_{50} of the other test methods. This is attributable to the lack of direct contact of luminescent bacteria with particle surfaces. Moreover, it is noticeable in the m-BSPT results, that samples with lower toxicity (LL and some LH samples) yielded toxicity results with relatively high EC_{50} values (up to 100, and this value means that a sample is non-toxic) even though the samples are certainly toxic in some degree since the results obtained from L-BT, as a lowest threshold, is presenting those samples' toxicity. The reason for this false measurement using the mBSPT will be considered in detail in the discussion section.

In particular, the EC_{50} relative to the range of turbidity of the BSPT and c-BSPT were comparable, but the effective concentration was slightly higher in c-BSPT. This is probably owing to elimination of effects on light transmission caused by turbidity particles (removed by centrifuging in the c-BSPT). This effect from control of turbidity with c-BSPT can be seen in Figure 8C. This trend of higher EC_{50} in c-BSPT was not observed in results of samples with turbidity that was too high ($turbidity_{15min} > 500$ NTU) or too low ($turbidity_{15min} < 100$ NTU). The reason for the smaller EC_{50} in c-BSPT will be also discussed in the next section.

Table 3 Results of EC₅₀ tested with five methods

	15min turbidity	L-BT	SPT	BSPT	mBSPT	c-BSPT
HH#1	684	0.68	HT ^a	0.17	0.4	0.15
HH#2	568	3.95	1.02	0.55	3.22	0.46
HH#3	342	0.44	0.58	0.37	0.54	0.41
HL#1	121	0.4	HT ^a	0.47	0.58	0.63
HL#2	153	0.74	0.57	0.28	0.45	0.32
HL#3	104	1.18	0.8	0.49	0.78	0.42
LH#1	173	10.84	2.53	1.81	NT ^b	1.98
LH#2	187	7.11	1.84	1.76	1.65	2.08
LH#3	194	10.22	4.37	2.16	17.24	1.52
LH#4	310	3.04	3.68	2.11	NT ^b	3.1
LL#1	27.2	31.37	30.1	4.24	NT ^b	3.3
LL#2	40.4	12.38	9.16	5.15	NT ^b	1.83
LL#3	39.9	17.82	4.11	2.05	NT ^b	1.74
LL#4	72	NT ^b	NT ^b	5.1	NT ^b	3.4
LL#5	20.2	13.38	4.55	6.27	NT ^b	5.41

^a HT = High Toxicity = too low EC₅₀^b NT = Non Toxicity = too high EC₅₀

Figure 8. EC_{50} in each test method. Samples were categorized in four groups depending on their turbidity: (A) $turbidity_{15min} \leq 100$ NTU, (B) $turbidity_{15min} \geq 500$ NTU, (C) HH & HL samples of $100 < turbidity_{15min} < 500$ NTU and (D) LH samples of $100 < turbidity_{15min} < 500$

Chapter 4 Discussion

4.1 Error-causing factor of the L-BT

The L-BT yielded higher EC_{50} in most cases, which means the results obtained from the L-BT yielded lower toxicity results than other test methods. This is primarily because the samples used for the L-BT contained only the water-soluble portion of toxicants and did not consider toxicants on the particles. An example of the toxicity of particle is shown in Figure 9. Given that Sample LL#2 had one of the lowest turbidities, it is logical to regard it as a turbidity-effect-free sample. Although use of the L-BT could eliminate the negative turbidity effect during the test, it resulted in a possible under-estimation of toxicity. It was noted that aqueous extracts from soil are less sensitive (tests of actual toxicity in un-extracted solid-phase samples) mainly because bacteria are not in direct contact with contaminants present in the bulk soil (Loureiro et al. 2005; Indiana DOT. 2008; Harkey et al. 2000). Thus, the L-BT and any other extraction method should be carefully examined with complete consideration of the potential for toxic contribution of solid particles.

Figure 9. Normalized light-intensity change of samples with series dilution (LL#2). The BSPT test process for samples without particles is identical to the L-BT method. The normalized Δ light intensity equation is:

$$Control = \left(\frac{I_t - I_0}{I_0} \right) * 100,$$

$$Normalized \ \Delta \ light \ intensity = \left\{ \left(\frac{I_t - I_0}{I_0} \right) * 100 \right\} - Control$$

4.2 Error-causing factor of the SPT

The SPT uses a specially designed commercial filter to isolate soil particles from the solution while allowing the bacteria to remain suspended. However, the filtration efficiency is low and it is questionable whether the filtration efficiency in the SPT is better than the condition provided by natural settling with the BSPT. Despite the questionable performance of the SPT filter in separating fine particles, the usage of this method in evaluating solid samples containing fine particles has been reported (for example: Serafim et al., 2013). Moreover, in contrast with other methods in which the light output is measured twice (initial step: I_0 and final step: I_t or I_{ct}), the SPT calls for only one measure of light output (after 15 min of reaction for each batch of serial dilution). With the Microtox[®] system, only 10 to 20 μL of bacterial reagent is transferred from a reagent-well to a sample tube or diluents tube. Because this is such a small amount, errors can easily occur. With the SPT, the turbidity particles were filtered out after contact between the bacteria and the sample. Then the supernatant was extracted and dispensed into a cuvette. This transfer is a possible source of errors in the result and Table 4 shows how it occurs.

For example, if the particle size were smaller than the pore size of the filter, fine particles would also pass into cuvette, so the turbidity could not be fully removed. Moreover, the filtering process after exposure of the bacteria to sample requires sophisticated pipetting technique. This is a second factor contributing to erratic results. In most cases, the SPT only measures the final light intensity; thus, it cannot be used to compare the change in light intensity induced by the sample toxicants. Table 4 shows examples of such irregularities. In the statistical analysis of the measured I_0 of BSPT (there were 300 batches), the I_0 values was ranged from 64 to 121, with an average value of 92 ± 9.6 .

Table 4. The irregularity inducing by unsophisticated pipetting technique

Concentration (mg/L)	I _t
0	95
6168.75	48
12337.5	101
24675	65
49350	51
98700	32
197400	54

4.3 Error-causing factor of the BSPT

When a sample is highly turbid, even after natural settling during the 15 min of reaction time, the light from bacteria can still be blocked by suspended particles, so the light detector cannot read the authentic light intensity in the BSPT. If the sample has turbidity of less than 100 NTU after 15 min of natural settling, the turbidity was well controlled, and did not disturb the measurement. However, when the sample has turbidity over 100 NTU, turbidity is a primary factor that should be regulated. Since BSPT and c-BSPT are similar methods, except that with c-BSPT the final light intensity is read after centrifugation, these methods can be compared to verify the effect from controlling turbidity. In Table 3, it was revealed that EC_{50} was lower in most cases with BSPT, when turbidity was between 100 NTU and 500 NTU. This is because turbidity particles that might have blocked the light from the bacteria were effectively settled by centrifugation.

4.4 Error-causing factor of the mBSPT

When the mBSPT method is applied on a sample with low toxicity and high turbidity, the light reduction caused by the turbidity may mask the toxicity-related reduction in light production. For instance, the measured initial light output (I_{mod}) of the disturbed sample is similar to the measured light output ($I_t = I_{t_disturbed}$) of the same sample after 15 min. However, the undisturbed I_t ($I_{t_undisturbed}$), of which the light output was measured just before shaking the sample, was higher than the I_t of the disturbed sample ($I_{t_disturbed}$). In this case, the reduction in the light output caused by the sample's real toxicity is masked by a false light reduction caused by the physical blocking of light by turbidity particles. The test result in Figure 10A show the case for which mBSPT would be ideal, and Figure 10B shows a case for which mBSPT cannot be applied. The graph in Figure 10A shows how the mBSPT corrected the possibility of over-estimation of toxicity of BSPT by separating the causes of light reduction into turbidity-induced and toxicity-induced parts. However, the graph in Figure 10B displays the typical case of toxicity screening by turbidity-induced light output. The turbidity-induced light reduction is greater than the toxicity-induced light output, so the change in light intensity was not noticeable. This demonstrates that this method may produce false results and this limitation is also elucidated in the recent research (Lee et al. 2012). Despite the obvious error causing factor of mBSPT, this method is still used by other researchers without profound understanding of possible false results (Jho et al., 2015).

Figure 10. Result of mBSPT in terms of normalized light intensity: The case presented in Graph A fits well with the concept of mBSPT (HL#3), but Graph B presented an inappropriate case resulting in serious under-estimation of toxicity (LL#2)

4.5 Error-causing factor of the c-BSPT

It turns out that the c-BSPT is suitable when the sample turbidity is in the range from 100 NTU to 500 NTU. The c-BSPT is similar to the BSPT, except the fact that samples are centrifuged to settle turbidity particles in advance of the final reading of the light output. For this reason, the light-intensity results with the former tend to be higher (Figure 11.), and this demonstrates well the positive effect of controlling turbidity particles by centrifugation and it appears the most effective result in low toxicity and high turbidity sample.

Figure 11. Difference between BSPT and c-BSPT (solid line: BSPT, dotted line: c-BSPT) in inhibition of bioluminescence

However, different trends were observed in the c-BSPT results of samples with too high or too low turbidity. Particularly the light intensity in the c-BSPT for samples with high turbidity ($\text{turbidity}_{15\text{min}} \geq 500 \text{ NTU}$) was lower than that from the BSPT. This phenomenon can be explained using the ‘density theory’. This theory provides an explanation of why more-turbid samples tend to have fewer microbes in the solid-particle suspension, and thus why less bioluminescence would be detected from the supernatant. In samples with many turbidity particles, the solid particles are more likely to adsorb or simply

drag the bacteria during the centrifugation procedure. This may cause the removal of active luminescent bacteria; thereby, resulting in a false rate of bacterial mortality. I found that if samples with extremely high turbidity were centrifuged, the intensity of the light from bacteria decreased because the settling turbidity particles dragged or adsorbed some of the bacteria and forced them to the bottom. Figure 12. includes data for cuvette of the same size (height and width), with the same amount of bacteria inside, but different amounts of turbidity particles. When all were centrifuged, the particles in the cuvette with few particles settled down effectively. However, in the cuvette with a higher density of particles, some of the bacteria producing light were also forced to settle, thereby reducing the light intensity. This may be a reason that the c-BSPT appeared less effective when it was applied to extremely high turbid samples.

Furthermore, the c-BSPT applied to samples with low turbidity and low toxicity may have another possible source of error. The centrifugation caused the activation of luminescent bacteria and this led to slightly higher I_0 values. Since the changes of light output before and after the sample addition will be small in samples with low turbidity and low toxicity, a small change in I_0 could bring about the over-estimation of toxicity. Moreover, in general, turbidity is not a problematic factor in toxicity testing of samples falling in the LL category, so the BSPT would be the most practical method due to its simplicity.

Figure 12. Concept of density theory assuming the same amount of bacteria in each cuvette: Left: with fewer turbidity particles, Center: with more particles, Upper right: adjacent particles confine a bacterium, Lower right: toxicants adsorbed onto solid particle

4.6 Hormesis

During the test, I found out an interesting fact that the light output of bacteria is actually getting intenser when it is used for some LL samples (Figure 13.). This is called as *hormesis* that somehow the bacteria's viability gets stronger than the initial state. Shen et al. (2009) reported the stimulatory effect of low concentrations of toxic chemicals on organismal metabolism is common in luminescent bioassay (Fulladosa et al., 2005, 2007). The increased light amount varies at exposure time and dose of toxicant. Like I mentioned earlier, LL samples are mostly low toxic and has low turbidity compared to other samples. I did not cover in this research about what the mechanism of this phenomenon or the probable impact factor of it. Some research revealed the explanation that the hatched-out juvenile (e.g. sepiolid squid) which is aposymbiotic state is infected by *A. fischeri* in 24 hours and then it proliferates in 20 minutes of doubling time. Also the population of symbionts grew inside the adult light organ with an average doubling time of about 5 hours. However in this research samples do not have any symbionts during the test, proliferation effect is ignored for the explanation of hormesis.

Figure 13. Hormesis indicating the increase of light intensity without the reaction of toxicants in filtering-out samples particularly (shaded part); LL#2, LL#3

4.7 Application of sample-specific methods

I have clarified that sample turbidity is a primary factor causing inaccurate results when turbid samples are evaluated using the Microtox[®] solid-phase test. It is not easy to rule out the particle-effect when considering the toxicity effect of the particles with the bioluminescence method. Therefore, a compromise strategy is necessary to obtain the results with the least bias. For toxicity tests of stream sediments, I suggest using the flow chart shown in Figure 14., for the screening of methods. One needs to measure the 15-min settling turbidity first with 1:200 diluted solid suspensions. If the measured turbidity falls in the range of 100 to 500 NTU, I recommend the use of the c-BSPT. If the measured turbidity is less than 100 NTU, use of the BSPT is recommended. After selecting the optimal test method, I recommend preparing a sample at 1:5 dilution using 2% NaCl diluent (or using commercial Microtox[®] Diluent). For samples with turbidity that is too high (> 500 NTU), more dilution is recommended. Upon careful analysis of toxicity results for this particular case, it seems rational to evaluate the sample toxicity after extra dilution, to lessen the influence of turbidity. The dilution factor is known to be another error-causing factor (Dombroski et al. 1996; Volpi-Ghirardini et al. 2009) since the bacterial response to the toxicant is not usually linearly proportional to the concentration in the suspension. To differentiate accurate sample results from the typical dilution-effect of chemicals, the same dilution factor is recommended for cross-comparison of results.

Figure 14. Screening flow chart for Microtox[®] Solid Phase toxicity measurement (^asample dilution at 1:200, ^bsample dilution at 1:5)

Chapter 5 Conclusions

In bioassays using bioluminescent microorganisms, the turbidity of samples is a potential error-causing factor in the assessment of soil or sediment-sample toxicity. The toxicity result of solid samples could be under or over-estimated depending on whether, and how, the turbidity of the samples is controlled. The application of five solid-phase toxicity test methods for the Microtox[®] system (four existing and one newly proposed), provides pros and cons for each, so no single method was identified for universal application. One of the difficulties of this research was that there was no standard sample of known toxicity and defined turbidity level. Therefore, the best-use recommendation among the five methods was selected based on the need to minimize errors in the toxicity results. Despite the limitations of this research already discussed, these results provide useful guidance for making better decisions in the selection of solid-phase toxicity-test methods by simply adding a new procedure for turbidity measurement. Further study should be conducted in order to verify the effectiveness of the suggested screening strategy.

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Abstract

최근 반 세기 동안 생태 독성을 평가하기 위해 다양한 방법들이 개발되어 발전되어 왔다. 그러나, 발광성 미생물을 이용한 독성 평가의 경우 시료 입자 자체의 탁도가 결과에 영향을 미쳐 문제가 된다. 특히 Microtox[®] 를 이용한 독성 평가의 경우 입자의 탁도를 제어하기 위한 많은 연구들이 진행되었지만, 아직 기준이 될 만한 방법이 확립되진 않았다. 본 연구에서는, 원심분리를 통해 탁도를 제어한 새로운 Microtox[®] 독성 평가 방법 c-BSPT (centrifuged-Basic Solid Phase Test)를 제안하고 기존의 통용되던 방법들과 그 결과를 비교하였다. 기존에 사용되던 4가지 방법은 다음과 같은 방법으로 탁도를 조절하였다. 1) 0.45- μm 필터링을 통해 입자를 완전 제거 시킨 후 용출액으로 실험 2) 입자를 자연 침강시킨 후 실험 3) 상대적으로 크기가 큰 입자만 제거 후 실험 4) 용액을 교란시켜 탁도를 최대한 높인 후에 이에 따른 발광 감소를 계산하여 독성에 의한 발광감소에서 제하는 방법 등 이다. 본 연구가 제안한 방법은 적절한 속도의 원심분리 과정을 통해 흙입자는 가라앉히되 발광성 미생물은 상등액에 남아있게 하는 방법이다. 그러나 입자의 용출 없이 고체 입자와 발광성 미생물의 직접적인 접촉을 통해 독성을 평가하던 기존의 방법들과 (SPT, BSPT, mBSPT) 본 연구를 통해 새롭게 제안된 c-BSPT 중 특정한 한가지의 방법이 특성이 각기 다른 모든 시료의 평가에 적용될 수는 없다. 따라서 시료의 탁도를 기준으로 가장 적절한 평가 방법을 제안하는 간단한 선행 선별 방법을 제안하였다. 이에 다양한 시료의 적용을 통해 이 선별 방법의 정확

성을 입증하는 후속 연구가 필요할 것이다. 본 연구는 발광성 미생물을 이용한 생태 독성 평가 시, 고체 입자 제어의 필요성을 조명하고 새롭게 제안한 방법을 포함하여 시료의 특성 (탁도)에 따른 최적의 방법을 선정하기 위한 방법을 제안하였다.

Keywords: Toxicity, Bioassay, Turbidity, Microtox[®], Centrifuging