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自然環境循環学分野

平成 24 年度　修士論文

Ecotoxicological assessment of river water and facility's wastewater using algae, daphnids and fish

藻類・ミジンコ・魚類を用いた河川水及び事業所排水の生態影響評価

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1 Introduction

Million chemicals exist in the world, maybe a lot of them need to be managed, however, there is only 0.42% been inventoried/regulated (www.cas.org (2013)). Innumerable number of chemicals benefits our life to greater extent; however, effects of those chemicals on human health and organisms in the ecosystem are of much concern and cannot be overlooked.

The Water quality monitoring that assesses the individual chemical concentration in environment also plays an important role as well as effluent regulation using discharge limits. Traditionally, effluents discharged into streams is mostly regulated the concentration of their individual toxicities. In Japan, government published public water area water-quality standard. With 50 years endeavor, the standards achieving rate raised up to nearly 90% except lake (figure 1), because the condition of lake is more complex. However, the water environment has long been managed on the basis of Water Pollution Control Law to monitor and set the limit of several items, mainly in terms of chemical compounds, in effluent and water bodies from the viewpoint of protecting human health and living environment. While this framework has contributed

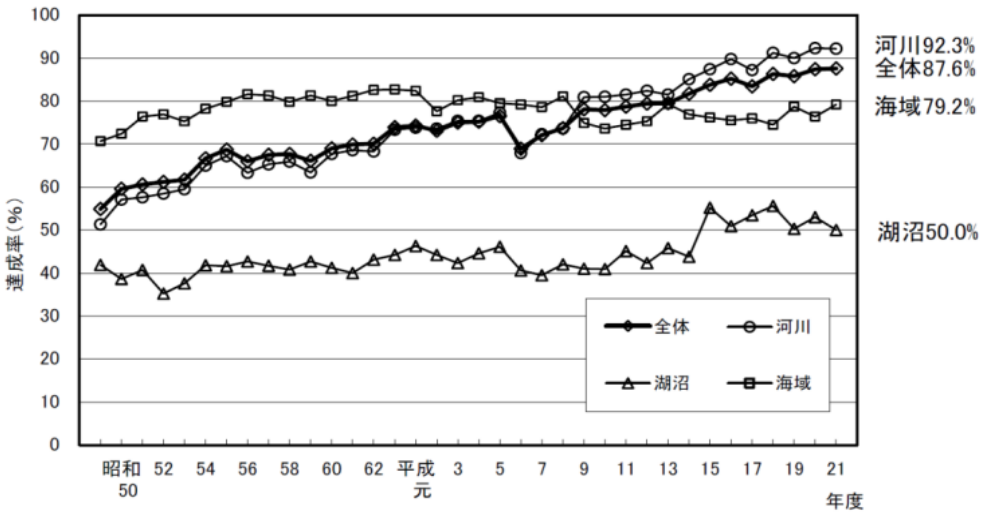


Figure 1 Public water area water-quality standard achieving rate of Japan

to diminish the severe water pollution, this “chemical compound-based management“ scheme is concerned to neither cover all the chemical compounds

increasing rapidly nor predict mixture effects of these numerous compounds. Namely, there could be a case for waters to satisfy all the existing limitations but they still exert adverse effects on aquatic organisms in the natural water environment. Moreover, the regulations oriented are different, in Japan the regulations are pointing at human healthy not ecosystem protection. The standards for aquatic organisms content only two chemicals Zinc and Nonyl phenol. Whether managed water has adverse effect to the aquatic organisms is unknown. In addition, it will be very difficult to make the standards as those for human one by one; therefore, to know whether chemicals have adverse effect to the organisms we need bioassay. Bioassay also has other advantages; it can evaluate the effect of mixtures and unregulated chemicals.

To evaluate the effect of water, people need to use biological test. Expose organisms to sample and evaluate the effect by the response of the organisms (figure 2).

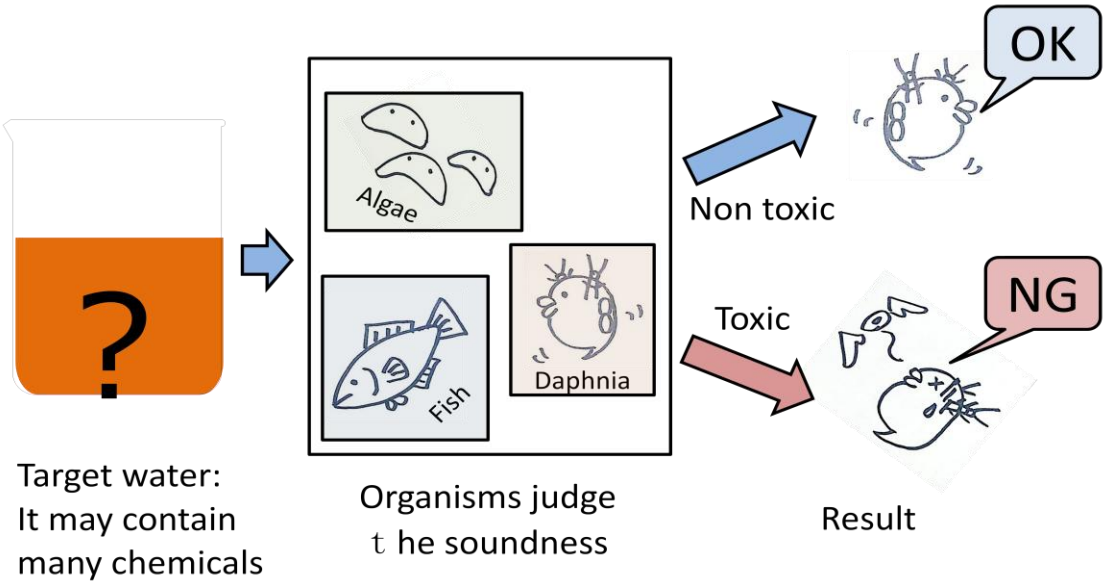


Figure 2 Biological test

We can date toxicity tests back at least to Aristotle, who collected ‘‘bloodworms’’ (most probably chironomids) from freshwater muds downstream of where Athenians discharged their sewage and observed the responses of these animals when placed into salt water (Hynes (1960)). Similar experimentation occurred on an investigator-

specific basis through to the present century (Anderson (1980)). However, if the sample was evaluated as toxic, it is very important to find out what caused the toxic, how to remove the toxic,and how to reduce the toxicity. Biological test cannot provide us the toxicant, methods to reduce the toxicity, so scientists need some methods else to identify the causes.

As we know, most environment invents were caused by the effluents, therefore, scientists tried to use bioassay to the effluents. Effluent toxicity testing relative to organized efforts to assess and control water pollution began in the 1940s; the first attempt at standardizing effluent toxicity tests occurred in the 1950s '(American Public Health Association (1996)).

In 1985, whole effluent toxicity (WET) testing was formalized by the USEPA, with the intent: ''To identify, characterize, and eliminate toxic effects of discharges on aquatic resources'' (U.S. Environmental Protection Agency (1955)). Via expose organisms to effluents, evaluate the toxicity by the response of organisms, whether they can survive, reproduce and multiple. After the toxicity evaluation, if the effluent shows no toxicity, effluent just need to normal monitoring. If the effluent shows toxicity, toxicity reduction procedure TRE/TIE will bring into operation. In the toxicity reduction evaluation /toxicity identification evaluation ((TRE/TIE) procedure, via several treatments confirm the toxicant group or the toxicants, and search the reduce methods for/by factory.

Over the intervening 25 years, extensive publications have been prepared regarding WET, including various manuals and interpretative guidance. Without question, WET tests have been extremely useful tools for identifying toxicity impacts in the environment (Grothe et al. (1996)). As noted by Mount (Mount (1998)), WET testing arguably led to the identification of organophosphate insecticides, surfactants, and treatment polymers in treated effluent. (Peter m. Chapman (2000)),(Cooper et al. (2009))

Also the whole effluent toxicity (WET) test has become a powerful tool in the monitoring of effluents (U.S. Environmental Protection Agency (1991)). The similar

systems are using in some countries. In the UK and Australia, the term 'Direct Toxicity Assessment' (DTA) is used, meaning bioassays in general, covering both effluent and receiving environment sample testing. In some European countries, the term WET is used but it can be applied more broadly than in the US, for example to include bioaccumulation, biodegradation and persistence testing. The term 'Whole Effluent Assessment' (WEA) has become increasingly common in Europe. And in The German term 'Integrating Controlling of Effluents' (ICE) and the Dutch term 'Whole Effluent Environmental Risk' (WEER) may also cover the use of effluent bioassays.(Power and Boumphrey (2004))

WET testing is an integrative tool that measures the toxicity of effluents and accounts for the uncharacterized sources of toxicity as well as their toxic interactions, but cannot explain the origin or identity of chemicals affecting toxicity (Aguayo et al. (2004)). Therefore, these chemical measurement and biological toxicity test methods should be used together for regulatory purposes. Many studies have recently been conducted on the toxicity of mixtures of toxicants from sewage effluents (Ra et al. (2006)), (Sarakinos et al. (2000)), which showed increased or decreased toxic effects on aquatic organisms according to the chemical characteristics when in combination.

In 2003, a TIE test (part of WET system) was performed in Hong Kong, (Kwok et al. (2005)). Through TIE procedures, sulfide (S^{2-}) was suspected as toxicant. However, during the procedures, they got some non-toxic unknown compounds enhanced the toxicity of sulfide. Therefore, using WET, people can know the causes and related matters.

Although WET is a very effective method of effluent management and water protection. However, in Japan, the environment condition is different; organisms are also different from America.

Moreover, there are two major concerns in implementing this system to Japan: there are few case studies to apply bioassays to river water samples both in terms of quality and in terms of quantity. Most researches about WET of Tatarazako are about toxicity

evaluation; by bioassay evaluate the toxicity of effluents and provide reports to factories, almost no research about toxicity reduction. In addition, there are few case studies to investigate how to reduce the toxicity if the significant toxicity is found. Cases are too less to cover all kinds of factories. Different factory has different condition, which means these cases are not enough; methods in the procedure are not systematic enough. (Tatarazako (2012), Yamamoto (2011)).

1.1 Objectives

In this study, water samples were collected to evaluate effects on the selected aquatic organisms in an urban stream where existing water quality standards are completely met, and the causes of the toxicity and the toxicity reduction majors were investigated. I tried to use bioassay to estimate impact of the river waters in Japan and investigate toxicity characterization of facility's wastewaters.

1.2 Mainly research contents

(1) Estimate samples' impact by bioassay

(2) Use several treatments to clean the samples

(3) Analyze samples with necessary instruments

(4) Combine results of biological test and analysis results infer the causes

2 Materials & Methods

2.1 Materials (samples)

2.1.1 River water

River K is a Class A river located in the Kanto region, Japan. Five samples (S1-S5) were collected from upper reaches to lower reaches including one **environment reference point**. There are some factories and farmland beside the river.

Water was collected with a soft plastic tank using plastic bucket. The water quality (Temperature, pH, DO, EC/Salinity, Turbidity, ORP, and Total solid) was measured with multi-parameter water quality sensor (W-20XD, HORIBA) in the field. Water quality data was summarized in Appendix, Table11). All samples had no smell or color.

2.1.2 Effluents

As point pollution sources to the water bodies, and as case study of toxicity characterization, six effluent samples (F1-6) were collected from four different factories that use different materials. Samples were collected by the manufacturers from outfall with clean glass bottles, and were delivered to the laboratory by cooling transport. Four of six samples (F1-4) were collected from four different factories in 2011 and the other two samples were collected from the same factory as F4 in 2012. All effluent samples were tested in 80, 40, 20, 10, 5, 2.5, 1.25% concentration to screening the impact. (Water quality in appendix, table 12)

2.1.3 Preparation of samples

After arrival in the laboratory, all samples were filtered with glass fiber filter (0.45um pore diameter) immediately, and stored at 4 °C until the toxicity test start. Just before bioassay, samples were heated at 27 ℃ in a water bath.

Dilution series of samples with a control solution (see 2.1.4) were subjected to bioassay. River water samples (S1-4) were tested at 40% and 80% concentration for screening at first, and tested again with additional dilutions to estimate NOEC (No

Observed Effect Concentration). The maximum concentration was set at 80% to provide enough nutrients for algae to grow by mixing with 20% of the control solution. Effluent samples (F1-6) were tested at 5 to 7 concentrations (i.e., 80% to 1.25%, 0.5 dilution factor) to estimate NOEC.

2.1.4 Control and culture solution

In an algal test (see 2.2.1.1), OECD medium (OECD, 2011) was used as control and dilution solution. In the bioassay using daphnids and fish (see 2.2.1.2 and 2.2.1.3), treated and dechlorinated tap water with activated carbon filter was used as control, dilution and culture water.

2.2 Methods (bioassay & toxicity identification)

2.2.1 Bioassay

To estimate the biological impact of samples, three aquatic organisms; green alga (*Psuedokirchnerlella subcapitata*), water flea (*Ceriodaphnia dubia*), and zebra fish (*Danio rerio*) were exposed to the samples. Each bioassay was conducted in conformity with the international standardized test guideline as below.

2.2.1.1 Green alga (*Psuedokirchnerlella subcapitata*)

This is a 72 hours growth inhibition test using green alga, *Psuedokirchnerlella subcapitata* conducted following OECD test guideline 201(OECD, 2011). Exponentially growing alga was exposed to test solutions (including control) during a period of 72 hours and growth rate and growth inhibition were quantified from measurement of the algal biomass (cell counts) as a function of time. Test conditions were listed below.

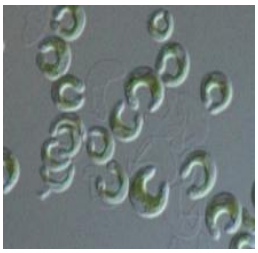


Figure 3 *Psuedokirchneriella subcapitata*

Test organisms: *Psuedokirchnerlella subcapitata* (figure 3)
Test duration: 72 hours
Test volume of solution: 100 ml/vessel
Initial biomass concentration: 5000 cells/ml in test solutions

No. of replicates: 3 for samples and 6 for control
Renewal of test solutions: None
Endpoint: Growth inhibition (growth rate)

2.2.1.2 Water flea (*Ceriodaphnia dubia*)

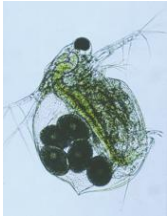


Figure 4 *Ceriodaphnia dubia*

This is a three-brood reproduction inhibition test with *Ceriodaphnia dubia* conducted following the test guideline published by Environment Canada (EPS 1/RM/21, Second Edition, Environment Canada, 2011). Each neonates (<24-h old) was exposed to each test solutions during around a week. The number of dead test (parent) organisms and the number of neonates reproduced in three broods was counted. Reproduction inhibition was determined by the decreased number of neonates compared with the number of neonates obtained in control. Test conditions were listed below.

Test organisms: <24-h old neonate of *Ceriodaphnia dubia*
Test duration: around a week (must end as soon as 60% (or more) of control organisms have three broods, maximum duration of test is 8 days)
Test volume of solution: 15 ml/vessel
No. of test organism: one neonate per vessel
No. of replicates: 10
Renewal of test solutions: Every two days
Endpoint: Reproduction (the number of neonates in three broods) and mortality of test organisms (parent organisms)

2.2.1.3 Zebra fish (*Danio rerio*)

Figure5 *Danio rerio*

This is a 9 days embryo and larval survival test using zebra fish, *Danio rerio* conducted following OECD test guideline 212(OECD, 1998). 20 fertilized eggs were exposed to each test solution per vessel and the number of live or dead embryo and larvae were observed daily. Hatching rate and

survival rate post-hatch were determined and as total endpoint, survival index was calculated by the product of hatching rate and survival rate post-hatch. The test conditions were listed below.

Test organisms: Fertilized eggs (<4 h after fertilization) of *Danio rerio* (figure 5)
Test duration: 9 days
Test volume of solution: 50 ml/vessel
No. of test organism: 20 eggs per vessel
No. of replicates: 4
Renewal of test solutions: Every two days
Endpoint: Hatchability, Post-hatch survival, Survival, survival index (hatchability×post-hatch survival)

2.2.2 Data analysis

No observed effect concentration (NOEC) were the highest concentration that does not cause statistically significant adverse effect on the organisms. NOEC for each endpoint was obtained using multiple comparison tests: Dunnett's test for homogenous variance data and Steel's test for heterogeneous variance data (US EPA, 2002). Toxic unit (TU=100/NOEC) was calculated and if TU of a sample was higher than 1, it was considered that the sample has biological impact. TU indicates the dilution rate needed to make a sample non-toxic (at NOEC).

ICx was the concentration of sample which exhibits x% inhibition.

TU_{IC25} =100/IC25 was also calculated.

2.3 Toxicity characterization

Following each treatment procedures, eliminate anticipatory target elements/matters and characterize the causes. Methods are referred TIE guideline. (US EPA (1991))

2.3.1 Methods and treatment procedures

2.3.1.1 Cation exchange test:

To eliminate heavy metal cation, I chose SIR-300 cation exchange resin that is made by Resintech.inc for cation exchange, could eliminate most free heavy metal cation from the sample. The capacity of SIR-300 is 1.1mmeq/ml as Na+. With this formula [1.1meq/ml * X ml = sample volume * element concentration * valence / atomic weight]. We could calculate how much resin is needed. In the treatment procedures, put X ml SIR-300 into 1mol/L NaCl solution for 24h, and wash the resin with MilliQ water to eliminate residue NaCl solution, then put in the sample and stir sample with magnet stirring for 30min. Separate resin from sample and store sample with clean glass bottle at 4℃ until test. (Figure 7)

Figure 6 SIR-300 ion exchange resin

Figure 7 C18 SPE column

2.3.1.2 C18 SPE column test:

For non-polar organics, some metals, and some surfactants can be removed from the sample I select Sep-Pak C18 plus short cartridges (WAT020515) made by Water® to eliminate organic matters. In the treatment procedures, because the capacity of C18 is

unknown, so I mean to filter the sample with many columns with suction filtration to eliminate the organic matter. The treated sample will be store with clean glass bottle at 4℃ until test.

In F4 treatment, 1.2L sample used four cartridges totally, two cartridges were joined together; and filtered 0.6L sample/30min; in F6 treatment, 1.2L sample used 10 cartridges (last cartridge does not show obvious color), five cartridges were joined together; and filtered 0.6L sample/120min. (Figure 6)

2.3.1.3 Aeration:

Aerate 1.2L sample with pure air for 24h, 2L/min. And store the treated sample with clean glass bottle at 4℃ until test.

2.3.1.4 Aluminum flocculants

Aluminum flocculants (AF) that used in the research are coming from an effluent flow of one factory, which is using aluminum in some produce procedures. Because we do not know the usage volume, we just copy the ratios using in the factories. The normal usage volume was calculated as 6ml AF per 1.2L sample. (Table 1)

Table 1 Usage volume of different ratio of AF addition

1.2L sample	0.5times of normal	1times of normal	2time of normal
AF usage volume	3ml	6ml	12ml

In the treatment procedure, AF was used directly, and because the AF shows weak acidity, after add AF, samples need to adjust pH to 8 and stirring 10min, and precipitating 20min. Recycle supernatant for test.

2.4 Chemical analysis

In the research water quality (pH, Do, conductivity and salinity), elements concentration, TOC and other items necessary.

TOC: Total organic carbon analyzer TOC-5000 made by SHIMADSU, the standard concentrations for measuring set as 0, 5, 10, 20ppm.

ICP: ICP-MS (Agilent 7500xc), ICP-AES we asked other research department to measure the elements concentration. Because some sample could over the limit of analyzer, some samples were diluted several times and recorded the dilution rate for final calculation.

Hardness &NH3 & Cl2: pack test made by HACH (2319900, 26045-45, 21056-69)

pH: pH meter D55 made by HORIBA, calibrate pH meter once a week with pH4, pH7, pH9 standard solution.

DO: DO meter HQ30d made by HACH

Conductivity and salinity: ES-51 made by HORIBA

2.5 Others

Data processing: ECOTOX V1.2; No Obvious Effect concentration (NOEC) and IC50 were calculated by this software.

Other experiment supplies: 50 &100ml glass dilute cup (organisms exposing), 300 & 500ml glass cup, 500ml measure flask, 300ml measuring cylinder, 15 & 50ml plastic tube, 200μl & 1,5 10ml pipettes, green alga (food for water flea).

Nickel standard solution: 1001mg/L (147-06461 Wako)

Sodium chloride: 191-01665 Wako

3 Experiment Results

3.1 Biological impact of River water (S1~5)

Because the biological impact of samples was unknown, screening test in which only 40% and 80% concentration of sample was conducted at first. Samples collected from S1 and S2 did not show any biological impact on three test organisms (water flea, Figure 8). On the other hands, significant growth inhibition of algae was found from the sample collected in S4, and the significant mortality of the daphnids was found from the samples collected in S3-5. Therefore, these samples were tested again with more dilutions to estimate NOEC. The results of bioassay with water fleawas summarized in Table 2.

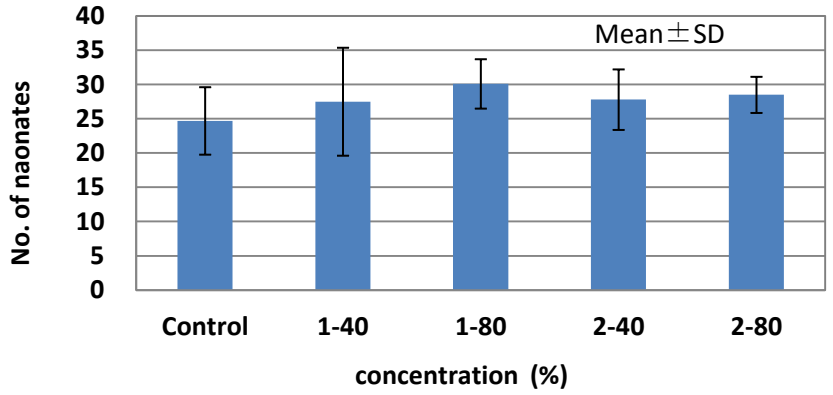


Figure 8 Reproduction (water flea) of S1&S2 (1-40 means S1 at 40% concentration of original)

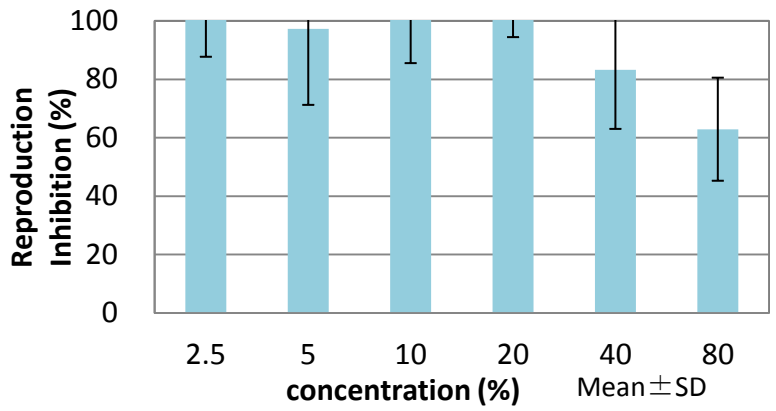


Figure 9 Reproduction inhibition rate of S3 (water flea)

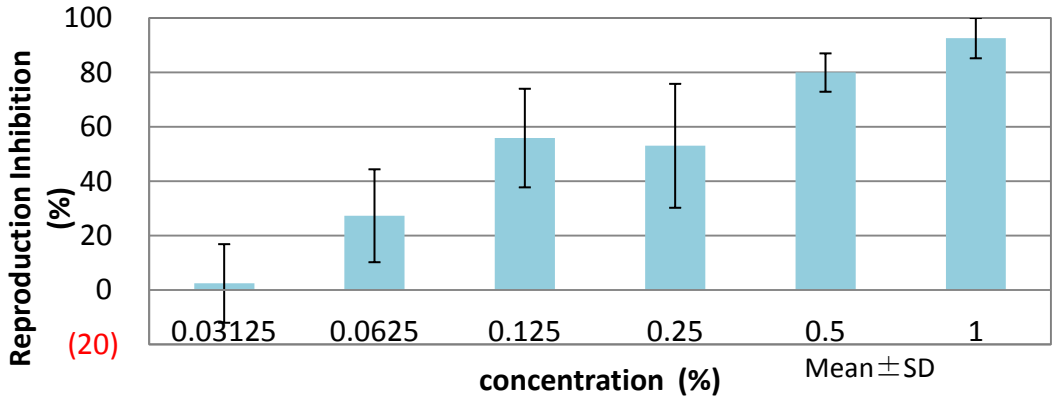
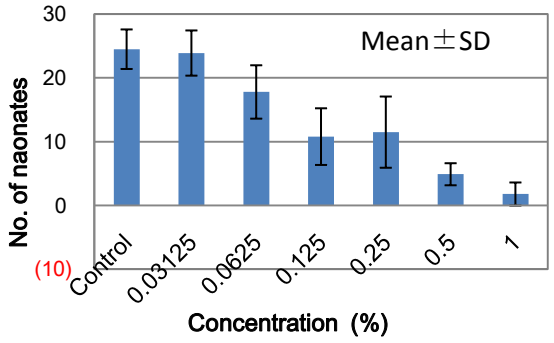


Figure 10 Reproduction Inhibition rate of S4 (water Flea)



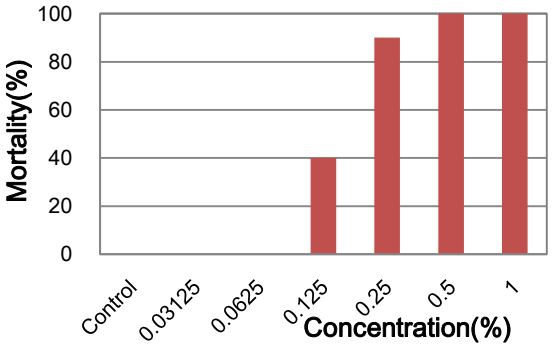


Figure 11 Reproduction of S4 (water flea)

Figure 12 Mortality of S4 (water flea)

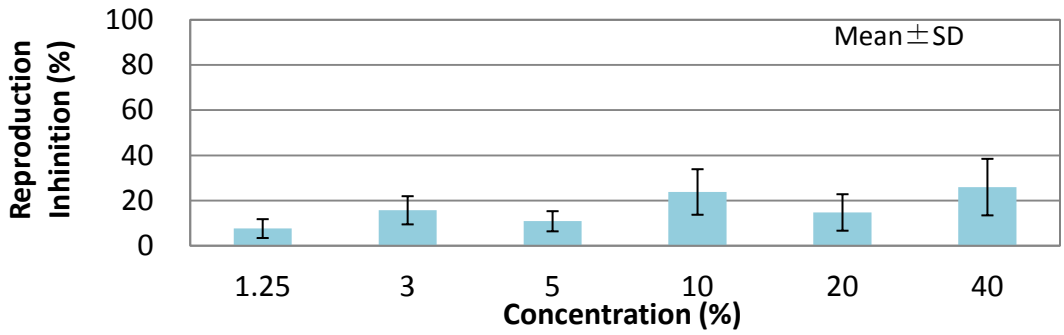


Figure 13 Reproduction inhibition rate of S5 (water flea)

Table 2 Biological impact of river water samples (S1-S5) on three test organisms

	sample	S1	S2	S3	S4	S5
Fish	NOEC	80%	80%	80%	80%	80%
	TU	-	-	-	-	-
Alga	NOEC	80%	80%	80%	10%	80%
	TU	-	-	-	10	-
Water flea	NOEC	80%	80%	40%	0.03125%	40%
	TU	-	-	2.5	3200	2.5
	LC50	-	-			

The result show water from river K has strong impact on water flea. S1 and S2 have no adverse effect to three organisms (figure 8). S3 and S5 have strong toxic to water flea (figure 9 &13). S4 even needs to dilute 3200 times until show no obvious effect (figure 10, 11 &12). River pollution is caused by discharge mostly (USEPA 2007-2009), around River K, there are a lot of farm lands and small factories. To confirm the effluents comes from which factories are very difficult. Therefore, six industrial effluents with license were taken for my research. (Water quality in chapter 6)

3.2 Biological impact of facility's wastewater (F1~6)

Some samples' NOEC were under 1.25%, they were tested again until get NOEC. The results are as below (table.3). Results showed three of six effluents have adverse effect to water flea (figure14, 15, 16); two of six effluents have adverse effect to fish and algae; all six effluents have no adverse effect to alga. F3 & F6 both have adverse effect to fish and water flea. (Water quality in appendix, table 12)

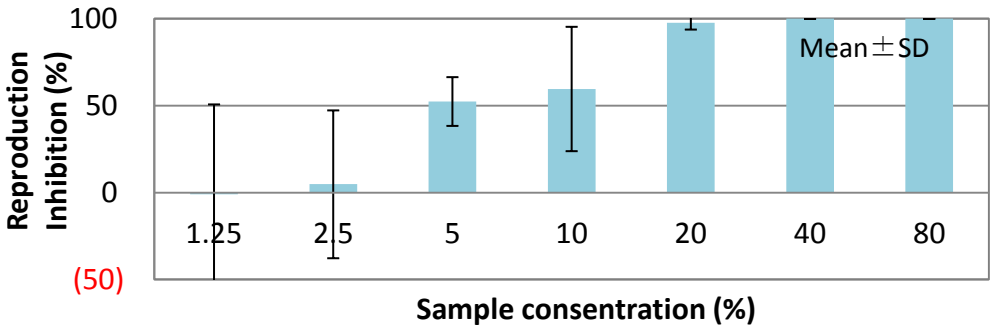


Figure 14 Inhibition rate of F4 (water flea)

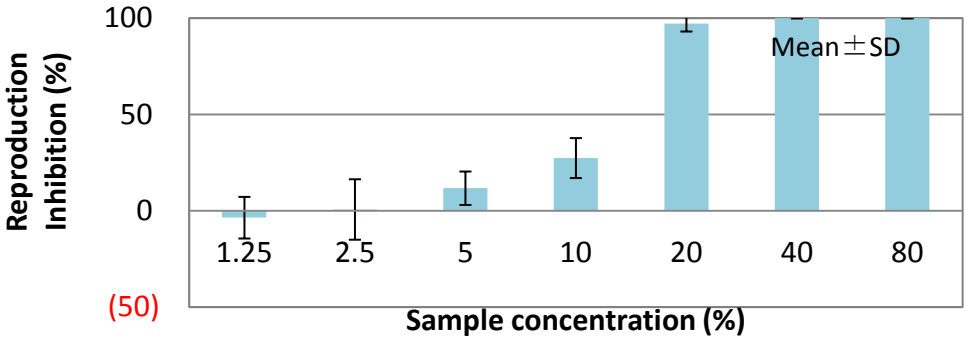


Figure 15 Inhibition rate of F5 (water flea)

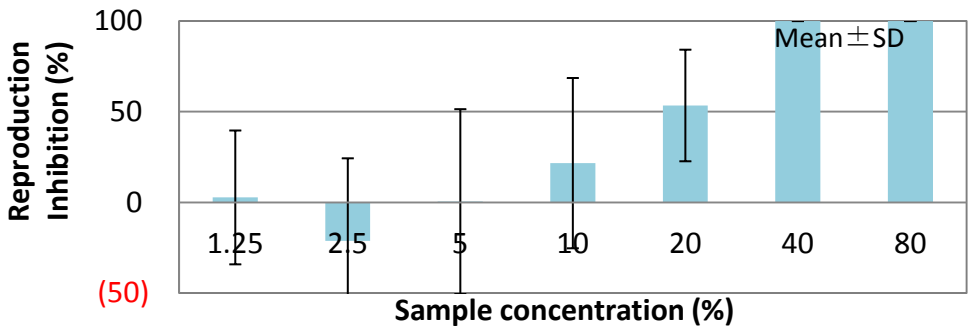


Figure 16 Reproduction inhibition rate of F6 (water flea)

Table 3 Impact result of industrial effluent

	sample	F1	F2	F3	F4	F5	F6
Fish	NOEC	80%	80%	80%	10%	80%	10%
	TU	-	-	-	10	-	10
Alga	NOEC	80%	80%	80%	20%	80%	20%
	TU	-	-	-	5	-	5
Water flea	NOEC	80%	80%	80%	2.5%	5%	5%
	TU	-	-	-	40	20	20

3.3 Results of toxicity characterization

To identify the impact causes of each impact confirmed samples, each sample was treated by different methods to character the causes.

3.3.1 Toxicity characterization of F4

The basic water quality data showed F4 had a very high salinity that is 0.41%. Could salinity be the main causes? Therefore, we did a sensitivity test of NaCl on water flea. In the experiment, the NaCl concentration was set as 0.4g/L(0.04%), 0.59g/L(0.059%), 0.89g/L(0.089%), 1.33g/L(0.133%), 2g/L (0.2%). the result as below (figure 17)

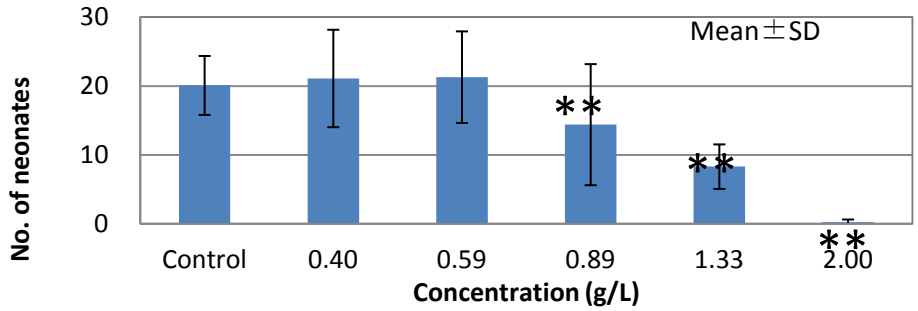


Figure 17 Reproduction test of NaCl. NOEC is 0.89g/L (0.089%)

Test result suggests salinity over 0.1% will cause impact to water flea, but zebra fish can tolerate over 0.2% salinity (OECD (1998))

Compare result of sensitivity test with F 4, salts were suspected as the main toxicants for impact. To confirm the contribution of salts to the effluent toxicity, dose-response curves for NaCl solution and F 4 were shown in the same Figure (Fig.18). Because all of the dose-response curves were nearly close to each other, the toxicity of effluent almost could be explained by salt. However, dose-esponse curve for Baseline was slightly above that for NaCl, was it possible that there are other toxicants except the salinity.

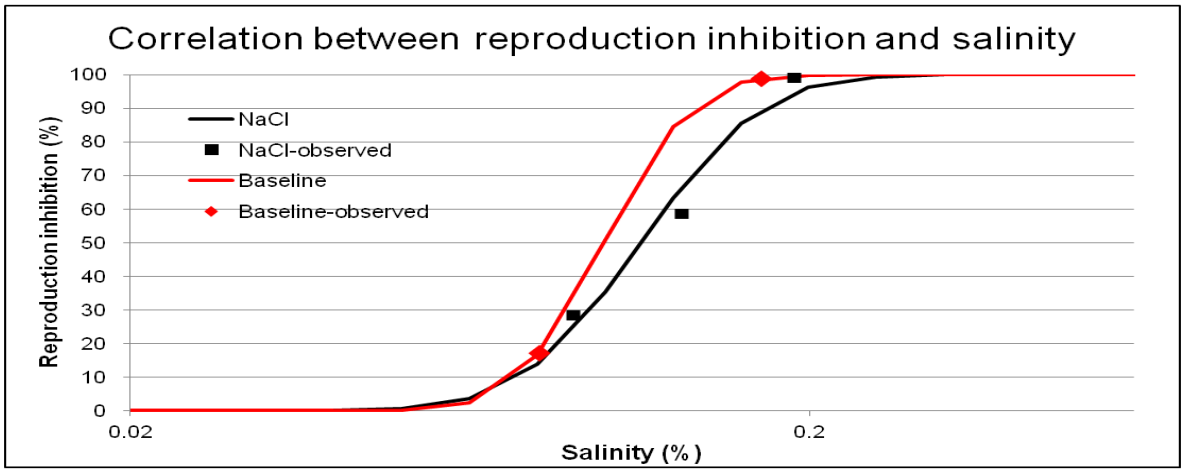


Figure 18 Correlation between reproduction inhibition and salinity

I referred the methods of TIE and chose aeration, C18 SPE column filtration and cation exchanging (SIR-300) for F4. For water flea tests, 1.2L F4 was aerated for 24h at

2L/min with pure air, 1.2L was filtered with C18 cartridge (300ml/2cartridge• 30min), and 1.2L sample was add prepared SIR-300 resin 15ml and stirred for15min. Treated samples were stored by clean glass bottle.

To know the effectiveness of treatments, the elements concentration was measured by ICP-MS, result as below (table.4)

Table 4 results of ICP-MS

Sample	TU	Heavy metals (unit: ppb)							
		Ni		Cu		Zn		Pb	
		Conc.	TU_{IC25}	Conc.	TU_{IC25}	Conc.	TU_{IC25}	Conc.	TU_{IC25}
Baseline	5.0	2.40	2.86	434.20	12.06	7.08	0.11	0.99	0.02
SIR-300	4.5	0.91	1.09	347.40	9.65	3.40	0.05	0.66	0.01
C18	3.8	1.58	1.88	34.20	0.95	5.09	0.08	0.70	0.02
Aeration	4.8	2.47	2.94	461.60	12.82	6.27	0.10	0.66	0.01
IC25		0.84	-	36.00	-	66.00	-	46.00	-

From table 4 we can see TU of Nickel and copper of baseline is over 1 which means both Ni and Cu could caused impact. After treatment, TU of Ni became 1, and TU of Cu became 0.95 from 12.06, means treated sample should show no impact or weak impact.

So each treated F4 were tested at 80, 40, 20, 10, 0% concentration of samples. The impact strength may changes during store. Therefore, the original effluent was also tested at the same concentration as baseline. The Result is as below (table.5)

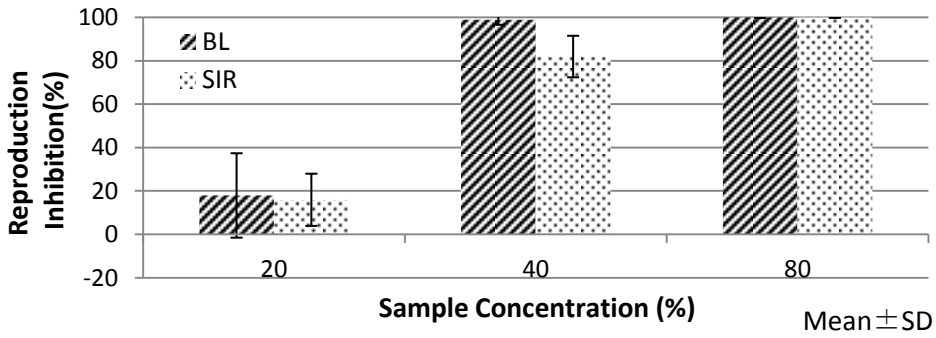


Figure 19 Inhibition of treated F4& Baseline

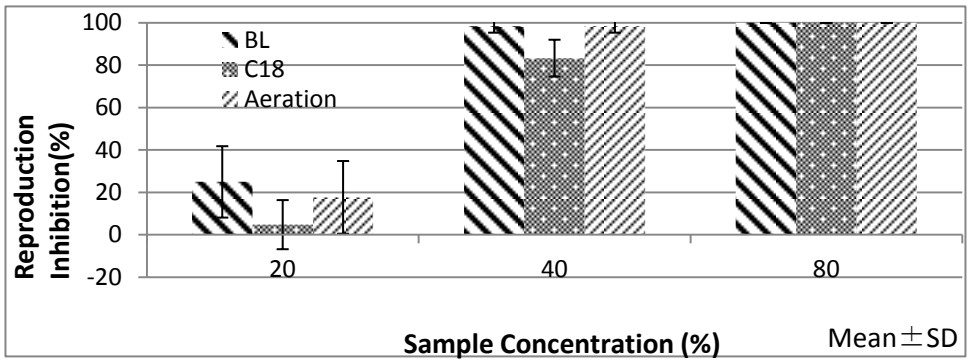


Figure 20 Inhibition rate of treated F4 with C18 & aeration

Table 5 results of each treatment for F4

sample	Aeration	C18	SIR-300	Baseline
NOEC	20%	20%	20%	20%
TU	5	5	5	5

Unfortunately, the NOEC of F4didn`t changed (table 5). Results of each treatment is almost the same (figure19 &20). However, the IC25 changed a little and No. of brood neonate in 40% concentration was no longer zero. (Table 6)

Table 6 IC25 of each treatment

sample	Aeration	C18	SIR-300	Baseline

IC25	21%	26%	22%	20%
TU_{IC25}	5	4	5	5
No. of brood neonate in 40% concentration	0	4	5	0

From the table 6 we can see, IC25 of C18 and SIR-300 increased 6% and 2%, and it is error in error range. However, neonate appeared in 40% concentration is truth, and the number is average that means treatment could have weak effect in reducing toxic. Compare these results with table 4, result suggested that almost all Cu existed in organic-bound fraction, which may not has toxic to the daphnids, and the toxic of Ni is weaken by hardness(NaomiL.Cooper et al. (2009), James Keithly et al. (2004))(appendix, table 13), so Ni and Cu were not considered as the main toxicants caused the impact.

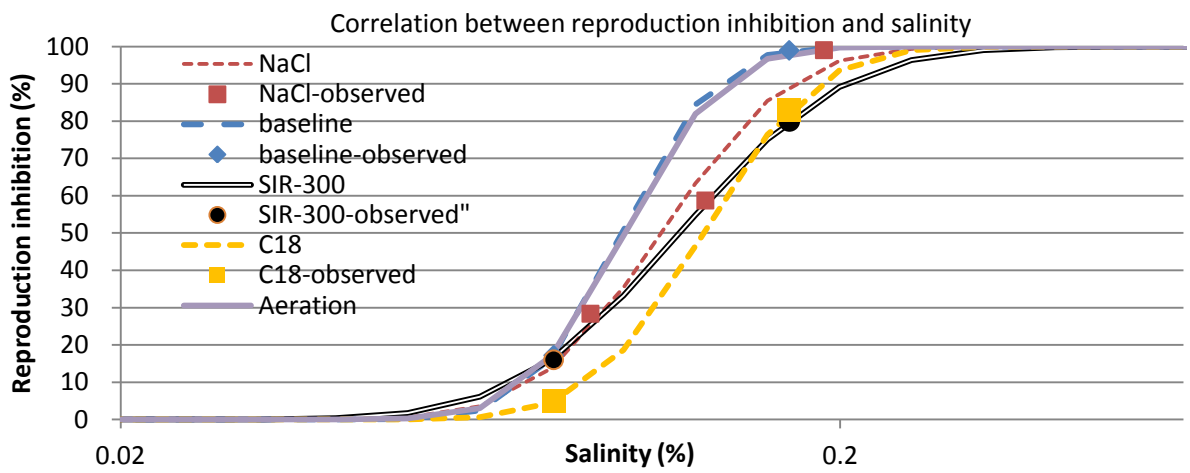


Figure 21 Correlation between reproduction inhibition and salinity

The figure 21 showed no matter which treatments, toxicity of F 4 did not changed much. The main causes could be the salts, however heavy metal, organic matter also could be the causes. And we know, according data of chemical analysis we cannot infer whether sample has adverse effect to the organisms or not.

3.3.2 Toxicity reduction methods of F5

The quantity of F5 limited cannot take plenty test, so for F5 I only did aluminum flocculants (AF) addition test. Aluminum flocculants is a very effective method to eliminate organic matters and SS, but the usage amount is unknown. I calculated the volume referring the usage ratio of factory where F5 came from. The usage volume is 6ml AF per 1.2L sample as basic volume. I designed the treatments as add 0.5, 1, 2 times of basic volume to samples and test treated sample (F5AF0.5, F5AF1, F5AF2) at 40, 20, 10, 0% concentration and original effluent as baseline. The results as below (table.7)

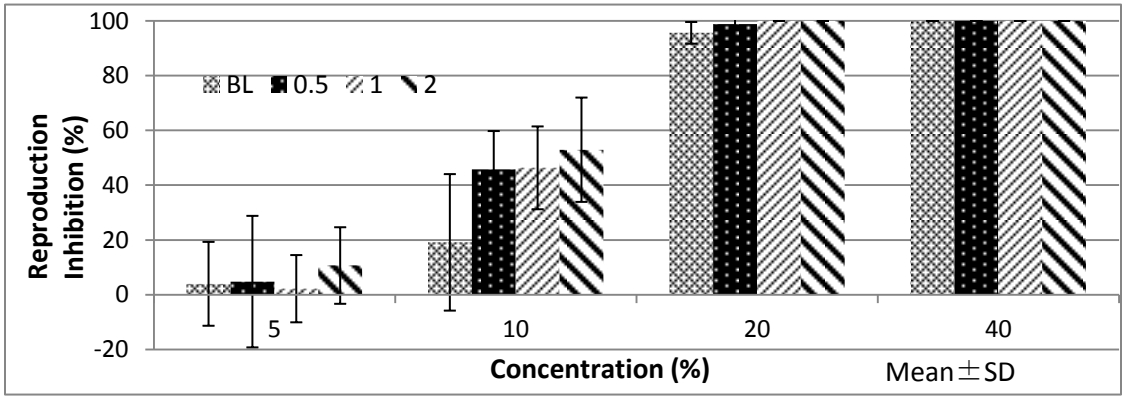


Figure 22 Reproduction Inhibition rate of AF added F5 (water flea) (BL-baseline, 0.5-half normal volume, 1-normal volume, 2-doubule volume)

Table 7 results of Aluminum Flocculants addition test

sample	F5AF0.5	F5AF1	F5AF2	Baseline
NOEC	5%	5%	5%	5%
TU	20	20	20	20
IC20	7.05	7.36	6.22	8.83
Inhibition rate at 10% concentration	46%	46%	53%	19%

Inhibition rate at 20% concentration	99%	100%	100%	96%

The results suggest in F5 condition, by add aluminum flocculants not only does the effluent not become clean, but also actually became worse (figure 22). So the impact of F5 was caused by something cannot be flocculated. Because of F5 ran out of sample, this experiment was suspended. But result of TOC shows AF decreased some organic matters, F5AF1 is 58.6ppm & F5AF2 is 53.0ppm, which means the impact should reduced a bit. However, compare with the result of ICP(table 8), we can see although TOC was decreased, but the concentration of Cu, Al became higher with the ratio increase that means the impact should become stronger which is as same as the test results showed.

Table 8 ICP result of treated F5 with AF

	Al	Fe	Cu
F5AF0.5	176.3ppb	0.931ppb	58.61ppb
F5AF1	201.2ppb	1.083ppb	70.98ppb
F5AF2	1380ppb	1.387ppb	73.33ppb
IC25	-	-	36ppb

3.3.3 Toxicity characterization of F6

Summarizing the methods used for F4 and F5, we found combine instrumental analysis with treatment could shorten the experiment, so I decided to analyze F6 by ICP-AES. According metal element analysis, F6 did not content enough high concentration metal elements that can cause impact. However, F6 has a golden color and strong smell and high TOC value, so test of F6 was designed as taken C18 cartridge flirtation test and AF addition application test.

AF addition test was designed as F5 add 0.5, 1, 2 times of basic volume, and test at 40, 20, 10, 0% concentration including baseline test. (6AF0.5, 6AF1, 6AF2, Baseline1)

C18 cartridge flirtation test was using C18 cartridge to eliminate the organic matter as clean as possible, in my experiment I used 10 cartridges to filter 1.2L F6, 5 cartridges were connected together and filtered 600ml F6 in120min. Treated sample was test at 80, 40, 20, 10, 0% concentration. Because these two tests did not tested at the same time, in C18 test there is another baseline test. (Baseline2, F6C) Tests results as below (Table 9, Figure 23, 24)

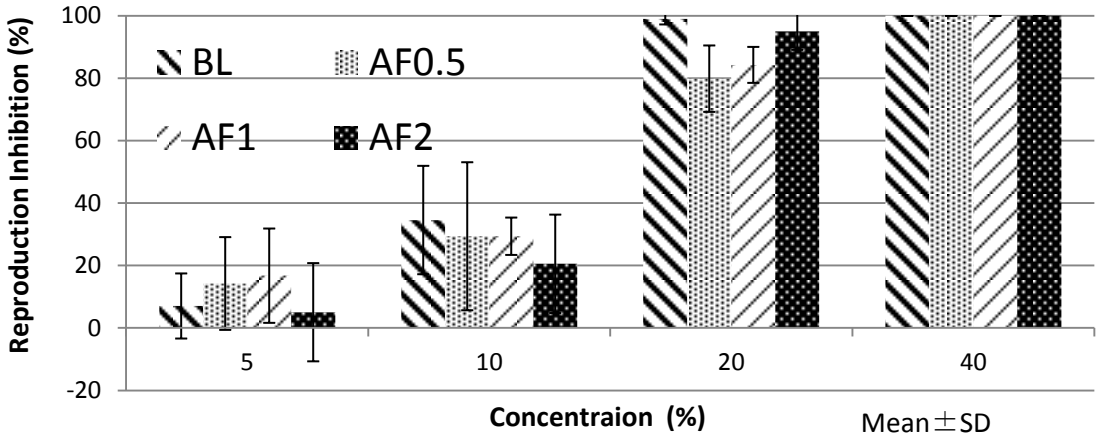


Figure 23 Reproduction Inhibition rate of treated F6 with AF (water flea) (BL-baseline, 0.5-half normal volume, 1-normal volume, 2-doubule volume)

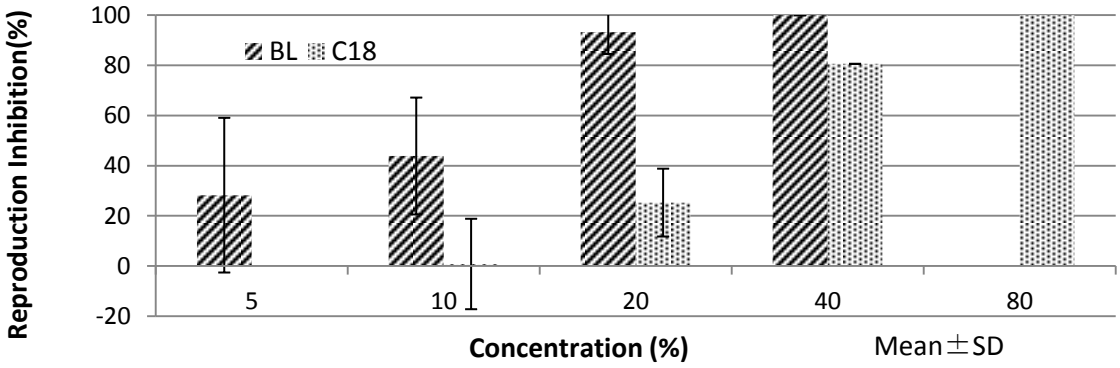


Figure 24 Reproduction Inhibition rate of treated F6 with C18 (water flea) II

Table 9 Results of Aluminum Flocculants addition test and C18 cartridge filtration test

sample	Baseline1	F6AF0.5	F6AF1	F6AF2	Baseline 2	F6C
NOEC	5%	5%	<5%	5%	5%	10%
TU	20	20	>20	20	20	10
IC50	8.74%	6.91%	6.56%	7.35%	8.37%	33%
inhibition rate at 10% conc.	35%	29%	29%	21%	44%	1%
inhibition rate at 20% conc.	99%	80%	84%	95%	93%	25%
inhibition rate at 40% conc.	100%	100%	100%	100%	100%	81%

The test results show AF addition test is effective in low concentration (figure 23); and the same as F5, more AF add, the worse result we got, but result was still better than baseline. Result of C18 test shows C18 cartridge eliminated most organic matters in F6, the impact was weaken by eliminate organic matters(figure 24), but F6 still has fairly strong impact that could caused by 0.11% salinity and other possibilities (metal ion). (Table 10)

Table 10 Metal ion concentration in F 6 baseline and treatment

unit: ppb	**52 Cr**	**55 Mn**	**56 Fe**	**60 Ni**	**63 Cu**	**66 Zn**
F6 Baseline	1.4910	4.2780	2.4580	1.4600	202.600	15.7300
F6 C18	1.5110	4.4370	12.2000	1.8150	14.110	3.0690
F6 AF0.5	0.6835	2.0610	1.2800	1.1700	158.900	29.5000
F6 AF1	0.6080	4.0050	1.3230	1.7310	149.100	56.9500
F6 AF2	0.5255	8.1490	2.2420	2.2840	116.400	129.7000

3.3.4 Toxicity characterization of S4

After toxicity characterization test for industrial effluent, results suggest by using bioassay and toxicity identification procedures we can estimate the impact in different water and conjecture what or what kind of matters caused the impact. So back to River K, what caused the extremely impact on water flea.

From the data of water quality of S4, we can know the salinity of S4 is 0.01% which cannot cause impact; S4 also only contain very low concentration organic matters, TOC is 1.86ppm, such strong impact couldn't caused by organic matters. According the report of river water quality from ministry of environment Japan 2010, the causes was suspected as Zn at first.

With the result of ICP analysis, we found an anomalous high concentration of Ni (table 11)

Table 11 ICP-MS result of S4

	Cu	Zn	Ni
S4	30ppb	28ppb	519ppb
IC25	36ppb	66ppb	0.84ppb

IC25 of Ni is based on the sensitivity test below (figure 25, 26).

Results shows in S4 the concentration of Nickel is as high enough as 500ppb, to know the toxicity character of Ni and Ni sensitivity on water flea, we did an expose test using Ni(NO3)2 on water flea. Test result as below (fig 25, fig 26)

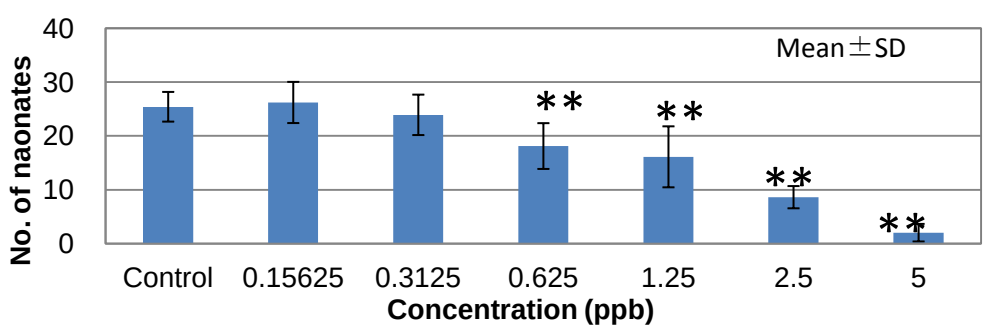


Figure 25 Reproduction test of Nickel. NOEC is 0.3125ppb

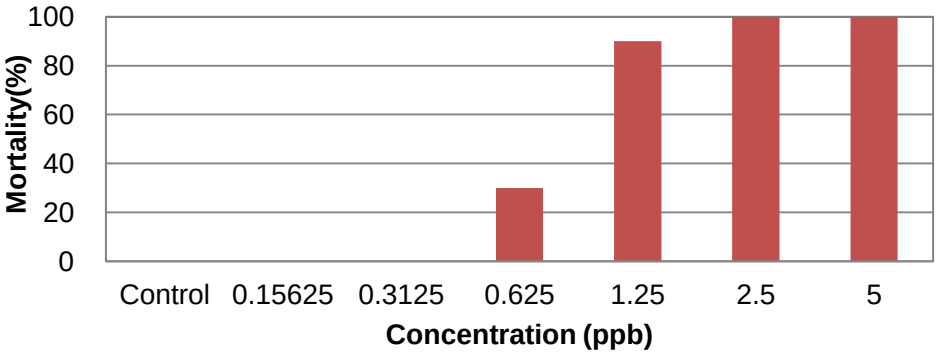


Figure 26 Mortality of each concentration

The Test result of Nickel suggests Nickel is a very toxic metal element; it can cause impact even at 0.625ppb, lower than most metal element. However, the toxic can be weakening by hardness, and when pH is over 10 the solubility of Ni^{2+} is less than 0.1ppb.

NOEC of Nickel acute toxic test on zebra fish is over 5ppm (Volker Scheil, Heinz-R. Ko¨hler (2009)), so in my research S4 does not caused impact to zebra fish.

Therefore, I selected cation exchange to try to eliminate the Ni^{2+} cation to weak the impact. According the formula [1.1meq/ml * X ml = sample volume * element concentration * valence / atomic weight] and data from ICP-AES, the volume of SIR-300 resin was calculated as 26ml to eliminate most heavy metal cation. Test result as below (figure 27, 28, 29)

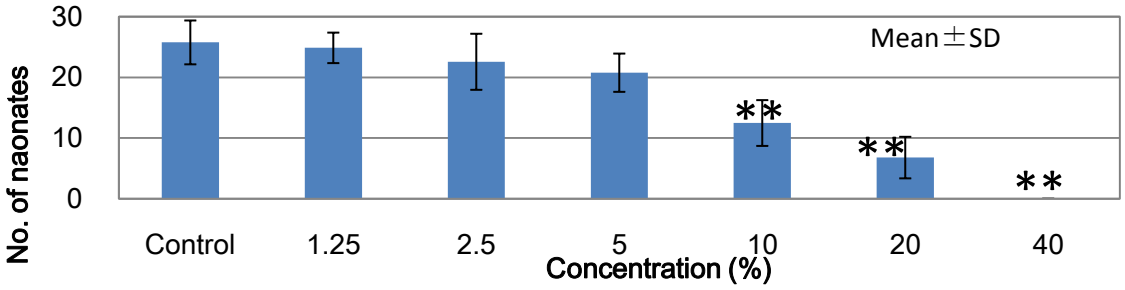


Figure 27 Reproduction of treated S4

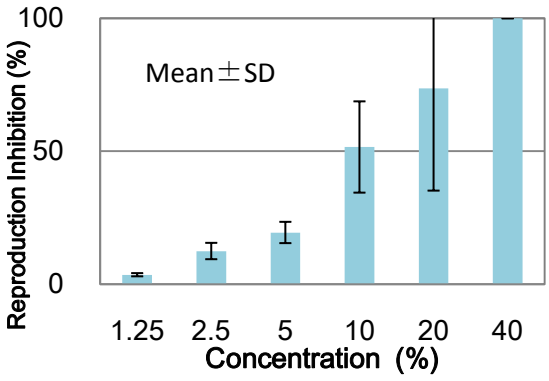


Figure 28 Inhibition of treated S4

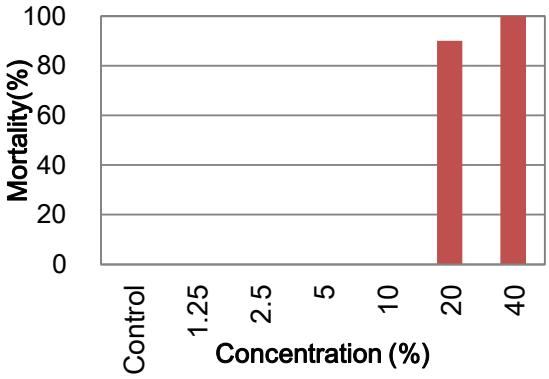


Figure 29 Mortality of treated S4

Test result shows after treatment the NOEC of S4 changed to 5%, and analysis result from ICP-MS showed high concentration nickel disappeared (table 11), which means SIR-300 resin is effective to eliminate Ni^{2+} cation to weak the impact. Compare with the result of nickel sensitivity test, concentration of Nickel of NOEC is almost the same.

Table 12 ICP result of treated S4

	Cu	Zn	Ni
Treated S4	10.04ppb	1.21ppb	21.56ppb
IC25	36ppb	66ppb	0.84ppb
NOEC	16ppb	-	0.3125ppb
Treated S4 at NOEC	0.5ppb	0.06ppb	1.078ppb

Therefore, Ni in S4 could be the causes of the impact. In addition, for the concentration of Zn and Cu were decreased either, so the Zn and Cu also have the possibility to be the causes.

However, according table 11, the concentration of Ni of treated S4 was higher than IC25, which means the TU of treated S4 should bigger than 20. Therefore, there might be something in treated S4 weaken the impact caused by Nickel, or in S4 the impacts caused by mixed chemicals But there is another possibility, in Nickel sensitivity test, we used Ni(NO3)2 standard solution to prepare the test water. However, in S4, the

existence form of Nickel is unknown, might be NiCl2 or other form. The sensitivity of different existence form might different. It needs future research about the relationship between sensitivity and existence form.

4 Discussion

Lower reaches (especially S4) of River K had strong impact on water flea. According the results of ICP-MS analysis data of S4, we found Nickel at the high concentration in S4; therefore, gave test of the addition with Daphnia about toxicity of the nickel (table12). Result of the test suggests nickel has very toxic to *Ceriodaphnia dubia*. After toxicity characterization procedure, combined results of each biological test, we found heavy metal element Nickel caused the main impact. It is necessary to remove nickel to reduce toxicity, and, for example, it is known for not less than pH 9.5 that large portion is removed as hydroxylation nickel from the effluent. However, when pH is over 10, sample will cause different impact to organisms by high value of pH.

In my research, because the C18 cartridges eliminated most organic matters, the main causes of F6 were suspected as organic matter. In toxicity characterization procedures of F4, according the data of ICP-MS, Cu was suspected as mail causes. However, although cation exchange & C18 cartridge filtration eliminated most heavy metal cation (Zn, Pb) and Cu, test results showed they only had weak effect to reduce toxicity. . As for what was guessed by a test result of the NaCl, it was suspected as a primary cause of F4 the salt which occurred by a neutral stage. However, strong impact of salts might cover impacts caused by other matters, for example cationic heavy metal such as Ni, Zn and free copper ion that eliminated by SIR-300 and some organic matters adsorbed by C18 cartridges. Therefore, in the toxicity characterization procedures or impact estimation procedures for high salinity facility's wastewater, people should pay attention to something besides salts.

Sometime we need to understand the toxic mechanism or toxic characters of chemicals. My research shows, one of toxicity characterization of Nickel is: when nickel concentration is between minimum lethal concentration and 0.625ppb, after water fleas brood some neonates, they died of a characterization that looks like internal hemorrhage. High concentration Nickel caused death to daphnids, but low concentration Nickel causes inhibiting to the reproduction instead death.

In my research, we can know using the bioassay to estimate the impact including potential impact is very effective. By combine bioassay, toxicity characterization and instrumental analysis together, whether any toxic chemical exists in effluent effectively.

Bioassays have frequently used for the evaluation of the toxicity of the sample waters but the results of this study suggest the combination of bioassays, water treatments and chemical analyses could comprehensively identify and reduce the toxicity of the waters. This combined approach could complement the conventional “chemical compound-based management” to develop more advanced water quality management. It is important to further collect the combined data of bioassays and chemical analysis, the confirmation of the effects of the effluent on ecosystem, and the restoration of the ecosystem by the improvement of the toxicity of the effluent.

When we get a sample, we need to estimate the impact first and at the same time collect basic water quality data (pH, DO, Salinity, NH3-N, residual chlorine, etc.). If the sample has impact to test organisms, we need to refer the character of toxicants that we have already known to conjecture the causes. If there is not matching character, we will need an instrumental analysis for the sample (TOC, element concentration analysis, etc.). According to analysis data, we can choose proper water treatments for sample. For example, if sample shows high TOC, we can eliminate organic matter first, then using bioassay to estimate effect and conjecture the causes. If sample shows high concentration heavy metal ion, we can eliminate the ions first, then estimate effect and conjecture the causes. After proper treatment and conjectured the causes, via instrumental analysis to verify the conjecture. If we get a suspected cause, we can spike the suspected causes into a sample with matrix similar to that of the test sample or treated sample to verify whether the impact was caused by the suspected causes. If the suspected causes were identified correctly, the result of spiking test should similar to the result of untreated sample.

5 Conclusion

In my research, water fleas are the most sensitivity among three organisms. Results of water flea tests might indicated potential of impact for the crustacean. In the environment, they are primary consumer. Therefore, adverse effect of effluents on water fleas could be a warning to alert people to pay attentions.

Organisms in the environment once disappeared or became extinct may not come back again. In that case, species existing in that environment may just the survived ones. Taking into account that current environment may be different from previous, when we evaluate the environment or choose organisms for research; we need to do it more cautious.

From test results, we could infer some river or lake might be polluted already, and the same situation could have happened in other country. For example, in China, a lot of rivers and underground water have been polluted (verified by people living around those rivers and organisms in the rivers). As we know, most of polluted ground water was polluted by the effluents. Therefore, to protect the environment, we need to manage the effluents. I believe that after we cleaned the effluents, ground water will become clean to some extent. In Japan, to protect the water quality, most effluents (effluent flow more than 50t/d) were regulated by the laws. However, biological impact of effluents was detected. And the impact might be combined, might caused by various substances, just like F4, salts, organic matters and heavy metal might caused the total impact.

By bioassay, we detected the combined impacts of effluent, but the concentration of each item in the effluents has satisfied the regulations already. Regulations and laws could regulate items in effluents, but they could not regulate impact in the mix zone. Mixed chemical might have combined impact. Therefore, these regulations and laws might not enough on protect ecosystem. However, we do not have enough research data about this part; we need more fieldwork research on different water body to collect the data.

On the other hand, for the factory, once the effluent had toxicity to aquatic organisms, to decrease the toxicity or clean the effluent will increase the cost. More cleanly the more expensive, and there is no need to purify the effluent to the cleanliness that has no adverse effect to the aquatic organisms. Via my research, we can almost know the toxicant; factories just need to eliminate the target materials in cleaning procedure.

In my research, I used bioassays and chemicals analysis; the combine of these two methods showed us the condition of river water and effluents. Therefore, if we combine field study with bioassay and chemicals analysis, it might be a brand new and more effective method to monitoring and protect the ground water.

I learned the technique in this study about estimating the impact for the water bodies by bioassay. So-called WET system that I referred in my study is one of the assessing and managing methods to control the environmental risk of water bodies. As a student from China, I strongly recommended Chinese government introduce this system into China to manage the effluents, to protect not only the national health but also the ecosystem.

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Appendix

Table 13 Water quality of River K

Sample name		S①	S②	S③	S④	S⑤
Temperature	℃	24.7	24.7	25.4	26.2	27.3
pH		7.85	7.92	7.79	7.92	7.93
DO	mg/L	8.5	8.6	9.1	9	9.1
EC	mS/m	28.5	37	38.3	37.5	41.6
Salinity	%	0	0	0	0	0
Turbidity	NTU	2	3	2	15	90(37～180)
ORP	mV	139	175	242	204	180
Total Solid	g/L	0.19	0.24	0.25	-	0.27

Table 14 Water quality of facility's wastewater (80% concentration)

Sample name	Temperature(℃)	pH	DO(mg/L)	Salinity (%)	EC(ms/m)
F1	25.6	8.09	9.63	0.01%	28.25
F2	24.7	8.03	10.05	0.01%	34.5
F3	24.3	8	10.37	0.15%	318
F4	23.7	7.91	9.85	0.41%	761
F5	25.8	8.05	9.66	0.16%	340
F6	26.2	7.77	9.13	0.11%	274

Table 15 Water quality (hardness, Cl2 NH3-H of facility's wastewaters)

	Hardness (mg/L)	Free Chlorine	Total Chlorine	NH3-N
S1	30.4	0	0	1.1
S2	33.8	0	0	0.95
S3	40.5	0.07	0.09	1.33
S4	121.2	0.09	0.09	0.85
S5	80.6	0.06	0.06	0.75
F1	277	0.75	0.13	0.19
F2	342	0.07	0.08	1.06
F3	550	0.1	0.08	1.13
F4	692	0.13	0.29	1.1
F5	102.2	0.54	0.05l	0.33
F6	104.4	0.44	0.14	0.31

Table 16 TOC of each samples

Sample name	TOC(ppm)	sample name	TOC(ppm)
S1	1.59	F4SIR15	60.57
S2	1.90	F4C18	88.29
S3	1.93	F4Aeration	63.47
S4	1.86	F5AF1	58.60
S5	2.13	F5AF2	53.00
		F6AF0.5	53.00
F1	25.59	F6AF1	44.80
F2	41.42	F6AF2	44.84
F3	77.72	F6C	54.65
F4Baseline	69.63		
F5Balseline	60.37		
F6Baseline	52.96		

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