보건학석사 학위논문

Mixture toxicity of three NSAIDs and hospital wastewaters using *Daphnia magna* and *Aliivibrio fischeri*

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병원폐수 및 비스테로이드성 소염진통제 3종의
혼합물 독성 연구

2019년 8월

서울대학교 보건대학원
환경보건학과 환경보건 전공
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이 논문을 석사 학위논문으로 제출함

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Abstract

Mixture toxicity of three NSAIDs and hospital wastewaters using *Daphnia magna* and *Aliivibrio fischeri*

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Pharmaceutical residues in water may cause ecological consequences. Pharmaceutical industries and hospitals are among the major sources of release of pharmaceuticals to ambient environment. In the aquatic environment, pharmaceuticals exist in mixture, however, their interactions and ecotoxicity are not well-characterized. In the present study, ecotoxicities of pharmaceutical mixtures were evaluated using three frequently used non-steroidal anti-inflammatory drugs (NSAIDs), i.e., diclofenac (DCF), ibuprofen (IBP), and naproxen (NPX), as model compounds. In addition, the influent and effluent samples were collected from wastewater treatment plants of two hospitals, and measured ecotoxicities of the complex mixture were compared with those predicted. For measurement of ecotoxicity, a standard acute 48 h *Daphnia magna* test and Microtox assay using *Aliivibrio fischerii* were used. In addition, the change of *D. magna* heartbeat rate measured following 1 h exposure was used as an observation endpoint. For mixture study, mixture ratios of selected pharmaceuticals were determined based on the measured ratio of each NSAID detected in the hospital wastewater. Following the exposure to individual

pharmaceuticals, luminescence was decreased in all Microtox assay. Similarly, *D. magna* immobilization test showed dose-dependent decrease of surviva. The Daphnia heartbeat was also dose-dependently increased. In the ternary mixtures, concentration addition (CA) assumption showed reasonable prediction of the toxicity. However, in the wastewater, toxicity predicted from each component of the mixture showed notable difference from the measured toxicity. The discrepancy might be caused by the other chemicals that may exist in the hospital wastewater. Moreover, in actual wastewater, NSAIDs exist in extremely low concentrations (in ng/L) compared to those tested in toxicity assessment (mg/L). Predicted toxicity extrapolated to lower range of concentration may lead to overestimation. The results of this study demonstrate that toxicity of simple NSAIDs mixture can be reasonably predicted by additive model, while that of complex mixture at low level of exposure concentrations can be difficult.

Key words: Mixture toxicity, *Daphnia magna*, *Aliivibrio fischeri*, Heartbeat rate, Hospital wastewater, Nonsteroidal anti-inflammatory drugs

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1. Introduction

Pharmaceuticals have been extensively used for treatment and prevention of diseases. Therefore, their environmental releases have grown more rapidly due to both population growth and aging (Fick et al., 2010). Numerous pharmaceuticals have been detected in ambient water at levels ranging between ng/L and μg/L (Jelic et al., 2011; Komori et al., 2013; Rivera-Jaimes et al., 2018). At environmental levels, some pharmaceuticals have been associated with adverse ecological outcomes (Kidd et al., 2007; Oaks et al., 2004). Accumulating evidences indicate potential adverse effects of these pharmaceuticals in aquatic environment (González-Pleiter et al., 2013; Santos et al., 2010; Stanley et al., 2006). In the aquatic environment, pharmaceuticals exist in mixtures, however, toxicological understanding of their toxicological interaction is limited. Several studies have been performed for pharmaceutical mixture toxicity, but generally focused on a few binary mixtures (Villa et al., 2014; Heys et al., 2016; Di Nica at al., 2017).

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the top-selling drugs worldwide for anti-inflammatory and antipyretic properties. This group of pharmaceuticals include ibuprofen, diclofenac, fenoprofen, and naproxen. In the United States, more than 100 million prescriptions are written annually for these pharmaceuticals (Nissen et al., 2016). Therefore, these chemicals have been detected frequently in waterways worldwide (Gómez et al., 2006; Sim et al., 2010; Ashfaq et al., 2017). Ecotoxicity of major NSAIDs such as diclofenac, diflunisal, ibuprofen, mefenamic acid, naproxen and piroxicam, have been evaluated extensively, employing *Daphnia*, fish, algae, and cyanobacteria (Cleuversetal., 2004; Hanetal., 2010; Memmertetal., 2013; Bácsietal., 2016).

Mixture toxicity of NSAIDs has been also evaluated (Cleuvers et al., 2004; Di Nica et al., 2017; Nieto et al., 2016). However, for the current understanding on the mixture toxicity of NSAIDs, several limitations are present. First, existing mixture studies do not reflect the composition of the mixture that are expected to encounter in real environment. As most of previous NSAIDs mixture studies employed the mixtures of pharmaceuticals with similar ecotoxicity levels, e.g., in levels with similar toxicity potential or in equitoxic ratio (Cleuvers et al., 2004; Di Nica et al., 2017; Gómez-Oliván et al., 2014; Neale et al., 2017).

Pharmaceutical mixtures that mimic their composition in the ambient water or wastewater outfall should be considered. Another limitation of the existing studies lies on choice of non-specific observation endpoints such as immobilization (Cleuvers et al., 2004) or reproduction of *Daphnia magna* (Cleuversetal., 2008). For pharmaceuticals of specific modes of action, mixture toxicity tests employing endpoints reflecting specific modes of action may provide better understanding of mixture interaction of the pharmaceuticals.

NSAIDs show their therapeutic functions through inhibiting cyclooxygenases, i.e., cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), which are prostaglandin synthesis enzymes (Gonzalez-Rey et al., 2011; Vane et al., 2003). One downstream effect of this activity is inhibition of platelet aggregation, proliferative effect of smooth muscle and vasodilation (García, 2001). Because of this mechanism, this group of pharmaceuticals may cause thromboembolic event, hypertension, and cardiovascular diseases (FitzGerald, 2004). Cardiovascular effects of NSAIDs may suggest that the use of heartbeat as an observation endpoint of their toxicity on *D. magna* is reasonable (Dilgardetal, 2006).

The aim of this study is to understand toxicity interaction of major NSAIDs using commonly used test organisms. To simulate real environmental exposure situation, the mixtures of several NSAIDs that mimic hospital wastewater as well as hospital collected effluents were evaluated. Both *Daphnia magna* with observation endpoints of acute immobilization (48 hr exposure) and heartbeat (1 hr exposure), and Microtox assay were employed. The results of this study will provide useful information for understanding toxicity of NSAIDs in the environment.

2. Materials and Methods

2.1. Selection of target chemicals, and mixture

Study NSAIDs were chosen based on the studies published between January 2000 and January 2018. For literature search, “pharmaceutical manufacture wastewater”, “hospital wastewater”, and “drug manufacture wastewater” were used as keywords. A total of 286 papers were identified, and were further screened by reviewing the titles and abstracts to narrow down to relevant reports. Initial list was developed based on the detection frequency and detection level. For mixture study, mixing ratio of the target NSAIDs was determined based on the measured concentrations of the monitoring research data of the hospital wastewater.

Diclofenac sodium salt (CAS. 15307-79-6) and naproxen (CAS. 22204-53-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ibuprofen sodium salt (CAS. 31121-93-4) was purchased from Fluka Analytical (St. Gallen, Switzerland).

2.2. Hospital wastewater samples and chemical characterization

2.2.1. Chemicals

Acetaminophen ($C_8H_9NO_2$), naporxen ($C_{14}H_{14}O_3$), ibuprofen ($C_{13}H_{18}O_2$), diclofenac ($C_{14}H_{11}Cl_2NO_2$), metoporol ($C_{15}H_{25}NO_3$), sulfamethoxazole ($C_{10}H_{11}N_3O_3S$), sulfamethazine ($C_{12}H_{14}N_4O_2S$), caffeine ($C_8H_{10}N_4O_2$), and carbamazepine ($C_{15}H_{12}N_2O$) were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA). All chemicals were analytical grade, and solutions were prepared with deionized water generated from a Milli-Q water system (R = 18.2 MΩ/cm, Millipore, St. Louis, USA).

2.2.1. Laboratory analyses

All pharmaceuticals were measured using Phenomenex Lunar column (3 μm; 150 × 20 mm) with offline SPE-LC-MS/MS system. We used Oasis HLB cartridge (200 mg; 30 μm) to concentrate hospital wastewater samples. Specific operation parameters of HPLC (Nexera, Shimadzu, Kyoto, Japan) were summarized in Table S1. Detailed conditions of triple quadrouple mass spectrometer (API4000, ABSCIEX, Foster city, CA, USA) and QA/QC results were shown in Table S2.

2.2.3. Sampling

Hospital wastewater samples used in this study were collected from hospital wastewater treatment plants (HWTPs) of two hospitals (number of beds around 100) two located in Seoul, South Korea. Wastewater treatment methods include three steps of treatment process; a pretreatment (grit-removal), flocculation tank, and disinfection using chlorine. Hydraulic retention time of HWTP is around 8 hours. Wastewater samples (2000 mL each) were collected from influent and effluent, and stored at 4 ℃ before measurement.

2.3. *Daphnia magna* 48 h acute toxicity assays

Daphnia magna were cultured in the Environmental Toxicology Laboratory of Seoul National University (Seoul, Korea), according to the U.S. EPA guideline (2002). *D. magna* maintained in 2 L glass beakers in M4 media at 20 ±1 ℃ under a 16:8 dark photoperiod. The conditions of target organism were checked monthly by reference test. Sodium chloride was used as a reference chemical.

The 48 h immobilization toxicity assays were conducted according to the OECD Test Guideline 202 (OECD, 2004). Four replicates of five neonates (< 24 h old) were placed in 50 mL volume for each treatment at 20±1 ℃. *D. magna* were exposed to five test concentrations, plus a control. Immobilization of *D. magna* was recorded .in 24 and 48 hours after exposure.

Exposure concentrations of each NSAID were determined by preliminary range finding tests. Both ibuprofen and naproxen were diluted 3, 10, 30, 100, and 300 mg/L. Diclofenac was diluted 0.1, 1, 10, 100, and 1000 mg/L. For naproxen, dimethyl sulfoxide (DMSO) was used as solvent and in the exposure media, final concentration was 0.1 % (v/v). For mixture toxicity assays, two ternary mixtures were prepared. The ternary mixture representing influent included 79.4 mg/L IBP, 31.8 mg/L DCF, 15.1 mg/L NPX. The aforementioned mixgture was considered as the 100% ternary mixture, and the dilution for the influent sample was determined as 6.25, 12.5, 25, 50, and 100 %. For ternary mixture representing effluent sample, a sample with 106.8 mg/L, 2.3 mg/L, 36.5 mg/L for IBP, DCF, and NPX, respectively, was prepared. This sample was diluted into 20, 40, 60, 80, and 100 %. The mixture ratios of DCF, IBP, and NPX were determined based on the ratio of each NSAIDs detected in the influent and effluent of hospital wastewater.

2.4. *Daphnia magna* 1 h heartbeat assays

For the 1 h *D. magna* heartbeat test, juvenile *Daphnia* aged between 7 - 10 d were used as test organisms. *D. magna* were placed in 96 well plates, and exposed to a given NSAID for 1 h in 25 ℃ incubator (Perkin Elmer, Waltham M.A., USA). The test was conducted with six concentrations including control, four replicates. Exposure concentrations for each NSAID were determined based on preliminary range finding tests, and these were 3, 10, 30, 100, 300, and 1000 mg/L for ibuprofen, and diclofenac, and 3, 10, 30, 100, and 300 mg/L for naproxen. For the mixture of three NSAIDs and actual wastewaters, toxicity tests were conducted with a series of concentrations of 20, 40, 60, 80, and 100 %. The 100 % concentration of influent ratio mixture is 79.4 mg/L, 31.8 mg/L, 15.1 mg/L for IBP, DCF, NPX each. For effluent ratio mixture, the 100 % concentration is 106.8 mg/L, 2.3 mg/L, 36.5 mg/L for IBP, DCF, and NPX each.

For the 1 h *D. magna* heartbeat test, one juvenile was placed in a well with 400 μL. Four replicates per treatment, and five concentrations and control were prepared. After 1 h exposure, heartbeat *D. magna* was recorded for 1 minute under a stereoscopic microscope using a digital CCD color camera (DAMI H 2000, DAMISYSTEM, Korea). The number of heartbeat was later counted manually. The same ternary mixtures used for the 48 h *D. magna* immobilization test were used for the 1 h *D. magna* heartbeat test.

2.5. Microtox assay®

The lyophilized *Aliivibrio fischeri* was activated with Reconstitution Solution and luminescence intensity was measured with Microtox 500® before and after exposure. The test was conducted according to the protocol of the International Standard Organization (ISO), 11348-3 (ISO, 2007). The luminescence intensity was measured after 5, 10, 15 minutes of exposure. Changes in luminescence emission over time were measured with 2 replicates. For single chemical toxicity test, *Aliivibrio fischeri* was exposed to 3, 10, 30, 100, and 300 mg/L for each NSAIDs. For ternary mixtures and actual wastewaters, exposure concentrations were set at the same as *D. magna* acute toxicity. Inhibition of measured luminescence was determined by comparing with control group luminescence changing rate. EC50 was calculated by the probit analysis method of SPSS version 23.

2.6. Prediction of mixture toxicities

To predict mixture compound toxicities, both concentration addition (CA) and independent action (IA) models were used. Because the mixture composition ratio is already known, the concentration of each component can be expressed as a fraction of the total concentration. Using the mixture fraction, concentration addition model can be described as Equation 1 (Berenbaum, 1985).

$$ECx_{(mix)} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1} \qquad (1)$$

ECx_{mix} is the total concentration of the mixture provoking x % effect and p_i denotes the fraction of component i in the mixture. ECx_i is the concentration of the component i provoking x % of effect.

For the 1 h *D. magna* heartbeat test, the CA model was slightly modified. This was because the change of heartbeat can occur in both directions of increased heartbeat or tachycardia or decreased heartbeat or bradycardia.To consider absolute levels of changes of both direction into the effect term, the opposite direction of heartbeat rate were subtracted from the other direction of response. For calculating heartbeat changes, responses observed in lethal concentrations were not considere, e.g., the concentrations that influenced *Daphnia* survival after 24 h exposure to *D. magna* because *D. magna* heartbeat test is sub-lethal toxicity test. 100 % changes of heartbeat rate at sub-lethal level was determined as double the results of highest heartbeat rate changes at sub lethal level among the results.

For independent action model, Equation 2 was run under the assumption that the susceptibilities of individual substances are not correlated with each other (Bliss, 1939). In IA model, every compound of mixture contributes to a given effect. But unlike CA model, each component is assumed to act via different mode of action (MOA).

$$E(C_{mix}) = E(C_1 + C_2 + C_3 + ... + C_n) = 1 - \prod_{i=1}^{n} (1 - E(C_i)) \qquad (2)$$

$E(C_{mix})$ is the predicted effect of the mixture ($0 \leq E \leq 1$) composed of n chemicals, each present at a concentration c_1, c_2, ⋯, c_n. $E(C_i)$ means the effect

of single chemical i at the concentration c_i.

2.7. Statistical analysis

Changes in endpoints among different treatments and control in the 48 h *D. magna* acute test, the *D. magna* heartbeat test, and Microtox assay®, were tested. One-way analysis of variance (ANOVA) with Dunnett's test was used. P-value <0.05 was considered as significant. Shapiro-Wilk's test and Levene's test were used for normality of the data and homogeneity of variance. Excel (version 2016; Microsoft corp., Redmond, Washington, USA) was used for Microtox assay® luminance inhibition ratio calculation, and mixture model calcula tion. Effective concentrations of *D. magna* acute, *Daphnia* heartbeat rate test, and Microtox assay® were analysed with probit analysis of IBM SPSS Statistics (version 22.0; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Target chemicals and mixture

Literature which reported ambient levels of major NSAIDs, show that diclofenac, ibuprofen, naproxen and ketoprofen are among the most frequently detected NSAIDs in aquatic environment. Among these pharmaceuticals, naproxen occurred at the highest levels in ambient water environment, and followed by ibuprofen and diclofenac. Among these chemicals, toxicity reported for *D. magna* is higher for ibuprofen, diclofenac, ketoprofen and naproxen. Therefore, ibuprofen (IBP), diclofenac (DCF), and naproxen (NPX) were chosen as target chemicals in this study.

For manufacturing ternary mixture samples, two ternary mixtures, i.e., IBP, DCF, and NPX at 62.8, 25.2, and 12.0 mg/L representing the influent sample, and IBP, DCF, and NPX at 73.4, 1.6, and 25.1 mg/L representing the effluent, were manufactured.

3.2. Individual chemical toxicity

The 48 h *D. magna* immobilization EC_{50} values were determined at 109.6 mg/L, 61.7 mg/L, and 98.1 mg/L, for ibuprofen, diclofenac, and naproxen, respectively. For diclofenac, the survival was significantly decreased at 100 mg/L, and for ibuprofen and naproxen, the survival of *D. magna* was significantly decreased at 300 mg/L (Figure 1).

Concentration dependent inhibition of luminescence was observed for the whole test pharmaceuticals in Microtox assay® (Figure 1). The luminescence EC_{50} values determined following 15 min exposure to ibuprofen, diclofenac, and naproxen were 50.3 mg/L, 20.2 mg/L, and 28.7 mg/L, respectively. Similar patterns of luminescence inhibition were observed between the 5, and 10 min exposures.

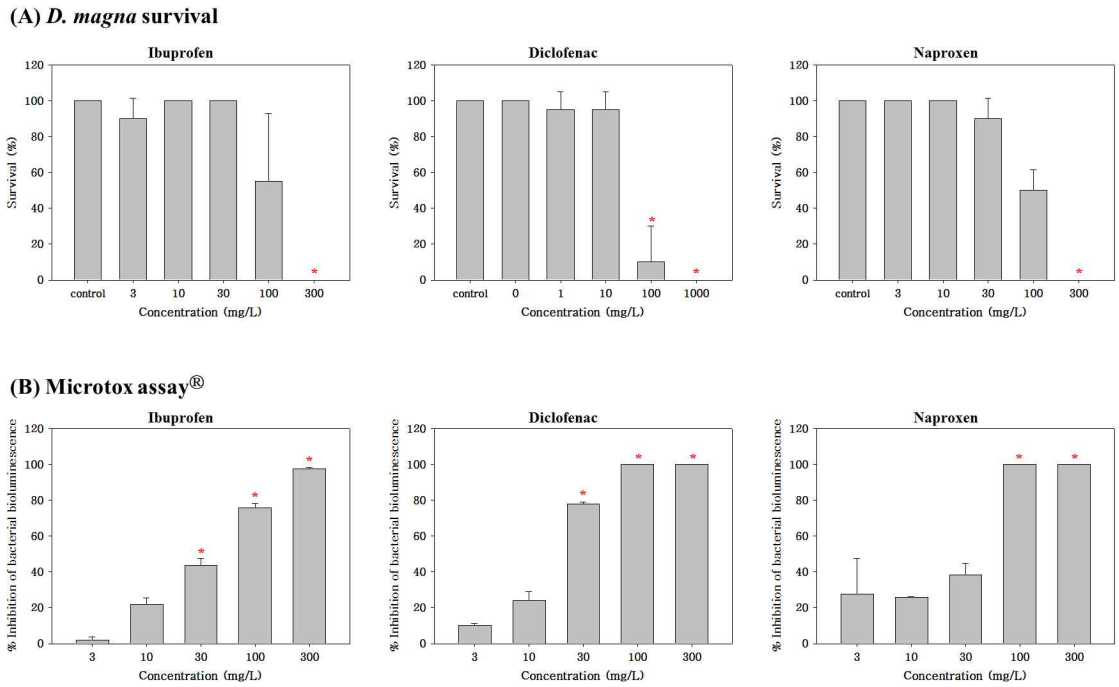


Figure 1. Concentration dependent response of (A) 48 h *D. magna* acute immobilization test, (B) Microtox assay® (15 min), following exposure to ibuprofen, diclofenac, and naproxen, from left to right. The results are presented as mean response ± SD (standard deviation) with significance (*) compared to control group ($p < 0.05$).

3.3. Toxicity of ternary NSAIDs mixtures

For the ternary mixture representing an influent ('influent mixture'), the 48 h *D. magna* acute immobilization EC_{50} value determined at 72.4 % (95 % CI: 62.3 - 84.7 %). For the ternary mixture representing an effluent ('effluent mixture'), the EC_{50} was 77.8 % (95 % CI: 70.5 - 85.9 %) (Figure 2). Concentration dependent decreases of *Daphnia* survival were observed in both ternary mixtures. With Microtox assay®, the EC_{50} value for the influent mixture was determined at 16.9 % (95 % CI: 13.7 - 20.3 %), and that for the effluent mixture was 12.4 % (95 % CI: 9.6 - 15.4 %). In Microtox assay®, the NOAEL for both ternary mixtures were found at 25 %.

The toxicities of the ternary mixtures measured in the 48 h *D. magna* acute immobilization test and Microtox assay® (15 min), were in a reasonable agreement with the predictions based on the concentration addition (CA) assumption. While both CA and IA models showed similarly good prediction in lower concentration ranges, as mixture concentration was increased, CA model resulted in better agreement with the observed toxicity (Figure 3).

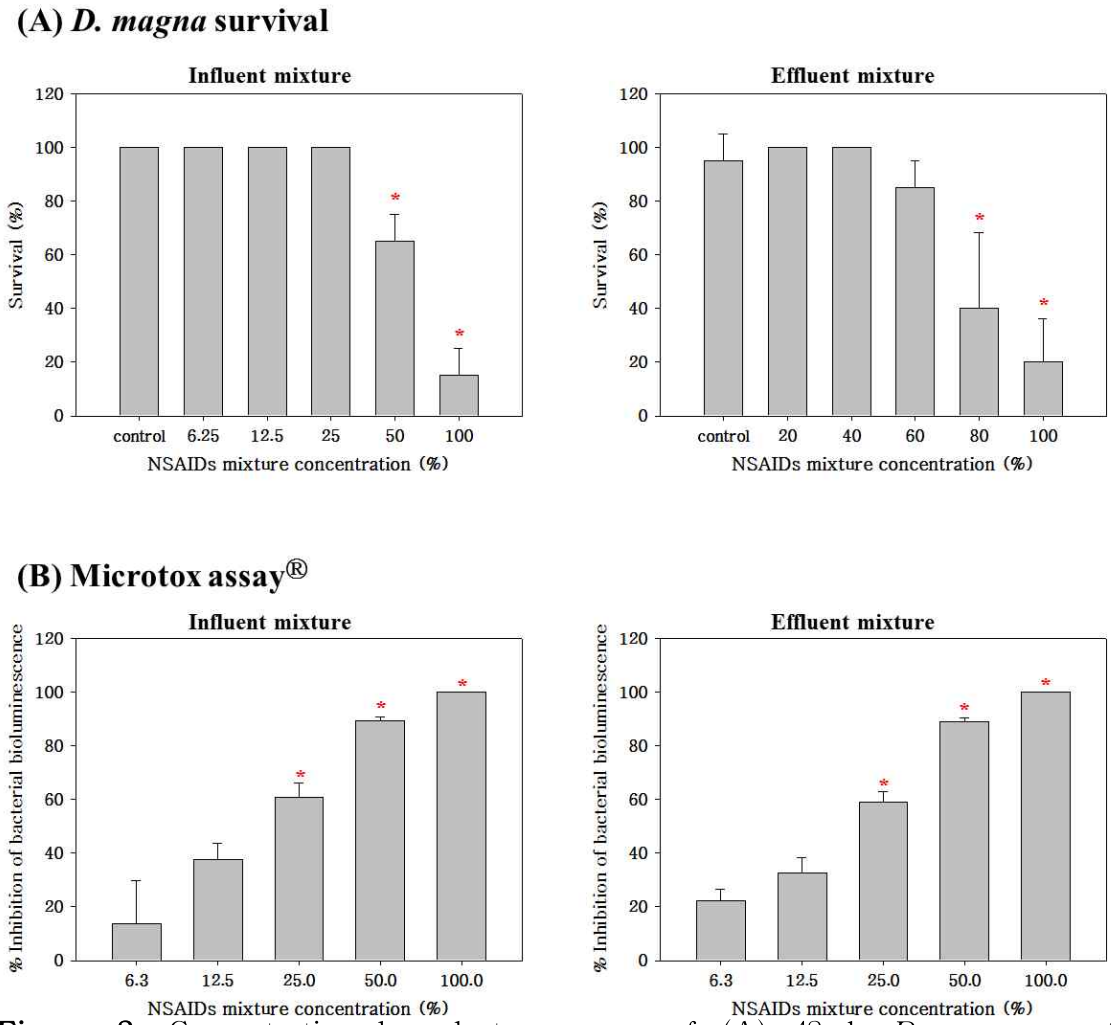


Figure 2. Concentration-dependent response of (A) 48 h *D. magna* acute immobilization test, (B) Microtox assay® (15 min), following exposure to the ternary mixture representing influent (influent mixture) and that representing effluent (effluent mixture). The results are presented as mean response ± SD (standard deviation) with significance (*) compared to control group ($p < 0.05$).

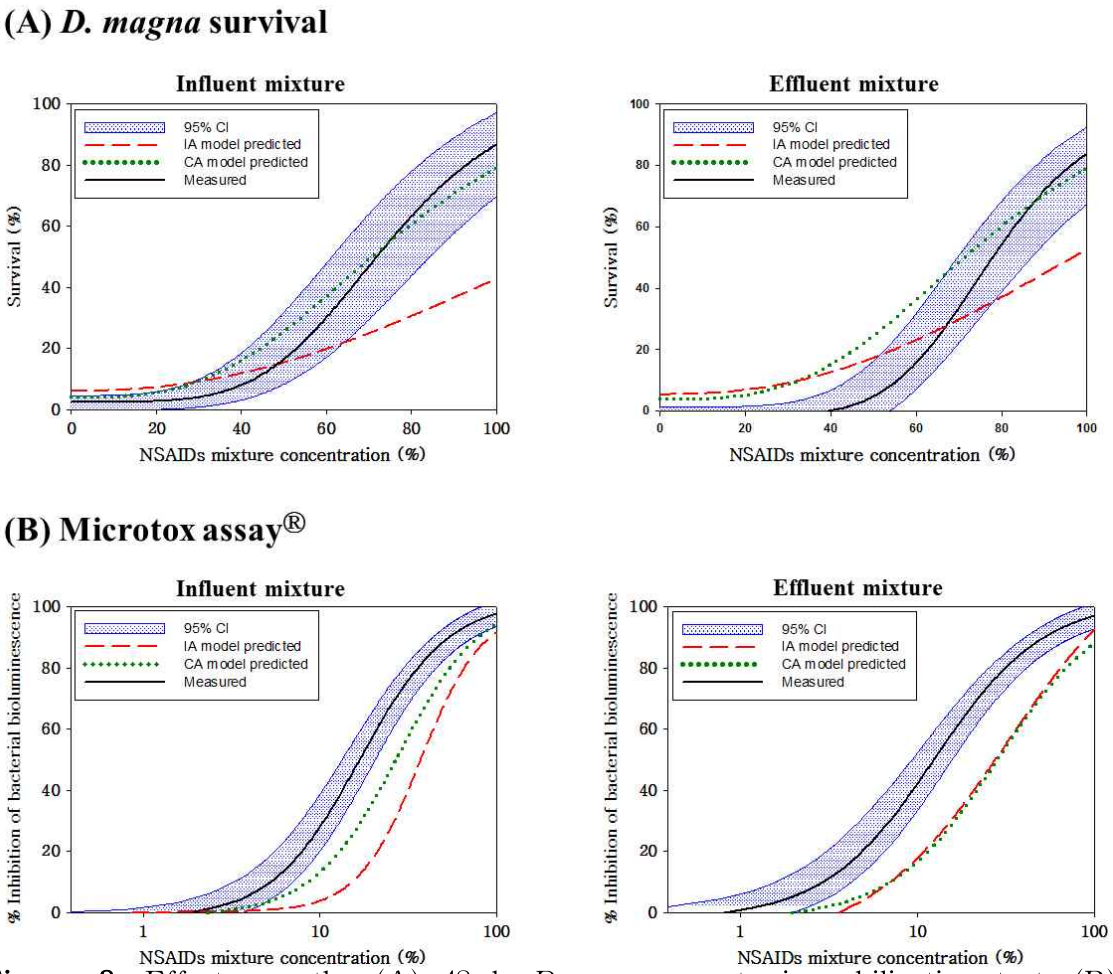


Figure 3. Effects on the (A) 48 h *D. magna* acute immobilization test, (B) Microtox assay® (15 min) predicted based on concentration addition (CA) and independent action (IA) models, in comparison with the measured toxicity (and its 95% confidence band) derived for the ternary mixture representing influent (influent mixture) and that representing effluent (effluent mixture).

3.4. Toxicity of site collected hospital wastewater

For the site collected hospital wastewater samples, i.e., influent and effluent, the 48 h *D. magna* acute immobilization EC_{50} value was found at 72.4 % (95 % CI: 62.3 - 84.7 %), and 77.8 % (95 % CI: 70.5 - 85.9 %), respectively (Figure 4).

Among the selected NSAIDs, NPX occurred at the highest concentration in wastewater (345 - 439.6 ng/L) followed by IBP (<LOD - 146.6 ng/L) and DCF (29.1 - 92.8 ng/L). The individual NSAIDs for each wastewater detected in hospital wastewater at level of ng/L (Table 1). However, concentrations of individual compounds in toxicity prediction of CA model or IA model were mg/L level.

Table 1. Concentrations (ng/L) of pharmaceuticals in hospital wastewater samples.

Pharmaceutical	Site 1		Site 2	
	Influent	Effluent	Influent	Effluent
Acetaminophen	516.4	494.6	27.7	102.5
Carbamazepine	7.3	13.4	14.7	12.6
Caffeine	997.7	1,059.7	60.7	65.9
Diclofenac	62.5	92.8	29.1	33.7
Ibuprofen	<LOD*	<LOD*	146.6	74.8
Metoprolol	2.8	2.1	<LOD*	<LOD*
Naproxen	439.6	356.6	345.0	419.5
Sulfamethazine	<LOD*	<LOD*	<LOD*	<LOD*
Sulfamethoxazole	49.0	71.3	<LOD*	<LOD*

* LOD: limit of detection (ng/L). The levels of LOD were described in Table S2.

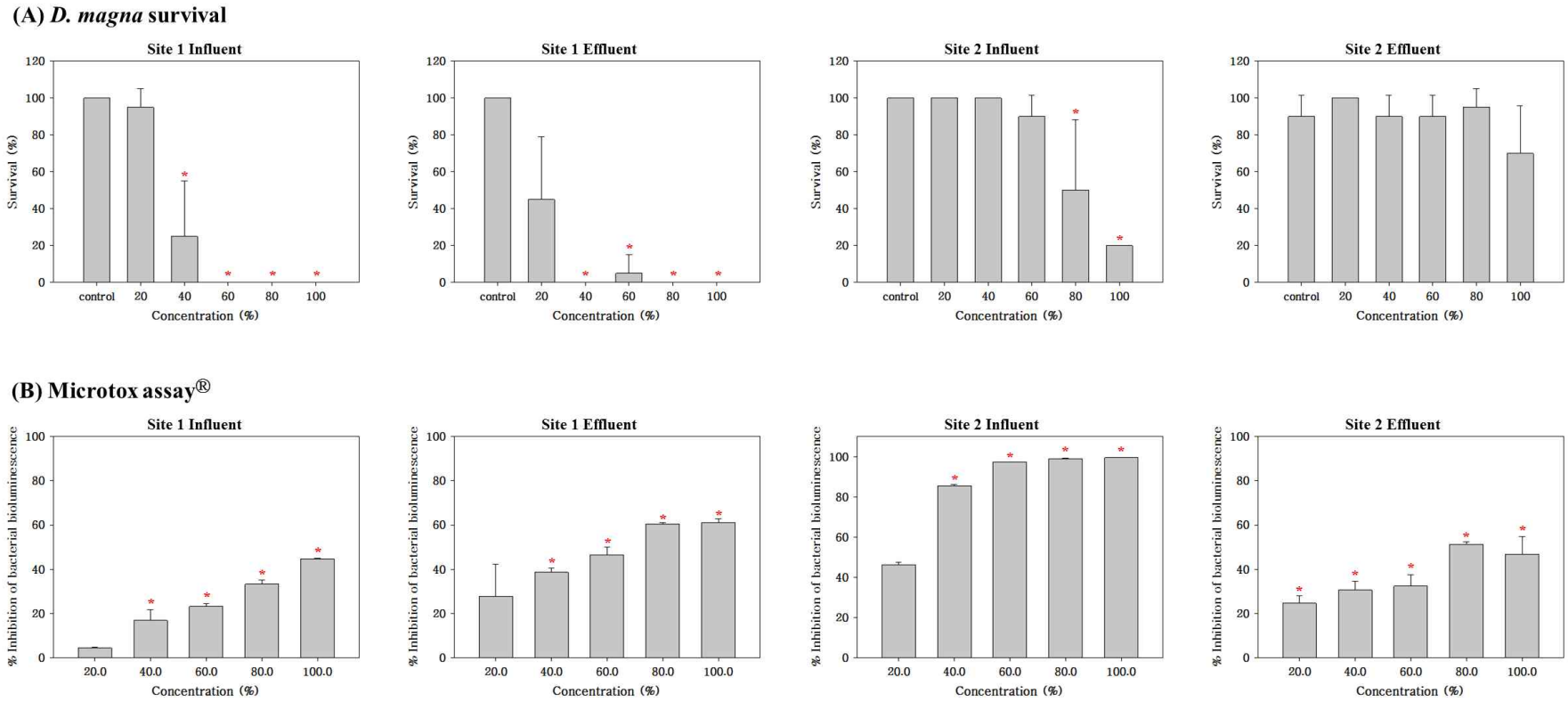


Figure 4. Concentration-dependent response of (A) 48 h *D. magna* acute immobilization test, (B) Microtox assay® (15 min) following exposure to the actual hospital wastewater influent and effluent samples collected from two sites. The results are presented as mean response ± SD (standard deviation) with significance (*) compared to control group ($p < 0.05$).

3.5. *Daphnia magna* heartbeat rate

For the *D. magna* heartbeat test, three water samples were used. These include individual NSAIDs, ternary mixtures, and hospital wastewater.

The heartbeat rate of *D. magna* measured after 1 h exposure showed different pattern depending among the selected individual NSAIDs which are ibuprofen, diclofenac, and naproxen. While ibuprofen exposure caused concentration dependent increasing of heartbeat rate, diclofenac and naproxen caused decreasing trends of heartbeat rate (Figure 5). No observed adverse effect level (NOAEL) of 1 h heartbeat rate test for ibuprofen and diclofenac were determined at 100 mg/L, and that for naproxen was at 30 mg/L.

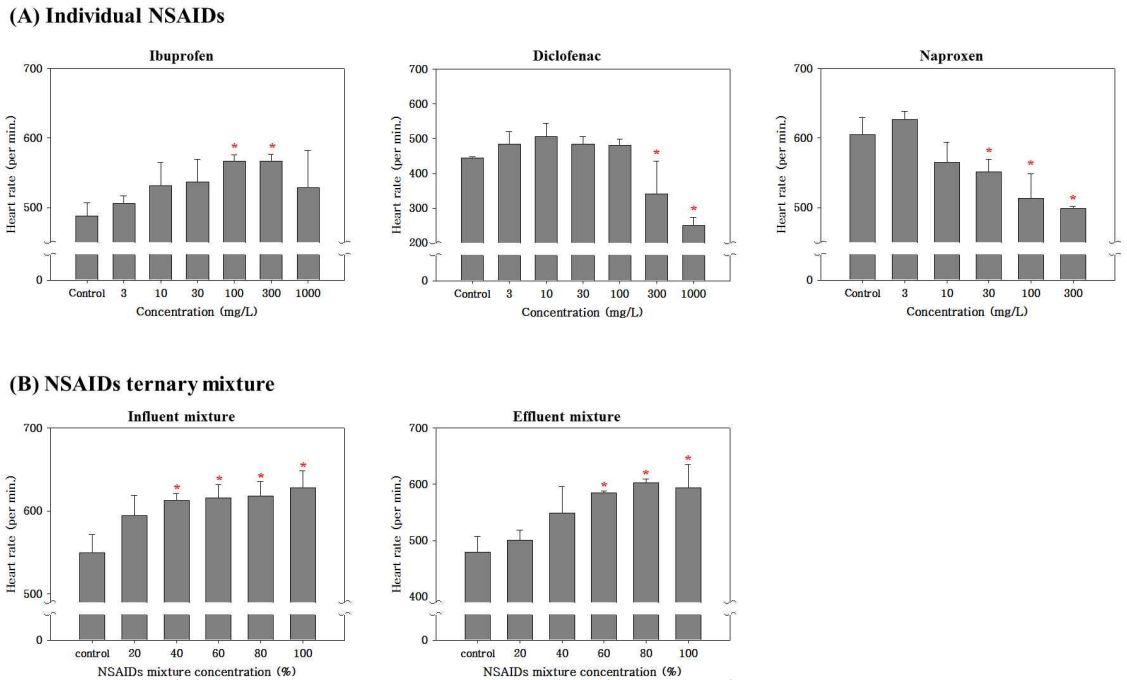
In the NSAIDs ternary mixture toxicities, concentration dependent increase of *D. magna* heartbeat rate was observed in both mixtures. For the influent ratio mixture, significant difference of heartbeat was observed at 40 % and higher. The effluent ratio mixture showed significant changes in heartbeat at 60 % and higher (Figure 5).

Based on the results of *D. magna* acute toxicity test and Microtox assay®, ternary mixtures of NSAIDs were found to follow CA model. For mixture toxicity estimation on *D. magna* heartbeat rate, as this response can be shown in two directions, i.e., increasing or decreasing heartbeat, mixture toxicity prediction was made in two approaches, predict+- considering only magnitude of the heartbeat change and predict+ considering also the direction (Figure 6).

The toxicities of the ternary mixtures measured in the 1 h *D. magna* heartbeat rate test, were in a reasonable agreement with the predictions based on the concentration addition (CA) assumption. While both Predict+ and Predict+- models showed similarly good prediction for the influent mixture, Predict+- model resulted in underestimation of toxicity for the effluent mixture (Figure 6).

For the site collected hospital wastewater samples, i.e., influent and effluent, the significant change of heartbeat was detected at relatively low concentrations, compared to the predicted levels.

Figure 5. Concentration dependent response of *D. magna* 1 h heartbeat test following exposure to the (A) Individual NSAIDs, (B) NSAIDs ternary mixture. The results are presented as mean response ± SD (standard deviation) with



significance (*) compared to control group ($p < 0.05$).

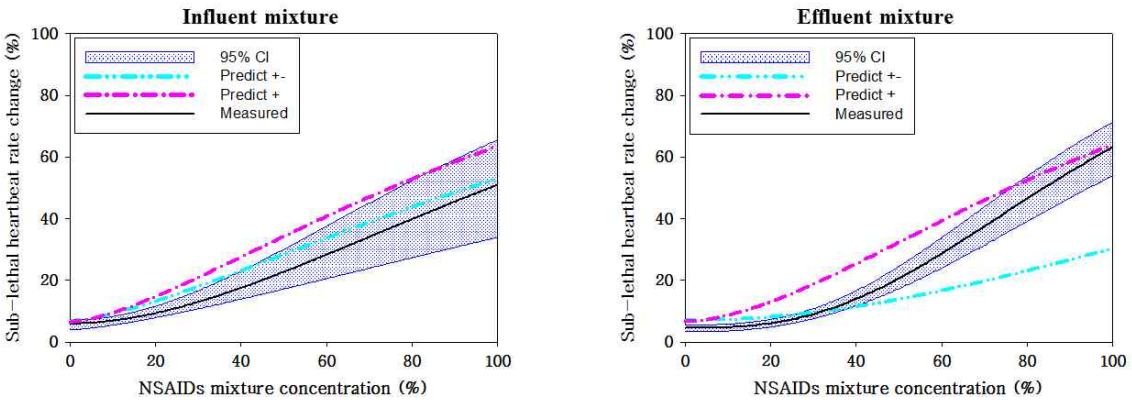


Figure 6. Effects on heartbeat rate predicted based on concentration addition (CA) model. In the 1 h *D. magna* heartbeat test. Predicted+- : CA prediction without the consideration of the magnitude of the heartbeat change but not the direction. Predicted+: CA prediction without the consideration of the heartbeat changing direction.

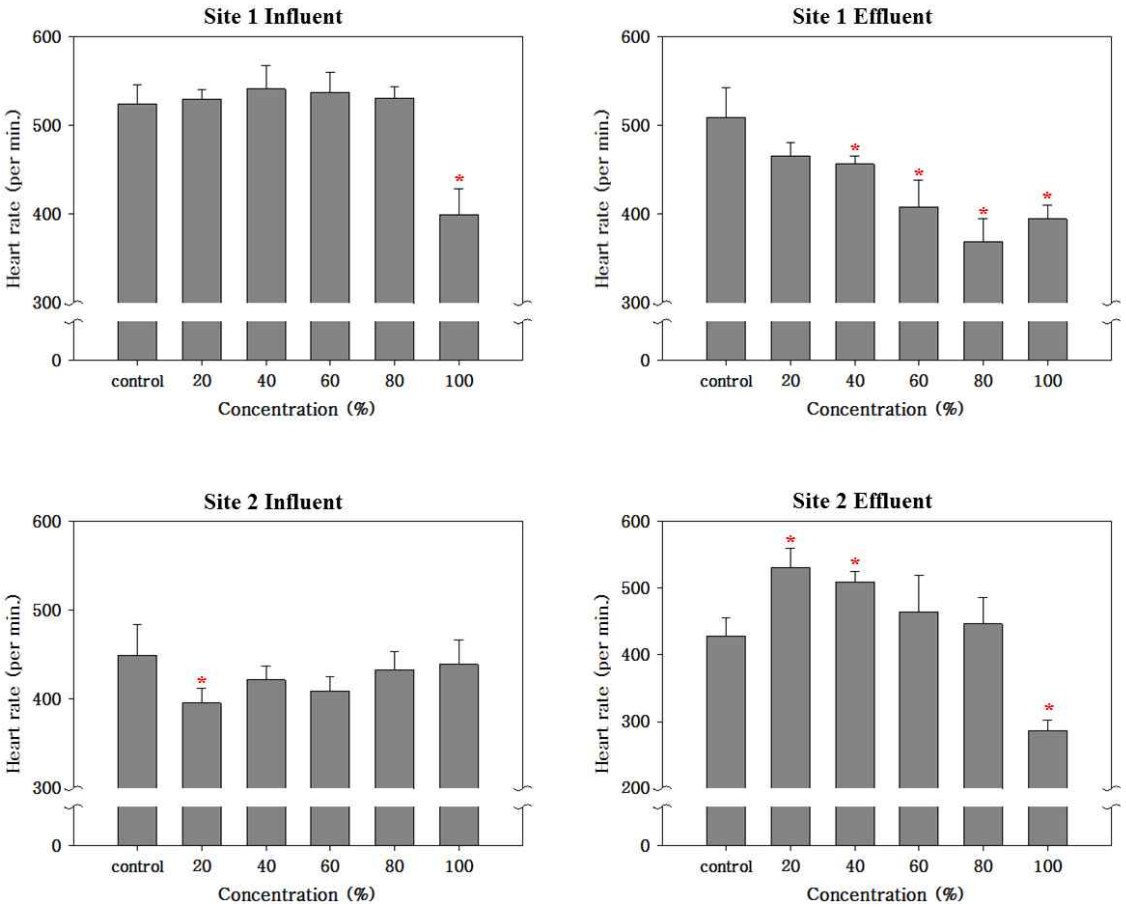


Figure 7. Concentration-dependent response of *D. magna* 1 h heartbeat test, following exposure to the actual hospital wastewater influent and effluent from 2 sites. The results are presented as mean response ± SD (standard deviation) with significance (*) compared to control group ($p < 0.05$).

4. Discussion

The NSAIDs selected in the present study showed mixture toxicity that can be reasonably predicted by CA model. Although some discrepancy exists, the measured toxicity of *D. magna* acute test and Microtox assay® generally fitted the line predicted by CA model (Figure 3). This observation may be due to that fact that the modes of toxicity of these NSAIDs are similar. Our observation shows clearly that the simple NSAIDs mixtures can be predicted for its toxicity by CA model.

All tested NSAIDs have the same mode of therapeutic action as a non-selective cyclooxygenase (COX) inhibitor (Bleumink et al., 2003). Cyclooxygenase is known to have two isoenzymes of COX-1 and COX-2. COX-1 is induced constitutively in normal cells for tissue homeostasis, and COX-2 is expressed in inflammatory cells (Oshima et al., 2002; Radi et al., 2006). COX inhibitor suppressed synthesis of prostacyclin (Ruan et al., 2011), and prostacyclin is important for protecting of vasoconstriction and blood clots in heart, kidneys and many other organs. As NSAIDs suppressed cyclooxygenase non-selectively, these drugs may influence cardiovascular system (Vane, 2000). Due to association with cardiovascular system and NSAIDs, heartbeat rate can be selected as a useful endpoint of NSAIDs toxicity test.

Prediction of actual hospital wastewater toxicity, however, was not inconsistent with the observed toxicity. The results of both *D. magna* acute test and Microtox assay measured toxicity showed notable discrepancies with those predicted by CA and IA models. The reason for these discrepancies may be other contaminants that exist in the hospital wastewater, e.g., other unmeasured pharmaceuticals and organic chemicals. It should be also mentioned that the uncertainty of prediction can be increased in the lower concentrations than those tested in toxicity assessment. Our observation has an implication that ecotoxicity prediction for complex mixtures may not be feasible unless further methodological advances are made.

For bioassay studies, *D. magna* are frequently used as test organisms because of their similarities with human. Also, *D. magna* has myogenic heart similar to mammals (Villegas-Navarro et al., 2003; Campbell et al., 2004). Because of similarity of heart between *D. magna* and mammals, *D. magna* heartbeat rate

could be a useful endpoint in cardiovascular toxicity effects of NSAIDs (Dilgard et al., 2006).

Changes in *D. magna* heartbeat rate have been reported for perfluorooctane sulfonate, copper, metoprolol, metaproterenol, and verapamil (Villegas-Navarro et al., 2003; Fernandez-Gonzalez et al., 2011; Liang et al., 2017). In addition, NSAIDs has been tested for zebrafish heartbeat rate (David et al., 2009; Li et al., 2016). However, for NSAIDs, *D. magna* heartbeat rate or their mixture are limited. The present study is the first study that evaluated NSAIDs mixture toxicity with *D. magna* heartbeat rate test.

In *D. magna* heartbeat rate test, NSAIDs mixture showed different predicted agreement depends on the mixing ratio (Figure 6). Measured toxicity of influent ratio mixture showed greater agreement with Predict+- line (modified CA model), however effluent ratio mixture showed a better fit with Predict+ line (conventional CA model). Although the measured and predicted toxicity lines showed a discrepancy, toxicities of both mixtures could be to certain extent explained by CA model.

The difference of heartbeat rate directions may be caused by other pathways that can affect the heartbeat regulation in *D. magna*. Ion channels of *D. magna* heart play a role in regulation of the heartbeat rate (Pirtle et al., 2018), and NSAIDs can modulate ion channels of which mechanisms are not related to COX inhibition (Gwanyanya et al., 2012).

Higher levels of certain pharmaceuticals in the effluent compared to those measured in the influent require an explanation (Table 1). These pharmaceuticals include diclofenac, naproxen, and sulfamethoxazole (Table 1). Negative removal efficiencies of pharmaceuticals in wastewater have been reported before (Ejhed et al., 2018). This can be explained by metabolized pharmaceuticals in the influent. Deconjugation of metabolites during wastewater treatment may lead to observation of higher concentrations of the pharmaceutical in the effluent than in the influent (Blair et al., 2015). Moreover, the grab sampling of the influent samples may be another reason. Due to hydraulic retention time (HRT) and diurnal variations in the influent wastewater, grab samples could cause some error of determination of removal efficiencies in the wastewater treatment (Ort et al., 2010; Sui et al., 2010).

5. Conclusion

Due to NSAIDs exist in aquatic environment in mixture form and may cause serious ecological effects, ecotoxicity interactions of NSAIDs warrant detailed studies.

Based on *D. magna* immobilization test and Microtox assay, we found that the toxicity of ternary mixture of major NSAIDs, i.e,. DCF, IBU and NPX, can be reasonably predicted using CA model. However, for complex mixtures such as hospital wastewater, we found that both CA and IA models could not predict the mixture toxicity. The toxicity prediction of wastewater which has complex composition of chemicals required further studies.

6. References

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Supplementary Information

Table S1. HPLC condition for analysis

Parameter	Condition
Column	C18 column (Luna 3 µm; 150 × 20 mm; Phenomenex)
Mobile phase	Positive mode; A:B = 5:95 (v/v, %) A: 10 mM ammonium formate with 0.3% formic acid B: Methanol Negative mode; A:B = 2:98 (v/v, %) A: 5 mM ammonium acetate in deionized water B: Methanol
Flow rate	200 µL/min (gradient)
Injection volume	10 Ml
Ionization mode	ESI negative/positive[a]
Curtain gas	10 psi
Column temperature	40 °C
Ion spray voltage	-4500V/5500V[a]
Ion source gas 1	40 psi/30psi[a]
Ion source gas 2	60 psi/10psi[a]
Collision gas	5

Table S2. LC-MS/MS condition for PPCP analysis

Compounds	Detection mode	Retention time (min)	LOD (ng/L)	LOQ (ng/L)	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (mV)	Collision energy (mV)	Collision cell exit potential (mV)
Acetaminophen	Negative	1.78	2.54	8.48	149.942	106.9 108	-65 -65	-24 -20	-5 -5
Naproxen	Negative	3.31	1.51	5.05	228.928	185 168.9	-30 -30	-10 -36	-11 -11
Ibuprofen	Negative	3.51	1.63	5.43	205.227	158.8 160.8	-45 -45	-10 -10	-9 -9
Diclofenac	Negative	4.14	3.62	12.06	293.794	249.9 214	-45 -45	-14 -28	-17 -15
Metoprolol	Positive	1.26	1.58	5.28	268.087	116.1 121.1	81 81	25 31	12 12
Sulfamethoxazole	Positive	1.77	0.81	2.7	254.011	156 92.1	61 61	21 37	16 10
Sulfamethazine	Positive	1.80	1.23	4.11	279.001	186	61	23	16
Caffeine	Positive	1.97	2.14	7.14	194.99	137.9 137.7	56 56	25 25	14 12
Carbamazepine	Positive	2.02	1.37	4.56	237.134	194.1 191.8	61 61	25 31	18 20

국문 초록

*Daphnia magna*와 *Aliivibrio fischeri*를 이용한 병원폐수 및 비스테로이드성 소염진통제 3종의 혼합물 독성 연구

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의약폐수는 제약 산업과 병원에서 주로 배출되며, 다양한 의약물질로 이루어진 혼합물 형태로 수생태에 존재한다. 의약폐수는 생태계에 악영향을 미칠 수 있으며 생태 독성연구가 이루어지고 있다. 하지만 환경 중 의약물질은 혼합물로 존재함에도 불구하고 의약폐수 내 혼합물간의 상호 작용 및 생태 독성은 연구는 부족한 실정이다.

따라서 본 연구에서는 의약품으로 널리 사용되고 있는 비스테로이드성 소염진통제(Non steroidal anti inflammatory drugs, NSAIDs) 중 다빈도로 사용되는 이부프로펜(IBP), 디클로페낙(DCF), 나프록센(NPX)을 대표물질로 선정하여 비스테로이드성 소염진통제의 혼합물 독성을 파악하고자 하였고, 2개의

지역에서 샘플링한 병원 폐수의 유입수와 방류수의 독성 역시 평가하고자 하였다. 생태 독성을 파악하기 위하여 OECD 지침에 따른 급성 생태독성 공정시험법인 *Daphnia magna* 48시간 급성 독성평가 실험과 *Aliivibrio fischerii*를 사용한 Microtox assay®를 진행하였다. 또한, *Daphnia magna*를 대상물질에 1시간 노출하여 심박수의 변화를 관찰하는 *Daphnia magna* 심박수 관찰 실험도 진행하였다. 혼합물 독성평가에서 IBP, DCF, NPX의 혼합물의 혼합비는 병원폐수에서 검출된 NSAIDs의 검출비를 기반으로 결정되었다. *D. magna*의 48시간 급성 독성평가 실험과 Microtox assay®에서 NSAIDs 혼합물의 독성은 모두 농도 의존적으로 증가하였다. *D. magna* 1시간 노출 실험의 경우, 준치사 농도에서 심박수의 농도 의존적 증가를 관찰하였다. 유사한 작용 기전을 지닌 물질의 독성예측에 사용하는 Concentratoin addition (CA) 모델이 NSAIDs 혼합물의 실제 독성 평가 결과와 대부분 높은 유사도를 보였고 이는 NSAIDs의 작용 기전이 비슷하기 때문으로 여겨진다. 하지만 실제 병원폐수의 독성은 독성 예측 모델과 상이한 결과를 보였다. 병원폐수의 실제 독성평가 결과와 예측값의 차이는 병원폐수 내에 존재하는 의약품 외 다른 화학물질 때문으로 여겨진다. 또한, 실제 폐수에 존재하는 NSAIDs가 독성학적인 영향이 나타나는 농도(mg/L)보다 훨씬 낮은 농도(ng/L)이기 때문에 독성 예측과 실제 독성 영향의 차이가 두드러질 수 있다.

본 연구는 *Daphnia magna* 48시간 급성 독성평가, Microtox assay® 및 *Daphnia magna* 심박수 관찰 실험을 통하여 비스테로이드성 소염진통제 혼합물 독성을 확인하였다. 복잡한 조성을 지닌 실제폐수의 독성 예측을 위한 연구가 추후 필요하다고 여겨진다.

주요어: 혼합물 독성, *Daphnia magna*, *Aliivibrio fischeri*, 심박수 변화, 병원폐수, 비스테로이드성 소염진통제

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