Towards a multi-bioassay-based index for toxicity assessment of fluvial waters

L.K. Pandey, I. Lavoie, Soizic Morin, S. Depuydt, J. Lyu, H. Lee, J. Jung, D.H. Yeom, T. Han, J. Park

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Title: Towards a multibioassay-based index for toxicity assessment of fluvial waters

Lalit K. Pandey[a,b], Isabelle Lavoie[c], Soizic Morin[d], Stephen Depuydt[e], Lyu Jie[f], Hojun Lee[g], Jinho Jung[h], Dong-Hyuk Yum[i], Taejun Han[g,j], Jihae Park[e,*]

[a]*Institute of Green Environmental Research Center, 169, Gaetbeol-ro, Yeonsu-gu, Incheon 21999, Korea.*
[b]*Department of Plant Science, Faculty of Applied Sciences, MJP Rohilkhand University, Bareilly, 243006, India.*
[c]*Institut national de la recherche scientifique, centre Eau Terre Environnement, 490 rue de la Couronne, Québec City, Québec, G1K 9A9, Canada.*
[d]*Irstea, UR EABX, 50 avenue de Verdun 33612 Cestas cedex, France.*
[e]*Lab of Plant Growth Analysis, Ghent University Global Campus, 119, Songdomunwha-ro, Yeonsu-gu, Incheon 21985, Korea*
[f]*Department of Life Sciences, Jilin Normal University, Siping City, Jilin Province, China*
[g]*Department of Marine Science, Incheon National University, 119, Academy-ro, Yeonsu-gu, Incheon 22012, Korea.*
[h]*Division of Environmental Science & Ecological Engineering, Korea University, Seoul 02841, Korea*
[i]*Ecotoxicology Team, Korea Institute of Toxicology, Daejeon 34114, Korea*
[j]*Ghent University Global Campus, 119, Songdomunwha-ro, Yeonsu-gu, Incheon 21985, Korea.*

*Corresponding author at:
Lab of Plant Growth Analysis, Ghent University Global Campus, 119, Songdomunwha-ro, Yeonsu-gu, Incheon 21985, Republic of Korea. E-mail address: jihae.park@ghent.ac.kr / Telephone number: +82 32 626 4305 / ORCID: 0000-0002-1873-1480 (J. Park).

Highlights

- An ecotoxicological assessment of natural waters based on a multi-organism trial was conducted in four South Korean rivers
- The tested organisms showed distinct levels of performance in their response to natural waters
- A scoring system is proposed to integrate biological responses into an overall toxicity category
- This bioassay approach identified more sites as potentially degraded as did water chemistry measurements alone

Abstract

Despite their proven reliability for revealing "acceptable" degrees of toxicity in waste- and reclaimed waters, bioassays are rarely used to assess the toxicity of hazardous contaminants present in natural waters. In this study, we used organisms from different trophic levels to assess the toxicity of water samples collected from four different South Korean rivers. The main objective was to develop a multi-descriptor index of toxicity for undiluted river water. The responses of six test organisms (*Aliivibrio fischeri*, *Pseudokirchneriella subcapitata*, *Heterocypris incongruens*, *Moina m acrocopa*, *Danio rerio*, and *Lemna minor*) after laboratory exposure to water samples were considered for this index, as well as the frequency of teratologies in diatom assemblages. Each individual test was attributed a toxicity class and score (three levels; no toxicity = 0, low toxicity = 1, confirmed toxicity = 2) based on the organism's response after exposure and a total score was calculated. The proposed index also considers the number of test organisms that received the highest toxicity score (value =2). An overall toxicity category was then attributed to the water sample based on those two metrics: A = no toxicity, B = slight toxicity, C = moderate toxicity; D = toxicity, E = high toxicity. The susceptibility of the test organisms varied greatly and the sensitivity of their response also differed among bioassays. The combined responses of organisms from different trophic levels and with different life strategies provided multi-level diagnostic information about the intensity and the nature of contamination.

Keywords: Aquatic plants; Bioassay; Biological indicators; Microorganisms; Multi-descriptor index; Multiple endpoints; Receiving water

1. Introduction

Fluvial ecosystems experience multiple anthropogenic disturbances such as industrial, municipal and agricultural effluents leading to eutrophication and chemical contamination, acidification, hydrological and hydro-morphological alterations, and invasion by non-native species (Li et al. 2010). In particular, wastewater effluents derived from industrial and municipal sources contain a mixture of chemicals which, once released into the receiving water bodies, can have deleterious effects on the biological integrity of the flora and fauna (Kim et al. 2015). These impacts are observable at different levels of biological organisation (e.g., molecular, individual, population, and community; Nedeau et al. 2003; Ntengwe and Maseka 2006; Tabrez and Ahmad 2012; Hassan et al. 2015) and may ultimately alter ecosystem function. The most commonly used approach for assessing water quality is the measurement of chemical substances and their metabolites. However, it is now generally accepted that there are potential limitations when relying solely on chemical profiling to evaluate ecosystem health status, regulate acceptable loads of wastewater effluents, and conduct risk assessments (Wolska et al. 2007). Routine chemical monitoring does not account for the bioavailability of chemicals and nutrients, the temporal changes in exposure, or the additive and synergistic effects of contaminants (Ahlf et al. 2002; Chu and Chow 2002). Most importantly, chemistry-based monitoring does not provide information regarding the effect of contaminants on the biota. Ecological surveillance of running waters based on an integrated assessment of the biological, chemical and physical properties of a system contributes to better water resource protection and conservation and helps water managers to plan rehabilitation.

Numerous water quality monitoring approaches based on organisms such as algae (mostly diatoms) (e.g., Ponader et al. 2007; Coste et al. 2009; Kelly 2013; Lavoie et al. 2014), macrophytes (Small et al. 1996; Thiebaut et al. 2002), invertebrates (e.g., Reynoldson et al. 1997; Lento et al. 2008; Canesi and Corsi 2016) and fish (e.g., Joy and Death 2001; Oberdorff et al. 2002) are commonly used worldwide. These indices are generally based on metrics such as species assemblage structure, diversity, % tolerant species, life-forms, traits, size distribution, etc. However, these monitoring approaches have mostly been developed to assess overall biological integrity, and often mostly reflect nutrient and organic matter enrichment and habitat degradation, not toxicity. Biological assessment of potential water toxicity from

various types of inorganic and organic contaminants is usually performed using single and multi-species toxicity tests which can effectively demonstrate causal relationships between the presence of contaminants and adverse effects on the biota. Bioassays are widely used to assess the toxicity of a substance or a combination of compounds in aquatic and terrestrial environments. As a general trend, this ecotoxicity assessment approach relies on stepwise dilutions of samples to determine effective or lethal concentrations (EC and LC, for example, the concentration of a substance or mixture giving half-maximal response of the test organism is the EC_{50}), with the objective to establish acceptable degrees of toxicity before wastes can be discharged in the environment or reclaimed. The information can then be used for monitoring and predicting the effects of chemical discharges and for deriving chemical-specific water quality guidelines (Ankley et al. 1992). Bioassays, such as bioluminescence in the bacteria *Vibrio fischeri*, the cell count of microalgae such as *Pseudokirchneriella subcapitata*, photosynthetic activity of microalgal communities (Kim Tiam et al. 2016), mortality and growth of small invertebrates such as ostracods and cladocerans, and survival of fish such as *Danio rerio* (zebrafish) are internationally standardized test methods commonly used in ecotoxicology. Bioassays can also be used to monitor potential toxicity of natural waters, although this approach is not as conventional and necessitates a different method for the determination of the contamination level than the EC or LC generally used for wastewaters.

In this paper, assessment of natural water toxicity is proposed using undiluted river samples and a battery of biological indicators. Including various organisms in toxicity evaluation ensure a better-integrated response by providing information at different levels (e.g., biochemical function, cellular growth, mortality, etc.). The various test organisms have different sensitivities to the suite of contaminants, which in turn maximises chances to detect a response after exposure to a sample with unknown chemical composition. The goal of our research was to develop a multi-species toxicity test procedure using six commonly used test organisms (*Aliivibrio fischeri* (bacterium), *Pseudokirchneriella subcapitata* (green microalga), *Heterocypris incongruens* (ostracod), *Moina macrocopa* (cladocera), *Danio rerio* (fish), *Lemna minor* (aquatic macrophyte)) for ecotoxicological assessments of river waters collected at different sites distributed among four watersheds impacted by industrial and municipal effluents in South Korea. In addition, the frequency of teratologies (abnormalities) in diatoms (siliceous brown microalga) *collected in situ* was also included in this multi-species toxicity assessment.

This study proposes an approach for a rapid screening of flowing waters with suspected toxic contamination, and provides valuable knowledge for further development of a bioassay-based index for future implementation in the national river water quality management program in South Korea.

2. Materials and methods

2.1. Water sample collection and on-site field measurements

Sites were selected based on an existing micropollutant dataset from an eight-year extensive monitoring program, conducted by the South Korean government, to assess river water quality at 159 sites receiving effluents from 34 industrial complexes (Cho et al. 2014) (Tables S1 and S2). The South Korean Ministry of Environment (MOE) also performs regular water quality assessments of streams and rivers, including measurements of metals (As, Ag, Cd, Cr, Cu, Hg, Ni, Pb, and Zn), total phosphorus (TP) and total nitrogen (TN), biological oxygen demand (BOD_5), total dissolved solids (TDS), pH, conductivity, and total coliforms. Based on the available information on river water quality, 16 sites were chosen to cover various sub-watersheds across South Korea. Additional information on the sampling sites selected and on the four watersheds is provided in supplementary tables (Tables S1 and S2).

Surface water samples were collected in plastic bottles (2 litres) at the 16 selected sites distributed along four major South Korean rivers in September 2015 (Fig. 1). The samples were kept cold during sampling and transportation and were stored at 4°C upon arrival at the laboratory. Temperature, pH, and dissolved oxygen were measured directly in the field with a multi-parameter display system (YSI 650, USA). Conductivity was also measured on-site using a portable metre (Milwaukee, USA), and total dissolved solids measurements were performed with a field amperometric graphite electrode (Hanna HI98301 DiST® 1, USA).

2.2. Chemical analyses

Biochemical oxygen demand was measured by dark and light bottle incubation for 24 h at 20°C in controlled light conditions, and Colilert-18 tests (IDEXX) were carried out to estimate the total number of coliforms. Water samples were filtered through 0.45 μm syringe filters prior to analyses of nutrients and metals. An automatic water analyser (Skalar/ Netherlands, SAN ++) was used to measure TN and TP. Analyses of metals were conducted by inductively coupled plasma-optical emission spectrometry (ICP-OES; Varian Vista PRO, CA, USA). Standard solutions were prepared fresh and calibration curves (r^2 >

0.995) were generated daily. Standard solutions were analysed after every 10 samples to verify their concentrations. Measurement precision ranged from 94 to 107%, and detection limits were calculated based on the standard deviations of blanks triplicates (range: 4 to 14 μg L^{-1}). Organic compounds were analysed by the South Korean Ministry of Environment, following the methods presented by Cho *et al*. (2014).

2.3. Toxicity assessment

2.3.1. Microtox bioassay (bacteria)

The Microtox® (Newark, DE, USA) bacterial acute toxicity assay was used to determine inhibition in the metabolism of *Aliivibrio fischeri* when exposed for 30 minutes to each of the 16 surface water samples. The cultures were maintained at 15°C throughout the experiment. The control treatment consisted of 2% NaCl water. Bacteria were exposed in triplicates to each treatment, according to the standard Microtox procedure (Tarkpea and Hansson 1989). Bioluminescence of the bacteria was measured with a Microtox photometer (Model 500). Results were expressed as % inhibition in bioluminescence between pre-exposure to the water samples and after 30 minutes of exposure, taking into account the temporal changes in luminescence occurring in control (ISO 11348-1:2009; Parvez et al. 2006). Confidence intervals (CI) were calculated for α-value = 0.05.

2.3.2. Microalgal bioassay

The test performed in triplicate on *Pseudokirchneriella subcapitata* was conducted using an Algaltoxkit (Microbiotests, Belgium). The methods followed standard operational procedures provided by the manufacturer and in accordance with the OECD Test Guideline 201 (OECD 1984). Microplates were filled with 900 μL of test water (for the control, 900 μL of MBL medium (Nichols 1973) was used) and 100 μL of an algal-inoculum solution, with the initial number of algal cells being adjusted to 10^5 cells ml^{-1}. The exposure tests were performed for 72 h at 25°C under light conditions of 60-80 μmol photons m^{-2} s^{-1}. Determination of algal growth was conducted using absorbance values measured at a 670 nm wavelength using a spectrophotometer (Scinco, S-3100, Korea). Optical densities of the blank and samples were taken at 670 nm and then converted into cell densities (cells ml^{-1}). The growth inhibition (% calculated as the percentage reduction in average specific growth rate compared to the control, following OECD 1984) was used as an indicator of the toxicity of the 16 river water samples. Confidence intervals (α = 0.05) were calculated.

2.3.3. Ostracod bioassay

Hatching of ostracod (*Heterocypris incongruens*) cysts was initiated 48 h prior to the start of the toxicity test, and freshly hatched ostracods were pre-fed (with Spirulina powder from Spirulina International, Tilburg, Holland) for 4h. The toxicity test followed the procedure described in the International Standard ISO 14371:2012. Pore sediment water was collected at each site and sand was used as reference sediment following the protocol of Chial and Persoone (2002a). Multi-well plates were filled with 100 µL of sediment and 2 ml of overlying water (distilled water for the control). *Pseudokirchneriella subcapitata* was provided as food source. Ten ostracod eggs per treatment were placed in the dark at 25°C for six days. Water was not replaced during the experiment—that is, the experiment was static. Each of the 16 river water treatments and the control was tested in triplicate. Ostracods were recovered from the multi-well plates at the end of the six-day exposure to determine % mortality and growth length inhibition and were compared to the controls. For both endpoints, confidence intervals ($\alpha = 0.05$) were calculated.

2.3.4. Cladocera bioassay

Toxicity effects of the 16 river water samples on *Moina macrocopa* ($\leq$ 24 h old) were tested after adaptation of the OECD Test Guideline 211 (OECD 2012) for *Daphnia magna* to better reflect conditions appropriate to *M. macrocopa* intrinsic population dynamics. Each toxicity test was conducted on 10 neonate female cladocerans, with one animal per well. Elendt M4 medium (Elendt 1990) was used as the control water. Exposure was performed for nine days at 20 ± 2°C with 16 h light and 8 h dark photoperiods. The test medium (river water) was renewed every three days and the cladocerans were fed daily *ad libitum* with green algae (*Chlorella vulgaris*). Offspring production per female was recorded and expressed as % of control values (CI: $\alpha = 0.05$). Growth inhibition induced by the treatment, compared to the control, was determined from individual length measurements. Mortality was determined as the percentage of dead individuals at the end of the exposure.

2.3.5. Fish embryo test

The fish embryo toxicity test (FET; OECD Test Guideline 236 2013) was conducted with *Danio rerio*. A total of 20 fertilised eggs for each treatment were incubated at 20°C for 96 h. Percent mortality was evaluated based on the numbers of coagulated embryos or lack of heartbeat at the end of the

200 experiment. Criteria to establish the validity of the test were reached, with > 90% survival in the negative
201 controls, and 100% mortality in the positive control (3,4-dichloroaniline, 4 mg L^{-1}).

202 **2.3.6. *Lemna* root bioassay**

203 A *Lemna* root bioassay was adapted from Park et al. (2017). Fresh green fronds of *Lemna minor*,
204 consisting of two fronds, were selected as the test material. Roots were excised, and rootless plants were
205 placed in a well plate with 3.0 ml of test water (3.0 ml of Steinberg medium for the control; Steinberg
206 1946). The experiment was conducted using 20 fronds per treatment. Toxicity tests were carried out in a
207 growth chamber for 72 h at 25°C under continuous light (90-100 µmol photons m^{-2} s^{-1}). Plants were
208 examined after the 72 h incubation period and the root lengths were measured using an imaging analyser
209 (Moticam 2500, Ted Pella Inc., USA). Root elongation inhibition was then expressed as the % reduction in
210 the root length of exposed *Lemna* (measured in mm), compared to the control (CI: $\alpha = 0.05$).

211 **2.3.7. Deformity assessment in diatom frustules**

212 The occurrence of deformities was assessed using on-site diatom assemblages chronically exposed
213 to river water impacted with industrial discharges. The methods for biofilm collection, sample preparation,
214 slide mounting, and deformity evaluation are explained in Pandey and Bergey (2016), Cerisier et al. (2018)
215 and Pandey et al. (2018). Briefly, biofilm samples were collected at each site by scraping hard substrates
216 such as stones and concrete walls (~ 25 cm^2) using a blade and a brush. Diatom valve deformities were
217 enumerated using digested material mounted onto permanent microscope slides and observed at 1000x
218 magnification under an oil immersion microscope (Carl Zeiss, Axiostar plus, Germany). The proportion of
219 deformed diatom valves at each site was estimated based on 500 valve counts and expressed as %
220 deformity (CI: $\alpha = 0.05$). For comparison purposes, biofilms were also sampled in least-impacted areas
221 upstream of the major sources of contamination (three sites and four samples per river), and these
222 "reference" diatom assemblages were examined for deformities. The average % deformity observed in the
223 upstream site samples was used as the control.

224 **2.3.8. Scoring of bioassay results**

225 For each test, three toxicity classes were established to estimate the level of contamination: no
226 toxicity, moderate toxicity, and high toxicity (Table 1). For all of the bioassays, > 50% response (i.e., above
227 the EC_{50} or LC_{50}) was considered as the lower boundary for "high toxicity" of the water samples, in line

with classical toxicity criteria. This 50% effect threshold is commonly reported as a significant acute toxicity value in the bioluminescence assay (Niemirycz et al. 2007; ISO 11348:2009), algal growth inhibition test (OECD 1984), *Lemna* root regrowth test (Park et al. 2017), invertebrate mortality bioassays (ISO-14371 2012; OECD 2012), and fish embryo toxicity test (OECD 2013). Samples resulting in a 20-50% toxicity response were assigned to the "moderate toxicity" class, in agreement with the test validity criteria defined for *A. fischeri* bioluminescence (Niemirycz et al. 2007) or for ostracod and cladocera mortality (ISO-14371 2012; OECD 2012). For the purpose of the present study, a 20% effect boundary was used for the *Lemna* re-growth tests for which validity criteria have not yet been established. This criterion ensures consistency with the results from the multiple-organisms trial and is in line with the hazard classification system proposed by Persoone et al. (2003), which recommends the use of a 20% effect level as the lowest significant toxic impact value. However, this threshold was lowered to 10% in the *Danio* embryo tests, as per OECD (2013). The criteria for abnormal diatom valves (% teratologies) included three categories with narrow ranges. The upper limit for the "no toxicity" class was set at < 0.5%, which is regarded as the baseline in natural conditions, as suggested by Morin et al. (2008a) and Arini et al. (2012). Teratology percentages ranging between 0.5-1% were assigned to the category "moderate toxicity" based on previous observations (Lavoie et al. 2012; Morin et al. 2012; Pandey et al. 2014, 2015; Pandey and Bergey 2016), while values above 1% were assigned to the category "high toxicity". The toxicity classes for diatom teratologies are subjected to modifications due to insufficient information and data regarding the presence and type of abnormalities as a response to stress along a contamination gradient (Lavoie et al. 2017).

2.4 Overall toxicity assessment

A score was assigned to each toxicity class ("no toxicity" = 0, "moderate toxicity" = 1, and "high toxicity" = 2), allowing for the calculation of a total score integrating the response of all organisms tested. Where multiple endpoints were tested on an organism (reproduction, growth, and mortality), it was the worst toxicity class observed that was considered for the calculation of the final score. Here, the maximum total score possible is 14, based on the seven biological descriptors included in the present study. However, this value will differ as a function of the number of bioassays tested in future bioassessments. The total toxicity value is then reported as a percentage. Low values indicate no or low toxicity. Finally, each sample

was assigned an overall toxicity category by considering both their toxicity class and the number of tests (expressed as %) that obtained a score of 2, "high toxicity" (Table 2). For example, a sample which would obtain the following scores for each of the seven tests: 0, 2, 1, 0, 1, 0, and 2, would have a total score of 6 out of a maximum of 14 (6/14*100 = 43%). Two out of the seven tests would have been classified as "high toxicity" (score value = 2), which represents 29% of the cases (2/7*100 = 29%). Following the criteria proposed in Table 2, this sample would be categorised as "toxic" (D).

3. Results and Discussion

3.1. Physico-chemical assessment of water quality

The purpose of this study was to evaluate the potential of various toxicity bioassays for testing water toxicity in natural freshwaters and to develop a scoring system allowing for an integrated assessment of aquatic ecosystems health. An exhaustive presentation of the chemical and physical properties of the tested waters is thus beyond the scope of this paper. Briefly, analysis of the physicochemical characteristics of the 16 sites sampled (Table 3) revealed that all waters were nutrient-enriched. Particularly elevated nitrogen concentrations and high conductivities were found at N2 and N3 (Nakdong River), as well as at H1 and H2 (Han River). Water quality data for these four sites (N2 and N3; H1 and H2) represented the most severely impacted conditions with particularly high suspended solids and elevated metal concentrations (zinc at H1 and H2, Han River; copper and nickel at N2 and N3, Nakdong River). The remaining sites were less impacted, based on the data available. However, it is worth highlighting the high TP values measured in the samples collected on the Geum River, in particular, G1, G2, G3, and G4 (TP = 0.28 ± 0.03 mg L^{-1}). Organic compound analyses were also conducted during this survey, and concentrations were below detection limits (data not shown). The fact that organic contaminants were not detected is surprising because these ecosystems receive wastewater effluents from 34 industrial complexes distributed along the four main rivers studied. Moreover, Cho et al. (2014) published the results from an extensive survey conducted by the South Korean government and reported the presence of numerous toxic chemicals (mainly organochlorine and organophosphate pesticides, volatile organic compounds, solvents, and plasticisers) in these environments. As stated previously, traditional water chemistry measurements are not always reliable to fully characterise environmental conditions because they do not integrate fluctuations in water quality, and they are susceptible to missing intermittent contamination unless samples are collected

during wastewater effluent release or high-resolution analyses are performed. It is also possible that the low concentrations of organic contaminants observed in the present study, compared with the values reported in Cho et al. (2014), reflect the efforts of the South Korean government to reduce contaminant inputs from industrial effluents.

3.2. Bioassay-based toxicity assessment

According to a report on a survey and evaluation of aquatic ecosystem health in South Korea (Hwang et al. 2011), some rivers and streams are considerably contaminated in the country and are in worse biological condition than predicted by conventional chemical analysis data. As a result, the country recently adopted the use of bioindicators to improve water quality evaluation as part of a more integrated concept of ecological status assessment. The use of biological descriptors is now part of the surface water management policy in South Korea with the creation of the "Nationwide Aquatic Ecological Monitoring Program (NAEMP)". This new monitoring program is similar to the Water Framework Directive established in the European Union (2000/60/EC), where the use of organisms from multiple biological compartments (different trophic levels and different life strategies) is recommended in addition to chemical, physical, and bacteriological parameters for examining the ecological status of fluvial ecosystems (Geiszinger et al. 2009). Data gathered from the multiple bioassays used in this study provide preliminary considerations for the development of a biological index of river water toxicity and serve as a foundation for future optimisation of the approach. The results from each test are presented in Table 4, along with CI and F-statistics from analysis of variance (ANOVA). The overall toxicity index values for the 16 tested waters are reported in Table 5, along with individual scores obtained for each test organism. The toxicity categories were attributed based on the criteria presented in Tables 1 and 2. The results for all biological descriptors, as well as the final toxicity categories, are presented in detail and discussed in the following paragraphs.

3.2.1. Inhibition of bioluminescence

Bacterial bioluminescence inhibition assays are commonly used to evaluate the toxicity of contaminants released by wastewater effluents (e.g., Rodrigues and Umbuzeiro 2011). Here, inhibition of bioluminescence in *Aliivibrio fischeri*, after exposure to the 16 river water samples, was used to evaluate its potential in determining the toxicity of natural river waters. Significant luminescence inhibitions were only

312 revealed for the N2 and G2 sites, with changes of -28% (CI = 3%) and -16% (CI = 3%), respectively (Table
313 4). Based on the criteria established for this bioassay (Table 1 and Table 2), almost all sites were
314 categorised as “no toxicity”, except N2, which exhibited “moderate toxicity” (Table 5). The higher metal
315 concentrations at N2 were probably responsible for the observed toxicity, potentially in combination with
316 other toxic organic compounds suspected to contaminate these rivers (according to Cho et al. (2014)).
317 Because the presence and concentrations of the various contaminants may vary greatly, depending on
318 various factors such as water level and timing of wastewater effluent release in the river water, it is possible
319 that the other water samples were not as toxic to bacteria as was previously observed (MOE 2007). It is
320 also possible that *Aliivibrio fischeri* is simply not very sensitive to the type and level of contamination
321 tested in this study, as was observed by Macken et al. (2009) with the same bacterium shown to be the least
322 sensitive indicator tested for Cd contamination. Stimulation of bacterial metabolism was also observed in
323 the present study, with a significant increase in bioluminescence for the water sample collected at H2 (19%,
324 CI = 15%). This increased bioluminescence, compared to the control, may result from interferences caused
325 by the presence of volatile or insoluble substances in the waters (ISO 11348-1), or from a biological
326 response. Indeed, the presence of higher (but non-lethal) concentrations of metals, nutrients, and organic
327 matter in the waters tested compared to the control may, in turn, favour bacterial metabolism. For example,
328 bioluminescence stimulation of *Aliivibrio* species was also observed under metal exposure in other studies
329 (Fulladosa et al. 2005, 2007; Shen et al. 2009) and was attributed to hormesis (Calabrese 2005).
330 Based on the lack of a clear response of the bacteria to the test waters in this study, i.e., no strong
331 inhibition observed and stimulation of bioluminescence noted for numerous samples, this bioassay does not
332 seem to be sensitive enough nor appropriate when used alone for routine biological assessments of flowing
333 water toxicity. Becouze-Lareure et al. (2016) came to the same conclusion when assessing water and
334 sediment quality of a peri-urban river subjected to combined sewer overflow, which is also supported by
335 others (Angerville 2009; Gonzalez-Merchan et al. 2014a, b). This acute bioluminescence test is usually
336 carried out with an exposure time of 30 minutes or less, which does not provide information on potential
337 adverse effects that may happen later (e.g., Backhaus et al. 1997). For example, Hsieh et al. (2004)
338 observed that chronic exposure (22 h) of *Aliivibrio fischeri* to seven priority pollutant metals showed a

toxic response at concentrations many-fold lower than for acute exposure (5 or 15 min). This suggests that toxicity assessments based on bioluminescence could be better evaluated with longer exposure times.

3.2.2. Algal cell density

The *Pseudokirchneriella subcapitata* bioassay was selected for toxicity assessment in the present study for various reasons. This species is easily available from culture collections and easily maintained under laboratory conditions. It is among the most widely recommended species for freshwater toxicity testing, with standard guidelines having been established (OECD 1984; Environment Canada 1992; USEPA 1994), and this bioassay is currently being endorsed for regulatory purposes. Moreover, the responses of *P. subcapitata* to a variety of contaminants and its relative sensitivity compared with other test organisms have been studied extensively (e.g., Radix et al. 2000; Weyers et al. 2000).

Algal cells exposed to the 16 test water samples showed significant growth inhibition (Table 4) when exposed to the water samples from H1 (-30%, CI = 8%) and N1 (-11%, CI = 5%), compared to the controls (growth rate: 0.035 div h^{-1}). Based on the selected toxicity thresholds, only H1 fell into the "moderate toxicity" class (Table 5). These results are in contrast with the numerous reports of good sensitivity of this test organism. For example, Katsumata et al. (2006) reported lower cell counts in *P. subcapitata* when exposed to two herbicides (simazine and 3,5-dichlorophenol). However, it is most likely that those pesticides have a more noticeable effect on algae (as their mode of action directly targets plant functions) than do contaminants of largely industrial and municipal origin, as in the present study. Moreira-Santos et al. (2004) reported a negative effect of a mine effluent (low pH, metals, and turbidity), with growth inhibition reaching 98%, also suggesting good sensitivity of algal bioassays. The metal concentrations in their study were much higher than the values observed in our selected South Korean rivers which could explain why the magnitude of the effects we observed was lower. Moreover, acid mine drainage (pH = 3) also likely inhibited algal growth in their study. The water samples from the Yeongsan and Geum Rivers significantly stimulated algal growth ($p < 0.005$), which can most likely be attributed to their nutrient-enriched waters (e.g., higher TP in the Geum River, see Table 3). This result suggests that the potential toxicity of the samples may be overshadowed by the positive effect of nutrients on algae. Although this algal bioassay does not seem to be very sensitive to the toxicity level and type of contamination characterising the tested waters in the present study, it is worth noting that an increase in cell

density may provide information on the eutrophication potential of the waters. In other words, the fact that most of the tested waters scored "no toxicity" does not guarantee good overall water quality.

3.2.3. Toxicity to Ostracoda

Freshwater ostracods are reported to be excellent bioindicators of surrounding physico-chemical conditions and anthropogenic stressors (pesticides, hydrocarbons, and metals) (Ruiz et al. 2013). Mortality in *Heterocypris incongruens* is a widely-used endpoint in ecotoxicology and constitutes a standardised bioassay recognised by the International Organization for Standardization (ISO 2012). Ostracods in bioassays are generally used to assess toxicity of solid phase matrices such as sediments or storm water run-off particles (e.g., Chial and Persoone 2002a, b; Watanabe et al. 2008, 2011; Angerville et al. 2013) because this organism spends most of its life in contact with sediments. However, this test has also been used to assess the toxicity of various chemicals and to evaluate the toxicity potential of natural waters (Toumi et al. 2015).

After six days of exposure to the tested waters, *Heterocypris incongruens* showed a mortality rate higher than the 20% of the control. Mortality was significantly higher at H1 (with 100% mortality on the date of recovery, $p < 0.0001$) and lower at G5 and G6 (≤ 10%, $p < 0.05$). Concerning growth, cell elongation in control was higher than expected, with final ostracod lengths 2.8 (± 0.2)-fold the initial sizes. With exposure to the tested waters, growth inhibition compared to the controls was highlighted at G2 (44%, CI = 19%), G3 (45%, CI = 37%), and Y1 (52%, CI = 19%). Based on the criteria presented in Table 1, H1 and Y1 received the "high toxicity" score, while G3 and G2 were classified in the "moderate toxicity" category. The remaining sites were classified as "no toxicity". Compared to the bacterial and algal bioassays, the response of the ostracods suggests that they have a higher sensitivity to the toxicity of the samples, with four sites highlighting toxicity, including two with the highest score (Table 5). In a study on the effects of metals on *H. incongruens*, Sevilla et al. (2014) observed that aquatic exposure to different concentrations of dissolved Cd (3.2-339 μg L^{-1}) and Cu (260-2600 μg L^{-1}) resulted in high mortalities (57-100% and 95-100%, respectively). Watanabe et al. (2011) also reported high mortality in ostracods exposed to elevated concentrations of metals (Cu, Zn, Ni, As, Cd, and Pb). Based on water chemistry measurements available for this study, metal concentrations were much lower than the above-mentioned values, which could partly explain the lower mortality rates observed. Moreover, the toxicity found using the ostracod

bioassay in this study may also result from exposure to other substances that were not measured by the targeted micropollutant analyses, or it may reflect an integrated response to a cocktail of multiple chemicals.

3.2.4. Toxicity to Cladocera

The *Daphnia magna* acute toxicity test is a common standard protocol to help regulate wastewater effluents. However, this organism has not been found in aquatic systems in Korea. Thus, it has been suggested that a domestic species, rather than the international standard species, be used for the Korean whole effluent toxicity criteria. *Moina macrocopa*, which has been widely used in ecotoxicology applications, is one of the most promising domestic species for this purpose. Kim et al. (2012) found that effluents discharged from wastewater treatment plants in Korea induce multi-level toxicity, including acute toxicity, feeding rate inhibition, and oxidative stress in *M. macrocopa*. Yi et al. (2010) found that *M. macrocopa* was more sensitive to toxins than *Daphnia magna*, based on an acute toxicity test with industrial effluents. Ji et al. (2008) noted significantly higher % mortality (50-100%) in *M. macrocopa* when exposed to PFOS (perfluorooctane sulfonic acid) and PFOA (perfluorooctanoic acid) than in the control treatment. These results show the sensitivity of this cladoceran species when exposed to high concentrations of contaminants or when directly incubated with wastewaters. However, in the context of the present study, the contamination levels of the tested waters were undoubtedly lower, which could explain the fact that most samples were categorised as “no toxicity” (Table 5). Mortality assessment was invalidated by the 22% mortality in the controls. Growth was significantly affected by exposure to the tested waters ($F = 4.49$; $p \leq 0.001$), with significant growth inhibition at N1, H2, Y3, Y1, N3, G5, G6, and Y4, compared to the control. However, despite significant differences with the control, growth inhibition was always lower than 20% and therefore did not suggest toxicity. Contrastingly, reproduction expressed as offspring production per female was inhibited by 44% (CI = 8%) at Y4, falling into the “moderate toxicity” category (Table 5). Nine-day exposure to this water sample also led to 50% mortality (although this endpoint was not considered due to >20% mortality in control). Interestingly, significant increases in offspring production were observed in G1, G3, and H1 ($p \leq 0.05$), where survival was 100%. The same pattern, although not significant, occurred at N3. This suggests that *Moina macrocopa* was indifferent to, or even stimulated by the substances present in these waters. It must be noted, however, that the test was not

replicated and was invalidated due to high mortality under control condition. Based on the reproduction of *M. macrocopa*, all the natural waters tested reflected a non-toxic environment, except site Y4 in the Yeongsan River.

3.2.5. Fish bioassay

Worldwide, zebrafish has proven to be popular for toxicity evaluations because they are easy to keep and to breed in the laboratory, are free of pathogenic microorganisms, and deliver eggs of high quality independent of the season (Bresch 1991). For the purpose of this study, fish embryos were incubated with river waters and toxicity was estimated based on % mortality. The results suggest that the tested waters are generally of "moderate toxicity", based on the selected criteria (Table 5), except for the samples from N1, Y4, G1, G4, and G6, which exhibited < 10% mortality (Table 4). It must be noted that this exposure test was not replicated. One hundred percent of surviving eggs hatched within 96 hours, irrespective of the treatment.

In line with numerous studies showing the sensitive response of zebrafish embryos to wastewaters of differing compositions (e.g., Şişman *et al*. 2008; Vincze *et al*. 2014), the results obtained here from exposure to natural waters showed some toxicity for more than 50% of the samples. This finding may reflect the sensitivity of fish embryos to moderate contamination and/or to a wide variety of substances. However, it is worth underscoring the fact that, following the OECD (2013) specifications, we used a more conservative effect value (10%) as the lower boundary for the "moderate toxicity" class.

3.2.6. Lemna root re-growth

The small size, structural simplicity, and rapid growth of *Lemna* are some of the characteristics that make it advantageous for use in laboratory toxicity tests (Park et al. 2017). Moreover, this plant is an essential primary producer and has a wide geographical distribution. Bioassays using *Lemna* as a test organism are traditionally based on endpoints such as the number of fronds, their growth rate and their wet or dry biomass, which require standard exposure durations of at least seven days to detect toxicity (Park et al. 2017). Roots of *Lemna minor* are highly sensitive to environmental stressors (Panda and Upadhyay 2003). However, there have been few studies incorporating root elongation as a test endpoint, partly due to the fragility of the roots, which introduces difficulties into measurements (Davis 1981), and to the difficulty of obtaining a sufficient number of test specimens with identical root lengths for exposure experiments.

The use of *Lemna* in ecotoxicology assessments has thus been re-evaluated (Gopalapillai et al. 2014; Park et al. 2017) using root re-growth (after cutting them at the base) as an endpoint. This method has been shown to be sensitive, precise, and ecologically significant in comparison with more traditional measurements such as frond growth and biomass.

Root elongation under control conditions was 34 ± 4 mm. Compared to the controls, exposure of *L. minor* to the 16 water samples collected in South Korean rivers showed significant toxicity for six sites (Table 4). Samples from the Han River (H1 and H2) fell into the category "high toxicity", and "moderate toxicity" was noted for N2, N3, Y4, and G1; while the remaining 10 sites were considered "non-toxic" (Table 5). The observed root re-growth inhibition may be a response to elevated zinc concentration in the Han River, as this bioassay was shown to respond to metal contamination. For example, Park et al. (2013) reported root elongation in three *Lemna* spp. exposed to Ag, Cd, Cr, Cu, and Hg to be a sensitive endpoint to assess metal toxicity. However, in the sites where "moderate toxicity" was observed, metals do not seem to represent a contamination concern, at least not based on measurements of punctual water samples taken for these experiments. We cannot exclude a contribution of organic contaminants (and additive/synergistic effects) to the observed toxicity, which may not have been detected owing to concentrations being below the detection limit and/or because they were not targeted in the analysis (unknown compounds such as degradation products).

3.2.7. Diatom teratologies

Percent deformed frustules were examined from periphytic diatom samples collected at the 16 sites (Fig. 2). Diatom deformities were found at high abundance at H2 (5.9 ± 0.7%) and H1 (4.9 ± 0.4%). N2, Y1, and Y3 also showed potential toxicity, with deformities present at > 1%. Based on the categories tentatively suggested for this study, these teratology occurrences would place these five samples into the category "high toxicity". A marginal increase in diatom teratologies was also noticed at N1 (0.8 ± 0.7%) and N3 (0.7 ± 0.1%), which were thus categorised as being of "moderate toxicity" (Table 5).

The approach based on diatom teratologies differs markedly from the six bioassays presented above because it is conducted with organisms collected *in situ*, and therefore reflects an integrated response to environmental fluctuations over a longer period of time. Moreover, the assessment of toxicity is not based on the level of inhibition or mortality compared with a control. Rather, control conditions are

estimated to be the % of teratologies generally encountered in natural systems with minimal disturbance (observed to be ≤ 0.5%, based on Morin et al. 2012). In this study, the % deformities averaged for three sites (four replicates per site) sampled upstream of contamination was 0.3 ± 0.1%. One particular advantage of using teratologies as indicators of contamination is that no exposure experiment is required because samples are collected *in situ* and examined, which allows for sampling of multiple sites in a short period of time. Moreover, analyses can be performed later if samples are properly stored and preserved.

The ecological status of the same 16 sites was also assessed based on a suite of diatom descriptors (cell size, frustule health, lipid bodies, frustules deformities, etc.) in previous studies (Pandey and Bergey 2016; Pandey et al. 2018). Overall, the authors found that water quality assessment based on diatom assemblages and diatom-based metrics had a good fit with the available physicochemical data (least versus most impacted sites). However, as was observed in the present study, a greater number of sites showed signs of degradation based on the diatom metrics used (biological descriptors) compared with the available physicochemical data. This suggests that the use of biotic indicators provides useful complementary information on ecosystem health status at the selected sites, and that *in situ* diatom assemblages are good assessment tools for monitoring rivers, alone or in combination with bioassays.

3.3. Bioassay scores and overall index values

3.3.1. Toxicity assessment of water samples: complementarity of the bioassays

The scores obtained for the six bioassays after exposure to the 16 test water samples as well as for the diatom teratology assessment are presented in Table 5, along with the overall index classes. The test organisms used covered different trophic levels, from decomposers (*A. fischeri*) and primary producers (*P. subcapitata*, *L. minor*, diatoms), to primary consumers (*H. incongruens* and *M. macrocopa*) and, ultimately, secondary consumers (*D. rerio*). The scores obtained for each test organism varied greatly, with certain bioassays indicating high toxicity while others showed no response. The bacterial and microalgal bioassays were the least sensitive because only one of the water samples was characterised as being of moderate toxicity. The fish embryo test was the most sensitive to the types of contamination present in the test waters because it showed a response in almost all samples. Different levels of responses to toxicity were observed in the present study, with only ostracods, duckweed, and diatoms evidencing “high toxicity” for certain samples. This heterogeneity in response sensitivity using multiple organisms was also noted in other

507 bioassay-based assessments (e.g., Persoone et al. 2003; Pandard et al. 2006; Mankiewicz-Boczek et al.
508 2008). Organisms from various trophic levels have also shown different responses to perturbations using
509 biological indices based on assemblage structure and other biological descriptors (e.g., Marzin et al. 2012;
510 Lainé et al. 2014). In fact, this combination of trophic levels allows for a better assessment of the overall
511 contamination of waters by substances with different modes of action as well as biological targets.
512 When contamination was high, such as at H1, most bioassays (five out of seven) highlighted potential
513 toxicity. This was also the case, to a lower extent, at N2 and H2. The advantages of this multi-bioassay
514 approach are more striking in the case of subtle water contamination. In the latter case, the type of
515 organisms affected, or the functions impaired, could provide valuable information to identify the potential
516 nature of contamination. For example, despite the low sensitivity of the *P. subcapitata* bioassay to water
517 toxicity, microalgal growth was significantly enhanced at 10 sites, where higher nutrient loads were found.
518 Analysing this response in light of the response of the other test organisms, two scenarios can be
519 hypothesised. First, growth could have been stimulated by higher nutrient availability at sites with low
520 concentrations of toxicants. This scenario may be valid at sites Y2, G2, G3, G5, G7, and G6, where no
521 toxicity was observed on other plant organisms tested (macrophytes and diatoms). In contrast, and given
522 the high sensitivity of the root re-growth assay to metal contamination (Park et al. 2013), and of diatom
523 teratologies (Morin et al. 2012 Lavoie et al. 2017), a second scenario likely occurred at Y1, Y3, Y4, G1,
524 and G4: the stimulating effects of nutrients on microalgae may have masked the potential toxicity of zinc
525 present in the test waters. Microalgal growth stimulation by nutrients in metal- (Cd/Zn) contaminated sites
526 was also observed in periphytic biofilms (Morin et al. 2008b). The first scenario, however, only discards
527 the presence of plant-targeting substances; indeed, some toxicity was observed in the growth of freshly
528 hatched ostracods and/or in fish embryo survival at G2, G3, G5, and G7. This suggests contamination by
529 compounds specifically affecting the first stages of animal cell development. In contrast, at N2 and H1,
530 moderate to high toxicity was found through diverse bioassays, covering different trophic levels; this may
531 be the consequence of contamination by narcotic toxicants (i.e., non-specific acting) or by a mixture of
532 dissimilarly acting compounds. The results from the present study underscore the significant benefit of
533 using a multi-organism approach allowing for a better integration of water quality testing, which in turn
534 results in a more complete assessment of complex environmental stresses. Moreover, this approach is of

particular significance in the goal to characterise the overall water toxicity of the freshwater ecosystem. Organisms have the major advantage of reflecting the toxic potential of waters, which cannot be sufficiently highlighted by chemical measurements alone due to the limitations stated below.

3.3.2. Toxicity scores versus water chemical analyses

Based solely on available water chemistry data, N2, N3, H1, and H2 are the sampling sites suggesting more severely degraded conditions, mostly due to high metal concentrations, total nitrogen, coliforms, BOD, and conductivity. No toxic contamination (metals or organic compounds) was found at the other sampling sites based on water chemistry (Pandey et al. 2018). The results from the present multi-bioassay study identified H1 as "highly toxic" to living organisms (class D). N2, H2, Y1, and Y3 were classified into the "moderate toxicity" class (C), and N3 into the "slight toxicity" class (B). Some sites, although exhibiting some impact (e.g., two assays indicating some toxicity at Y4, G2, and G3), fell into the "no toxicity" category. All samples from the Geum River, as well as N1, Y2, and Y4, were considered not toxic to aquatic life. The fact that additional sampling sites suggested some toxicity when using biological descriptors compared to chemistry alone underscores that (i) chemical analyses cannot be exhaustive (some compounds with toxicity may not have been targeted), (ii) quantification limits may be higher than the toxic concentrations, and (iii) mixture effects are not considered. In fact, toxicity often results from the effects of a cocktail of compounds and their degradation products (Kim Tiam et al. 2016), which renders the task of water quality assessment even more challenging due to additive, synergistic, and antagonistic interactions.

3.3.3. Scoring approach: preliminary considerations

Classification based on the "harmfulness potential" of natural waters is relatively uncommon as bioassays are generally used to derive EC_{50} for particular chemicals or for wastewaters. The most similar approach found for comparison is the scoring system presented by Persoone et al. (2003) and also used by Mankiewicz-Boczek et al. (2008), where the scores obtained from the bioassays are expressed as an overall degree of hazard or toxicity. Although the toxicity classes differ as well as the overall method for indexing hazardous potential of the sample, the approach proposed in the present study is generally comparable to that of Persoone et al. (2003). As mentioned in the methods, the criteria used to establish the toxicity categories and scores might be subjected to change in the future as the use of this approach becomes more

popular and experience is gained. For example, the criteria were set using similar boundaries between biotests for simplicity, but this may be refined as similar studies multiply. The final overall toxicity classes may also have to be refined in the future, but as of now, this classification system seems to be appropriate to adequately qualify the tested waters. This type of investigation is still in its infancy and it is presently difficult to identify the most sensitive and reliable test organisms. As an example, bioluminescence does not seem to be an appropriate bioassay based on the results from this study. However, this does not necessarily mean that bacteria-based bioassays are inefficient. The potential toxic effects of natural waters on bioluminescence need to be tested using a larger array of water samples covering a range of different types of contamination. This statement is also valid for the other organisms tested. With experience, it should become easier to select the suite of organisms that is most appropriate for the type of water tested, based on the nature of the suspected contamination (when this applies). The main objective of this preliminary investigation was to lay the foundation for this biomonitoring approach in South Korean rivers, and the results are promising in the way that the test organisms used complemented each other and supplemented traditional chemistry measurements.

Conclusion

South Korean rivers, as with many rivers worldwide, receive a great deal of various chemicals from urban, industrial, and agricultural activities, which makes the measurement of all substances practically impossible (e.g., Vörösmarty et al. 2010). Moreover, new chemical compounds are constantly detected in surface waters, rendering the task of water toxicity assessment even more challenging and costly. The toxicity index proposed in this study is a valuable tool for preliminary screening of water contamination as an alternative or in addition to traditional chemistry-based assessments. Based on the 16 water samples collected, the various bioassays tested provided complementary information to chemical analyses by flagging additional sites as being potentially degraded. The variability in the response of each organism to water exposure underscores the need for testing toxicity based on a multi-organism approach, which can possibly highlight the kind of toxic substance that is most responsible for water degradation. As it would be utopic to recommend using all organisms in routine monitoring, suggesting to at least test water samples using representative organisms from different trophic levels seems appropriate at this time.

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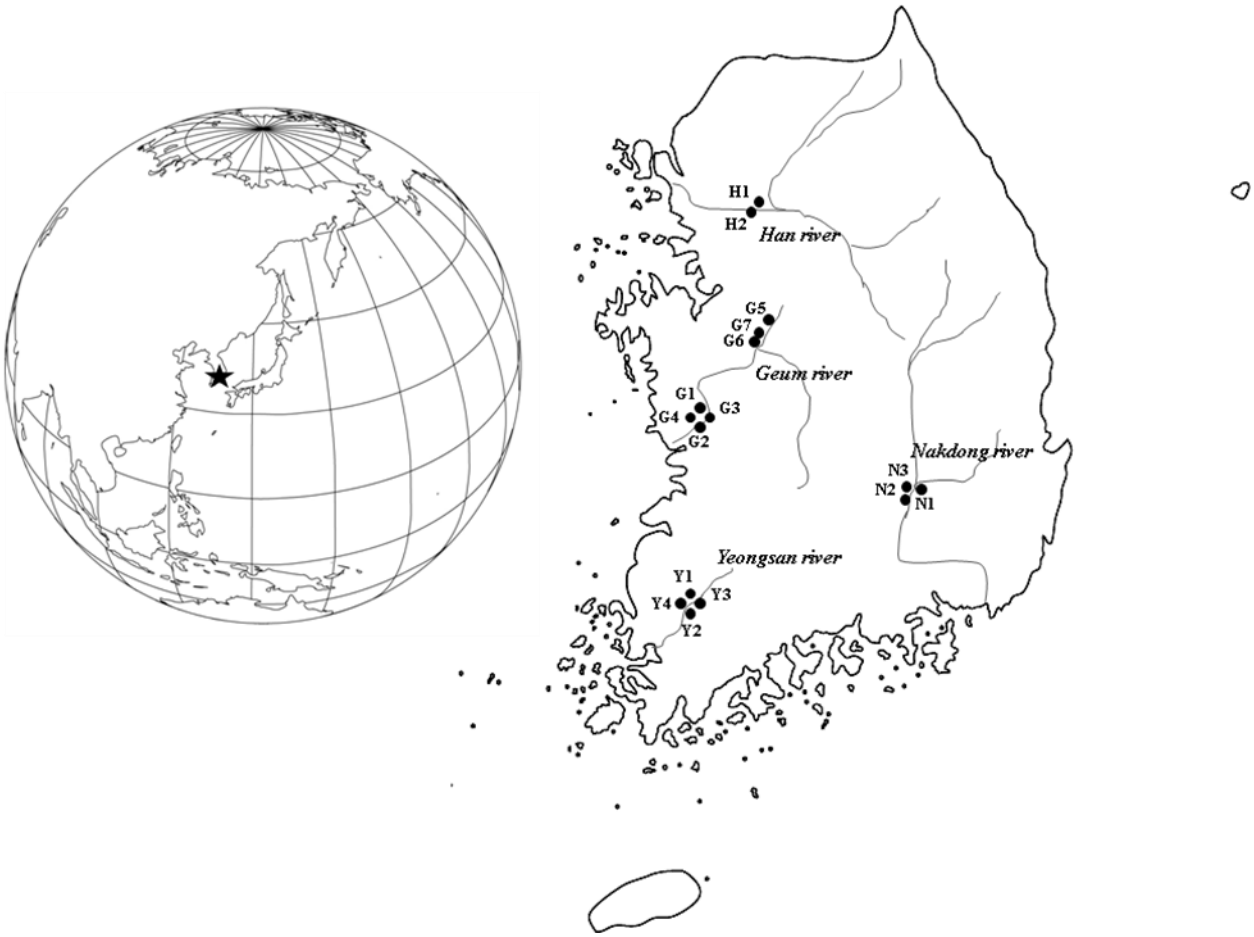

Fig. 1. Site distribution along four major rivers of South Korea.

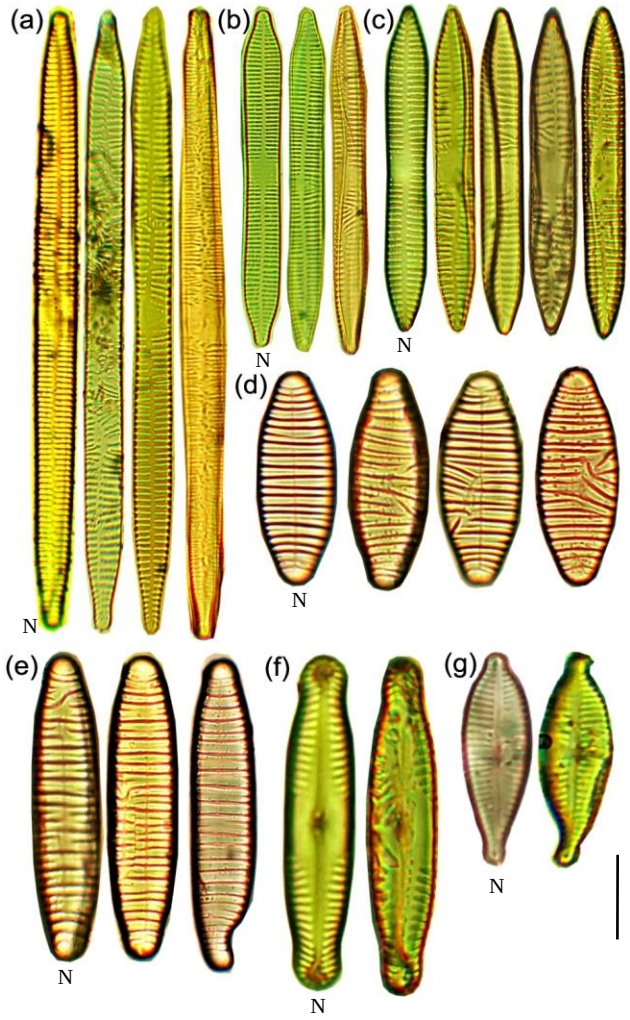

Fig. 2. Normal and deformed frustules of the diatoms *Ulnaria ulna* (a-c), *Diatoma vulgaris*, (d,e), *Pinnularia biceps* (f) and *Gomphonema parvulum* (g) examined from the16 sites. 'N' denotes normal frustules.

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Table 1. Toxicity classes and attributed scores for each biological descriptor and endpoints.

	Toxicity class and score		
	No toxicity (%)	Moderate toxicity (%)	High toxicity (%)
Bioassay/biological descriptor	Score = 0	Score = 1	Score = 2
% inhibition in bioluminescence of *Aliivibrio fischeri*	[0-20]	[20-50]	>50
% growth inhibition in *Pseudokirchneriella subcapitata*	[0-20]	[20-50]	>50
% mortality in *Heterocypris incongruens*	[0-20]	[20-50]	>50
% growth length inhibition in *Heterocypris incongruens*	[0-20]	[20-50]	>50
% mortality in *Moina macrocopa*	[0-20]	[20-50]	>50
% growth length inhibition in *Moina macrocopa*	[0-20]	[20-50]	>50
% inhibition in reproduction of *Moina macrocopa*	[0-20]	[20-50]	>50
% mortality in *Danio rerio* embryos	[0-10]	[10-50]	>50
% inhibition in root elongation of *Lemna minor*	[0-20]	[20-50]	>50
% deformities in diatoms	<0.5	0.5-1	>1

Table 2. Proposed cross-tabulation approach for the calculation of an overall toxicity category (index) based on (1) the total score obtained by adding individual scores from each test (column), and (2) the number of tests that received a score = 2 suggesting “high toxicity” (rows). Because the number of tests performed may change depending on the project, the values used for this tabulation are reported as percentages.

		Total score (%)				
		[0-20]	[20-40]	[40-60]	[60-80]	[80-100]
% of tests with "high toxicity" score	0					
	[0-20]					
	[20-40]					
	[40-60]					
	[60-80]					

Overall toxicity category (index)
A = no toxicity
B = slightly toxic
C = moderately toxic
D = toxic
E = very toxic

Table 3. Physico-chemical measurements averaged (±SD) per river (Nakdong, 3 sites; Yeongsan, 4 sites; Geum, 7 sites; Han, 2 sites).

Parameter \ River	Nakdong	Yeongsan	Geum	Han
pH	8.2 ± 0.4	8.2 ± 0.4	8.0 ± 0.2	8.0 ± 0.4
Temperature (°C)	25.7 ± 1.4	22.2 ± 0.7	22.8 ± 0.4	26.2 ± 2.1
Conductivity (µS/cm)	1982 ± 932	251 ± 44	458 ± 45	3293 ± 1010
Dissolved Oxygen (mg/L)	11.5 ± 0.0	11.6 ± 0.2	11.4 ± 0.1	10.6 ± 0.4
Total Nitrogen (mg/L)	9.0 ± 1.9	3.9 ± 0.8	7.6 ± 1.6	12.0 ± 3.0
Total Phosphorus (mg/L)	0.07 ± 0.03	0.09 ± 0.03	0.21 ± 0.04	0.10 ± 0.00
TDS (mg/L)	1421 ± 660	223 ± 25	284 ± 51	1515 ± 873
BOD_5 (mg/L)	1.8 ± 0.2	1.7 ± 0.2	1.8 ± 0.4	6.3 ± 4.1
Coliforms (CFU/mL)	7 ± 3	26 ± 18	16 ± 6	152 ± 124
Cu (µg/L)	39 ± 13*	nd	nd†	43‡
Ni (µg/L)	57 ± 16*	nd	nd	19 ± 4
Zn (µg/L)	42 ± 21	66 ± 6	71 ± 6	370 ± 99

*: Not detected in N1

†: Except in G7: 4µg/L

‡: Not detected in H1

Table 4. Results of the six bioassays and the diatom teratology assessment. Inhibition is calculated in comparison to the controls (positive values: inhibition, negative values: stimulation), while mortality rates (number of dead individuals scaled to the initial size of the population) and diatom teratologies are given as raw percentages. Average response (Avg), confidence intervals (CI, 95%), significant differences with the controls (p-values after post-hoc Tukey test; * <0.05, ** <0.01, *** <0.001) and ANOVA outputs are given for the tests that were replicated. Bold indicates toxicity. See tables S1 and S2 for complete information about sites.

Rivers	Bioassay / Sites	Bacteria	Microalgae	Ostracods		Cladocera	Fish	Macrophytes	Diatoms
		Inhibition of bioluminescence	Inhibition of average growth rate	Mortality	Growth inhibition	Inhibition of reproduction	Embryo mortality	Inhibition of root re-growth	Teratologies
		Avg (CI)	Avg (CI)	Avg (CI)	Avg (CI)	Avg (CI)		Avg (CI)	Avg (CI)
Control		1 (2)	0 (1)	35 (17)	0 (15)	0 (7)	5	0 (22)	0.3 (0.1)
Nakdong	N1	9 (18)	11 (5)**	32 (13)	-31 (23)	3 (7)	5	8 (29)	0.8 (0.7)
	N2	**28 (3)*** **	-7 (3)	27 (20)	-15 (33)	6 (13)	**15**	**40 (9)*** **	**1.5 (0.2)*** **
	N3	9 (4)	-1 (3)	37 (17)	22 (24)	-31 (7)	**10**	**24 (24)***	0.2 (0.2)
Yeongsan	Y1	0 (0)	-15 (12)***	42 (20)	**52 (19)** **	-11 (5)	**10**	2 (14)	**2.1 (0.4)*** **
	Y2	-6 (1)	-28 (5)***	43 (18)	18 (26)	-28 (9)	**10**	11 (14)	0.2 (0.0)
	Y3	13 (6)	-13 (10)	45 (15)	20 (22)	1 (4)	**10**	9 (11)	**3.1 (0.7)*** **
	Y4	13 (13)	-32 (1)***	30 (16)	0 (22)	**44 (8)** **	5	**49 (12)*** **	0.2 (0.0)
Geum	G1	-2 (3)	-21 (4)***	20 (13)	-11 (11)	-60 (6)***	5	**41 (7)*** **	0.2 (0.0)
	G2	16 (3)*	-26 (0)***	40 (18)	**44 (19)***	-25 (8)	**15**	8 (10)	0.4 (0.0)
	G3	-6 (21)	-17 (8)***	56 (24)	**45 (37)***	-36 (5)*	**40**	-1 (20)	0.1 (0.0)
	G4	-11 (4)	-37 (2)***	25 (13)	-13 (17)	-28 (7)	5	-5 (19)	0.3 (0.0)
	G5	-5 (12)	-23 (6)***	10 (7)*	-35 (13)	-20 (6)	**25**	10 (3)	0.3 (0.0)
	G6	-6 (8)	-9 (8)	5 (7)**	-37 (5)	-33 (7)*	0	-1 (16)	0.4 (0.1)

	G7	2 (9)	-34 (3)***	23 (12)	4 (20)	-44 (9)**	**10**	17 (6)	0.5 (0.1)
Han	H1	11 (24)	**30 (8)*** **	**100 (0)*** **	/	-77 (5)***	**20**	**52 (11)*** **	**4.9 (0.6)*** **
	H2	-19 (15)*	7 (3)	15 (15)	-33 (18)	-22 (10)	**10**	**62 (10)*** **	**5.9 (0.9)*** **
ANOVA		**F=4.18, *p*<0.0001**	**F=37.38, *p*<0.0001**	**F=6.02, *p*<0.0001**	**F=7.21, *p*<0.0001**	**F=9.10, *p*<0.0001**	/	**F=7.20, *p*<0.0001**	**F=85.58, p<0.0001**

Table 5. Scores obtained for the six bioassays and the diatom teratology assessment after exposure to the 16 water samples tested, and final toxicity class (index). See table 2 for more detail on overall toxicity classes and tables S1 and S2 for complete information about sites.

Rivers	Bioassay / Sites	Bacteria Bioluminescence	Microalgae Growth	Ostracods Mortality	Cladocera Mortality	Fish Embryo mortality	Macrophytes Root regrowth	Diatoms Teratologies	Total score	Number of tests with score = 2	Overall toxicity class
Nakdong	Ayang (N1)	0	0	0	0	0	0	1	1 (7%)	0 (0%)	A
	Dalseo(N2)	1	0	0	0	1	1	2	5 (36%)	1 (14%)	C
	Keumho(N3)	0	0	0	0	1	1	1	3 (21%)	0 (0%)	B
Yeongsan	Gw.gong(Y1)	0	0	2	0	1	0	2	5 (36%)	2 (29%)	C
	Gwangju-1(Y2)	0	0	0	0	1	0	0	1 (7%)	0 (0%)	A
	GJ-2(Y3)	0	0	0	0	1	0	2	3 (21%)	1 (14%)	C
	PD-1(Y4)	0	0	0	1	0	1	0	2 (14%)	0 (0%)	A
Geum	Ohryang(G1)	0	0	0	0	0	1	0	1 (7%)	0 (0%)	A
	Bangchuk(G2)	0	0	1	0	1	0	0	2 (14%)	0 (0%)	A
	Masan(G3)	0	0	1	0	1	0	0	2 (14%)	0 (0%)	A
	Sucheol(G4)	0	0	0	0	0	0	0	0 (0%)	0 (0%)	A
	Miho-5(G5)	0	0	0	0	1	0	0	1 (7%)	0 (0%)	A
	Miho-8(G6)	0	0	0	0	0	0	0	0 (0%)	0 (0%)	A
	Miho-7(G7)	0	0	0	0	1	0	0	1 (7%)	0 (0%)	A
Han	Soyo(H1)	0	1	2	0	1	2	2	8 (57%)	3 (43%)	D
	Daejeon(H2)	0	0	0	0	1	2	2	5 (36%)	2 (29%)	C