Toxicity prediction: An application of alternative testing and computational toxicology in contaminated groundwater sites in Taiwan

by Trias Mahmudiono

Research article

Toxicity prediction: An application of alternative testing and computational toxicology in contaminated groundwater sites in Taiwan

Rachelle D. Arcega [a,1], Rong-Jane Chen [a,1], Pei-Shan Chih [a], Yi-Hsuan Huang [a], Wei-Hsiang Chang [a,b], Ting-Khai Kong [a], Ching-Chang Lee [b,c], Trias Mahmudiono [d], Chun-Chih Tsui [e], Wen-Che Hou [f], Hsin-Ta Hsueh [g], Hsiu-Ling Chen [a,d,*]

[a] Department of Food Safety/Hygiene and Risk Management, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
[b] Department of Environmental Trace Toxic Substances Research Center, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
[c] Department of Environmental and Occupational Health, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan
[d] Department of Nutrition, Faculty of Public Health, Universitas Airlangga, Surabaya 60115, Indonesia
[e] Toxic and Chemical Substances Bureau, Environmental Protection Administration Executive Yuan, Taipei City,106, Taiwan
[f] Department of Environmental Engineering, National Cheng Kung University, Tainan City,701, Taiwan
[g] Sustainable Environment Research Laboratories, National Cheng Kung University, Tainan City,701, Taiwan

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ABSTRACT

Groundwater contamination remains a global threat due to its toxic effects to humans and the environment. The remediation of contaminated groundwater sites can be costly, thus, identifying the priority areas of concern is important to reduce money spent on resources. In this study, we aimed to identify and rank the priority groundwater sites in a contaminated petrochemical district by combining alternative, non-animal approaches – chemical analysis, cell-based high throughput screening (HTS), and Toxicological Priority Index (ToxPi) computational toxicology tool. Groundwater samples collected from ten different sites in a contaminated district showed pollutant levels below the detection limit, however, hepatotoxic bioactivity was demonstrated in human hepatoma HepaRG cells. Integrating the pollutants information (i.e., pollutant characteristics and concentration data) with the bioactivity data of the groundwater samples, an evidence-based ranking of the groundwater sites for future remediation was established using ToxPi analysis. The currently presented combinatorial approach of screening groundwater sites for remediation purposes can further be refined by including relevant parameters, which can boost the utility of this approach for groundwater screening and future remediation.

1. Introduction

Groundwater makes up 97% of our freshwater and is a vital source of drinking water for the global population (Schmoll et al., 2006). In Taiwan, groundwater is a major water resource in the southwestern region that is used for agricultural, industrial, and domestic purposes (Liu et al., 2004). However, the increase in industrial development has aggravated the use of chemicals involved in complex manufacturing and petrochemical industries such as aromatic compounds, monomers (e.g., acetaldehyde), and solvents (e.g., acetone) (Hu and Chen, 2012). As a consequence of their use in several industries, many of these chemicals find their way to groundwater systems causing groundwater contamination worldwide (Ali et al., 2016; Chao et al., 2020; Lin et al., 2015;

Shankar and Shanker, 2014; Vu et al., 2017). In Taiwan, contamination of groundwater has been majorly linked to the industries, sites containing storage tanks, gas stations, farmland, and illegal dumping sites (Yeh, 2021). These anthropogenic activities can result in underground penetration of the chemicals, which may seep through nearby aquifers and threaten groundwater quality and human health (Li et al., 2021; Yeh, 2021). Specifically, toxic compounds in the groundwater can enter the human body via the food chain (e.g., via drinking of contaminated water or ingestion of crops irrigated with contaminated groundwater) and implicate hazardous health effects (Li et al., 2021). For this reason, agencies across the globe such as the United States Environmental Protection Agency (USEPA) and the European Commission (EC) have developed groundwater quality standards to protect public health

* Corresponding author. Department of Food Safety/Hygiene and Risk Management, College of Medicine, National Cheng Kung University, Tainan 701, Taiwan.
E-mail address: hsiulinchen@mail.ncku.edu.tw (H.-L. Chen).
[1] These authors contributed equally to the article.

(USEPA, 2009; EC, 2006).

There are over 5500 contaminated soil and groundwater sites in Taiwan to date (Yeh, 2021). According to Article 6 of the Soil and Groundwater Pollution Remediation (SGPR) Act of Taiwan, pollutants in groundwater including polycyclic aromatic hydrocarbons (PAHs), monocyclic aromatic hydrocarbons (MAHs), chlorinated hydrocarbons (CAHs), heavy metals, agricultural chemicals, and others must meet the recommended standard values to secure its sustainable use and to protect human health (Taiwan Ministry of Justice, 2014). Over a decade ago, only about 17% of the groundwater samples collected from remediation sites in various cities in Taiwan were contaminated with pollutants including tetrachloroethylene, trichloroethylene, vinyl chloride, and so on (Fan et al., 2009). Benzene, toluene, ethylbenzene, and xylene (collectively known as BTEX) were also detectable in groundwater samples in a tank farm area and in a naphtha-cracking plant in southern and central Taiwan, respectively that have histories of leakage leading to groundwater contamination (Chen et al., 2010; Yang et al., 2019). Moreover, the levels of several CAHs (vinyl chloride, perchloroethylene, and trichloroethylene) in groundwater samples collected from villages located around a polyvinyl chloride factory in Kaohsiung city were found to be higher than the Taiwanese regulatory control standards (Chen and Wu, 2017). However, chemical analyses alone do not provide information about the potential hazards of contaminated environmental matrices and can miss unexpected toxic compounds due to its targeted nature (Logeshwaran et al., 2018; Schroeder et al., 2016). Additionally, the toxicity of the contaminated groundwater needs to be further investigated using conventional animal models to extrapolate the potential toxic effects to humans (Gustavson et al., 2000; Siddiqui et al., 2011; van Leeuwen, 2000). However, with the current 3 R concept of "Replacement, Reduction, and Refinement" to reduce the quantity of animals used in experiments and to protect animal welfare (Tornqvist et al., 2014), there have been numerous efforts to promote alternative methods for assessing pollutant hazards.

In 2007, the US National Research Council (NRC) issued a challenge to transition away from the use of animal models for toxicity testing (NRC, 2007). In response, the National Toxicology Program, the USEPA, and the National Institutes of Health Chemical Genomics Center collaborated and established the Toxicity Testing in the 21st Century (Tox21) program to develop cost-efficient and high-throughput technologies and computational models for prioritizing compounds for further studies and predicting adverse health outcomes in humans (Attene-Ramos et al., 2013; Shukla et al., 2010; Thomas et al., 2018; Tice et al., 2013). High-throughput screening (HTS) capable of testing hundreds of thousands of chemical compounds across numerous bioassays in a variety of *in vitro* models, therefore, become an integral aspect of the Tox21 program (Richard et al., 2016; Shukla et al., 2010; Tice et al., 2013). The cell-based approach has been conducted by previous researchers to investigate the toxicity of environmental water samples including groundwater (Helma et al., 1998; Masood et al., 2021; Pan et al., 2015; Schirmer et al., 2004). Nevertheless, the number of new chemicals that need to be tested and prioritized is rapidly increasing. Hence, computational modelling is introduced to complement the HTS experiments (Shukla et al., 2010). Such models can predict the toxicity of a compound by integrating the information generated from HTS *in vitro* assays or from existing high throughput toxicity databases such as the USEPA's Toxicity Forecaster (ToxCast) and Ecotoxicology Knowledgebase (ECOTOX) (Rogers et al., 2021; Zhu et al., 2014); thus decreasing the time spent for testing and expediting the prioritization of chemicals (Knudsen et al., 2013; Zhu et al., 2014). The Toxicological Priority Index (ToxPi) is a software program that integrates different readily available information from such databases into a dimensionless index score, known as ToxPi score, visualized as slices in a pie chart (Reif et al., 2010; Rogers et al., 2021). Each slice corresponds to a single component (i.e., toxicity data) wherein a higher toxicity score (characterized by a wider and a bigger slice) suggests a greater potential for toxicity relative to the other compounds in a dataset (Rogers et al.,

2021). In recent years, the ToxPi approach has been utilized to rank potentially toxic chemicals in water systems for more in-depth investigations (Danforth et al., 2020; Hedgespeth et al., 2019) and prioritize environmental sites of concern for future environmental management (Chen et al., 2021a, 2021b).

Environmental monitoring of pollutants and remediation techniques are costly. Therefore, in this study, we collected groundwater samples from a targeted petrochemical district in Taiwan, analyzed the samples for chemical contamination, and performed several HTS assays to evaluate their *in vitro* toxicity. Finally, the results of chemical analysis and *in vitro* toxicity testing were integrated into ToxPi to predict the contaminated groundwater sites that should be prioritized for remediation. With this combined alternative testing and computational toxicology approach, the cost for remediation can be reduced by allotting it to priority sites, thus, consequently impeding the potential health impacts of contaminated groundwater to the residents of the surrounding communities.

2. Materials and methods

2.1. Chemicals

Hydrochloric acid (HCl), sodium hydroxide (NaOH), bovine calf serum (BCS), neutral red dye (product no. 861251), William's E medium (product no. SLCH0504), hydrocortisone, beta-naphthoflavone (β-Na, product no. N3633), CITCO (product no. C6240), rifampicin (RIFA, product no. R3510), doxorubicin (product no. D1515), and cytochalasin B1 (product no. C6762) were purchased from Sigma-Aldrich (Missouri, US). The JC-1 mitochondrial membrane potential (MMP) assay kit (product no. ab113850, containing JC-1 dye and carbonyl cyanide 4-(tri-fluoromethoxy) phenylhydrazone [FCCP]) was procured from Abcam (Cambridge, UK). Hoechst 33258 dye (product no. 16756) and staurosporine (product no. 81590) were from Cayman Chemical Company (Michigan, US). MitoSOX™ Red (product no. M36008) and Mito-Tracker™ Green (product no. M7514) dyes were supplied by Thermo Fisher Scientific (Massachusetts, US).

2.2. Groundwater samples

The groundwater samples were collected from 10 different sites in a declared contaminated petrochemical district in Yunlin County, Taiwan, and were designated as samples S1, S2, S3, S4, S5, S6, S7, S8, S9, and S10, respectively. The sampling strategy was carried out according to the Taiwan EPA (TEPA)'s Monitoring Well Groundwater Sampling Method (NIEA W103.56 B) (TEPA, 2021). The concentration of the groundwater pollutants including PAHs, MAHs, CAHs, and others (as required in the SGPR Act of Taiwan) in the samples were analyzed in accordance with the Detection of Volatile Organic Compounds in Water by Purge-and-Trap/Gas Chromatography Mass Spectrometry Analysis (NIEA W785.57 B) method of TEPA (TEPA, 2020). Several water quality parameters including water temperature, conductivity, dissolved oxygen, and turbidity were also measured (Table S1). The samples were stored at 4 °C until use for HTS.

2.3. Assessment of groundwater toxicity by HTS

2.3.1. Sample preparation

The groundwater samples were filtered using a 0.45 μm polytetra-fluoroethylene (PTFE) membrane filter and mixed with the appropriate assay medium (10X concentration) at a 9:1 ratio accordingly. The pH of the mixtures was adjusted to 7.2 ± 0.2 using HCl or NaOH. The mixtures were filtered using a 0.22 μm PTFE membrane filter and stored at 4 °C until use. Before performing the following assays, the groundwater mixtures were diluted 2X, 4X, and 8X (for 3T3 neutral red uptake [NRU], cell viability, and cytochrome P450 [CYP] activity assays) and 2X and 4X (for MMP, mitochondrial superoxide, cell cycle, and

apoptosis assays) with the appropriate assay medium.

2.3.2. 3T3 NRU cytotoxicity assay

The 3T3 NRU cytotoxicity assay was performed according to the database service on alternative methods to animal experimentation (DB-ALM)'s protocol No. 139 (EU, 2007). Briefly, Balb/c 3T3 cells (Bio-resource Collection and Research Center, Hsinchu, Taiwan) were seeded in 96-well plates at a seeding density of 3×10^3 in Dulbecco's modified Eagle medium (Gibco™, Massachusetts, US) supplemented with 10% BCS 24 h prior to treatment. The cells were treated with the groundwater solutions and incubated for 48 h (37 °C and 5% CO$_2$). After treatment, the groundwater solutions were discarded the cells were incubated with 25 µg mL^{-1} of NR staining solution for 3 h. The NR staining solution was removed after incubation and the wells were washed with PBS. Freshly prepared NR desorption solution (comprising 49% H$_2$O, 50% ethanol, and 1% acetic acid) was added to the wells to dissolve the NR taken up by the cells. The plate was shaken for 30 min and left to stand for 5 min. The absorbance was read at 540 nm with a multimode microplate reader (SpectraMax 340PC384, Molecular Devices, California, US).

2.3.3. CYP activity assay

Cell viability was assayed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test using doxorubicin (at 8 µM, the cell viability was 30%–70%) as the control before performing the CYP activity assay. The method for the CYP activity assay was referred from the DB-ALM protocol No. 194 (EU, 2009). Briefly, HepaRG cells, which were kindly provided by H. Sunny Sun laboratory (Institute of Molecular Medicine, National Cheng Kung University), were seeded in 96-well plates at a density of 6.5×10^4 in William's E medium with 5 µg mL^{-1} insulin (PeproTech, New Jersey, US) and 50 µM hydrocortisone 72 h before the treatment. The cells were treated with the groundwater solutions and incubated for 48 h. The positive controls for analyzing CYP1A2, CYP2B6, and CYP3A4 were β-Na (25 µM), CITCO (1 µM), and RIFA (10 µM), respectively. Finally, the CYP activity was measured according to the manufacturer's protocol.

2.3.4. Adverse outcome pathways

2.3.4.1. MMP assay.
The MMP was measured using a JC-1 MMP assay kit according to the manufacturer's instructions. Briefly, HepaRG cells were seeded in black 96-well plates with clear bottoms. Each well contained 100 µL of cells at a density of 2×10^4. After growing to a suitable density, the cells were treated with the groundwater solutions and incubated for 24 h. FCCP (100 µL) served as the positive control. After incubation, the groundwater solutions were discarded and the wells were washed. Then, 100 µL of PBS containing JC-1 (2 µM) and Hoechst 33258 (4.5 µg mL^{-1}) was added to the wells in the dark and then incubated for 30 min. The wells were again washed and filled with 100 µL of PBS. Finally, images were captured using the 20× magnification objective of ImageXpress® Micro Confocal HTS system and analyzed using MetaXpress software (Molecular Devices, California, US). The Texas Red/Fluorescein isothiocyanate (FITC) fluorescence ratio was used to compare the results of the control and the samples.

2.3.4.2. Mitochondrial superoxide assay.
The mitochondrial superoxide assay was performed following the procedure described in Section 2.4.4.1 with modifications. Doxorubicin (100 µM) was used as the positive control. PBS solution (100 µL) containing MitoSOX™ Red (10 µM), MitoTracker™ Green (100 nM), and Hoechst 33258 (4.5 µg mL^{-1}) was added to the wells in the dark before incubation for 30 min.

2.3.4.3. Cell cycle analysis and apoptosis detection.
The cell cycle and apoptosis were detected following the procedure described in Section 2.4.4.1 with modifications. HepaRG cells were seeded in 96-well plates.

The positive controls for analyzing cell cycle and cell apoptosis were cytochalasin B1 (1 µM) and staurosporine (5 µM) respectively. PBS solution (100 µL) containing Cell Event™ Caspase-3/7 green detection reagent (10 µM, product no. C10427, Invitrogen™, Massachusetts, US) and Hoechst 33258 (4.5 µg mL^{-1}) was added to the cells in the dark before incubation for 30 min. The cell cycle distribution was detected by DAPI fluorescence. The G2/M phase arrest percentage was used to compare the difference between the results of the control and samples. For cell apoptosis, the FITC fluorescence intensity percentage of the control and samples were compared.

2.4. Prioritization of contaminated groundwater sites using ToxPi

2.4.1. Pollutant information and data sources

In addition to the chemical analysis data of the groundwater pollutants (PAHs, MAHs, CAHs, and other pollutants), ToxPi analysis included cytotoxicity, environmental fate, physicochemical properties, and toxicity domains as well (Table 1). The components in each domain, their definitions, data sources (Table 1), and corresponding values (Table S2) have been provided.

2.4.2. Weighting scheme

For the chemical ranking of groundwater pollutants, the environmental fate and physicochemical property domains were each given a weight of 35% while the cytotoxicity and toxicity domains were each

Table 1
The chemical information (domains, slice components, and data sources) of groundwater pollutants considered for ToxPi prioritization.

Domain	Components	Component definition	Data sources
Cytotoxicity	MAD	The median absolute deviation of the "burst" endpoint logAC$_{50}$ values	invitroDB v3.3^a
	MED	The median of the "burst" endpoint logAC$_{50}$ values	
	Cyto_pt	The cytotoxicity point, or the value in "MED" when the number of active "burst" endpoints is at least 5% of the total assay endpoints	
Environmental fate	BCF	Bioconcentration factor	RAIS^b
	K$_{oc}$	Organic carbon-water partition coefficient	
Physicochemical properties	H	Henry's law, unitless	RAIS and TRRP^c
	LogK$_{oa}$	Octanol-air partition coefficient	
	LogK$_{ow}$	Octanol-water partition coefficient	
	Sol	Water solubility, mg/L	
Toxicity	IARC	Standard IARC classification based on existing scientific evidence for carcinogenicity: four points for Group 1, three points for Group 2 A, two points for Group 2 B, one point for Group 3, and no point for unclassified substances	IARC^d
	RfD_oral	Reference dose	RAIS and TRRP
	RfC_inhalation	Reference concentration	RAIS and TRRP

^{a} US EPA ToxCast Database (invitroDB file v3.3), accessible at https://www.epa.gov/chemical-research/exploring-toxcast-data-downloadable-data.
^{b} RAIS (Risk Assessment Information System), accessible at https://rais.ornl.gov/.
^{c} TRRP (Texas Risk Reduction Program), accessible at https://www.tceq.texas.gov/remediation/trrp.
^{d} IARC (International Agency for Research on Cancer), accessible at https://www.iarc.who.int/.

given 15% (Table 2). The weight of the slice components was distributed equally in each domain (Fig. S1A), and the slice components were scaled using the equations provided in Table 2. Meanwhile, the domains for the chemical analysis and HTS profiles were each given an equal weight and the "hit count" and "linear(x)" scaling equations were applied, respectively. In the overall profile (containing chemical analysis and HTS domains), each domain accounted for 50% and the same scaling equations were applied for each domain as in the individual profiles (Table 2).

2.4.3. ToxPi analysis

The ToxPi™ graphical user interface software version 2.3 was used to carry out the prioritization (Marvel et al., 2018) using the general formula as follows:

$$\text{ToxPi score} = \sum_{1}^{A} w_a \times \text{slice}_a + \ldots + \sum_{1}^{n} w_n \times \text{slice}_n$$

where *slice* refers to a component while *w* corresponds to the weight of each slice. First, the groundwater pollutants were ranked on the basis of their chemical information (Figs. S1B–D). The ToxPi scores of the groundwater pollutants were then incorporated to the pollutant concentration data to generate the final chemical analysis ToxPi profile. To obtain the HTS ToxPi profile, the results of the seven HTS assays were used. Finally, integration of the two ToxPi profiles generated the overall ToxPi profile. The ToxPi results were presented as a hierarchical cluster to identify the similarity between samples. Additionally, the numerical ToxPi value in the overall ToxPi profile was used to rank the groundwater samples collected at different sites from lowest to highest priority

Table 2
The weight of the domains and scaling equation of the slice components.

Domain	Weight	Components	Scaling equation
Groundwater pollutant ranking			
Cytotoxicity	15%	MAD	$-\log10(x)+\log(\max(x))$
		MED	$-\log10(x)+\log(\max(x))$
		Cyto_pt	$-\log10(x)+\log(\max(x))$
Environmental fate	35%	BCF	$-\log10(x)+\log(\max(x))$
		K_{oc}	$\log10(x)$
Physicochemical properties	35%	H	$-\log10(x)+\log(\max(x))$
		$LogK_{oa}$	$-\log10(x)+\log(\max(x))$
		$LogK_{ow}$	$-\log10(x)+\log(\max(x))$
		Sol	$\log10(x)$
Toxicity	15%	IARC	$\text{linear}(x)$
		RfD_oral	$-\log10(x)+\log(\max(x))$
		RfC_inhalation	$-\log10(x)+\log(\max(x))$
Chemical analysis ToxPi profile			
PAH	25%	n/a	hit count
CAHs	25%	n/a	hit count
MAHs	25%	n/a	hit count
Others	25%	n/a	hit count
Bioactivity analysis ToxPi profile			
Cell cycle	14.3%	n/a	$\text{linear}(x)$
Apoptosis	14.3%	n/a	$\text{linear}(x)$
CYP activity	14.3%	n/a	$\text{linear}(x)$
Mitochondrial damage	14.3%	n/a	$\text{linear}(x)$
MMP	14.3%	n/a	$\text{linear}(x)$
Liver cytotoxicity	14.3%	n/a	$\text{linear}(x)$
NRU	14.3%	n/a	$\text{linear}(x)$
Overall ToxPi profile			
Chemical analysis	50%	Chemical analysis domains	hit count
Bioactivity analysis	50%	Bioactivity analysis domains	$\text{linear}(x)$

(lowest to highest score) for remediation.

3. Results

3.1. Pollutant levels in the groundwater samples

A total of 38 chemicals including one PAH, seven MAHs, twenty-one CAHs, and nine other pollutants were analyzed (Table S1). The concentration of these pollutants in the 10 groundwater samples were lower than the detection limit, in exception of chloroform in S3 and total petroleum hydrocarbons (TPHs) in S2, S3, S5, S6, S8, and S10. Additionally, the chloroform and TPHs concentrations in those samples were low. All groundwater samples did not exceed the control standards for groundwater pollutants indicated in the SGPR Act of Taiwan.

3.2. Toxicity of the groundwater samples

3.2.1. Cytotoxicity

The viability of Balb/c 3T3 cells after 48 h of exposure to groundwater samples is depicted in Fig. 1. Of the 10 undiluted (i.e., 1X dilution) groundwater samples, two samples (S2 and S9) were cytotoxic (i.e., cell viability was less than 50%). Furthermore, S2 were notably cytotoxic even at 2X dilution, suggesting that attention should be paid to this site. Meanwhile, groundwater samples that were diluted 4X and 8X did not exert any cytotoxic effects (i.e., cell viability was more than 50%). The results indicate that S2 and S9 groundwater samples may cause greater cytotoxicity than those collected from the other sites in this petrochemical district.

3.2.2. Liver cytotoxicity

The viability of hepatic HepaRG cells was assayed by MTT test to investigate the hepatotoxicity of the groundwater samples (Fig. 2A). Particularly, the cell viabilities were less than 20% after exposure to undiluted S2, S5, and S8 samples, indicating that the original groundwater from these sites can cause severe toxicity to liver cells.

To further verify the hepatotoxicity of the groundwater samples, the expression of the CYP enzymes, namely, CYP1A2, CYP2B6, and CYP3A4, in HepaRG cells were investigated (Fig. 2B–D). Relative to the control, S2 (diluted 2X) notably increased the expressions of CYP1A2 (Fig. 2B) and CYP3A4 (Fig. 2D) by 36- and nearly 100-fold, respectively. For CYP2B6, its expression did not change or was inhibited after exposure of the cells to 2X diluted samples (Fig. 2C). However, S2 and S9 (both diluted 4X) enhanced CYP2B6 expression by about 2-fold and was higher compared to the positive control CITCO (Fig. 2C).

3.2.3. Mitochondrial damage

To investigate whether the groundwater samples can induce mitochondrial damage, the change in the MMP and the production of mitochondrial reactive oxygen species, particularly superoxide, in HepaRG cells were assessed. The ratio of Texas Red fluorescence intensity to that of FITC decreased in cells treated with S4, S5, S6, S7, S9, and S10 diluted 4X and in S8 and S9 diluted 2X compared to those in the control group, indicating a disruption in the MMP (i.e., mitochondrial depolarization) (Fig. 3A). By contrast, the fluorescence ratio in S1- and S2-treated cells increased compared to that of the control, suggesting that the cells were highly polarized (Fig. 3A), however, all the samples were not significantly different from the control group.

Furthermore, the level of superoxide was significantly elevated, as reflected by the increase in Texas Red percentage, in cells treated with the groundwater samples (in exception of S1, S3 diluted 4X, and S9 diluted 2X) compared to those in the control and positive control (Doxorubicin) groups (Fig. 3B) suggesting those groundwater samples can induce a large amount of superoxide production, thus, causing mitochondrial damage.
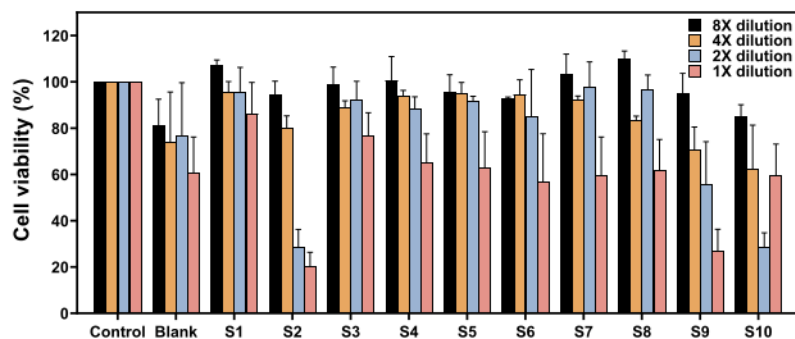
Fig. 1. The cytotoxicity of the groundwater samples assessed by Balb/c 3T3 NRU assay (48 h exposure). 1X dilution represents cells treated with undiluted groundwater-medium mixture. Untreated cells served as the control group.
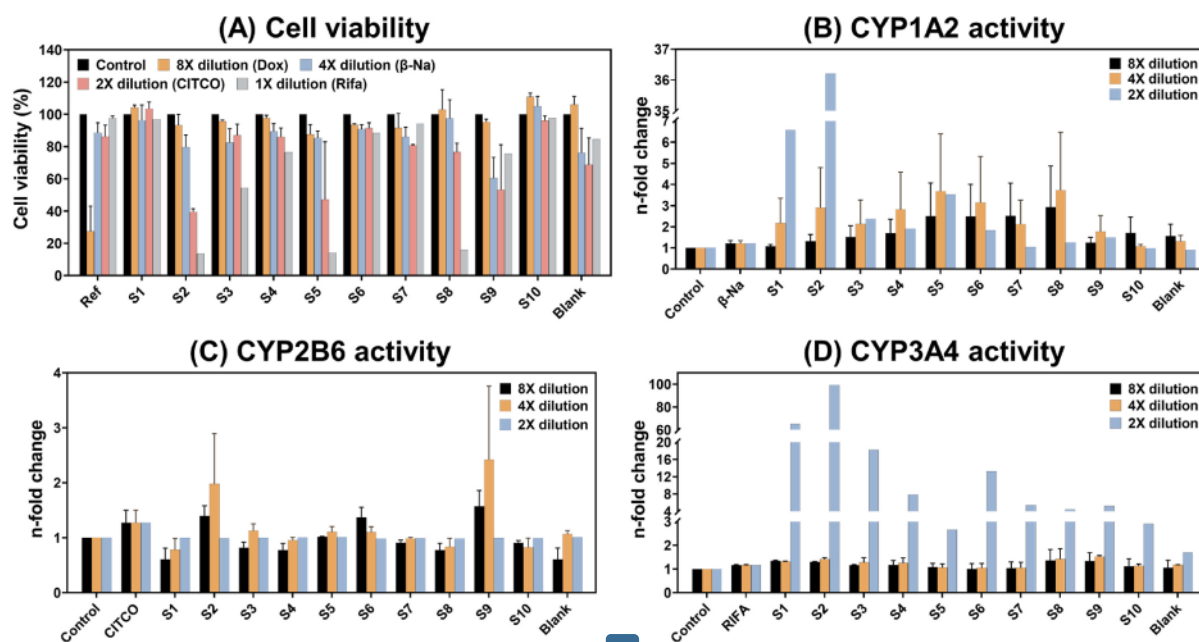

Fig. 2. The liver toxicity of the groundwater samples. The (A) viability and (B–D) CYP1A2, CYP2B6, and CYP3A4 activities of HepaRG cells after 48 h of exposure to groundwater samples. Untreated cells served as the control group.

3.2.4. Cell cycle and apoptosis

The effects of the groundwater samples on the cell cycle distribution in treated HepaRG cells are shown in Fig. 4. Treatment with S9 that was diluted 2X (Fig. 4A) and 4X (Fig. 4B) resulted in a notable accumulation of cells in the G0/G1 and G2/M phases, respectively, subsequently decreasing the percentage of cells in the other phases compared with the control. These results demonstrated that the contents of S9 groundwater sample can induce G0/G1 and G2/M phase arrests in HepaRG cells, which might inhibit cell proliferation and ultimately lead to cell death. For cell apoptosis (Fig. 5), S2, S6, and S9 demonstrated significant increases in the percentage of apoptotic HepaRG cells compared with untreated cells. Particularly, the 2X diluted S2 and S9 greatly induced apoptosis, indicating their superior toxic effects than those of the other samples. The apoptosis induction also significantly increased in the 2X diluted S3, S4, S5, S8 and S10 groups. By contrast, the percentage of apoptotic cells in the S1 and S7 groups were not significantly different from that of the control.

3.2.5. Overall cytotoxicity evaluation

The results of the seven *in vitro* toxicity assays are summarized in Table 3. For the purpose of visualization and integration of data for ToxPi analysis, the result of each assay was recorded as either bioactive (marked with ✓) or inactive (marked with ×) groundwater sample. In the "Overall" column, any groundwater sample which was bioactive for ≥4 of the assays was deemed hepatotoxic (marked with ✓). Based on the overall evaluation, the groundwater samples S2, S5, S6, S8, S9, and S10 were found to induce hepatotoxicity.

3.3. Prioritization of the groundwater samples

3.3.1. Individual ToxPi profiles

The priority of the groundwater samples or sites for remediation was ranked using ToxPi and evaluated based on their score (Fig. 6). S9 (ToxPi score: 0.5), (ToxPi score: 0.258), and S4 (ToxPi score: 0.167) were the priority sampling sites on the basis of the chemical analysis

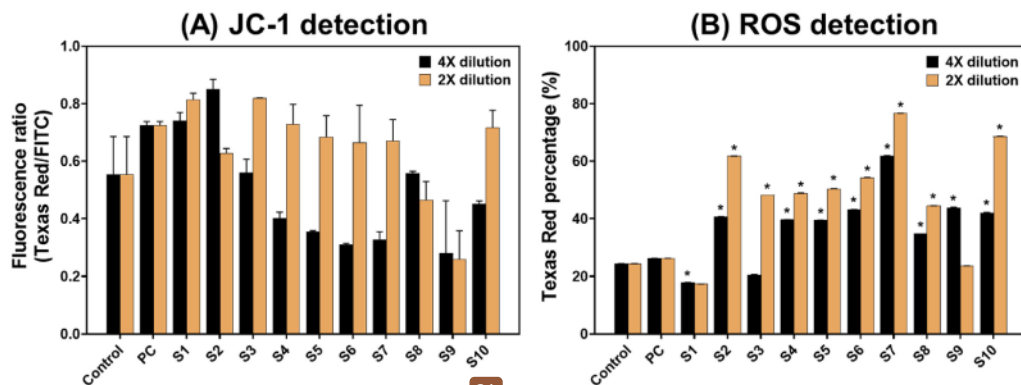**Fig. 3.** The effects of groundwater samples on the mitochondria of HepaRG cells after 48 h of exposure. Detection of (A) MMP disruption and (B) ROS formation ($p < 0.05$ was considered statistically significant denoted by *). Untreated cells served as the control group.
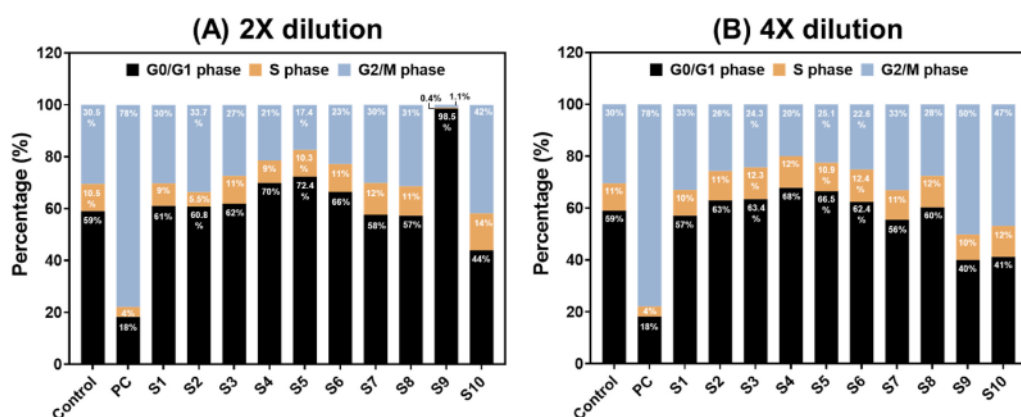

Fig. 4. The cell cycle inhibition analysis of (A) 2X and (B) 4X diluted groundwater samples. Untreated cells served as the control group.
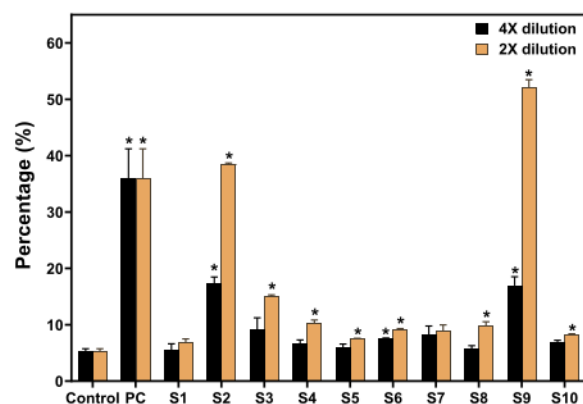

Fig. 5. The cell apoptosis induction analysis ($p < 0.05$ was considered statistically significant denoted by *). Untreated cells served as the control group.

results (Fig. 6A). Meanwhile, S2 (ToxPi score: 0.857) and S5 (ToxPi score: 0.857) were the highest priorities, followed by S6 (ToxPi score: 0.714), S8 (ToxPi score: 0.714), and S9 (ToxPi score: 0.714), when the results of the seven toxicity assays were considered (Fig. 6B). Moreover, hierarchical clustering of the ToxPi profiles revealed the groundwater

samples with similar chemical and bioactivity profiles. As examples, a similar chemical profile was evident in S1–S3, S5, S7, S8, and S10 (Fig. 6A) while S2 and S5 as well as S4 and S10 elicited striking similarities in terms of bioactivity (Fig. 6B).

3.3.2. Overall evaluation

An overall evaluation of the groundwater samples based on the combined biological and chemical analyses information revealed that S9 (ToxPi score: 0.607) was ranked the highest and, thus, should be given the highest priority for remediation (Figs. 6C and 7). S9 was followed by S6 (ToxPi score: 0.486), S2 (ToxPi score: 0.442), S5 (ToxPi score: 0.431), S8 (ToxPi score: 0.376), S4 (ToxPi score: 0.298), S7 (Toxpi score: 0.287), S10 (ToxPi score: 0.214), S3 (ToxPi score: 0.160), and S1 (ToxPi score: 0.077), respectively (Fig. 7).

4. Discussion

Currently, contamination of groundwater with the groundwater pollutants analyzed in this study (Table S1) has been poorly investigated. Despite Yunlin County housing the largest petrochemical industry complex in Taiwan (Jobin, 2021), none of the 36 groundwater pollutants were detected in almost all samples collected from the pollution source area in this study. The probable cause could be due to natural attenuation processes, for instance, the pollutants were biodegraded by *in situ* bacterial populations in the groundwater and formed metabolites (Farhadian et al., 2008).

Table 3
The overall results of the *in vitro* toxicity screening of the groundwater samples.

Sample	NRU cytotoxicity assay	Hepatocytes cell viability	CYP activity	MMP	Mitochondrial damage	Cell cycle arrest	Cell apoptosis	Overall
S1	✗	✗	✗	✗	✗	✗	✗	✗
S2	✓	✓	✓	✓	✗	✗	✓	✓
S3	✓	✓	✓	✗	✗	✗	✗	✗
S4	✗	✗	✗	✓	✓	✗	✓	✗
S5	✗	✓	✓	✓	✓	✗	✓	✓
S6	✗	✗	✓	✓	✓	✗	✓	✓
S7	✗	✗	✓	✓	✓	✗	✗	✗
S8	✗	✓	✓	✓	✓	✗	✓	✓
S9	✓	✓	✓	✓	✓	✓	✓	✓
S10	✓	✗	✗	✓	✓	✗	✓	✓

Fig. 6. The ToxPi profiles of the groundwater samples presented as hierarchical clusters sorted based on (A) chemical analysis, (B) bioactivity analysis, and (C) overall (combined chemical and bioactivity analyses) testing results.
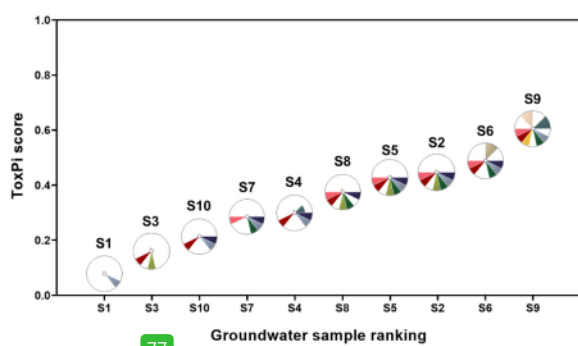

Fig. 7. The final ranking of the groundwater samples based on the overall (combined chemical and bioactivity analyses) ToxPi score. A higher ToxPi score indicates a higher priority for remediation.

An alternative approach through the use of cell-based toxicity assays was performed in this study to act as a screening method for the toxicity testing of contaminated groundwater sites (i.e., samples) while simultaneously reducing the use of animals for laboratory experiments. Our results revealed that some groundwater samples (S2, S5, S6, S8, S9, and S10) demonstrated a potential for hepatotoxicity despite the non-detectable levels of majority of the pollutants (Table 3). The toxicity of the groundwater samples in the current study could be partly contributed, to a minor extent, by those pollutants that were detected above the detection limit such as TPHs and chloroform (Table S1). For instance, TPHs typically found in gasoline can cause severe intoxication at small doses (Kuppusamy et al., 2020). Another possible reason could be that the targeted groundwater pollutants did not induce the toxicity but rather the other contaminants that may also be present in the groundwater samples (e.g., metabolites or other toxic compounds not investigated in this study) that may have additional biological effects. As an example, groundwater in Yunlin has been documented to contain high concentrations of arsenic over the past years (Liao et al., 2011; Liu et al., 2006), which is a principal groundwater pollutant that can induce hepatotoxicity and is associated with liver disease (Renu et al., 2021; Shankar and Shanker, 2014; Zhang et al., 2020). Additionally, metabolites of the compounds investigated that can possibly arose from biodegradation with unknown toxicological profiles may have also contributed to the displayed toxicity of the groundwater samples (Logeshwaran et al., 2018; McGuire et al., 2018). Examples of metabolites that may be found in groundwater are catechol related to benzene degradation, which has been found to be more toxic than benzene itself (Boyd et al., 1997) as well as tert-butyl formate and tert-butyl alcohol related to methyl tert-butyl ether degradation (Rosell et al., 2003). Thus, remediation of contaminated groundwater is of immediate concern due

to the possible toxic effects of such areas on human health.

Several studies have emphasized the necessity to combine chemical analyses with toxicity data for an efficient assessment and remediation of contaminated sites (Baderna et al., 2011; Gustavson et al., 2000; Müller et al., 2018). Therefore, this combined approach was performed in the current study to predict the groundwater sites that require immediate attention in a petrochemical district in Yunlin, Taiwan by integrating the chemical concentration and *in vitro* toxicity data of groundwater samples via the ToxPi tool. Currently, ToxPi is mostly applied for chemical prioritization using data available from libraries or databases (Chen et al., 2020; Danforth et al., 2020; Reif et al., 2013; Tilley et al., 2017) and for prioritizing of pollutants or contaminants in the environment (Danforth et al., 2020; Rogers et al., 2021; Tilley et al., 2017) and in food (dos Santos and Nardocci, 2019; Luo and Wu, 2021). Meanwhile, its use for the identification and prioritization of real environmental samples like the groundwater samples in the current study has emerged recently (Chen et al., 2021a, 2021b). For instance, Chen et al. (2021b) demonstrated the utilization of ToxPi in identifying the possible areas of concern in a post-flooded residential area using *in vitro* bioactivity and chemical concentration data of surface soil samples. Interestingly, the results of the current study revealed that the variety of information (e.g., bioactivity data, pollutant concentration data, or combined data) inputted to ToxPi analysis affected the results of the ranking, that is, the order of the priority groundwater sites (i.e., samples) were different between bioactivity and chemical analyses-based rankings (Figs. 6 and 7). One reason might be because the pollutant concentrations were below the detection limit, thus, the true concentration of most pollutants in each sample is unknown. Second, the pollutant composition may be different between samples and whether they contain other types of pollutants is also unknown. Therefore, the inclusion of multiple and additional information about a chemical or environmental sample in the ranking process can increase the risk-based transparency of the ToxPi score and can benefit the post-management strategies for specific targets or objectives (Danforth et al., 2020; Tilley et al., 2017). For environmental management, the important parameters that can be taken into consideration in the ranking system majorly include environmental characteristics (e.g., water quality indicators such as pH, conductivity, biological and chemical oxygen demands, color, or total organic carbon content, pollutant concentration data) along with toxicity effects (*in vitro*, *in vivo*, or predicted human toxic effects of the individual pollutants and of the sample itself). Overall, our results provided additional evidence on the utility of ToxPi to identify and rank real environmental samples, those with or without prior knowledge of contamination and toxic potential, using pollutant concentration and bioactivity data for future environmental management, especially for groundwater.

This study has some limitations. First, the chemical analysis data focused on the targeted analysis of several known groundwater pollutants, which underestimates the possibility of the presence of other chemical contaminants. Given that groundwater may be contaminated due to leaching of various chemicals from different sources, it is recommended that future studies should consider additional chemicals. Second, the *in vitro* assays only considered the bioactivity of the groundwater samples in liver cells. Contaminated groundwater is a complex mixture of chemicals and different chemicals have different primary or secondary target organs. Therefore, future studies should consider different kinds of cells in toxicity testing to determine whether the groundwater samples elicit biological responses and toxic effects in other organs or systems.

5. Conclusions

In this study, a combination of chemical analysis, bioassays, and computational toxicology approaches was performed to provide a priority ranking of groundwater sites in a known contaminated petrochemical district in Taiwan. Although none of the targeted groundwater pollutants were detected in almost all of the samples after chemical analysis, the results of the *in vitro* toxicity tests demonstrated that the groundwater collected from certain sites displayed a potential for hepatotoxicity, thus, indicating the need for follow-up investigations. By integration of the chemical analysis and *in vitro* toxicity data in the ToxPi tool, the groundwater sites were ranked and the priority sites of concern were identified. However, further studies are recommended to validate our current findings. Nonetheless, the current results can aid the responsible parties in allocating resources for the subsequent remediation of the studied groundwater sites.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2022.116982.

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