2022 年臺灣國際科學展覽會
優勝作品專輯

作品編號　**180015**

參展科別　地球與環境科學

作品名稱　**The Use of Brine Shrimp to Test for Water Pollutants**

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關鍵詞　**Brine shrimp、organic pollutants、heavy metals**

作者照片

Abstract

The use of brine shrimp nauplii to test for the overall toxicity of sediment samples is proposed. Brine shrimp nauplii were cultured with different concentrations of heavy metals, including chromium (III), copper (II), nickel, lead and zinc, and organic pollutants, including triclosan, oxybenzone, octinoxate and bisphenol A. The brine shrimp nauplii were observed under a dissection microscope to determine the death rate. Results showed that brine shrimp nauplii are more sensitive to copper, cadmium, bisphenol A and oxybenzone. The LC_{50} (24h) are 55.5, 24.9, 5.6 and 2.7 ppm respectively. Zinc is likely to have synergistic toxic effect with nickel or lead. The synergistic toxic effects of other heavy metals and organic pollutants should be confirmed with further investigations.

Brine shrimp nauplii were treated with extracts from sediment samples collected from three sites of the oyster culture zone of the Deep Bay, namely Pak Nei, Sha Kiu Tsuen and Hang Hau Tsuen. The sediment samples were extracted with neutral sodium acetate to dissolve the exchangeable heavy metal ions and some organic pollutants. The death rate of brine shrimp nauplii treated with the sediment extract of Hang Hau Tsuen was similar to 1 ppm PBA. It was also about 10 to 20% higher than that of the other two sites (Pak Nei and Sha Kiu Tsuen). Since Hang Hau Tsuen is closer to the residential area and Lau Fau Shan Seafood Market than the other two sites, its sediment sample is likely to have a higher level of environmental pollutants. The results suggest that brine shrimp nauplii may be used as a biomarker to monitor the environmental changes in the overall level of pollutants in sediment samples.

1. Introduction

Oyster culture is one of the global aquaculture productions for more than two millennia and 86% of global oysters by mass was produced in China (Botta et al., 2020). The Pearl River Delta is one of the important oyster culture regions in China for more than 1000 years and it is the second largest contribution to China's total in 2018 (Peng et al., 2021). Deep Bay is located near the Pearl River Delta and it is a well-known oyster culture zone in Hong Kong, as shown in **Figure 1**. Lau Fau Shan Seafood Market is one of the major markets of oyster products, such as oyster meat, oyster sauce and dried oyster.

Oyster culture industry in Deep Bay was started around 200 to 700 years ago and it is still an important local economic activity (Morton and Wong, 1975). According to the information provided by the Agriculture, Fisheries and Conservation Department of the Hong Kong SAR Government, oyster production in 2020 was about 119 tonnes (meat only) valued at 56 million TWD. But according to our interview with local oyster farmers, most of the oyster meat were sold to the Mainland China and therefore the economic revenue should be much higher than this.

The oyster culture zone is located between Pak Nei and Sha Kiu Tsuen. Oysters in Deep Bay are either cultured in oyster posts (**Figure 2**) or oyster rafts (**Figure 3**). Before the 1960s, most oyster farmers used concrete plates or posts to culture oyster around the lower tidal zone. After 1960s, oyster raft culture method was introduced by China oyster farmers and it is now the major culture method in the Deep Bay. Local oyster farmers estimated that there are about 8000 oyster rafts in the Deep Bay.

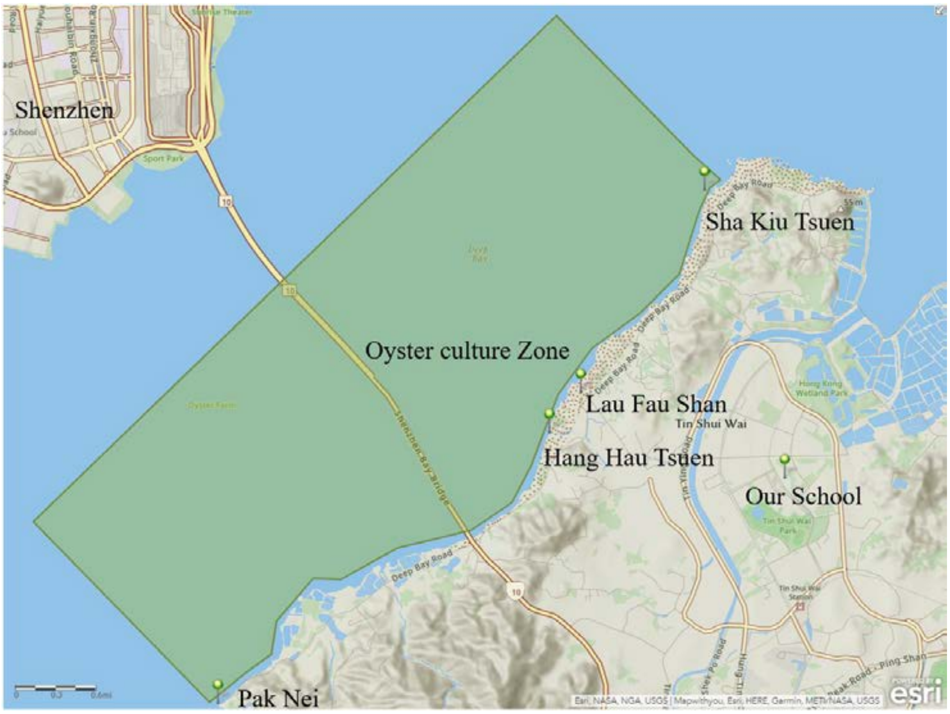


Figure 1 Oyster culture zone in Deep Bay

Figure 2 Oyster posts in Pak Nei

Figure 3 Oyster rafts float on Deep Bay

Oyster products in Lau Fau Shan was an important export in 1960s and 1970s. However, water pollution was a serious threat to the oyster industry at the end of 1970s. Heavy metals and peristant organic pollutants are the two major group of water pollutants. Some examples of the persistent environmental pollutants are shown in **Figure 4**.

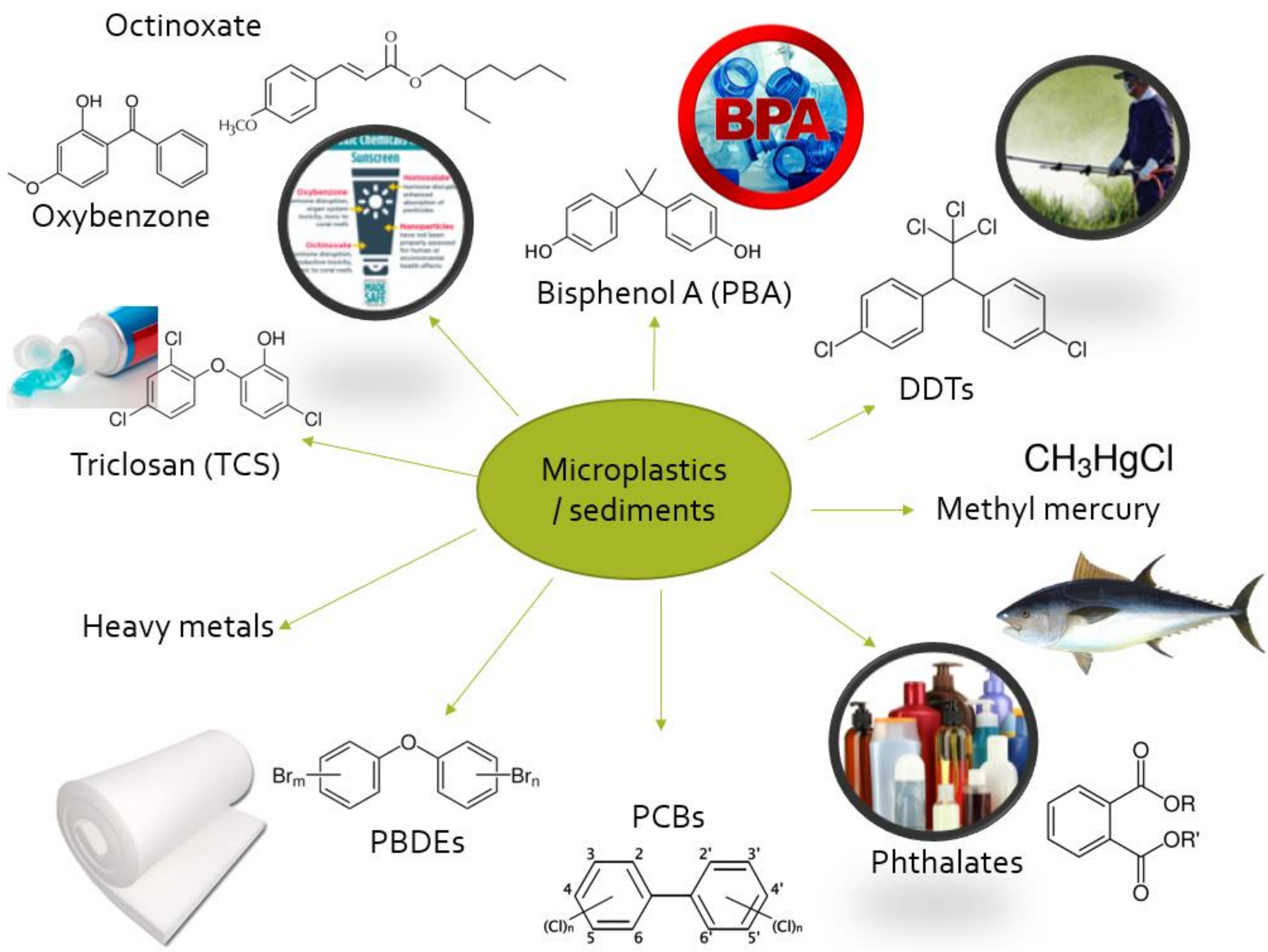


Figure 4 Different types of persistent water pollutants

The term 'heavy metals' is not well defined but it is in general regarded as a group of metallic chemical elements that have a relatively high density (usually higher than 3.5-5 g/cm^3) and is toxic or poisonous at low concentrations (Duffins 2001). Reports about the high level of heavy metals contaminations in oyster samples from Deep Bay seriously lowered the confidence in the consumption of local oyster (Philips, 1982; Cheung and Wong, 1992; Yau, 2008; Liu & Den, 2007). The negative health impacts of heavy metals, include neurotoxicity, mental retardation, and carcinogenicity (Jaishankar et al., 2014).

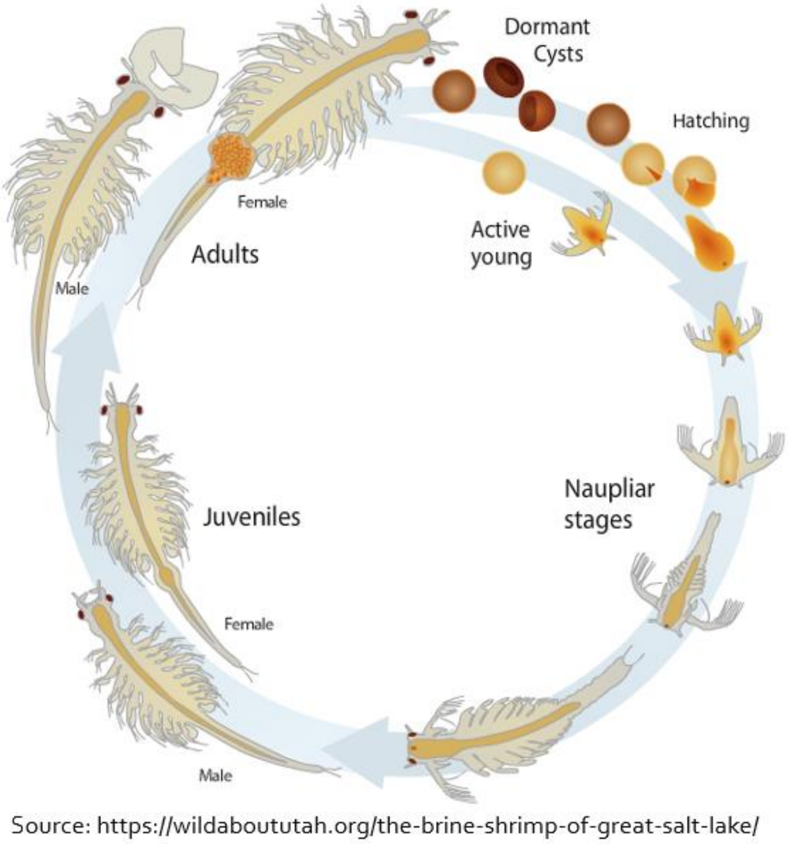
Microplastics are another health threat. Oyster samples were reported to contain 6.4-10.8 pieces of microplastics per gram fresh oyster tissue, which was much higher than the imported oyster samples from the Atlantic Ocean (Hong Kong 01, 27th Nov 2017). The surface of microplastics may contain high level of persistent organic pollutants like methyl mercury and DDTs (Santillo et al., 2017). Pollutants adsorbed on the surface of sediment and microplastics may be accumulated along food chain can impose food security problem to human health.

Regular monitor program to detect the sediment heavy metal and organic pollutants would help the oyster farmers, consumers, and the public to identify the potential food safety problems and environmental changes. Atomic absorption spectrometry (AAS) and inductively coupled plasma (ICP) are the techniques that can detect the heavy metals in environmental samples. In addition, high performance liquid chromatography (HPLC) and mass spectrometry are commonly used to identify and detect the presence of persistent organic pollutants. These methods are very sensitive and specific, but are also very expensive and are not commonly installed in secondary school laboratories. The objective of the present study is to develop simple, low-cost and reliable methods to detect the overall toxicity of environmental samples by using brine shrimp, which acts as a biomarker species.

A biomarker is a change in biological response in different level of biological organization, such as enzymatic reaction, which can be related to exposure to or toxic effects of environmental chemicals (Peakall and Walker, 1994; Kaviraj et al., 2014). Brine shrimp (*Artemia* spp) is a crustacean that naturally lives in salt lakes (0.45% salt water). As the salt lake environment may change rapidly, brine shrimp reproduce by cyst formation to overcome an extreme dry, hot or cold environment. When the rainfall comes, brine shrimp cysts hatch in 1 day and the nauplii develop into adults in around 9 days (Dockery and Tomkins, 2009). The exposure of environmental pollutants may affect its behavior and even cause death. Therefore, it is one of the model organisms used in toxicity and drug testing (Mohamed and Sheir, 2014; Lewan et al., 1992).

Different level or organization of brine shrimp can be used as biomarkers for environmental pollutants. For example, Hohamed et al. (2014) used brine shrimp superoxide dismutase, hydrogen peroxidase, glutathione peroxidase and metallothionein as biomarkers of some heavy metals. Kokkali et al. (2011) reported the used of movement pattern to compare the toxicity of different heavy metals. The death rate of brine shrimp was affected by salinity and the presence of heavy metals (Umarani et al., 2012). In the present study, death rate was used as a measurement of toxicity of sediment samples as it is cost-effective and less technologically demanding.

The toxic effects on brine shrimp on some heavy metals and organic pollutants were firstly determined. Five heavy metals, namely chromium (Cr^{3+}), copper (Cu^{2+}), nickel (Ni^{2+}), lead (Pb^{2+}) and Zinc (Zn^{2+}) were chosen in this research (**Table 1**), as they are commonly used in different industrial processes and are some of the major heavy metal contaminants in the environment. They are also available in the school laboratory.



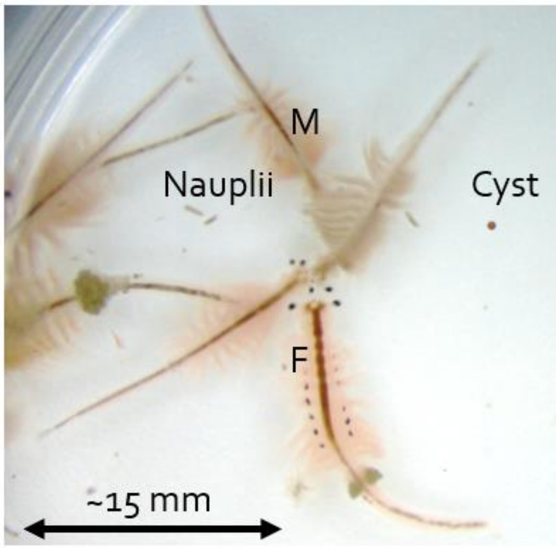


Figure 5 Brine shrimp life cycle (left) and brine shrimp adults (right)

For the persistent organic pollutants, triclosan (TCS), bisphenol A (BPA), oxybenzone (OXY) and octinoxate (OMC) were chosen, as shown in Table 1. TCS (Jaishankar et al., 2014) and BPA (Weatherly and Gosse, 2018) are environmental hormones that would disrupt the endocrine system, whereas OXY and OMC are active ingredients in sun lotion that can cause coral bleaching (Schneider et al, 2018).

Sediment samples were also collected from the three sites of the oyster culture region of the Deep Bay. Hang Hau Tsuen is close to Lau Fau Shan Pier and market, whereas Pak Nai is far from village and is also located near some accredited fish farms. Therefore, the sediment of Hang Hau Tsuen is assumed to have a higher level of environmental contaminants.

The exchangeable heavy metals of the sediments were extracted by treating with neutralized sodium acetate (adapted from Nowrouzi et al., 2014). The extracts were used to treat the brine shrimp nauplii and compare the toxicity with heavy metals and organic pollutants.

Table 1 Types of water pollutants and their uses and impacts on the environment

Types	Selected persistent water pollutants	Uses and impacts
Heavy Metals	Cr, Cu, Ni, Pb, Zn (Jaishankar, et al., 2014)	● used in many industrial processes ● destroy internal organs
Persistent organic pollutants	Triclosan (TCS) (Weatherly and Gosse, 2018)	● disinfectant used in personal health products ● disrupts endocrine system
	Bisphenol A (BPA) (Rochester, 2013)	● production of plastics ● disrupts endocrine system
	Oxybenzone (OXY) & octinoxate (OMC) (Schneider, et al, 2018)	● active ingredients in sun lotion causes coral bleaching

2. Methodology

2.1 Culture of brine shrimp

Brine shrimp adults were bought from local market. Brine shrimps nauplii were cultured in a 0.4% saline solution from brine shrimp cysts. The cysts hatch in room temperature in one day and activated yeast was used to feed the nauplii. Brine shrimp nauplii were used immediately after hatched.

2.2 Toxicity test with brine shrimp adult

Around 15-20 brine shrimp adults in 13.5 mL culture solution (0.4% saline solution) were put in 50 mL centrifuge tube. 1.5 mL heavy metal solution (1000 ppm Cr^{3+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}) was added to each tube. Triplicates were done for each type of heavy metal. The number of living and dead brine shrimp adults in each tube was counted every day, so as to calculate the overall death rate, for 4 consecutive days.

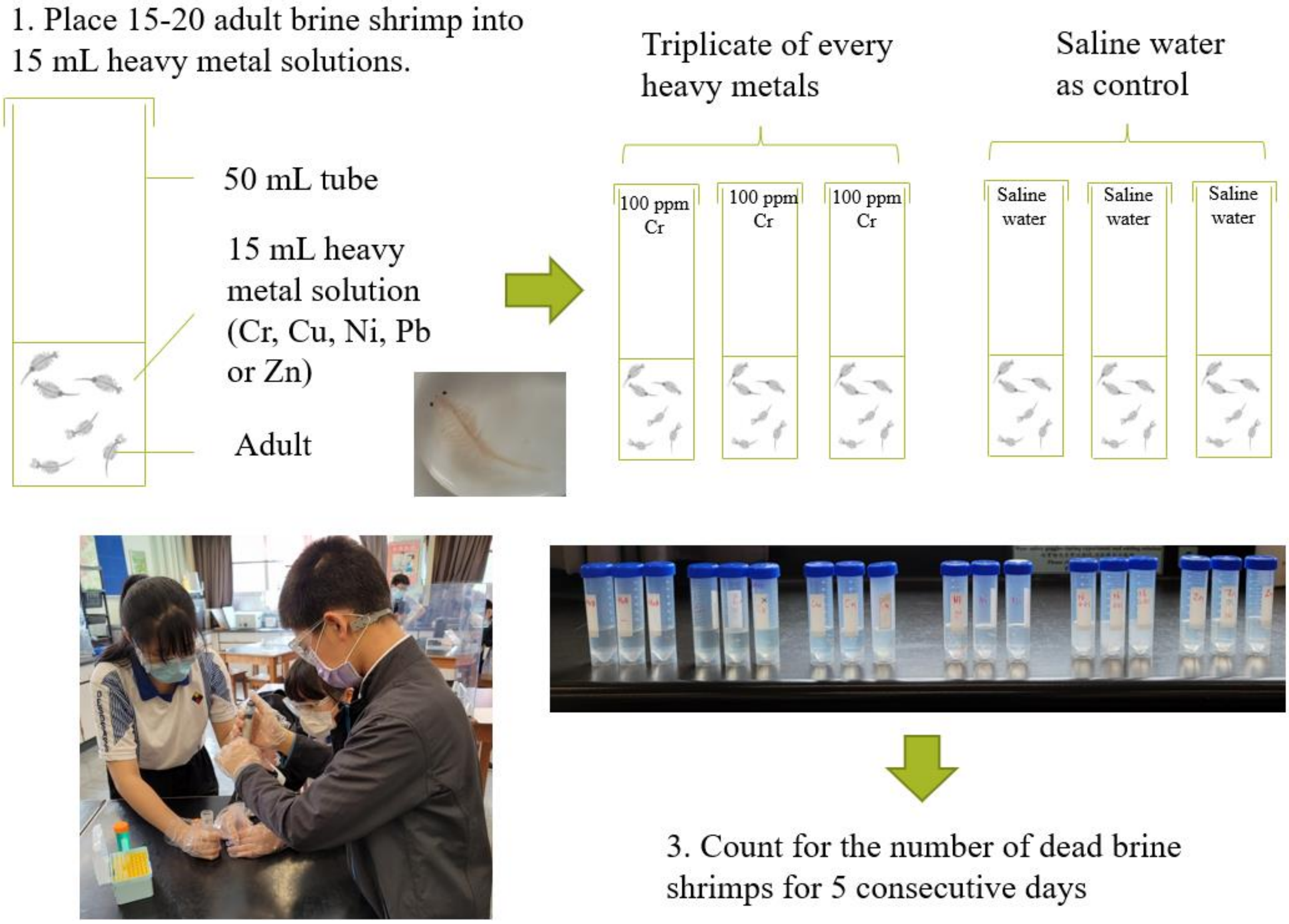


Figure 6 Brine shrimp adult toxicity test

2.3 Toxicity test with brine shrimp nauplii

Around 50 to 300 brine shrimp nauplii, in 2.7 mL culture solution (0.4% saline solution) was put in a 3.5 mm petri dish. 0.3 mL water pollutant solutions (10000 ppm, 1000 ppm, 300 ppm, 100 ppm, 30 ppm, 10 ppm, 1 ppm Cr^{3+}, Cu^{2+}, Ni^{2+}, Pb^{2+} or Zn^{2+}, BPA, TCS, OXY and OMC) was added to the petri dish. The nauplii were observed under a dissection microscope with video recording function. The number of living and dead brine shrimp nauplii in each dish was counted, so as to calculate the death rate from 1 hour to 36 hours. Lethal concentration 50 (LC_{50}) of different environmental pollutants were determined with the use of a web-based platform known as AAT Bioquest LC_{50} Calculator.

Using similar method, the combined toxicity effects of two different combinations of heavy metals or organic pollutants were carried out. The death rates were calculated to see if the combined effect is synergistic or not.

2.4 Collection of sediment samples

Sediment samples were obtained in three different areas of the oyster culture zone in Deep Bay in a sunny day (for three consecutive days) during the low tide (0.5m) period on 10 May 2021. The three sites are Sha Kiu Tsuen, Hang Hau Tsuen and Pak Nei (**Figure 1**). Sha Kiu Tsuen and Pak Nei are at the north-east and south-west margins of the oyster culture zone respectively, and they are far away from residential areas. Hang Hau Tsuen is close to residential area and Lau Fau Shan Fish Market, which are the sources of water pollutants. Three samples were collected in each site with a tubular soil sampler. The samples were placed in refrigerator before use.

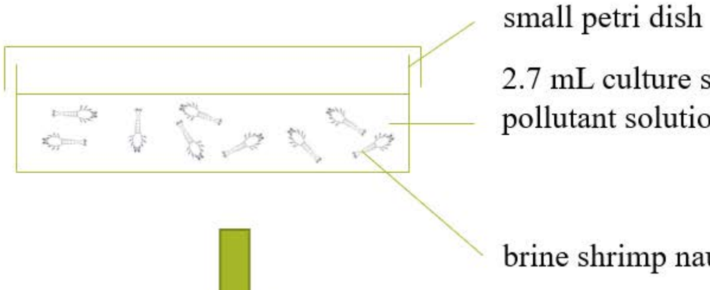
2.5 Extraction of sediment extract

The exchangeable heavy metals, as well as some organic pollutants were extracted by the method modified from Nowrouzi et al (2014). Each sediment samples were put in an acid washed evaporating dish and then dried at 60°C overnight. The dried sediment samples were sieved by a soil sieve and only the particle with size smaller than 0.25 mm were collected and weighed. 1 M neutral sodium acetate (1g/5 mL) was added to each of the dried sieved sediment sample in an acid-washed 100 mL conical flask. The flasks were placed at 37 °C shaking incubator for 1 hour. The mixtures were centrifuged at 5000 rpm for 5 minutes and the supernatants were obtained for toxicity test.

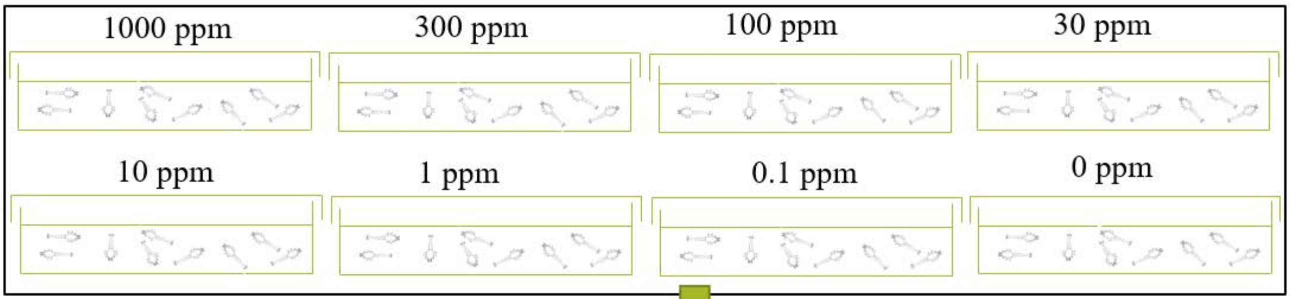
2.6 Sediment extract toxicity test

The toxicities of the sediment extracts were tested with brine shrimp nauplii as mentioned in 2.2. Chromium, bisphenol A and oxybenzone (1 and 10 ppm) were also tested for toxicity comparison. Triplicates were done for each sample.

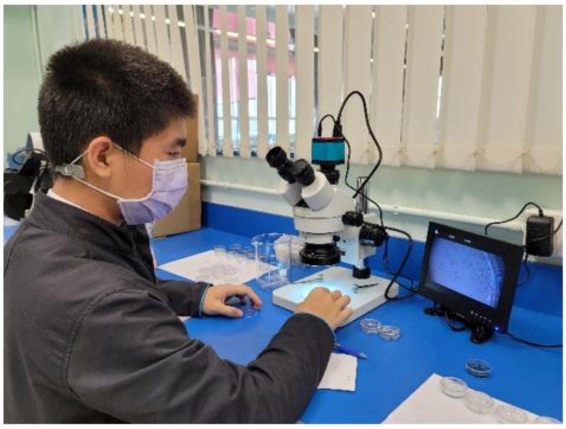
1. Put different concentration of heavy metal or organic pollutants to the brine shrimp nauplii culture solution

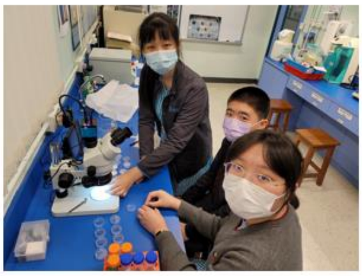


2. Wait for 1 hour to several days



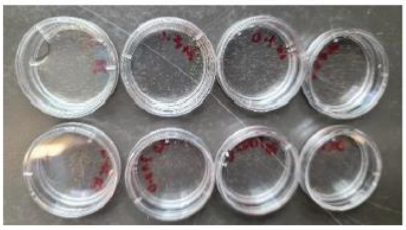
3. Use a dissection microscope to record the number of active, inactive and dead brine shrimp nauplii



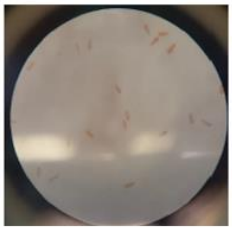


AAT Bioquest®

4. Use of *AAT Bioquest* to plot the dose and effect relationship and determine the LC_{50}



Brine shrimp nauplii in petri dish



Photomicrograph of brine shrimp nauplii

Figure 7 Toxicity test with brine shrimp nauplii

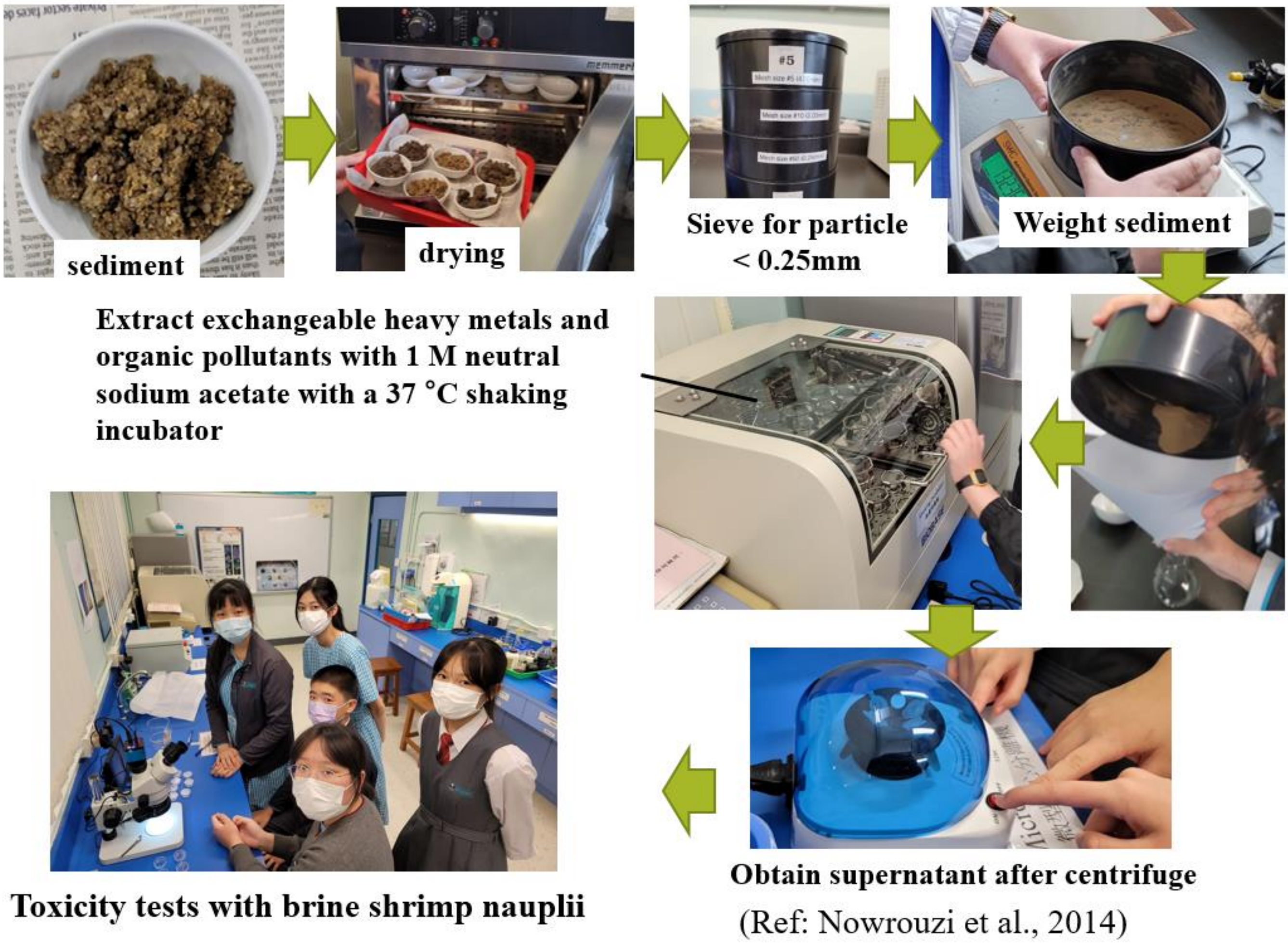


Figure 8 Extraction of sediment extract

3. Results and Discussion

Figure 9 shows the change in death rate of the brine shrimp adults for 4 consecutive days. Brine shrimp adults showed different sensitivity to different heavy metals. The adults were the most sensitive to Cr and Cu and then Zn, but not very sensitive to Ni and Pb.

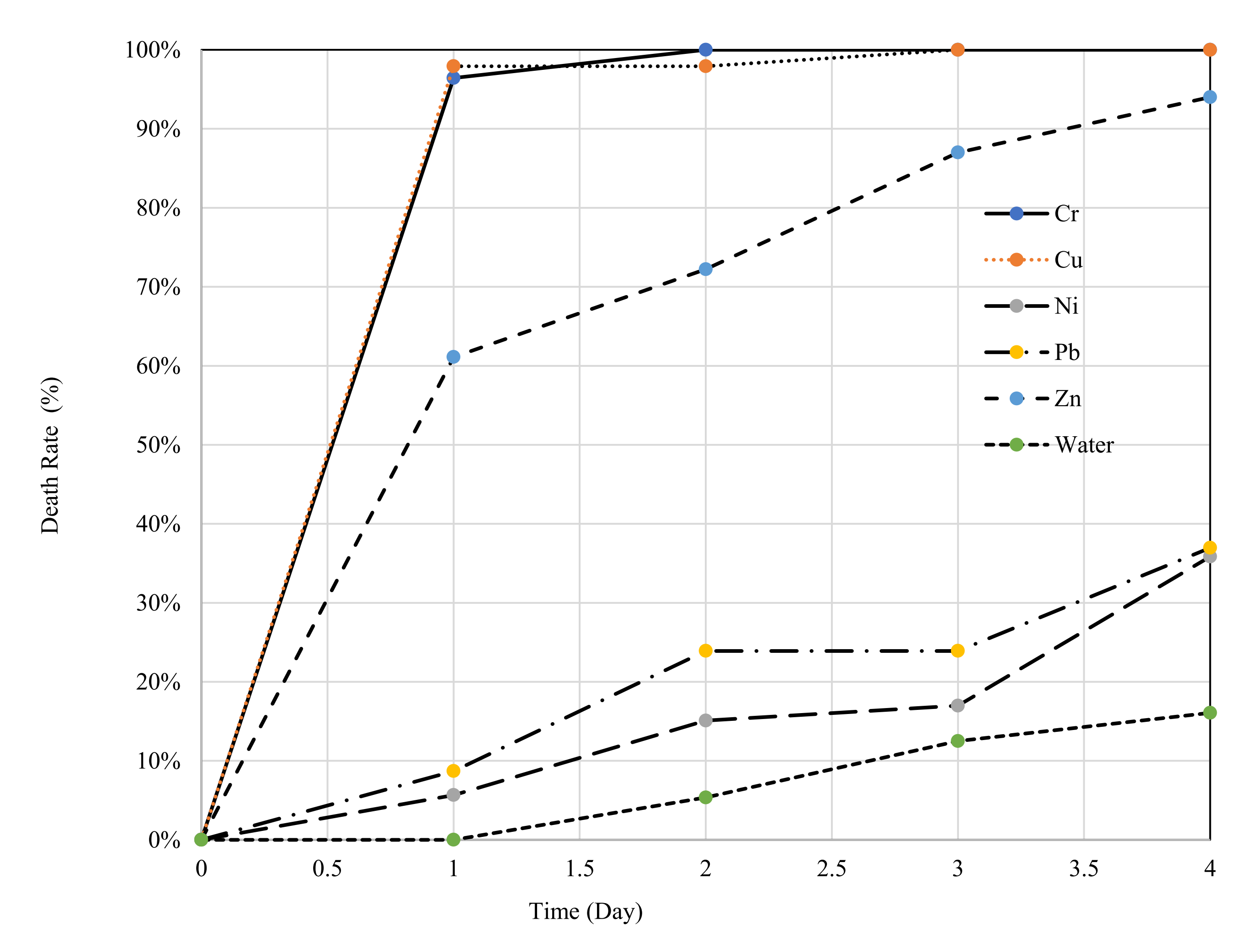


Figure 9 Death rate of brine shrimp adults in 100 ppm heavy metal solutions

Table 2 summarizes the LC_{50} of Brine shrimp nauplii to different heavy metals in 24 hours. LC_{50} drops significantly after 24 hours and therefore LC_{50} (24 hr) was used for the comparison of the toxicity of environmental pollutants in this paper. Both brine shrimp adults and nauplii were the most sensitive to Cr and Cu. The relative insensitivity of brine shrimp to Zn may be due to the use of Zn in different processes (Beyersmann and Haase, 2001). Since the death rate of the brine shrimp nauplii treated with heavy metals were in general higher than the adult, and the cost is much higher, the brine shrimp nauplii were chosen for toxicity tests.

Table 2 Effect of culture time on the LC_{50} of brine shrimp nauplii

	LC_{50} (ppm)				
	1.5 hr	3 hr	5 hr	7 hr	24 hr
Cr	67.6	45.9	45.6	40.2	24.9
Cu	*	210	206	135	55.5
Ni	*	*	*	*	*
Pb	*	*	843	778	495
Zn	*	*	*	*	335

* less than 50% death rate and LC_{50} cannot be determined

Table 3 showed the LC_{50} of brine shrimp when treated with different environmental pollutants. Brine shrimp nauplii are the most sensitive to BPA and OXY, and the LC_{50} values are 5.6 and 2.7 respectively. It is also sensitive to Cr and Cu. Brine shrimp has lower sensitivity to other pollutants tested in this study.

Table 3 LC_{50} of brine shrimp nauplii to different pollutants

	Pollutants	LC50 (24 hr) (ppm)
Heavy metals	Cr	24.9
	Cu	55.5
	Ni	*
	Pb	495
	Zn	335
Organic pollutants	BPA	5.6
	OMC	1037
	OXY	2.7
	TCS	2428

* less than 50% death rate and LC_{50} cannot be determined

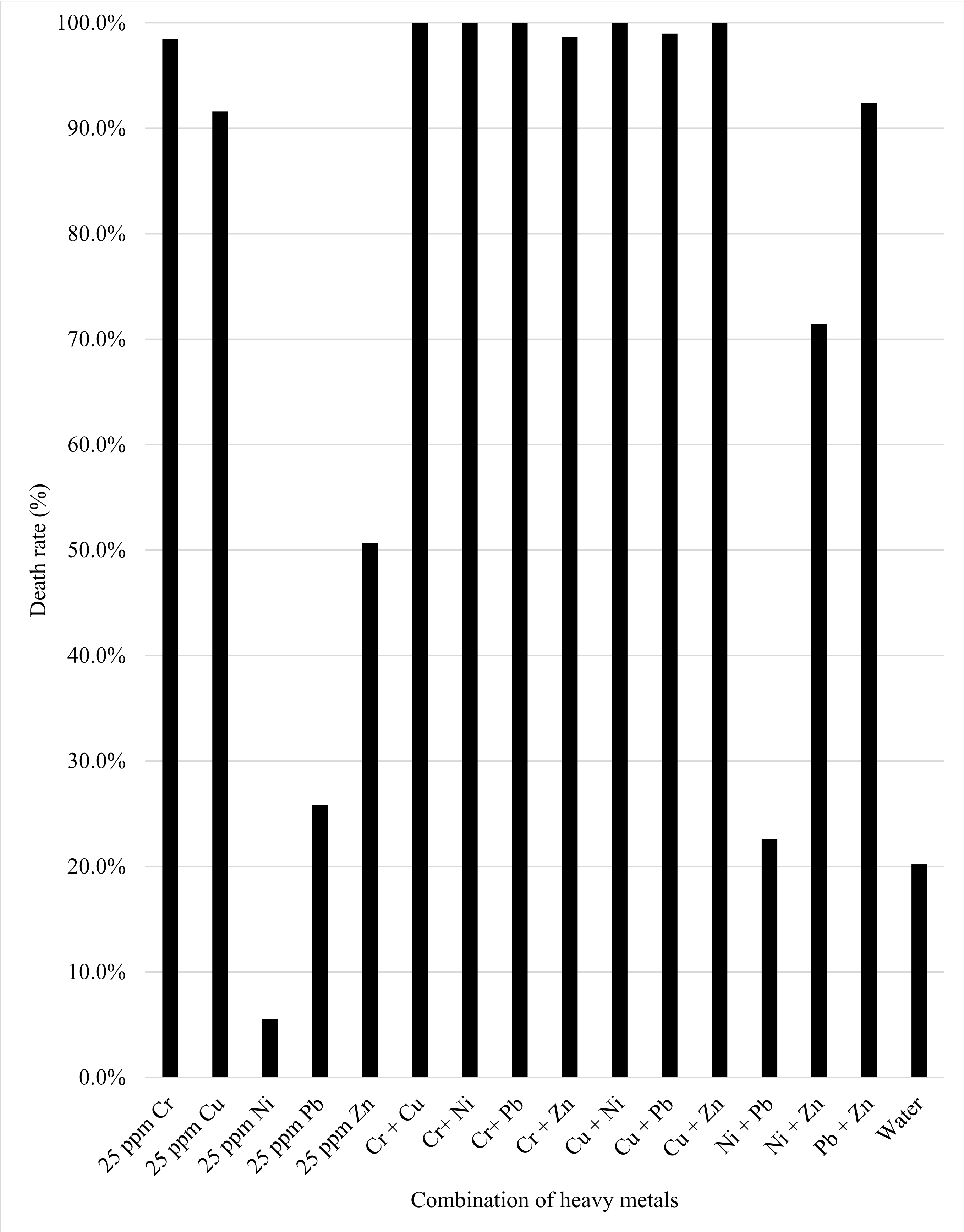


Fig. 10 Study of combined toxic effect of heavy metals on brine shrimp nauplii

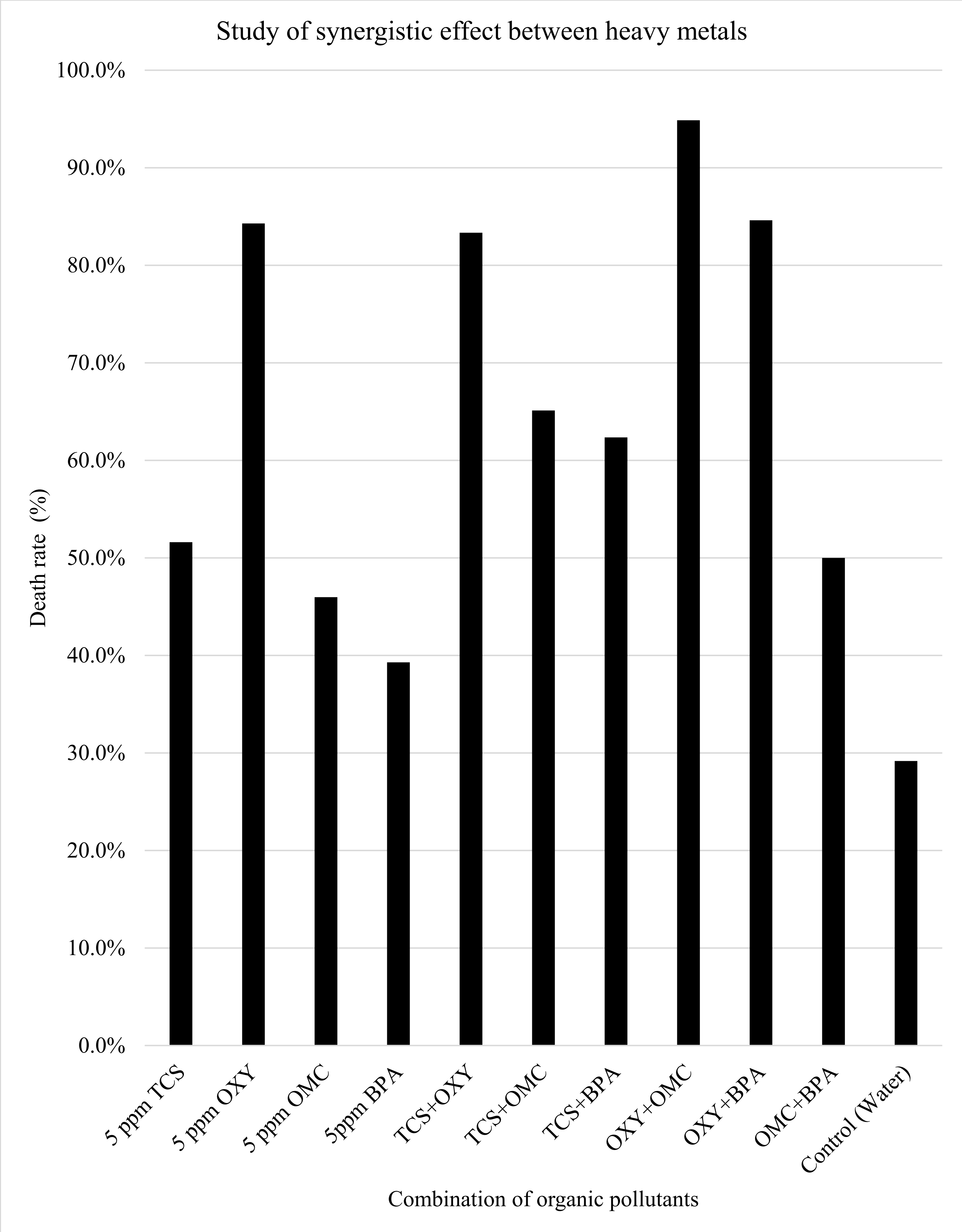


Figure 11 Study of combined toxic effect between persistent organic pollutants

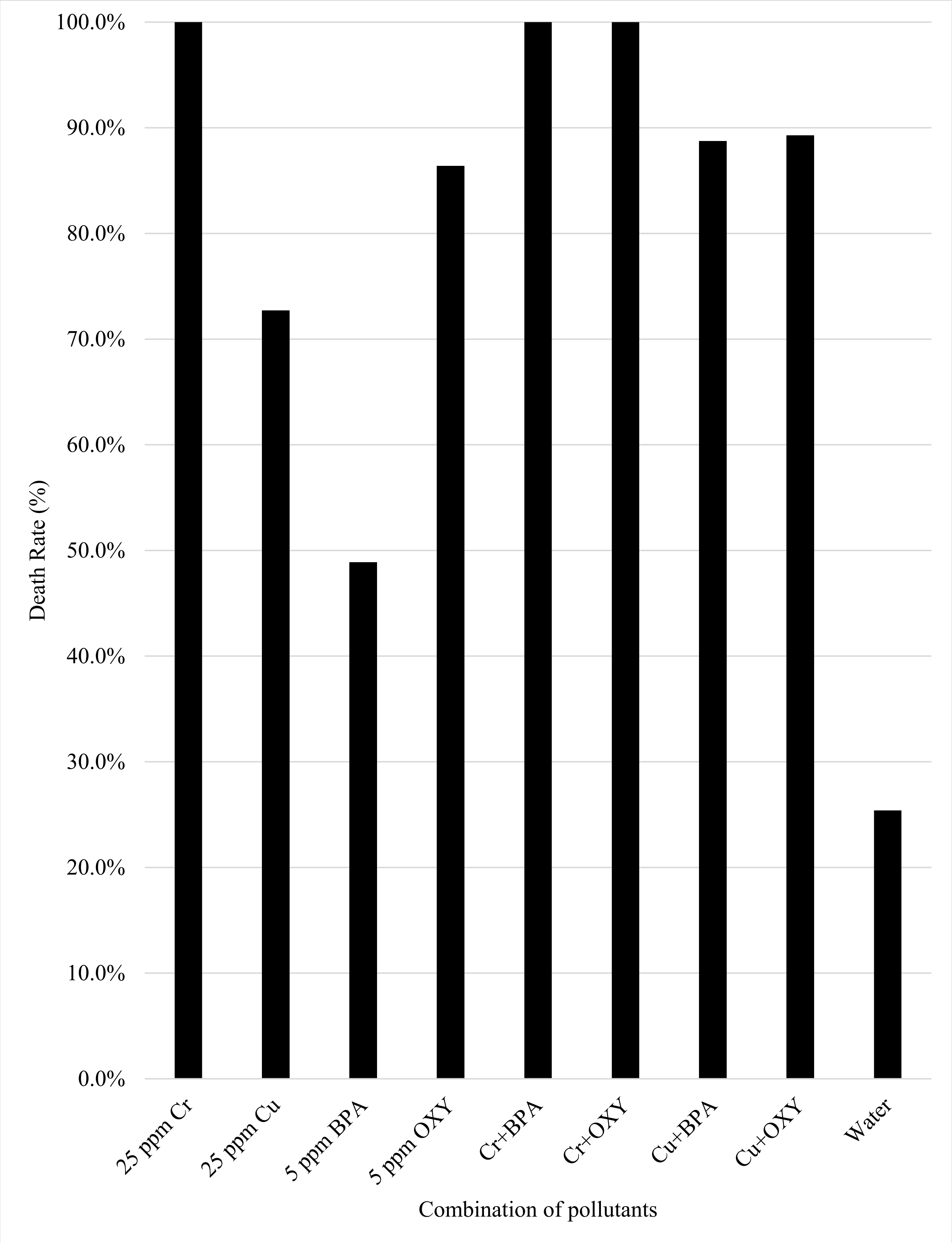


Figure 12 Study of combined toxic effect between heavy metals and persistent organic pollutants

Fig. 13 Test for some heavy metals in the sediment extract using commercial test kits, which showed undetectable level of Cu, Cr and Ni

Further study of the combined toxic effects of the pollutants are shown in **Figure 10 to 12**. In **Figure 10**, the combined death rate of Ni and Pb was higher than the sum of the individual death rate of Ni and Pb. Therefore, Ni and Pb may have a synergistic toxic effect on brine shrimp nauplii. The results did not provide strong evidence about the synergistic toxic effect of other combinations of water pollutants, since the death rate of the concentrations of the pollutants led to a high death rate. More investigations need to be carried out to determine whether other combinations of pollutants have synergistic or antagonistic toxic effects, either by reducing the concentration of the pollutants, or do the toxicity test in a shorter time.

When the sediment extracts were tested with commercial test kits, the concentrations of chromium, copper and nickel were undetectable (**Figure 13**). In **Figure 14**, the death rate of brine shrimp nauplii treated with the sediment extract of Hang Hau Tsuen was similar to 1 ppm PBA. It was also about 10 to 20% higher than that of the other two sites (Pak Nei and Sha Kiu Tsuen). Since Hang Hau Tsuen is closer to the residential area and Lau Fau Shan Seafood Market than the other two sites, its sediment extract is likely to have a higher level of environmental pollutants. The results suggest that brine shrimp nauplii may be used as a biomarker to monitor the overall toxicity of pollutants in sediment samples.

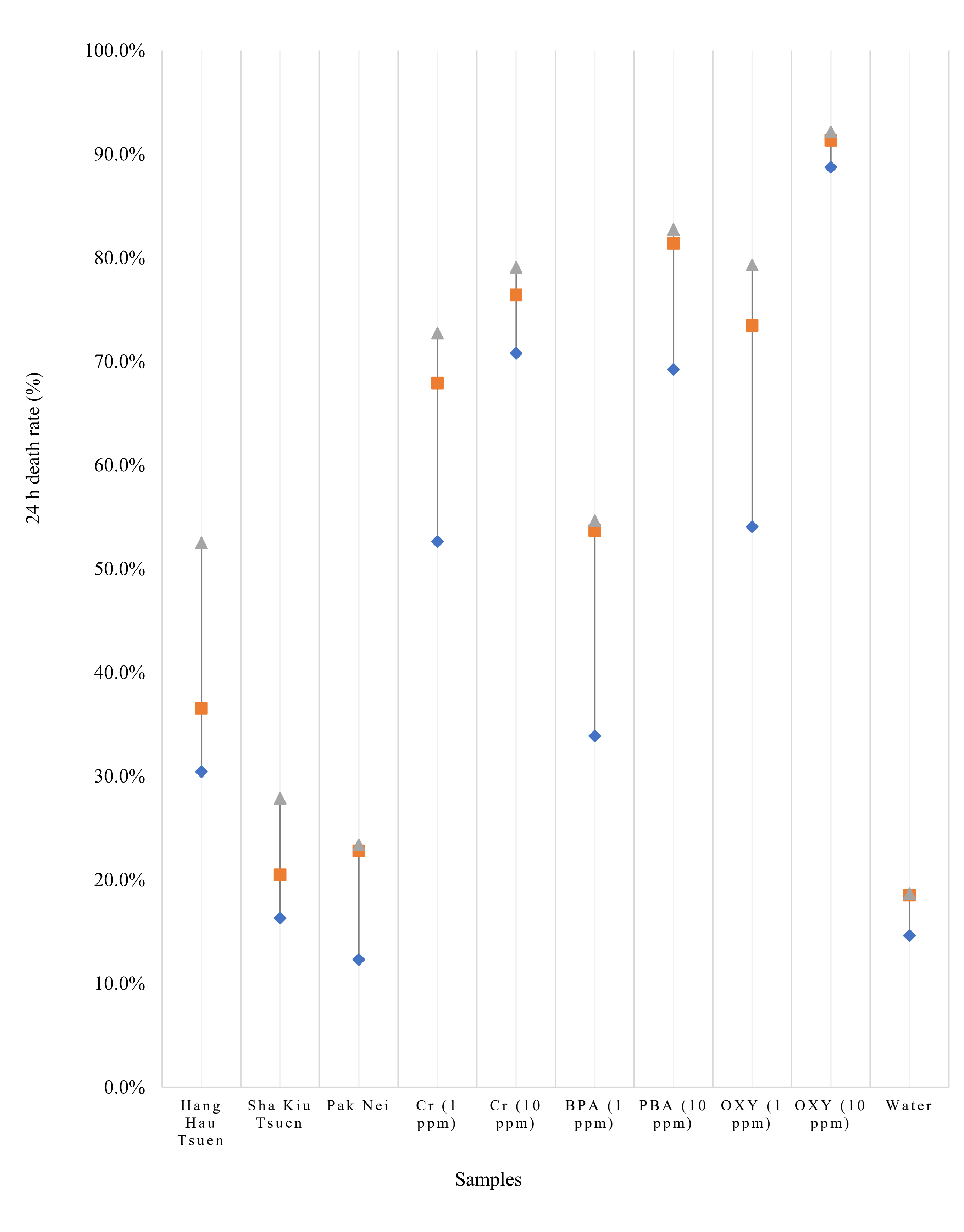


Fig. 14 Effect of sediment extracts and different pollutants on the death rates of brine shrimp nauplii

The use of brine shrimp nauplii to test for water pollutants has the following advantages:

1. Low-cost: The consumables and eqipment used in this study are in general not expensive. The brine shrimp cyst can be bought from the online shop easily. For the consumables, the cost for each test should be less than \$0.4 TWD.

2. Simple test: The testing method is simple. Just put the brine shrimp into petri dish with different concentration of pollutants and then observe the death rate under a dissection microscope.

3. Test for overall toxicity: In the present study, the overall combined effects of environmental pollutants may be tested by brine shrimp nauplii. Environmental samples should contain various type of environmental pollutants. Since it is not quite possible to test for all environmental pollutants in each sediment sample, the use of brine shrimp to test for the overall toxicity of the sediment extract offer an alternative to identify the potential change in the marine environment, and then to evaluate the potential ecological and health threats.

As oysters may not only take up sediment but also microplastics when they filter the organic matters as food, further investigations about the microplastics will be carried out. Methods for detecting the microplastics in surface water and sediemt was suggesgted by Tsang et al. (2017) and Wenjie et al. (2020). The microplastic in the living organisms, such as oyster, mudskipper and other consumers can be detected by the method suggested by Teng et al. (2019).

4. Conclusion

The death rate of brine shrimp nauplii is a better biomarker of water pollutants than the adults. The nauplii are especially sensitive to copper, chromium, bisphenol A and oxybenzone. The sediment extract of Hang Hau Tsuen, a region closer to the source of water pollutants, is higher than that of Pak Nei and Sha Kiu Tsuen. The proposed method to test for the overall toxicity of sediment extracts with brine shrimp nauplii is simple, low-cost and safe.

5. References

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Heavy metals and organic pollutants are serious contamination issue worldwide. Brine shrimp nauplii was used for the testing of the overall toxicity of sediment samples. The proposed contaminants were extracted from sediment samples from three sites of the oyster culture zone of the Deep Bay. The brine shrimp nauplii should influenced by water contamination from sediment dissolution. The collection of sediments should be address with more details such as location, depth.. etc.