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Concentration of pathogenic bacteria and trace metals in bivalve mollusk *Anadara granosa* (Bivalvia: Arcidae) harvested from East Java Coast, Indonesia

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Abstract: A study to evaluate the level of *Escherichia coli, Salmonella* and trace metals in bivalves *Anadara granosa* collected from East Java Coast has been conducted. The results showed that all stations demonstrated undetectable values of *Salmonella* in bivalve tissues. In contrast, the *Escherichia coli* values in bivalves ranged from undetectable level to 4800 MPN.100 g^{-1}. Measurement for lead of all samples of bivalves from all sampling sites was always below the detection limit of 0.0405 $mg.kg^{-1}$. The concentrations of cadmium in bivalves highly varied from 1.234 to 2.404 $mg.kg^{-1}$, and mercury levels were between < 0.0014 and 0.048 $mg.kg^{-1}$. *Escherichia coli* level in bivalves from some locations evaluated exceeded the acceptable limit for shellfish for human consumption. This study reported that the concentrations of lead and mercury in *Anadara granosa* was below the range acceptable for consumption. However, the concentrations of cadmium in bivalves harvested from some locations of East Java Coast were higher than the maximum acceptable limit for consumption.

Résumé : *Concentration en bactéries pathogènes et en métaux traces chez le mollusque bivalve* Anadara granosa *(Bivalvia : Arcidae) récolté sur la côte est de Java, Indonésie.* Une évaluation du niveau de contamination en *Escherichia coli, Salmonella* et métaux traces a été réalisée sur la côte est de Java. Les résultats montrent à toutes les stations des niveaux indétectables de *Salmonella* dans les tissus des bivalves. Au contraire, les valeurs de concentration d'*Escherichia coli* varient du seuil de détection jusqu'à 4800 MPN.100 g^{-1}. Les mesures de plomb sont sous la limite de détection de 0,0405 $mg.kg^{-1}$ pour tous les échantillons. Les concentrations de cadmium varient fortement, de 1,234 à 2,404 $mg.kg^{-1}$, et les niveaux du mercure de < 0,0014 à 0,048 $mg.kg^{-1}$. Les niveaux de contamination en *Escherichia coli* à certaines stations dépassent le seuil acceptable pour la consommation humaine de coquillages. Cette étude montre que les concentrations de plomb et de mercure chez *Anadara granosa* restent en dessous du seuil de comestibilité. Toutefois, les concentrations de cadmium de bivalves récoltés à certaines stations de la côte est de Java sont supérieure à ce seuil.

Keywords: Escherichia coli ● *Salmonella* ● Metals ● Bivalve ● East Java Coast

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Introduction

Bivalve mollusks are mass-consumer products, which constitute relatively cheap sources of animal protein for people living in coastal region of East Java. Bivalves are found abundantly in the intertidal and shallow subtidal coastal zone. Rapid population growth and development in coastal area deteriorated the quality of coastal waters which served as their habitat. Recently, most of East Java coastal waters received a large amount of pollutants from different sources.

Bivalves are filter-feeding animals that can concentrate many different types of pollutants including trace metals and microorganisms from large volumes of water. Pollution by trace metals is a serious problem due to their toxicity and ability to accumulate in the biota. The progressive and irreversible accumulation of trace metals in various organs of marine creatures ultimately leads to metal-related diseases in the long run, thereby endangering the aquatic biota and other organisms (Reddy et al., 2007). Moreover, consumption of bivalves harvested from contaminated area can cause illness associated with pathogenic organisms (Kronkvist, 2006).

Bivalves have been well established as bioindicators for monitoring the concentration of trace metals in many areas in the world (Rasmussen & Williams, 1975; Rainbow, 1990; Etim et al., 1991; Schuhmacher et al., 1995; Lang et al., 1996; Haynes et al., 1997; Miramand et al., 2001; Usero et al., 2005; Beldi et al., 2006; Sidoumou et al., 2006). Some surveys on the microbiological quality of shellfishes have shown that shellfishes played major role as harbor of pathogenic organisms (Adebayo-Tayo et al., 2006). Since shellfishes are found in bodies of waters containing untreated human and industrial waste, there is a tendency that they may concentrate and accumulate high level of metals and pathogens which can pose a significant health hazard to consumers.

Considering the commercial and nutritional importance of shellfishes, there is important to create awareness to the public on health risks of consuming bivalves, as this could be a channel of ingesting pathogenic microorganisms and toxic substances into the body. This study was undertaken to evaluate the level of *Escherichia coli* (Migula, 1895), *Salmonella* and trace metals in bivalves *Anadara granosa* (Linnaeus, 1758) collected from East Java Coast.

Materials and Methods

Sample collection and preparation

Bivalve samples were collected from fishermen from five selected sites of East Java Coast between October and December 2007 (Fig.1). The bivalves were rinsed, but not immersed, and drained at the time of sampling. The samples were then directly placed in plastic bag, properly packed in a cool box and brought to the laboratory. During transportation the temperature of ice box maintained near 4ºC. The microbiological analysis started no later than 24 h after collection. The selected bivalves were opened with a flame sterilized shucking knife and the flesh put into a pre-weight glass cup. Approximately 20 bivalves, which weights around 80-100 g were used for each analysis.

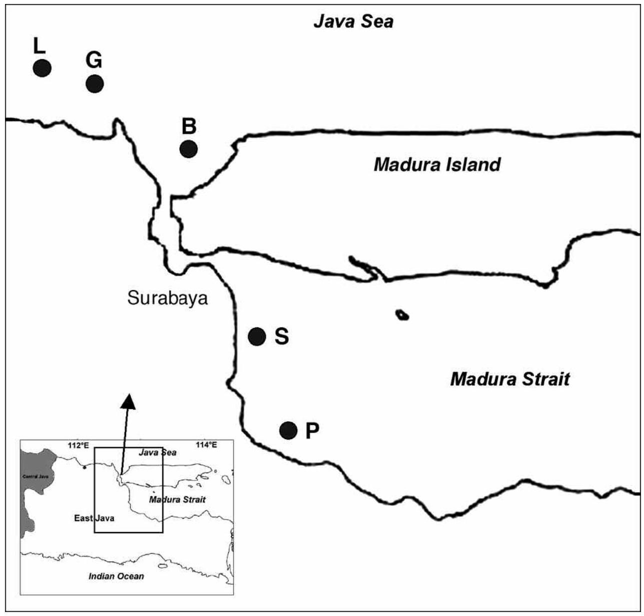

Figure 1. *Anadara granosa.* Location of sampling stations (B: Bangkalan, G: Gresik, L: Lamongan, P: Pasuruan and S: Sidoarjo).

Figure 1. *Anadara granosa.* Localisation des stations de prélèvement (B : Bangkalan, G : Gresik, L : Lamongan, P : Pasuruan and S : Sidoarjo).

Detection and analysis of Escherichia coli

After the general sample preparation, 225 ml Butterfield's phosphate buffered was added to 25 g of mussel tissue. The total was put in a blender jar and homogenized at high speed for approximately two minutes.

The most probable number (MPN.100 g^{-1}) enumeration of *Escherichia coli* in the samples was determined using the multiple tube method with five tubes and three dilutions in lauryl tryptose broth. The tubes were incubated in 35 ± 1ºC for 48 ± 2 h. For *Escherichia coli* presumptive test, the positive sample was inoculated onto enrichment coliform (EC) broth tube, and incubated in water bath at 45 ± 0.5ºC for 48 ± 2 h. For *Escherichia coli* confirmation, the positive sample from EC broth was spread onto a Levine's eosin-methylene blue (LEMB) agar and incubated in 35 ± 1ºC for

24 ± 2 h. Colonies from LEMB agar were then inoculated onto plate count agar slants and incubated in 35 ± 1ºC for 24 ± 2 h. For further analysis, the samples were subjected to subsequent biochemical standard test using indole, Voges-Proskauer, methyl-red, and gas from lactose tests.

Detection and analysis of Salmonella

One ml of the homogenized samples was spread to *Salmonella Shigella* agar plate and incubated in 37.5 ± 1ºC for 24 ± 2 h. For confirmation, suspicious *Salmonella* colonies were inoculated onto natrium agar slants and incubated in 37.5 ± 1ºC for 24 ± 2 h. The typical *Salmonella* colonies were subjected to subsequent biochemical standard test using lysine-iron agar, triple sugar iron agar, and urea broth. The concentration of *Salmonella* in bivalves was quoted as colony forming unit ($CFU.g^{-1}$).

Analysis of trace metals

Whole tissues were extracted from the shells for metals analysis, and then pooled for each station and each period to form a single sample. External water from each sample was absorbed using tissue papers. Approximately 20 bivalves, which weights around 80-100 g were used for each analyse. After the general sample preparation, sufficient deionized water was added to 25 g of bivalve tissue, and put in a blender jar and homogenized at high speed for approximately two minutes.

Cadmium and lead

A subsample of homogenized bivalves from each location and each period was dried at 60ºC to a constant weight. All reagents were obtained from Merck and were of analytical grade. All samples were digested in acid solutions 2 ml of HNO_3 and 3 ml H_2SO_4 in glass flasks at 100ºC for 3 h. After cooling, samples were diluted to 50 ml with deionized water. An aliquot was taken for lead and cadmium detection using atomic absorption spectrophotometer (Shimadzu tipe AA-6200).

Mercury

The homogenized tissues were digested in 3 ml H_2SO_4 and 2 ml HNO_3 in glass flask at 80°C for 3 h. After cooling, 15 ml of $KMnO_4$ solution was added until the purple color of the solution stabilized. Sufficient hydroxylamine sulphate/sodium chloride solution was added to neutralize the excess potassium permanganate as a preservative. The solution was adjusted to 50 ml with deionised water. An aliquot was taken for mercury determination using mercury vaporizer unit tipe IA connected to Shimadzu atomic absorption spectrophotometer.

Analysis of reference material

Analytical blanks were run in the same way as the samples and concentrations were determined using standard solutions prepared in the same acid matrix. All metal concentrations of samples were analyzed in duplicate and quoted as $mg.kg^{-1}$ wet weight. Validity of analytical methods was checked using dogfish muscle reference materials (DORM-2) provided by the National Research Council of Canada. The recoveries for Cd, Pb and Hg in the tissue standard reference material DORM-2 were 107, 95 and 111% respectively.

Results

Table 1 shows the levels of *Escherichia coli* and *Salmonella* in the bivalve *Andara granosa* from the five different sites of East Java Coast during two different periods. The *Escherichia coli* values in bivalves ranged between 47 and 4800 $MPN.100\ g^{-1}$ in October 2007, and ranged from undetectable (negative) level to 3300 $MPN.100\ g^{-1}$ in December 2007. In October 2007, the lowest value of *Escherichia coli* noted at Station L and the highest value recorded at Station S. In December 2007, the negative level of *Escherichia coli* recorded at Station S, P and B, and the highest level of *Escherichia coli* documented at Station L. In contrast, all stations

Table 1. *Anadara granosa.* Microbiological analysis results of bivalves.
Tableau 1. *Anadara granosa.* Résultats de l'analyse microbiologique des bivalves.

Sampling Site	***Escherichia coli*** **($MPN.100\ g^{-1}$)**		***Salmonella*** **($CFU.g^{-1}$)**	
	Oct. 2007	Dec. 2007	Oct. 2007	Dec. 2007
Lamongan (L)	47	3300	Negative	Negative
Gresik (G)	130	2700	Negative	Negative
Sidoarjo (S)	4800	Negative	Negative	Negative
Pasuruan (P)	1100	Negative	Negative	Negative
Bangkalan (B)	210	Negative	Negative	Negative

Table 2. *Anadara granosa.* Trace metal concentrations ($mg.kg^{-1}$ wet weight) in bivalves.
Tableau 2. *Anadara granosa.* Concentrations en métaux traces ($mg.kg^{-1}$ de poids frais) des bivalves.

Sampling Site	Pb		Cd		Hg	
	Oct. 2007	Dec. 2007	Oct. 2007	Dec. 2007	Oct. 2007	Dec. 2007
Lamongan (L)	< 0.0405	< 0.0405	2.404	1.864	< 0.0014	< 0.0014
Gresik (G)	< 0.0405	< 0.0405	1.234	2.206	0.012	< 0.0014
Sidoarjo (S)	< 0.0405	< 0.0405	1.364	1.448	< 0.0014	0.008
Pasuruan (P)	< 0.0405	< 0.0405	1.640	1.676	< 0.0014	0.016
Bangkalan (B)	< 0.0405	< 0.0405	1.476	2.286	< 0.0014	0.048
Acceptable limits	2 (Indonesia[1]), 0.2 (EU[2]) 2.5 (Australia[3])		0.05 (EU[2]), 2 (Australia[3])		0.5 (Indonesia[1], EU[2], Australia[3])	

Note: [1] Decree of General Directorate of Drug and Food Surveillance No. 03725/B/SK/VII/89 concerning acceptable limit of metals in food.
[2] European Regulation 466/2001/EC concerning maximum acceptable limit of metals in marine foodstuffs.
[3] Food Standards Code/1987, Australian Government Publishing Service.

demonstrated undetectable values of *Salmonella* during October and December 2007.

Concentrations of trace metals measured in bivalves are presented in Table 2. Measurement for lead (Pb) of all samples of bivalves from all sampling sites was always below the detection limit of 0.0405 $mg.kg^{-1}$. The concentrations of cadmium in bivalves were highly varied from 1.234 and 2.404 $mg.kg^{-1}$. Although variability of cadmium concentrations was quite high from bivalve to bivalve, the concentrations of Cd at Sidoarjo (S) and Pasuruan (P) were relatively constants. In October 2007, the highest level of cadmium noted at Station L and the lowest level recorded at Station G. In December 2007, the highest level of cadmium noted at Station B and the lowest level recorded at Station S. The concentrations of mercury in *Anadara granosa* ranged between < 0.0014 and 0.048 $mg.kg^{-1}$. In October 2007, four stations (Station L, S, P and B) showed undetectable level of Hg, and Station G presented the mercury level of 0.012 $mg.kg^{-1}$. In December 2007, Station L and G showed the mercury level below the detection limit of 0.0014 $mg.kg^{-1}$, and the highest level of Hg noted at Station B.

Discussion

There are no previous data available on the level of bacteria in bivalves from East Java Coast. The present study indicated that the *Escherichia coli* levels in *Anadara granosa* showed a high variation among all stations and periods. Values of *Escherichia coli* in same location were changed from one period to other. The occurrence of enteric organisms in *Anadara granosa* was an indication of the pollution of their overlaying water with untreated faecal waste and sewage. There are many factors affecting the quality of habitat and life of bivalve mollusk. Climatic changes, rainfalls, rivers and development of rural areas were considered as pollution factors of harvesting area of shellfishes (Rozak & Clowell, 1987; Pommepuy et al., 1992). Some studies reported that in general, the level of bacterial contamination of shellfish depends on the extent of pollution in the growing waters (Adebayo-Tayo et al., 2006).

Indonesian food products governmental guideline and other guidelines (EU, Turkey) (Yilmaz el al., 2005; Kronkvist, 2006) have suggested a maximum *Escherichia coli* level of not greater than 230 MPN.100 g^{-1} of shellfishes for consumer safety, and there should not be any *Salmonella*. Our results showed that *Escherichia coli* level in *Anadara granosa* from some locations evaluated (station S and P during October 2007, station L and G during December 2007) exceeded the acceptable limit for shellfish. The similar results have been reported by Sulaj et al. (2004) who noted that *Escherichia coli* level in bivalves from Albanian Coast ranged between 2 and 91000 MPN.100 g^{-1}. Kronkvist (2006) reported that *Escherichia coli* levels in bivalves collected from Maputo Bay Mozambique ranged between 110 and 170000 MPN.100 g^{-1}. In Marmara Sea Turkey, the level of *Escherichia coli* in bivalves varied from < 10 to 960 MPN.100 g^{-1} (Yilmaz et al., 2005) (Table 3). Shellfish contamination from polluted water is a serious and continuous problem. Some strains of *Escherichia* are highly pathogenic. Diseases caused by *Escherichia coli* include for example diarrhea, dysentery, haemolytic uremic syndrome (kidney failure), bladder infection, pneumonia and meningitis. Enterophatogenic strains of *Escherichia coli* are also becoming more frequently implicated in dysentery-like infections and generalized fevers, and are major causes of infant diarrhea in developing countries (DeVinney et al., 1998).

Table 3. Comparison of *Escherichia coli* and *Salmonella* levels in bivalves from different regions.
Tableau 3. Comparaison des niveaux de contamination en *Escherichia coli* et *Salmonella* de bivalves de différentes régions

Location	Species	*Escherichia coli* (MPN.100 g^{-1})	*Salmonella* (CFU.g^{-1})	Reference
Maputo Bay, Mozambique	*Meretrix meretrix*	110 - 170000	Positive (33% samples)	Kronkvist (2006)
	Eumarcia paupercula	400 - 3100	Positive (67% samples)	
Marmara Sea, Turkey	*Mytilus galloprovincialis*	< 10 - 590	Negative (100% samples)	Yilmaz et al. (2005)
	Venus gallina	48 - 960	Negative (100% samples)	
Albanian Coast	*Mytilus galloprovincialis*	200 - 91000	Positive (2% samples)	Sulaj et al. (2004)
Northern Ireland	*Bivalve mollusks*	-	Positive (8% samples)	Wilson & Moore (1996)
East Java Coast, Indonesia	*Anadara granosa*	Negative - 4800	Negative (100% samples)	This study

Regarding to *Salmonella* presence, none of the bivalve samples contained *Salmonella*. The absence of *Salmonella* in bivalves confirmed that this pathogen bacterium was more susceptible to coastal water salinity compared to *Escherichia coli*. Madigan et al. (2000) reported that when the coliform bacteria are excreted in the water, they die at a slower rate than pathogenic bacteria as *Salmonella* and *Shigella*. Pathogen accumulation in shellfish is reduced in salinity waters. The results of present study were in accordance with Yilmaz et al. (2005) who recorded that the bivalves harvested from the Marmara Sea did not contained *Salmonella,* but they were contaminated with high level of *Escherichia coli*. However, our results are in disagreement with Kronkvist (2006) who reported that 33 to 67% of bivalve samples from Maputo Bay Mozambique contained *Salmonella*. Sulaj et al. (2004) also reported that 2% bivalve mollusks collected from coastal waters contained *Salmonella* spp. in their tissues. Other finding reported that 8% *Salmonella* spp. isolation from bivalves collected from authorized harvesting beds in Northern Ireland (Wilson & Moore, 1996). *Salmonella* spp. are generally pathogenic to humans. The most common diseases are typhoid fever and gastroenteritis. In developing countries, these diseases occur and are most severe in children under ten years of age (Ambrus & Ambrus, 2004).

Results obtained from this work revealed that *Anadara granosa* harbour a lot of pathogenic microorganisms (especially *Escherichia coli*) that pose serious health risks to man. Considering the public health implications of the poor bacteriological quality of these shellfishes, particular attention should be paid to their safety through proper processing, storage and handling procedures. The

Table 4. Comparison of trace metal concentration (mg kg^{-1} wet weight) in bivalves from different regions.
Tableau 4. Comparaison des concentrations en métaux trace (mg kg^{-1} de poids frais) de bivalves de différentes régions.

Location	Species	Pb	Cd	Hg	Reference
Cross River, Nigeria	*Egeria radiata*	0.19 ± 0.18	0.064 ± 0.026	-	Etim et al. (1991)
Atlantic Coast of Southern Spain	*Donax trunculus*	0.22 - 0.72	0.038 - 0.040	-	Usero et al. (2005)
Senegal Coast	*Donax rugorus*	-	0.13 - 0.20	-	Sidoumou et al. (2006)
Victoria Beach, Australia	*Donax deltoides*	17 - 29	-	-	Haynes et al. (1997)
Gulf of Annaba, Algeria	*Donax trunculus*	0.30 - 0.70	0.02 - 0.04	-	Beldi et al. (2006)
Puget Sound, USA	*Mytilus* sp.	0.03 - 0.19	0.06 - 0.21	-	Acker et al. (2005)
Bellingham Bay, Puget Sound, USA	*Mytilus edulis*	-	-	0.12 ± 0.02	Rasmussen & Williams (1975)
	Crassostrea gigas	-	-	0.1 ± 0.02	
	Acema persona	-	-	0.12 ± 0.02	
Sungei Buloh and Sungei Khatib Bongsu, Singapore	*Polymesoda expansa*	0.28 - 0.64	0.03 - 0.06	-	Dang et al. (2005)
Mocha, West Coast of India	*Perna viridis*	30.56 - 113.30	1.08 - 16.81	ND - 0.15	Tewari et al. (2001)
Lake Geneva, Europe	*Dreissena polymorpha*	0.62	0.18	-	Berny et al. (2003)
Delta of Ebro River, Spain	*Cerastoderma edule*	0.14 - 0.55	0.07 - 0.28	-	Schuhmacher et al. (1995)
Seine Estuary, Europe	*Cerastoderma edule*	< 0.02 - 0.28	0.20 - 0.40	-	Miramand et al. (2001)
East Java Coast, Indonesia	*Anadara granosa*	< 0.0405	1.234 - 2.404	< 0.0014 - 0.048	This study

Note: ND= not detected, - = no data

introduction of enforceable microbiological guidelines as a way of protecting consumers appears to be highly desirable.

There are no previous data available on trace metal levels in bivalves from East Java Coast. The previous data presented only the concentration of Cd in shrimp and fish. The levels of cadmium in shrimp and fish were 0.003 to 0.013 mg.kg^{-1} and 0.003 to 0.009 mg.kg^{-1} respectively (Soegianto & Hamami, 2007).

The concentration of toxic metals (Pb, Cd and Hg) detected in bivalves was compared with reported values for other regions in an effort to determine the degree of contamination in the study area (Table 4). The concentrations of mercury found in *Anadara granosa* of the East Java Coast were lower than those recorded in bivalves from Puget Sound, USA (Rasmusssen & Williams, 1975) and West Coast of India (Tewari et al., 2001). Cd accumulations in *Anadara granosa* were higher than those reported in Cross River Nigeria (Etim et al., 1991), Delta of Ebro River, Spain (Schuhmacher et al., 1995), Seine Estuary (Miramand et al., 2001), Lake Geneva (Berny et al., 2003), Puget Sound USA (Acker et al., 2005), Singapore (Dang et al., 2005), Atlantic Coast of Southern Spain (Usero et al., 2005), Senegal Coast (Sidoumou et al., 2006), and Gulf of Annaba, Algeria (Beldi et al., 2006). On the other hand, Tewari et al. (2001) reported that Cd accumulations in bivalves collected from West Coast of India were higher compared to those from East Java Coast, Indonesia. The concentrations of Pb in *Anadara granosa* harvested from East Java Coast were relatively low as compared with values reported in some mollusks species from other countries (Etim et al., 1991; Schuhmacher et al., 1995; Haynes et al., 1997; Miramand et al., 2001; Tewari et al., 2001; Berny et al., 2003; Usero et al., 2005; Acker et al., 2005; Dang et al., 2005; Beldi et al., 2006).

The bivalves are important protein sources for some peoples who live at the coastal regions of East Java. It is necessary to note that bivalves are able to accumulate metals in excess of what occurs in the aquatic environment (Etim et al., 1991). Accumulation of trace metals can cause toxic reactions along the food chain (Clark, 1992). It has already been documented that mercury caused the death of many people in Minamata Japan from intake of seafood contaminated with toxic level of mercury (Kurland et al., 1960; Nitta, 1972). Similarly, the devastating effects of cadmium on animals were amply demonstrated by itai-itai disease in humans (Elinder & Jarup, 1996). Our studies reported that the concentrations of Pb and Hg in *Anadara granosa* were below the maximum acceptable limits of Indonesia, European Union (EU) and Australia (Table 2). However, the concentrations of Cd in shellfishes harvested from some locations of East Java Coast were higher than the maximum acceptable limit of EU (0.05 mg.kg^{-1}) and Australia (2 mg.kg^{-1}). This could lead to some adverse effects on human health. To anticipate the detrimental effects of metals to human health, therefore it is important to conduct a monitoring program which needed for health and environmental risk assessment.

Conclusion

The level of *Escherichia coli* in bivalves from some locations of East Java Coast exceeded the acceptable limit for shellfish for human consumption. In contrast, none of the bivalve samples from this region contained *Salmonella*. This study reported that all bivalves contain lead and mercury in their tissues below the range acceptable for consumption. Meanwhile, the concentrations of cadmium in some bivalve tissues were higher than the maximum acceptable limit. To anticipate the detrimental effects of pathogenic bacteria and trace metals to human health, a monitoring program should be conducted in order to assess the health and environmental risk.

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