Doctoral Dissertation

Health Risk Evaluation and the Investigation of the Potential Human Foetal Subacute Developmental Toxicities upon Exposures to Mercury-Contaminated Food Crops from Artisanal and Small-Scale Gold Mining Communities – A Case Study of Obuasi, Ghana

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Health Risk Evaluation and the Investigation of the Potential Human Foetal Subacute Developmental Toxicities upon Exposures to Mercury-Contaminated Food Crops from Artisanal and Small-Scale Gold Mining Communities – A Case Study of Obuasi, Ghana

小規模金採掘コミュニティーにおける水銀汚染農作物の健康リスク評価ならびに潜在的な亜急性発生毒性に関する研究 (ガーナ、オブアシのケーススタディ)

Sylvester Addai-Arhin

(シルベスタ アッダイアルヒン**)**

A dissertation submitted to the Graduate School of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, in partial fulfilment of the requirements for the Degree of Doctor of Philosophy (PhD) in Environmental and Symbiotic Sciences

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Abstract

Mercury (Hg) is one of the potent environmental toxicants with devastating effects on human health and the ecosystem. Its human health effects include cardiotoxicity, hepatotoxicity, nephrotoxicity, neurotoxicity, teratogenicity, gastroenteritis, haematological effects, etc. following short-term and/or long-term repeated exposures. It also affects the health of the ecosystem by destroying ecosystem functions. This study was aimed at evaluating the potential risks of Hg to humans and the entire ecosystem using soil and food crop (plantains and cassava) samples sourced from farms located near artisanal and small-scale gold mining (ASGM) facilities in selected ASGM communities around Obuasi, Ghana. Specifically, the study **1.** Evaluated the contamination levels of soils and the food crops and assessed the potential ecological risks using the Hakanson model, **2.** Evaluated the potential human health risks of long-term repeated exposures to total mercury (THg) and methylmercury (MeHg) levels of the soil and the food crop samples using the United States Environmental Protection Agency (USEPA) risk assessment models, and **3.** Investigated the potential subacute developmental toxicities and the genotoxicity of very low MeHg and high inorganic mercury (InHg) levels of Hg-contaminated food crops following repeated prenatal exposures using the Organization for Economic Cooperation and Development (OECD) bioassay method for medaka extended one generation reproduction test (MEOGRT) and the real-time quantitative polymerase chain reaction (RT-qPCR) for gene expression analysis, respectively. Results showed that the Hg levels of the soil samples were above the 500 µg/kg reference value for agricultural soils by the Swiss Environmental Regulation while the edible parts of plantains from Odumase and the edible parts of all cassava samples were above the 100 µg/kg reference value for food by the World Health Organization (WHO). Exactly 50% of plantain peels and the peels of all cassava samples also had Hg levels above the 100 µg/kg reference value for plants used as animal feed by the United Nations Economic Commission for Europe. Assessment of the contamination levels and the ecological risk of samples showed that all samples from the studied communities had some degree of contamination, hence were associated with some levels of ecological risks ranging from low to very high risks. Assessment of the potential human health risks indicated that residents of the studied communities are at risk of the non-carcinogenic human health effects of Hg since the hazard quotient (HQ) values for THg of plantains from Odumase and cassavas across the study areas were above 1. The HQ values for THg of the soil samples,

despite their higher Hg levels were below 1, hence may not pose any non-carcinogenic human health risks to residents. HQ values for MeHg of all the samples were also below 1, hence long-term repeated exposures to MeHg levels of the samples may not cause any non-carcinogenic human health effects. This meant that the health effects associated with plantains from Odumase and all cassava samples would be specific to InHg due to its extremely higher levels (~99.5%) in the food crops. The hazard indices of the selected farms were also above 1, hence long-term repeated exposures to the samples, particularly the food crops may pose potential non-carcinogenic human health effects to residents across the studied communities. The subacute toxicity study also showed that the maternal and cord blood MeHg levels of 0.20 – 1.63 µg/L and 0.33 – 2.77 µg/L, respectively, following repeated prenatal exposures to 0.0035 – 0.029 µg/kg bw/day of the food crops were below the 5.8 and the 3.6 µg/L blood MeHg reference values by USEPA and other researchers, respectively. This indicated that repeated prenatal exposures to MeHg levels of the food crops may not be associated with any subacute developmental toxicities to the human foetus or the new-born. For InHg, although very low but significant percentage of the prenatal exposure amount in the range of 1.00 – 5.14 µg/kg bw/day could reach cord blood and possibly the foetus, it was uncertain whether such low but significant levels can cause subacute developmental toxicities to the foetus or the new-born due to the non-applicability of the one-compartment dose conversion model and the lack of guideline value for blood InHg levels. However, considering the 5.8 and the 3.6 µg/L blood MeHg reference values by USEPA and other researchers, and the fact that the body Hg burden is always made with reference to MeHg, it can be concluded that the very low MeHg and high InHg levels of the food crops may not be associated with any subacute developmental toxicities to the human foetus or the new-born following repeated prenatal exposures. For the genotoxicity assessment, both MeHg and InHg caused the suppression of cyclin B1 gene. Cyclin B1 suppression led to disruption in cell cycle and mitotic cell division, which in turn resulted in cell growth and development and eventual death of the medaka embryos, particularly those exposed to the 148.2 – 338.3 µg/L solutions of InHg. These deductions meant that the MeHg and InHg levels of the food crops may be toxic to the human foetus at the genetic level. Additionally, the decreased in expression level of cyclin B1 by InHg showed that InHg may be associated with tumorigenesis or may be a potential tumour initiator at the genetic level. Generally, Hg contamination of the samples and the entire ecosystems resulted from ASGM activities. Despite the higher probability of no subacute developmental toxicities to the human

foetus or the new-born, it is evidently clear from the study, particularly the ecological risks, human health risks, and the genotoxicity assessments that Hg releases and subsequent contaminations have detrimental effects on human health and ecosystem functions, particularly upon long-term repeated exposures. Such detrimental effects can take several years to manifest and when they manifest the aetiology is usually unidentifiable. Therefore, since there are no treatment processes for Hg within the catchment areas and Ghana at large, regular and strict monitoring of ASGM activities, particularly Hg releases from ASGM facilities is required to preserve the integrity of the ecosystem and prevent the future occurrence of any toxic effects of Hg to humans, particularly the younger generation.

Key words: Plantain, Cassava, MeHg, InHg, Developmental toxicity, Genotoxicity, Cyclin B1, ASGM

Dedication

This research work is dedicated to my wife, children and my entire family, both nuclear and extended, PhD advisors, friends and loved ones including Dr Huiho Jeong, Miss Nana Hirota, and Mr. Viorel Ristea and to the ever loving memories of my late father, Mr. Daniel Akwasi Addai and my late father-in-law, Mr. Stephen Akwasi Boakye.

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Chapter One

Introduction

1.1 Background

Mercury (Hg) has been in use since antiquity although its uses such as in dental amalgams and other industrial applications such as in pharmaceuticals, wood preservation, dry battery depolarization, leather tanning, etc. (Broussard et al., 2002) are gradually reducing recently due partly to the implementation of the Minamata Convention on Mercury in 2017. The Minamata Convention on Mercury is a global treaty that was adopted in 2013 with the main aim to protect humans and the environment from the adverse effects of Hg by reducing or possibly eliminating the anthropogenic sources of Hg, especially in ASGM operations, coal-fired power plants, etc. (USEPA, 2021a). It has been approximately five (5) years after the implementation of the treaty, Hg is still largely being used in Artisanal and small-scale gold mining (ASGM) operations, especially in the developing world for gold purification (Novirsa et al., 2020; Addai-Arhin et al., 2021, 2022) despite its toxic effects. However, it is expected that through the treaty, the next decades will see a considerable reduction in anthropogenic pollution of Hg, especially from the ASGM sectors, coal-fired power plants, non-ferrous metal production, etc. (USEPA, 2021a).

Hg pollution results from both natural and anthropogenic emission sources with ASGM constituting the largest (about 37%) of anthropogenic Hg emissions globally (Fig. 1) (Pirrone et al., 2010; UNEP, 2013; USEPA, 2016, 2021b). Although natural emission sources such as volcanoes, bushfires, evaporation from ocean surfaces, etc. may be significant, anthropogenic sources are extremely enormous and have contributed significantly to increased Hg levels in the global environment (Fig. 1). This has been due mainly to humans' quest for industrialization and economic growth (Eze et al, 2018; Anyanwu et al., 2020), especially in most developing countries where there is virtually no proper treatment processes for the generated waste. However, it is believed that reduction or elimination in anthropogenic releases of Hg such as in ASGM and other industrial applications will drastically reduce Hg pollution and the associated adverse health effects on humans and the environment.

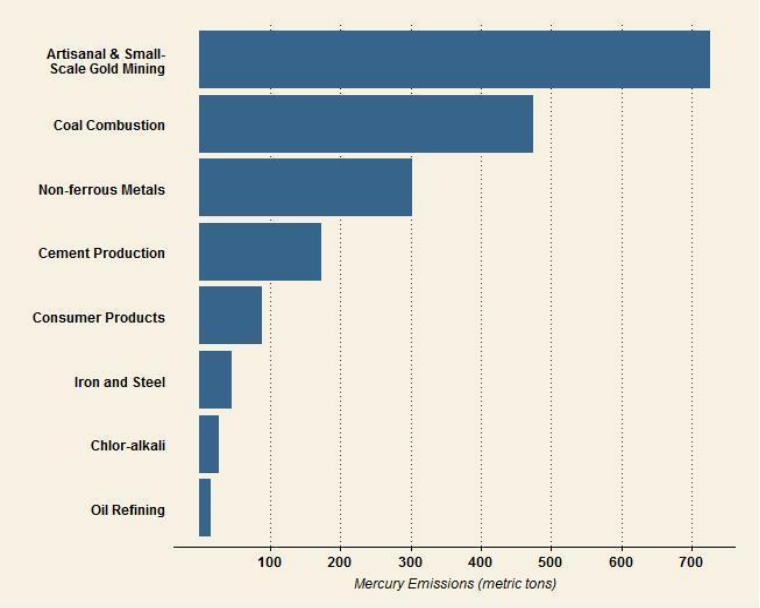

Fig. 1.1: Mercury emissions from the eight highest anthropogenic sources. Total global anthropogenic emissions estimated around 1960 metric tons (UNEP, 2013; USEPA, 2016).

The long-range transboundary transport especially in air (Durnford et al., 2010; Huang et al., 2016), non-biodegradability, high bioaccumulation potential, toxicity even in low doses, and the high methylation potential, especially within the aquatic biota have made Hg one of the most potent environmental toxicants. Hg is classified by the Agency for Toxic Substances and Disease Registry (ATSDR) as the third most toxic substance besides arsenic and lead (Rice et al., 2014) which occupy the first and the second positions, respectively. This means that the toxicity of Hg is well documented, especially after the Minamata disease in the mid-1950s which resulted from methylmercury (MeHg) poisoning through ingestion of Hg-contaminated fish and shellfish (Broussard et al., 2002; Hachiya, 2006; Yorifuji and Tsuda, 2014) and the Iraq MeHg poisoning which also occurred in the early 1970s through ingestion of bread produced from seed grains that were treated with a fungicide containing MeHg (Broussard et al., 2002). Nevertheless, there is little or virtually no information on Hg toxicities related to food crops such as plantains and cassavas since most studies on Hg risks or toxicities have centred on fish. Moreover, studies on Hg risk or toxicity still remains relevant due to the continuous use of Hg in ASGM operations and the incessant discharge of its waste into the environment.

Generally, Hg causes toxic effects by precipitating proteins (Broussard et al., 2002; Bernhoft, 2012) and inhibiting or interfering with DNA synthesis (Bernhoft, 2012), inhibition of enzymes (Broussard et al., 2002) and being corrosive, especially to the intestines (Broussard et al., 2002; Bernhoft, 2012). The affinity for sulfhydryl or thiol (SH), carboxyl, phosphoryl, amide, and amine groups ensure easy reaction of Hg with proteins including enzymes having any of such groups. The reaction, therefore, causes inactivation of proteins or enzyme inhibition (Broussard et al., 2002). This leads to any of the effects such as nephrotoxicity, neurotoxicity, immunotoxicity (Bernhoft, 2012; Oliveira et al., 2012; Ou et al., 2014), gastrointestinal toxicity (Broussard et al., 2002; Bernhoft, 2012), mutagenicity, genotoxicity, hepatotoxicity, teratogenicity, cardiotoxicity, haematological effects, and in some cases carcinogenicity (Ou et al., 2014).

Incessant exposure to Hg or its compounds may cause any of the indicated detrimental health effects and the long-term or short-term occurrence and the severity of such toxic effects may depend on the chemical form (i.e., elemental Hg (Hg^o), inorganic Hg (InHg) or MeHg), the dose, frequency of exposure, duration of exposure, route of exposure (Bernhoft, 2012; Rice et al., 2014; Teixeira et al., 2018), and probably the health status of the individual under exposure.

Hg^o is widely distributed and accumulated within the body tissues but the brain is the major site for its toxicity (Langford and Ferner, 1999; Bernhoft, 2012). It is highly lipid soluble (Broussard et al., 2002) and has high affinity for biomolecules with SH groups such as cysteine, glutathione and some enzymes such as the sodium-potassium ATPase and porphobilinogen synthase (Oliveira et al., 2012). This accounts for its wide distribution, especially within the lipophilic compartments of the body and its ability to cross the placenta and the blood brain barrier (BBB) (Broussard et al., 2002). The major route of exposure of Hg^o is inhalation (through the lungs) due to its high volatility even at room temperature, hence the atmosphere is the major exposure source contributing to about 70 – 85% absorption rate of Hg^o (Broussard et al., 2002; Bernhoft, 2012; Ou et al., 2014) but dental amalgams, to some extent, may be another exposure source (Ou et al., 2014). The average half-life of Hg^o is 60 days in a range of 35 – 90 days. This is suggestive of high retention time within the brain upon exposure and its toxicity usually results in neurological disorders due to its effects on brain cells (Broussard et al., 2002).

InHg, which is majorly available as salt of either Hg (I) or (II) compounds have different levels of toxicity due to differences in solubility. Hg (II) compounds are usually more soluble and toxic than Hg (I) compounds (Langford and Ferner, 1999; Bernhoft, 2012; Teixeira et al., 2018). InHg also causes toxicity to the renal, cardiovascular, reproductive, hepatic and gastrointestinal tract systems (Peixoto and Pereira, 2007; De Freitas et al., 2012; Gado and Aldamash, 2013; Kalendar et al., 2013; Omanwar et al., 2011, 2013; Joshi et al., 2014). Teixeira et al., (2018) stated that repeated exposure to low doses of InHg can result in oxidative stress, cell death and functional deficits within the motor cortex. Generally, InHg salts have low lipid solubility and do not easily cross the BBB and the placenta, hence InHg deposits are rarely found in the brain (Broussars et al., 2002; Bernhoft, 2012; Park and Zheng, 2012; Teixeira *et al*., 2018). However, the ionization of Hg^o in the brain, the slow elimination of InHg and long-term repeated exposures to InHg (Broussard et al., 2002) as well as its transport by amino acid transporters such as the cysteine (Bernhoft, 2012) can cause InHg deposits in the brain (Broussard et al., 2002). According to Broussard et al., (2002), the inability of InHg to easily cross the BBB and the placental barrier may also be attributed to its charge since charge particles have low membrane permeability. The primary exposure pathway is ingestion (through food or water) and only approximately 7 – 15% of the ingested amount is absorbed from the gastrointestinal tract upon exposure. InHg has high affinity for metallothionein (Broussard et al., 2002) but can also bind with SH groups on erythrocytes within the bloodstream or be suspended in plasma (Bernhoft, 2012). The high affinity for metallothionein accounts for its high accumulation in renal cells (Broussard et al., 2002; Bernhoft, 2012) since renal cells have high levels of metallothionein. Besides the kidneys, some percentage of InHg, especially in the form of Hg^{2+} can be found in the liver, epithelial tissues, choroidal plexus, and testes (Bernhoft, 2012). The half-life of InHg is about 40 – 42 days and its major excretory pathways are through faeces and urine (Broussard et al., 2002; Bernhoft, 2012) although some percentage can be excreted through sweat, saliva, and breast milk (Bernhoft, 2012).

Organic mercury compounds include both alkyl organic mercurial (OMs) such as methylmercury (MeHg), dimethyl mercury, ethyl mercury, etc. and aryl OMs e.g., phenyl mercury. The OMs are also highly lipid soluble or lipophilic and have high affinity for SH groups on cysteine, glutathione and other biomolecules (Broussard et al., 2002; Li et al., 2010; Bernhoft, 2012; Rice et al., 2014; Chan, 2019). The high lipophilic nature of OMs and their high affinity for

SH groups enhance their absorption from the GIT more readily than InHg. This is achieved by complexing with cysteine, which enables their passage across cell membrane on larger amino acid carriers (Broussard et al., 2002). Moreover, like Hg^o, the high lipophilic nature and SH affinity of OMs ensure their passage across the BBB and the placenta as well as their wide distribution and accumulation within body compartments such as the brain, liver, skin, hair, kidney, etc. but the brain is the major target organ (Broussard et al., 2002; Li et al., 2010; Bernhoft, 2012; Rice et al., 2014; Chan, 2019), hence OMs are mainly neurotoxic but can also be teratogenic, nephrotoxic, mutagenic, genotoxic, hepatotoxic as well as cause haematological and immunological effects (Broussard et al., 2002; Ou et al., 2014). MeHg and dimethyl mercury are the most toxic of all OMs but MeHg is the most prevalent and abundant (Bernhoft, 2012) probably due to its easy formation from the methylation of available InHg through the action of some microorganisms, particularly within the aquatic biota (Ou et al., 2014). Therefore, toxicity of OMs is always made with reference to MeHg (Bernhoft, 2012). The higher toxicity of MeHg than InHg is due to its lipophilicity which in turn, enhances its absorption and wider distribution within the human body. Moreover, ability to bio-accumulate also contributes to its higher toxicity. The absorption rate of MeHg is around 85 – 95% upon exposure and its elimination half-life is around 65 – 70 days with approximately 90% excreted through faeces (Broussard et al., 2002; Bernhoft, 2012). The major exposure pathway of MeHg is ingestion, especially through seafood such as fish and shell fish (Broussard et al., 2002; Ou et al., 2014) although it has been shown recently that rice may be another exposure source, particularly in regions where rice is a staple food (Ou et al., 2014; Novirsa et al., 2020).

Repeated exposures to Hg during pregnancy can have devastating nervous effects on the embryo since Hg, particularly MeHg or Hg^o easily crosses the placenta to the foetus. Foetal or childhood exposures to Hg begins from gestation through maternal – foetal transfer, and food consumption is the major exposure source (WHO, 2008). According to Mandana, (2016), an embryo's nervous system is very vulnerable to toxicants and exposure to these toxicants, especially during the third week of pregnancy can cause central nervous system (CNS) problems such as defects in attention, behaviour, cognition, and motor skills. Pregnant women may not show signs of Hg intoxication but the effects which result from mother – foetal transfer during gestation are likely to be seen in the foetus or the new-born soon or sometime after birth.

Studies on human embryonic or foetal developmental toxicities upon exposure to InHg is not well established in literature due to its poor passage across the placenta and the BBB. The poor passage of InHg across the placenta and the BBB, therefore, suggests that some amount of InHg, irrespective of how low, can be transferred from the mother to the foetus upon repeated prenatal exposures. This is evidenced in studies by Ask et al., (2002), Walker et al., (2006), Kim et al., (2015), etc. which directly and indirectly indicated levels of InHg in maternal and cord blood. Moreover, the percent maternal-foetal transfer of InHg may be dependent on the prenatal exposure doses. This means that prenatal exposures to very high levels such as in plantains and cassavas used in this study, can result in significant percentage of maternal-foetal transfer, and such levels may be detrimental to the foetus (WHO, 2008) either prenatally or postnatally or both.

The developmental toxicities of Hg is characterised with several neurophysiological and mental defects following prenatal exposures (Boischio, 2015; Mandana, 2016). Mandana, (2016) indicated how researchers from Kumamoto University, Japan, described two cases of children who were affected by cerebral palsy after the MeHg poisoning from the Minamata bay in the mid-1950s. According to Mandana, (2016), the researchers observed that the affected children were underdeveloped, could not move purposefully, had poor mental development and experienced convulsions. The researchers also observed a severe impact of MeHg on the CNS, which caused degeneration and decreased number of neurons in the brains of the children. Particularly, there was a disappearance of granule and pyramidal cells, narrowing of the molecular layer, reduction in central white matter, diffused atrophy of the foliar within the hemisphere of the cerebellum, myelination of the hindbrain, especially in the pons and the medulla oblongata and the elimination of Purkinje cells, which ensure proper functioning of the cerebellum, which in turn, is responsible for movement coordination and posture maintenance.

Another case of developmental defects of Hg was reported by researchers from Baghdad University, Iraq and Rochester Medical Centre, Rochester, New York, of two infants who were prenatally exposed to MeHg during the MeHg intoxication in Iraq in the early 1970s. The researchers stated that one of the infants died after thirty-three days and the other immediately after birth. Post-mortem analysis showed high MeHg levels in their blood which were responsible for the severe abnormal neuronal migration in the cerebral and cerebellar white matter. There were also disorganization and misalignment of cerebral cortex neurons (Mandana, 2016).

Bland and Rand, (2006) also showed that MeHg alters the notch signalling between developing nerve cells in fruit fly embryos. The notch signalling pathway mechanism in developing organisms has effect on cell fate, proliferation, migration, and neurite growth.

Tamm et al., (2006), reported that cells died when neural stem cell line from mice and primary embryonic cortical neural stem cells from rats were used to investigate the effects of MeHg exposure. Tamm et al., (2006) concluded that the cell death caused a reduction in the number of progenitor cells in cell population.

In 2008, some Brazilian and USA researchers also concluded that MeHg caused impaired glutathione antioxidant system in new-born mice when they were exposed to MeHg as embryos.

Another group of researchers from Egypt and USA also observed that zebrafish embryos had delayed neuronal development at low concentrations while there was extremely higher mortality especially in embryos which could not develop neural tubes upon exposure to higher levels of MeHg (Mandana, 2016).

According to Bernhoft, (2012), higher prenatal intoxication of MeHg induces cerebral palsy while exposure to lesser doses prenatally is characterised by neurodevelopmental delays and cognitive defects.

Engel and Rand, (2014) reported that MeHg caused a disruption in the production of a key protein in developing muscles, which in turn, caused a disruption in the signals between motor neurons and the muscles.

Drwiega, (2016) also reported that children below 12 years whose mothers have been involved in ASGM activities in some ASGM hotspots in Indonesia, had devastating health effects which included frequent seizures, cleft lips and palates, hydrocephalus, microcephaly, born without anus, born without complete fingers, toes and limbs, clubfoot, cerebral palsy, and leukaemia.

Additionally, Wang et al., (2019) found a significant decrease in neonatal behavioural development upon prenatal exposures to low Hg levels and concluded that there is the need to reduce exposure levels of Hg through fish consumption, particularly for women of childbearing age.

Llorente Ballesteros et al., (2020) in their study found that about 12% of pregnant women in Madrid, Spain, had MeHg levels above the USEPA reference level of 5.8 µg/L while 31% were above the 3.6 µg/L benchmark suggested by other researchers. They stated that Spanish women of childbearing age continue to have increased MeHg and there is a probability that about 19,000 infants born within Madrid, Spain, each year may be at risk of MeHg upon repeated prenatal exposures through fish consumption. Hence, it was advisable for these women to regulate the intake of fish per week.

Other studies including Ursinyova et al., (2019), Sekovanić et al., (2020), Stratakis et al., (2020), Sakamoto et al., (2021), and Shih et al., (2021) estimated the Hg levels in maternal and cord blood of pregnant women to assess the potential prenatal exposure to Hg through fish consumption. These and many other studies suggest that Hg causes serious developmental defects or risks upon frequent prenatal exposures, particularly through food consumption with majority of the defects, if not all, directed towards the CNS.

With ASGM as the largest Hg emission source globally and ingestion especially food consumption as the commonest exposure pathway of mercury, this study primarily aimed at evaluating the potential ecological and human health risks following exposures to Hg-contaminated plantains (Fig. 1.1) and cassavas (Fig. 1.2) (staple food) sourced from farms located near ASGM facilities in selected ASGM communities around Obuasi, Ghana.

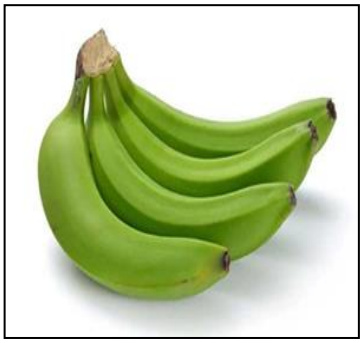

Fig. 1.2: Plantain (*Musa paradisiaca*)

Fig 1.3: Cassava (*Manihot esculenta*)

1.1.1 Problem of the Study

Ghana has several gold deposits as shown in Fig. 1.3. As a result, ASGM facilities are scattered mostly within the rural communities of the regions with gold deposits. According to Clifford, (2017), Ghana has about 500,000 to 1,000,000 miners in the ASGM sector. The ASGM sector produced about 1123 tons of gold between 1980 and 2008 and contributed about 25% of the total gold production in 2010. The 1123 tons of gold led to about 1123 – 2245 tons of Hg emissions into the environment between 1980 and 2008 with total emissions of about 79 – 158 tons only in 2008 considering a gold production: Hg emissions ratio of 1:1-2. Additionally, an estimated 50 and 40 tons of gold were produced by the ASGM sector in 2018 and 2019, respectively. These also resulted in an estimated release of 50 – 100 and 40 – 80 tons of Hg into the environment in 2018 and 2019, respectively. These releases of Hg raise serious environmental and toxicological concerns, particularly when global average emission of Hg by the ASGM sector is estimated around 727 (410 – 1040) tons annually (UNEP, 2013).

The proliferation of ASGM activities in Ghana has resulted in ASGM facilities being sited near food crop farms, particularly in rural communities where crop farming is also considered one of the major economic activities. The miners with little or no knowledge of the environmental and the human health effects of Hg (UNEP, 2013; Novirsa et al., 2020), ignorantly and incessantly discharge Hg waste from the facilities into the farms. This may lead to contamination of the food crops, especially plantains (*Musa paradisiaca*) and cassavas (*Manihot esculenta*), which are among the staple food crops eaten by about 80 – 85% Ghanaians daily, hence are commonly cultivated in most farms within the rural communities. Long-term frequent releases of Hg into the environment can have detrimental effects on the entire ecosystem. Additionally, long-term human exposures to Hg-contaminated food crops through ingestion may be associated with adverse human health effects, particularly to the human foetus following repeated prenatal exposures.

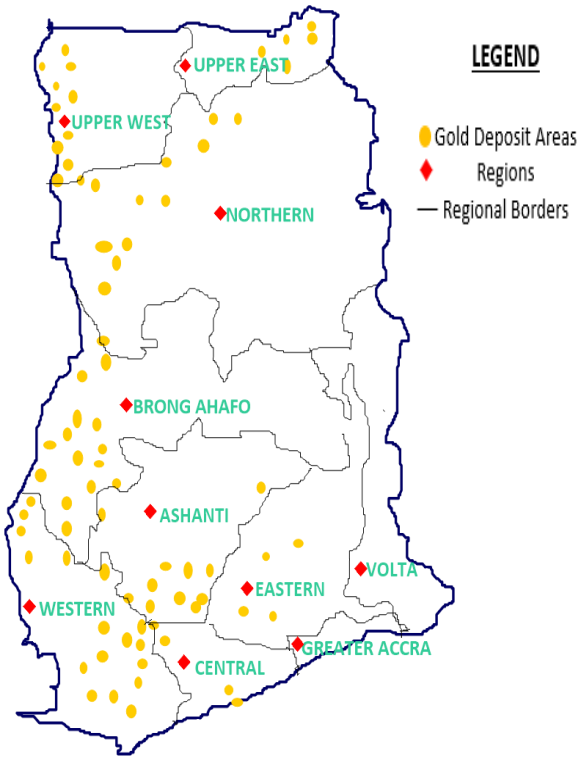

Fig. 1.4: Map of Ghana showing the various gold deposit areas. Greater Accra and the Volta are the only regions without gold deposit.

Fig. 1.5: Some ASGM activities in Ghana (Ghana Environmental Protection Agency, 2018; Swira, 2019).

1.1.2 Objectives of the Study

The specific objectives were to:

a. Determine the contamination levels of soil, plantain, and cassava samples and assess their potential ecological risks using the Hakanson, (1980) model. Ecological risk in this context referred to risk of Hg contamination to the entire ecosystem i.e., risk to soil, water bodies, plants, animals, and humans.

b. Evaluate the potential human health risks of long-term repeated exposures to total mercury (THg) and the MeHg levels of the soil, plantain, and cassava samples using the United States Environmental Protection Agency (USEPA, 2004 and 2007) risk assessment models.

c. Investigate the potential subacute developmental toxicities and the genotoxicity of very high InHg and low MeHg levels of Hg-contaminated plantains and cassavas upon repeated prenatal exposures using Japanese medaka (*Oryzias latipes*) embryos as models.

These three specific objectives constituted the chapters 2, 3, and 4 of this study, respectively.

1.1.3 Significance/Justification of the Study

The inevitable use of Hg in ASGM in Ghana and its frequent releases into the environment indicate that humans and the entire ecosystem are potentially at risk of the devastating effects of Hg. The Hg poisoning in Minamata, Japan and Iraq also suggest that food ingestion has been the major cause of Hg poisoning. This means that long-term repeated exposures to Hg-contaminated plantains and cassavas can cause serious adverse human health effects of Hg. Many studies on developmental toxicities of Hg, particularly MeHg have centred on fish due probably to the higher MeHg levels and its higher bioaccumulation in Fish. There are virtually no studies on the developmental toxicities of Hg in food crops such as plantains and cassavas, which are staple food in Ghana, and contain very low and high levels of MeHg and InHg, respectively.

Although MeHg levels in these food crops are extremely lower compared to fish (Addai-Arhin et al., 2021, 2022), the levels may be significant enough to cause prenatal or postnatal foetal abnormalities upon frequent or repeated prenatal exposures due to its higher absorption rate (85 – 95%) (Broussard et al., 2002; Bernhoft, 2012; Rice et al., 2014), higher bioaccumulation (Broussard et al., 2002; Bernhoft, 2012), and its ability to cross the placenta and the BBB (Broussard et al., 2002; Bernhoft, 2012; Ou et al., 2014). For InHg, although it has extremely lower absorption rate (7.5%), and poor passage across the placenta and the BBB, the extremely higher levels in the food crops used in this study also suggest that some percentage, irrespective of how low, can be transferred from the mother to the foetus upon repeated prenatal exposures. Such maternal-foetal transfer amount of InHg can potentially cause foetal developmental abnormalities as indicated by WHO, (2008).

Additionally, the higher vulnerability and sensitivity of the developing foetus to Hg intoxication (Health and Environment Aliance, 2002; Patel et al., 2019), and the fact that there is no known safe level of exposure to environmental Hg, particularly during pregnancy (Health and Environment Aliance, 2002; Bose-O'Reilly, 2010) suggest that very low Hg levels can be detrimental to the developing foetus or the new-born through gradual bioaccumulation. Therefore, there is the need to continually monitor the Hg levels in these food crops and evaluate the potential human health risks associated with such levels upon long-term repeated exposures or repeated prenatal exposures. Furthermore, the inevitable and/or continuous use of Hg in ASGM requires

constant monitoring of Hg releases into the ecosystem to protect the health of humans and the ecosystem.

1.1.4 Scope/Limitations of the Study

The study wholly centred on the potential risks of Hg-contaminated plantains and cassavas sourced from farms located near ASGM facilities in selected ASGM communities around Obuasi, Ghana. As stated in the specific objectives, the risks included **1.** Ecological risks of the farms from which samples were obtained, **2.** Potential human health risks of Hg-contaminated samples, and **3.** Potential subacute developmental toxicities and the genotoxicity which focused on repeated prenatal exposures to low MeHg and high InHg levels through frequent ingestion of Hg-contaminated plantains and cassavas. The human health risk of Hg was narrowed down to the developmental toxicities and the genotoxicity due to the vulnerability and the sensitivity of the foetuses and new-borns to Hg intoxication and its irreversible effects on them. The study was limited to ASGM because it is the only major source of Hg emissions in Obuasi and its surrounding communities. Moreover, plantains and cassavas were used as main samples in this study because they are staple diets for many Ghanaians, hence considered suitable tools for Hg risk assessment.

Chapter Two

Ecological Risk Assessment of the Samples and Farm Ecosystems

Publication

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2.1 Introduction

This chapter detailed the effects of Hg waste from ASGM activities on the ecosystems of the study areas under consideration in this study. The effects of Hg on the components of a particular ecosystem are interconnected i.e., the effect on one component may lead to the effect on another component (Fig. 2.1) since these components are inter-dependent. The totality of these effects show up on ecosystem functions which can later lead to the destruction of the ecosystem integrity. Therefore, this chapter evaluated the Hg contamination levels of samples and the study areas (farms) and their associated potential ecological risks using the Hakanson, (1980) model for ecological risk assessment.

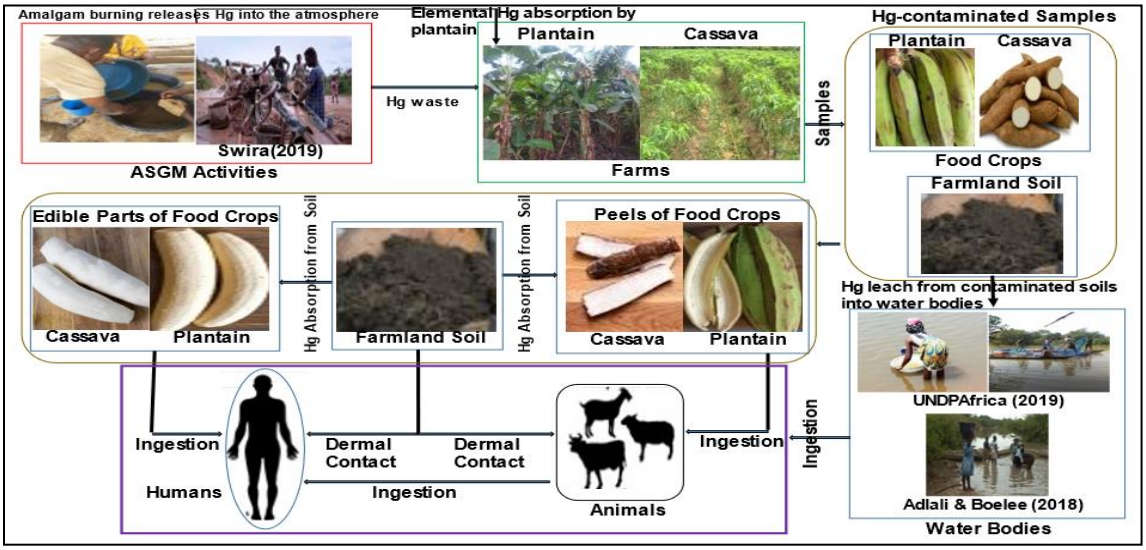

Fig. 2.1: Hg Waste Flow within the Ecosystems under consideration (Addai-Arhin et al., 2021).

2.2 Materials and Methods

2.2.1 Study Area

Obuasi, one of the largest gold mining towns in Ghana, is the capital of Obuasi Municipal. It is part of the Ashanti Region of Ghana (Fig. 2.2) and harbours Anglogold Ashanti, one of the largest and lucrative mining companies in Africa (Bedu-Addo et al. 2018; Akoto et al., 2018). It is located at latitude 5.35 ^{o}N and 5.65 ^{o}N (Bedu-Addo et al. 2018; Akoto et al., 2018) and has a total land mass of approximately 162.4 km^2 (Bedu-Addo et al., 2018; Akoto et al., 2018) with about 287,000 inhabitants (Akoto et al., 2018). It has around 63 urban communities (Bedu-Addo

et al., 2018) and surrounded by several rural communities including Tweapease, Nyamebekyere, Ahansonyewodea, and Odumase (Fig. 2.2). Obuasi has a very mountainous landscape (Akoto et al., 2018) with savanna climate (Bedu-Addo et al., 2018; Akoto et al., 2018). It has two rainy seasons in a year with an average annual precipitation of 1450 mm (Bedu-Addo et al., 2018). The average annual temperature is 25.5 ^{o}C with 23 ^{o}C and 30 ^{o}C as the minimum and the maximum, respectively while relative humidity falls between 75 and 80 % in wet seasons (Bedu-Addo et al., 2018; Akoto et al., 2018). Obuasi is counted among the richest gold deposits in West Africa. The gradual transformation of Obuasi into a modern mining town began in the latter part of the 19th century and is currently one of the densely populated towns in Ghana (Akoto et al., 2018). Mining is the main economic backbone of the inhabitants of Obuasi and its surrounding communities with a greater percentage of the ASGM facilities located in the surrounding rural communities.

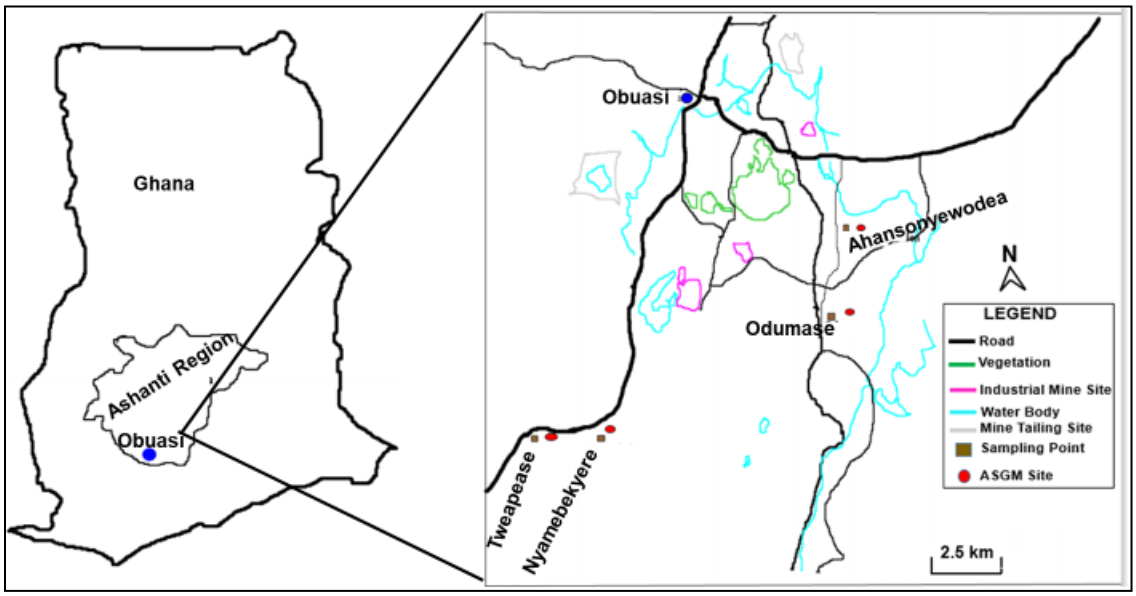

Fig. 2.2: Map of the Study Area (Addai-Arhin et al., 2021, 2022b).

2.2.2 Sampling

Sampling was done in August 2019. A total of twelve (12) farms and four (4) ASGM facilities with three (3) farms and one (1) ASGM facility from each selected community were used in this study. The selection of the farms was based on their proximity to the ASGM facilities. Farmland soils, plantains and cassavas were randomly sampled from five (5) different sampling locations in each farm. Plantains and cassavas were used as samples because they are staple diets

for many Ghanaians, hence cultivated in most farms. Farmland soil samples were sampled from 15-20 cm below the topsoil using a new stainless-steel machete (40 cm length x 7 cm width) from Crocodile Machete Ltd, Tema, Ghana. The farmland soils were used as samples because they serve as sinks for contaminants, hence a source of contaminants bioaccumulation in plants. A total of 165 samples with five (5) samples from each sample group (farmland soils, plantains, and cassavas) were obtained from each farm at Tweapease, Nyamebekyere, Ahansonyewodea, and Odumase but no cassava samples were obtained from the farms at Odumase.

2.2.2.1 Sample Preparation

The plantain and cassava samples were washed and peeled. The edible parts and the peels were cut into pieces for easy drying. The farmland soils, edible parts, and the peel samples were dried at room temperature for three weeks. The edible parts and the peels of plantains and cassavas were powdered, and all the samples homogenized or sifted using sieve of mesh size 120 µm. The same samples from each farm were composited and re-homogenized or resifted. Approximately 1 kg of each re-homogenized or resifted composite sample was prepared. A total of 54 composite samples including the peels were obtained. The plantain and the cassava peels were also used as samples because they are used as feed for both free and non-free-range animals such as goats, sheep, and cattle, hence form an integral part of the farm ecosystems under consideration (Fig. 2.1) in this study. The 54 composite samples were resifted the second time using a sieve of mesh size 150 µm to obtain finer particles and kept in a cold room at -23 ^{o}C for Hg content analysis. All the samples were dried at 35 ^{o}C using Isuzu auto-tuning control drying oven system, DS type (Isuzu Seisakusho Co. Ltd, Niigata, Japan) prior to instrumental analysis.

2.2.3 Experimentation

2.2.3.1 Cleaning and Decontamination

All glassware and plastic-ware were washed in detergent solution, rinsed severally with milli-Q water (Barnstead Smart2Pure, ThermoFischer Scientific, USA), sonicated for ten (10) minutes using ultrasonic cleaner AU-166C from Aiwa Medical Industry Co. Ltd, Tokyo, Japan and soaked in 10 % (v/v) nitric acid for 48 hours. They were again rinsed severally with milli-Q water after removal from nitric acid solution and kept in a dryer at 35 ^{o}C before analysis.

Sample boats were washed in detergent solutions, rinsed twice with distilled water, sonicated for 15 minutes using the ultrasonic cleaner and soaked in 10 % (v/v) nitric acid solution for 24 hours. The sample boats were rinsed thrice with milli-Q water after removal from nitric acid solution and dried using a hand dryer. The sample boats were then burnt in an Isuzu auto-tuning control furnace system, AT-S13 (Isuzu Seisakusho Co. Ltd, Niigata, Japan) at 750 ^{o}C for six (6) hours. The same burning temperature and time were used for the burning of additive B. Additive B is used as a cover for solid samples and as an absorbent for liquid samples, hence use as a blank in Hg analysis with the MA-3000 equipment. It is a white potassium iodide granule manufactured by FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan and supplied by Nippon Instruments Corporation, Tokyo, Japan.

2.2.3.2 Reagents and Standard Solutions for Method Calibration

All reagents used were of analytical grade and obtained from FUJIFILM Wako Pure Chemical Industries, Osaka, Japan. Calibration standard solutions of concentrations 10 ppb, 100 ppb and 1 ppm Hg^{2+} in 5 % (v/v) nitric acid solution were prepared serially from 10 ppm Hg^{2+} standard stock solution. The calibration standards were stored at 4 ^{o}C prior to analysis.

2.2.3.3 Soil Organic Matter (SOM) and pH Determinations

SOM content was determined using the method described by Akoto et al., (2018). Approximately 1 g of dried farmland soils was weighed into ceramic crucible using AS ONE analytical/electronic balance, (AS ONE Corporation, Osaka, Japan) and burnt using the auto-tuning furnace control system at 600 ^{o}C for five (5) hours. The % loss in weight was calculated as SOM using the equation:

$\frac{W_1 - W_2}{W_1} \text{x } 100\ \% = \text{SOM } (\%). - - - -$ equation 2.1 Where: W_1 = weight before burning and W_2 = weight after burning.

The pH of the farmland soils was determined using a calibrated AS ONE pH meter, model AS 800 (AS ONE Corporation, Osaka, Japan) to measure the pH of a sample-milli-Q water suspension (2 g of soil: 50 mL of milli-Q water) at temperatures ranging from 23.7 ^{o}C to 24.9 ^{o}C of the suspensions.

2.2.3.4 Hg Content Analysis

About 30 mg of each dried sample was weighed into sample boats in triplicates and put in a direct Hg analyser, MA-3000 (Nippon Instruments Corporation, Tokyo, Japan) for Hg content analysis. Farmland soils were analysed separately from the edible parts and the peels of food crops after obtaining suitable low and high calibration curves using the calibration standard solutions indicated above.

2.2.3.5 Quality Control

Instrumental analysis was triplicated for each sample with a triplicate determination for each instrumental analysis. A blank analysis after triplicate determination of each 3 samples was also carried out. Recovery analysis was carried out by spiking some of the samples. About 2 g of farmland soil samples were spiked with 7 µg of 1 ppm Hg^{2+} standard solution for Hg recovery analysis. For plantain and cassava samples, approximately 2 g were spiked with 0.7 µg of 100 ppb Hg^{2+} standard solution for Hg recovery analysis. For reliability of the method used and accuracy of the results, two certified reference materials (CRM), NMIJ 7302-a (National Metrology Institute, Japan) and ERM-CC580 (Institute of Reference Materials and Measurements, Belgium) were used.

2.2.4 Bioaccumulation Factor (BAF)

The BAF is an important parameter for consideration in risk evaluation of environmental samples involving soils and food samples (UK Environment Agency, 2009). The BAF defines the absorption and bioaccumulation of contaminants in plants and may be used as a measure of the level of risk or toxicity associated with plants (Addai-Arhin et al., 2021). The BAF was, therefore, used to determine the bioaccumulation of Hg in plantains and cassavas (both the edible and the peels) as a first step in assessing the ecological risk of Hg contamination to the farm ecosystems. It was evaluated using the equation:

$$BAF = \frac{\text{Hg content}_{(\text{edible or peels})}}{\text{Hg content}_{(\text{soil})}} - - - - \text{equation 2.2}$$

2.2.5 Ecological Risk Assessment of Samples and Farms (Study Areas)

Ecological risk assessment was done using the Hakanson, (1980) model. This model is based on the monomial ecological risk (M_{er}) and the potential ecological risk (P_{er}) indices. The M_{er} was used to assess the risk associated with the samples while the P_{er} was also used to assess the risk associated with the farms. The contamination and risk levels of the samples were used as a measure of the level of contamination and risk associated with the farms in the respective study areas. Applying the Hakanson, (1980) model, the associated ecological risk values of samples and farms were obtained from the equations below:

$C_f = \frac{C_m}{C_{rf}}$ ---- equation 2.3 (For individual samples from the farms in each study area),

$C_{deg} = \sum C_f$ ---- equation 2.4 (For all farms from each study area),

$M_{er} = C_f \times T_f$ ---- equation 2.5 (For individual samples from the farms in each study area) and

$P_{er} = \sum M_{er}$ ---- equation 2.6 (For all farms from each study area).

Where:

C_f = contamination factor of a metal (Hg) which may reflect the contamination behaviour of the environment but does not show any ecological risk and classified as; $C_f < 1$ = low contamination, $1 < C_f \leq 3$ = medium contamination, $C_f > 3$ = high contamination, C_m = mean concentration of Hg in a sample, C_{rf} = background or reference value of Hg [in this case, 500 µg/kg for farmland soil by the Swiss Environmental Regulation (Ritscher, 2018), 100 µg/kg for edible parts of plantains and cassavas by the World Health Organization (WHO) (WHO, 2000) and 100 µg/kg for plantain and cassava peels by the United Nations Economic Commission for Europe (UNECE)-European Directive 2002/32/EC (Li et al., 2017a)], C_{deg} = degree of contamination which indicates the combined contamination factors (C_f) of all metal/metalloids in the environment (in this case, C_f of all samples from the farms in each study area) and classified as; $C_{deg} < 5$ = low degree of contamination, $5 \leq C_{deg} < 10$ = medium degree of contamination, $10 \leq C_{deg} < 20$ = high degree of contamination, $C_{deg} \geq 20$ = very high degree of contamination, M_{er} defines the risk posed by an individual metal/metalloid (Hg) in the environment and is classified as; $M_{er} < 40$ = low risk, $40 \leq M_{er} < 80$ = moderate risk, $80 \leq M_{er} < 160$ = considerable risk, $160 \leq M_{er} < 320$ = High risk, $M_{er} \geq$

320 = very high risk and P_{er} defines the integrated or combined ecological risk of all heavy metals in an investigated environment (in this case, M_{er} of all samples from the farms in each study area) and is classified as; $P_{er} \leq 150$ = low risk, $150 < Per \leq 300$ = moderate risk, $300 < P_{er} \leq 600$ = considerable risk, $P_{er} > 600$ = very high risk and T_f = toxic factor of Hg = 40. The P_{er} is a common indicator that is used to assess the ecological risks or effects of heavy metals/metalloids on the environment, hence applicable to most environmental samples such as water, sediment, soil, and food crops.

2.2.6 Statistical and Data Analysis

Results were statistically analysed using statistical software IBM SPSS statistics version 26 from IBM Corporations, New York, USA and Microsoft Office Excel 2013 from Microsoft Corporations, USA. Descriptive statistics were used to obtain the mean Hg contents of the samples and the mean pH and SOM values of the soil samples. Regression analysis at significant probability (p) ≤ 0.05 and 0.001 were used to evaluate the effect of Hg content of farmland soils on Hg contents of the food crops, effect of pH and SOM on Hg content of farmland soils, effect of pH and SOM on Hg content of the food crops, and the effect of pH and SOM on the BAF of the food crops. Paired sample t-test (2-tailed) and non-parametric test using the Wilcoxon sign ranked test and the sign test at 95 % confidence interval (CI) were also used to evaluate the statistical difference ($p \leq 0.05$) in Hg contents of the same samples from different study areas, pH and SOM among farmland soils from the study areas, and the BAF of the food crop samples from the same and different study areas. The BAF values were presented in a bar graph with error bars representing standard error of the mean (SEM). The study area map was done using Surfer Golden Software, version 16 by Golden Software Inc., Colorado, USA.

2.3 Results

2.3.1 Hg Contents in Samples

Table 1 shows the results for Hg contents (µg/kg dw) of samples from the farms in each study area. The Hg contents of the edible parts and the peels of plantains ranged from 39.1 ±4.6 from farms at Nyamebekyere to 579.8 ±8.1 from farms at Odumase and 41.1 ±5.5 from farms at Ahansonyewodea to 274.8 ±12.6 from farms at Odumase, respectively. The Hg contents of the edible parts of plantains from farms at Tweapease and Ahansonyewodea were relatively equal, hence the difference was not statistically significant ($p > 0.05$) but the difference between the peels was statistically significant ($p < 0.05$). Additionally, there were statistically significant differences ($p < 0.05$) in Hg contents among the plantain samples (both the edible parts and the peels) from the farms in other study areas.

The Hg contents of the edible parts and the peels of cassavas also ranged from 114.7 ±9.8 from farms at Ahansonyewodea to 331.2 ±8.7 from farms at Tweapease and 338.4 ±19.5 from farms at Ahansonyewodea to 984.3 ±5 from farms at Tweapease, respectively (Table 1). This meant that farms at Ahansonywodea had the lowest Hg content while those at Tweapease had the highest for both the edible parts and the peels. However, like plantains, there were statistically significant differences ($p < 0.05$) in Hg content between the peels and the edible parts of cassavas. The Hg contents of the edible parts and the peels of cassavas from farms at Tweapease, Nyamebekyere, and Ahansonyewodea were extremely higher than those of plantains.

The Hg content of farmland soils was highest in farms at Odumase (16346 ±289.4) and lowest in farms at Ahansonyewodea (1335 ±27.5) (Table 1). The Hg contents of farmland soils from farms at Odumase, were on average, approximately 86 % higher than those from the farms in other study areas. This probably explained the extremely higher Hg content of the edible parts and the peels of plantains from farms at Odumase. Like plantains and cassavas, there were statistically significant differences ($p < 0.05$) in Hg contents among farmland soils across the study areas.

Upon comparison with guideline values, the edible parts of plantains from farms at Odumase and the edible parts of cassavas from farms at Tweapease, Nyamebekyere, and Ahansonyewodea are contaminated with Hg since the Hg content exceeded the 100 µg/kg

guideline limit by WHO for food other than fish (WHO, 2000). Plantain peels from the farms at Tweapease and Odumase as well as those of cassavas from farms at Tweapease, Nyamebekyere, and Ahansonyewodea had Hg content values (Table 1) above the 100 µg/kg guideline limit by the United Nations Economic Commission for Europe (UNECE), European Directive 2002/32/EC for plants used as animal feed (Li et al., 2017a), hence are also contaminated with Hg. The farmland soils from all the farms are also contaminated with Hg due to Hg contents above the 500 µg/kg guideline limit or reference value by the Swiss Environmental Regulation (Ritscher, 2018). Averagely, farmland soils, plantains (edible parts), plantain peels, cassava (edible parts), and cassava peels were about 11.5, 1.8, 1.3, 2.3 and 6.6-fold higher than their respective guideline values.

Table 1: Hg contents (µg/kg dw) of samples (Addai-Arhin et al., 2021, 2022a).

	Tweapease			Nyamebekyere		
	Min	Max	Mean ±SD	Min	Max	Mean ±SD
Plantain - Edible	39.5	64.6	50.0 ±10.1	33.2	46.8	39.1 ± 4.6
Plantain – Peels	107.1	145.4	130.2 ±17.0[c]	79.3	101.3	90.6 ± 8.6
Cassava – Edible	320.9	345.4	331.2 ±8.7[b]	236.3	248.1	242.6 ±4.2[b]
Cassava - Peels	978.3	990.9	984.3 ±5.0[c]	627.4	668.0	648.1 ±15.6[c]
Farmland Soils	3535.5	3880.3	3684.0 ±126.3[a]	2013.7	2045.0	2029.0 ±11.0[a]
	Ahansonyewodea			**Odumase**		
	Min	Max	Mean ±SD	Min	Max	Mean ±SD
Plantain - Edible	46.7	54.1	49.7 ±2.4	567.7	586.7	579.8 ± 8.1[b]
Plantain – Peels	33.3	48.0	41.1 ±5.5	262.7	292.8	274.8 ± 12.6[c]
Cassava – Edible	100.0	130.3	114.7 ±9.8[b]	-	-	-
Cassava - Peels	305.6	370.3	338.4 ±19.5[c]	-	-	-
Farmland Soils	1294.8	1387.1	1335.0 ±27.5[a]	15964.7	16692.8	16346.0 ±289.4[a]

Note:

Number of Determinations (n) = 9.

Guideline Values:

[a] 500 µg/kg for Farmland Soils by the Swiss Environmental Regulation.
[b] 100 µg/kg for edible parts of plantains and cassavas by WHO.
[c] 100 µg/kg for plantain and cassava peels by UNECE-European Directive 2002/32/EC.
[a], [b], and [c] superscript letters represent Hg content values above their respective guideline values.

2.3.2 Effect of pH and SOM on Hg Contents of the Soils and the BAF of the Food Crops

pH and SOM were evaluated since they influence Hg uptake from the soil by plants. The uptake of Hg may have effect on the BAF of plants. The BAF, therefore, defines the toxicity of Hg to plants, animals, and humans through the food chain (UK Environment Agency, 2009; Chang et al., 2014; Bortey-Sam et al., 2015a; Bedu-Addo et al., 2018). This is the reason the effect of Hg contents of farmland soils on Hg contents of the food crops, effect of pH and SOM on Hg contents of farmland soils and the food crops, and the effect of pH and SOM on the BAF of the food crops were evaluated. These regression studies helped in establishing whether soil Hg was available for uptake by the food crops. The availability of soil Hg for uptake by the food crops is well explained in 2.4.2.

Results for SOM and pH are shown in Table 2. The pH range (7.03 – 7.34) of farmland soils was very narrow, hence the differences in pH among the farmland soils were not statistically significant ($p > 0.05$). The pH values were near or within the neutral region and thus, showed the neutrality of the farmland soils. For SOM, the range (4.00 – 7.83 %) of farmland soils was relatively wider, hence the differences were statistically significant ($p < 0.05$) between Tweapease and Ahansonyewodea, Tweapease and Odumase, Nyamebekyere and Ahansonyewodea as well as Nyamebekyere and Odumase. However, the differences in SOM between Tweapease and Nyamebekyere as well as Ahansonyewodea and Odumase were not statistically significant ($p > 0.05$). The highest SOM of Ahansonyewodea and Odumase might be due to the proximity of the selected farms in these two study areas to garbage dumpsites, hence the significant differences in SOM of these two study areas from Tweapease and Nyamebekyere.

Table 2: pH and SOM (%) of the farmland soil samples (Addai-Arhin et al., 2021, 2022b).

	Soil Characteristics					
	pH			**SOM (%)**		
	Min	**Max**	**Mean ±SD**	**Min**	**Max**	**Mean ±SD**
Tweapease	6.90	7.38	7.20 ±0.23	3.98	4.02	4.00 ±0.02*
Nyamebekyere	6.73	7.20	7.03 ±0.22	4.08	4.37	4.24 ±0.13*
Ahansonyewodea	6.74	7.43	7.15 ±0.31	7.42	8.17	7.83 ±0.33
Odumase	7.27	7.45	7.34 ±0.08	6.42	7.08	6.72 ±0.29

Note:

Number of Determinations (n) = 9

$\frac{W_1 - W_2}{W_1} \times 100\ \% = \text{SOM}\ (\%)$. Where: W_1 = weight before burning and W_2 = weight after burning.

* Indicates statistically significant difference ($p < 0.05$) in SOM of Tweapease and Nyambekeyere from Ahansonyewodea and Odumase.

2.3.3 Bioaccumulation Factor (BAF)

BAF results are shown in Fig. 2.3. For farms at Tweapease and Nyamebekyere, the BAF values of plantain peels were higher than those of the edible parts. This meant that the peels bio accumulated more Hg than the edible parts. For farms at Ahansonyewodea and Odumase, the reverse was the case, indicating that the edible parts of plantains had more Hg bio accumulated in them than the peels. The farms at Nyamebekyere had the highest BAF for the peels of the food crops while those at Ahansonyewodea also had the highest BAF for only the edible parts of plantains. This probably accounted for the highest BAF of plantains (edible parts + peels) from the farms in these two study areas. This meant that proportionally, plantains from the farms with lower Hg contents of farmland soils absorbed and bio accumulated more Hg than those from farms with the higher Hg contents of farmland soils. The differences in BAF of the edible parts of plantains from farms at Ahansonyewodea and Odumase, and the BAF of the peels of plantains from farms at Tweapease and Ahansonyewodea were not statistically significant ($p > 0.05$).

For cassavas, there were statistically significant differences ($p < 0.05$) in BAF among the edible parts. Additionally, the BAF of the peels of cassavas from farms at Nyamebekyere was statistically significantly different from those of the farms at Tweapease and Ahansonyewodea. However, the difference in BAF of the peels of cassavas from Tweapease and Ahansonyewodea was not statistically significant. Averagely, the BAF of the edible parts and the peels of cassavas were approximately 5 and 7.6-fold higher than those of the edible parts and the peels of plantains, respectively.

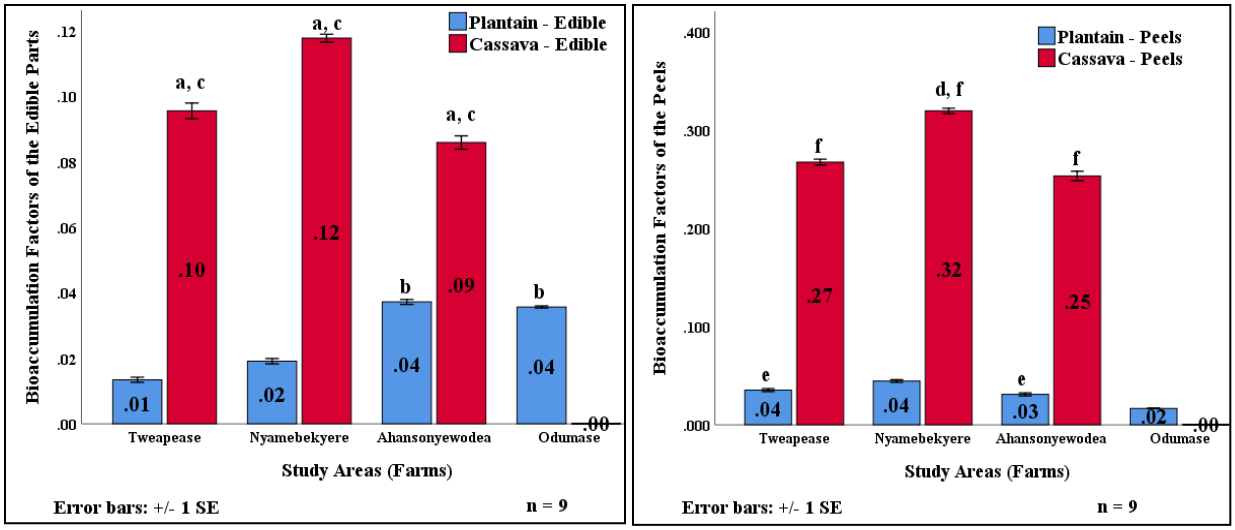

Fig. 2.3: Bioaccumulation factors of the edible parts and peels of plantains and cassavas. a = statistically significant difference ($p \leq 0.05$) in BAF among the edible parts of cassavas from different study areas, b = statistically significant difference ($p \leq 0.05$) in BAF of the edible parts of plantains from Ahansonyewodea and Odumase from those of Tweapease and Nyamebekyere, c = statistically significant difference ($p \leq 0.05$) in BAF of the edible parts of cassavas from those of plantains of the same study area, d = statistically significant difference ($p \leq 0.05$) in BAF of the peels of cassavas from Nyamebekyere from those of Tweapease and Ahansonyewodea, e = statistically significant difference ($p \leq 0.05$) in BAF of the peels of plantains from Tweapease and Ahansonyewodea from those of Nyamebekyere and Odumase, f = statistically significant difference ($p \leq 0.05$) in BAF of the peels of cassavas from those of plantains of the same study area, n = number of determinations, and error bars represent SEM (Addai-Arhin et al., 2022b).

$$\text{BAF} = \frac{\text{Hg content}_{(\text{edible or peels})}}{\text{Hg content}_{(\text{soil})}}$$

2.3.4 Ecological Risk Assessment of Samples and Farms (Study Areas)

The results for the contamination levels (C_f and C_{deg}) are shown in Table 3. Based on the assessment, the edible parts of plantains from the farms at Odumase, the edible parts of cassavas from farms at Tweapease, and the peels of cassavas from farms at Tweapease, Nyamebekyere, and Ahansonyewodea had $C_f > 3$, indicating high contamination factors of these samples. The edible parts of plantains from the farms at Tweapease, Nyamebekyere, and Ahansonyewodea had $C_f < 1$, indicating low contamination factors while the edible parts of cassavas from the farms at Nyamebekyere and Ahansonyewodea had medium contamination factors with $C_f = 2.4$ and 1.1, respectively. However, there was no significant difference in the C_f of the edible parts of plantains from farms at Tweapease and Ahansonyewodea. Plantain peels from the farms at Nyamebekyere and Ahansonyewodea had low contamination factors of 0.9 and 0.4, respectively while those from the farms at Tweapease and Odumase had medium contamination factors of 1.3 and 2.7, respectively. Except for farmland soils from farms at Ahansonyewodea which had medium

contamination with a C_f = 2.7, the farmland soils from farms in the other study areas had C_f > 3, indicating high contamination factors. The C_f for all the samples across the study areas showed that all the cassava peels from farms at Tweapease, Nyamebekyere, and Ahansonyewodea and the edible parts of plantains from farms at Odumase were the highest contaminated. Averagely, farmland soils had the highest contamination with C_f = 11.7 while plantain peels had the lowest contamination with C_f = 1.3 (Table 3). Based on Table 3, farmland soils were approximately 6.5 and 9-folds and 5 and 2-folds more contaminated than the edible parts and the peels of plantains and cassavas, respectively. These figures, therefore, showed that the edible parts and the peels of cassavas, were on average, about 1.3 and 4.7-folds more contaminated than those of plantains, respectively.

The C_{deg} also showed that the farms at Tweapease and Odumase with C_{deg} = 22.3 and 41.2, respectively had very high degree of contamination while those at Nyamebekyere and Ahansonyewodea had high and medium degrees of contamination with C_{deg} = 14.3 and 8.1, respectively. The farms at Odumase were approximately 2, 3 and 5-folds more contaminated than farms at Tweapease, Nyamebekyere, and Ahansonyewodea, respectively. This meant that farms at Ahansonyewodea had the lowest degree of contamination.

For M_{er}, the edible parts of plantains from farms at Tweapease, Nyamebekyere, and Ahansonyewodea as well as plantain peels from farms at Nyamebekyere and Ahansonyewodea had low monomial ecological risk indices with M_{er} < 40 (Table 3). The edible parts and the peels of plantains from farms at Odumase had high and considerable ecological risk indices with M_{er} = 232 and 108, respectively while the plantain peels from farms at Tweapease had a moderate monomial ecological risk index with M_{er} = 52. The edible parts of cassavas from farms at Tweapease and Nyamebekyere had considerable monomial ecological risk indices with M_{er} = 132 and 96, respectively while those from farms at Ahansonyewodea had a moderate monomial ecological risk index with M_{er} = 44. For cassava peels, farms at Tweapease, Nyamebekyere, and Ahansonyewodea had Mer = 392, 260, and 136, indicating very high, high, and considerable monomial ecological risk indices, respectively. The farms at Odumase had a very high monomial ecological risk index (M_{er} = 1308) for farmland soils while those at Tweapease and Nyamebekyere also had high monomial ecological risk indices of = 296 and 164 for farmland soils, respectively. The farms at Ahansonyewodea, however, had a considerable monomial ecological risk index of 108 for farmland soils. Averagely, the C_f and the M_{er} values of samples are, therefore, in the order:

farmland soils > cassava peels > cassava (edible parts) > plantain (edible parts) > plantain peels, indicating that plantain peels had the least contamination factor and the least monomial ecological risk index.

For potential ecological risk (P_{er}) index, the farms at Nyamebekyere and Ahansonyewodea had considerable potential ecological risk indices with P_{er} = 572 and 324, respectively while the farms at Tweapease and Odumase also had very high potential ecological risk indices with P_{er} = 892 and 1648, respectively. The C_{deg} and P_{er} of the farms are, therefore, in the order: Odumase > Tweapease > Nyamebekyere > Ahansonyewodea, indicating that Odumase, even with no cassava samples, had the highest degree of contamination and the highest potential ecological risk index.

Table 3: Contamination status and risk levels of samples and farms (study areas) using the Hakanson, (1980) model (Addai-Arhin et al., 2022b).

	Tweapease		Nyamebekyere		Ahansonyewodea		Odumase		Average	
	[a] C_f	[b] M_{er}	[a] C_f	[b] M_{er}	[a] C_f	[b] M_{er}	[a] C_f	[b] M_{er}	C_f	M_{er}
Plantain - Edible	0.5^{L}	20.0^{LR}	0.4^{L}	16.0^{LR}	0.5^{L}	20.0^{LR}	5.8^{H}	232.0^{HR}	1.8^{M}	72.0^{MR}
Plantain - Peels	1.3^{M}	52.0^{MR}	0.9^{L}	36.0^{LR}	0.4^{L}	16.0^{LR}	2.7^{M}	108.0^{CR}	1.3^{M}	53.0^{MR}
Cassava – Edible	3.3^{H}	132.0^{CR}	2.4^{M}	96.0^{CR}	1.1^{M}	44.0^{MR}	-	-	2.3^{M}	93.3^{CR}
Cassava - Peels	9.8^{H}	392.0^{VHR}	6.5^{H}	260.0^{HR}	3.4^{H}	136.0^{CR}	-	-	6.1^{H}	242.7^{HR}
Farmland Soils	7.4^{H}	296.0^{HR}	4.1^{H}	164.0^{HR}	2.7^{M}	108.0^{CR}	32.7^{H}	1308.0^{VHR}	11.7^{H}	469.0^{VHR}
[c] $C_{deg} = \sum C_f$	**22.3^{VH}**		**14.3^{H}**		**8.1^{M}**		**41.2^{VH}**		**21.2^{VH}**	
[d] $P_{er} = \sum S_{er}$		**892.0^{VHR}**		**572.0^{CR}**		**324.0^{CR}**		**1648.0^{VHR}**		**846.0^{VHR}**

Note:
Number of Determinations (n) = 9.

Superscript capital letters show contamination and/or risk levels of samples and farms (study areas).

[a] C_f: Contamination Factor, $C_f = \frac{C_m}{C_{rf}}$: [C_f < 1 = low, 1 < C_f ≤ 3 = medium and C_f > 3 = high contamination].

[b] M_{er}: Monomial Ecological Risk Index, $M_{er = C_f \times T_f}$: [M_{er} < 40 = low risk, 40 ≤ M_{er} < 80 = moderate risk, 80 ≤ M_{er} < 160 = considerable risk, 160 ≤ M_{er} < 320 = high risk and M_{er} ≥ 320 = very high risk. T_f of Hg = 40].

[c] C_{deg}: Degree of Contamination, $C_{\deg = \sum C_f}$: [C_{deg} < 5 = low, 5 ≤ C_{deg} < 10 = medium, 10 ≤ C_{deg} < 20 = high and C_{deg} ≥ 20 = very high degrees of contamination].

[d] P_{er}: Potential Ecological Risk Factor, $P_{\text{er} = \sum M_{er}}$: [$P_{er}$ ≤ 150 = low risk, 150 < P_{er} ≤ 300 = moderate risk, 300 < P_{er} ≤ 600 = considerable risk and P_{er} > 600 = very high risk.

Background or Reference Values:

500 µg/kg for farmland soil by Swiss Environmental Regulation; 100 µg/kg for plantain (edible parts) by WHO; 100 µg/kg for plantain peels by UNECE-European Directive 2002/32/EC.

Abbreviations Definitions:

^{L} = Low contamination/Low degree of contamination; ^{M} = Medium contamination/Medium degree of contamination; ^{H} = High contamination/ High degree of contamination; ^{VH} = Very high degree of contamination; ^{LR} = Low risk; ^{MR} = Moderate risk; ^{CR} = Considerable risk; ^{HR} = High risk and ^{VHR} = Very high risk.

2.4 Discussion

2.4.1 Hg Contents in Samples

Except for the edible parts of plantains from farms at Tweapease and Ahansonyewodea as well as cassava peels from farms at Tweapease, Nyamebekyere, and Ahansonyewodea with no statistical differences, all other samples of the same category from farms in different study areas showed significant statistical variations. The differences in the Hg contents of farmland soils across the study areas probably resulted from the differences in the amount of Hg waste discharged into the farms through ASGM activities, which in turn, may be due to differences in the number of ASGM facilities available in each study area, the differences in the amount of fertilizer used during cultivation (Pirrone et al., 2010; UNEP, 2013) and the differences in the natural characteristics of the soil such as pH, soil organic matter, cation exchange capacity, texture, etc. which to some extent, may influence the soil-Hg binding (Wang et al., 2006; Wilson et al., 2014; Khan et al., 2015). For both the edible parts and the peels of food crops, differences in Hg content might have resulted from differences in the Hg content of the farmland soils, differences in the amount of bioavailable Hg (Wang et al., 2006; Wilson et al., 2014; Khan et al., 2015), and the differences in the ages of the plantains and the cassavas (Montagnac et al., 2009; Khan et al., 2015). The differences in bioavailable soil Hg may account for the differences in the amount of Hg that can be absorbed and bio accumulated by the food crops. Moreover, the differences in growth stage (age) of plants, to some extent, may also affect the absorption and the distribution of nutrients and contaminants within plants. These probably were among the major factors that accounted for the differences in Hg contents of the edible parts and the peels of the same food crops across the study areas.

The results of this study far exceed the 600 ±0.1 and the 300 ±0.20 µg/kg dw of farmland soils and plantains (edible parts), respectively from farms at Odumase obtained by Amonoo-Neizer et al., (1995) in Hg and arsenic pollution in soil and biological samples around the mining town of

Obuasi, Ghana. Additionally, the average results of farmland soils, the edible parts of plantains and cassavas in this study are far above the Hg contents of 320 ±0.36 µg/kg dw, 1 ±0.001 and 4 ±0.003 µg/kg fw of agricultural soils, plantains and cassavas, respectively obtained by Bortey-Sam et al., (2015a, 2015b) in a similar study of heavy metal/metalloids accumulation in foodstuffs from farms within mining communities around Tarkwa Municipality, Ghana and the ecological risks of heavy metals and a metalloid in agricultural soils in Tarkwa, Ghana. Averagely, the result of farmland soils in this study is again far above the 150 µg/kg obtained by Zhou et al., (2018) for agricultural soils across China but below the 80,000 µg/kg by Li et al., (2017b) for farmland soils in Hainan, China and the 150,000, $2.61x10^6$, and 47,000 µg/kg for soils from mining areas in China, Spain, and Slovenia, respectively indicated by Natasha et al., (2020) in their review article. Differences in results might have resulted from differences in the levels from which soil samples were taken (top soil for Amonoo-Neizer et al., (1995), 0-10 cm for Bortey-sam et al., (2015a, 2015b) and 15-20 cm below the topsoil for this study), differences in the proximity of selected farms to ASGM sites, seasonal variations, and time differences (in years), especially in the case of Amonoo-Neizer et al., (1995) might have contributed to increased anthropogenic activities such as ASGM operations and fertilizer application (UNEP, 2013; Pirrone et al., 2010). According to USEPA, (1997a), Soil Hg level depends largely on depth of soil as greater percentage of Hg is found approximately 20 cm below the topsoil. Moreover, geographical differences coupled with the differences in natural sources of Hg emissions such as bushfires, volcanoes, evaporation from ocean surfaces, and the differences in anthropogenic activities such as mining operations, fertilizer application, chlor-alkali processes, and other industrial activities (UNEP, 2013; Pirrone et al., 2010) may be the reasons for the differences in Hg levels of farmland soils between this study and those of Li et al., (2017b), Zhou et al., (2018), and those indicated by Natasha et al., (2020).

The higher Hg contents of farmland soils than food crops may be due to the ability of the soils to serve as a reservoir or sink for Hg (USEPA, 1997a; Birkefield et al., 2005; Masindi and Muedi, 2018). However, the nature of plantains and cassavas might have accounted for the significant differences in Hg contents of these food crops. Cassavas are root crops and found approximately 15 to 20 cm below the topsoil, hence in direct contact with the soil while plantains are also fruit crops and exposed directly to the atmosphere. This means that the Hg in cassavas may be entirely from the soil while the Hg in plantains may be from both the soil and the

atmosphere but with a greater percentage from the atmosphere. According to Azevedo and Rodriguez, (2012) and Yu et al., (2018), the shoot part or leaves of plants can absorb elemental Hg from the atmosphere. Moreno et al., (2005) and Ahammad et al., (2018) also indicated that the transfer of Hg from the roots to the shoots is limited. These studies suggest that a greater percentage of Hg in plantains resulted from absorption of elemental Hg from the atmosphere. Elemental Hg is the gaseous Hg released into the atmosphere when the gold-Hg amalgam is burnt (Gyamfi et al., 2020). The direct contact of cassavas with the soil (major Hg reservoir), therefore, might have accounted for its significantly higher Hg content than plantains. The relatively equal Hg content of the edible parts of plantains from Tweapease and Ahansonyewodea might have resulted from relatively equal absorption rate and equal bioaccumulation of available soil Hg.

2.4.2 Effect of pH and SOM on Hg Contents of the Soils and the BAF of the Food Crops

Due to their effects on BAF of plants, the relationship of pH and SOM with Hg concentration must be studied in any environmental risk assessment involving soil and food samples (UK Environment Agency, 2009; Chang et al., 2014; Bortey-Sam et al., 2015). Hg adsorption to soil particles and its subsequent uptake by plants may either increase or decrease depending on pH and SOM (UK Environment Agency, 2009; Bortey-Sam et al., 2015a; Yu et al., 2018). This, therefore, determines the bioavailability and toxicity, which in turn defines the BAF of heavy metals/metalloids in plants. Soil pH within a threshold range of 4.50 - 6.42 (acidic region) enhances the absorption or uptake of more Hg by plants since Hg becomes readily soluble and available in soil solution by existing as a free Hg^{2+} ion (Bortey-Sam et al., 2015a; Akoto et al., 2018; Yu et al., 2018).

Based on this study, soil pH range (7.03-7.34) was very narrow, near or within the neutral region and above the pH threshold range of 4.50 – 6.42 (Table 1). This meant that soil Hg probably existed in insoluble forms, hence was unavailable for uptake by the food crops. This largely reduced the percentage of Hg available for uptake, hence the low BAF of the food crops (Fig. 2.3). Moreover, the regression analysis showed that there were no relationships between SOM/pH and Hg content of farmland soils (appendix, Fig. 1A), SOM/pH and Hg content of the food crops (appendix, Fig. 1B), and SOM/pH and BAF of the food crops (appendix, Fig. 1C). This, therefore, indicated that soil pH and SOM had no significant effect on the bioavailability or uptake of Hg by the food crops. This is consistent with the results obtained by Xu et al., (2014), Bortey-Sam et al.,

(2015a) and Akoto et al., (2018) in a similar study of pH and SOM effects on heavy metal/metalloids bioavailability in soil. However, Hg uptake and its accumulation in plants are not only influenced by pH and SOM but other factors such as the cation exchange capacity, redox potential, type of plants, age of plant, and the total metal content available for uptake (Wilson et al., 2014; Khan et al., 2015; Wang et al., 2016; Bentum et al., 2017). Nevertheless, the Hg contents of the plantains and cassavas were largely influenced by the Hg contents of the farmland soils (appendix, Fig. 1D) despite the assertion that a greater percentage of the Hg in plantains significantly resulted from atmospheric Hg.

2.4.3 Bioaccumulation Factor (BAF)

The BAF determines the potential of plants to accumulate contaminants from the soil, hence defines the toxicity of contaminants to humans and animals through the food chain. BAF < 1 means low bioaccumulation and toxicity while BAF > 1 also means high bioaccumulation and toxicity (Bedu-Addo et al., 2018). From Fig. 2.3, all the BAF values are below 1, an indication of low Hg bioaccumulation and toxicity. This means that the associated ecological risks or the toxicity of both the edible parts and the peels of food crops to humans and animals, respectively may be low or may take several years to occur upon repeated exposures through ingestion (Addai-Arhin et al., 2021, 2022a, 2022b). The significant differences in BAF of the edible parts and the peels of plantains and cassavas (Fig. 2.3) show differences in Hg bioaccumulation and toxicity while the relatively equal BAF values across the study areas (Fig. 2.3) also indicate equal Hg bioaccumulation and toxicity. Differences in BAF mean differences in ecological risks and vice versa. Therefore, the higher the BAF value the higher the bioaccumulation and toxicity, hence the higher the associated ecological risks. This means that the edible parts and peels of cassavas may have the highest toxicity and ecological risks than those of plantains while the peels of cassavas may also have higher toxicity and ecological risks than the edible parts. However, there may be equal toxicity and ecological risks associated with the edible parts of plantains from farms at Ahansonyewodea and Odumase, plantain peels from farms at Tweapease and Ahansonyewodea, and the cassava peels from farms at Tweapease and Ahansonyewodea since differences in BAF were not statistically significant (Fig. 2.3).

The differences in the nature (crop type) (Natasha et al., 2020) of cassavas (root crops) and plantains (fruit crops) might have accounted for the higher BAF of cassavas than plantains.

According to Natasha et al., (2020), the roots of plants provide a larger surface area that can absorb and accumulate nutrients and contaminants. Moreno et al., (2005) and Ahammad et al., (2018), indicated that many plant roots can absorb and accumulate Hg but its translocation to the shoot is limited. Although some studies (Xun et al., 2017; Yu et al., 2018), indicated otherwise, the significantly lower BAF of plantains than cassavas might have resulted from limited transfer of absorbed Hg from the roots to the shoots of plantains since a greater percentage of absorbed Hg is stored in the root (Moreno et al., 2005; Natasha et al., 2020). The higher BAF of plantains and cassavas (edible + peels) (Fig. 2.3) from the farms at Nyamebekyere and Ahansonyewodea despite their lower Hg levels probably resulted from a higher percentage of soil Hg that was available for uptake, which in turn, might have been due to Hg speciation and the soil characteristics such as pH, soil organic matter, cation exchange capacity, etc. (Natasha et al., 2020).

Generally, the differences in the BAF values might have resulted from the differences in the ages or growth stage of the food crops (Montagnac et al., 2009; Khan et al., 2015), differences in the amount of soil Hg available for uptake and the differences in the Hg content of the farmland soils (Wilson et al., 2014; Khan et al., 2015; Wang et al., 2016; Bentum et al., 2017). The growth stage influences the distribution of contaminants and/or nutrients in a plant while differences in available soil Hg and the total metal content of the soil are also affected by the natural characteristics of the soil such as the pH, SOM, cation exchange capacity, etc.

2.4.4 Ecological Risk Assessment of Samples and Farms (Study Areas)

Based on the C_{deg} and P_{er}, all the farms across the study areas are contaminated and may pose potential ecological risks to humans, animals, and the entire farm ecosystem although differences exist in the contamination and risk levels. Contamination of the farms resulted from contaminated samples, especially farmland soils, cassava peels, the edible parts of cassavas, and the edible parts of plantains from farms at Odumase. Mercury contaminated soils may lead to detrimental effects on plants, human, and animal health through the food chain over time (Srivastava et al., 2017; Masindi and Muedi, 2018). Higher Hg levels in soil may be detrimental to soil health by affecting activities of soil microorganisms and microbial diversity (Chibuike and Obiora, 2014; Xie et al., 2016). This may lead to detrimental effects on soil microflora and soil fertility, which in turn, may lead to reduction in agricultural land quality, reduction in plant growth, crop productivity and marketability (Wuana and Okieimen, 2011; Chibuike and Obiora, 2014; Xie

et al., 2016; Srivastava et al., 2017), reduction in food safety and quality (Wuana and Okieimen, 2011; Oppong et al., 2018), negative effect on food security, and land tenure problems (Wuana and Okieimen, 2011).

The effect of Hg contamination on plant growth and development may result from cytoplasmic enzymes inhibition and cell structure damage due to oxidative stress as well as essential nutrients replacement at cation exchange sites of the plants (Chibuike and Obiora, 2014; Xie et al., 2016). These effects may be more pronounced in root crops such as cassava, potatoes, yams, cocoyam, etc. and root vegetables such as carrot, onions, ginger, etc. since their edible parts are formed from the roots. Therefore, higher Hg levels may accumulate in the edible parts of plants in this category since the roots have higher potential to absorb and accumulate available soil Hg (Moreno et al., 2005; Ahammad et al., 2018; Natasha et al., 2020). With limited transfer of Hg from the roots to the shoots of plants (Moreno et al., 2005 and Ahammad et al., 2018), the effect of Hg contaminated soils on fruit crops such as plantains, etc., leafy vegetables such as lettuce, cabbage, spinach, etc., fruit vegetables such as tomatoes, pepper, garden eggs, okra, etc. may be comparably lower. However, the shoots or leaves of such plants can also absorb elemental Hg from the atmosphere (Yu et al., 2018) in Hg-contaminated environments. This may, therefore, increase Hg levels in the plants with subsequent detrimental effect on their growth and development.

The associated ecological risks of Hg to humans and animals such as goats, sheep, cattle, etc. and birds may result through the soil-plant-animal-human or soil-plant-human food chain upon long-term repeated exposures through dermal contact with the contaminated soils or the ingestion of the contaminated food crops or contaminated water. Although the edible parts of plantains and cassavas are eaten in processed forms which may have reduced Hg levels due to the application of heat, the long-term incessant ingestion may lead to bioaccumulation of Hg (Tchana et al., 2018) to levels above the provisional tolerable weekly intake of 4 µg/kg bw/week (FAO/WHO, 2003). Hg levels above this guideline value may, however, lead to human health risk. Animals such as goats, sheep, and cattle may even be exposed to higher risk of Hg since the peels of cassavas and plantains are consumed by these animals in their raw state. This may tend to increase the risks of Hg to humans through the soil – plant – animal – human food chain due to humans' dependence on both plants and animals for food (Addai-Arhin et al., 2022b). Additionally, Hg from

contaminated soils can leach into rivers, streams and underground water systems such as wells, boreholes, etc. (Tchana et al., 2018) especially in areas where these waters are used as drinking water sources. This may also pose ecological risk to aquatic life, the water system and pose subsequent detrimental health effects on humans, animals, and plants, especially when these water sources are used for irrigation. The totality of all these negative effects of higher Hg contamination may result from its effects on ecosystem functions such as fluxes of energy and materials processing e.g., decomposition, productivity; stocks of energy and materials e.g., biomass, genes (Kinzig et al., 2002); and habitat and regulatory functions (De Groot et al., 2002).

Based on the ecological risk assessment, the risks associated with the edible parts and peels of cassavas may be higher than those of plantains due to higher M_{er} values. The higher M_{er} values of Cassavas resulted from higher absorption and bioaccumulation of the available soil Hg. Moreover, farms at Nyamebekyere and Ahansonyewodea may also pose potential ecological risks to humans, animals, and the ecosystem such as those elaborated above but not as much as the risks associated with farms at Tweapease and Odumase. Although the farms at Tweapease and Odumase may pose very high ecological risks, the risks associated with farms at Odumase may be higher due to P_{er} which is about 2-fold higher than that of Tweapease. The highest contamination level and the highest ecological risk of farms at Odumase probably resulted from the highest amount of Hg waste discharged into the immediate environment. This in turn, might have resulted from the highest number of ASGM facilities at Odumase and the proximity of the selected farms to the ASGM facility. Comparably, the farms at Odumase were more proximal to the respective ASGM facility than those in other study areas. However, continuous release of Hg waste through ASGM activities into the least contaminated farms may result in higher contamination of the farms and their produce in future. This may, therefore, pose ecological risks to the soil, plants, animals, humans, and the entire ecosystems with time.

2.5 Conclusion

The Farmland soils, the edible parts and the peels of the food crops had Hg values above their respective guideline values, hence are contaminated with Hg. This suggests contamination of the entire farm ecosystems within the studied communities, primarily through ASGM operations. This may pose future risks to the farm ecosystems by affecting ecosystem functions. Therefore, regulatory bodies should ensure strict control of ASGM operations in all the study areas to prevent Hg-associated ecological risks to humans, animals, and the entire ecosystem in future.

Chapter Three

Potential Human Health Risks Assessment of Mercury-Contaminated Samples

Publications

1. **Sylvester Addai-Arhin**, Randy Novirsa, Huiho Jeong, Quang Phan Dinh, Nana Hirota, Hideki Shiratsuchi, Yasuhiro Ishibashi, and Koji Arizono. (2021). The Human Health Risks Assessment of Mercury in Soils and Plantains from Farms in selected Artisanal and Small-Scale Gold Mining Communities around Obuasi, Ghana. *Journal of Applied Toxicology*, *42*(2); 258 – 273. https://doi.org/10.1002/jat.4209

2. **Sylvester Addai-Arhin**, Randy Novirsa, Huiho Jeong, Quang Phan Dinh, Nana Hirota, Hideki Shiratsuchi, Yasuhiro Ishibashi, and Koji Arizono. (2022). Potential Human Health Risks of Mercury-Contaminated Cassavas – Preliminary Studies. *Fundamental Toxicological Sciences*, 9(2); 61 – 69. https://doi.org/10.2131/fts.9.61

3.1 Introduction

Prolong exposures to mercury through ingestion, inhalation or dermal absorption can lead to various detrimental human health effects such as any of the effects indicated in chapter one. Depending on the dose and the period of exposures, such potential detrimental human health effects which are usually non-carcinogenic may manifest early or in distant future. The reason many studies including Amonoo-Neizer et al., (1995), Bortey-Sam et al., (2015), Bentum et al., (2017), Bedu-Addo et al., (2018), Oppong et al., (2018), Addai-Arhin et al., (2021, 2022), etc. have all assessed the risks of Hg and other heavy metals/metalloids to humans through food ingestion. In chapter two, the risk of Hg contamination to the entire ecosystem was evaluated. This chapter, therefore, evaluated the potential human health risks of THg and MeHg to the residents of the studied communities following prolong exposures to Hg through ingestion of plantains and cassavas as well as dermal absorption of soil.

Moreover, the detrimental human health effects of Hg can take several years to occur following repeated exposures or even after cessation of exposures. Therefore, using the first order elimination kinetics formula, this chapter also evaluated the period (years) within which there may be potential human health risks of Hg following repeated exposures to the food crops and the period that may be required for elimination of a greater percentage of ingested Hg. Results obtained in the elimination kinetic studies were compared with USEPA reference values for InHg and MeHg to determine the probable period within which risks of Hg may manifest following repeated exposures or cessation of exposures.

3.2 Materials and Methods

The materials and methods section of this chapter considered the analysis method for only MeHg since that for THg was detailed in chapter two. A total of thirty-three (33) samples which included the edible parts of the food crops and the farmland soil samples were used in this chapter. Farmland soil samples were used because it was necessary to estimate the BAF for MeHg contents of the food crops, and evaluate the human health risk associated with the farmland soils upon long-term repeated exposures through dermal absorption.

NB: For study area, sampling, sample preparation, and cleaning and decontamination, refer to 2.2.1, 2.2.2, 2.2.2.1, and 2.2.3.1, respectively.

3.2.1 Experimentation

3.2.1.1 Reagents and MeHg Standard Solution for Calibration.

All reagents used were of analytical grade and purchased from FUJIFILM Wako Pure Chemical Industries, Osaka, Japan. Calibration standard solutions of concentrations 10 ppb CH_3HgCl in 5 % (v/v) nitric acid solution was prepared from 100 ppb CH_3HgCl standard stock solutions using the serial dilution approach and kept at 4 ^oC until analysis **(refer to 2.2.3.2 for THg calibration standard solutions)**.

3.2.1.2 MeHg Extraction and Content Determination

The extraction and determination was carried out according to the method described by Maggi et al., (2009) but was slightly modified to optimize the samples under consideration. Approximately 0.5 g of each dried sample was weighed into a 50 mL corning tube and 10 mL of 6 M HCl solution was added, shaken for 5 minutes at 250 rpm using horizontal recipro shaker SR-2s (Taitec, Nagoya, Japan) and centrifuged at 2400 rpm for 10 minutes using high-speed cooling Kubota 7000 centrifuge (Kubota Corporation, Japan) (for the food crop samples, 48 % (w/w) Hydrogen bromide (HBr) was used). The HCl and HBr solutions were decanted, and 20 mL toluene (99.5 % w/w) was added to the residue, the resultant mixture was shaken, and centrifuged at 250 rpm and 2400 rpm, respectively for 20 minutes. The toluene extract containing MeHg was decanted into another corning tube and 6 mL of 1 % (w/v) solution of L-cysteine (98 % w/w) containing 1.25 % (w/v) of anhydrous sodium sulphate and 0.775 % (w/v) of anhydrous sodium acetate was added. The resultant solution was shaken and centrifuged for 20 minutes as above. Approximately 100 µL of the L-cysteine phase (aqueous phase) containing MeHg was injected into sample boats, placed in DMA (MA-3000) for MeHg content analysis after obtaining a suitable calibration curve using the calibration standard solution of 10 ppb CH_3HgCl **(refer to 2.2.3.3 for THg analysis method)**.

3.2.1.3 Quality Control

Approximately 2 g of farmland soil samples were spiked with 3 ng of 10 ppb CH_3HgCl standard solution while about 2 g each of plantain and cassava samples were also spiked with 2 ng of 10 ppb CH_3HgCl standard solution for MeHg recovery analysis. For reliability and accuracy of the MeHg analysis method and the results, a certified reference material (CRM), ERM-CC580 in estuarine sediments from the Institute of Reference Materials and Measurements, Belgium was used. For quality control of THg analysis method, **refer to 2.2.3.4**.

3.2.2 Human Health Risk Assessment

Human health risk assessment was carried out based on the internationally accepted United States Environmental Protection Agency (USEPA) human health risk assessment model and the basic kinetics of Hg elimination and retention using the formula for first order elimination kinetics. BAF for MeHg levels of the food crop samples was calculated to determine the amount of MeHg absorbed and accumulated from the soil by the food crops. The BAF for MeHg, estimated average daily intake for ingestion ($eAvDI_{ing}$) of plantains and cassavas, estimated average daily intake for dermal absorption ($eAvDI_{der}$) of the farmland soils, hazard quotients (HQ) and hazard indices (HI) were evaluated using the equations below:

$$BAF = \frac{\text{MeHg Content}_{(crop)}}{\text{MeHg Content}_{(Soil)}} - - - - \text{ equation 3.1}$$ **(Refer to 2.2.4 for the BAF of THg)**

$$eAvDI_{(ing)} = \frac{\text{MC x IR x ED x EF}}{\text{AvBW x AvT}} - - - - \text{ equation 3.2}$$

$$eAvDI_{(der)} = \frac{\text{MC x SA x FE x AF x ABS x ED x CF x EF}}{\text{AvBW x AvT}} - - - - \text{ equation 3.3}$$

$$HQ = \frac{eAvDI}{R_f D} - - - - \text{ equation 3.4}$$

$$HI = \sum HQs\ [\text{i.e.}, HQ_{(\text{farmland soil})} + HQ_{(\text{plantains})} + HQ_{(\text{cassavas})}] - - - - \text{ equation 3.5}$$

The basic kinetics of Hg elimination and retention was carried out by determining the initial (N_o) and final (N) amounts for both InHg and MeHg following long-term repeated exposures and the cessation of exposures. The N_o and N were calculated by applying the formula for first order elimination kinetics; $N = N_o e^{-kt}$ – – – – equation 3.6 and $k = \frac{In2}{t_{\frac{1}{2}}}$ – – – – equation 3.7

Where:

HQ = Hazard quotient, HI = Hazard index, eAvDI = Estimated average daily intake (mg/kg bw/day) for either ingestion or dermal absorption (USEPA, 2004), MC = Mean concentration of the contaminant (Hg), IR = Ingestion rate for plantain obtained as 0.370kg for adults and 0.20kg for children per day (Bortey-Sam et al., 2015a), ED = Exposure duration (years) = 30 and 10 years for adults and children, respectively, EF = Exposure frequency (days/year) = 365, AvBW = Average body weight (kg) = 70 and 35 kg for adults and children, respectively, AvT = Averaging time (days) = ED x 365 for non-carcinogens (USEPA, 2004), SA = Skin surface area (cm^2) = 5700 and 2800 for adults and children, respectively (USEPA, 2004), FE = Dermal exposure ratio = 0.61 for both adults and children (USEPA, 2007), AF = Soil adherence factor (mg/cm^2) = 0.07 and 0.2 for adults and children, respectively (USEPA, 2004), ABS = Dermal absorption factor = 0.1 for both adults and children (USEPA, 2007), CF = Conversion factor (kg/mg) = $1x10^{-6}$ for both adults and children (USEPA, 2004), R_fD = Oral Reference dose of mercury given as $3x10^{-4}$ mg/kg bw/day for THg and $1x10^{-4}$ mg/kg bw/day for MeHg (USEPA, 2004), N_o = Initial amount (exposure amount), N = Final amount (retention amount), k = elimination constant, $t_{1/2}$ = half-lives of elimination = 45 and 75 days for InHg and MeHg, respectively, t = time (years) and In 2 = 0.693.

3.2.3 Statistical and Data Analysis

Paired sample t-test and non-parametric test using the Wilcoxon signed rank test and the sign test at 95% CI were used to evaluate the differences in MeHg contents, methylation ratio, and BAF among same samples from different study areas. To determine the statistical significance of the extremely lower MeHg contents of the samples, a box plot comparison was made between the THg and InHg contents of the samples and the statistically significant difference between the two was evaluated using a paired sample t-test and a non-parametric test at $p \leq 0.001$.

3.3 Results

3.3.1 MeHg Contents and the Percentage Methylation of the Samples

The MeHg contents (Table 4) were extremely lower than the THg contents of the samples **(refer to Table 1 for THg contents of the samples)**. Averagely, the percentage of MeHg in the samples was about 0.5% (Table 5), indicating that about 99.5% of THg in samples was available as InHg. However, a statistically significant difference ($p < 0.001$) between THg and InHg contents of the samples occurred (Fig. 3.1) despite their relatively equal Hg contents. The differences in MeHg contents of plantains from Tweapease and Nyamebekyere, Tweapease and Ahansonyewodea as well as Nyamebekyere and Ahansonyewodea were not statistically significant ($p > 0.05$) but statistically significant differences ($p < 0.05$) occurred among cassava samples and farmland soil samples. For percentage methylation, the differences were statistically significant ($p \leq 0.05$) among same samples from different study areas except for cassava samples from Tweapease and Nyamebekyere. Additionally, although cassavas had higher MeHg contents (Table 4) but the percentage methylation (Table 5) was lower than those of plantains. Comparably, of all the three sample types, farmland soils had the lowest percentage methylation despite the highest MeHg contents.

Table 4: MeHg contents (µg/kg dw) of the samples (Addai-Arhin et al., 2021, 2022a)

	MeHg Content (µg/kg dw)					
	Tweapease			**Nyamebekyere**		
	Min	**Max**	**Mean ±SD**	**Min**	**Max**	**Mean ±SD**
Plantains	0.40	0.64	**0.48 ±0.11**	0.40	0.47	**0.43 ±0.03**
Cassavas	0.80	1.02	**0.94 ±0.07**	0.63	0.72	**0.69 ±0.04**
Farmland Soils	3.19	3.48	**3.33 ±0.13**	2.63	2.99	**2.77 ±0.17**
	Ahansonywodea			**Odumase**		
	Min	**Max**	**Mean ±SD**	**Min**	**Max**	**Mean ±SD**
Plantains	0.40	0.45	**0.43 ±0.02**	4.20	5.00	**4.48 ±0.32**
Cassavas	0.55	0.60	**0.57 ±0.02**	-	-	**-**
Farmland Soils	2.21	2.50	**2.38 ±0.13**	17.05	17.87	**17.45 ±0.36**

Note:

Number of Determinations (n) = 9

Refer to **Table 1** for THg contents of the samples

Table 5: Ratio of MeHg to THg (percentage methylation) of samples (Addai-Arhin et al., 2021, 2022a).

	Percentage (%) Methylation								
	Plantains - Edible			Cassavas - Edible			Farmland Soils		
	Min	Max	Mean ±SD	Min	Max	Mean ±SD	Min	Max	Mean ±SD
Tweapease	0.85	1.04	**0.95 ±0.08**	0.25	0.30	**0.28 ±0.02**	0.09	0.09	**0.09 ±0.00**
Nyamebekyere	1.01	1.26	**1.11 ±0.08**	0.26	0.29	**0.28 ±0.01**	0.13	0.15	**0.14 ±0.01**
Ahansonyewodea	0.81	0.88	**0.86 ±0.01**	0.43	0.58	**0.50 ±0.05**	0.17	0.19	**0.18 ±0.01**
Odumase	0.72	0.86	**0.77 ±0.06**	-	-	-	0.10	0.11	**0.11 ±0.01**
Average	**0.85**	**1.01**	**0.92 ±0.15**	**0.31**	**0.39**	**0.35 ±0.13**	**0.12**	**0.14**	**0.13 ±0.04**

Note:

Number of Determinations (n) = 9

$$\text{Percentage Methylation} = \frac{\text{MeHg Content}}{\text{THg Content}} \text{ x } 100\%$$

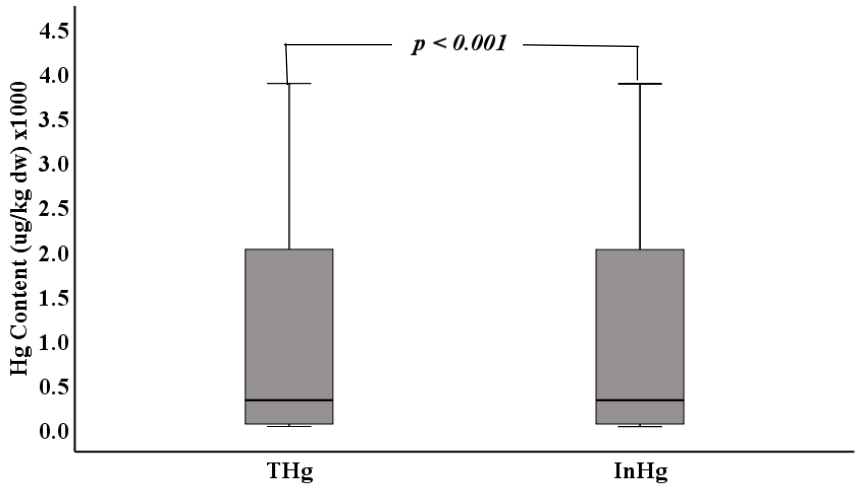

Fig. 3.1: Boxplot comparison between THg and InHg contents of samples. The difference in Hg content between THg and InHg was statistically significant ($p < 0.001$). InHg content = THg content – MeHg content.

3.3.2 BAF

The BAF values for MeHg contents of the food crops are shown in Table 6. The BAF values of MeHg were higher than those of THg **(refer to Fig. 2.3 for the BAF for THg of the edible parts of the food crops)** across all the study areas despite the low percentage methylation (Table 5). However, all the BAF values were below 1, indicating low absorption and accumulation. The differences in BAF–MeHg were statistically significant ($p < 0.05$) among plantain samples except for plantains from Nyamebekyere and Ahansonyewodea. For cassava samples, except for Tweapease and Ahansonyewodea, the differences in BAF–MeHg between Tweapease and Nyamebekyere and between Nyamebekyere and Ahansonyewodea were not statistically

significant ($p > 0.05$). Averagely, the BAF of cassavas was 1.4-fold higher than that of plantains. This meant that cassava can absorb and accumulate more MeHg from the soil than plantains.

Table 6: BAFs for MeHg contents of the edible parts of the food crops (Addai-Arhin et al., 2021, 2022a).

	BAF					
	Plantains			Cassavas		
	Min	**Max**	**Mean ±SD**	**Min**	**Max**	**Mean ±SD**
Tweapease	0.120	0.183	**0.143 ±0.028**	0.240	0.303	**0.282 ±0.021**
Nyamebekyere	0.142	0.180	**0.157 ±0.016**	0.210	0.274	**0.248 ±0.028**
Ahansonyewodea	0.167	0.203	**0.180 ±0.017**	0.225	0.272	**0.241 ±0.021**
Odumase	0.243	0.280	**0.257 ±0.021**	-	-	-

Note:

Number of Determinations (n) = 9

BAF = $\frac{\text{MeHg Content}_{(\text{Crop})}}{\text{MeHg Content}_{(\text{Soil})}}$ (refer to Fig. 2.3 for the BAF-THg of the edible parts of the food crops).

3.3.3 Human Health Risk Assessment

3.3.3.1 Hazard Quotient (HQ) and Estimated Average Daily Intake (eAvDI)

The results for HQ and eAvDI of soil samples and the food crops are shown in Table 7. For farmland soils, both the HQ and $eAvDI_{(der)}$ of THg across the study areas were below the recommended reference values of 1 and $3x10^{-4}$ mg/kg bw/day by USEPA, respectively. Plantains from Odumase and all cassava samples had HQ and $eAvDI_{(ing)}$ of THg for both adults and children above the USEPA reference values (Table 7). Tweapease, Nyamebekyere, and Ahansonyewodea had HQ and $eAvDI_{(ing)}$ of plantains below the USEPA reference values of 1 and $3x10^{-4}$ mg/kg bw/day, respectively for both adults and children. For MeHg, the HQ and the eAvDI values of farmland soils and the food crops for both adults and children across the study areas were below the USEPA reference values of 1 and $1x10^{-4}$ mg/kg bw/day, respectively (Table 7).

3.3.3.2 Hazard Index (HI)

HI results are also shown in Table 7. HI values in this study were a sum of the HQ values for farmland soils, plantains, and cassavas, hence represented the human health risks associated with the farms in each study area. The HI–THg values of the farms (study areas) for both adults and children were above the USEPA reference value of 1 while those of MeHg were below 1.

Table 7: HQ, HI, $eAvDI_{(ing)}$ of food crops, and $eAvDI_{(der)}$ of farmland soils for both THg and MeHg (Addai-Arhin et al., 2021, 2022a).

	HQ, HI, and eAvDI (mg/kg bw/day) for ingestion of food crops and dermal absorption of farmland soils											
	Tweapease			Nyamebekyere			Ahansonyewodea			Odumase		
	Pt	Cv	F. Soils	Pt	Cv	F. Soils	Pt	Cv	F. Soils	Pt	Cv	F. Soils
eAvDI (10^{-4}) – THg												
Adults	2.64	28.39	0.013	2.07	20.79	0.007	2.63	9.83	0.005	30.65	-	0.057
Children	2.86	37.86	0.036	2.24	27.73	0.019	2.84	13.11	0.013	33.13	-	0.160
HQ – THg												
Adults	0.88	9.46	0.0043	0.69	6.93	0.0024	0.88	3.28	0.0015	10.22	-	0.019
Children	0.95	12.62	0.0120	0.75	9.24	0.0070	0.95	4.37	0.0043	11.04	-	0.050
eAvDI (10^{-6}) – MeHg												
Adults	2.53	4.96	0.0012	2.29	3.62	0.0010	2.24	3.02	0.00083	23.69	-	0.0061
Children	2.74	5.36	0.0033	2.48	3.91	0.0027	2.43	3.26	0.00230	25.61	-	0.0170
HQ – MeHg												
			(10^{-6})			**(10^{-6})**			**(10^{-6})**			**(10^{-6})**
Adults	0.025	0.050	11.59	0.023	0.036	9.64	0.022	0.030	8.26	0.237	-	60.69
Children	0.027	0.054	32.53	0.025	0.039	27.05	0.024	0.033	23.19	0.256	-	170.35

	Tweapease	Nyamebekyere	Ahansonyewodea	Odumase
HI – THg				
Adults	10.34	7.62	4.16	10.24
Children	13.58	10.00	5.32	11.09
HI – MeHg				
Adults	0.075	0.059	0.052	0.237

Note:

Pt = Plantains, Cv = Cassavas and F. Soil = Farmland Soils.

$$HQ = \frac{eAvDI}{RfD}$$

$$eAvDI_{(der)} = \frac{MC \times SA \times FE \times AF \times ABS \times ED \times CF \times EF}{AvBW \times AvT}$$

$$eAvDI_{(ing)} = \frac{MC \times IR \times ED \times EF}{AvBW \times AvT}$$

$$HI = \sum HQs\ [i.e., HQ_{(farmland\ soil)} + HQ_{(plantains)} + HQ_{(cassavas)}]$$

Guideline values:

Hazard Quotient (HQ < 1) = Unlikely non-carcinogenic human health risks.

Hazard Quotient (HQ ≥ 1) = Likely non-carcinogenic human health risks.

Hazard Index (HI < 1) = Unlikely Integrated or combined non-carcinogenic human health risks.

Hazard Index (HI ≥ 1) = Likely Integrated or combined non-carcinogenic human health risks.

USEPA Reference Dose (R_fD) for THg = $3x10^{-4}$ and MeHg = $1x10^{-4}$ mg/kg bw/day

3.3.3.3 Basic Kinetics of Hg Elimination and Retention

Results for basic kinetics of Hg elimination and retention are shown in Table 8a. From the results, there was no significant difference between N_o and N for both InHg and MeHg, especially from the 3rd to the 5th year following repeated exposures. Plantains from Odumase and all cassava samples had retention amounts (N) for InHg above the extrapolated value of 0.394 µg/kg bw/yr of the USEPA reference dose while the N values of MeHg for both plantains and cassavas were below the extrapolated value of 1.252 µg/kg bw/yr of the USEPA reference dose after the 5th year of long-term repeated exposures in both adults and children. However, plantains from Odumase and cassavas from Tweapease had the highest N values for InHg in adults and MeHg in children, respectively while plantains from Nyamebekyere and Ahansonyewodea also had the lowest N values for InHg and MeHg, respectively in both adults and children after the 5th year upon long-term repeated exposures through consumption.

The estimated periods (yrs) for complete elimination upon exposure cessation (Table 8b) were approximately 3 yrs for InHg and between 4 and 4.5 yrs for MeHg in both adults and children. However, no significant differences occurred between the elimination periods for both adults and children. The elimination period for InHg in plantains from Tweapease, Nyamebekyere, and Ahansonyewodea were within the range of the reference dose while those for plantains from Odumase and all cassava samples exceeded that of the reference dose. For MeHg, the elimination periods were below the reference dose (Table 8b). Comparably, despite its extremely lower levels, the elimination periods for MeHg were higher than those for InHg.

Table 8a: Initial and final amounts (µg/kg bw/yr) of Hg following exposure and elimination using the first order decay kinetics formula (Addai-Arhin et al., 2021).

		InHg ($t_{1/2}$ = 45 days)				MeHg ($t_{1/2}$ = 75 days)			
		Plantains							
		Adults		Children		Adults		Children	
Study Area	Time (yrs)	Concentration (µg/kg bw/yr)							
		N_0	N	N_0	N	N_0	N	N_0	N
Tweapease	1	95.80	0.347	102.80	0.372	0.924	0.0317	0.997	0.0342
	2	96.20	0.348	103.20	0.374	0.955	0.0328	1.031	0.0354
	3-5	96.20	0.348	103.20	0.374	0.956	0.0328	1.031	0.0354
Nyamebekyere	1	74.90	0.271	81.10	0.294	0.851	0.0292	0.902	0.0309
	2	75.20	0.272	81.40	0.295	0.880	0.0302	0.933	0.0320
	3-5	75.20	0.272	81.40	0.295	0.881	0.0302	0.934	0.0320
Ahansonyewodea	1	94.70	0.343	102.80	0.372	0.829	0.0284	0.876	0.0301
	2	95.00	0.344	103.20	0.374	0.857	0.0294	0.906	0.0311
	3-5	95.00	0.344	103.20	0.374	0.858	0.0294	0.907	0.0311
Odumase	1	1106.80	4.008	1197.30	4.336	8.651	0.2970	9.344	0.3205
	2	1110.80	4.023	1201.70	4.352	8.948	0.3070	9.665	0.3315
	3-5	1110.80	4.023	1201.70	4.352	8.958	0.3070	9.676	0.3318

		InHg ($t_{1/2}$ = 45 days)				MeHg ($t_{1/2}$ = 75 days)			
		Cassavas							
		Adults		Children		Adults		Children	
Study Area	Time (yrs)	Concentration (µg/kg bw/yr)							
		N_0	N	N_0	N	N_0	N	N_0	N
Tweapease	1	1030	3.73	1374	4.97	1.81	0.062	1.96	0.067
	2	1034	3.74	1379	4.99	1.87	0.064	2.02	0.069
	3-5	1034	3.74	1379	4.99	1.87	0.064	2.02	0.069
Nyamebekyere	1	754	2.73	1006	3.64	1.32	0.045	1.43	0.049
	2	757	2.74	1009	3.65	1.37	0.047	1.48	0.051
	3-5	757	2.74	1009	3.65	1.37	0.047	1.48	0.051
Ahansonyewodea	1	357	1.29	476	1.72	1.10	0.038	1.19	0.041
	2	358	1.30	478	1.73	1.14	0.040	1.23	0.042
	3-5	358	1.30	478	1.73	1.14	0.040	1.23	0.042

Table 8b: Estimated period (years) for complete elimination of Hg following cessation of exposure (Addai-Arhin et al., 2021).

		Estimated Time (years) for Complete Elimination of Hg							
		Tweapease		Nyamebekyere		Ahansonyeowdea		Odumase	Reference Dose
		Plantains	Cassavas	Plantains	Cassavas	Plantains	Cassavas	Plantains	For both Crops
InHg	Adults	2.68	3.10	2.64	3.05	2.68	2.91	3.12	**2.70**
	Children	2.69	3.15	2.65	3.10	2.69	2.97	3.13	**2.70**
MeHg	Adults	3.77	3.97	3.74	3.87	3.73	3.82	4.43	**4.86**
	Children	3.79	3.99	3.76	3.89	3.75	3.84	4.45	**4.86**

Note:

Daily USEPA reference dose (R_fD) for THg = 0.30 µg/kg bw/day and MeHg = 0.10 µg/kg bw/day.
Based on 99.4% average of InHg in food crop samples (Table 5), the $R_fD_{(InHg)}$ = ($R_fD_{(THg)}$ x 99.40)/100 = (0.30 x 99.4)/100 = 0.298 µg/kg bw/day. Therefore,
The final amount per year for $R_fD_{(InHg)}$ = 0.394 µg/kg bw/yr using first order decay kinetics formula; $N = N_oe^{-kt}$.
The final amount per year for $R_fD_{(MeHg)}$ = 1.252 µg/kg bw/yr using first order decay kinetics formula; $N = N_oe^{-kt}$.

N_o: Initial or exposure amount (µg/kg bw/yr) of InHg; $N_o = {}_eAvDI_{(ing)}$ x 0.994 x EF and MeHg; $N_o = {}_eAvDI_{(ing)}$ x EF

N: Final amount (µg/kg/yr) i.e. after elimination or excretion of InHg or MeHg

k: Decay constant = $In2/t_{1/2}$ (In2 = 0.693) and EF = Exposure Frequency (days/year) = 365.
Estimated time (years) for complete elimination of mercury was evaluated using $In\frac{N}{No} = -kt$, where t = time (days) and converted to years through division by 365.

3.4 Discussion

3.4.1 MeHg Contents and the Percentage Methylation of Samples

The low percentage methylation and the subsequent lower MeHg contents of samples were probably due to low levels of available soil InHg. This meant that a greater percentage of THg was InHg in insoluble form, hence was not readily available for methylation by the microbial methylators in the soil. Moreover, pH and SOM (Table 2), and probably other soil characteristics such as cation exchange capacity could not favour higher availability of soil InHg and higher percentage methylation, hence the lower MeHg contents of samples. The Low SOM levels led to insufficient carbon substrate for microbial activities. This reduced the concentration of microbial methylators leading to reduced methylation of available soil Hg as indicated by Su et al., (2021). Additionally, soil pH within neutral or slightly alkaline region did not favour the solubility, mobility and availability of Hg in soil solution and its subsequent availability to plants as stated by Akoto et al., (2018) and Yu et al., (2018). Therefore, the neutral or slightly alkaline soil pH and the low SOM were not favourable for Hg methylation, hence the low MeHg levels. The 0.5% average of MeHg in the samples is, therefore, consistent with USEPA, (1997a) and Horvart and Kotnik, (2019) findings which reported 1 – 3% and < 2% of MeHg in terrestrial ecosystems and in soils, respectively. This range suggests that MeHg levels in terrestrial ecosystems are very lower compared to the aquatic and paddy fields ecosystems. Gilli et al., (2018) had percentage methylation around 0.1% for most soils in a speciation and mobility studies of Hg-contaminated soils by legacy emissions in the Rhone valley in Canton of Valais, Switzerland. Findings by Gilli

et al., (2018) is also consistent with the results for percentage methylation of farmland soils in this study and suggests that MeHg levels depends primarily on the amount of available soil InHg and not THg.

Although percentage methylation was generally low, food crop samples had higher percentage methylation than farmland soils which are sinks for Hg. This meant that some percentage of MeHg in the food crops might have been methylated by the food crops, hence the total MeHg contents were not directly and entirely absorbed from the soil in which the food crops are cultivated. According to USEPA, (1997a), plants have the potential to methylate available InHg absorbed or accumulated by them. Mauro et al., (2002), Achá et al., (2005), Correira et al., (2011) and Gentes et al., (2017) also indicated that plant roots promote Hg methylation because the roots contain more Hg methylating microorganisms such as the sulphur reducing bacteria. This probably accounted for the higher percentage methylation of the food crops than farmland soils despite their relatively lower MeHg levels. Moreover, the higher percentage methylation of plantains despite their relatively lower MeHg levels compared to cassavas also showed that plantains have higher methylation potential due to higher iron levels. Plantains contain about 9.2 – 23.3% iron (Adepoju et al., 2012) while cassavas contain about 0.3 – 1.4% iron (Montagnac et al., 2009), hence the higher probability of iron reducing microorganisms enhancing available InHg methylation in plantains than in cassavas.

The lack of statistically significant difference in MeHg contents of plantains from Tweapease, Nyamebekyere, and Ahansonyewodea might be due to equal absorption and/or methylation rates of MeHg. This in turn resulted from probably the relatively equal age or growth stage of plantains as reported by Montagnac et al., (2009) and Khan et al., (2015). The statistically significant difference between THg and InHg contents (Fig. 3.1) means that the MeHg contents of the samples, although are relatively lower but may be significant enough to potentially cause human health risks upon long-term repeated exposures.

3.4.2 BAF and Mercury Toxicity

The bioavailability and toxicity of heavy metals in plants are determined by the bioaccumulation factor (BAF). The BAF is, therefore, an important parameter in the human health risk evaluation involving soil and food samples. The low Hg bioaccumulation and toxicity due to BAF < 1 doesn't necessarily mean there may not be any detrimental human health effects of mercury upon long-term repeated exposures through incessant consumption of Hg-contaminated plantains and cassavas but the effects may be lower or insignificant or may take several years to occur (Addai-Arhin et al., 2021, 2022a, 2022b). The higher BAF values of MeHg than THg may be due to the relatively higher bioaccumulation potential of MeHg (Bernhoft, 2012; Rice et al., 2014; Kumar and Ghosh, 2016; Chan, 2019). The higher bioaccumulation of MeHg, generally, explains the reason for its higher toxicity when compared to InHg.

3.4.3 Human Health Risks Assessment

3.4.3.1 Hazard Quotient (HQ) and Estimated Average Daily Intake ($_e$AvDI) of Samples

The associated human health risk assessment of Hg through food crops consumption or dermal absorption of farmland soils was evaluated using hazard quotient (HQ), defined as the ratio of the estimated average daily intake (eAvDI) to the reference dose (R_fD). HQ > 1 is an indication of potential non-carcinogenic human health risks while HQ < 1 indicates that there may be no associated non-carcinogenic human health risks, particularly upon long-term repeated exposures (USEPA, 2004; 2007).

For THg, plantains from Odumase and all cassava samples can cause significant non-carcinogenic human health risks such as renal, hepatic, cardiovascular, reproductive, and gastrointestinal problems (Peixoto and Pereira, 2007; De Freitas et al., 2012; Gado and Aldamash, 2013; Kalendar et al., 2013; Omanwar et al., 2013; Joshi et al., 2014) as well as oxidative stress and functional deficit in the motor cortex even at low doses (Teixeira et al., 2018) over an exposure period of 30 and 10 years for adults and children, respectively. The higher HQ-THg values of cassavas than plantains from Tweapease, Nyamebekyere, and Ahansonyewodea was due to probably their nature as root crops. The direct contact of cassavas with the soil (sink for Hg) and their root crop nature as indicated by Natasha et al., (2020) enhanced greater absorption and accumulation of Hg in them. Additionally, the highest THg-HQ values of plantains from Odumase

than all cassava samples could also be due to the highest Hg absorption from the soil and probably the atmosphere. Farms from Odumase had the highest Hg content of farmland soils, and this probably resulted from the very high amount of Hg released into the immediate environment due to the comparably higher number of ASGM facilities.

Plantains from Odumase and cassava samples from Tweapease, Nyamebekyere, and Ahansonyewodea had $_{e}AvDI_{(ing)}$ of THg above the USEPA reference dose of $3x10^{-4}$ mg/kg bw/day (Table 7). This means that residents of the communities ingest more Hg daily than expected through consumption of the plantains or cassavas. The HQ and $eAvDI_{(ing)}$ values for plantains from Tweapease, Nyamebekyere, and Ahansonyewodea (for both adults and children) were below 1 and $3x10^{-4}$ mg/kg bw/day, respectively. This means they may not cause any non-carcinogenic human health risks to residents even upon long-term repeated exposures.

Despite its higher BAF as shown in Table 6, the HQ values and the $eAvDI_{(ing)}$ of MeHg for all samples across the study areas were far below 1 and the reference dose of $1x10^{-4}$ mg/kg bw/day, respectively (Table 7). This means MeHg may not cause any non-carcinogenic human health risks to residents of these communities. Based on the HQ evaluation, children may experience higher non-carcinogenic human health risks than adults. This may be due to the relatively smaller body mass per higher ingestion rate (European Environment and Health Information Systems, 2007; Bortey-Sam et al., 2015a).

Human health risk of farmland soils was evaluated using only $eAvDI_{(der)}$ because soil ingestion is very rare and not likely to occur daily (UK Environment Agency, 2009). HQ values of farmland soils were far below 1 (Table 7), hence there may not be any likelihood of the soils causing non-carcinogenic human health risks to residents in the study areas following long-term repeated exposures for 30 and 10 years for adults and children, respectively.

3.4.3.2 Hazard Index (HI)

HI refers to the sum of HQs of all contaminants, hence expresses the combined or integrated human health risks of two or more contaminants in a particular sample (USEPA, 2004; 2007). In this study, HI was evaluated using all samples from each study area to assess the combined or integrated human health risks associated with the farms in the study areas. Samples were used for HI evaluation because Hg was the only contaminant considered in this study. HI ≥

1 indicates possible combined non-carcinogenic human health effects of all samples within each study area over the exposure period while HI < 1 indicates the reverse (USEPA, 2004; 2007).

For THg, farms across the study areas may cause significant combined or integrated non-carcinogenic human health risks to residents over exposure periods of 30 and 10 years for adults and children, respectively. This is due to the higher HI values (> 1) (Table 7) of the samples from the selected farms. The higher HI values of farms from Tweapease, Nyamebekyere, and Ahansonywodea resulted from higher HQ values of cassava samples while that for Odumase was due to the higher HQ value of only plantains. Although farmland soils were used in the HI evaluation, their effects were insignificant across the study areas due to the extremely lower HQ values (HQ slightly above zero) (Table 7). Therefore, the combined non-carcinogenic human health risks may result from long-term repeated exposures to both plantains and cassavas from farms at Tweapease, Nyamebekyere, and Ahansonyewodea and only plantains from farms at Odumase.

HI-MeHg of the study areas were far below 1 (Table 7), hence there may not be any likelihood of an associated integrated non-carcinogenic human health risks of MeHg over exposure periods of 30 and 10 years for adults and children, respectively. This is because the total average percentage of MeHg in samples was extremely low (0.50 %) (Table 6), an indication that a greater percentage (~ 99.50 %) of THg may be InHg. Therefore, the potential non-carcinogenic human health risks may result from ingestion of InHg levels above the reference dose of $3x10^{-4}$ mg/kg bw/day. Like HQ, HI values for children were higher than that for adults, an indication that children might experience more combined non-carcinogenic human health effects than adults.

3.4.3.3 Kinetics of Hg Elimination and Retention

The kinetics was carried out to theoretically estimate the final amount (µg/kg bw/yr) of InHg and MeHg during continuous exposures and the period (years) that will be required for complete elimination (excretion) of InHg and MeHg following cessation of exposure. The estimation was based on the assumption that the body is a homogenous unit, daily exposure amount was constant, elimination followed a first order kinetics, equal rate of elimination in both adults and children, males and females, equal elimination rate irrespective of the elimination pathway

such as through sweat, urine, faeces, etc. (WHO, 2000) and equal rate of elimination for both metabolized and unmetabolized forms of Hg.

The highest retention (N) values (above extrapolated value of the USEPA reference dose) of the InHg levels of all cassava samples and plantains from Odumase suggest the probability of an individual experiencing risks of Hg within the first year of repeated exposures to the food crops (Table 8a). Conversely, residents of Tweapease, Nyamebekyere, and Ahansonyewodea are not likely to experience risks of Hg even after 5 or more years of repeated exposures to the plantains from these areas. This is due to the lower N values of InHg compared to the extrapolated value of 0.394 µg/kg bw/yr of the USEPA reference dose of 0.3 µg/kg bw/day (refer to extrapolations below Table 8b). The N values of MeHg levels of both plantains and cassavas are extremely below the extrapolated value of 1.252 µg/kg bw/yr of the USEPA reference dose of 0.1 µg/kg bw/day (refer to extrapolations below Table 8b), hence repeated exposures to the food crops for 5 or more years may not cause any human health risks to residents. However, the extremely lower N values of both InHg and MeHg (Table 8a) is an indication that a greater percentage (≥ 90%) of Hg is eliminated or excreted upon ingestion (WHO, 2000; Bernhoft, 2012). The basic kinetics is consistent with the HQ and HI estimations in terms of the level of human health risks posed by Hg-contaminated food crops from each study area, hence may be a useful tool in evaluating the amount of Hg retained and the period within which one may experience the risks of Hg following repeated exposures for many years.

Based on the maximum period (years) that may be required for the complete elimination of almost all the Hg from the human body following cessation of exposure, an assumed final amount (N) of $1x10^{-7}$ µg/kg bw/yr for both InHg and MeHg was used. This amount may be so insignificant to be detected within the body as well as cause any Hg related health problems. The estimated final amount, N (µg/kg bw/yr) after the 5^{th} year of repeated exposures was used as N_o. Based on the assumptions, the highest elimination period (years) of InHg levels (above USEPA reference dose) of plantains from Odumase and all cassava samples for both adults and children (Table 8b) is an indication of a possible non-carcinogenic human health risks even after cessation of repeated exposures. The InHg levels of plantains from Tweapease, Nyamebekyere, and Ahansonyewodea, and the MeHg levels of both the food crops across the study areas had elimination periods within those of the reference doses, hence may not cause any non-carcinogenic

human health risks even after cessation of repeated exposures. However, MeHg had higher elimination period than InHg despite its extremely lower values (Table 8b). The higher elimination period of MeHg after exposure cessation may be due to the longer half-life of MeHg which may lead to lower or slower elimination rate. The longer half-life may result from the ability of MeHg to bio accumulate (Bernhoft, 2012; Rice et al., 2014; Kumar and Ghosh, 2016; Chan, 2019). The Lower or slower MeHg elimination rate may also be due to the conversion of MeHg to InHg before elimination (WHO, 2000). The conversion, therefore, may delay the elimination period, hence increasing the $t_{1/2}$ of MeHg.

3.5 Conclusion

The human health risk assessment showed that plantains from farms at Odumase and all cassava samples may pose significant non-carcinogenic human health risks to residents (both adults and children) following prolong exposures to THg levels. Additionally, farms across the study areas may be associated with significant integrated or combined non-carcinogenic human health risks to both adults and children upon prolong dependence on samples, particularly plantains and cassavas. The human health risks associated with THg levels of the food crops may result from prolong ingestion of InHg levels above the USEPA reference dose of $3x10^{-4}$ mg/kg bw/day. This means the associated human health risks of Hg may be specific to InHg. MeHg levels, however, may not cause any non-carcinogenic human health risks to both adults and children following prolong exposures to the food crops and soil samples. The elimination and retention kinetics may be useful in evaluating the estimated period within which one is likely to experience the risks of Hg following prolong exposures.

Chapter Four

Investigation of the Potential Subacute Developmental Toxicities and the Genotoxicity of Very Low and High Mercury Levels of Mercury-contaminated Food Crops following repeated Prenatal Exposures – A Model Study

4.1 Introduction

Chapters 2 and 3 detailed the potential risks of Hg to the ecosystems and the residents of ASGM communities, respectively. Based on chapter 3, adults and children are potentially at risk of the non-carcinogenic human health effects of Hg, particularly InHg following long-term repeated exposures to either cassavas or plantains or a combination of both. In this chapter, the risks of Hg to a more sensitive group i.e., the foetal population is investigated through a model study. The significant levels of MeHg due to the statistically significant difference between THg and InHg levels of the food crops (Fig. 3.1) and the higher sensitivity or vulnerability of the foetal population to Hg intoxication (Bjørklund et al., 2019; Patel et al., 2019) have necessitated investigations into the risks of the Hg levels of the food crops to the foetal population.

Additionally, some studies have indicated that lower blood Hg levels, particularly MeHg levels (< 5.8 µg/L) may cause developmental defects in foetuses or new-borns upon repeated exposures. Myers et al., (2015) indicated that prenatal exposures to low levels of MeHg may be associated with developmental deficits in children. Chen et al., (2014) reported low birth weight and premature delivery at Hg levels ranging from 1.82 – 3.03 and 3.76 – 4.65 µg/L in maternal and cord red blood cells, respectively. Ou et al., (2015) reported 2.73 µg/L, 2.29 µg/L, and 572.98 µg/kg Hg levels in cord blood, maternal blood and foetal hair in women and neonates across North China, respectively, and concluded that low-level prenatal Hg exposure could be responsible for reduction in foetal and infant growth, and that the effects of MeHg and InHg are not the same.

These studies suggest that the extremely lower MeHg levels of the food crops can potentially cause human foetal developmental toxicities upon repeated prenatal exposures due to the higher absorption rate (≥ 90%) (Broussard et al., 2002; Bernhoft, 2012; Rice et al., 2014), the easy passage across the placenta and the BBB (Broussard et al., 2002; Bernhoft, 2012; Ou et al., 2014), the higher foetal or cord blood levels than mother's (Chen et al., 2014; Kim et al., 2015; Ou et al., 2015), and the higher bioaccumulation of MeHg (Broussard et al., 2002; Bernhoft, 2012). Furthermore, the higher InHg levels of the food crops (Addai-Arhin et al., 2021, 2022), its limited passage across the placenta (WHO, 2008) coupled with the extremely lower MeHg levels of the food crops suggest that very low but significant amount of Hg may reach the foetus. Such very low Hg levels of the food crops can be detrimental to the developing foetus upon repeated prenatal exposures since there is no known safe level of foetal exposure to Hg (Health and Environment

Aliance, 2002; Bose-O'Reilly, 2010). Therefore, using Japanese medaka embryos as models, this chapter comprehensively investigated the potential human foetal subacute developmental toxicities and the genotoxicity of InHg and MeHg levels of the food crops upon repeated prenatal exposures.

4.1.1 Japanese Medaka as a Model Tool

To accurately predict the toxic effects of very low levels of Hg to the developing foetus requires the use of suitable experimental animal models. Japanese medaka (*Oryzias latipes*) is one of such models which are recently being used in toxicity testing of chemicals. It is a small non-mammalian vertebrate fish. The adult has an average length of about 3 – 4 cm and weighs between 700 – 1000 mg (Laboratory data). It lives in the rice paddy fields and ponds throughout Japan. The medaka, like the zebrafish, is a popular animal model for the study of early developments and in understanding the processes associated with organ development (Gladys et al., 2015). Its resemblance to other vertebrates, particularly the mammalian vertebrates in genetics and organogenesis (Gladys et al., 2015) makes it a suitable tool in various toxicological studies, particularly in humans. Additionally, the microscopic transparency of the embryo and the generational period of about 60 – 90 days allow easy monitoring of organogenesis during embryonic development and post-hatch morphological changes or defects. The reason most studies involving the use of medaka have centred on embryonic development and post-hatch assessment of the larvae.

4.2 Materials and Methods

4.2.1 Reagents

Mercury chloride (purity = 99.5%), sodium chloride (purity = 99.5%), potassium chloride (purity = 99.5%), calcium chloride (purity = 95%) and sodium bicarbonate (purity = 99.5 – 100.3%) were purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan while methylmercury chloride (CAS number: 115-09-3, purity = 75%) was also purchased from Tokyo Chemical Industries Ltd, Toshima-Kita-Ku, Tokyo, Japan.

4.2.2 Preparation of Exposure Test Solutions

The isotonic (control) solution was prepared by dissolving 7.538g of sodium chloride, 0.201g of potassium chloride and 0.211g of calcium chloride in milli-Q water (Barnstead Smart2Pure, ThermoFischer Scientific, Massachusetts, USA) to make a 1L solution. The pH of the solution was adjusted to 7.3 ±0.03 using 1N sodium bicarbonate solution and a calibrated pH meter, model AS 800 (AS One Corporation, Osaka, Japan). Nominal concentrations of 1, 2, 3, 4.5, 20, and 26 μg/L of MeHg (CH_3Hg^+) and 15, 20, 23, 150, 230, and 380 μg/L of InHg (Hg^{2+}) were prepared from 83 and 153.5 ppm stock solutions of methylmercury chloride (CH_3HgCl) and mercury chloride ($HgCl_2$), respectively by a serial dilution method using the isotonic solution as a diluent. The nominal concentrations were selected based on the $eAvDI_{(ing)}$ of MeHg and InHg of the Hg-contaminated plantains and cassavas in chapter 3. Due to interspecies variability and differences in body masses, the $eAvDI_{(ing)}$ values of InHg were multiplied by the absorption factors of 0.9 and 0.075 for MeHg and InHg, respectively. This meant that the concentrations of the test solutions of CH_3Hg^+ and Hg^{2+} used in this study corresponded to the dose or amount of $eAvDI_{(ing)}$ that may be absorbed upon repeated prenatal exposures to the Hg-contaminated plantains and cassavas. The actual concentrations of CH_3HgCl and $HgCl_2$ exposure solutions including those of the stock solutions were determined by measuring the equivalent concentrations of Hg using the direct mercury analyser, MA-3000 (Nippon Instruments Corporation, Tokyo, Japan). For CH_3Hg^+ exposure test solutions, the equivalent concentration of Hg was used to compute the actual concentrations.

4.2.3 Brood stock

The brood stocks with average weight between 600 and 800 mg, which have been kept in the laboratory of the faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, Japan, comprised 1 – 2 years old 500 adult Japanese medaka (Hd-rR strain) which were separated into 5 groups of 100 medaka in a ratio of 2 males: 1 female and kept in a semi-flow through 100L plastic aquaria containing about 85L of culture water. The animals were maintained at a temperature of 25 ±1^{o}C and pH = 7.5 ±0.02 of the culture water, a photoperiod of 16:8 hours light: dark cycle and fed with only *Artemia nauplii* twice daily. The culture water was changed weekly upon cleaning of the aquaria.

4.2.4. Exposure and Toxicity Assessment of the Embryos

The toxicity assessment or test followed the Organization of Economic Cooperation and Development, OECD, (2013, 2014) guidelines for the Medaka Extended One Generation Reproduction Test (MEOGRT) for the testing of chemicals. Approximately 4 – 5 hours post-fertilized embryos spawned by the female adult medaka were collected within 4 – 5 hours after morning feed. The fertilized embryos were screened for viability using the stereoscopic zoom microscope (3.0-15.0 magnification, model SMZ25) with NIS-elements BR version 5.30.00 (Build 531) software (Nippon Instruments Corporation, Tokyo, Japan). A total of 60 viable embryos for each concentration of CH_3Hg^+ and Hg^{2+}exposure test solutions including their respective controls were selected for the toxicity assessment and these corresponded to 15 embryos per replicate (i.e., 4 replicate determinations). The 15 embryos were then exposed to the test solutions in a 96-well plate (1 embryo/well) and placed in a cool incubator, model PCI 300 at a temperature of 25 ±1^{o}C. Change of exposure test solutions and microscopic observation of the cultured embryos using the stereoscopic zoom microscope were done once daily for 18 days exposure period. To ensure optimum development of the embryos, the pH, temperature, and concentrations of the exposure test solutions were determined weekly. This ensured the values did not fall below or above the optimum ranges. During the 18 days exposure or observational period, end points such as heart rates, hatching time, hatching, hatchability, delays, deformities, and mortalities (deaths) of the developing embryos were noted and recorded.

Heart rate referred to the number of heart beats per minute (bpm) during embryogenesis and it was done by recording the heart beats of eight randomly selected embryos for each exposure test solution on days 4, 6, and 8 of 3 replicate determinations; hatching time referred to the period (day) at which the embryos of a particular exposure solution began to hatch; hatching referred to the complete emergence of the embryo from the chorion (Yamagami, 1981); hatchability referred to the hatching success i.e., number or percentage of embryos that successfully hatched. This excluded embryos that were already dead upon emergence from the chorion; Delays referred to the normally developed embryos that could not hatch within the exposure period; deformities (malformations) referred to any abnormal development of an organ or whole embryo or larva. These included thrombus, lack of eye pigment, unabsorbed yolk, slow heart beat and blood circulation, reduced eyes, reduced head, small body, arrested growth, bent tail, stunted or arrested

growth of embryo and/or larva, etc.; and mortalities (deaths) referred to the opaqueness of the embryo, absence of heart beat and/or blood circulation, presence of fungal growth around the embryo and the inability of the larva to move upon gentle touch after hatching. The delays, deformities, and mortalities were all classified as developmental toxicities.

4.2.5 Post-Hatch Survival, Mortalities and Growth of Larvae

To reduce competition for space and food, and ensure optimum growth of larvae, the hatched larvae of each replicate for each exposure test solution of CH_3Hg^+ and Hg^{2+} were separately put into a 10L aquaria containing approximately 9L of culture water at same temperature and pH as the brood stock. The larvae were also fed with only *Artemia nauplii* twice daily (feeding began within 24 hours post-hatch) and maintained at 16:8 hours light: dark cycle. The larvae were assessed daily for abnormal behaviour, stunted or arrested growth, mortalities, survival, and normal growth based on total body length and body weight for 8 weeks. Daily survivals and mortalities were noted and the total recorded weekly for 8 weeks post-hatch (wph) while the total body length and the body weight were also determined at 8 wph. Change of culture water was done weekly upon cleaning of the aquaria.

4.2.5.1 Concentration–Response Analysis

The concentration–response analysis was done to evaluate the lethal concentration (LC_{50}), no observed adverse effect level (NOAEL) and the low observed adverse effect level (LOAEL). These parameters were evaluated graphically by plotting log concentration of the exposure solutions of CH_3Hg^+ and Hg^{2+} against their respective percentage responses (mortalities). These parameters were evaluated at the embryonic stage for Hg^{2+} and at the larval stage for CH_3Hg^+ because it was at these respective stages that the maximum toxicities of Hg^{2+} and CH_3Hg^+ were observed.

4.2.5.2 Body Weight and Total Length Determination

Fifteen larvae of each exposure test solution except for the 26 μg/L of CH_3Hg^+ and the 230 μg/L Hg^{2+} were randomly selected from the four replicates, put in petri dishes, and immobilized according to the method described by Gladys et al., (2015). Briefly, the larvae were immobilized by placing them on ice for 1 – 2 minutes. The body weight (mg ww) and the total length (mm) of

each larva for each exposure test solution of CH_3Hg^+ and Hg^{2+} were then taken using the Practum 124-1sjp analytical/electronic balance (Satorius Lab Instruments, GmbH, and Co., 37070, Goettingen, Germany) and a ruler respectively. Each larva was revived by putting it in a volume of culture water in another petri dish immediately after the measurement. The phenotypic sex of each selected larva for each exposure solution was also determined by examining the shape of the dorsal and anal fins.

4.2.6 Genotoxicity Assessment of the Embryos

4.2.6.1 Gene Expression Analysis

The gene expression analysis was done to evaluate the genetic effects of the Hg levels on selected growth and development genes following repeated prenatal exposures to the food crops. Eight genes including RPL7 as a reference gene (Table 9) were used for the gene expression analysis. The isolation/purification of total RNA and the gene expression analysis using the real - time quantitative polymerase chain reaction (RT-qPCR) followed the procedures described by Ishibashi et al., (2020).

4.2.6.2 Isolation, Purity and Quality Assessment of Total RNA

The total RNA was isolated from 6 days post-fertilized medaka embryos using an RNeasy Micro Kit (Qiagen Co. Ltd, Tokyo, Japan) and treated with an RNase-Free DNase Set (Qiagen Co. Ltd, Tokyo, Japan) to eliminate genomic DNA contamination. The concentration/purity and quality of the isolated total RNA was determined by optical density (Absorbance) measurement using the nana drop (ND 1000) spectrophotometer (NanoDrop Technologies, Inc, Wilminton, USA). The concentrations and the absorbance ratios i.e., A_{260}/A_{280} and the A_{260}/A_{230} were used to evaluate the purity and quality of the isolated RNA.

4.2.6.3 Gene Expression by RT-qPCR Analysis

Approximately 1µg of total RNA was reverse transcribed into complementary DNA (cDNA) using the ReverTra Ace qPCR RT Kit (Toyobo; Osaka, Japan), and the gene expression was analyzed by quantitative PCR (qPCR) using the Fast SYBR Green Master Mix and Mx3000P Real-Time PCR System (Agilent Technologies). The specific primers for the genes including the reference gene (Table 9) used were as previously described by Zhang and Hu, (2007), Yamaguchi

et al., (2018) and Ishibashi et al., (2020). The reaction mixtures were incubated at 95 °C for 20 s, followed by 40 PCR cycles at 95 °C for 30 s, and 60 °C for 30 s. Following RT-qPCR, the specificity of gene amplification was assessed using melting curve analysis.

Table 9: Selected genes and their corresponding sequenced primers

	Sequenced Primers	
Gene	Forward Primer	Reverse Primer
Cyclin B1	5'-ACTACGACAACCCCATGCTC-3'	5'-CCACTTGTACCAGCCAGTCA-3'
	5'-GAGCTACCAAAAGCTGCTGTG-3'	5'-CTCACATCCAGAGGTCTCCA-3'
FGF8	5'-AACGCCCACTACAACGACTG-3'	5'-CGGGTGCGTTTAGTCCTTTG-3'
Bglap	5'-ACTGAGATTAACGGACTGCGA-3'	5'-CTCGGGTTCCACAACTGACA-3'
Hoxd9	5'-AGGACACCTCTTTTTCCTCGT-3'	5'-CGAGTCACGGAGAGCCAAA-3'
CYP3A	5'-ACCCCGAGGTCATGAAGAAAC-3'	5'-TTTCCAACAGTGGTGCGTAC-3'
CYP2P3	5'-CCCTTGGTGTCCTCCGTAT-3'	5'-TGGAGTCTCCCACATGGAC-3'
CYP1A	5'-TTCCACACTGCACAACAAGG-3'	5'-CTTCACTCAGGAAACGATCTGG-3'
RPL7 (ref. gene)	5'-CGCCAGATCTTCAACGGTGTAT-3'	5'-AGGCTCAGCAATCCTCAGCAT-3'

Note:

Ref = reference

4.2.7 Statistical and Data Analysis

The data were statistically analysed using statistical software IBM SPSS statistics version 26 (IBM Corporation, New York, USA) and Microsoft Office excel 2013 (Microsoft Corporation, USA). Paired sample t-test at 95% CI was used to obtain the statistical difference in rates of mortality, delays, deformities, hatching time, and hatchability of the embryos and the differences in survival and/or mortalities, body weights and lengths of the larvae of the exposure test solutions from their respective controls. Non-parametric test using the Wilcoxon signed rank test and the sign test was also used as a post hoc test to confirm the reliability of the statistical significance of the results. SEM and standard deviation were used to evaluate the variability of individual values from the mean for parameters presented in graphs and in tables, respectively. The expression levels of the genes were also analysed statistically using paired sample t-test and non-parametrically using the Wilcoxon signed rank and the sign tests at a significance probability (p) ≤ 0.05.

4.3 Results

4.3.1 Exposure Test Solutions

Tables 10a and 10b show the actual concentrations and the percentage deviations of the exposure test solutions of CH_3Hg^+ and Hg^{2+}, respectively. The initial actual concentrations of CH_3Hg^+ and Hg^{2+} were relatively equal to the nominal concentrations. This showed that the differences between the initial actual and the nominal concentrations were not significant since factor was almost equal to 1 for all the exposure solutions of CH_3Hg^+ and Hg^{2+}. Additionally, the percentage deviations of the final actual concentrations from the initial actual concentrations were within the range of ±20% as indicated by OECD, (2013, 2014).

Table 10a: The initial and final concentrations of exposure test solutions of CH_3Hg^+, and their corresponding percentage deviations

Initial Concentration (µg/L)						Final Concentration (µg/L)		
Nominal			Actual			Actual		
CH_3HgCl	Hg	CH_3Hg^+	Hg	CH_3Hg^+	Factor	Hg	CH_3Hg^+	Dev. (%)
83000	**66466**	**71426.3**	**65137**	**69998**	**0.98**	-	-	-
Control	-	-	-	-	-	-	-	-
1.2	0.96	1.00	0.88	0.94	0.92	0.81	0.87	8.0
2.3	1.84	2.00	2.00	2.2	1.09	1.95	2.10	2.5
3.5	2.80	3.00	3.00	3.2	1.07	2.79	3.00	7.0
5.2	4.20	4.50	4.50	4.7	1.07	4.09	4.40	9.1
23.2	18.60	20.00	18.20	19.7	0.99	17.87	19.20	1.8
30.2	24.20	26.00	23.50	25.3	0.97	21.96	23.60	6.6

Table 10b: The initial and final concentrations of exposure test solutions of Hg^{2+}, and their corresponding percentage deviations

Initial Concentration (µg/L)				Final Concentration (µg/L)	
Nominal		Actual		Actual	
$HgCl_2$	Hg^{2+}	Hg^{2+}	Factor	Hg^{2+}	Deviation (%)
153500	113923.5	114220	1.00	-	-
Control	-	-	-	-	-
20.20	15	16.8	1.12	15.6	7.1
27.00	20	20.7	1.04	20.1	2.9
31.00	23	25.6	1.11	24.1	5.9
202.10	150	157	1.05	148.2	5.6
310.00	230	222.5	0.97	206.6	7.1
512.00	380	364.2	0.96	338.3	7.1

Note:

The actual initial and final concentrations of CH_3Hg^+ were computed from the corresponding Hg concentrations

$$\text{Factor} = \frac{Initial\ actual\ concentration_{(Hg)}}{Initial\ nominal\ comcentration_{(Hg)}} \qquad \text{Deviation (\%)} = \frac{Final\ (actual)_{(Hg)} - Initial\ (actual)_{(Hg)}}{Initial\ (actual)_{(Hg)}} x\ 100$$

4.3.2 Stages of Embryonic Development

The stages of embryonic development are shown in Fig 4.1a and 4.1b. Embryogenesis started on the 1 day post-fertilization (dpf) and by the 8 dpf, all organs of all normally developed embryos had been formed and the embryos could hatch if there was effective enzymatic or biophysical process to mediate hatching (Yamagami, 1981). For CH_3Hg^+, although some embryos (6.5%) of the exposure solutions hatched on 8 dpf, majority (32.4%) hatched on the 9 dpf as shown in Fig. 4.2. For Hg^{2+}, majority (23.6%) of the embryos hatched on the 10 dpf while none of the embryos hatched on the 8 dpf as shown in Fig. 4.2.

Fig 4.1a: Stages of embryonic development of Japanese medaka – for CH_3Hg^+ exposure solutions

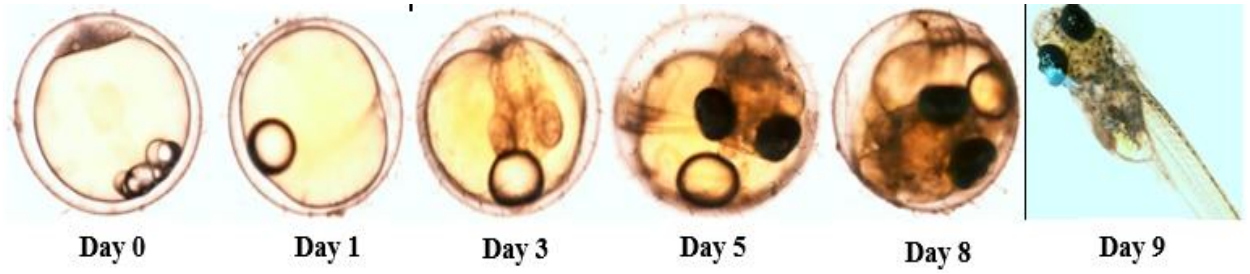

Fig 4.1b: Stages of embryonic development of Japanese medaka – for Hg^{2+} exposure solutions

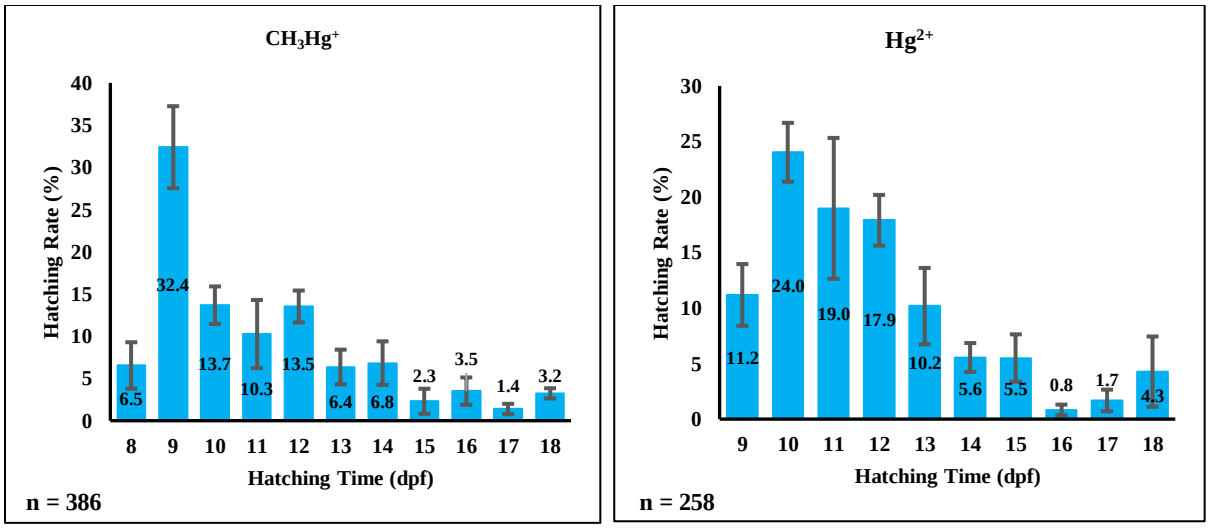

Fig 4.2: Variability in hatching time of medaka embryos. n = total number of hatched embryos in the 4 replicate determinations for all exposure test solutions and error bars represent SEM.

4.3.3. Exposure and Toxicity Assessment of the Embryos

4.3.3.1 Effect of CH_3Hg^+ and Hg^{2+} on Embryonic Development of Medaka

Throughout the exposure period, end points such as Hatchability, heart rate, hatching time, and developmental toxicities (teratogenic effects) such as delays, deformities or malformations, and mortalities were monitored. The teratogenic effects common to both CH_3Hg^+ and Hg^{2+} were thrombus, death, arrested growth or delayed development, slow heartbeat, and general body malformation which included small head and eyes or virtually no eyes, shrunk body, etc. (Fig. 4.3a and b). The teratogenic effect that was specific to CH_3Hg^+ was deformed swim bladder (Fig. 4.3a, F, J, and K) while reduced eye pigmentation was also specific to Hg^{2+} (Fig. 4.3b, F and L). Delays were observed in test solutions of 0.87 – 3 µg/L of CH_3Hg^+ and 15.6 – 24.1 µg/L of Hg^{2+} including their respective controls (Fig. 4.4A).

For CH_3Hg^+, mortalities were seen in all exposure solutions except the control with increased mortality rate (10%) in the 2.1 µg/L solution. This led to a statistically significant difference ($p \leq 0.05$) in mortality rate of the 2.1 µg/L solution from its control (Fig. 4.4A). However, the 2.1 µg/L solution had no deformities but deformities were seen in other solutions except the control. For Hg^{2+}, mortalities were seen in all exposure solutions including the control with increased mortalities (56.7 – 100%) in 148.2 – 338.3 µg/L solutions (Fig. 4.4A). Although the

148.2 – 338.3 μg/L solutions showed increased mortality rates, only the mortality rate of the 338.3 μg/L solution was statistically significantly different ($p \leq 0.05$) from the control. However, like CH_3Hg^+, the Hg^{2+} solutions with the highest mortality rates had no deformities or malformations but deformities were observed in the 15.6 – 24.1 μg/L solutions (Fig. 4.4A). Most embryos with deformities or malformations died before the end of the exposure period, particularly between the 8 and 12 dpf while few were already dead upon hatch. All the embryos exposed to the 338.3 μg/L solution died by the 4 dpf. The major teratogenic effects observed in the control of CH_3Hg^+ were delays (8.3%) while mortalities (5%) were also the major teratogenic effects in the control of Hg^{2+} (Fig. 4.4A). The delays in the exposure solutions of CH_3Hg^+, particularly the 4.4, 19.2, and 23.6 μg/L solutions were 0%, hence the statistically significant difference ($p \leq 0.05$) in delay rates of these solutions from their control. Only the 338.3 μg/L solution of Hg^{2+} showed a statistically significant difference in rate of hatchability from its control but there was no statistically significant difference ($p > 0.05$) in rates of deformities and hatchability (Fig. 4.4A) of exposure test solutions of CH_3Hg^+ and the 15.6 – 206.6 μg/L solutions of Hg^{2+} from their respective controls. The hatchability (%) of the controls of CH_3Hg^+ and Hg^{2+} were 91.7 and 93.3 while the average hatchability of their exposure test solutions were 91.7 and 67.3%, respectively.

The hatching time (Fig. 4.4B) of CH_3Hg^+ ranged from 8 – 9.3 dpf while that of Hg^{2+} ranged from 9.3 – 11.5 dpf. For CH_3Hg^+, only the 19.2 μg/L solution showed a statistically significant difference ($p \leq 0.05$) in hatching time from the control although all the exposure solutions of CH_3Hg^+ had lower hatching times compared to the control. The differences in hatching time of the 15.6 – 206.6 μg/L exposure solutions of Hg^{2+} were not statistically significant from their control.

The differences in heart rates (Fig. 4.4C) of the embryos were not statistically significant ($p > 0.05$) although the heart rates of test chemicals were higher than their respective controls except for 148.2 and 206.6 μg/L solutions of Hg^{2+}, particularly on the 6 and 8 dpf. For CH_3Hg^+, heart rates of the embryos increased from the 4 to 8 dpf while those of the embryos exposed to Hg^{2+} were in the order 4 dpf > 8 dpf > 6 dpf except for the 206.6 μg/L solution where the heart rates of embryos on 6 dpf were higher than those on 8 dpf (Fig. 4.4C). This showed that the heart rates of embryos in Hg^{2+} exposure test solutions did not follow a particular trend.

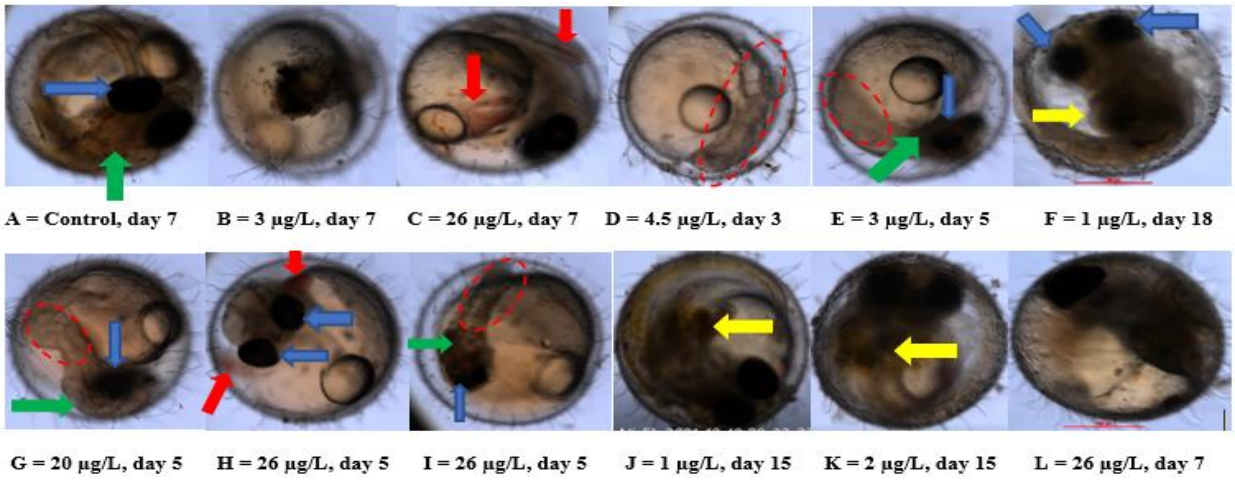

Fig 4.3a: Some observed developmental toxicities (teratogenic effects) of CH_3Hg^+ during embryogenesis. A = normal, B = dead, C = thrombus, general body malformation, D = general body malformation, absence of head and eyes, E = general body malformation, extremely reduced head and eyes, F = reduced eyes, deformed swim bladder, rough skin, and death, G = general body malformation, reduced eyes, small head, and small body, H = thrombus, small eyes, and small head, I = general body malformation, extremely small head, virtually no eyes, and extremely small body, J = deformed swim bladder, K = deformed swim bladder, and L = general body malformations.

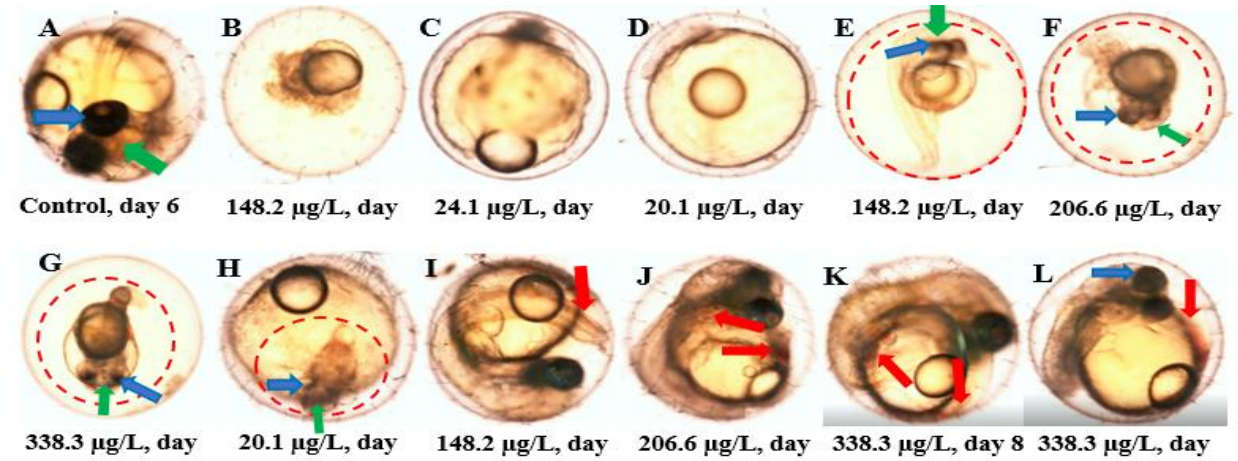

Fig. 4.3b: Some observed developmental toxicities (teratogenic effects) of Hg^{2+} during embryogenesis. A = normal embryogenesis, B = dead, C = dead, D = arrested growth, E – G, and H = general body malformations: extremely reduced head, virtually no eyes, shrunk body, lack of eye pigment, I – K = thrombus, and L = thrombus, small eyes, and reduced eye pigment.

Key: Blue arrow = eyes, green arrow = head, red arrow = thrombus, dotted circle = general body malformation, yellow arrow = deformed swim bladder.

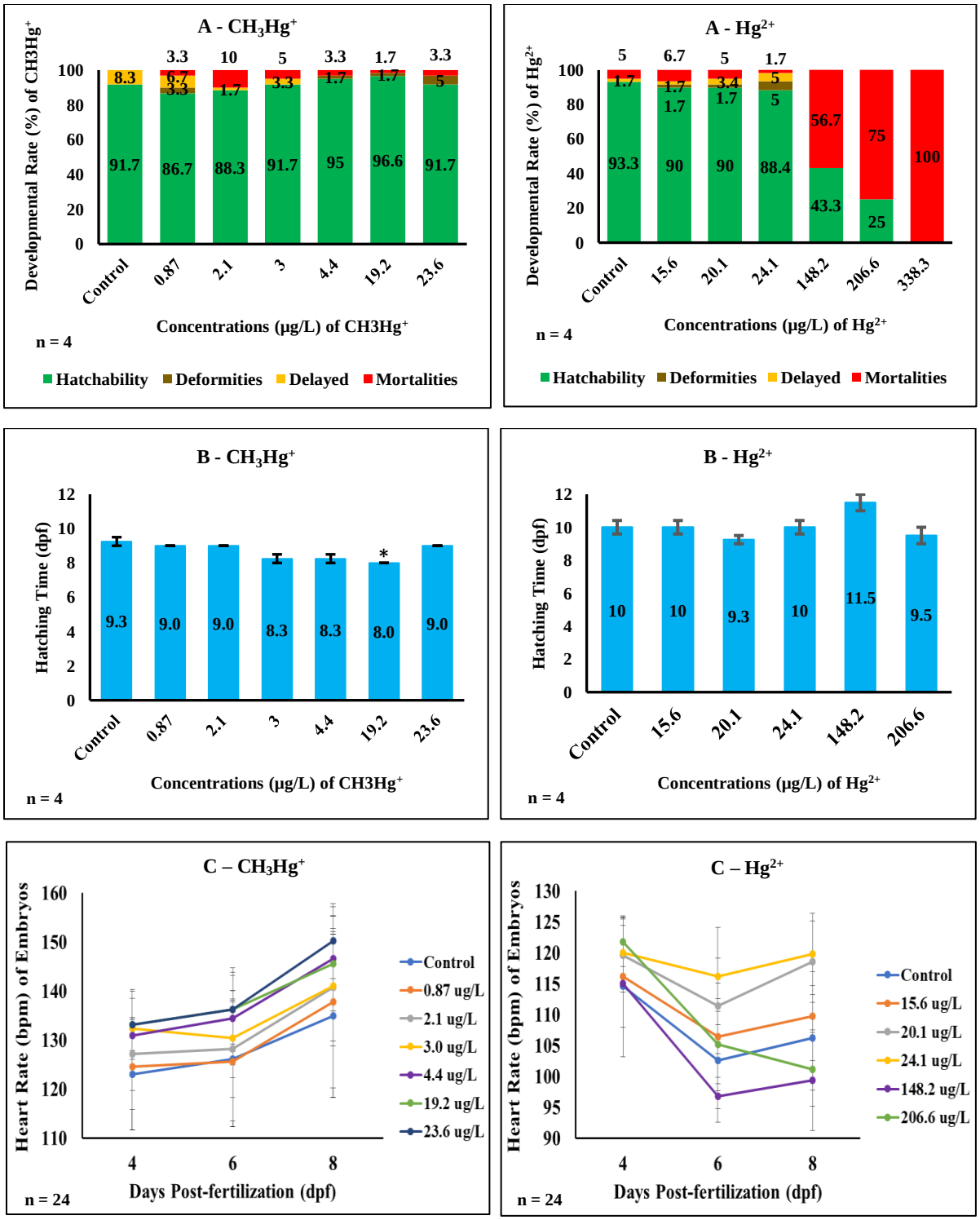

Fig 4.4: Experimental end points of CH_3Hg^+ and Hg^{2+}. A = Rate of development comprising hatchability and teratogenic effects, B = hatching time, and C = heart rate. Error bars represent SEM, n = number of replicate determinations but n for the heart rate is the number of randomly selected embryos from 3 replicate determinations, and * denotes statistically significant difference ($p \leq 0.05$) of a test solution from its control.

4.3.4 Post-Hatch Survival, Mortalities and Growth of Larvae

4.3.4.1 Effect of CH_3Hg^+ and Hg^{2+} on Larval Survival and Mortality

Fig. 4.5a and 4.5b show the developmental toxicities that were observed in the larvae during the post-hatch assessment. Besides larval mortalities, other developmental toxicities were very specific to the chemical form of Hg. The developmental defects that were specific to CH_3Hg^+ included deformed mouth, intestinal thrombus (Fig. 4.5a; C), deformed or uninflated swim bladder, and protruded yolk sac (Fig. 4.5a; C-E). These toxicities were observed in very few individuals on the 1 day post-hatch (dph). Individuals with such deformities died within the 1 wph. Larval mortality rate of CH_3Hg^+ increased from the 3 – 23.6 μg/L solutions (Fig. 4.6; Table 11a). This resulted in statistically significant difference ($p \leq 0.05$) in mortality rates of these solutions from their control (Fig. 4.6; Table 11a).

For Hg^{2+}, the only developmental defect seen was bent tail which occurred in the 148.2 and 206.6 μg/L solutions (Fig. 4.5b). This defect was observed in 50 and 93.3% individuals of the 148.2 and 206.6 μg/L solutions of Hg^{2+}, respectively. This led to a statistically significant difference ($p \leq 0.05$) in mortality rate of the 148.2 μg/L solution from its control (Fig. 4.6; Table 11b). The statistical difference between the 206.6 μg/L solution and its control was not evaluated since only a larva survived in all the four replicate determinations after the 1 wph (Table 11b). Out of the 13 larvae that survived in the 148.2 μg/L solution after the 1 wph, only 2 died by the 8 wph. Like CH_3Hg^+, individuals with bent tail deformities died within the 1 wph.

Additionally, during the 8 wph assessment, death (Fig. 4.5a, B) was observed in all exposure solutions of CH_3Hg^+ and Hg^{2+} including their controls but the survival rates in the controls of CH_3Hg^+ and Hg^{2+} at 8 wph were ≥ 80 (Fig. 4.6; Tables 11a and b). This was consistent with the 80% or higher survival rate at 60 dph in controls indicated by OECD, (2013, 2014). The differences in mortality and survival rates of the 0.87 and 2.1 μg/L solutions of CH_3Hg^+ and the 15.6 – 24.1 μg/L solutions of Hg^{2+} were not statistically significantly different ($p \leq 0.05$) from their respective controls (Fig. 4.6; Tables 11a and b).

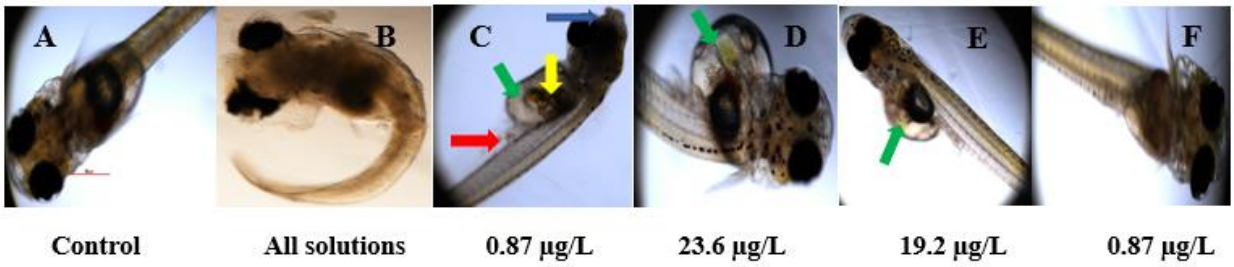

Fig 4.5a: Some observed developmental toxicities of CH_3Hg^+ during the 8 wph assessment of the larvae. A = normal, B = dead, C = intestinal thrombus, deformed mouth, and deformed swim bladder (uninflated swim bladder), C – E = protruded yolk sac, and F = normal. Except for death which occurred in almost all the weeks of post-hatch assessment, the other defects (C – E) were observed within the 1 wph, and all larvae with such defects died within the 1 wph.

Key: Blue arrow = deformed mouth, red arrow = intestinal thrombus, green arrow = protruded yolk sac, and yellow arrow = deformed or uninflated swim bladder.

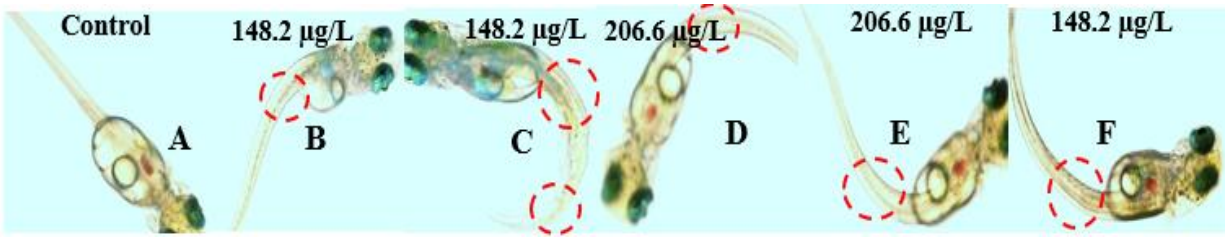

Fig. 4.5b: Major post-hatch developmental toxicities of Hg^{2+} after 18 dpf. A = normal and B – F = bent or curved tails. Dotted red circle indicates the point of curvature. The bent tail defect was observed on 1 dph and all larvae with such defect died within the 1 wph.

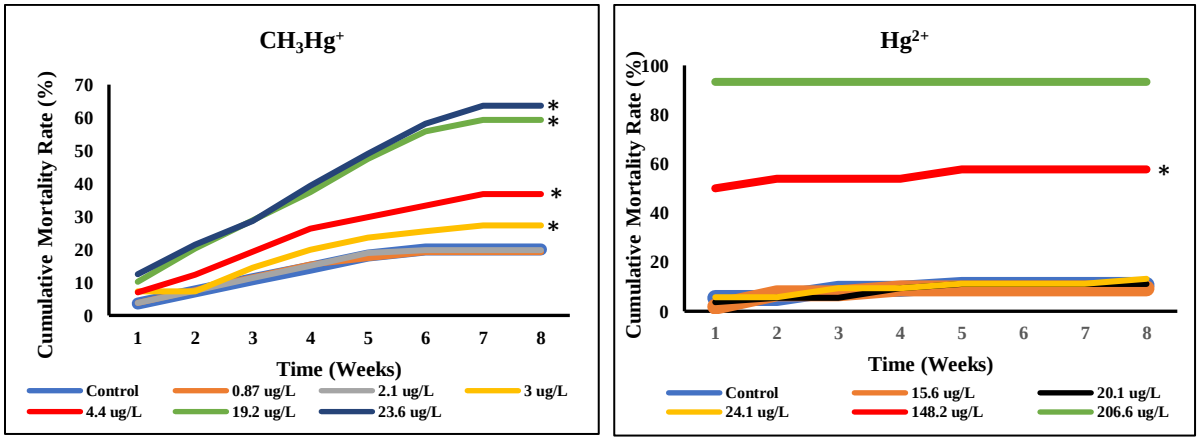

Fig. 4.6: Cumulative mortality rate of CH_3Hg^+ and Hg^{2+}. * Represents the statistically significant difference ($p \leq 0.05$) of exposure test solutions from their respective controls.

Table 11a: Survival and mortality rates of the larvae of CH_3Hg^+ test solutions at 8 wph

	Concentrations (µg/L) of CH_3Hg^+						
	Control	**0.87**	**2.1**	**3**	**4.4**	**19.2**	**23.6**
No. of Larvae (1st wph)	55	52	53	55	57	59	55
No. of Larvae (8th wph)	44	42	42	40	36	24	20
Survival Rate (%)	80.0	80.8	79.2	72.7*	63.2*	40.7*	36.4*
Mortality Rate (%)	20.0	19.2	20.8	27.3*	36.8*	59.3*	63.6*

Table 11b: Survival and mortality rates of the larvae of Hg^{2+} test solutions at 8 wph

	Concentrations (µg/L) of Hg^{2+}						
	Control	**15.6**	**20.1**	**24.1**	**148.2**	**206.6**	**338.3**
No. of Larvae (1st wph)	56	54	54	53	26	15	-
No. of Larvae (8th wph)	50	49	48	46	11	1	-
Survival Rate (%)	89.3	90.7	88.9	86.8	42.3*	6.7	-
Mortality Rate (%)	10.7	9.3	11.1	13.2	57.7*	93.3	-

Note

* Denotes statistically significant difference ($p \leq 0.05$) of test solution(s) from the control

4.3.4.1.1 Concentration–Response Analysis

The LC_{50}, NOAEL and the LOAEL are shown in Fig. 4.7. The LC_{50} was evaluated to determine the concentrations of CH_3Hg^+ and Hg^{2+} that caused the death of 50% of the larvae and the embryos at 18 dpf exposures and subsequent 8 wph assessment. The NOAEL was the concentrations at which no adverse effects were observed while the LOAEL corresponded to the concentrations at which low or minimal adverse effects likely occurred. From Fig. 4.7, the LC_{50}, NOAEL and the LOAEL of CH_3Hg^+ were 11 (antilog of 1.04), 2.1 (antilog of 0.32) and 3.1 µg/L (antilog of 0.49) while those of Hg^{2+} were 126 (antilog of 2.1), 20 (antilog of 1.3) and ≥ 24.5 µg/L (antilog of 1.39).

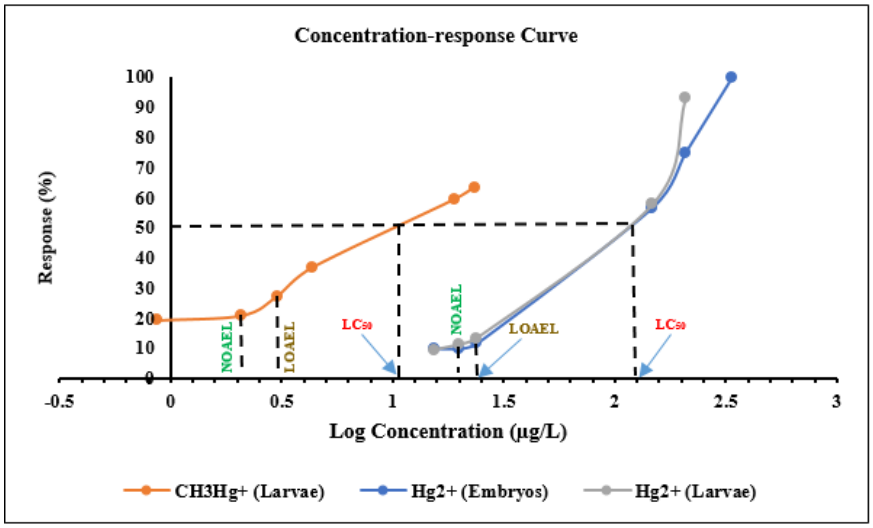

Fig 4.7: Concentration–response curves for embryos and larvae following 18 dpf exposures to CH_3Hg^+ and Hg^{2+} and subsequent 8 wph assessment.

4.3.4.2 Effect of CH_3Hg^+ and Hg^{2+} on Larval Growth

The body weight, total length and the sex ratios of the larvae of embryos that were exposed to CH_3Hg^+ and Hg^{2+} are shown in Tables 12a and 12b, respectively. For CH_3Hg^+, only the 4.4 µg/L solution showed a statistically significant difference ($p \leq 0.05$) in body weight and total length from the control. Although the differences in body weight and total length of larvae in the 23.6 µg/L solution of CH_3Hg^+ were not statistically significant from the control but the larvae in the 23.6 µg/L solution had the highest body weight and total length than those of the control and other exposure solutions despite the fewer number of larvae present (Table 12a). The male: female sex ratio was relatively balanced in all exposure solutions including the control (Table 12a).

For Hg^{2+}, only the 148.2 µg/L solution showed a statistically significant difference ($p \leq 0.05$) in body weight from its control (Table 12b). No statistical difference was evaluated for the 206.6 and the 338.3 µg/L solutions due to the 1 and 0 larval numbers, respectively. However, the 1 larva that remained in the 206.6 µg/L solution had the highest body weight and total body length than those of the control and other exposure solutions of Hg^{2+} at 8 wph (Table 12b). Compared to the control, the sex ratios for the embryos exposed to Hg^{2+} were also relatively balanced in all exposure solutions.

Table 12a: Body weight, total length, and sex ratio of the Larvae of CH_3Hg^+ at 8 wph

	Concentrations (µg/L) of CH_3Hg^+						
	Control	**0.87**	**2.1**	**3**	**4.4**	**19.2**	**23.6**
Weight (mg ww)	111.7 ±22.4	96.7 ±25.6	96.4 ±25.7	110.8 ±39.2	95.9 ±19.0*	118.5 ±22.0	161.8 ±60.1
Length (mm)	22.4 ± 1.6	21.5 ± 2.2	21.3 ± 2.4	22.0 ± 3.0	21.0 ± 1.8*	22.8 ± 1.9	24.3 ± 3.0
Male: Female	7:8 (15)	7:8 (15)	9:6 (15)	9:6 (15)	7:8 (15)	10:5 (15)	5:4 (9)

Table 12b: Body weight, total length, and sex ratio of the Larvae of Hg^{2+} at 8 wph

	Concentrations (µg/L) of Hg^{2+}						
	Control	**15.6**	**20.1**	**24.1**	**148.2**	**206.6**	**338.3**
Weight (mg ww)	89.3 ±18.0	88.7 ±31.3	77.4 ±20.5	77.0 ±20.8	106.2 ±14.6*	139.7	-
Length (mm)	20.7 ± 1.5	20.3 ± 2.8	19.8 ± 2.0	19.7 ± 2.3	22.1 ± 1.5	24.7	-
Male: Female	9:6 (15)	7:8 (15)	7:8 (15)	6:9 (15)	7:4 (11)	0:1 (1)	-

Note:

Numbers in parentheses shown against the male to female sex ratios refer to the number of randomly selected larvae for the post-hatch assessment of body weight and total length. * Indicates exposure solutions of the test chemicals that are statistically significantly different ($p \leq 0.05$) from their respective controls.

4.3.5 Genotoxicity Assessment

4.3.5.1 Concentrations, Purity and Quality Assessment of Total RNA

The concentrations, A_{260}/A_{280}, and A_{260}/A_{230} ratios are shown in Fig. 4.8. The concentrations of the controls and test solutions of both CH_3Hg^+ and $Hg^{2+} \geq 100$ ng/µL while the A_{260}/A_{280} and A_{260}/A_{230} ratios ranged from 2.05 – 2.09 and 1.03 – 1.48, respectively, for both CH_3Hg^+ and Hg^{2+}.

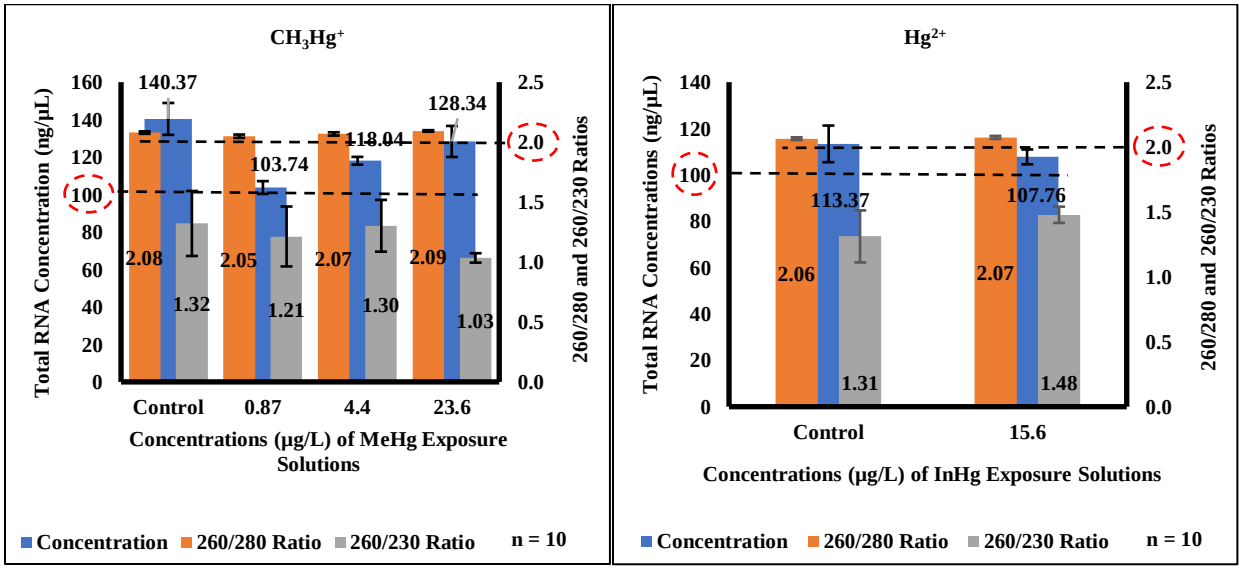

Fig. 4.8: Quantification and quality assessment of isolated total RNA of embryos exposed to CH_3Hg^+ and Hg^{2+}. Error bars represent SEM and n = total number of measurements for 4 replicate determinations.

4.3.5.2 Gene Expression by RT-qPCR Analysis

The results for the gene expression analysis are shown in Fig. 4.9. Out of the seven tested genes, only cyclin B1 showed an increase or decrease in expression levels following 6 dpf exposures to both CH_3Hg^+ and Hg^{2+}. The remaining genes showed no expressions as their cycle threshold (Ct) values ≥ 40. However, the expression levels of cyclin B1 by both CH_3Hg^+ and Hg^{2+} were not statistically significantly different from their respective controls but there was an apparent concentration-dependent increase and decrease in cyclin B1 expression, particularly by the 0.87 and 23.6 µg/L solutions of CH_3Hg^+ respectively, and a concentration-dependent decrease in cyclin B1 expression by the 15.6 µg/L solution of Hg^{2+}.

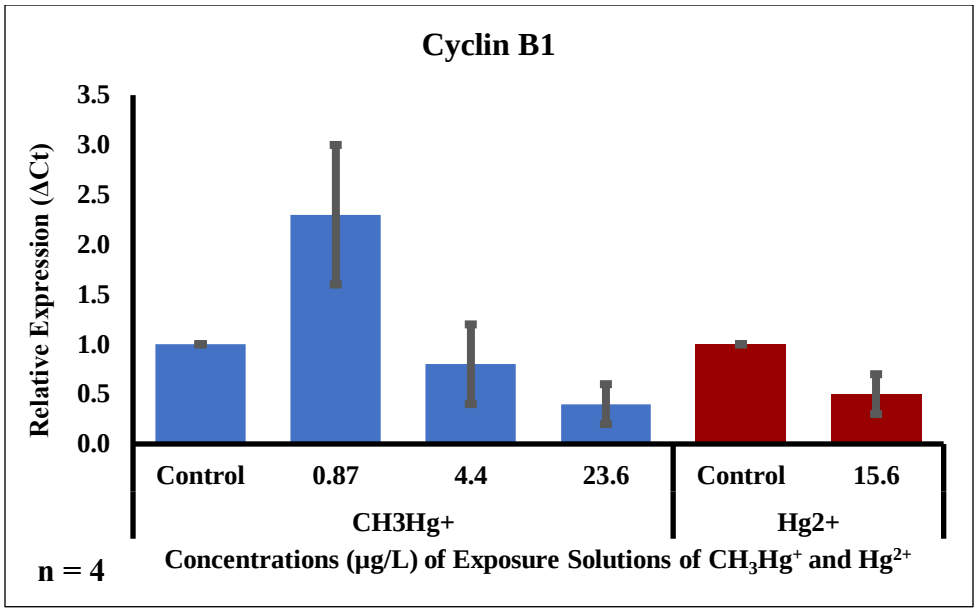

Fig. 4.9: Relative expression of cyclin B1 gene following 6 dpf exposures of medaka embryos to CH_3Hg^+ and Hg^{2+} test solutions. n = number of replicate determinations. No statistically significant difference ($p > 0.05$) was observed between test solutions of the test chemicals and their respective controls but an apparent concentration-dependent increase or decrease was observed.

4.4 Discussions

4.4.1 Exposure Solutions

The percentage deviations of the exposure solutions of CH_3Hg^+ and Hg^{2+} (Tables 10a and b) were within the ±20% range specified by OECD, (2014). This meant that the solutions were stable during the exposure period despite the minimal reduction in concentrations. Moreover, the factor values around 1 showed the relative equality between the initial nominal and the initial actual concentrations of the solutions, hence confirmed the suitability of the test solutions for the toxicity studies.

4.4.2 Stages of Embryonic Development

Embryonic development was consistent in all the replicates of CH_3Hg^+ and Hg^{2+} throughout the exposure period except for the 148.2 and 206.6 µg/L solutions of Hg^{2+}. These concentrations had 100% mortality rates in one and two replicate determinations, respectively. Nevertheless, the start of embryogenesis, the formation of various organs and the hatching times

of normally developed embryos were consistent with the stages described by Iwamatsu, (2004) and OECD, (2013, 2014). Although most of the normally developed embryos hatched between days 9 and 12 (Fig. 4.2), the variability in hatching time of the embryos probably resulted from the differences in enzymatic or biophysical processes that soften the chorion (Yamagami, 1981) to cause the release of the embryo.

4.4.3 Exposure and Toxicity Assessment of the Embryos

4.4.3.1 Effect of CH_3Hg^+ and Hg^{2+} on Embryonic Development

The embryonic toxicities of CH_3Hg^+ and Hg^{2+} were evaluated based on hatchability, hatching time, heart rate, and teratogenic effects such as delays, deformities (malformations), and mortalities. The controls of both CH_3Hg^+ and Hg^{2+} had hatchability > 90% (Fig. 4.4A). This was consistent with the ≥ 80% (OECD, 2013) and/or ≥ 90% (Ministry of Environment, Japan, MEJ, 2002; OECD, 2014) hatchability in controls. All the exposure test solutions of CH_3Hg^+ and Hg^{2+} except for the 148.2 and 206.6 µg/L solutions of Hg^{2+} had hatchability within the ranges indicated by MEJ, (2002) and OECD, (2013, 2014). This showed that both the controls and the test solutions of CH_3Hg^+ and Hg^{2+} in concentration ranges of 0.87 – 23.6 and 15.6 – 24.1 µg/L, respectively had no effect on hatchability of medaka embryos. Additionally, the lack of statistically significant difference in hatchability of the 0.87 – 23.6 µg/L solutions of CH_3Hg^+ and 15.6 – 206.6 µg/L solutions of Hg^{2+} from their respective controls meant that these concentrations had no effect on the hatchability of medaka embryos. However, the extremely lower hatchability (Fig. 4.4A) of the 148.2 and 206.6 µg/L solutions and the zero hatchability of the 338.3 µg/L solution showed that Hg^{2+} concentration of 148.2 µg/L or higher will lead to higher rate of embryonic toxicities including mortalities.

Yu et al., (2019) indicated higher hatching success in embryos of the large yellow croaker at MeHg concentrations of 5 – 20 µg/L. Abbott et al., (2017) also reported a 100% hatchability of zebrafish embryos at MeHg concentrations of 5 and 10 ppb at 48 hours post-fertilization (hpf) whereas Dong et al., (2016) indicated 80 – 100% hatchability of medaka embryos in concentration range of 0.001 – 0.1µM (0.25 – 25 µg/L) of MeHg at 10 dpf. The concentration range of CH_3Hg^+ in this study falls in the range of the studies indicated above, hence the consistency in hatchability

(86.7 – 96.7%) of exposure solutions of CH_3Hg^+ in this study with those of the studies indicated above.

Abbott et al., (2017) reported 62.5 – 83.6% and 0 – 38.3% hatchability at 96 hpf of zebrafish embryos exposed to $HgCl_2$ in concentration range of 5 – 100 µg/L and 200 - 500 µg/L, respectively. Abbott et al., (2017) indicated that the differences in hatchability of the 5 – 100 µg/L solutions from the control (72.9%) were not statistically significant while those of 200 - 500 µg/L were statistically significant. Dong et al., (2016) also reported about 85% or higher hatchability at 10 dpf of medaka embryos exposed to $HgCl_2$ at concentration range of 0.272 – 27.2 µg/L while those exposed to 272 µg/L and 2720 µg/L solutions had 70 and 0% hatchability, respectively. These studies suggest that $HgCl_2$ in concentration range of 5 – 100 µg/L and 0.272 – 27.2 had no effect on embryonic toxicity of zebrafish and medaka, respectively while the 200 – 500 µg/L concentrations had severe embryonic toxicities. These findings are, therefore, consistent with the findings for Hg^{2+} in this study.

Although some developmental toxicities (teratogenic effects) upon exposures to CH_3Hg^+ occurred in few embryos during embryonic development, the higher hatchability of the embryos confirmed that CH_3Hg^+ in concentration range of 0.87 – 23.6 µg/L had no toxic effects on embryonic development despite the statistically significant difference in rates of delay and mortality of the 4.4, 19.2, and 23.6 µg/L solutions and the 2.1 µg/L solution from their control, respectively (Fig. 4.4A). Moreover, the lack of statistically significant difference in deformities of test solutions of CH_3Hg^+ from their control also proved the no toxic effect of MeHg (0.87 – 23.6 µg/L) on embryonic development of medaka. This meant that the few embryos that had these teratogenic effects were probably naturally non-viable considering the highest rate of delay in the control. Additionally, studies by Dong et al., (2016) and Abbott et al., (2017) reported no developmental toxicities of MeHg to medaka and zebrafish embryos in concentration ranges of 0.25 – 25 µg/L and 5 – 10 ppb, respectively. This, therefore, indicated that CH_3Hg^+ (0.87 – 23.6 µg/L) caused no developmental toxicities to medaka embryos.

Like CH_3Hg^+, the higher hatchability and the lack of statistically significant difference in rate of teratogenesis of the 15.6 – 24.1 µg/L solutions of Hg^{2+} also meant that Hg^{2+} in the above concentration range had no toxic effects on embryonic development of medaka despite the few

teratogenic effects that occurred in these concentrations. However, the 56.7 – 100% mortality rate of embryos in the 148.2 – 338.3 µg/L solutions clearly showed that such concentration range of Hg^{2+} had severe effects on embryonic development of medaka. The no toxic effects of the 15.6 – 24.1 µg/L solutions of Hg^{2+} is consistent with the findings of Dong et al., (2016) and Abbott et al., (2017) in a similar study with relatively equal concentration ranges.

Generally, the hatching time of medaka embryos in control is around 9 – 10 days as indicated by Iwamatsu, (2004) and MEJ, (2002). Wang et al., (2006b), Cho et al., (2013) and Le Bihanic et al., (2014) reported hatching times of medaka embryos at 7.5, 8 – 9, and 10.4 – 11.9 dpf, respectively for controls and test chemicals under similar experimental conditions used in this study. Additionally, Yokota et al., (2001) and Hano, (2012) also reported hatching times of 9.4 – 10.2 and 7.9 – 8.7 dpf, respectively for controls and test chemicals under similar experimental conditions. The above studies suggest that the hatching time of cultured medaka embryos is usually within 8 – 12 dpf and falls in the range of this study as shown in Fig. 4.2. Therefore, the hatching times of 8 – 9.3 dpf for CH_3Hg^+ and 9.3 – 11.5 dpf for Hg^{2+} (Fig. 4.4B) of medaka embryos in this study are consistent with the above studies. The hatching times of 9.3 and 10 dpf of the controls of CH_3Hg^+ and Hg^{2+}, respectively fall in the range indicated by MEJ, (2002), Iwamatsu, (2004) and OECD, (2013, 2014).

Additionally, although the 19.2 µg/L solution of CH_3Hg^+ showed a statistically significant difference in hatching time from the control, the hatching time at 8 dpf was consistent with other studies, particularly those of Wang et al., (2006b), Hano, (2012) and Cho et al., (2013). Therefore, early hatching time and the statistically significant difference in hatching time of the embryos in the 19.2 µg/L solution of CH_3Hg^+ could not be attributed to the effect of MeHg in concentration range of 0.87 – 23.6 µg/L.

For Hg^{2+}, the hatching times of embryos in the 148.2 and 206.6 µg/L solutions were within the acceptable range and not statistically significantly different from the control but these concentrations showed severe toxic effects on embryonic development of medaka. This meant that the few embryos that survived without any or minimal embryonic toxicities hatched within the acceptable hatching time since the delay rate was 0% in these concentrations compared to the

control and the lower concentrations of Hg^{2+}. Therefore, Hg^{2+} in concentration range of 15.6 – 206.6 µg/L had no effect on the hatching time of medaka embryos.

The lack of statistically significant difference in heart rates (Fig. 4.4C) of embryos exposed to CH_3Hg^+ and Hg^{2+} from their respective controls also showed that both CH_3Hg^+ and Hg^{2+} in concentration ranges of 0.87 – 23.6 and 15.6 – 206.6 µg/L, respectively had no effects on heart rates of the embryos despite the extremely higher mortality rates of embryos exposed to 148.2 and 206.6 µg/L solutions of Hg^{2+}. The heart rates of embryos exposed to CH_3Hg^+ increased from 4 to 8 dpf with a huge increase at 8 dpf (at or near hatching time). The increased heart rates from 4 to 8 dpf, particularly on 8 dpf of embryos in exposure solutions of CH_3Hg^+ including the control was due probably to the development of heart muscles as the embryos developed. Heart muscles of the embryos were almost completely developed by 8 dpf, hence the extreme increase in heart rates of the embryos at 8 dpf (at or near hatch) compared to the 4 and 6 dpf. The increase in heart rates is consistent with the findings of Yu et al., (2019) who reported increased heart rates of the larvae of large yellow croaker at MeHg concentrations between 5 and 20 µg/L. Therefore, the increased heart rates of medaka embryos could not be attributed to the effect of MeHg in concentration range of 0.87 – 23.6 µg/L.

The heart rates of embryos exposed to Hg^{2+} did not follow a particular trend but the lower heart rates of embryos at 6 and 8 dpf, particularly of embryos in the 15 – 24.1 µg/L solutions could not be attributed to the effect of Hg^{2+} since embryos of the control also had lower heart rates. However, the decrease in heart rates of embryos exposed to 148.2 and 206.6 µg/L solutions of Hg^{2+} at 6 and 8 dpf compared to the control and other test solutions were very much pronounced despite the lack of statistically significant difference. This, therefore, could be attributed to the effect of Hg^{2+} on embryonic development. Cho et al., (2013) reported a decrease in heart rate at 7 dpf of medaka embryos exposed to highest concentration of Ag nanoparticles and concluded that the decreased heart rates of the embryos were due to the increased toxicity of the Ag nanoparticles. This is consistent with the decreased heart rates of embryos that were exposed to 148.2 and 206.6 µg/L solutions of Hg^{2+}. However, this trend was not observed in embryos that were exposed to CH_3Hg^+. The difference probably resulted from the differences in the test chemical and the concentration used because the nature of the chemical and the exposure dose are among the factors that determine the extent of toxicity of contaminants.

Cho et al., (2013), Watanabe-Asaka et al., (2014) and Puybareau et al., (2015) reported normal heart rates (bpm) of medaka embryos of 110 – 125 at 3 – 7 dpf, 116 , and 130 for control in their studies under similar experimental conditions of temperature and pH. The heart rates (bpm) of medaka embryos in this study were 123 – 135 and 102.6 – 114.6 for the controls of CH_3Hg^+ and Hg^{2+}, respectively while those for the test solutions were 124.6 – 150.2 for CH_3Hg^+ and 96.8 – 121.8 for Hg^{2+}at 4 – 8 dpf. These are consistent with those of the studies indicated above.

4.4.4 Post-Hatch Survival, Mortalities and Growth of Larvae

4.4.4.1 Effect of CH_3Hg^+ and Hg^{2+} on Larval Survival and Mortality

According to OECD, (2013, 2014), the survival rate of larvae in the control within or at 60 dph (8 wph) should be ≥ 80%. This means that mortality rate > 20% in any exposure solution of a test chemical can be attributed to the toxic effect of the toxicant if all experimental parameters such as pH, temperature, feeding, photoperiod, etc. are duly monitored. The embryos in the control and those that were exposed to 0.87 and 2.1 µg/L of CH_3Hg^+ had larval mortality rate ≥ 80% at 8 wph. This meant that the increased mortality rate of the larvae in the 3 – 23.6 µg/L solutions resulted from the toxic effect of CH_3Hg^+. Additionally, the statistically significant difference in mortality rate of the 3 – 23.6 µg/L solutions from the control (Table 11a) confirmed the toxicity of CH_3Hg^+ to the larvae in the above concentration range.

The increased mortality rate of the larvae in the 3 – 23.6 µg/L solutions resulted from the increasing concentration of CH_3Hg^+ because the exposure dose is one of the major factors that determine the toxicity level of a toxicant. The toxicity of CH_3Hg^+ probably resulted from the gradual bioaccumulation of CH_3Hg^+ in the embryos right from the onset of embryogenesis. The repeated exposures to higher concentrations of CH_3Hg^+ in embryogenesis led to higher bioaccumulation and subsequent post-hatch toxicity which resulted in higher deaths, particularly in the 19.2 and 23.6 µg/L solutions. Lemly, (2002) in his study of selenium toxicity to fishes reported of post-hatch mortality due to bioaccumulation of selenium in eggs. Additionally, some few larvae, particularly in the 19.2 and 23.6 µg/L solutions had protruded yolk sac (Fig. 4.5C–E). Although, larvae with swim bladder deformities or protruded yolk sac could not swim or move actively and died within the 1 wph, such post-hatch toxicities observed in this study are similar in

nature and consistent with those reported by Dong et al., (2016) in a similar study of CH_3Hg^+ toxicity to medaka embryos.

The higher larval mortality rates of the embryos that were exposed to the 148.2 and 206.6 µg/L solutions of Hg^{2+} within the 1 wph resulted from embryos which were already deformed (bent tail or bone curvature) upon hatch. Such larvae could not swim effectively or move actively, hence could not survive beyond the 1 wph. The bent tail or bone curvature observation is consistent with the finding by Abbott, (2017) when he reported of curved tails in zebrafish embryos that were exposed to 100 – 200 µg/L solutions of $HgCl_2$ for 96 hpf. However, there was significantly reduced mortalities, particularly of the 148.2 µg/L solution from the 2 to 8 wph (Fig. 4.6). Additionally, the larval mortality rates of the 15.6 – 24.1 µg/L solutions of Hg^{2+} at 8 wph were below 20% (Table 11b). This, therefore, suggests that unlike CH_3Hg^+, Hg^{2+} does not bio accumulate or its bioaccumulation potential is extremely lower (Addai-Arhin et al., 2021), hence the extremely lower mortality rates in the exposure solutions of Hg^{2+} from the 2 to 8 wph (Fig. 4.6).

4.4.4.1.1 Concentration–Response Analysis

The increased post-hatch mortality rate of the larvae, particularly in the 19.2 and 23.6 µg/L solutions of CH_3Hg^+ and the increased mortality rate of embryos in the 148.2 – 338.3 µg/L solutions of Hg^{2+} necessitated the estimation of the LC_{50}, NOAEL, and LOAEL (Fig. 4.7). The gradual bioaccumulation of CH_3Hg^+ in embryos during embryogenesis coupled with the sensitivity or vulnerability of the larval population to CH_3Hg^+ intoxication probably resulted in the high rate of post-hatch or larval mortality. Yu et al., (2019) reported that the larvae of large yellow croaker were more sensitive to CH_3Hg^+ than their embryos when they obtained LC_{50} of 18.27 and 28.39 µg/L for larvae and embryos, respectively at 48 hpf. Additionally, Yu et al., (2019) indicated lower survival rate of the larvae of large yellow croaker at CH_3Hg^+ concentrations $\geq$ 5 µg/L, which is consistent with the findings of this study. Cho et al., (2013) reported 0.80 and 0.84 ppm LC_{50} at 96 hpf of medaka larvae and embryos exposed to Ag nanoparticles, respectively. This also confirmed the higher vulnerability or sensitivity of larvae to toxicants. Yokota et al., (2001) also stated that 4-nonylphenol caused subacute toxicities to larvae of fishes at concentration lower than that which caused toxicities to embryos, hence also confirming the larval vulnerability or sensitivity to toxicants.

Conversely, Hg^{2+} was more toxic to the embryos than the larvae. The higher mortality rate of the larvae within the 1 wph as shown in Fig. 4.7, resulted from embryos, particularly in the 148.2 – 206.6 µg/L solutions that were already deformed before hatch. This meant that the larvae were not sensitive to Hg^{2+} considering the relatively higher survival rate after the 1 wph. The larval insensitivity to Hg^{2+} resulted probably from the very low or zero bioaccumulation of Hg^{2+}. Wang et al., (2006b) had 568 mM LC_{50} at 10 dpf of medaka embryos exposed to ethanol. Yokota et al., (2001) indicated 183 µg/L LC_{50} at 8 dpf of embryo-larvae upon exposure to 4-nonylphenol. The differences in LC_{50} values of CH_3Hg^+ and Hg^{2+} in this study from those of the studies indicated above might have resulted from the differences in the kind or type of test organisms, stages of development of the test organisms, exposure durations and the exposure doses.

Based on Fig. 4.7, CH_3Hg^+ concentrations of 2.1 and 3.1 µg/L may be considered the no observed adverse effect level and the low observed adverse effect level, respectively. This means that repeated 18 dpf exposures of medaka embryos to CH_3Hg^+ concentration ≥ 3 µg/L can cause toxic effects including deaths of less than 50% of medaka larvae from the 1 to 8 wph while the 11 µg/L solution can cause deaths of about 50% of medaka larvae. For Hg^{2+}, the 20.0 µg/L solution may be considered the no observed adverse effect level. The low observed adverse effect level may be ≥ 24.5 µg/L but below 126 µg/L since the 24.5 µg/L solution did show any toxicity to the medaka embryos. However, concentrations of 126 µg/L is enough to cause curved or bent tails and subsequent deaths in about 50 % or more of developed medaka embryos following 18 dpf exposures.

4.4.4.2 Effect of CH_3Hg^+ and Hg^{2+} on Larval Growth

Despite the increased larval mortality rate, particularly by CH_3Hg^+, both CH_3Hg^+ and Hg^{2+}, compared to their controls, had no significant toxic effects on the growth (body weight and the total length) of the larvae that survived by the 8 wph. This meant that the statistically significant differences in body weight and total length of the 4.4 and 148.2 µg/L solutions from their respective controls (Tables 12a and b) did not result from the effects of CH_3Hg^+ and Hg^{2+}. The body weights at 8 wph in this study were consistent and within the range of 85 – 145 mg (males) or 95 – 150 mg (females) indicated by the OECD, (2014). Yokota et al., (2001) and Powe, (2018) obtained body weight (mg) and total length (mm) in the range of 167 – 197; 25.2 – 26.4 and 60 – 120 mg; 21 – 24 mm of medaka larvae exposed to nonylphenol and oxyfluorfen, respectively at

60 dph. Despite the differences in the test chemicals, these results are also consistent with the body weight (mg ww) and total length (mm) of 95.9 – 161.8 and 21.3 – 24.3 for CH_3Hg^+ and 77 – 139.7 and 19.7 – 24.7 for Hg^{2+} of medaka larvae in this study.

The higher body weight and total length of the 23.6 µg/L solution of CH_3Hg^+ and the 148.2 and 206.6 µg/L solutions of Hg^{2+} probably resulted from the fewer numbers of larvae in the respective aquaria. According to Magnuson, (2011), increase in medaka numbers in a particular aquarium leads to limited space and increase in competition for food. This results in uneven growth (body weight and total length). Therefore, the fewer numbers probably led to a reduction in competition for space and food, hence the higher body weight and total length of larvae in these solutions.

The lack of statistically significant difference in phenotypic sex ratios of the test solutions of CH_3Hg^+ and Hg^{2+} from their respective controls (Table 12a and b) meant that CH_3Hg^+ and Hg^{2+} in concentration ranges of 0.87 – 23.6 µg/L and 15.6 – 206.6 µg/L, respectively had no significant effect on the sex differentiation of medaka larvae. The sex ratios were more balanced in all exposure solutions, hence there was no skewness towards males nor females. Hano, (2012) also indicated no differences in phenotypic sex ratios of test chemicals from the controls of medaka larvae at 100 dph after injection of the embryos with nonylphenol in concentration range of 2 – 125 ng. Yokota et al., (2001), however, reported significant differences in sex ratios based on gonadal histology of the F_o and F_1 generations of medaka larvae exposed to 51.5 and 17.7 µg/L of 4-nonylphenol, respectively at 60 dph. Differences in results probably resulted from differences in the test chemicals, the doses, and the method of sex ratio determination.

4.4.5 Genotoxicity Assessment

4.4.5.1 Concentrations, Purity and Quality Assessment of Total RNA

Concentrations ≥ 100 ng/µL were considered as ideal for purity of the isolated total RNA. According to Thermo Scientific, (2012), a very low total RNA concentration < 10 ng/µL contains high levels of impurities which may negatively affect downstream applications such as RT-qPCR, microarray, etc. Additionally, a pure and quality total RNA should have A_{260}/A_{280} and A_{260}/A_{230} ratios around 2.0 (Thermo Scientific, 2012; Biotek, 2012; Qiagen, 2018) and between 2.0 – 2.2 (Qiagen, 2018), respectively but A_{260}/A_{230} ratio > 1is considered acceptable if only the A_{260}/A_{280}

ratio is around 2. Total RNA with absorbance ratios around these values contain low levels of contaminants such as proteins and other contaminants including phenols, guanidine salts which are contents of the buffers that are employed in the extraction or isolation process. Low contaminants may, therefore, have no effect on downstream applications (Thermo Scientific, 2012; Biotek, 2012). Moreover, these ratios, particularly the A_{260}/A_{230} also depend on the concentration of the total RNA (Qiagen, 2018), hence higher total RNA concentration corresponds to lower levels of contaminants. However, ratios extremely below or above these values may have effect on downstream applications which usually involve the synthesis of cDNA. With reference to Fig. 4.8, the total RNA concentrations and the absorbance ratios meant that the isolated total RNAs contained low levels of contaminants, hence were ideal for the intended downstream application (RT-qPCR analysis).

4.4.5.2 Gene Expression by RT-qPCR Analysis

Although the difference in cyclin B1 expression levels of the test chemicals was not statistically significant but the apparent concentration-dependent increase or decrease in cyclin B1 expression levels (Fig. 4.9) suggests toxicity of CH_3Hg^+ and Hg^{2+} to medaka embryos at the genetic level. Cyclin B1 functions to initiate mitotic cell division by complexing with p34 (Cdc2 or Cdk1) to form the maturation promoting factor (MPF) i.e. Cdc2-cyclin B1 complex, which is necessary for the proper control of the G2/M transition phase of the cell cycle (Jin, 1998; Schatten, 2013). This suggests that decreased in cyclin B1 expression levels led to disruption of the cell cycle and mitotic cell division of the medaka embryos following 18 dpf exposures to CH_3Hg^+ and Hg^{2+}. This in turn led to suppression in cell growth and development and eventual cell death which were common developmental toxicities observed in the embryos, particularly those that were exposed to higher concentrations of Hg^{2+}. This finding is consistent with Burke et al., (2006) who reported 75% decrease in expression levels of cyclin E in rats' developing brain by MeHg and identified G1/S transition as an early target for MeHg toxicity, indicating cyclin E suppression by MeHg may cause decreased cell proliferation and eventual cell death.

Additionally, although the lowest concentration of MeHg caused no developmental toxicities to the medaka embryos but the approximately 2.5-fold increase in expression level of cyclin B1 by the 0.87 μg/L solution of CH_3Hg^+ (Fig. 4.9) also meant that lower concentrations of MeHg can cause toxicity to medaka embryos at the molecular or genetic level.

4.5 Deductions from the Toxicity Studies

4.5.1 Human Foetal Subacute Developmental Toxicities

Based on the toxicity studies, the LOAEL of 3.1 µg/L, the LC_{50} of 11 µg/L, and the highest exposure concentration of 23.6 µg/L of CH_3Hg^+ correspond to human prenatal exposure amounts of 0.0035, 0.0132, and 0.029 µg/kg bw/day, respectively (appendix, Fig. 2). Application of the dose conversion using the one-compartment model by USEPA, (2001) (appendix, equation 1) gives corresponding maternal blood MeHg levels of 0.20, 0.74, and 1.63 µg/L, respectively. Additionally, studies on maternal blood and cord blood MeHg concentrations among human populations, especially those who eat fish suggest that the cord blood concentration is averagely 1.7-fold higher than the maternal blood (Ask et al., 2002; WHO, 2008; Chen et al., 2014; Kim et al., 2014; Llorente Ballesteros et al., 2020; Pinheiro et al., 2020). Therefore, modifying equation 1 to include the ratio as indicated by Stern, (2005) gives the corresponding cord blood MeHg levels of 0.33, 1.26, and 2.76 µg/L, respectively (appendix, equation 2).

According to Myers and Davidson, (1998), exposure to MeHg levels in the range of 10 – 20 ppm can adversely affect the foetus. Broussard et al., (2002) stated that daily ingestion above 0.3 mg MeHg leads to chronic Hg poisoning in a 70 kg adult with a steady-state blood and hair levels of 0.2 mg/L and 60 mg/kg, respectively. Such levels have devastating effects on the foetus. The prenatal exposure amounts in this study are below the USEPA reference dose of 0.1 µg/kg bw/day and those of Myers and Davidson, (1998) and Broussard et al., (2002), hence repeated prenatal exposures to the food crops may not be associated with any human foetal subacute developmental toxicities.

Jędrychowski et al., (2006) reported delays in neurocognitive performance of one year old infants due to Hg levels above 800 and 500 µg/L in cord and maternal blood, respectively. Bjørklund et al., (2019) indicated that plasma MeHg levels above 2 µg/L corresponding to erythrocyte and whole blood MeHg levels above 40 and 42 µg/L, respectively according to a 1: 20 MeHg ratio between plasma and erythrocytes (WHO, 1990; Rice et al., 2010) and a 21: 20 MeHg ratio between whole blood and erythrocytes (Rice et al, 2010) can cause premature birth, low body weight children, and spontaneous abortion. According to WHO, (2000), blood MeHg levels of 40 – 50 µg/L in a pregnant woman could cause toxicity to the developing foetus. In the Faroes study,

Hg levels in maternal and cord blood were 15.30 and 22.95 µg/L, respectively. In the mothers' and children environmental health study, Hg levels in the maternal and cord blood were 15.45 and 25.90 µg/L, respectively (Kim et al., 2014). Some studies have suggested a maternal blood MeHg level of 3.6 µg/L due to the 1.7-folds higher MeHg levels in cord blood (Llorente Ballesteros et al., 2020). The maternal and cord blood MeHg levels in this study are below the 5.8 µg/L blood MeHg reference value by USEPA, the 3.6 µg/L suggested by some studies, the 40 - 50 µg/L by WHO, (2000), the 500 and 800 µg/L by Jędrychowski et al., (2006), and the corresponding 40 – 42 µg/L by Bjørklund et al., (2019) within or above which foetal developmental abnormalities are likely to occur. Therefore, based on the maternal and cord blood MeHg levels in this study, repeated prenatal exposures to Hg levels of the food crops may not be associated with any subacute developmental toxicities to the human foetus or the new-born. This finding of very-low doses of MeHg not being associated with developmental abnormalities in foetuses and/or new-borns is consistent with those of Hibbeln et al., (2018) and Patel et al., (2019).

For Hg^{2+}, the LC_{50} of 126 µg/L and the highest exposure concentration of 338.3 µg/L correspond to prenatal exposure amounts of 1.80 and 5.14 µg/kg bw/day, respectively (appendix, Fig. 2). The one-compartment dose conversion model is not applicable to InHg since body Hg burden is always made with reference to MeHg but considering an average absorption factor of 0.075 results in corresponding absorption amounts of 0.135 and 0.386 µg/kg bw/day, respectively, which are above the USEPA reference dose of 0.3 µg/kg bw/day. However, its high affinity for the kidneys (USEPA, 1997b; Broussard et al., 2002; Park and Zheng, 2012), its higher elimination rate compared to MeHg, and the protective barrier of the placenta to InHg (Pinheiro et al., 2020) suggest limited transfer of InHg from the mother to the foetus as stated by USEPA, (1997b). This means that a very low amount of the absorbed doses may reach the foetus.

Additionally, data on InHg levels in maternal and cord blood is limited (WHO, 2008) but Ask et al., (2002); Walker, (2006) and Ou et al., (2015) reported 0.34; 0.32, 0.83; 0.78, 1.23; 1.09 and 0.62 – 0.86; 0.95 – 1.10 of InHg in cord and maternal blood, respectively in populations where fish is staple. Although InHg levels in fishes are lower compared to the food crops but the data above showed significant levels of InHg in cord and maternal blood. This means that considering the food crops with higher levels of InHg than fish, significantly higher levels of InHg can be transferred from the mother to the foetus. However, due to the factors indicated above coupled

with its lower or zero bioaccumulation potential, the limited data on InHg levels and the lack of reference values of InHg in maternal or foetal blood, it cannot be concluded if the limited maternal–foetal transfer amount of InHg levels of the food crops may be associated with subacute developmental toxicities following repeated prenatal exposures.

Summarily, based on the 5.8 μg/L by USEPA and the 3.6 μg/L blood Hg levels suggested by other researchers, and the fact that the body Hg burden is always made with reference to MeHg, it can be summarized that the very low MeHg and the high InHg levels of the food crops may not be associated with any subacute developmental toxicities to the human foetus or new-born despite its sensitivity or vulnerability to Hg intoxication. However, further studies on the developmental toxicities of very low Hg levels are still needed.

4.5.2 Genotoxicity to the Human Foetus

The increased or decreased expression of cyclin B1 by MeHg and InHg suggests that these contaminants are toxic at the genetic level since their expression of cyclin B1 leads to disruption in cell cycle and mitotic cell division, hence genotoxic. Some studies including Burke et al., (2006) reported of cyclin E suppression in rat's developing brain and Khan et al., (2013) also reported of increased expression of cyclin B1 in breast cancer patients. Cyclin B1 can be expressed in many organs/tissues of the human body (appendix; Table 1) and the expression of cyclin B1, particularly overexpression as shown by the 0.87 μg/L solution of MeHg has been linked with cancers including non-small cell lung cancer (Soria et al., 2000; Yoshida et al., 2004; Khan et al., 2013), renal or adrenal cancer (Ikuerowo et al., 2006; Khan et al., 2013), oesophageal cancer (Murakami et al., 1999; Nozoe et al., 2002; Khan et al., 2013), head and neck squamous cell carcinoma (Hassan et al., 2002; Takeno et al., 2002; Khan et al., 2013), breast cancer (Kawamoto et al., 1997; Aaltonen et al., 2009; Agarwal et al., 2009; Khan et al., 2013), etc. This means that either increase or decrease expression of cyclin B1 in any of the indicated organs/tissues (appendix, Table 1) can lead to cancer. The cancer may result from growth of malignant cells (Tumorigenesis) due to suppression of the cell cycle and mitotic cell division.

With reference to the Hg levels of the food crops, it is possible that repeated prenatal exposures to the food crops may be genotoxic to the human foetus despite interspecies variability. This is because genotoxic agents may have no safe or threshold dose due to their interactions with

nucleic acid as stated by Nohmi, (2018). Additionally, molecular effects such epigenesis, genotoxicity, etc. of environmental contaminants such heavy metals can occur at very low concentrations even below the NOAEL as stated by Alyea, (2012). These findings by Alyea, (2012) and Nohmi, (2018) are consistent with the 2.5-fold increase in expression levels of cyclin B1 by the 0.87 μg/L solution of MeHg in this study. The lack of developmental defects and the potential genetic defect of the 0.87 μg/L solution of CH_3Hg^+, however, suggest that the genetic effects of very low doses of contaminants or toxicants may be inherent, hence can take several years to manifest phenotypically or may show at all due to the very low exposure doses.

Furthermore, the decrease in expression levels of cyclin B1 by InHg suggests that InHg may be associated with tumorigenesis or can cause genetic tumour initiation in any of the body organs or tissues where cyclin B1 expression can occur. This finding is also consistent with that of Zefferino et al., (2017) who stated that Hg^{2+} is an epigenetic tumour promoter despite their assertion that Hg^{2+} is not a genotoxic compound that directly affects gene expression. The assertion, therefore, contradicts the findings regarding the genotoxicity of InHg in this study. However, further research on the genotoxicity and subsequent tumorigenesis of InHg or the potential of InHg, particularly Hg^{2+} as a genetic tumour initiator is still needed.

4.6 Conclusion

Although MeHg and InHg were toxic to the larvae and embryos in concentration ranges of 3 – 23.6 and 148.2 – 338.3 μg/L, respectively, deductions from the toxicity studies showed that there may not be any subacute developmental toxicities of the current Hg levels of the food crops to the developing human foetus or the new-born. The huge difference in body mass due to interspecies variability between the medaka embryos or larvae and the human foetus was the reason for the no Hg toxicity to the human foetus or the new-born. However, despite the differences in interspecies variability, the current Hg levels of the food crops may be toxic to the human foetus at the genetic level. This is because genotoxic agents may have no safe or threshold dose or can cause genotoxicity even at concentrations below the NOAEL. Additionally, there is a possibility of InHg-associated tumorigenesis or InHg being a genetic tumour initiator due to its ability to cause decrease in expression levels of cyclin B1.

Chapter Five

Conclusion and Recommendations

5.1 Conclusion

The study evaluated risks of Hg to humans and the entire ecosystem using selected ASGM communities around Obuasi, Ghana as case studies. The risks of Hg covered **1.** Ecological risks which involved Hg risk to the ecosystem, particularly the farms where samples were obtained, **2.** Potential human health risks that were associated with the plantains and cassavas following long-term repeated exposures, and **3.** Associated subacute developmental toxicities and the genotoxicity of Hg to the human foetus upon repeated prenatal exposures to mercury contaminated plantains and cassavas. The ecological risk assessment showed that the incessant releases of mercury from ASGM facilities pose serious potential risks to the ecosystems. The major effect of these risks is ecosystem functions which are essential for the survival of the biotic components of the ecosystem.

The human health risks assessment showed that long term-repeated exposures to Hg-contaminated plantains and cassavas from the selected farms may cause non-carcinogenic human health effects in the near or distant future. These potential human health effects may result from long-term repeated exposures through ingestion of InHg levels above the USEPA reference dose of $3x10^{-4}$ mg/kg bw/day. This means the nature of the non-carcinogenic human health effects following long-term repeated exposures to the food crops may be specific to InHg.

The toxicity study also showed that the human foetus may be exposed to very low but significant levels of MeHg and InHg in the Hg-contaminated food crops. These very low foetal exposure levels of MeHg and InHg in the food crops may not be associated with any subacute developmental toxicities to the human foetus or new-born due to blood Hg levels, particularly MeHg below the 5.8 μg/L and the suggested 3.6 μg/L reference values by USEPA and other researchers, respectively. This suggests that the developmental toxicities of MeHg and InHg to the medaka larvae and embryos, respectively resulted from the wide differences in body masses due to interspecies variability. This also means that the application of the uncertainty factor to the interspecies variability puts the human foetus at higher risk of developing abnormalities upon repeated prenatal exposures but such higher Hg levels, particularly MeHg levels, upon the application of the uncertainty factor cannot be found in the food crops used as samples in this study.

Following genotoxicity assessment, the increased or decreased in expression levels of cyclin B1 at the genetic level by both MeHg and InHg suggests that the very low human foetal

exposure doses of Hg following repeated prenatal exposures to the food crops, may be genotoxic to the human foetus or the new-born despite interspecies variability. This is because genotoxic agents may have no safe or threshold dose due to their interaction with nucleic acid or can cause genetic or molecular toxicities at very low concentrations, even below the NOAEL. Molecular toxicities at such very low concentrations of environmental contaminants may be inherent, hence can take several years to manifest phenotypically or may not manifest at all. The decreased expression of cyclin B1 may lead to the disruption in the initiation of the cell cycle and mitotic cell division and subsequent suppression of cell growth and development. The suppression of cell growth and development can trigger a series of cellular events which can lead to tumorigenesis and eventual cell death. Furthermore, the genotoxicity study has shown that there is a possibility of InHg-associated tumorigenesis or being a genetic tumour initiator due to its ability to cause decrease in expression level of cyclin B1. Therefore, further research is needed to ascertain such possibility. The genotoxicity of Hg to the human foetus at such very low Hg levels confirms the statement that **''there is no known safe level of exposure to environmental Hg, particularly in pregnancy''**.

Finally, the study has shown that the incessant releases of Hg from ASGM facilities within the studied communities are detrimental to the ecosystem and human health. Such detrimental human health effects can take several years to occur, and when they occur the etiology is mostly unidentifiable. Therefore, since there are no treatment processes for Hg within the catchment areas and Ghana at large, regular and strict monitoring of ASGM activities, particularly Hg releases from the facilities is required to preserve the integrity of the ecosystem and prevent the future occurrence of any toxic effects of Hg to humans, particularly the younger generation.

5.2 Recommendations

- Due to the extremely low levels of Hg, particularly MeHg in cord and maternal blood following repeated prenatal exposures to the food crops and the potential of MeHg to bio accumulate, it is recommended that post-hatch assessment be done for about 12 – 24 weeks. This will enable a better evaluation of the toxicity of very low Hg levels to human foetal development, particularly postnatal development.
- To accurately evaluate the toxic effects of InHg on human embryonic development, the use of rodents such as mice is recommended. This may provide a better estimation of the level

of maternal blood InHg that can reach the foetus, and whether such level may be detrimental to the developing foetus.

- Further studies on the developmental and the genetic toxicities of very low Hg levels as well as the possibility of InHg-associated tumorigenesis are recommended.

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Appendix

Table 1: Organs/Tissues in which Cyclin B1 gene is expressed (National Library of Medicines, 2022).

Bone marrow	Cortex	Artery	Kidney	Pancreas	Skin
Whole blood	Cerebellum	Smooth muscle	Lung	Thyroid	Ovary
WBC	Retina	Skeletal muscle	Liver	Salivary gland	Uterus
Lymph nodes	Spinal cord	Small intestine	Spleen	Adrenal gland	Placenta
Thymus	Tibial nerve	Colon	Stomach	Pituitary gland	Prostate
Brain	Heart	Adipocyte	Oesophagus	Breast	Testis

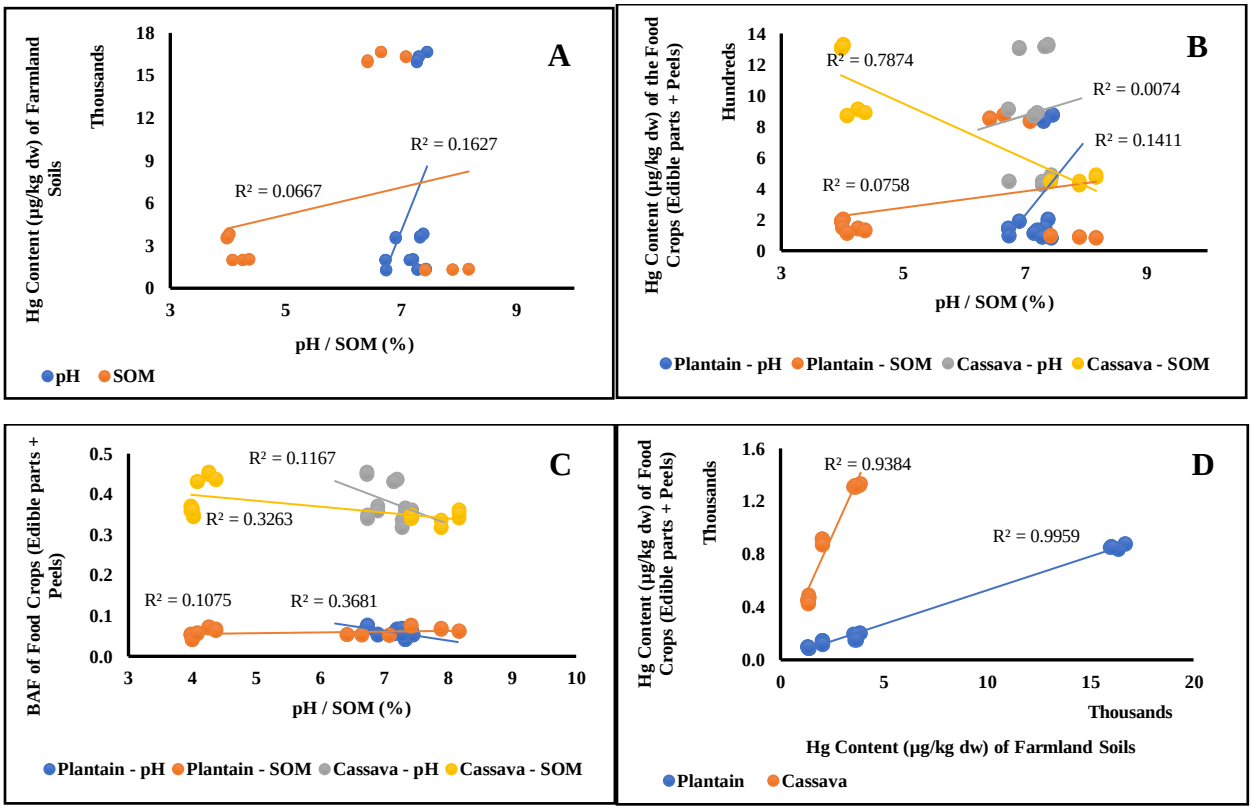

Fig. 1: Correlational graphs showing the effects of SOM and pH on the uptake of Hg by soils and the food crops as well as the relationship between soil Hg content and the Hg content of the food crops. A = Effect of pH/SOM on Hg content of farmland soils, B = Effect of pH/SOM on Hg content of food crops, C = Effect of pH/SOM on the BAF of the food crops, and D = Effect of Hg content of farmland soil on Hg content of the food crops (Addai-Arhin et al., 2021).

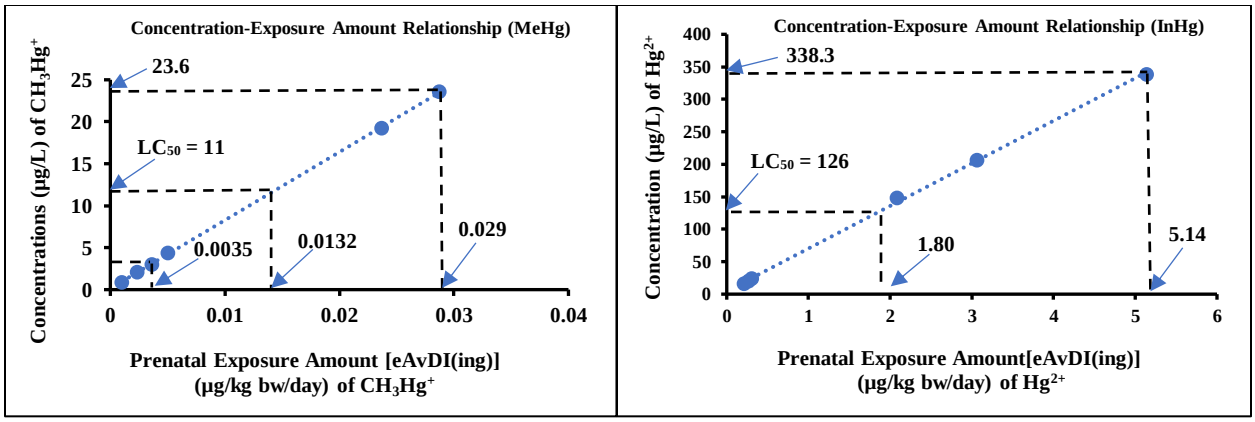

Fig. 2: Estimation of the prenatal exposure amounts of CH_3Hg^+ and Hg^{2+} using the LOAEL, LC_{50} and the highest concentration values.

Formula for the one-compartment dose conversion model (USEPA, 2001)

Equation 1

$$d(\mu g/kg\ bw/day) = \frac{c \times b \times V}{A \times f \times bw}$$

Where;

d = daily dietary intake (expressed as µg of methylmercury) = 0.0035, 0.0132, and 0.029

c = concentration in blood (expressed as µg/L)

b = elimination constant (expressed as $days^{-1}$) = 0.014

V = volume of blood in the body (expressed as liters) = 5

A = absorption factor (expressed as a unitless decimal fraction) = 0.95

f = fraction of absorbed dose taken up by blood (unitless) = 0.059

bw = body weight (expressed in kg) = 70

Modifying equation 1 to include the 1:1.7 maternal to cord blood MeHg ratio as indicated by Stern, (2005) gives umbilical cord blood MeHg as shown in **equation 2**;

$$d(\mu g/kg\ \ bw/day) = \frac{c \times \left(\frac{1}{R}\right) \times b \times V}{A \times f \times bw}$$

Where;

R = maternal to cord blood MeHg ratio and c = concentration of MeHg in cord blood.