CHAPTER 2

Literature Review

2.1. Point Source - Gold Mine.

Due to the recent gold rush, many Asian countries had opened their resources to both international and local mining. Artisanal gold mining is a crude method of gold dust collection from the sediment. The sand and the ore with high concentrations of arsenic and other metals associated with gold (Karimi et al. 2010) are crushed, homogenised and washed in a sediment collection machine. The washed sand and water flow out to a sediment trap on the slide. This trap retains the gold dust and the sediment which will be panned (Figure) with a little bit of mercury which forms the amalgam with the gold. The amalgam collected from bottom is heat treated to evaporate the mercury to get the gold.

2.1.1. Pollution and Mining

Anthropological activities such as mining can often pollute the water, air, and soil. Soil as a medium of contamination can cause trace concentrations of the metals in the biota. In a study by Rashed (2010), which monitored old gold mine tailings in mines that used mercury-gold amalgamation method in Egypt showed that the abandoned mining sites had metal pollutants in the soil and tailings including arsenic and mercury. Where, the concentration of metal increased in the soil and wild plants when we get closer to the tailing. The study also cautioned the use of the area for grazing due to the potential hazard. This study is proof contaminated soil affecting the biota surrounding the soil due to mining activity.

In an aquatic ecosystem where the main medium of contamination is, water. The water eventually becomes the medium of transport of the contaminant. In an aquatic ecosystem,

contamination can be detected in the water, aquatic flora and fauna (Förstner and Wittmann 1983). It is known that primary producers both pelagic and free floating can bio-transform metals (Kelly, Budd, and Lefebvre 2007) absorbing them. The 1994 spring phytoplankton bloom in San Francisco where the concentration of dissolved metals dropped supports this fact (Luoma et al. 1998). Practices like phytoremediation and phytomining shows that exposed biota will have trace amounts of pollutants in them. Natural phytoremediation in the wild due to an exposure from a pollutant will lead to bioaccumulation in the food chain. Studies also shows that this bioaccumulation is a direct result of the ability for the biota to adopt to survive in high concentrations of pollutants. This high exposure also results in defects in the cellular level. Promsid et al. (2015), speaks of snakehead fish living in reservoirs with contaminated waters, and contaminated soil showing metal concentration in their tissue. The study is proof of the contamination of the medium resulting in the contamination of the biota. The study also talks of bioaccumulation of the pollutants resulting in chromosomal aberrations.

Figure 1 one of the steps of the process of Artisanal mining; the panning, magnetic metal dust removed before the introduction of mercury into the pan.

2.2. Mercury

Mercury or Hg, is a heavy metal with oxidation states +1 and +2 with the molecular weight 200.59 g/mole. It has a solubility of 5.6×10^{-5} g/L at 25°C with a density of 13.534 g/cm^3 at 25°C. it is also known as metallic mercury (National Research Council (US) Committee on the Toxicological Effects of Methylmercury 2000). The major natural sources of mercury are degassing of the earth's crust, emissions from volcanoes, and evaporation from natural bodies of water.

2.2.1. Mercury in Mining

Mercury emissions are associated with both large scale and small-scale gold mining. Pure mercury, at room temperature is a liquid that can be extracted by roasting of the metal's ore Cinnabar. The elemental mercury forms an amalgam with gold making it the ideal candidate for the extraction of gold dust. The elemental mercury in the extraction of gold from the amalgam is released in trace amounts into the atmosphere. Some 650-1000 tonnes of mercury are released annually, with estimated 350 tonnes entering the atmosphere directly and the rest released into the water systems (UNEP Chemicals Branch 2008) and sediment. In the sediment mercury gives rise to substances such as the more toxic methylmercury (CH_3Hg). (Krabbenhoft and Sunderland 2013). The generation of substances like CH_3Hg is favoured by conditions such as low salinity and high pH (Compeau and Bartha 1984).

Mercury vapour is more soluble in plasma, whole blood, and haemoglobin than in distilled water, where it dissolves only slightly. The worldwide mining of mercury yields about 10,000 tons/year. The activities lead to some losses of mercury and direct discharges to the atmosphere. Other important sources are fossil fuel combustion, metal sulphide ore smelting, gold refining, cement production, refuse incineration, and industrial applications of metals.(National Library of Medicine HSDB Database 2009)

2.2.2. Mercury Standards

The accepted standard concentration of mercury in drinking water in Thailand is 0.005 mg/l. The accepted minimum concentration mercury in the soil of the habitat and agriculture zone should not exceed 23 mg/kg (PCD Thailand 2014). Similarly the accepted maximum value for mercury in edible biota should be less than 0.02 mg/kg (PCD Thailand 2017). According to US EPA (2002), The accepted concentration of mercury in agricultural soil is less than 1 mg/kg and as for the level of mercury in edible biota, according to US FDA (1979) the mercury concentration should not exceed 1mg/kg.

2.2.3. Mercury Toxicity

The toxicity of mercury depends on the exposed specimen and the route of exposure. according to NOISH (2018a), records show one of the lowest published toxic concentration for a mouse, exposed through inhalation as 2.71mg/m^3/for 1 hour. As a response, the lungs and thorax showed acute pulmonary edema and had a decrease in the cellular immune response. Another record shows that lowest published toxic concentration for a man exposed through inhalation is 44.3 mg/m^3/for 8 hours where, the individual showed muscle weakness followed by an increase in the body temperature. Another record shows the lowest published concentration as 5 mg/ m^3/ for 3 hours where, the individual as a result became nauseated and vomited followed by a high fever.

Rice et al.(2014) cited Clifton (2007) and US Department of Health and Human Services (1999) supporting the claim that the primary pathways of entry of mercury into the wildlife can be through exposure and the food they eat. Mercury being used in the process of gold mining is a by-product of the mining process (UNEP Chemicals Branch 2002). Artisanal gold mining that includes panning and heating for the recovery of gold leads to about 18% of the global atmospheric emission of mercury. This will cause a large deposition in these areas and surrounding areas (UNEP Chemicals Branch 2008). A study was carried out on mosquitos where the traced MeHg concentrations in some of the specimens were related to loadings of inorganic mercury, mostly from wet atmospheric deposition (Hammerschmidt and Fitzgerald 2005). The existence of mercury in the water can lead to eutrophication and the eventual uptake of mercury into the biota. (Chen et al. 2008). Which shows a direct pathway of entry of the mercury into the biota. Surviving in these conditions also will lead to exposure through the skin as well. In the book, mercury

from gold and silver mining: a chemical time bomb by Lacerda and Salomons (2012) cited D'Itri and D'Itri (1977) and Nriagu (1979) as reporting the ban of importing mercury for gold and silver mining from Spain during the Roman era, most likely due to the environmental health problems.

Most human exposure occurs through inhalation and dental amalgam. Acute inhalation exposure causes chest pains, dyspnea, coughing, haemoptysis, and interstitial pneumonitis leading to death. The central nervous system is the main area of mercury vapour exposure. Sub-acute exposure causes psychotic reactions characterized by delirium, hallucinations, and suicidal tendency. Occupational exposure causes erythrism and many other diseases such as that referred to as the mad hatter's disease. Mercury compounds also give raise to contact dermatitis. Mercurial pharmaceuticals have been responsible for Pink disease (acrodynia) in children, and mercury vapour exposure may be a cause of "Kawasaki" disease. Results of both human and animal studies indicate that about 80% of inhaled metallic mercury vapour is retained by the body, whereas liquid metallic mercury is poorly absorbed via the gastrointestinal tract (Fawer, et al. 1983). One of the most well documented conditions of mercury exposure through food is the Matamata disaster of 1956, which lead to generations with conditions such as sensory disturbances (glove and stocking type), ataxia, dysarthria, constriction of the visual field, auditory disturbances, tremor, etc. (Harada 1995).

2.3. Arsenic

Arsenic or As which has a molecular weight of 74.92 g/mole is the 20th most abundant element in the earth's crust it is associated with igneous and sedimentary rocks. Arsenic in nature is rarely in its pure form. The most abundant form of arsenic is arsenopyrite a compound of iron, arsenic, and sulphur.(Minerals Education Coalition 2014). This heavy metal although not soluble in water has salts with varying solubility depending on the pH and the ionic environment (World Health Organization 2004). Arsenic is a natural component of the earth's crust, and found in all environmental media (Gomez-Caminero et al. 2001)

2.3.1. Arsenic in Mining

Since valuable metals such as copper and gold can also be found in sulphide mineral deposits, mining exploration companies will often look for soil and water with a naturally high arsenic content as a means of locating an ore body (Health Canada 2007). These water-soluble sulphide mineral deposits usually contain valuable metals such as copper and gold. Thus natural erosion or processing for gold inevitably releases arsenic into the environment (Wang and Mulligan 2006). These mobilized arsenic has 2 major forms the less toxic organic form and the more harmful inorganic form (Vahter 2002).

2.3.2. Arsenic Standard

As arsenic found in the environment, most commonly enters the biota through the food, drinking water, and air. Among the sources, food is the most prominent. The predominant dietary source of arsenic is seafood, followed by rice, cereal, mushrooms, and poultry. While seafood contains the greatest amounts of arsenic, for fish and shellfish, this is mostly in an organic form of arsenic called arsenobetaine that is much less harmful (ATSDR 2007). The accepted standard concentration of arsenic in drinking water in Thailand is 0.25 mg/l. The accepted minimum concentration arsenic in the soil of the habitat and agriculture zone should not exceed 3.9 mg/kg (PCD Thailand 2014). Similarly the accepted maximum value for arsenic in edible biota should be less than 2 mg/kg (PCD Thailand 2017). According to US EPA (2002), The accepted concentration of arsenic in agricultural soil is less than 0.11 mg/kg and as for the level of mercury in edible biota, according to US FDA (1979), the arsenic concentration should not exceed 0.5 mg/kg.

2.3.3. Arsenic Toxicity

The toxicity of arsenic depending on the subject used. According to NOISH,(2018b) the lethal dose for acute exposure of a mouse injected with arsenic intraperitoneally is 1 mg/kg, and for a rat is 13.30 mg/kg. For a child the lowest published toxic dose taken orally is 4 mg/kg this lead to a change in the leukocyte count. For a man the lowest published lethal dose taken orally is 7857 mg/kg/55 Years and observable changes in the structure and the function of the oesophagus, blood haemorrhage, and dermatitis.

Inhalation is the main route of arsenic exposure in occupational settings, while use of contaminated drinking water is the predominant source of significant environmental

exposure globally. Acute and chronic arsenic exposure via drinking water is the main form of exposure in many countries of the world, where a large proportion of drinking water has high concentrations of arsenic. The main health effects that are associated with arsenic exposure include, cardiovascular and peripheral vascular disease, developmental anomalies, neurologic and neurobehavioural disorders, diabetes, hearing loss, portal fibrosis, hematologic disorders (anemia, leukopenia and eosinophilia) and multiple cancers: significantly higher standardized mortality rates and cumulative mortality rates for cancers of the skin, lung, liver, urinary bladder, kidney, and colon in many areas with arsenic pollution (Tchounwou, Patlolla, and Centeno 2003).

As arsenic which is found with the ore of gold and other minerals (Karimi et al. 2010) is mobilised in the water releasing the gold. The run off arsenic has a high affinity towards the soil and its minerals which immobilises the arsenic in the sediment (Shiowatana et al. 2001). This opens up the possibility of exposure to benthic animals and consequently their predators (Woodward et al. 2011). The algal accumulation of arsenic is another pathway of exposure and infiltration into the food chain to eventually reach the animals (Chen et al. 2008).

2.4. Boi-indicators

Tissue concentrations of metals in aquatic organisms is a reflection of the habitat exposure to the pollutants. However, predators such as frogs and humans on the top of the food chain may obtain their pollutants by ingestion increasing the possibility for bioaccumulation (Richter and Nagel 2007). Since amphibians are experiencing a rapid global decline (Stuart et al. 2004), they function as excellent bio indicators because of their high sensitivity to environmental modifications and pollution (Hopkins 2007).

2.5. Selected Frog Species.

According to the data by (International Union for Conservation of Nature 2004a; International Union for Conservation of Nature 2004b) , both the frogs *Fejervarya limnocharis* (Figure 2) and *Fejervarya cancrivora* (Figure 3) categorized under least concern are synonyms of each other. They are widely distributed throughout South Asia

and Southeast Asia, ranging from Kalimantan to the foothills of the Himalayas and from India to the islands of Taiwan.

The data also shows that both the species are very tolerant to a range of habitats. They inhabit most open wet habitat types, including river floodplains, wet agriculture areas such as rice fields, ditches, marshes, parks, gardens and other habitats and in closed-canopy forest. They breed and spend their larval stage in various wetland habitats. The records also shows that these species are a part of the diet for some communities.

Figure 2 a defrosted specimen of the species *Fejervarya limnocharis*

Known as the Chinese Edible Frog, East Asian Bullfrog and the Taiwanese Frog, *Hoplobatrachus rugulosus* (Figure 4) is a synonym of *Rana rugulosa* and *Rana tigrina.* The distribution of these frogs ranged from central, southern, and southwestern China including Taiwan, Hong Kong, and Macau to Myanmar through Thailand, Lao People's Democratic Republic, Viet Nam, and Cambodia south to the Thai-Malay peninsula. They breed in paddy field, diches, fish pools, and wetlands. They are effective predators at most of their life stages. These frogs are a farmed in Thailand for consumption (International Union for Conservation of Nature 2004c).

Figure 3 a defrosted specimen of the species *Fejervarya cancrivora*

Figure 4 A defrosted specimen of the species *Hoplobatrachus rugulosus*.

2.6. Amphibian Husbandry

The enclosures used is often the acclimatization unit of the specimens. As per the enclosure systems of Poole and Grow, (2012). The enclosure should satisfy the physiological and behavioural needs of the specimen. At the same time, promote hunting or feeding. It should also be easy to monitor, easy to maintain and difficult to escape.

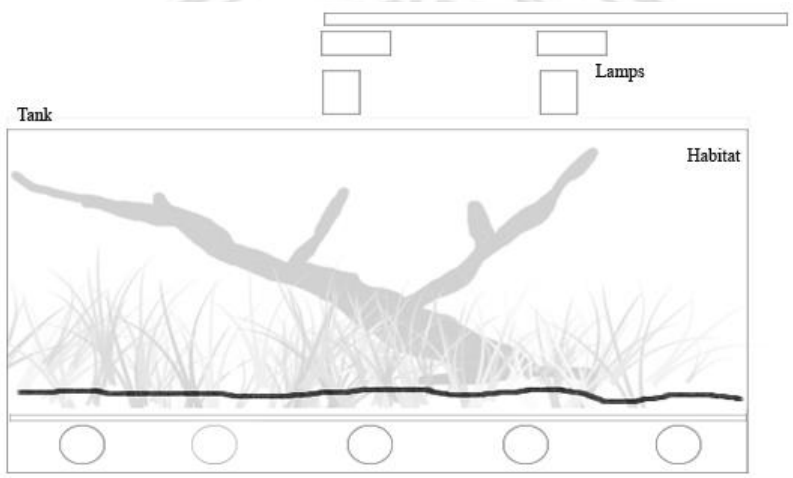

Figure 5 an example of a closed system enclosure. The system is not plumbed, therefore needs extra attention and care for cleaning

While the ideal material differs from enclosure to enclosure, most amphibian enclosures are of glass or fiberglass. The enclosures usually fitted with lights and temperature regulated. The enclosure should be complete with a lid with ventilation.

The enclosures are of two main types. Open system (Figure 7) which allows plumbing and outflow of the input of the system. These systems are ideal for long-term use where generations can breed and live undisturbed. The design of the system allows the system to clean itself. The water and the ambient temperature has is regulated separately as the water flows out. Therefore, the system requires less maintenance. However, it is more expensive to make due to the required pluming.

Closed systems (Figure 5 and Figure 6) do not allow plumbing and outflow. These systems are ideal for maintaining and observing of a knot of frogs of the same age. The design of the system does not clean itself. Therefore, the water and the ambient

temperature can be maintained together. This system requires constant attention as bio-waste will accumulate. Unlike the open system, it is cheaper to make.

Figure 6 an example of a closed system enclosure, with two frogs of the species *Sylvirana nigrovittata* housed inside as a trail system in the lab.

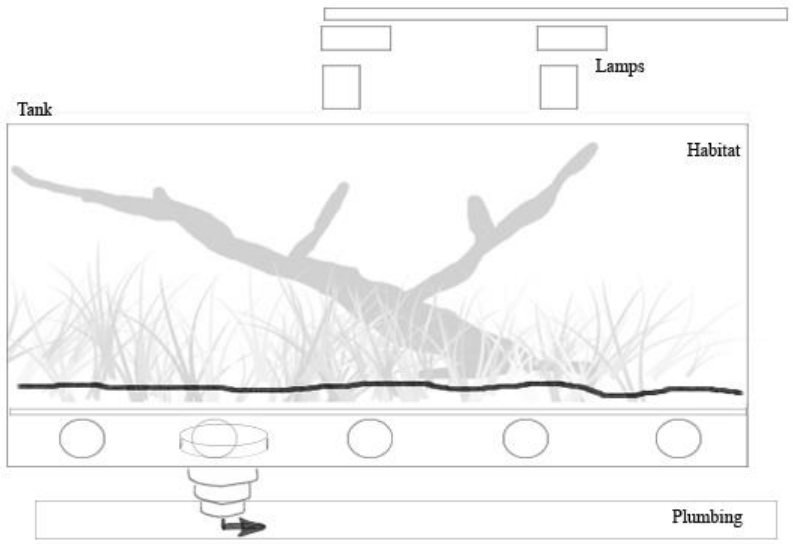

Figure 7 an example of an open system enclosure. The system is plumbed, therefore cleans itself

2.6.1. Cleanliness and Disease Control

One of the major treats to a knot of frogs in a system, specially a closed system is the treat of the colonization of harmful bacteria in the tank. *Bacillus hydrophilus fuscus*, an

anaerobic bacterium that is gram negative, and non-spore forming which can be found in the soil and in gastro intestines of fish and reptiles and can cause gastroenteritis (Embrey et al. 2004). The bacteria is responsible for "red-leg" disease in the frog (Figure 8). The disease is characterized by congestion of the ventral surfaces of the body, with more or less ulceration in, and haemorrhage beneath, the skin, bloating due to serous exudation into the lymph sacs, gradual failure to respond to stimuli, which symptoms are followed by coma and death (Emerson and Norris 1905).

Often the death of one frog to this disease sets out a domino effect resulting in the loss of the entire tank. In a closed system, the easiest way to avoid such an outbreak is to clean daily and remove the droppings from the habitat as soon as it is spotted. If an individual shows suspicious signs, the best thing to do would be to quarantine that individual and clean the tank.

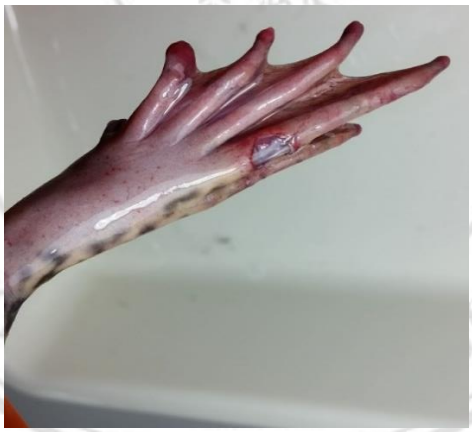

Figure 8 an example of the "red-leg" disease observed in *Hoplobatrachus rugulosus*

2.6.2. Segregation and Maintenance.

The groups within the knot of frogs must be according to the size of the frogs. This will prevent to a certain degree predation and competition within the tank. Sufficient space per an individual will avoid territorial disputes reducing aggression and stress. It is also important to segregate the specimens in takes specific to the same site of origin. This practice will reduce diseases and infections. (Poole and Grow S 2012).

2.7. Age and Lines of Arrested Growth (LAG).

In frogs the age can be calculated as lines of arrested growth (LAG)s on the leg bones of the frogs. These show the time of favourable conditions where the frog goes through a growth phase and time of arrested growth where the frogs is hibernating showing growth inhibition on the bones. However, in the tropics where the seasonal changes in the area are not as drastic *Fejervarya limnocharis* and *Fejervarya cancrivora* do not go into hibernation and in case of unfavourable conditions the frogs migrate. Therefore, they do not have LAGs in their bones and the size of the frogs is not ideal to determine the age. (Kusrini 2006; Liao et al. 2011). While sampling frogs form an exposed area frogs of a uniform size can represent uniform food intake and skin surface area to ensure uniform exposure.

2.8. Mode of Admission

Various modes of admission exist such as enteral, intravenous, cutaneous, epidural and intrathecal, intraperitoneal and intranasal admission. Each mode being unique has its disadvantages and advantages. Enteral admission requires the controlled feeding of the animal. This is a major disadvantage, as the controlled mixing of the food pallets with the test substances will vary the desired concentrations. Furthermore, the requirement of restraining, which can lead to undesired stress may alter the controlled nature of the experiment (Cinelli et al. 2007; Meijer et al. 2006; Turner et al. 2012).

Intravenous admission requires admission of the injection to the blood stream. A major disadvantage of this mode is that the slightest imbalance in the volume, pH, electrolyte and fluid level may cause discomfort, illness and even death for the specimens which may impact the controlled nature of the study (Mazzaferro 2008).

Subcutaneous administration is the injection of the substance under or to the skin layer. The slow rate of absorption and sustained effect of subcutaneous administration makes it a good form of admission. The pathways although not certain, seems to take the small capillaries underling the skin which absorbs the molecules (Kagan et al. 2007). However, for frogs subcutaneous injection is not ideal due to the permeability of the skin which may not provide the desired result (Smith and Stump 2000).

Epidural and intrathecal admission requires anaesthetized or restrained precise injection of the intrathecal space of the spinal cord which impacts the central nervous system (Valverde 2008). The requirement of an anaesthesia is a major disadvantage of this method. Similarly intranasal administration required the animals to be sedated or anesthetized. (Hall, Clarke, and Trim 2001). Hence, a major disadvantage.

Intraperitoneal administration due to the similarities to oral exposure where the primary route is the mesenteric pathway, to the portal vein (Lukas, Brindle, and Greengard 1971) has an advantage over all other modes of administration. A limitation is the possibility of hepatic metabolism before systemic circulation and exposure to lacunae and into the thoracic lymph (Abu-Hijleh, Habbal, and Moqattash 1995). However, as this mode allows the effective re-hydration of dehydrated specimens. This also allows the release of excess fluid reducing extra stress (Turner et al. 2011).

Ringer and Murrell, in (1878) conducted a series of experiments where they injected different forms of arsenious acid to an undefined species of frogs, testing a pervious study and testing weather the chemical was a protoplasmic poison which can destroy the function of nitrogenous tissue. The high concentrations used mostly ensured the paralysis and the death of the animal.

Peritoneal injection in frogs is through the abdominal side of the frog. The legs stretched out and held ensures unwanted movement. The injection area is low on the ventral side. Depending on the size of the frog, on the back of the animal the point finger or a surface, aids the process by countering the injection pressure. The injection needle should be just long enough to break the peritoneum and reach the body cavity. The needle should not puncher the internal organs (Poole and Grow S 2012). If the frog is large enough, holding the frog upside down helps.

2.9. Staining

G-and R-banding are the most commonly used techniques for karyotyping and for identifying abnormalities of chromosomes. Nearly all methods of chromosome banding rely on harvesting chromosomes in mitosis. This is usually achieved by treating cells with tubulin inhibitors, such as colchicine or demecolcine (colcemid), that depolymerize the

mitotic spindle and so arrest the cell at this stage (Bickmore 2001). G banding stains chromosomes with a dye that reacts the G positive bands. Different bands stain in different intensities for different staining techniques (Francke 1994). In G-bands, the dark regions tend to be AT rich and the bright regions tend to be GC rich (Bickmore 2001).

2.10. Frogs as Food

As per G. Pendrageon (2015), Thai cuisine consists of different types of frogs. M. Wiens (2011), also reported that different stages of frogs are enjoyed in the Thai cuisine. According to IUCN (2004b; 2004a; 2004c) the selected species of the frog are harvested for human consumption. The smaller *Fejervarya Spp.* which, is mostly caught, is fried and eaten whole with the intestinal content. The larger *Hoplobatrachus rugulosus*, which is usually farmed, varies in preparation depending on the size of the frog. This is a point of human exposure to mercury and arsenic through the food chain. Hassold on (1986) reported that mercury and arsenic exposure can lead to liver damage, damage to the nervous system kidney and induce chromosomal abnormalities in developing foetuses leading to fatal loss.

2.11. Cytogenetic testing

Chromosomal aberration analysis gives an overview of the genotoxic effects of pollutants in the habitat (Leme and Maria Aparecida 2008; Leme, Angelis, and Maria A 2008; Márcia. M, Angelis, and Maria Aparecida 2008; Márcia. M and Maria Aparecida 2009). All chromosomal aberration study rely on the mitotic metaphase stage (Bickmore 2001) for clear observation. Studies carried out by Suttichaiya et al. (2016), shows that using the metaphase stage the aberrations of exposure to contaminations can be shown. The published data such as the cytotoxic evaluation of rice field frogs (*Fejervarya limnocharis*) from gold mine area with arsenic contamination by Intamat et al.(2016) and other literature such as chromosome aberrations of East Asian bullfrog (*Hoplobatrachus rugulosus*) around a gold mine area with arsenic contamination by Suttichaiya et al.(2016) provides a peer reviewed karyotype as reference. Both the studies reports trace amounts of the pollutant in the habitat, which results in the specimens with a certain level of tissue

concentrations. From the specimens, the analysed chromosomes showed different types of breaks, fragments, and gaps.

Similarly studies by Krishnaja and Rege (1982), shows that induction of cromosomal abberations are possiable by exposure to the pollutant in a controlled environment. In the study they used two different modes of exposure to heavy metals Hg, Se and Cr, injections or “Direct exposure” and dissolved in the meduim “in direct exposure”. The sesults showed that both modes were effective in inducing aberrations. However, the specimens injected was observed for 72 hours and the specimens in the injected medium was observed for 92 hours.

Preetpal and Tripathi (2014) on their extensive study of genotoxic effects of some heavy metals on frogs where they observed other types of fragment modifications. The study reported fragments, minutes, and pulverised chromosomes.

On other studies, among the impacts of exposure to ionizing radiation and genotoxic agents in humans, chromosomal aberrations are the most significant. Several researches show people with elevated chromosomal aberrations in their blood has a high risk of developing cancer. (Bonassi et al. 1995; Bonassi et al. 2000; Hagmar et al. 1994; Hagmar, Bonassi, Strömberg, Brøgger, et al. 1998; Hagmar, Bonassi, Strömberg, Mikoczy, et al. 1998).