CHAPTER 4

Results and Discussion

4.1. Field Study of the Mining Area

4.1.1. Concentrations of Metals in the Sediment

Sediment from all three sites had concentrations of arsenic (Figure 24). According US EPA (2002) all the sites are unfit for agriculture as they cross the limit of acceptable arsenic concentration in agricultural soil. However, according to (PCD Thailand 2017). only site 1 crosses the limit of acceptable arsenic concentration in agricultural soil and is unfit for agriculture.

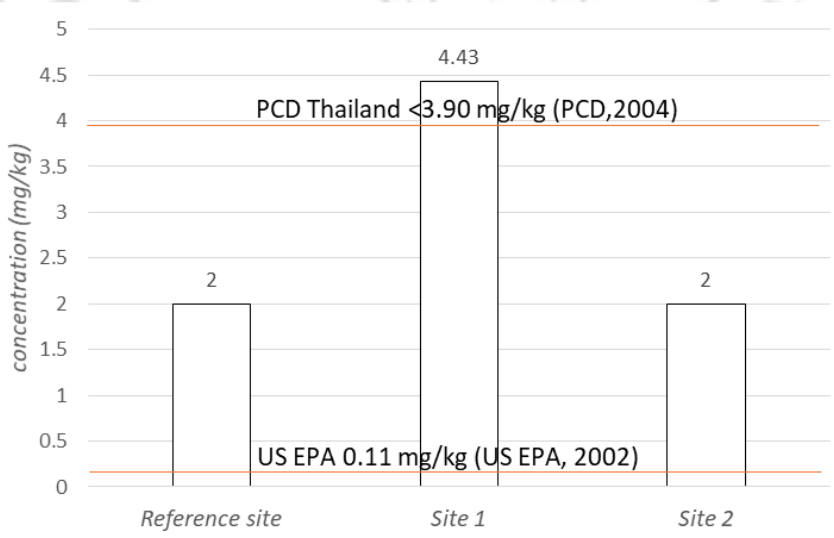

Figure 24 sediment arsenic concentration from all the study sites.

Gold is found as an ore with arsenic (Karimi et al. 2010). Since Pichit is known for the presence of gold dust, suggests that the area will have high levels of arsenic. This explains the arsenic detected in the sediment of the reference site, which was not a mining site.

The guardian (2015) reported of an increased arsenic concentration in the blood of the community in this area due to the mining process.

Similar results had been reported in studies that analysed the urine of the communities living in the mining areas (Hinwood et al. 2004). Therefore, we can assume that the arsenic detected in the mining area is from the processed gold ore . Shiowatana et al. (2001) reported that arsenic has a strong affinity towards different elements in soil which immobilizes arsenic in a general area.

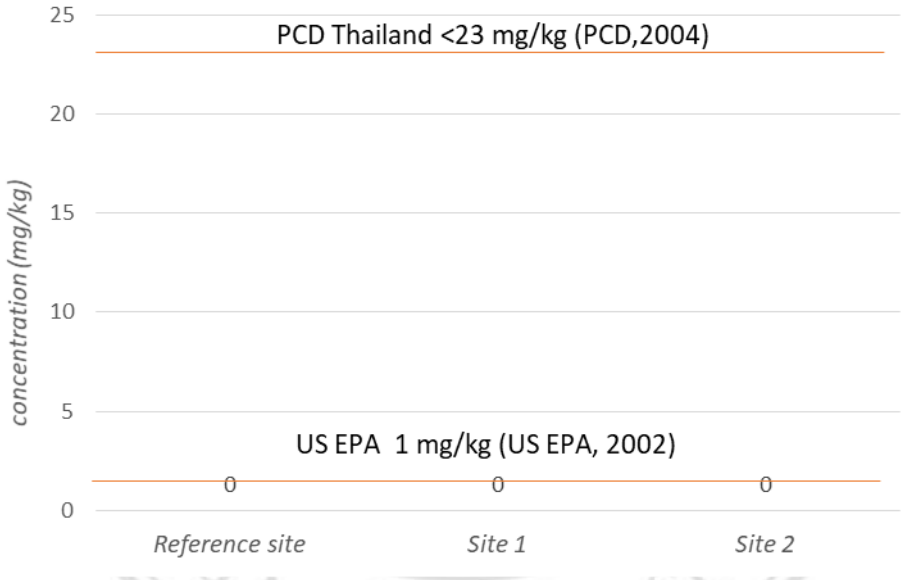

Figure 25: sediment mercury concentration from all the study sites

Therefore, arsenic is absorbed strongly into the soil of the initial point of contact. Hence, this explains the drop in the concentration of arsenic in site 2 as the sampling location was a water drain point, which brings arsenic in direct contact with the sediment. The fixed number of sediment collectors in the area also contributes to this drop. For site 1, since the area is a deeper pool it enables the mobilized arsenic to be transported to the edges to settle in the sediment. It must be noted that this area is open for all to use, therefore the number of sediment collectors are not fixed and can access the area in all locations. Hence, the arsenic concentration of the sediment is higher than site 2. As for mercury, sediments from the study sites had no traceable concentration of mercury (See Table 10 in the appendix)

A unique characteristic of the Pichit miners is the use of an amalgamating procedure where mercury is introduced in the sediment collector to trap potential gold dust in the initial process. This is a point source of release of the mercury to the environment before the panning of the sediment and the heating of the amalgam. However, the sediment do not have detectable levels of mercury in the study area. Another point of release that is evident is through evaporation as mercury vapour as the final amalgam that is gathered from the panning process is heated to release the mercury and extract the gold. This would deposit as either dry deposition or wet deposition in the waterways. The works of Chen et al. (2008) in an open lake system in China reports an increase in eutrophication with the increase in the concentration of mercury. The excessive algal growth and to the eutrophic nature of the pools in the mining sites would absorb the mercury and explains the lack of mercury in the sediment.

The only known professional gold miner in the area was the Akara gold mine. This was functional from 1993 to 2016 (Fernquest 2016). This closing of the commercial gold mine led to the boom of the Artisanal and Small-scale Gold Mining industry as a direct result of the many-trained locals losing their jobs due to the closing of the commercial gold mine. However, the practices by the local gold miners would understandably lack important mitigation measures (Sulc 2000). This practice of the artisanal scale small gold mines eventually becomes the main source of the mercury found in the area.

4.1.2. Concentrations of Metals in the Frogs

Tissue of *Fejervarya limnocharis* has varying concentrations of mercury in them (Figure 26). According to PCD Thailand (2017), the frogs from the sites are not fit for consumption. It is alarming that the frog tissue samples from the reference site too has concentrations of mercury in them. However according to US FDA (1979), the samples are fit for consumption. As for the tissue concentration of arsenic from the same frogs, only trace amounts of arsenic concentration was detected from site 1. However, the detected amount did not cross any standards from the US FDA and PCD Thailand (Figure 27). Similarly, tissue of *Fejervarya cancrivora* has varying concentrations of mercury in them (Figure 28). According to PCD Thailand (2017), the frogs from the sites are not fit

for consumption. It is alarming that the frog tissue samples from the reference site too has concentrations of mercury in them.

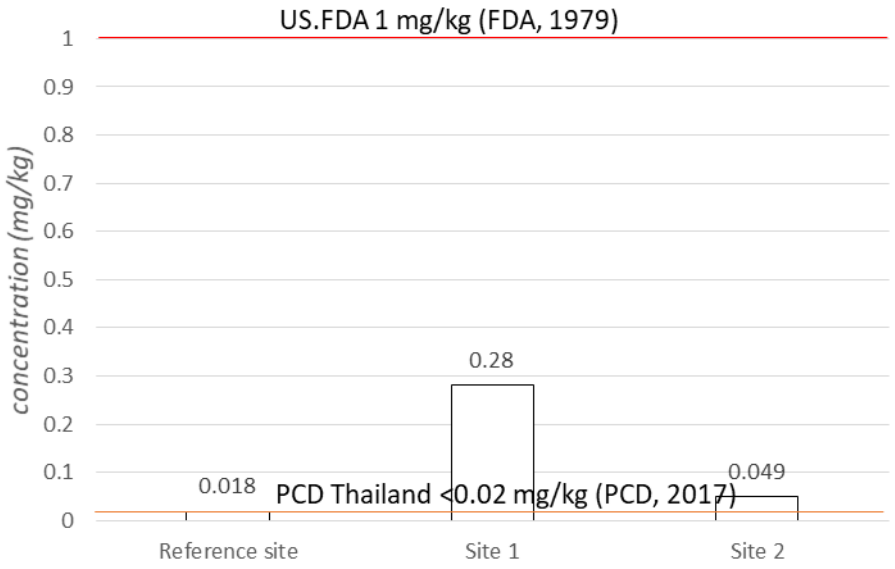

Figure 26 mercury tissue concentration of the *Fejervarya limnocharis* collected from all the sites

However according to US FDA (1979), the samples are fit for consumption. As for the tissue concentration of arsenic from the same frogs, only trace amounts of arsenic concentration was detected from site 2. However, the detected amount did not cross any standards from the US FDA and PCD Thailand (Figure 29). (See Table 11 in the appendix)

The highest levels of mercury was detected from Fejervarya limnocharis at the site 1. The amalgam is often processed in the open, often in the kitchens releasing mercury vapours in to the atmosphere. Traces of mercury detected in the reference site samples can be a result of the vapour released in to the atmosphere. This can be explained by literature that reported atmospheric deposition of mercury (Mason, Laporte, and Andres 2000; Hammerschmidt and Fitzgerald 2005) and the bioaccumulation of mercury through the food chain due to exposure (Tirkey, Shrivastava, and Saxena 2012).

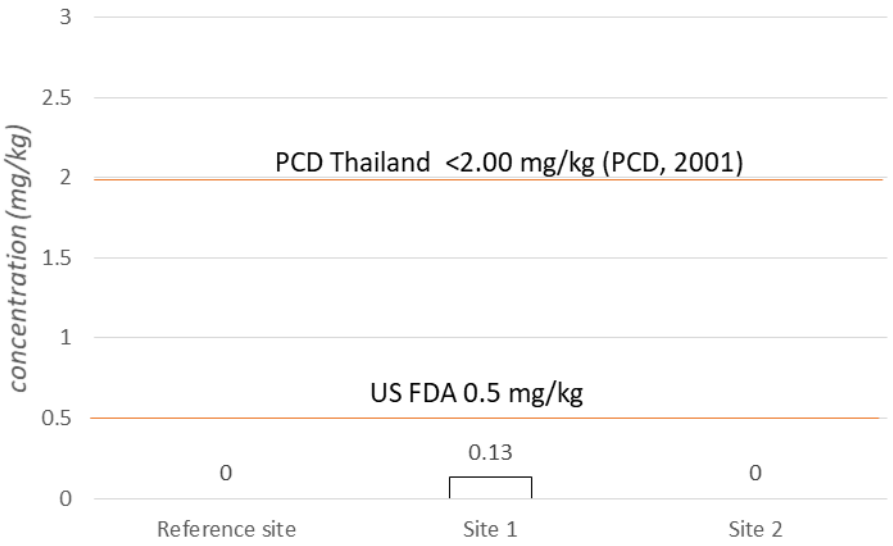

Figure 27 arsenic tissue concentration of the *Fejervarya limnocharis* collected from all the sites.

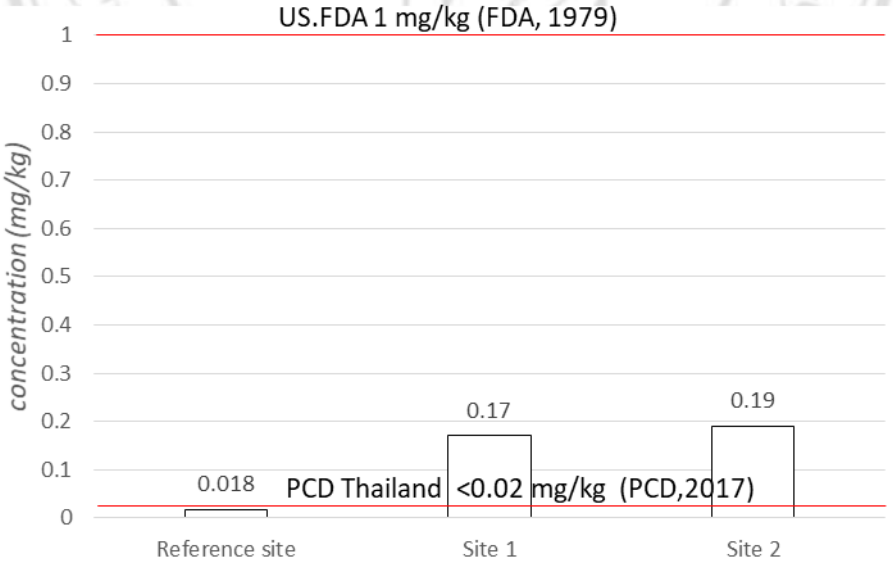

Figure 28 mercury tissue concentration of the *Fejervarya cancrivora* collected from all the sites

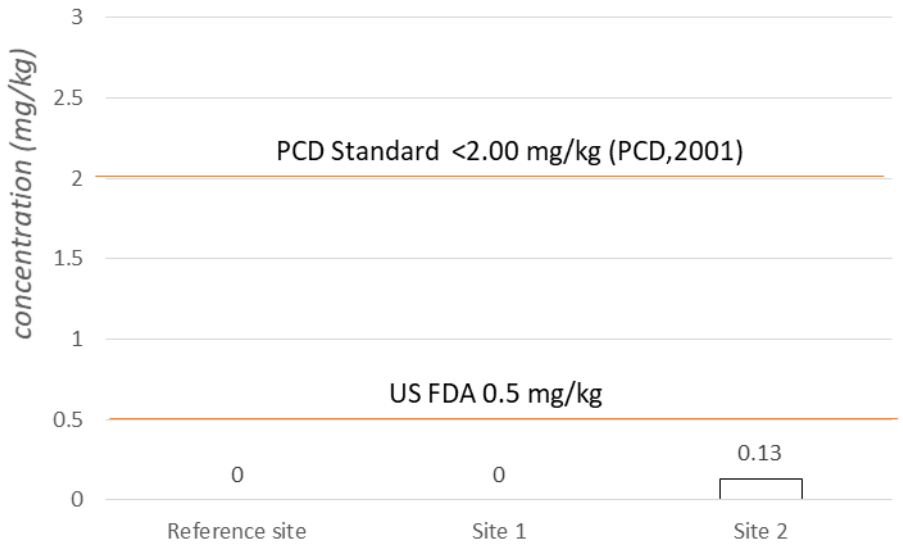

Figure 29 arsenic tissue concentration of the *Fejervarya cancrivora* collected from all the sites

The presence of mercury in the frogs in all the sites shows the extent of impact on the area and is of concern for us. Apart from sorption through the skin, the food chain is the main source of entry of the mercury into the frogs (Mason, Laporte, and Andres 2000). However, this can only be confirmed by the analysis of the vegetation of the area.

Only trace amounts of arsenic was detected from the frog tissues. This is understandable as most of the arsenic was in the sediment due to properties of arsenic which has an affinity towards soil (Shiowatana et al. 2001). Although arsenic has a direct impact on the chromosomes in cases where a significant tissue concentration is detected (Suttichaiya et al. 2016; Intamat et al. 2016), due to lack of field evidence we dismiss that relationship. Hence, concluding that since only trace concentrations of mercury can be detected form the tissue, it is the only cause of aberration with enough evidence.

4.1.3. Chromosome Analysis

As per the studies of Joshy and Kuramoto (2008), the genus *Fejervarya* has a diploid number 2n = 26. According to the studies of Patawang et al. (2014) the karyotype can be arranged as 6 metacentric pairs, 5 sub meta centric pairs, 1 acro centric pair and a metacentric X chromosome and an sub metacentric Y chromosome. The template for both

the species from the reference site as can be seen on Figure 30 and Figure 31. Karyotypes of metaphase cells with aberrations from the two species from the test site 1 and test site 2 are on Figure 32, Figure 33, Figure 34 and Figure 35 respectively. During the analysis of the chromosomes, care was taken to avoid counting of procedural artefact as gaps. To do so analysis was carried out according to the published data (Savage 1976; Paris Conference -1971: Standardization in Human Cytogenetics 1972)From the study we have observed that with the presence of concentrations of mercury in the frog's body, chromosomal aberrations were observed.

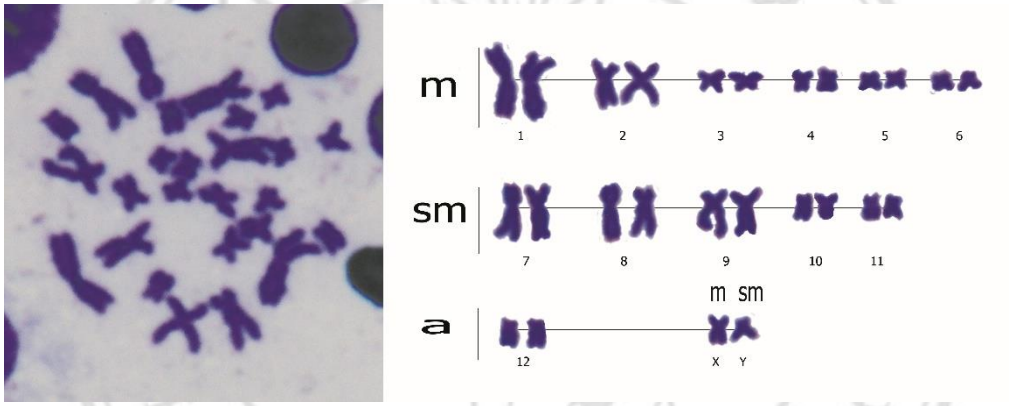

Figure 30 the karyotype of *Fejervarya limnocharis* collected from the reference area where 2n = 26 chromosomes are arranged according to the centromere arrangement

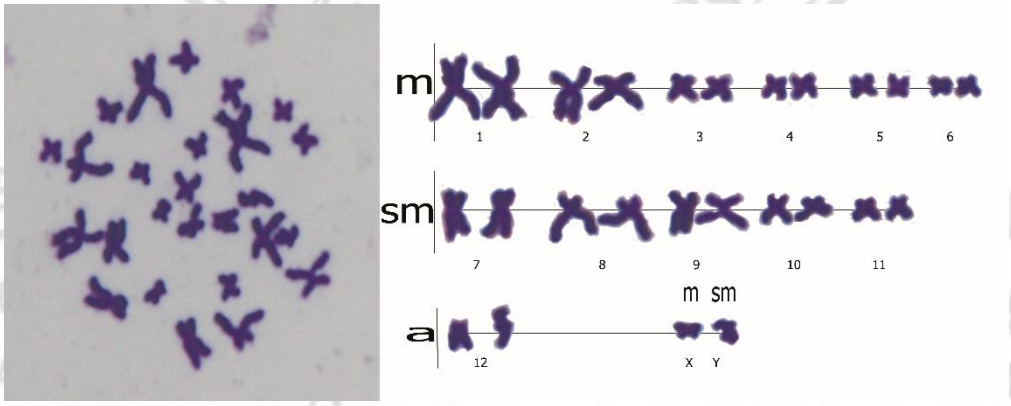

Figure 31 he karyotype of *Fejervarya cancrivora* collected from the reference area where 2n = 26 chromosomes are arranged according to the centromere arrangement

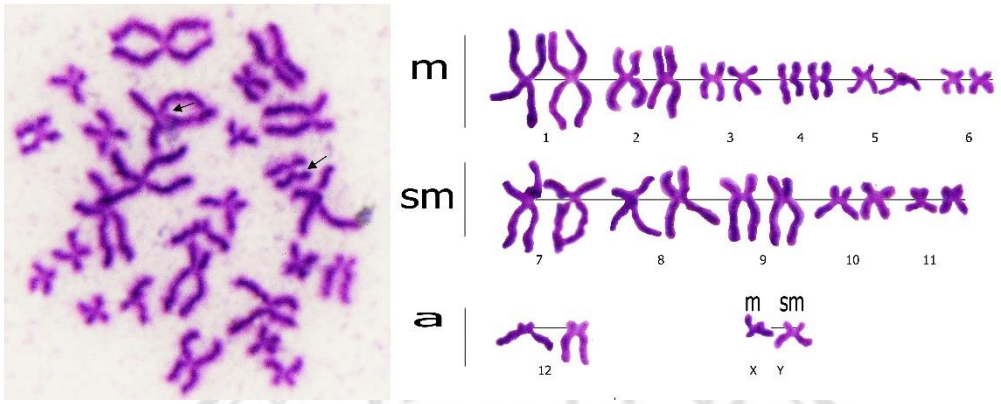

Figure 32 a metaphase karyotype of *Fejervarya limnocharis* from site 1 showing centromere break at one of the metacentric 4th pairs and single chromatid break at the q- chromatid on one of the sub metacentric 7th pair.

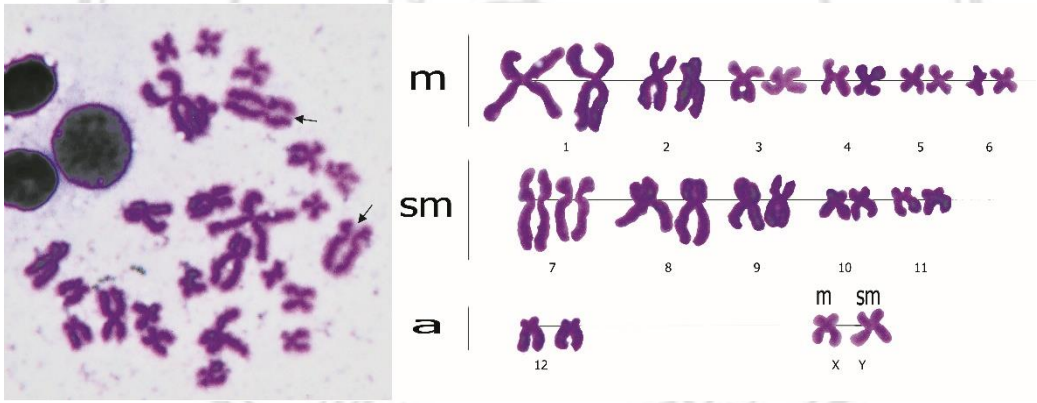

Figure 33 a metaphase karyotype of *Fejervarya cancrivora* from site 1 showing a centromere break at the sub metacentric 7th pair

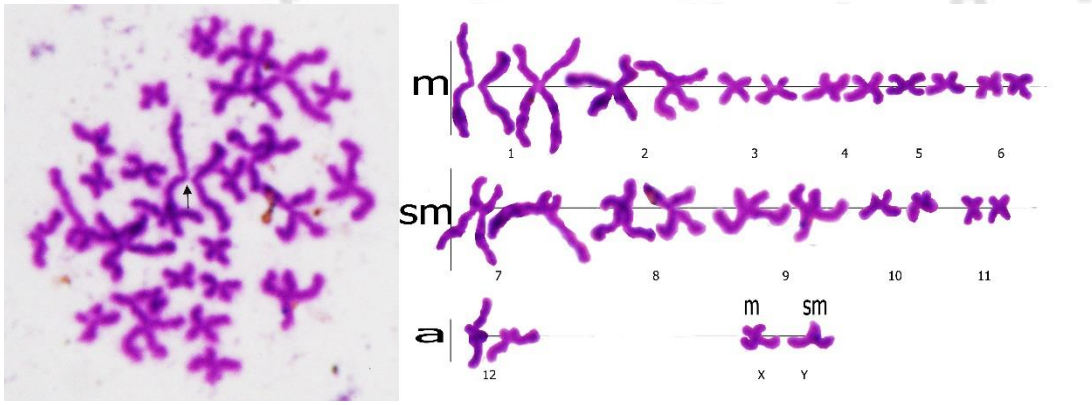

Figure 34 a metaphase karyotype *of Fejervarya limnocharis* from site 2 showing a centromere break at one of the 1st pair.

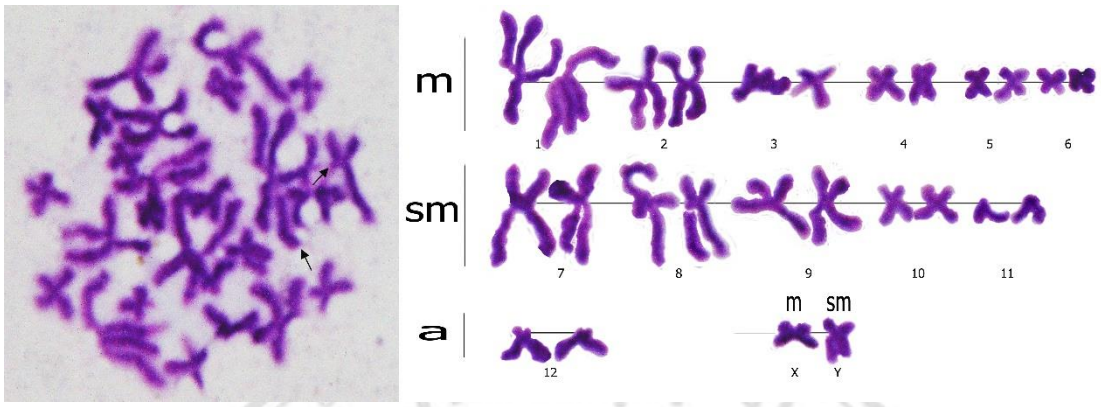

Figure 35 a metaphase karyotype of *Fejervarya cancrivora* from site 2 showing a single chromatid break at the q- chromatid on one of the sub metacentric 8th pair

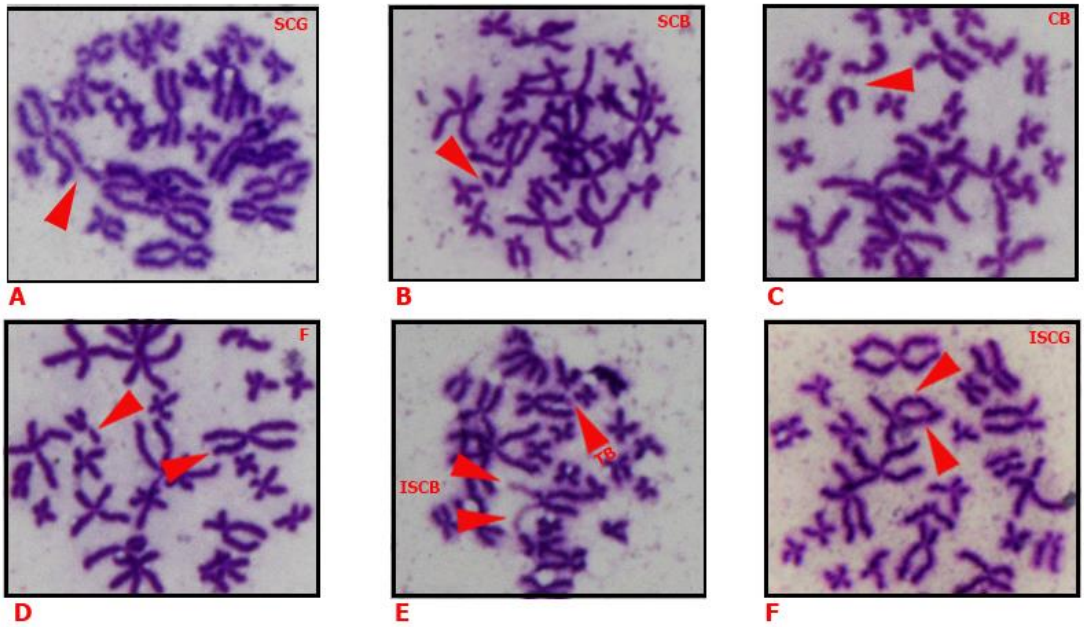

Figure 36 the different types of chromosomal aberrations observed in metaphase spread of both the species from A till F.

This shows that there is a relationship between the mercury in the body and the chromosomal aberrations. This has been studied and reported in literature (Promsid et al. 2015).

As shown on Figure 36 and Table 12 (appended), chromosomal aberrations looked into were fragments (F), deletions (D), single chromatid breaks (SCB), iso-chromatid break

(ISCB), Single chromatid gap (SCG), iso-chromatid gap (ISCG), terminal break (TB) and centromere breaks (CB).

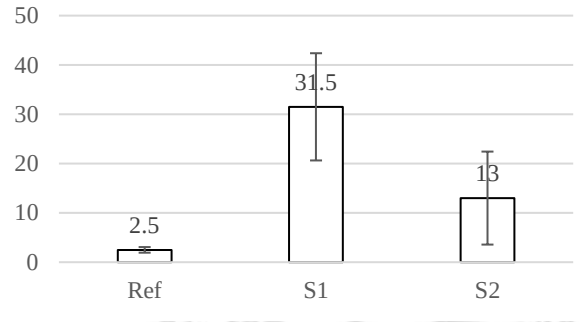

Figure 37 total number of aberrations per site

Table 1 summery, total number of aberrations

	Ref	S1	S2
Total	10	126	52
Average	2.5	31.5	13
SD	0.58	11.09	9.63
95% Confidence Interval	0.57	10.87	9.43
P-Value		0.014*	0.107

The total number of aberrations increased with the mining activity. It must be noted that the total number of aberrations were generally more in site 1 in comparison to site 2 (Figure 37). Statistical analysis (Table 1) of the difference between the mean number of aberrations in the reference site and site 1 was significant (P = 0.014). However, that of site 2 was not significant.

The percentage number of number of cells with chromosomal aberrations (See Table 12 in the appendix) shows, that, with the mining activity the percentage increased (Figure 38). Site 1 which had more activity and is larger, which, had unlimited access points to the sediment collectors shows a higher percentage aberration in comparison to site 2. The statistical analysis of the differences between the means of percentage chromosomal aberrations of the reference site and site 1 showed a statistical significance (P=0.047). However, that of the reference and site 2 was not significant (Table 2).

The highest percentage of aberrations seen on site 1 is mostly due to the uncontrolled number of sediment collectors used which will lead to more mercury and arsenic released to the area, in comparison to the fixed sediment collectors housed in site 2. Another factor that will affect the percentage of aberrations is the size of the sediment flow area. The small and muddy sediment collection area in site 2 ensures that the waste water is concentrated in a small space and allows for other modes of mitigation such as

eutrophication.At site 2, the percentage of chromosomal aberrations is less in comparison to site 1. This is mostly due to the large size of the area and the sampling location of the frogs. This is demonstrated in aberration induction experiments where the dosage has a direct relationship with the percentage aberrations (Krishnaja and Rege 1982).

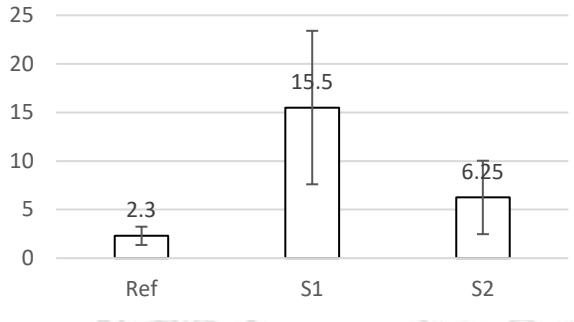

Figure 38 percentage number of aberrations per site

Table 2 summery, percentage number of aberrations per site

	Ref	S1	S2
percentage	9	62	25
Average percentage	2.3	15.5	6.25
SD	0.96	8.06	3.86
95% Confidence Interval	0.94	7.9	3.78
P-Value		0.047*	0.138

Literature suggests that the algal biota and plant biota can influence the exposure of the animals by mitigating the concentrations (Chen et al. 2008). This can explain the difference in the percentage aberration of the frogs from site 1 and 2. In site 2 the algal growth must have mitigated the frogs from exposure reducing the impact on them. However, this will open up to the possibilities of exposure through the food chain (Tirkey, Shrivastava, and Saxena 2012). The lack of an algal bloom can be used to explain the high percentage of aberration in the frogs from site 1. The location of the collection point being exactly in the flow out area further increases the chance of exposure of these frogs.

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high percentage of aberration in the frogs from site 1. The location of the collection point being exactly in the flow out area further increases the chance of exposure of these frogs.

The reference with its trace concentrations due to either wet deposition (Hammerschmidt and Fitzgerald 2005) or other mercury based chemicals such as pesticides (Krieger 2001), shows aberrations. A certain level of aberrations in the reference is often reported due to unforeseen external factors. (Suttichaiya et al. 2016; Promsid et al. 2015). This often acts as a benchmark for the background aberrations. However, this is relatively less in comparison to the mines where the concentrations of mercury and arsenic are high and the source of mercury and arsenic is known.

Further statistical analysis of the aberrations shown in between the species analysed from reference site (Figure 39), site 1 (Figure 40), and site 2 (Figure 41) shows that both *F. limnocharis* and *F. cancrivora* are not significantly different (Table 3, Table 4, and Table 5 respectively).

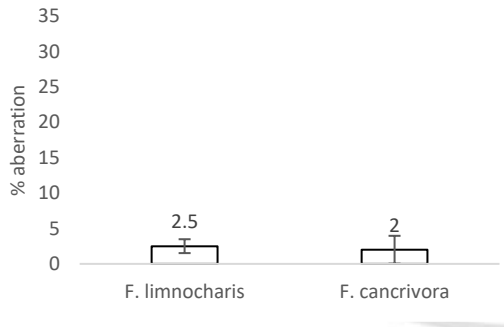

Figure39 percentage aberrations per species on the reference site.

Table 3 summery, percentage aberrations per species on the reference site.

	F. limnocharis	F. cancrivora
Average	2.5	2
SD	0.71	1.414
95% Confidence Interval	0.98	1.96
P-Value	0.698	

Although a resistant species was not established, we believe that in general the both *Fejervarya limnocharis* and *Fejervarya cancrivora* would have developed a resistance to the pollutants in the area explaining breeding and thriving of these frogs in the area. The most alarming observation is that the farmers use the wastewaters of mining as fish and shrimp farming waters. The fact that they heat the amalgam indoors in their kitchen is another alarming observation.

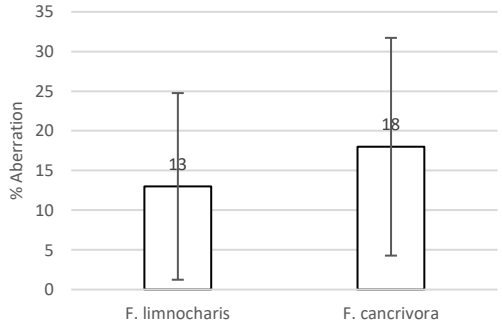

Figure 40 percentage aberrations per species on site 1.

Table 4 summery, percentage aberrations per species site 1.

	F. limnocharis	F. cancrivora
Average	13	18
SD	8.49	9.9
95% Confidence Interval	11.76	13.72
P-Value	0.641	

Labour exposure is often mentioned in the literature (Basri, Sakakibara, and Sera 2017). And if a community is known to harvest and eat animals from these areas (Pendrageon 2015; Wiens 2011) the risk further increases. We speculate that the individuals living in that area, especially children will have a high level of mercury in their body (Sarikaya et al. 2010). This can lead to disastrous problems in the future.

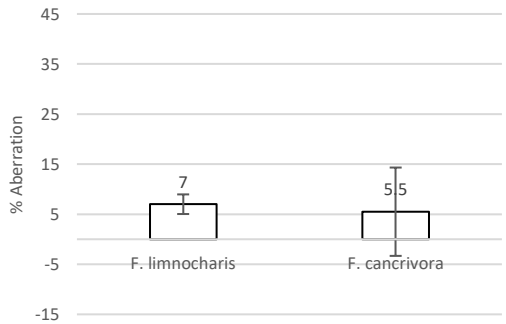

Figure41 percentage aberrations per species on site 2.

Table 5 summery, percentage aberrations per species site 2.

	F. limnocharis	F. cancrivora
Average	7	5.5
SD	1.41	6.37
95% Confidence Interval	1.96	8.82
P-Value	0.775	

In addition to the mercury and arsenic that the study is limited to, other pollutants will also be mobilized in the mining process and needs to be analysed. Regardless of the small sample size, the aberrations were observed in the frogs which raise a need for better

mitigation of the areas. This calls for a controlled and continuous bio monitoring protocol to be established in this area.

4.2. Laboratory Experiment

4.2.1. Acute Exposure for 4 days.

An introduction to the experiment

A through examination of the field study would be to look into the impact of chronic and acute exposure to mercury and arsenic on species similar to the observed *Fejervarya limnocharis* and *Fejervarya cancrivora* which are farmed on the site. This being the initial plan of the study, it was later informed that the use of methyl mercury would not be allowed due to the risk of exposure. This was a limitation and drawback to the initial research plan. Nevertheless, the question of acute and chronic exposure to arsenic can be explored.

It must also be noted that the fact that gold – arsenic ore is being targeted as the main mining element and the fact that arsenic is detected form the individuals from the area (The Guardian 2015), analysis of the impact of arsenic on a selected species will show the general impact of exposure. Thus, the current and the next e deals with the analysis of acute and chronic arsenic exposure.

i. Chromosome Analysis

The diploid chromosome number of all the exposed samples of analysed *Hoplobatrachus rugulosus* used was 2n = 26 with 8 metacentric and 18 submetacentric chromosomes. Suttichaiya et al. (2016) studied the same species from a mine and reports the same structure. The following karyotype (Figure 42) was used as a template in this study.

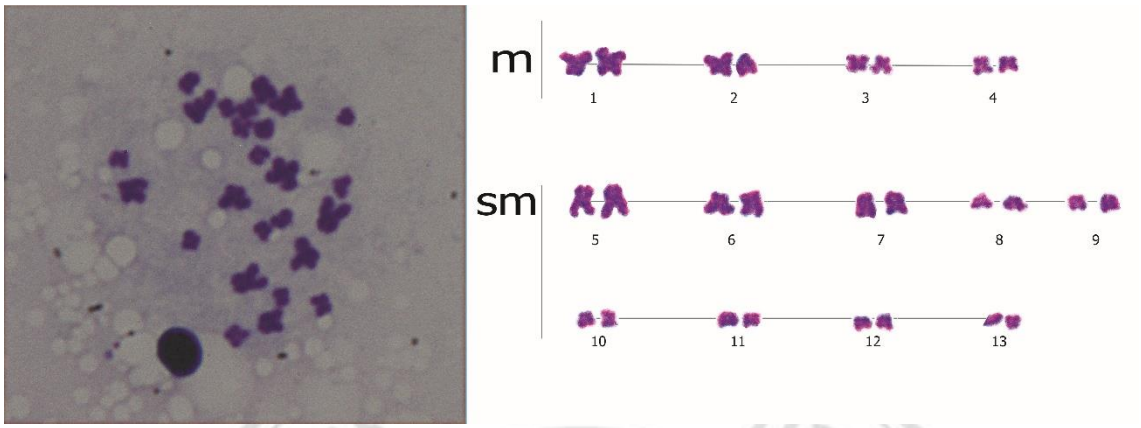

Figure 42 a karyotype of one of the samples from the control

ii. The Total number of Aberrations per Trial of Concentrations.

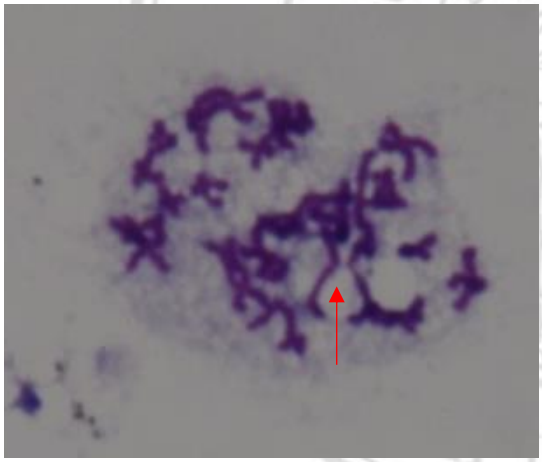

Figure 43 centromere breaks resulting in fragments

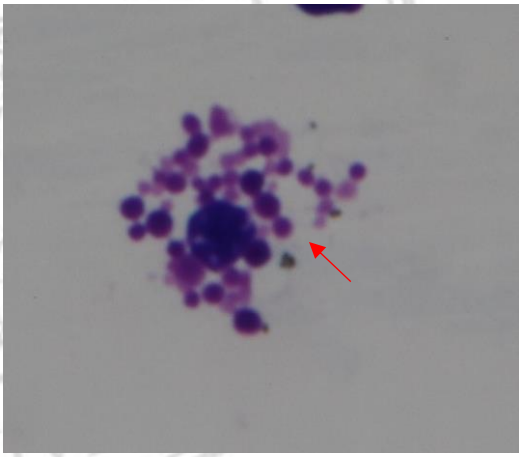

Figure 44 pulverised chromosomes observed

The general types of aberrations observed were analysed, cross checked and confirmed with the literature available. Table 14 (appended), shows that for acute exposure of the aberrations observed were Ring Chromosomes (RC), Minutes (M), Fragments (F) (Figure 43), Pulverised Chromosomes (P) (Figure 44), and breaks (B). Among the observed, few cells had more than one type of aberration.

Table 6 summery, the average total and the average percentage number of aberrations per trial of acute exposure

Concentration mg/l	Average Total	Average percentage
0		
Average	3.0	2.0
Standard deviation	1.4	0.0
Standard error	1.0	0.0
95% confidence interval	2.0	0.0
0.5		
Average	4.0	5.0
Standard deviation	0.0	1.4
Standard error	0.0	1.0
95% confidence interval	0.0	2.0
P=	0.5	0.204
1		
Average	6.5	10.0
Standard deviation	0.7	0.0
Standard error	0.5	0.0
95% confidence interval	1.0	0.0
P=	0.089	…
1.5		
Average	8.5	13.0
Standard deviation	2.1	1.4
Standard error	1.5	1.0
95% confidence interval	2.9	2.0
P=	0.092	0.057*
2		
Average	11.0	14.0
Standard deviation	2.8	5.7
Standard error	2.0	4.0
95% confidence interval	3.9	7.8
P=	0.07	0.205

$\alpha = 0.05$

The average total number of aberrations per trial of acute exposure (Table 6) increased as the concentration of arsenic increased. The visualized trend of the acute exposure to arsenic (Figure 45) shows that the the averaged total number of aberrations increased as the concentration increased. Statistical analysis of the means shows that none of the average total aberrations due to the acute exposure to arsenic against that of the control was significantly different (Table 6). The range of the values may have been contributing

factors to this. Reflecting this, at all concentrations the error bars shows an overlap of the 95% confidence interval with that of the control.

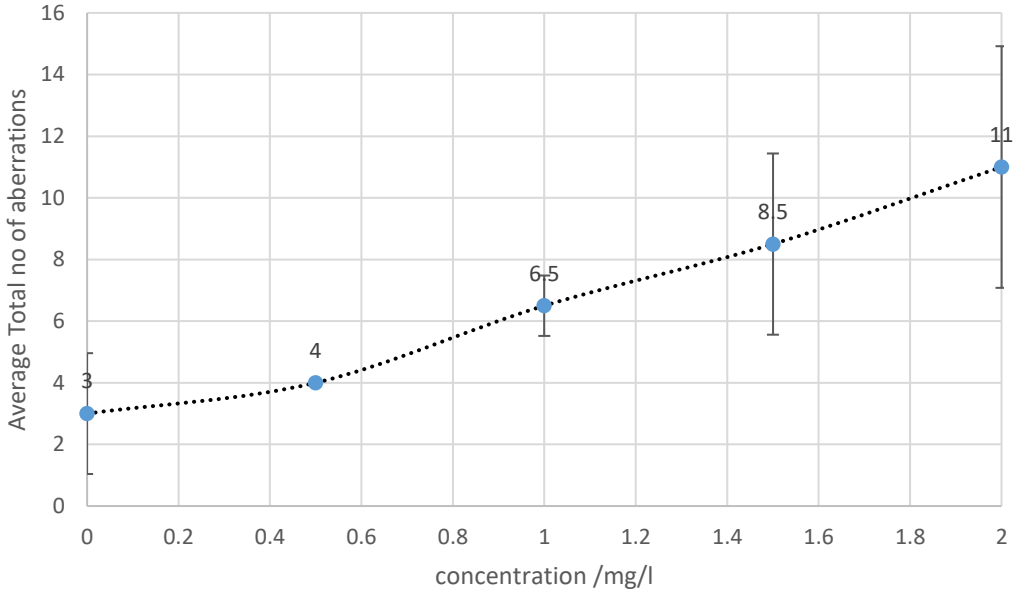

Figure 45 illustrated trend in the averaged total number of aberrations per trial.

Although a general increasing trend of the impact of acute exposure to arsenic was observed by using the average total number of aberrations, a major limitation of the mode of analysis was that this tends to show multiple aberrations in one cell. For example, while counting fragments some can be traced back to the source break, while others were difficult to trace as the whole cell or half of the cell would appear to be fragmented. In these cases, the multiple fragments were counted as one unit while in cases the fragment was traced to the origin, the fragment was linked and counted. As for the ring chromosomes the same rule of counting a complete cell in this condition as one unit and separate units as fragments was applied. Therefore to standardize the data, the number of cells with aberrations as a percentage was calculated from within 50 cells per individual.

iii. Percentage Number of Cells with Aberrations per trial of Concentrations

Table 6 shows the percentage aberrations of the exposure to acute arsenic concentrations show an increasing trend (Table 14, appended). Despite the increase, a resistive

acceleration was seen in the averaged percentage aberrations from 0.5 mg/l to 1mg/l (Figure 46).

Statistical analysis of the means shows that the impact of the acute exposure to concentration of 1.5 mg/l of arsenic was statically different from the control (P= 0.057) while others were not. Due to the "perfect" data on the control and 1mg/l, the data was not analysable. Therefore, an increase in the percentage aberrations of cells with aberrations was seen with the concentration without a statistical significance until 1.5 mg/l. Despite the increase in the percentage aberration at 2 mg/l, due to the large range of the data the mean was statically not different from the control (Table 6).

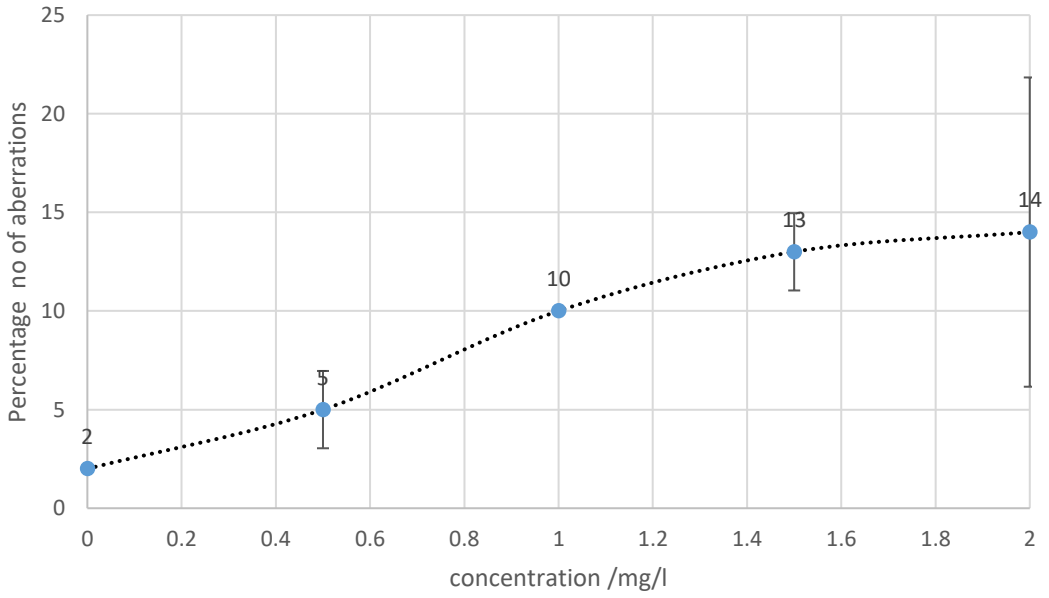

Figure 46 illustrated percentage of aberrations per trial

Preetpal and Tripathi (2014), conducted an experiment where they exposed a selected species of frogs to different heavy metals for 4 days. They too report a similar trend where the chromosomal aberrations increased as the concentration increased. The types of aberrations observed were also similar, where they reported fragments, breaks, rings and minutes as their primary findings slimier to ours further reinforcing our findings. It is interesting to see the average percentage aberration showing signs of reaching an asymptote. The expected curve form the experiment is a straight line. if the experiment

could have been carried on further with increasing concentrations, this relationship could be explored.

4.2.2. Chronic Exposure.

An introduction to the experiment

Referring to the preliminary study, we observed arsenic to be in the soil but not in the tissue. Exposing the animals to an acute dose of arsenic shows a definite increase in the percentage of aberrations.. This further raises the question of the impact of extended chronic exposure of the above tested concentrations. The exposure that the field specimens would have been exposed to. The current section explores the impact of chronic exposure.

i. Chromosome Analysis

The diploid chromosome number of all the exposed samples of analysed *Hoplobatrachus rugulosus* used was 2n = 26 with 8 metacentric and 18 sub metacentric chromosomes. Similar to the acute experiment, the template for comparison is by Suttichaiya et al. (2016) whom studied the same species from a mine. The keriotype on Figure 47 was used for the analysis of the other metaphases.

Throughout the study, variations of aberrations were observed while fragments (Figure 48), minutes (Figure 49) and rings dominated (Figure 50). It must also be noted that pulvarised chromosomes (Figure 51) were also observed (Table 16, Table 17, and Table 18, appended). Chou et al. (2001) in their study titled, arsenic inhibition of telomerase transcription leads to genetic instability reported chromosomal breaks, rings, fragments and end to end fusions.

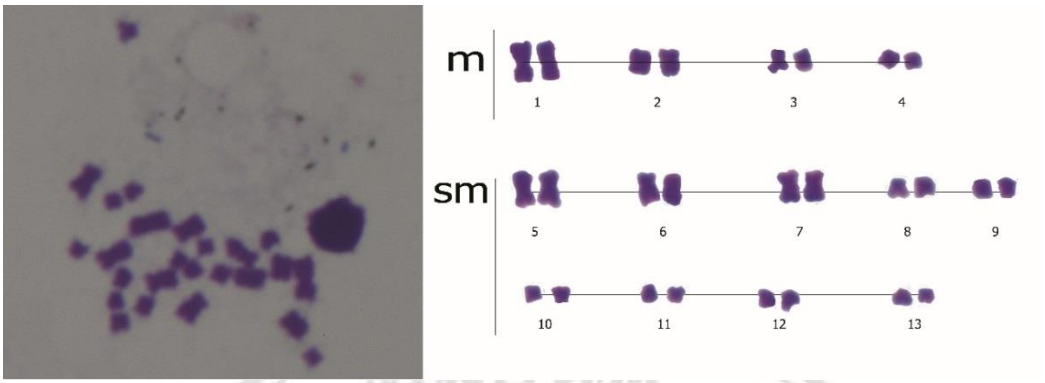

Figure 47 Sample karyotype of the metaphase of *Hoplobatrachus rugulosus*

.The experiment exposed arsenic to cultured human cells in the laboratory to induce aberrations. These further supported our findings of arsenic exposure inducing chromosomal abnormalities as we were able to identify all of the above reported aberrations in both chronic and acute exposure except for end to end fusions supporting our findings and reinforcing the types of aberrations to be expected in the experiment

A field study analysing the types of aberrations seen in 33 copper smelter workers exposed to arsenic in Sweden shows similar aberrations as our field study such as such as fragments, and breaks (Nordenson and Beckman 1982) further supporting and reinforcing the types of aberrations expected in a long-term exposure, much like our study. Furthermore, studies such as that of Preetpal and Tripathi (2014), Chou et al. (2001), Yadav and Trivedi (2009; 2006) shows gapa, breaks, fragments, and ring chromosomes, hence, showing the types of aberrations expected in a field study and supporting our chronic and acute experiment. Analysis of exposed human specimens against a control shows a major increase in aberrations such as fragments and ring chromosomes on the exposed humans (Beckman, Beckman, and Nordenson 1977) The same trend was seen on the acute exposure . hence this can be a point to explore further

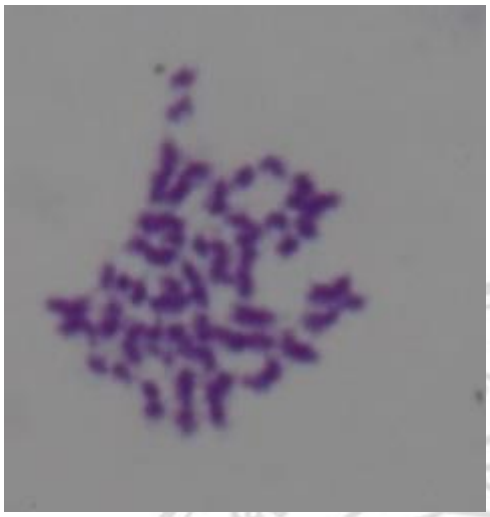

Figure 48 fragmented cells observed in the chronic exposure

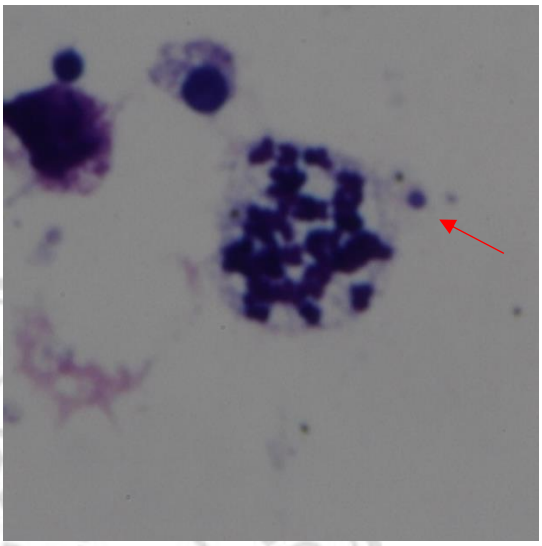

Figure 50 ring chromosomes observed in the chronic exposure

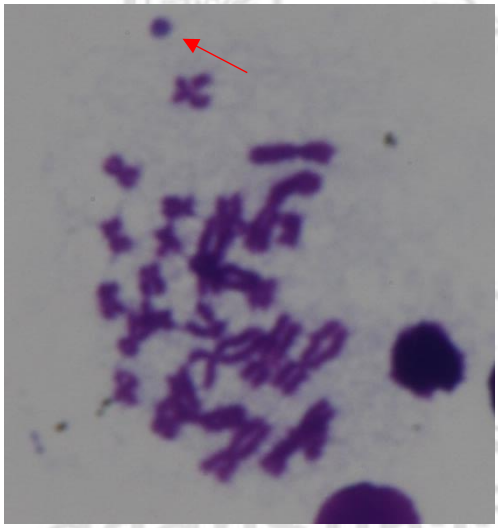

Figure 49 minute chromosome observed in the chronic exposure.

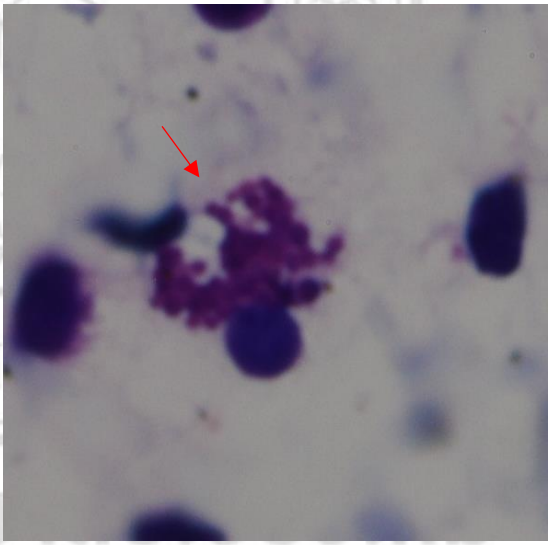

Figure 1 clumping and pulverised chromosomes observed in chronic exposure.

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ii. The Total number of Aberrations per Trial of Concentrations

On the first day of analysis, that is the 12^{th} day, the data shows that as the concentration increases, the average total number of aberration increased (Table 16, appended).

The difference between the concentrations 1 and 2 mg/l is considerable the increasing trend is similar to the acute exposure and is the expected result. This also shows a statistical difference between the means of the control and the dose concentrations 1 and 2 mg/l (P=0.033* and $1.7X10^{-0.5}$* respectively) (Table 6). On the second day of analysis that is the 16^{th} day (Table 17, appended), like the acute exposure and the 12^{th} day of chronic exposure, an increase of the average total aberration can be seen. It must be noted that the average increase of the concentrations seen on the 16^{th} day was a little bit higher from the 12^{th} day. Statistical analysis of the means of the concentrations against the control shows a difference (P=0.0049* and 0.0005* respectively) (Table 6).

On the third day of analysis, that is the 20^{th} day (Table 18, appended) A general increase was seen in the average total number of aberrations, much like the acute, 12^{th} and 16^{th} day of the exposure. However, we expected to see a general increase from the 16^{th} day onwards. However, a slight drop is seen. Statistical analysis of the difference between the means of control 2 mg/l was significant while that of 1mg/l was not ($6.44X10^{-6}$*) (Table 6). It must be noted that the main contributing factors of the above stated similarity of the means between 1mg/l and control was due to the drop in the average brought about by the loss of 2 specimens and the incredibly high value at the specimen named A1 that brought about a high standard error.

The overall trend of the average total number of aberrations observed in the chronic exposure to arsenic show that as the concentrations increased number of aberrations increased. Due to the previously mentioned irregularities in counting, the aberrations (see page 53), the chronic exposure also uses the percentage drawn from 50 identifiable cells.

Table 1 summery, the total number of aberrations per trial per day.

Concentration mg/l	Specimen	12, Total	16 ,Total	20, Total
0	A1	0	3	2
	A2	4	5	4
	A3	2	2	5
	B1	4	1	3
	B2	1	3	2
	B3	6	3	3
Average		2.833	2.833	3.167
Standard deviation		2.229	1.329	1.169
95% confidence interval		1.783	1.064	0.935
1	A1	18	0	36
	A2	3	37	8
	A3	6	23	17
	B1	13	7	0
	B2	4	11	28
	B3	11	26	0
Average		9.167	17.333	14.833
Standard deviation		5.845	13.721	14.919
95% confidence interval		4.677	10.979	11.937
P=		0.033*	0.049*	0.114
2	A1	16	27	32
	A2	10	40	27
	A3	16	48	25
	B1	11	27	35
	B2	14	25	32
	B3	13	29	25
Average		11.500	32.667	29.333
Standard deviation		6.058	9.223	4.227
95% confidence interval		4.847	7.380	3.382
P=		1.7 E-05*	0.0005*	6.44 E-06*
$\alpha = 0.05$				

iii. Percentage Number of cells with Aberrations per Trial of Concentrations

a. Intra Concentration Analysis

The percentage aberrations observed on the chronic exposure to 1 mg/l, 2 mg/l and control is shown on the Table 8. Before analysing the data, the difference between the trials had to be considered. This intra concentration analysis was key in identifying the difference between the trials A and B of all the exposed concentrations within the duration..

Table 2 percentage aberrations observed in chronic exposure

Concentration mg/l	Day / Specimen	Day 12 %	Day 16 %	Day 20 %
0	A	1.82	3.64	3.70
		7.27	7.27	3.70
		3.64	3.64	9.26
Average		4.24	4.85	5.56
Standard deviation		2.78	2.10	3.21
Standard error		1.60	1.21	1.85
	B	5.45	1.82	3.70
		1.82	5.45	5.56
		9.09	5.45	5.56
Average		5.45	4.24	4.94
Standard deviation		3.64	2.10	1.07
Standard error		2.10	1.21	0.62
P=		0.67	0.7415	0.7676
		Over all		
Average		4.85	4.55	5.25
Standard deviation		2.97	1.91	2.16
Standard error		1.21	0.78	0.88
1	A	24.07	0.00	50.00
		5.56	46.43	14.29
		11.11	30.00	17.65
Average		13.58	25.48	27.31
Standard deviation		9.50	23.54	19.72
Standard error		5.49	13.59	11.39
	B	7.41	11.11	38.89
		14.81	20.00	0.00
		0.00	28.89	0.00
Average		7.41	20.00	12.96
Standard deviation		7.41	8.89	22.45
Standard error		4.28	5.13	12.96
P=		0.425	0.7254	0.4524
		Over all		
Average		10.49	22.74	20.14
Standard deviation		8.34	16.20	20.47
Standard error		3.40	6.61	8.36
0 mg/l vs 1 mg/l P=		0.1692	0.04*	0.1366
2	A	20.37	31.11	39.66
		12.00	35.56	34.55
		20.37	55.56	25.45
Average		17.58	40.74	33.22
Standard deviation		4.83	13.02	7.19
Standard error		2.79	7.52	4.15
	B	14.81	31.11	38.98
		18.52	26.67	33.33
		0.00	44.44	32.14
Average		11.11	34.07	34.82
Standard deviation		9.80	9.25	3.65
Standard error		5.66	5.34	2.11
P=		0.36	0.5098	0.7483
		Over all		
Average		14.35	37.41	34.02
Standard deviation		7.77	10.74	5.18
Standard error		3.17	4.39	2.11
0 mg/l vs 2 mg/l		0.0312*	0.0007*	$5.00E^{-06}$ *
$\alpha = 0.05$				

Statistical analysis of the difference between the trials shows that the means are not significantly different. The main contributing factor for this is the fact that most of the data fall in the same data range with a few exceptions providing a large variation. Therefore, the difference in between the trials shows the similarity of the impact of the arsenic exposed. It is important to point out that on the 20th day, in the exposed concentration of 1 mg/l of arsenic in trial B, due to the premature death of a sample and the contamination of another, a proper intra concentration statistical analysis was not possible.

The range of aberrations seen between the specimens of the control group was of a particular interest. Although not much in comparison to the exposure groups, it is noteworthy. This hints a background aberration to an exposed substance. One possibility is a past exposure such as those frogs of which live in mines and other hazardous environments (Suttichaiya et al. 2016; Intamat et al. 2016).

However, due to the long acclimatization period of ideal conditions the specimens were, treated to counter for such an exposure such as clean filtered water, timed temperature and sufficient food, the aberrations observed is less likely to be the case of a past exposure. Another possibility of the abovementioned aberration in the control is the possibility of volatilization of the arsenic (Frankenberger 2001) from the exposed concentrations and impacting the control frogs in the same room

b. Inter Concentration Analysis

There are two ways of looking at the percentage aberrations; the average aberrations against time, and against concentration.

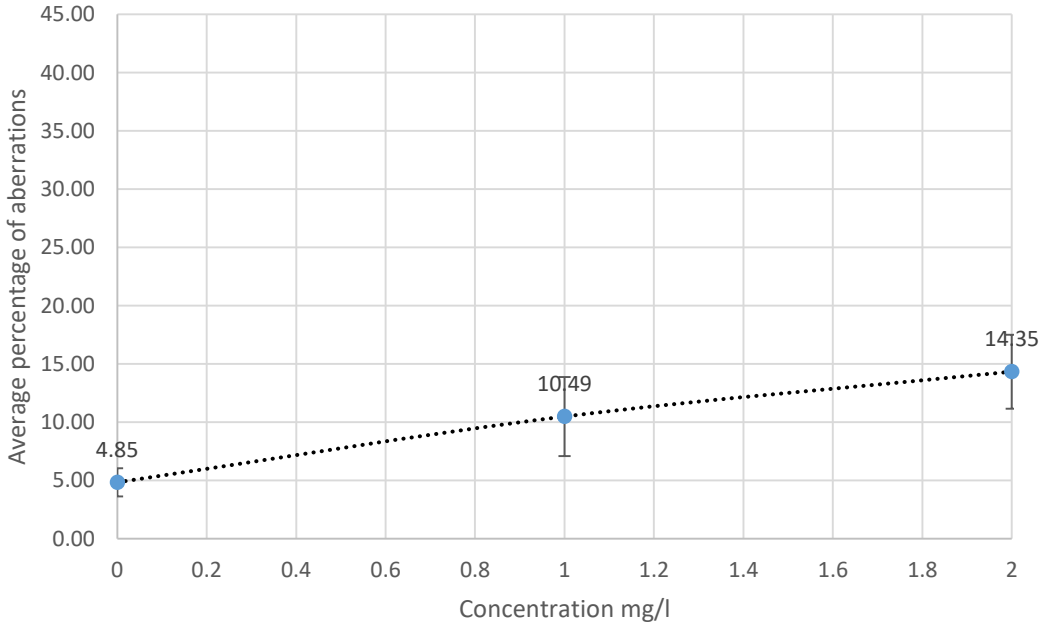

Figure 52 the visualised change in trend of the aberrations for three concentrations of exposure for the 12th day.

Looking at the average percentage against concentration for the 12th day, a slight increase in the average percentage is seen (Figure 52). Statistical analysis of this increase in the concentrations against control shows that the mean of the average percentage aberration of 1mg/l was not statistically significant from the control while 2 mg/l was (P=0.0312). The main reason for the statistical similarity seems to be due to the spread of the data, which can be seen by the standard deviation (Table 8).

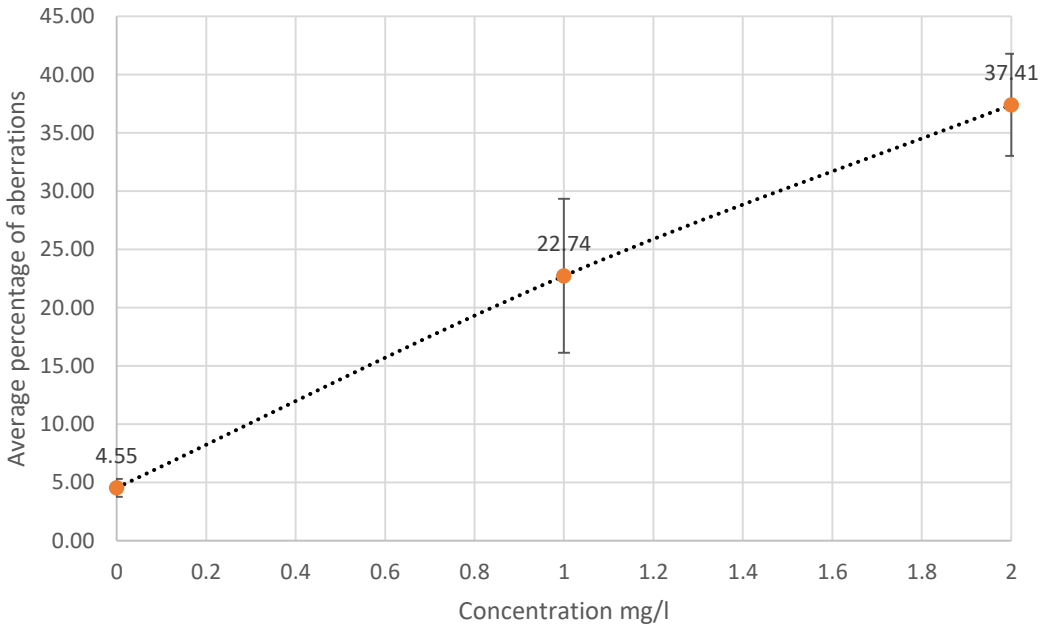

Figure 53 the visualised change in trend of the aberrations for three concentrations of exposure for the 16th day.

Similarly, the average percentage aberrations of the 16th day (Figure 53) shows the same increase as the 12th day. However the general percentage is higher than the 12th day. Statistical analysis of this increase in the concentrations against control shows that the mean of the average percentage aberration of all the exposed concentrations were statistically significantly different (P=0.04 and 0.0007 respectively (Table 8).

The average percentage aberrations of the 20th day (Figure 54) shows an increase as concentration increases. However, the general percentage is lower than the 16th day. Statistical analysis of the increase in the concentrations against control shows that the mean of the average percentage aberration of 2 mg/l was statistically significantly different while 1mg/l was not. (P= 5.00 $X10^{-06}$) (Table 8).

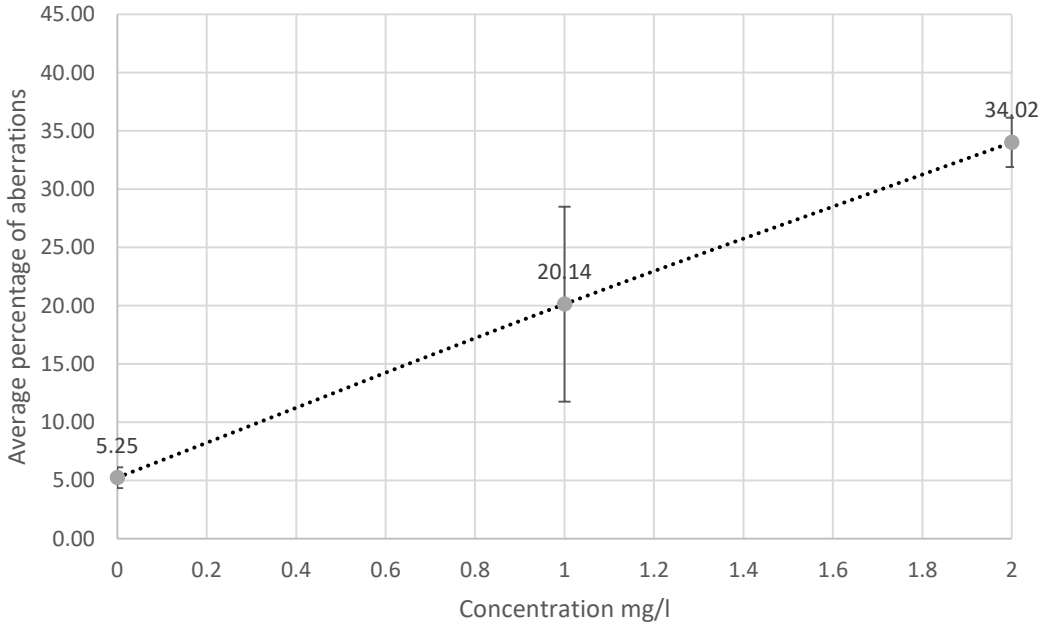

Figure 54 the visualised change in trend of the aberrations for three concentrations of exposure for the 20th day.

The data shows that the impact was the highest on the 16th day and a considerable drop was seen on the 20th day. The expected results for such an exposure to increasing concentrations regardless of time would be to see a constant increase. However, a trend that we have seen in analysing the data for the average percentage aberrations against increasing concentrations for both acute and chronic data is a decrease in the highest concentrations. A speculation is that this might be due to the build-up of a tolerance to the new conditions. The observation can be appreciated by, analysing the data taking each concentration against time.

Looking at the percentage aberration against time, we see the percentage aberration change due to different concentrations of arsenic over the timeline. The results averaged per tank and represented against time would have the origin of time as “day 0”. However, this does not report the aberration at that period nor will it be used in any calculation. All the analysis will be carried out from the 16th day, which is the first day of analysis.

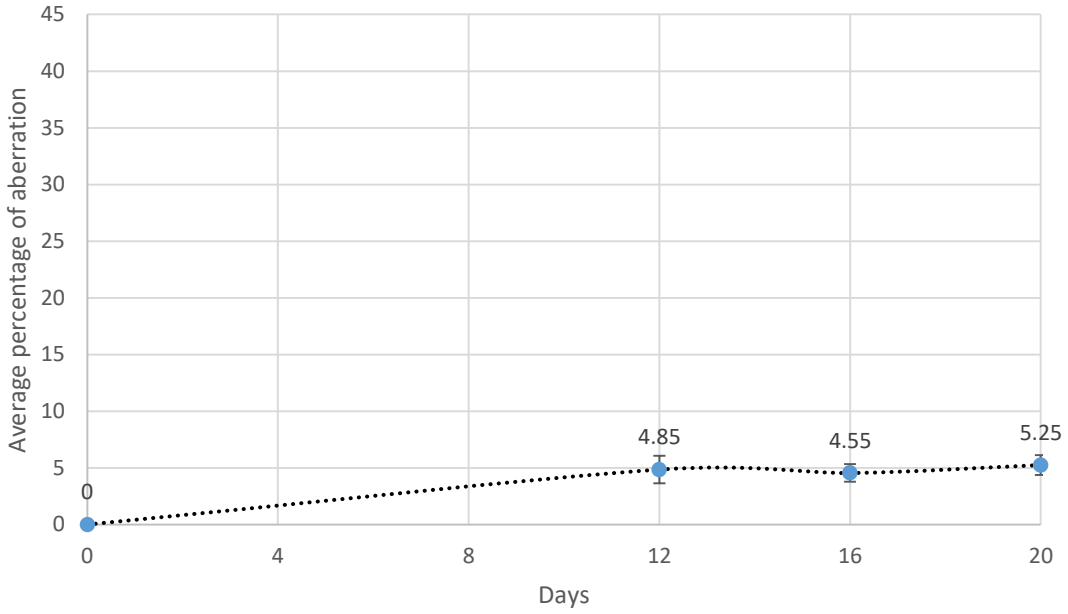

Figure 55 the visualised change in trend of the aberrations over the three days of exposure for control.

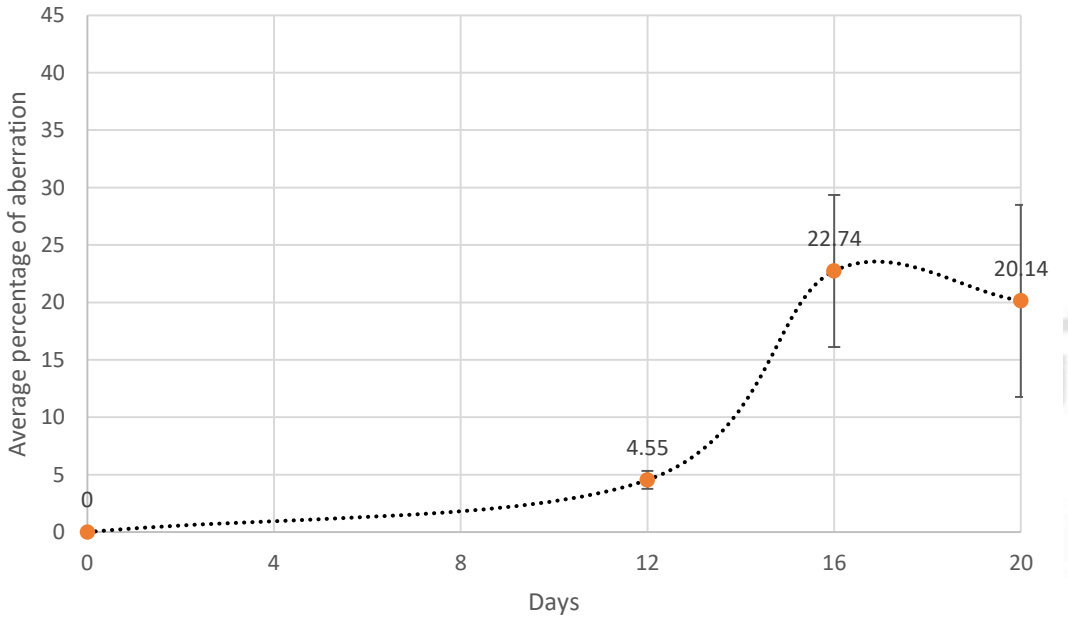

Figure 56 the visualised trend of the aberrations over the three days of exposure for 1mgl.

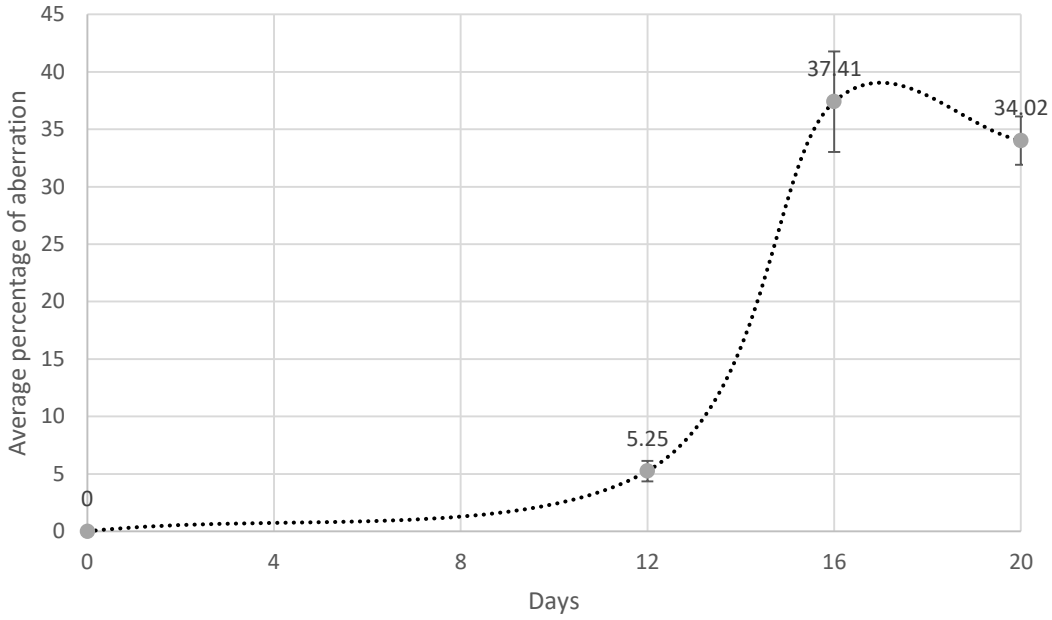

Figure 57 the visualised trend of the aberrations over the three days of exposure for 2mg/l.

On the control concentration (Figure 55) although a straight line was expected form the 12th day till the 20th day, a dip was observed on the 16th. This dip is due to an increase in the average percentage aberrations of the 20th day. A minimal percentage of aberration is seen in most field studies that deal with an effected site and a reference or unaffected site.

This is mostly because, although the targeted pollutant does not affect the animal, the animal may have been exposed to other pollutants (Tengjaroenkul et al. 2017; Intamat et al. 2016; Suttichaiya et al. 2016; Promsid et al. 2015). That being a field example, the same pattern is seen in controlled experiments. Preetpal and Tripathi (2014) in there study where they exposed frogs to different heavy metals report a similar aberration similar to the aberration we reported in our study. This can be regarded as a background aberration in the experiment.

On the specimens exposed to 1 mg/l and 2 mg/l of arsenic although a positive relationship to time was observed until the 16th day, the trend shows that there was a drop on the 20th day (Figure 56 and Figure 57).

The general analysis and the visualizations shows that there is a considerable difference between the aberrations observed over different concentrations of arsenic and the aberrations observed over different days of exposure to arsenic. An ANOVA (Table 9) had to be carried out in order to identify the significance of the difference between the test groups as this approach lacked a control.

Table 3 statistical analysis of the percentage aberrations against concentration and time.

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Concentration	5072.996	2	2536.498	23.5244	$1.02E^{-07}$	3.204317
Time	1424.099	2	712.0494	6.603804	0.003057	3.204317
Interaction	937.4748	4	234.3687	2.17362	0.087235	2.578739
Within	4852.086	45	107.8241			
Total	12286.66	53				

The analysis of the statistical difference between the concentrations 0 mg/l, 1 mg/l and 2 mg/l of arsenic shows that there was a significant difference between the means of the aberrations seen in all the three concentrations ($P=1.02X10^{-07}$). Hence, proving that the exposure concentrations affect the percentage aberrations significantly.

The same test was carried out to find out if the difference observed within the percentage aberrations between the exposure periods was significant. The test showed that there is an overall significant difference between the means of the percentage aberrations of all the exposed days (P=0.0031). Hence, proving that exposure period also has an impact on the percentage aberration.

Analysing the interaction between the concentrations and time shows that there is no interaction between the two factors supporting the previous speculation of adopting to the change overtime (P= 0.087).

Analysing which of the factors, concentration or time had a greater impact can be answered statistically by comparing the f critical and the f value of the data (Table 9). The analysis shows that the concentration has a greater impact on aberration in comparison to time. As the results show that as time goes by the average percentage aberration decreases. As speculated, this may have been due to a build-up of tolerance

The fact that animals can be seen thriving in pools of high concentrations of arsenic (Intamat et al. 2016) is a point that must be taken into account while discussing these facts. Another proof of adoptability is that these species are known to live in areas with contamination of other material as well (Suttichaiya et al. 2016). Similarly studies show that frogs can survive in hostile conditions (Marc 2004) further supporting this data. Similarly, in a non-related study held at Mangualde, Central Portugal in an abandoned Uranium mine pond, it was observed that frogs were found to be thriving despite the high concentrations of toxic substances despite chronic exposure and the damage due to this (Marques et al. 2009). The fact that the impact decreased on the 20th day shows us what can be speculated as a response to prolonged exposure where, the frogs show a high level of adoptability. However, evidence must be gathered from literature to explain the speculation as it is out the scope of the current study.

In a study by Yadav and Trivedi (2006) reported the same pattern of an increase and a decrease in the percentage of aberrations induced due to chromium exposure. The main cause of this pattern they reported in the study is an initial stage of chromium damage to the cell and the genetic material inducing chromosomal aberrations. However, overtime with increase in exposure, the animal develops a mechanism by which they accumulate chromium in their body and the cells in which the chromosomal aberrations formed. And eventually they fail to divide and multiply. Further explaining the trend of our results overtime and supporting our findings.

Yadav and Trivedi (2009) also showed that aberration can be induced by exposure to heavy metals including arsenic to aquatic organisms reporting aberrations such as rings, breaks fragments and gaps further supporting our study. Much like our findings, the pattern of their data shows a decrease in the aberrations as time progresses. Validating the findings of our study. They also cited Brunetti et al. (1998) as reporting that the higher concentration of toxicant might inhibit normal cell division, damage chromosome and interdict DNA duplication, thus showing cytotoxic damage to be less. They also cited Huang et al. (1995) found that arsenic significantly delays mitotic division inhibits assembly of the mitotic spindle and induction of chromosome endoreduplication.

In a similar but non related study of analysing the frequency of micronuclei in exposed fish De Lemos et al.(2001) reported a similar pattern of decreased number of micronucleus with time. He cited Das and Nada (1995), as reporting inhibition of cellular division as the cause of this decrease. This showed that the phenomena is seen across different disciplines of study, and widely accepted as a major cause of the trend reinforcing our study.

Overall, The general trend of the percentage aberrations shows an increases with the increase in concentration of the exposure showing a correlation. Studies such as the Induction of chromosomal aberrations in fish *Boleophthalmus dussumieri* after exposure in vivo to mitomycin C and heavy metals mercury, selenium and chromium by Krishnaja and Rege (1982) supports the findings of this experiment that exposure to heavy metals can induce chromosomal aberrations. the study also , shows that induction of cromosomal abberations are possiable by exposure to the pollutant in a controlled environment. using two different modes of exposure to heavy metals injections or dissolved in the meduim. Their study 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 meaning that the injection mode works faster and is more effective bringing about an effect.