EFFECT OF CHLORINE WATER CONSUMPTION ON PHENOTYPIC AND MICROBIOME DEVELOPMENT

By

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And approved by

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ABSTRACT OF THE THESIS

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Urbanization sanctions the use of sanitation and health practices that have helped to control the transmission of infectious diseases. One of the widely used methods of disinfection is water chlorination, which achieves the purpose of killing pathogenic microbes, but may could also affect the human microbiome. In this pilot study, experiments were conducted in mice, to investigate the effects of chlorinated water consumption from early life, on growth and microbiome development. The experiment involved 10 pregnant dams, 5 in each group, and it was repeated twice (cohort 1 and 2). Body weight changes were measured, and fecal samples were collected weekly for microbiome structure determination. The results showed that there was a significantly higher developmental weight gain in mice (both males and females) that consumed chlorinated water, and the effect was consistent in the two cohorts. The effect was observed after weaning, when the young animals started drinking the chlorinated water. Chlorination significantly altered the microbiome, lowering fecal alpha diversity. The results suggest that drinking chlorinated water at early ages may significantly affect development, by microbiome-meditated mechanisms, with important implications for human health.

ACKNOWLEDGEMENT

I could have not accomplished the work of my thesis without the training and support of many colleagues and mentors over the past two years. Most importantly, I would like to thank my thesis advisor, Gloria, who generously gave time and much patience to make me a better scientist and professional. With her guidance, I have learned more about the way I think and approach science than I thought possible. She has taught me how to ask the right questions and how to manage multiple projects. Additionally, I would like to thank the members of my thesis committee, Dr. Liping Zhao and Dr. Max Haggblom, for the advice, questions, and encouragement. I have deep gratitude for Dr. David Reimer, Nancy Rossi who provided me a technical foundation in mouse handling and husbandry, without whom, this work would have not been possible, and I would like to acknowledge other members of Rutgers Animal Facility who provided so much support to my project. I would like to thank Dr. Martin Blaser and Dr. Clifford Weisel for their help in the conception and early design of the project. Finally, I would like to thank and acknowledge the efforts of my fellow members of the Dominguez-Bello lab, who have helped in every step of the way. I relied on constant assistance from Keith Martinez and Jincheng Wang and would like to extend my gratitude to all the undergraduate students who assisted me on this project namely, Sherry Wong, Sami Eiraket and Jared Lim.

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CHAPTER 1: INTRODUCTION

Living multicellular organisms have species – specific microbiomes that have organ specific structures (GI tract, vaginal and skin) (Ley, Hamady et al., 2008, Ley, Lozupone et al., 2008, Godoy-Vitorino, Goldfarb et al., 2012, Yildirim, Yeoman et al., 2014), which are not only important for the colonized host, but also hold adaptive value for the offspring. They are responsible for important functions that include developmental (Stappenbeck, Hooper et al., 2002, Cox, Yamanishi et al., 2014, Malmuthuge, Griebel et al., 2015), immune (Round, Mazmanian et al., 2010, Littman and Pamer 2011, Olszak, An et al. 2012), ecological, and defense, such as protection against invading pathogens (Buffie and Pamer 2013, Yilmaz, Portugal et al., 2014, Buffie, Bucci et al., 2015, Lewis, Buffie et al., 2015). Over the years multiple studies have helped us understand gut-microbe symbiotic relationships, however, little remains known about the health consequences of dysbiosis.

1.1 Urbanization and Water Disinfection

Water disinfection has been key to control urban infectious diseases. Urbanization -defined as the transitional changes in lifestyle from traditional to industrial societies (Hawley, 1967)- entitle practices that effectively control infections, including methods traditionally used to improve water quality, disinfection by chlorination (Richardson and Postigo, 2012). Chlorination has proven effective in removing pathogens, making drinking water safe for consumption (Yuan, Guo, & Yang, 2015), and reducing the incidence of waterborne diseases in industrialized cities. Snow first used it in 1850 to attempt to disinfect London's water supply during the infamous cholera epidemic and since the 1900's, and it has been widely used. Chlorination is a method practiced the US Centers for Disease Control and Prevention ("Public Health And National Security: The Critical Role Of Increased Federal Support," 2002), and according to the American Water Works Association, chlorination of water has virtually wiped out instances of waterborne diseases like typhoid fever, cholera, dysentery, gastroenteritis, cryptosporidiosis and cysticercosis which are caused by pathogenic bacteria, viruses, protozoa and helminthes ("Public Health and National Security: The Critical Role of Increased Federal Support," 2002).

As other halogenated compounds, chlorine is a potent antimicrobial. Other halogenated compounds are used in products of personal hygiene, such as fluoride in toothpaste (Maltz and Emilson, 1982).

1.2 Chlorination and Toxin Production

Chlorine is a potent antimicrobial (Scott, Gratz et al., 2013), but in the presence of organic matter, it produces trihalomethanes (THMs) (Richardson, 2003), which have been associated with cancer (Richardson at al., 2007), reproductive and developmental disorders such as miscarriages and birth defects (Waller et al.,1998). EPA has permissible levels of THMs in the drinking water. However, despite these regulations, chlorine and subsequently THMs exposure is common in the life of urban children and adults in many cities (Richardson and Postigo, 2012).

The recommended concentration of chlorine in the US in the drinking water is up to 5mg/L (Obolensky et al.,2007) and in swimming pools, the recommended concentration is at least 1-3mg/L (Weaver et al.,2009). The maximum limit in drinking water is 33% higher than chlorine used in pool water and the justification for this is that under federal law water companies must ensure a minimum of 0.02 mg/L to consumers living at the end of sometimes very lengthy water pipes. In some cities across the world, a method called hyperchlorination is used where the chlorine concentrations are maintained between the range of 10-20 mg/L (Lin et al.,2015) to disinfect water from disease causing bacterial species like *Legionalle spp, Cryptosporidum spp* (Hlvasa et al.,2015).Thus, exposure to chlorinated water in urban settings is common, extensive and starts from early ages.

1.3 Urbanization and Modern Diseases

The microbiomes from multiple human body sites change with urbanization (Yatsunenko et al., 2012, Clemente et al., 2015, Smits et al., 2017), and use of antibiotics, cleaning products and disinfectants might underlie antimicrobial effects on the microbiome (Richardson, 2003). Immune and metabolic diseases in the industrialized world, which include allergies, asthma, autoimmune diseases, and obesity (Henao-Mejia et al., 2012), have been related with inappropriate education of the immune system in early life (Dominguez-Bello et al., 2019), and the mechanism could be via the microbiome. There are instances where the microbiome is an important factor in the protection against development against allergies. (Seiskari et al.,2007)

Little work has been done on environmental stressors of the microbiota, other than antibiotics (Foster and McVey Neufeld, 2013), and on the physiological consequences of altering the microbiota for adult health. A normal and healthy microbiome depends on environmental stability, individual's genetics and health status (Bäckhed et al., 2012), and certainly, it would be expected that there are microbiome alterations after antimicrobial exposures.

Work on mice has shown causality in the obesogenic effect of antibiotics (Cox et al., 2014), and farmers have used low doses of antibiotics for decades, because animals grow bigger and fatter (Cho et al., 2012)

1.4 Hypotheses and Aims

We hypothesize that exposure to chlorinated water decreases the diversity of the gut microbiota and leads to phenotypic developmental changes in the host, specifically increased developmental growth and weight deposition.

To test this hypothesis, we proposed the following aims:

Aim 1- To characterize the developmental growth in mice drinking chlorinated or non-chlorinated water.

Aim 2- To determine the fecal bacterial microbiota structure during development, in mice drinking or chlorinated or non-chlorinated water.

Aim 3- To determine the effect of drinking water chlorination on high fat diet response.

We used approaches that have successfully demonstrated causation in the relation to microbiota stressors leading to physiological changes, such as increased obesity after antibiotics exposure (Cox et al.,2014), or C-section birthing (Martinez et al.,2017). Identifying the developmental effects of environmental exposures during early development – when many host-physiological responses are programmed – is crucial for prevention and restoration strategies. This work will contribute to understanding the effect of chemical-induced perturbations of the gut microbiota on disease risks.

CHAPTER 2: METHODS

2.1 Animals

We used C57BL/6J mice purchased from Jackson Laboratory. Pregnant dams (n=20 in total) were used in two replicate experiments, each with 10 mothers (5 in each treatment/control arms). Mice were received in the animal facilities at day 15-17 of gestation (E15-E17 of gestation). During 2 days of acclimation they were fed standard diet and water. At E 17-19, mice were randomly separated into 2 groups of 5 dams, one group receiving chlorinated water (25mg/L and pH 7-7.5) and the other group received distilled water (pH 7-7.5). The concentration of chlorine in the water was determined using a Lovibond colorimeter and the method was followed as specified in the appendix. The chlorine concentration was higher than the EPA recommended concentrations (4 mg/L), but some cities in the world can reach high chlorine concentrations, similar to those used here. All animals were fed standard chow diet (PicoLab Rodent Diet 20 (5053), which contains 0.51% chlorine).

In order to avoid cage-effect, dams and litters were randomly placed in the facility on cage racks, with mice receiving chlorinated water having green labels, and mice that received the non-distilled, chlorine free water having pink labels as shown in **Figure 2**. At gestation day E19-E21 the dams gave birth to litters of varying sizes (**Table 1**). Litters continued to receive chlorinated or distilled water during the rest of the experiment that lasted 18 weeks.

High fat diet (D12451) from Research Diets, Inc was provided to all the animals in both groups, at week 12 of life for 7 weeks, until week 18, Animals were weaned at 3 weeks of age, and separated based on sex into individual cages of 5 per cage and remained in the same treatment or control group.

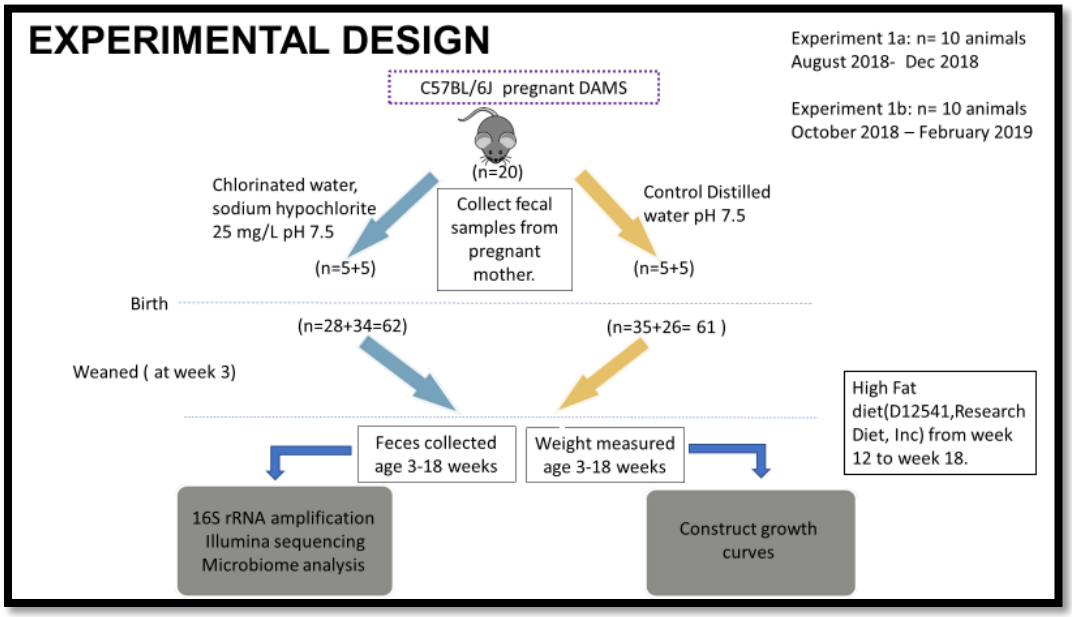


Fig 1. Experimental Design. Pregnant dams are split into two groups and provided either chlorinated water or non-chlorinated water.

2.2 Fecal Sample Collection: One to two fecal pellets were collected from each individual directly from the anus into sterile pre labelled 2mL cryotubes and frozen at -80 °C, at different time points of development, namely weekly from week 3 to week 18.

2.3 Body weight measurements:

Body weight was measured inside a biosafety cabinet using an OHAUS^{TM} CS series compact portable scale with a capacity of 200g.

2.4 Terminal Sampling of blood and organs

After sacrifice at week 18, blood was collected through aseptic cardiac puncture using a polypropylene syringe. The blood sample was left undisturbed for 2 hours and then centrifuged in order to collect the serum which was stored at -80°C. Liver and ileum were collected and sectioned into 2 pieces. One was placed into ~700 µL RNAlater and one snap-frozen at -80°C. Blood and samples were not analyzed in the current study.

Fig 2. Cages housing the dams and litters with the green(chlorinated) and pink(control) labels on them on ventilated racks.

Table 1. Number of pups at birth and the number of pups that were sampled for cohort 1 and 2.

Experiment replicate (cohort) #	Family	Group	No of pups at birth	No of current pups
1	A	Chlorine	4	4
1	C	Chlorine	5	5
1	E	Chlorine	7	4
1	G	Chlorine	7	7
1	I	Chlorine	1	0
1	K	Chlorine	8	8
2	N	Chlorine	6	6
2	P	Chlorine	9	8
2	R	Chlorine	6	6
2	T	Chlorine	8	7
2	V	Chlorine	7	7
1	B	Control	8	8
1	D	Control	7	7
1	F	Control	6	0
1	H	Control	6	6
1	J	Control	6	6
1	L	Control	9	8
2	M	Control	6	6
2	O	Control	4	4
2	Q	Control	6	6
2	S	Control	7	5
2	U	Control	6	3

2.5 Fecal microbiome analyses

DNA extraction and sequencing.

Total DNA was extracted, sequenced and analyzed (**Figure 3**). Extraction of DNA from feces was performed using the MoBio Powersoil Kit according to the manufacturer's instructions, modified as described in the Earth Microbiome Project protocol http://www.earthmicrobiome.org/emp-standard-protocols/dna-

extraction-protocol/) and the V4 region of the 16S rRNA gene was amplified by PCR using barcoded primers (Caporaso, Lauber et al., 2012). The chosen primer set is used in the Earth Microbiome Project and amplifies a phylogenetically informative region of the 16S rRNA gene with few selective biases against known bacterial or archaeal taxa and has been successfully applied to examine bacterial (Bergmann et al., 2011) and archaeal (Bates et al., 2010) communities in complex ecosystems. We anticipate, in most cases, to obtain genus to family resolution. Reagents for DNA extraction and for PCR amplification were sequenced as controls (Salter, Cox et al., 2014). Amplicons were pooled in equimolar ratios and were sequenced on the Illumina MiSeq platform (Genome Technology Center of NYU Medical Center, NY) using a paired-end technique.

QIIME2 is a widely used next generation platform for microbiome analysis. The QIIME2 workflow started with the raw sequencing data from the NYU server and the metadata file created for this experiment. This metadata file included multiple ways to describe the samples in order to provide tabular output for the different analyses. Next the data from the sequences and the metadata were combined to de-multiplex the barcoded reads from the samples and quality filtering was performed. (Caporaso et al., 2010). The samples were split into individual per sample fastq files and the non-biological nucleotides were removed. These reads were then trimmed to create high quality sequence variants. Sequence variants (SVs) are referred to the changes in DNA, RNA and protein sequences. The sequence reads were used to pick SVs with an open-reference picking method

based on 97% identity to entries in the Greengenes database. The next step performed was denoising which was done using DADA2(Divisive Amplicon Denoising Algorithm). This was then used to build a feature table summarizing the representative sequences on the table as well as a phylogenetic tree for further phylogeny analysis. Samples were rarefied at 10,000 sequences per sample. The studies generated large data sets that needed to be manipulated and analyzed rigorously to determine if statistically significant associations between compared groups exist.

With the study design, comparison both within and across groups was possible. Group means were compared using PERMANOVA (Martinez et al., 2017), with statistical significance defined as $p \leq 0.05$ after correction for false discovery rate from multiple comparisons.

Community composition and structure was assessed using species richness within samples (α diversity; **Figure 4.)** to detect species richness by counting the number of distinguishable taxa (bacterial variants) in each sample. Alpha diversity (bacterial variants diversity) between treatment (chlorinated) and control (non-chlorinated distilled water) mice separately for females and males were compared with t tests. All statistical tests were two-sided and a p value of less than 0.05 was considered to be statistically significant.

Comparison of diversity between samples (β diversity, **Figure 5**) (Lozupone et al., 2007, Kuczynski et al., 2010) was also assessed, by comparing the microbial

composition, relative abundance and phylogenetic diversity accounted by in UNIFRAC distances, between samples and groups were analyzed using Principal co-ordinates analysis statistical method.

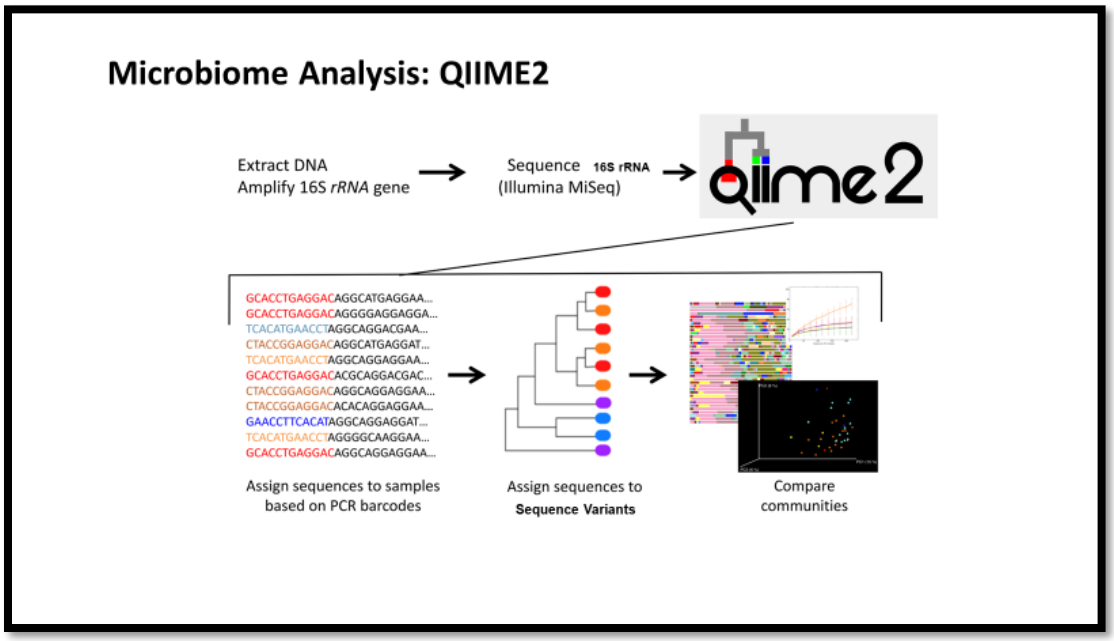


Fig 3. Microbiome analysis. Using QIIME2, a next generation microbiome bioinformatics platform.

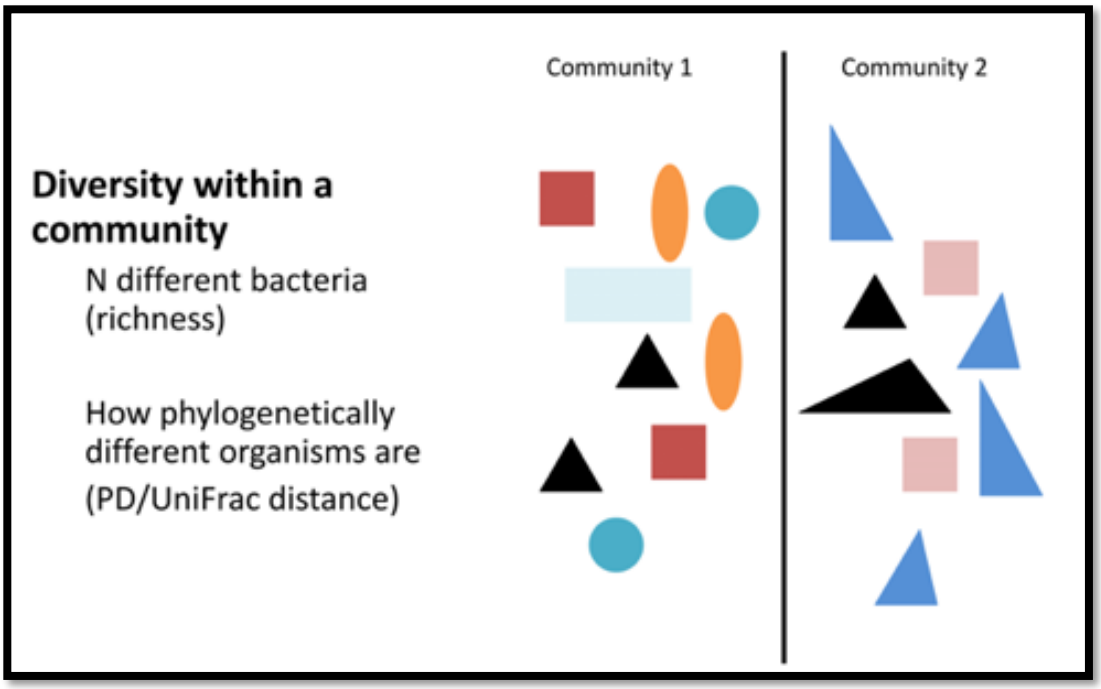


Fig 4. Alpha Diversity concept. 2 communities showing richness (number of species within a sample) and diversity (difference in distribution)

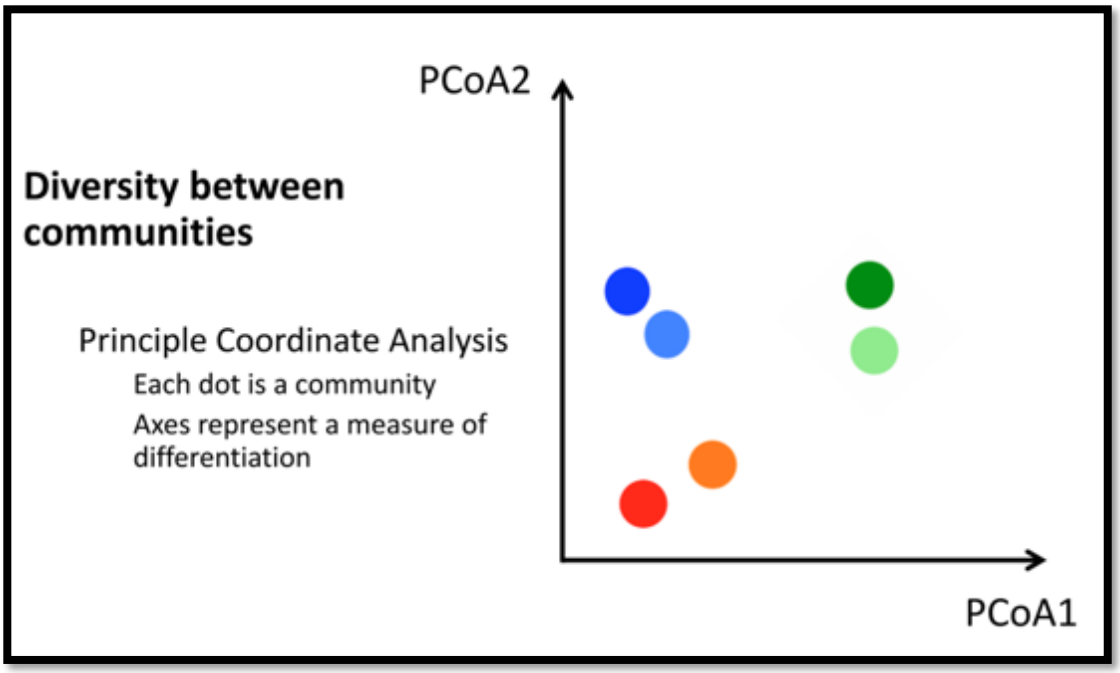


Fig 5. Beta Diversity concept. Diversity between communities where each dot represents a community, and the coordinates indicate the components that explain the variance between samples.

CHAPTER 3: RESULTS

This experiment was carried out as two separate cohorts where the second cohort was a replicate of the first cohort. **Table 3** shows the total number of animals per group, from the litters of the first and second cohort. There was a total of 123 baby mice, 62 from 10 litters, which consumed chlorinated water, and 61 from 10 litters, which did not.

3.1 Developmental body mass

C57Bl/6J treatment mice were exposed to chlorinated water through their mother at E17 gestation date and were on the same treatment until age 18 weeks. Control mice were exposed to non-chlorinated water similarly. Mice that received chlorinated water showed a significant increase in body weight after week 6 of life (they gained ~ 7-10% more weight than the controls), as shown in **Figure 6**. The effect was observed in both males and females.

3.2 Microbiome

Fecal samples from week 3,5 and 7 were examined. The total number of fecal samples and frequency of sequences per sample is shown as a histogram in **Figure 7**. Sequencing depth was set at greater than 10,000 sequences in order to remove the bacterial variants found in blank samples. The total number of sequences and number of bacterial variants in the sequences was measured which can be seen in **Table 4** separately for females, males and for the

contaminated blanks. The contaminated blanks were suspected to be due to fecal contamination from week 7 samples belonging to both cohort 1 and 2 as can be seen in from the DNA extraction plate map in **Table 5** and from **Figure 9** showing the beta diversity.

3.2.1 Alpha Diversity

Drinking chlorinated water decreased the alpha diversity in the fecal samples. Females show a significant decrease in microbial diversity in chlorinated at weeks 3,5 and 7 and males show significant decrease in microbial diversity at week 7.

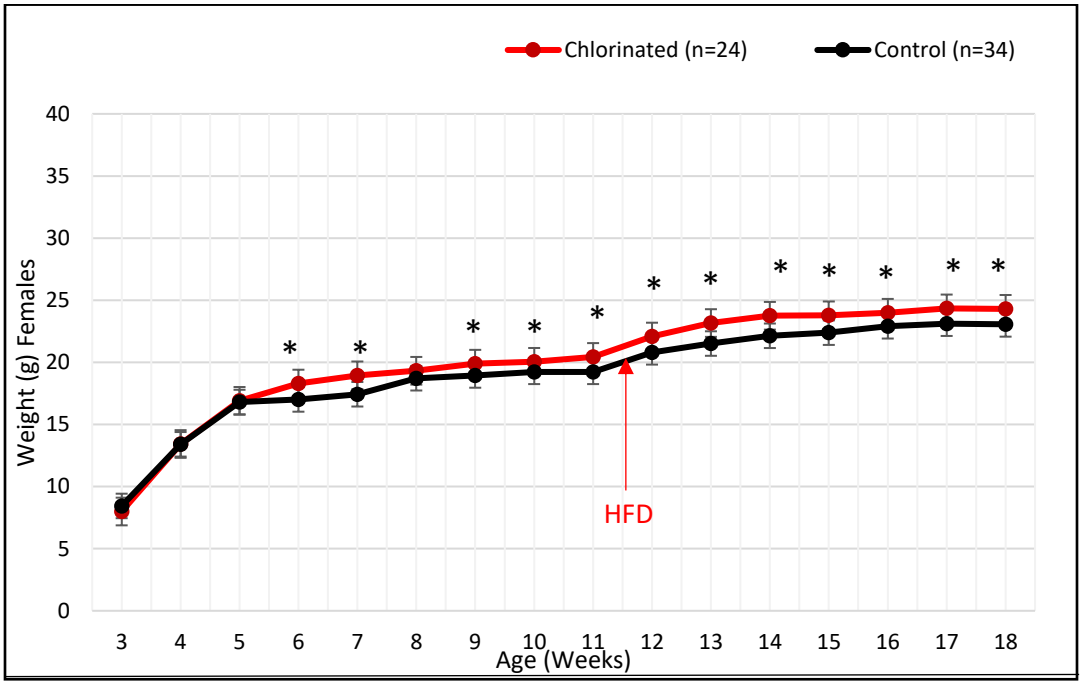
3.2.2 Beta Diversity

Beta diversity in feces from consuming chlorinated water or non-chlorinated water from birth show that the communities segregate significantly based on cohort, while no significant segregation was observed based on chlorination as seen in **Figure 8.** Further analysis was done in feces from mice separately in weeks 3, 5 and 7 for cohort 1 and 2 which showed that there was no significant difference observed at weaning. However, with development there was significant difference observed between chlorinated and control at week 5 for both cohorts and only for cohort 2 at week 7 as seen in Figure 10.

Table 2. N of families and litters by gender in the 2 sets of experiments (cohorts)

N	Chlorinated (1st +2nd cohort)	Non chlorinated (1st +2nd cohort)
Families /litters	**5+5 =10**	**5+5 =10**
Litter size	**28+34 = 62**	**35+26 = 61**
N females	**14+10= 24**	**18 + 16= 34**
N males	**14+24= 38**	**17 +10 = 27**

(A)



(B)

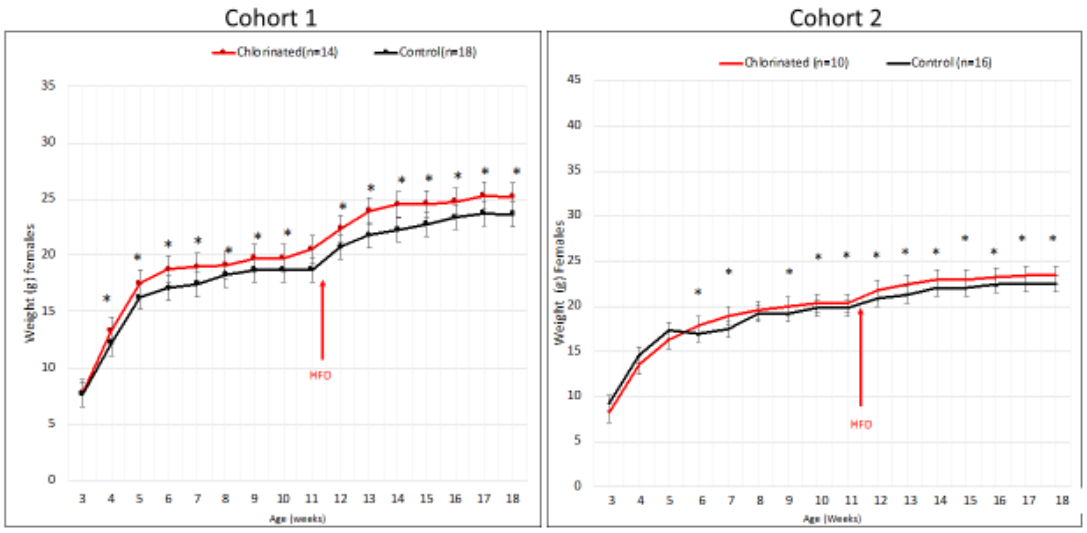
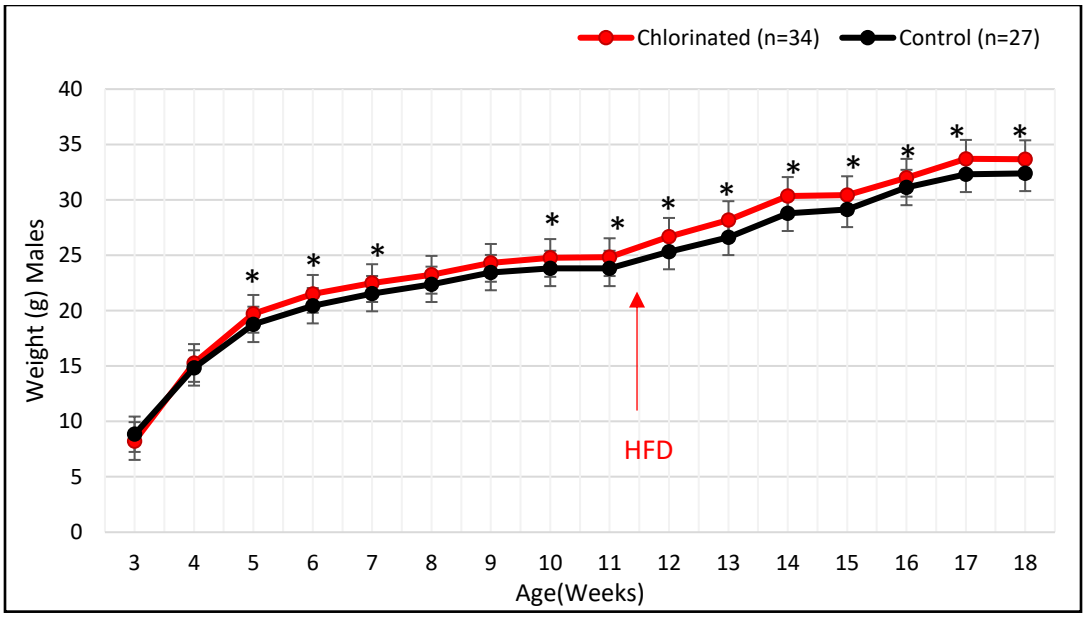


Fig 6. Effect of chlorination on developmental body mass in females (A) Both cohorts (B) Cohort 1(Left) and Cohort 2(Right). Significance was determined by t-test (*p <0.05). Mice that received chlorinated water showed increased body weight after week 6 of life when cohort results were combined.

(A)



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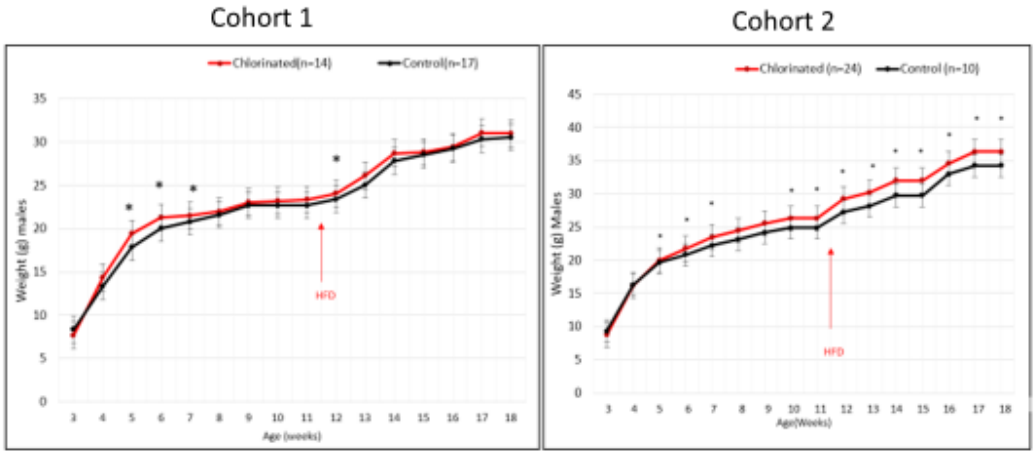


Fig 7. **Effect of chlorination on developmental body mass in males (A) Both cohorts (B) Cohort 1 (Left) and Cohort 2 (Right).** Significance was determined by t-test (*p <0.05). Mice that received chlorinated water showed increased body weight after week 6 of life when cohort results were combined.

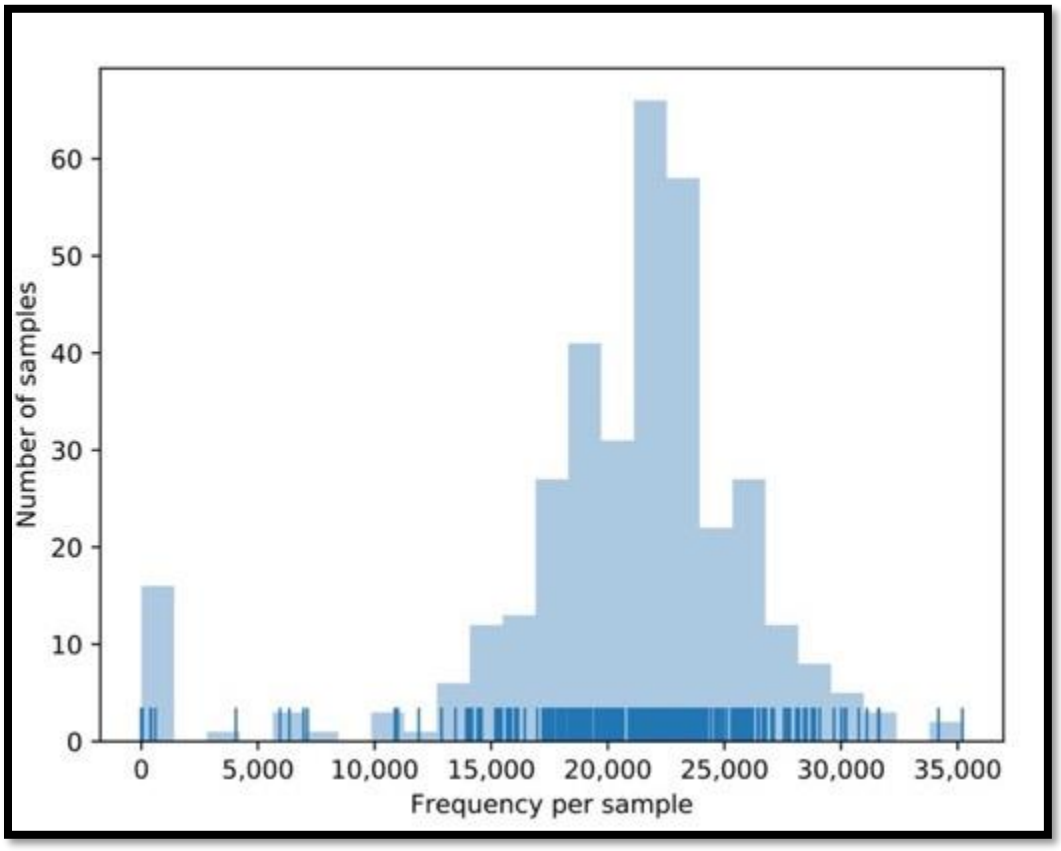
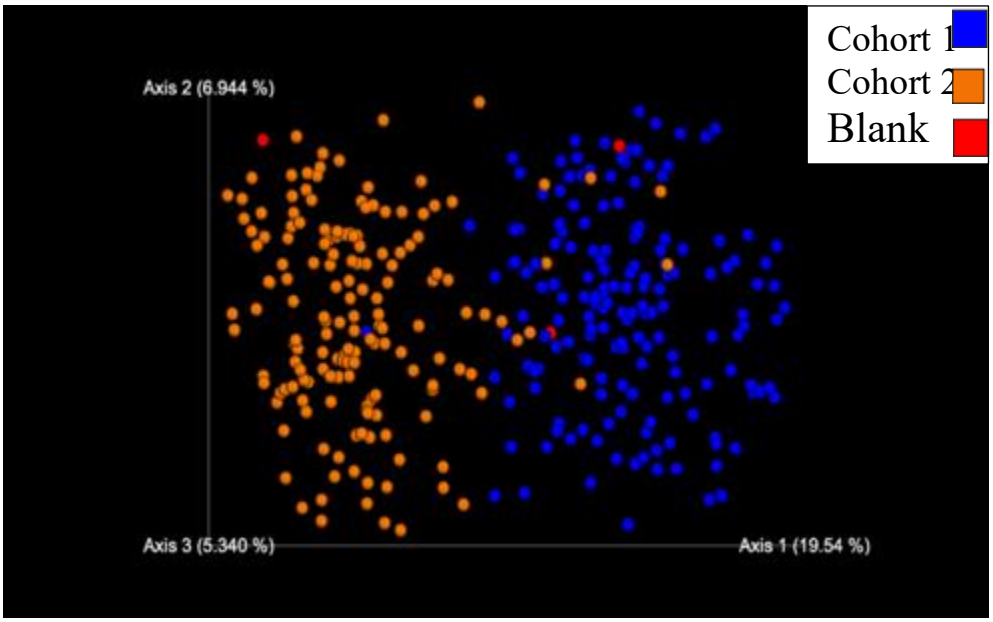


Fig 8. Histogram showing Number of Samples and Frequency of sequences per sample. Sequencing depth was set at >10000. Histogram shows that highest percentage is in the range of 15000 – 30000.

Table 3. Number of samples, sequences and Bacterial variants. Rarefaction at 10000 sequences per sample

Sample Type	Male		Female		Blanks	
	Chlorinated	Control	Chlorinated	Control	PCR Blank	DNA Blank
Number of samples	103	76	66	89	1	2
Total # Sequences to pick Bacterial variants	21,69,921	17,18,870	13,07,012	20,25,981	34181	65,428
Mean # of Sequences ± St Dev.	20898 ± 3894	22617 ± 3736	19,803 ± 2968	22,764 ± 3674	34181	32714 ± 3520
# of Bacterial variants represented	11,110	7542	6252	8749	136	257
Mean # Bacterial variants ± St Dev.	108 ± 28	99 ± 27	95 ± 21	98 ± 25	136	129 ± 59

(A)



(B)

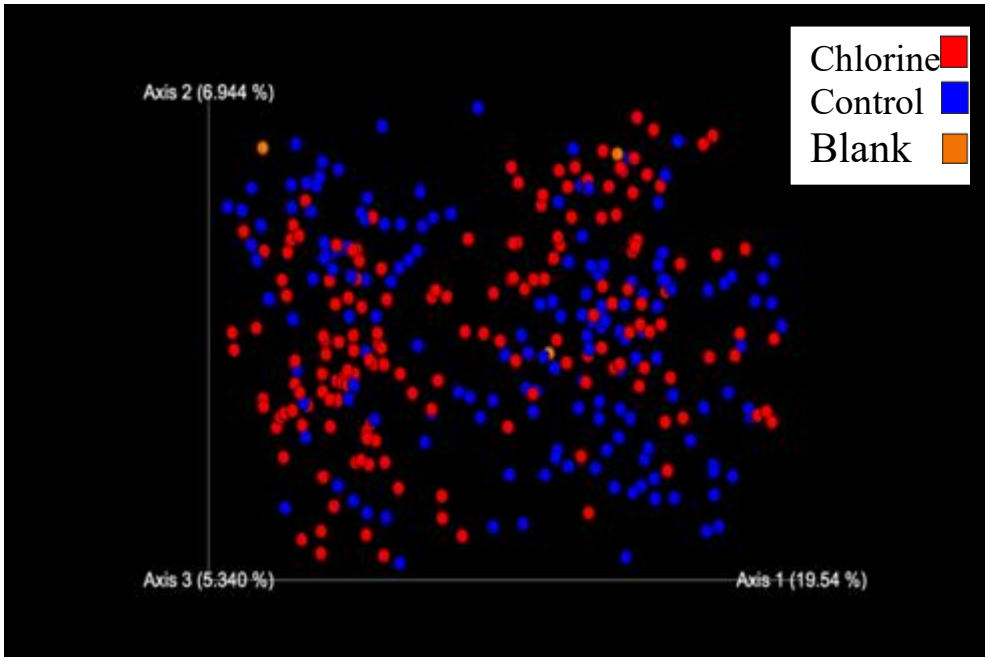


Fig 9. PCoA plot showing beta diversity in feces from mice consuming chlorinated water or non-chlorinated water, from birth (ages 3, 5 and 7 weeks) (A) Communities segregate significantly based on cohort ($p < 0.001$) Each blank corresponds in its own cohort. (B) No significant segregation was observed based on chlorination ($p > 0.05$)

Table 4. Two of the four DNA extraction plate maps, showing contaminated blanks highlighted in yellow.

(A) Plate #3

	1	2	3	4	5	6	7	8	9	10	11	12
A	CHL81	CHL94	CHL116	CHL124	CHL140	CHL216	CHL229	CHL248	CHL264	CHL339	CHL370	CHL371
B	CHL82	CHL95	CHL117	CHL125	CHL141	CHL217	CHL230	CHL249	CHL265	CHL340	CHL369	CHL372
C	CHL83	CHL96	CHL118	CHL126	CHL142	CHL218	CHL242	CHL250	CHL266	CHL341	CHL368	CHL373
D	CHL84	CHL97	CHL119	CHL135	CHL211	CHL224	CHL242	CHL251	CHL267	CHL342	CHL356	CHL374
E	CHL85	CHL98	CHL120	CHL136	CHL212	CHL225	CHL244	CHL252	CHL268	CHL343	CHL355	CHL375
F	CHL86	CHL99	CHL121	CHL137	CHL213	CHL226	CHL245	CHL253	CHL269	CHL344	CHL354	CHL376
G	CHL87	CHL100	CHL122	CHL138	CHL214	CHL227	CHL246	CHL262	CHL337	CHL350	CHL353	DNA blank
H	CHL88	CHL115	CHL123	CHL139	CHL215	CHL228	CHL247	CHL263	CHL338	CHL351	CHL352	PCR blank

(B) Plate #4

	1	2	3	4	5	6	7	8	9	10	11
A	CHL377	CHL393	CHL0033	CHL0049	CHL0072	CHL0147	CHL0169	CHL0183	CHL0263	CHL0285	CHL0299
B	CHL378	CHL394	CHL0034	CHL0050	CHL0073	CHL0148	CHL0170	CHL0191	CHL0264	CHL0286	CHL0300
C	CHL379	CHL0021	CHL0035	CHL0058	CHL0074	CHL0155	CHL0171	CHL0192	CHL0265	CHL0287	CHL0301
D	CHL388	CHL0022	CHL0036	CHL0059	CHL0075	CHL0156	CHL0172	CHL0194	CHL0266	CHL0288	CHL0309
E	CHL389	CHL0023	CHL0045	CHL0060	CHL0143	CHL0157	CHL0179	CHL395	CHL0273	CHL0289	CHL0310
F	CHL390	CHL0024	CHL0046	CHL0061	CHL0144	CHL0158	CHL0180	CHL243	CHL0274	CHL0290	CHL0311
G	CHL391	CHL0025	CHL0047	CHL0062	CHL0145	CHL0167	CHL0181	CHL0261	CHL0275	CHL0297	DNA blank
H	CHL392	CHL0026	CHL0048	CHL0071	CHL0146	CHL0168	CHL0182	CHL0262	CHL0276	CHL0298	PCR blank

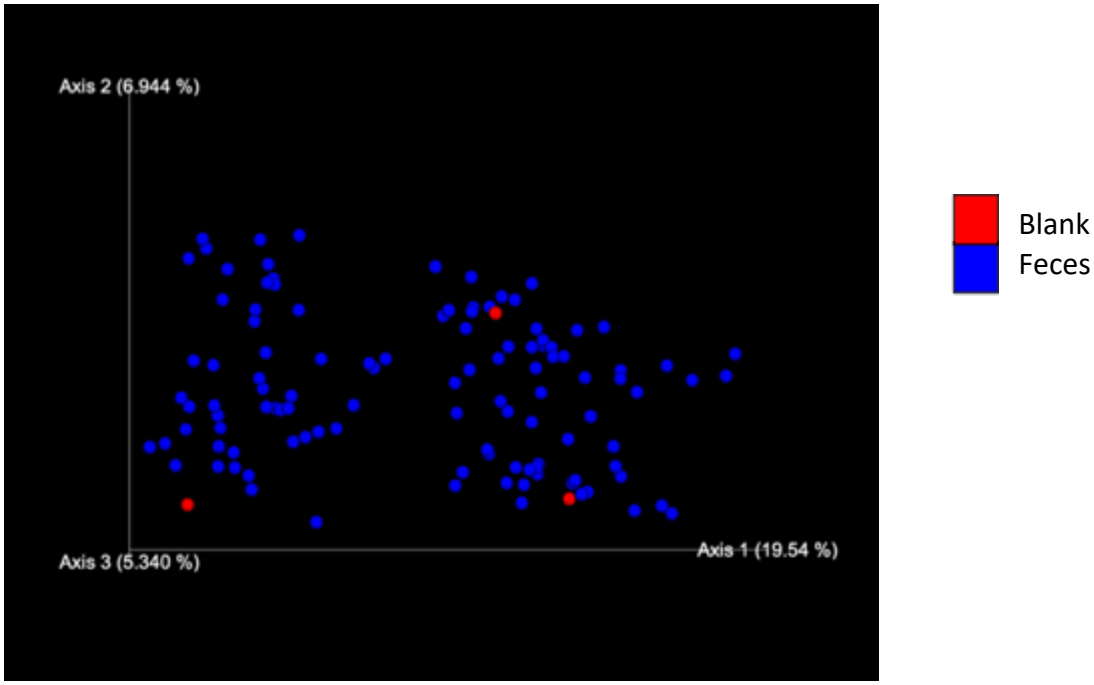


Fig 10. PCoA plot showing beta diversity in feces from mice and blanks at week 7. 3 wells were contaminated with feces from mice. No significant difference can be observed between samples from week 7 of both cohort 1 and cohort 2 and the blanks. ($p > 0.05$)

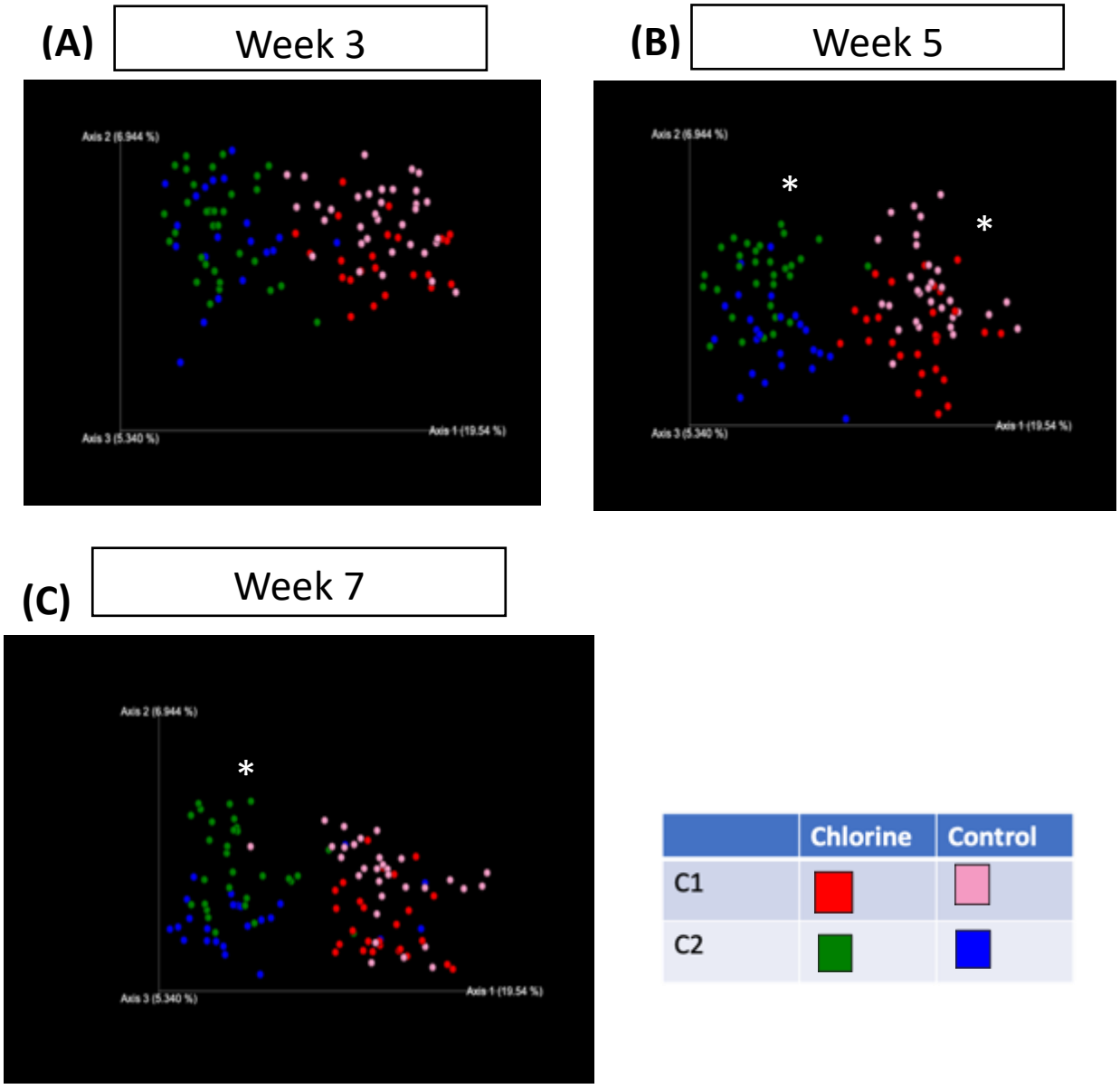


Fig 11. PCoA plot showing beta diversity in feces from mice consuming chlorinated or non-chlorinated water from weeks 3 to 7. (*$p < 0.05$) (A) No significant difference was observed between chlorinated and control groups in both cohorts at weaning stage (week 3). (B) At week 5 there was significant difference observed between chlorinated and control in both cohort 1 and cohort 2. (C) At week 7 there was significant difference observed between chlorinated and control in both cohort 1 and 2.

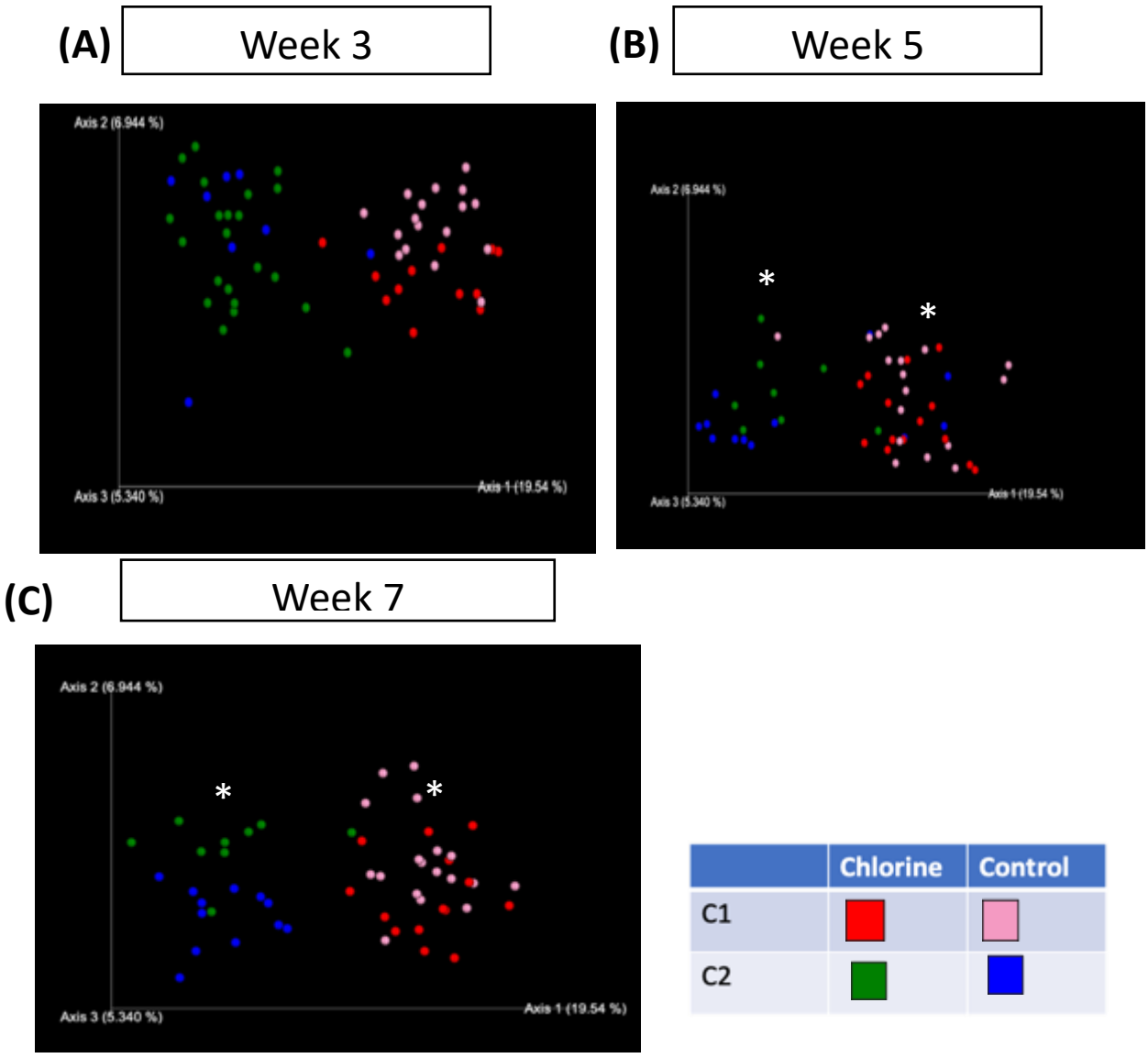


Fig 12. PCoA plot showing beta diversity in feces from mice consuming chlorinated water or non-chlorinated water from weeks 3 to 7 in females. (*p < 0.05) (A) No significant difference was observed between chlorinated and control groups in both cohorts at weaning stage (week 3). (B) At week 5 there was significant difference observed between chlorinated and control in both cohort 1 and cohort 2. (C) At week 7 there was significant difference observed between chlorinated and control in both cohort 1 and 2.

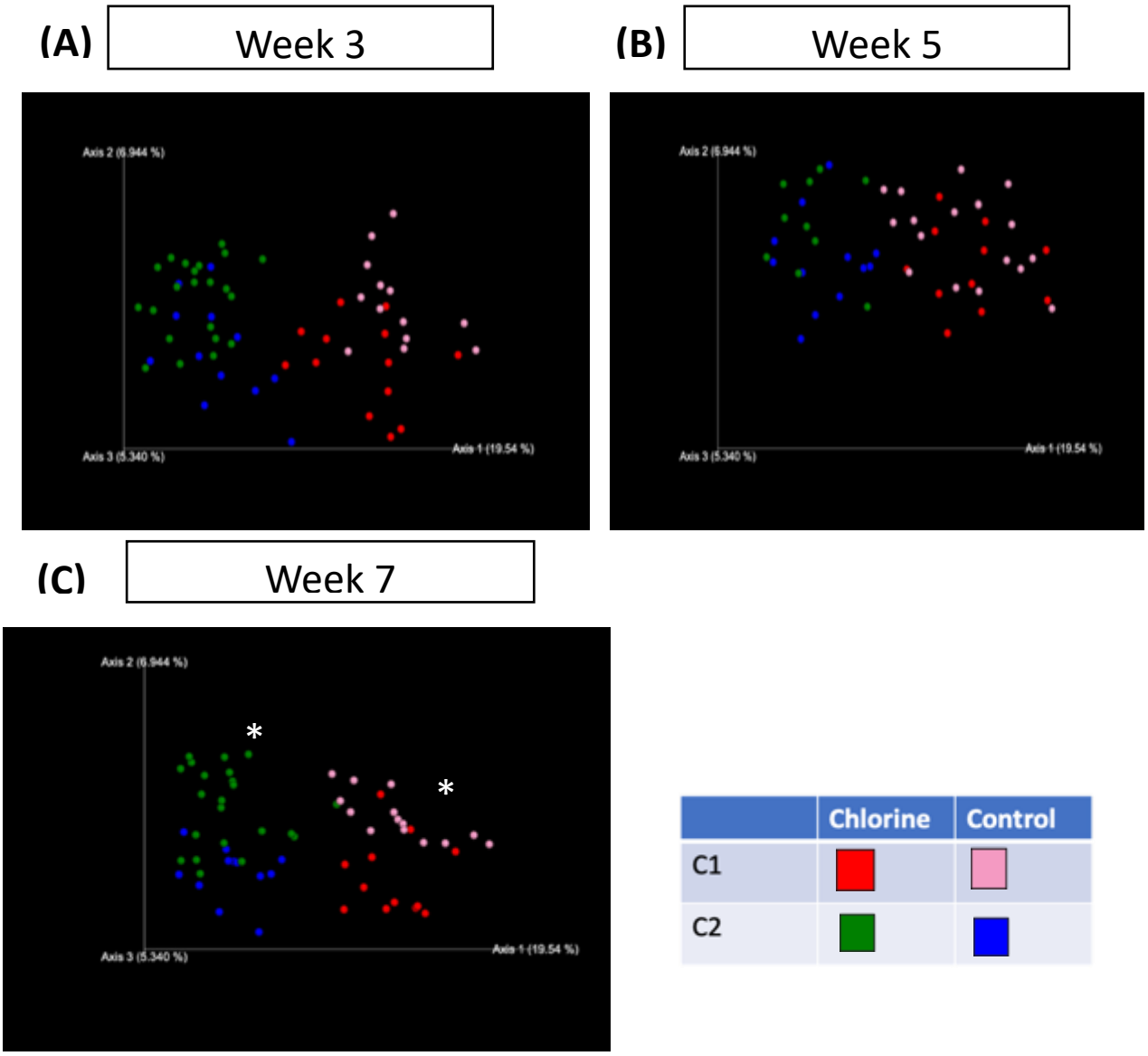


Fig 13. PCoA plot showing beta diversity in feces from mice consuming chlorinated water or non-chlorinated water from weeks 3 to 7 in males. (*p < 0.05) (A) No significant difference was observed between chlorinated and control groups in both cohorts at weaning stage (week 3). (B) At week 5 there was no significant difference observed between chlorinated and control in both cohort 1 and cohort 2. (C) At week 7 there was significant difference observed between chlorinated and control in both cohort 1 and 2.

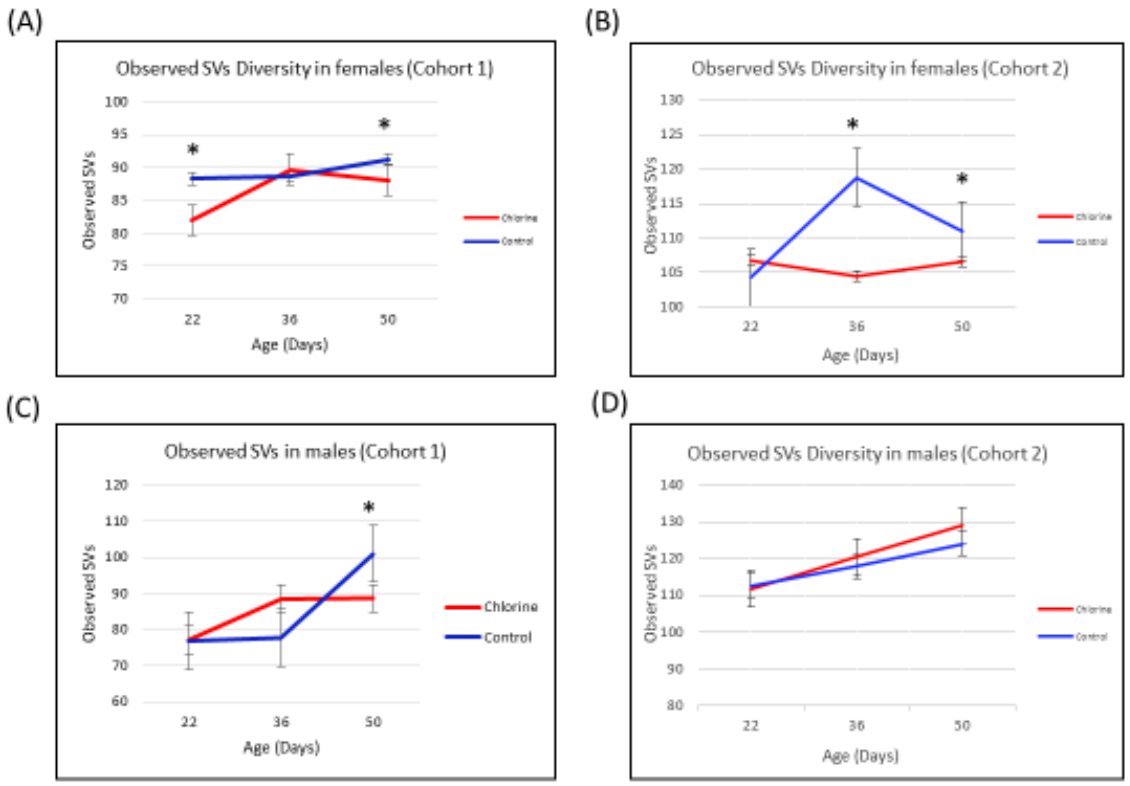
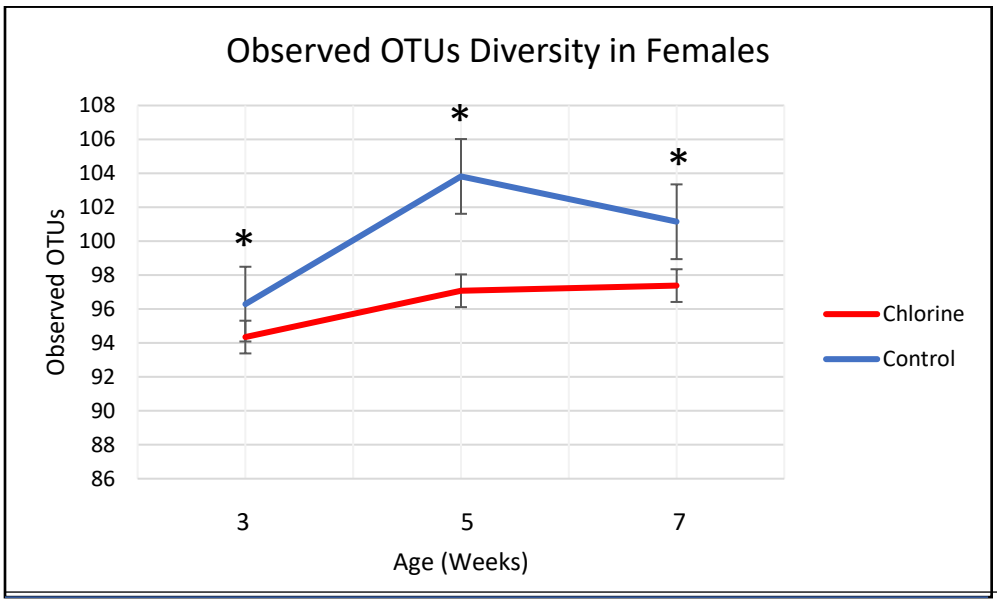


Fig 14. Alpha diversity of bacterial DNA from week 3,5 and 7 consuming chlorinated water or non-chlorinated water. (*p < 0.05) (A) Females from cohort 1 show a significant decrease in microbial diversity in chlorinated at weeks 3 and 7. (B) Females from cohort 2 show a significant decrease in microbial diversity in chlorinated at weeks 5 and 7. (C) Males show significant decrease in microbial diversity at week 7 for cohort 1. (D) No significant decrease in microbial diversity in males for cohort 2 can be observed.

(A)



(B)

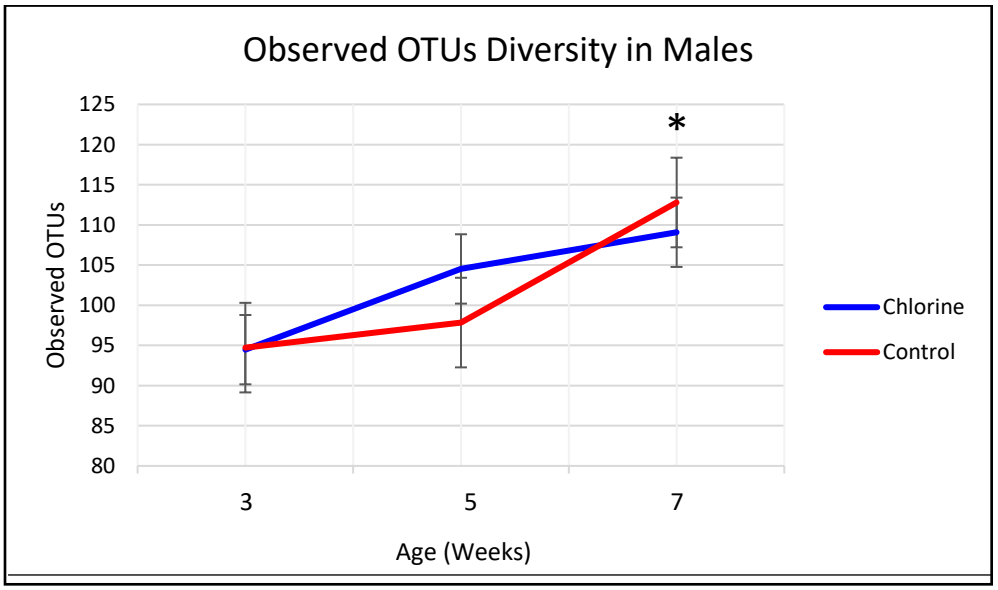


Fig 15. Alpha diversity of bacterial DNA from week 3,5 and 7 treated with chlorinated or control water. (*p < 0.05) (A) Females show a significant decrease in microbial diversity in chlorinated at weeks 3,5 and 7. (B) Males show significant decrease in microbial diversity at week 7.

CHAPTER 4: DISCUSSION

Chlorination had a significant increase in developmental weight. Based on the studies that showed the causal nature of antibiotics for an obesogenic effect on mice (Cox et al.,2014) the mechanisms underlying the effects of chlorination could be similar to those of antibiotics and involve the microbiome. The mechanisms of microbially induced obesity (MIO) are not clear. Possible involvement are changes in hormones such as decrease in peptide YY and leptin (Morrison et al., 2009). Peptide YY is one of the major anorexigenic gastrointestinal peptides (Grudell, Camilleri et al.,2007) which has been found to be associated with gut physiology and obesity.

A deeper phenotyping is needed to characterize the effect of chlorination. It would be interesting to examine body composition to determine alterations in lean mass, fat mass, bone mineral content, bone area and bone mineral density using DEXA (Dual energy x-ray absorptiometry). The alterations could be mediated by alterations in the liver or the intestine gene expression. Altered hepatic gene expression, increased adiposity, reduced epithelial tight junction proteins (Cani et al.,2008), loss of inflammasome function (Henao-Mejia et al.,2012) or thinning of the intestinal mucus layer (Everard et al., 2013) can increase translocation of bacterial products which can lead to systemic inflammation and obesity (Cox et al., 2014).

The preliminary results in this study also show that chlorination alters the development of the microbiota. Females show a significant chlorinated water-associated decrease in microbial diversity at weeks 5 and 7, while males show it at week 7. Beta diversity and PERMANOVA results show that there was a significant difference in beta diversity in feces from cohort 1 and cohort 2. Since there are sex differences, it is important to determine hormonal profiles. Also, further analysis needs to be done to identify the keystone taxa and understand which ones are associated with the phenotypes.

4.3 SIGNIFICANCE

Obesity is a complex disease which can increase the risk of diabetes, heart disease and cancer (Vucenik et al.,2012). Apart from the diet and genetic polymorphisms, there are environmental factors that may trigger changes in microbiota diversity. The antimicrobial effects of disinfectants and specifically chlorine, might underlie the observed phenotypic effects during development.

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APPENDIX

1 Preparation of chlorinated water

1.1 Requirement:

We need 3L of water weekly to give to approximately 30 mice.

30 mice x 50 ml per week= 1.5 L per week. We prepare twice this volume to account for losses.

1.2 Background

Chlorination of water is the process of adding chlorine in the form of gas or as sodium hypochlorite to water. Chlorination is usually carried out to prevent the spreading of waterborne diseases. However, chlorine water is prepared in this case in order to provide this to mice and check the effects of its consumption on development of gut microbiota and phenotypically in terms of weight gain.

The following is the chemical reaction to make chlorine water which is typically green in color:

$$NaOCl + 2HCl \rightarrow Cl_2 + H_2O + NaCl$$

1.3 Materials:

1. Sodium hypochlorite (NaOCl)
2. Hydrochloric acid (HCl) 1N
3. Sodium Hydroxide (NaOH) 1N
4. Deionized water
5. Brown/amber glass bottles (1 L)
6. pH strips

7. Free chlorine measuring strips

1.4 Method:

1. Add 2.5 mL of sodium hypochlorite to 990 mL of deionized water in the 1 L amber bottle.
2. Adjust solution to a pH 7.5 with HCl or NaOH, using the pH strips.
3. Add deionized water until the final volume of solution is 1 L.

1.5 Storage conditions:

1. pH should be maintained at 7.5.
2. Stored in brown bottles as it is light dependent.
3. Stored in cold conditions (5°C) to slow down any unnecessary reactions.
4. Prepare new solutions every week.

2. Testing the pH and concentration of chlorine:

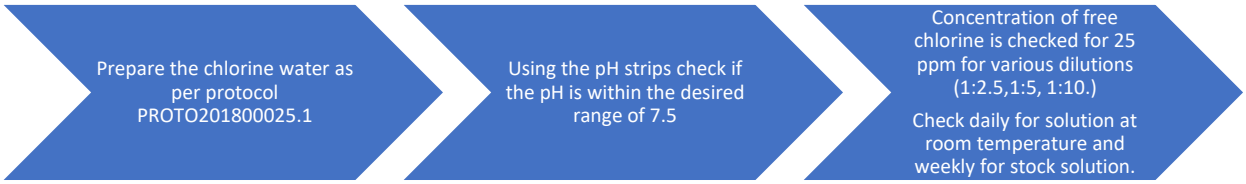
To test the stability of the prepared chlorine water for concentration and pH.

- Over a period of 1 week at 4 degrees Celsius for a stock solution
- Daily at room temperature in the amber mouse drinking bottles.

2.1 Background:

Chlorine reacts to form hypochlorites and hypochlorous acid in water. It is also known that in very dilute solutions at pH above 4.0 very little free chlorine exists in solution which shows that chlorine undergoes degradation depending on concentration, temperature and pH. Previously stability studies have showed that chlorinated water retains approximately 96% of its concentration after storage for 7 days.

2.2 Procedure



2.3 Methods:

2.3a Measurement of chlorine:

1. The chlorine water prepared (25ppm) needs to be diluted into multiple dilutions of 1:2.5, 1:5 and 1:10 and this needs to be done in triplicates.
2. Dip the chlorine strip in the water and observe the change in color.
3. Using the chart match the color change to the concentration of chlorine. Take photo.
4. Check this weekly for the stock solution and every 24 hours for the water in the amber drinking bottles at room temperature.

2.3b Measurement of pH:

1. Dip the pH strip into the stock solution and observe the change in color.
2. Using the chart match the color change to the pH. Take photo.
3. Ensure that it is within the desired pH range.

3. Lovibond Water Testing Procedure:

3.1 Materials:

- Lovibond Photometer-System MD100
- Phenol Red Photometer tablet OR Phenol Red solution
- Stirring rod
- Chlorine Free-DPD/F10 powder pack
- Distilled water for diluting
- Testing vials (24 mm and 10 mm)

Turn on device and select the desired test by pressing the [MODE] key.

Zero Calibration:

1. Fill a clean vial with 10 mL of your water sample if using the 24 mm vials, or 5 mL of your water sample if using the 10 mm vials and screw the cap on. Wipe down outside of the vial with a Kimwipe until dry and clean.
2. Place the vial in the sample chamber, making sure to align the arrows.
3. Press the [ZERO/TEST] key. The display should read 0.0.0.

Measuring pH: Use the 24 mm vial

1. Perform Zero Calibration
2. Remove vial from sample chamber. If using the phenol red tablet, add one table to the vial, crush and mix with stirring rod, swirl until dissolved, then cap again. If using the phenol red solution, add six drops to the vial, mix, and cap.
3. Clean the outside of the vial to ensure no marks.
4. Place sample vial back into sample chamber, making sure to align the arrows.

5. Press the [ZERO/TEST] key.
6. Record the display value.

Preparation of dilutions:

Prepare three different dilutions 1:5, 1:25, 1:50.

For 1:5 dilution:

- Take 2 mL of your 25 mg/L chlorine solution and add 8 mL of distilled water to dilute.
- Use 5 mL of the resulting dilution to measure.

For 1:25 dilution:

- Use 2 mL of the remaining 1:5 dilution and add 8 mL of distilled water to dilute
- Use 10 mL of the resulting dilution to measure.

For 1:50 dilution:

- Use 1 mL of the remaining 1:5 dilution and add 9 mL of distilled water to dilute.
- Use 10 mL of the resulting dilution to measure.

Measuring Free Chlorine Concentration:

At 0.02 – 2.0 mg/L: use the 24 mm vial (For dilutions 1:50 and 1:25)

1. Perform Zero Calibration
2. Remove vial from sample chamber.
3. Add one pack of the Chlorine Free-DPD/F10 Powder Pack to the vial and mix approximately 20 seconds until dissolved, and cap.

4. Clean the outside of the vial to ensure no marks.
5. Place sample vial back into sample chamber, making sure to align the arrows.
6. Press the [ZERO/TEST] key
7. Record the display value (which is in mg/L).

At 0.1 – 8.0 mg/L: use the 10 mm vial (For 1:5 dilution)

1. Perform Zero Calibration
2. Remove vial from sample chamber. Add two packs of the Chlorine Free-DPD/F10 Powder Packs to the vial and mix approximately 20 seconds until dissolved, and cap.
3. Clean the outside of the vial to ensure no marks.
4. Place sample vial back into sample chamber, making sure to align the arrows.
5. Press the [ZERO/TEST] key
6. Record the display value (which is in mg/L).