RELATIONSHIP BETWEEN THE CONCENTRATION OF MICROCYSTIN IN SERUM AND PRIMARY LIVER CANCER IN PATIENTS ATTENDING JOOTRH, KISUMU, KENYA

BY

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SCHOOL OF PHYSICAL AND BIOLOGICAL SCIENCES MASENO UNIVERSITY

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DECLARATION.

I affirm that this thesis is my own authentic work that has not been presented in any other institution of higher learning for the award of MSC degree or any other academic qualification in any university or institution of higher learning.

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I am much grateful and may Almighty God bless you all abundantly.

DEDICATION

I commit this thesis to entire communities' around Lake Victoria for whom my desire is for them to have a clean source of drinking water.

ABSTRACT

Microcystins (MCs) are toxins released by unicellular micro-organism known as cyanobacteria. These unicellular micro-organisms flourish in aquatic habitats with warm temperatures and nutrients rich water bodies. Microcystins are known to cause Liver cancer via inhibition of serine-threonine protein phosphatases (PP1 & 2A). Previous studies in China and Serbia have shown an association between MCs and occurrence of primary liver cancer (PLC). However, in Kenya, there is no data that links MCs and occurrence of PLC in humans despite there being an existence of harmful cyanobacteria within Nyanza gulf. This cross- sectional study was therefore carried out to investigate the association of serum microcystins to occurrence of PLC. The specific objectives were to determine the levels of MCs and nutrients within the Nyanza gulf; to determine the levels of serum serine-threonine protein phosphatases (PP 1 & 2A) in PLC patients and controls and to determine the correlation between *mcyE* and *mlrA* genes. A total of 40 study participants were enrolled; 20 PLC patients confirmed from Jaramogi Oginga Odinga Teaching and Referral Hospital were recruited and 20 controls obtained from the source population giving rise to PLC cases. Their demographic information was obtained through questionnaire. Blood samples were collected and analyzed for total serum-serine-threonine PP1 and 2A levels using enzyme-linked immunosorbent assay. Water samples were collected from three sites along Nyanza gulf; Dunga, Homabay, Mbita; and filtration was done using 0.45 µm membrane filter using vacuum pump to concentrate phytoplanktons. MC-toxin levels in water were measured using MC-ELISA Kit, whereas Nutrients analysis was done using photometric methods. Total RNA was extracted and quantification of *mlrA* and *mcyE* gene expression was done using RT-PCR software. MCs levels were detected using ELISA reader, Nutrients levels were quantified using spectrophotometer; whereas total serine-threonine protein phosphatase quantification were done using ELISA reader and analyzed using independent t-test, while correlation of gene expression analysis was done using Pearson correlation. RESULTS: MCs toxins were present in all samples sites, and their levels exceeded recommended WHO guidelines of 1.0µg/l for quality water safety. Highest MCs levels were detected in Homa/Bay B site (21.4 µg/L) which corresponded to high nitrogen and phosphorus levels within the same station (498.64 µg/L: 136.145 µg/L) respectively. This was followed by Mbita A (13.1 µg/L; TP = 46.14 µg/L; TN = 327.32 µg/L), Homa/Bay A (7.9 µg/L; TP = 100 µg/L; TN = 395.21 µg/L), as well as Dunga B (7.1 µg/L; TP = 84.71 µg/L; TN = 308.37 µg/L) and Mbita B (5.9µg/L; TP = 59 µg/L; TN = 330.47 µg/L). However, Dunga site A had the low level of toxin detection of about (2.2 µg/L, TP = 71.14 µg/L; TN = 247.58 µg/L) despite having high levels of nitrogen. Higher levels of serine-threonine protein phophatase activities were observed in liver cancer patients (103.9 ± 17.83 µg/ml) than in controls (25.91 ± 3.342 µg/ml). The variation was statistically significant ($P < 0.05$; $t_{38} = 4.298$;). Water sample analysis provided evidence of expression of MC-synthesizing gene (mcyE) and MC-biodegrading gene (*mlrA*). Pearson correlation revealed an insignificant moderate positive relationship between mcyE and mlrA genes, ($r = 0.625$, P (two-tailed) = 0.053). In conclusion, Nyanza gulf presented a high levels of MCs beyond recommended WHO values (1 µg/L) hence posing a great health risk to riparian communities. This could be attributed to variations in the levels of serine-threonine liver enzyme in PLC patients in the study, due to consumption of MC-contaminated water. Therefore, it is necessary to strengthen the protection and monitoring of drinking water source for effective control of water pollution and safeguarding of human health.

TABLE OF CONTENTS

DECLARATION..ii

ACKNOWLEDGEMENT..iii

DEDICATION..iv

ABSTRACT..v

TABLE OF CONTENTS..vi

LIST OF ABBREVIATIONS AND ACRONYMS..ix

LIST OF TABLES..x

LIST OF FIGURES..xi

CHAPTER ONE : INTRODUCTION..1

Background Information..1

1.2 Statement of the Problem..4

1.3 General Objective..5

1.4 Null Hypothesis..5

1.5 Justification and Significance of the Study..6

CHAPTER TWO: LITERATURE REVIEW..7

2.1 Occurrence of microcystin and the factors influencing its concentration within Nyanza Gulf..7

2.2 Implications of microcystin to ecosystem health..9

2.3. Relationship between MC-producing bacteria (mcyE) and MC-biodegrading bacteria (mlrA)..9

2.4. Primary Liver Cancer and its associated risk factors..13

2.4.1 Introduction..13

2.4.2 Risk factors associated with PLC development..13

2.5. Molecular mechanism of microcystin toxicity in animals and human health..15

CHAPTER THREE : MATERIALS AND METHODS..18

3.1 Study methodology..18

3.2 Study Area..18

3.3 Study Design..19

3.4 Target Population..19

3.5 Inclusion and Exclusion Criteria:..20

3.5.1 Inclusion Criteria:..20

3.5.2 Exclusion Criteria:..20

3.6 Sampling Design ... 20
3.6.1 Sample size determination. ... 20
3.7 Variables ... 21
3.7.1 Independent variable ... 21
3.7.2 Dependent variable ... 21
3.8 Sample collection ... 22
3.8.1 Water sampling ... 22
3.8.2 Blood sample collections ... 22
3.9 Sample Analysis ... 22
3.9.1 Microcystin toxin analysis ... 23
3.9.2 Phytoplankton Sampling, Identification and Nutrients analysis ... 23
3.9.2.1 Nutrients analysis ... 24
3.9.2.2 Isolation and analysis of Total RNA from phytoplanktons ... 24
3.9.2.2.1 Total RNA isolation ... 24
3.9.2.2.1 Reverse transcriptions (RT) of isolated Total-RNA into c-DNA ... 25
3.9.2.2.3. Quantification of microcystis mcyE and mlrA gene expression ... 27
3.9.3.1 Preparation of working solutions ... 28
3.9.3.1.1 Assay procedure ... 28
3.10 Data Management/ Data Analysis ... 29
3.11 Ethical considerations and Informed consent ... 29
CHAPTER FOUR : RESULTS ... 31
4.1 Levels of extracellular Microcystin toxin and Nutrients along Nyanza gulf. ... 31
4.2 Quantification of mcyE and mlrA gene expressions ... 31
4.2.1 Expression of mcyE and mlr A genes ... 32
CHAPTER FIVE: DISCUSSION ... 37
5.1 Determination of microcystin concentration and nutrients levels within Nyanza gulf ... 37
5.2 Correlation between mcyE and mlrA gene expression in cyanobacteria. ... 39
5.3 Total serine-threonine protein phosphatase (PP1andPP2A) enzyme levels in serum samples of primary Liver cancer patient and controls ... 41
5.4 Conclusion ... 43
5.5 Recommendations for Further Studies ... 43
REFERENCES ... 45
APPENDICES ... 53
Appendix I; Map of Lake Victoria, Nyanza gulf showing Sampling Stations. ... 53
Appendix II: Letter of Consent. ... 54
Appendix III: Study Questionnaire ... 56
Appendix IV: Research Approvals ... 59
Appendix V: Cyanobacteria species viewed in water samples collected. ... 60

Appendix VI: Amount of Total RNA extracted from phytoplankton measured using Nanodrop. 61
Appendix VII: Table 4.5; MeanCt values for 16sRNA 62
Appendix VIII: Total serine-threonine protein phosphates concentration PLC patients attending JOOTRH 63

LIST OF ABBREVIATIONS AND ACRONYMS.

CT………………….. Threshold cycle

DM…………………. Diabetes Mellitus

DNA/RNA…………. Deoxyribonucleic Acid / Ribonucleic Acid

HBV………………... Hepatitis B Virus

HCC………………... Hepatocellular Carcinoma

HCV………………... Hepatitis C Virus

MAPK……………… Mitogen Activated Protein Kinase

MCs…………...... Microcystins

McyE………………… Microcystin Sythetase gene

MlrA…………………. Microcystin biodegrading gene

NAFLD……………... Non-alcoholic fatty liver disease

PLC…………………. Primary Liver Cancer

PP1 & 2A……………Protein Phosphatase1 and 2A liver enzymes

ROS…………………. Reactive Oxidative Species

RT-PCR………………Real Time Polymerase Chain Reaction

TDI…………………. Total Daily Intake

WHO…………………World Health Organization

LIST OF TABLES

Table 3.1-: Types of primers used in the current study……………………………………….25

Table 4.1 -: Socio-demographic features…………….……………………………….….…33

Table 4.2 -: Sampling sites toxins and total dissolved phosphorus and nitrogen levels………28

Table 4.3-: Total amount of RNA extracted from phytoplanktons, measured using a nanodrop..55

Table 4.4-: Microcystin mean CT values for mcy E gene and their levels of expression values..30

Table 4.5-: Microcystin mean CT values for mlrA gene and their levels of expression values...30

Table 4.6-: Mean CT values for 16SsRNA reference gene……………………………………...55

Table 4.7-: A summary of total levels of serine-threonine protein phosphatase among the study Participant……………………………………………………………………………………..33

Table 4.8 -: Total serine-threonine protein phosphatase in study participants (PLC &N-PLC)…56

LIST OF FIGURES

Figure2.1 -: General structure of microcystin molecule……………………………………11

Figure 2.2 -: Identified routes for microcystin intake, bioprocessing, lethality and elimination in animal cells…………………………………………………………………16

Figure 4.1 -: Picture showing toxic cyanobacteria and water hyacinth clogging the KMFRI Kisumu Pier; b) ……………………………………………………………………..…54

Figure 4.2 -: Correlation between mcyE and mlrA genes in cyanobacteria…………………..…31

Figure 4.3 -: Mean change levels in total serine protein phosphatase (PP1&PP2A) among the study participants (PLC & N-PLC)…………………………………………………………33

CHAPTER ONE

INTRODUCTION

Background Information

Universal growth of the harmful cyanobacteria algae bloom in large fresh water bodies has raised a concern internationally (Mchau *et al.,* 2019). Cyanobacteria, is a class of phytoplankton micro-organism found in large reservoirs such as lakes, ponds and rivers globally (Massey *et al.,*2020). A sub-family of cyanobacteria such as: Anabaena, Microcystis,Oscillatoria,Planktothrix and Nostoc (Otoigo *et al.,* 2018) are able to produce harmful secondary metabolites known as cyanotoxins; (Microcystin (hepatotoxin; Neurotoxin ,cytotoxin); posing a substantial threat to livestock, humans together with aquatic animal species who depend on such water bodies for domestic use (Panksep *et al.,*2020). Fresh water bodies in Africa such as Lake Victoria (LV), has undergone a lot of quality water challenges due to increase in nutrients/minerals levels (Otoigo *et al.,* 2018). The increase in levels of nutrients and minerals is caused by anthropogenic and industrial activities within the lake regions (Onyango *et al.,* 2020). These has led to an increase in harmful blue-green algae bloom in the entire course of the year , with the potential of synthesizing dangerous cyanotoxins (Simiyu *et al.,* 2018). This has been linked with death of fish and short-term closure of Lake Victoria in 2004, i.e., from January to March 2004 (Sitoki *et al.,* 2012). However, negative effects of cyanotoxins exposure to riparian population of L. Victoria remained poorly described (Simiyu *et al.,* 2018), hence this study.

Among the several hazardous substances produced by cyanobacteria, microcystins (MC) is known to be the prevalent cyanotoxins in large natural reservoirs (Mchau *et al.,* 2019). They are synthesized by cyanobacterium *Microcystis aeruginosa, Anabaena and Plankthorix* (Rantala *et al.,* 2014), and are stored in the outer cells of cyanobacteria. Once these cells are ruptured due to

cell death or environmental stress, the toxins (microcystin) are released into water leading to contamination of water bodies (Panksep *et al.,* 2020). Microcystin are synthesized by microcystin synthetase enzyme; encoded by *mcyE* gene in the cells of cyanobacteria (Lezcano *et al.,* 2016). Among the many encoded genes in cyanobacteria, *mcyE* is commonly used as a specific marker for microcystin-producing cyanobacteria. These is due to its regions being highly conserved among the species and also the responsibility of *mcyE* genes of activating and condensing the amino acids residues of MCs responsible for toxicity i.e the D-glutamate and Adda moiety (Ngwa *et al.,* 2014). The toxins synthesized are biodegraded by microcystinase metallopeptidase enzyme encoded by *mlrA* gene in other natural bacteria found in water bodies (Dexter *et al.,* 2018) however there is still scarce information about biodegrading bacteria.

MC are transferred to humans through drinking of contaminated water, inhalation, recreational activities and ingestion of aquatic organism such as (fish, crusteaceans and molluscs) (Massey *et al.,* 2018). Exposure of microcystin to human as well as animals has been proposed to contribute to, liver failure and liver death in many countries (Drobac *et al.,* 2016) thus making it a public health concern. MCs, once ingested are transported to Liver organ as a paramount organ of interest. They bind onto serine-threonine protein phosphatases 1and 2A and hinder the phosphatases from playing their physiological functions of reversing the activated kinases, hence causing impairment of the liver cells (Greer *et al.,* 2018). The hindrance brought about by MCs, will result into an increase in addition of phosphate groups in cellular signaling pathways hence several proteins involved in cell homeostasis are produced and not regulated (Liang *et al.,* 2011) resulting into increase in oxidative stress in the cells which eventually causes cell necrosis (Massey *et al.,* 2018). These suggest that MCs molecules can act as potent liver tumor promoter (Zegura, 2016). However, adequate documentation in human is still needed (Zhou *et al.,* 2018),

to confirm its process of initiating liver cancer occurrence.

Several epidemiological cases have revealed signs of liver damage and injury among the inhabitants who have come into contact with MCs levels beyond the WHO recommended tolerable daily intake (0.04 µg/ml) (TDI). For instance, Chen *et al.,*(2009) showed a direct relationship allying levels of microcystin in serum sample of local fishermen and the impact on the role of liver enzymes. Furthermore Zi, (2001) noticed a remarkable increased in liver enzymes levels among children who took MCs contaminated Lake water compared to controls. Human contact with MCs has also been associated with remarkable rise in number of PLC cases, for instance, studies done in Serbia, United states and China reported a positive correlation between chronic exposure to MCs in consumed water and number of cases of PLC (Ueno *et al.,* 1996). In addition, it was observed that PLC cases in China reduced after cyanotoxin-free drinking water was introduced in the affected regions (Li *et al.,* 2011).

Basing on this, there is a high possibility that microcystin acting as initiator and promoter maybe the major risk that acts synergistically with other risk factors such as HBV, HCV, aflatoxin, alcoholism; leading to increase incidence rate in PLC (Zheng *et al.,* 2017). However, this has not been ascertained among population living along Nyanza gulf which is contaminated with microcystin algae in Kenya where PLC cases is on the increase (Otedo *et al.,* 2018). To ascertain this, the study embarked on determining the levels of MCs and nutrients within Nyanza gulf and its effects of on levels of serine-threonine PP 1 and 2A liver enzymes among patients attending Jaramogi Oginga Odinga Teaching and Referral Hospital Kisumu Western Kenya. Moreover, the study also aimed at finding out the relationship between microcystin producing bacteria (*mcyE* genes) and MC-biodegrading bacteria (*mIrA*) genes using real time RT-PCR

1.2 Statement of the Problem

Primary Liver Cancer (PLC) is a global public health problem which is poorly addressed in developing countries. It is ranked the sixth most common neoplasm and the second leading cause of cancer-related mortality worldwide. About 8.2% of total cancer-related deaths in 2020 were due to PLC. Majority of the incidence rates occurred in regions such as Asia and Africa. According to Burden et al., 2019, in Kenya, Primary liver cancer ranked 6th in males and 5th in females as per the Nairobi cancer registry 2016. The high numbers of cases (5.2%) were reported in Nyanza region, with countries bordering the lake region reporting the high prevalence rate, with no confirmed causes. According to Otedo *et al*, 67.7% prevalence rate of PLC cases in western Kenya were reported in his study, out of this 39.4% in their study had no known risk factors, suggesting a need for unraveling these unknown factors. Environmental factors like microsystin levels in the population have not yet been explored as contributors to PLC occurrence in the region. However, other epidemiological cases in China have shown a direct relationship allying number of PLC cases and levels of MCs in consumed water.

Lake Victoria is known as the main source of water used in domestic chores among the surrounding population. However, the Lake has experienced a lot of negative drastic alterations in both water and biological level of quality measure, in the recent past. This could be associated with eutrophication brought about by climate change and anthropogenic activities in the nearby surroundings. This has enhanced cyanobacteria algal booming in the region leading to excess release of MCs into Lake Waters. Hence inadequate clean drinking water for the population that drives them to use of contaminated lake water thus subjecting them to health risk.

In this regard, there has been interest/concern in evaluating the impacts of human exposure to microcystin and associated health effects in PLC development globally. However, this had not been ascertained among population within the Nyanza region in Kenya where PLC has been on the increase. It was therefore, important to investigate the possible association between the numbers of cases of PLC with microcystin as a risk factor in the population residing along the Lake Victoria region in Kisumu County, Western Kenya.

1.3 General Objective

To determine the relationship between the concentration of microcystin in serum and primary liver cancer in patients attending Jaramogi Oginga Odinga Teaching and Referral Hospital.

Specific Objectives

1. To determine the levels of microcystins (MCs) and nutrients within Nyanza gulf.
2. To determine the correlation between microcystin-producing bacteria (*mcyE*) and microcystin-biodegrading bacteria (*mlrA*) within Nyanza gulf.
3. To compare the total serine-threonine protein phosphatases (PP1&2A) serum levels between PLC cases and controls.

1.4 Null Hypothesis

1. There is no MCs and Nutrients detected within Nyanza gulf.
2. There is no correlation between microcystin-producing bacteria (mcyE) and microcystin-biodegrading bacteria (mlrA) genes within Nyanza gulf.
3. There is no comparison in the total levels of serine-threonine protein phosphatses PP1and 2A liver enzymes in PLC cases and controls.

1.5 Justification and Significance of the Study

Several epidemiological studies have shown that exposure to microcystin toxin via ingestion of contaminated drinking water and aquatic animals could be associated with high incidence cases of PLC. This is due to their mechanism of toxicity which is based on the MCs binding on serine-threonine proteins and inhibiting the roles of protein phosphatases 1 (PP1) and 2A (PP2A) of eukaryotic cells, enzymes that catalyse the dephosphorylation (removal of a phosphate group) of the serine/threonine amino acids from proteins and are important for many signal transduction pathways in the cell (cytoskeletal rearrangement, cell movement, apoptosis, etc). There was therefore a need for extensive research on analyzing the levels of microcystin along the Nyanza gulf region of L. Victoria and its effects on the levels serine-threonine protein phosphatase among PLC patients attending JOOTRH, who depend on Lake Victoria as a source of water for consumption. The findings of this study can help medical professionals together with the public health officers in every dimension of managing, diagnosis and treatment of liver cancer disease patients associated with microcystin exposure thus reducing the burden in health sector and improving the lifespan of the patient affected.

CHAPTER TWO

LITERATURE REVIEW

2.1 Occurrence of microcystin and the factors influencing its concentration within Nyanza Gulf

Microcystin are globally the most commonly occurring fresh water cyanotoxins, which are synthesized by cyanobacterium species such as Microcystins aeruginosa (Otoigo *et al*.,2018). Cyanobacteria, are structurally single cell (prokaryote), photoautotrophic true bacteria that inhabits in natural and saline water bodies globally (Babu *et al.,* 2021). Excessive growth of cyanobacteria has resulted into a visible accumulation of cells, commonly referred to as a harmful algal bloom (HAB) or more specifically, a CyanoHAB (Mclellan & Manderville, 2017). Several studies on toxins have reported harmful algal blooms to cause weighty threat to the population and animals because of the release of fatal substances recognized as cyanotoxins (Microcystin, Saxitoxins, Cytotoxins, dermatitis and alimentary tract irritants) (Panksep *et al.,* 2020) . There is therefore, an urgent need to precisely keep on track the existence of the harmful cyanotoxin and enhance on the comprehension of the surrounding conditions that boost the formation of hazardous cyanobacteria, so as to come up with workable mitigation measures to safeguard our water points

Eutrophication, a process that occurs when the environment receive excess nutrient input; is caused by anthropogenic activities and global climate change has continued to be a critical environmental problem on freshwater ecosystem worldwide (Mwamburi *et al.,* 2020). Lake Victoria, being one of the fresh water Lake in Africa, is reported to face eutrophication challenges which has resulted to an increase in invasive growth of harmful cyanobacteria in the entire year, with the potential of releasing cyanotoxins into the Lake (Simiyu *et al*., 2018). This

has been linked to the death of aquatic animals and short-term closure of L. Victoria as a source of drinking water supply in 2004 (Sitoki *et al.,*2012). Epidemiological studies has shown that increment in nutrients-load alters the population of phytoplankton organisms into increase in number of hazardous cyanobacteria with an ability to produce cyanotoxins (Kimambo *et al.,* 2019). Nevertheless, manifestation of siltation is influenced by an increase in temperature change globally.

Therefore the degree and how often the hazardous blue - green alga is hypothesized to depend on warm climate and high temperatures (Babu *et al.,* 2021). Anthropogenic development and excessive inputs of sewage and fertilizer run off due to agricultural activities render the superficial water bodies ecosphere at risk to change in the surrounding conditions, consequently leading to elevated harmful cyanobacteria bloom (Mwamburi *et al.,* 2020) a scenario that is currently observed in Lake Victoria (Babu *et al*.,2021).

Generally, cyanobacterial harmful bloom occurrence is significantly associated with three environmental parameters e,g: nutrients, light and temperature (Kimambo *et al.,* 2019). High temperatures above 25°C are reported to boost the growth of various blue-green algae micro-organism species (Scherer *et al.,* 2017). On the other hand, nutrient variations in reservoirs are highly influenced by the elevated levels of phosphorus (P) and nitrogen (N) elements from external sources as explained by Sitoki *et al.,* (2012). Traditionally, high concentration of Phosphorus is reported as the core contributor of invasive growth of lethal blue-green algae-bloom (Mchau *et al.,* 2019). However, recent studies have also identified Nitrogen elements to have a role to play in accelerating the growth of cyanobacteria bloom (Babu *et al.,* 2021). There is therefore insufficient data on which of the elements boost the cyanoHab bloom, However, monitoring of the two nutrient elements has shown the need to manage water quality (Kimambo *et al.,* 2019; Mwamburi *et al.,* 2020). Due to continuous increase in cyanoHAB in Lake Victoria

there is therefore an urgent need to precisely monitor environmental factors that promote the bloom and monitor the levels of cyanotoxin released by cyanobacteria to enable health sector to evaluate the public health risk faced by the riparian communities.

2.2 Implications of microcystin to ecosystem health

The most common cyanotoxin group is microcystin(MCs) (Massey *et al*.,2020; Mclellan & Manderville,2017). Within the sub-family microcystin-LR (MC-LR) variant is the most fatal (Drobac *et al.,* 2016) whose adverse implication in health is highly reported and needs to be studied. Harmful blue-green algae micro-organism and their poisonous products causes a negative impact on the population, animals and plants (Massey *et al*., 2018). These cyanotoxins may develop impairment/sickness among individuals (He *et al.,* 2018), or loss of life when one come into contact through haemofiltration (Melaram, 2021) in the kidneys. Exposure of microcystin to individuals may lead to manifestations of the following signs: bellyache, vomiting, looseness of the stomach, itchiness of the skin among others to long-term diseases such as those related to liver cancer and colorectal malignancies which has been observed in animal models (Massey *et al.,* 2018). In this regard, the physiological effects and implications of microcystins association to human and animals needs to be address based on its potential to provoke cancer through acute doses and promote cancer prognosis through long-term exposure in drinking water as described from previous studies (Blaha *et al.,* 2009; Massey *et al.,* 2018; Melaram, 2021) hence this study.

2.3. Relationship between MC-producing bacteria (mcyE) and MC-biodegrading bacteria (mlrA)

Excessive growth of cyanobacteria has resulted into a visible accumulation of cells, commonly referred to as a harmful algal bloom (HAB) or more specifically, a CyanoHAB (Mchau *et al.,*2019). Several studies on toxins have reported cyanobacterial blooms to pose a substantial

health risk to humans and animals due to the production of highly active toxic compounds known as cyanotoxins (Panksep *et al.,* 2020). Cyanotoxins are diverse in chemical structure and toxicity. Depending on the human organ affected, cyanotoxins are classified as hepatotoxins (microcystins, nodularin, cylindrospermopsin) , neurotoxins (saxitoxins, anatoxin-a, anatoxin-a(s), homoanatoxin-a), cytotoxins (aplysiatoxin, debromoaplysiatoxin, lingbyatoxin, lipopolysaharide endotoxin), and skin and gastrointestinal irritants (Oliveira *et al.,* 2019). The most common cyanotoxin group are microcystins (MCs) (Stelzer *et al.,* 2013) while microcystin-LR (MC-LR) is their most toxic structural variant (Drobac *et al.,* 2016) whose adverse implication in health is highly reported and needs to be studied.

Microcystins are the toxins which are synthesized by blue-green algae belonging to sub-family Microcystis, Planktothrix, and Anabaena (Massey *et al.,* 2020). However, not all these variants have the potential to synthesize the toxin. Therefore, Knowledge on toxin-producing genera is helpful for mitigating bloom formation and evaluating factors that affect cyanobacterial growth and toxin production which needs to be provided as early aspossible in-order to evaluate public health risk (Ngwa, 2013). The earliest conventional methods used in studying cyanobacteria, such as traditional microscopy; chemical methods and assays; spectrophotometric measurements, cannot be used to differentiate toxin-producing genera but the latter methods can only quantify the toxin concentration levels (Kristel *et al.,* 2020). Both methods are insufficient to detect toxin-producing cyanobacteria because toxic and non-toxic strains within the same species are often morphologically identical and might co-exist within the same sample (Ngwa, 2013). The blue-green algae, that has the potential of synthesizing microcystin contains the microcystin synthetase (mcy) genes which are unavailable in similar species (Ngwa *et al.,* 2014). Hence, identification of these synthetase genes in samples confirms the presence of potentially toxic cyanobacteria. This has led to evolvement of quantitative polymerase chain reaction (qPCR) assays which are used for identification of harmful species (Massey *et al.,* 2020). The

Microcystin toxin biosynthetic gene cluster consists of genes encoding peptide synthetase, polyketide synthase, or modifying enzymes andnapped to a 55 kb cluster. Genes in the 55 kb cluster include mcyA through mcyJ (Yu *et al.,* 2019). Among the several functional mcy genes, mcyE gene represent a combined group of enzymes known as; polyketide synthase/peptide synthetase which are used to generate Adda molecule and initiation and incorporation of D-glutamate into microcystin molecule (Ngwa *et al.,* 2014). As a result, mcyE gene region is recognized as a specific target for identifying microcystin synthesizing bacteria (Ngwa *et al.,* 2014).

Microcystins production involves the translation of mcy genes into mcy mRNAs, transformation of mRNAs into polyketide synthases, non-ribosomal peptide synthetases, mixed peptide synthetases, together with customizing enzymes which combined different constituents of protein building blocks to form MC structure (Ngwa *et al.,* 2014). Microcystins are very steady and counter the common chemical breakdown (Massey *et al.,* 2018). Despite the chemical stability of the MCs, there are specific bacteria that co-exist with cyanobacteria within same aquatic environment. These bacteria can degrade these toxins, using an enzyme known as microcystin as e which is encoded by microcystin methylopeptidase (mlrA) gene. Only few studies have reported MC degradation capacities by other microorganisms, such as fungi (Isaac, 2020) or ochrophyta (Poterioochromonas sp), (Lezcano *et al.,* 2016), thus conferring to bacteria as the major biological MC degradation in nature. The *mlrA* gene encrypting methylopeptidase (*mlrA* enzyme) digest initial steps of cyanobacterial degradation of microcystin molecule and is linked with hydrolysis and ring opening of structure of the molecule at the Adda-Arg peptide-bond formation site; **Fig.2.1** (Krausfeldt *et al.,* 2019). However, there is still scarce information about the degrading bacteria species, and it is for this reason that this study was muted.

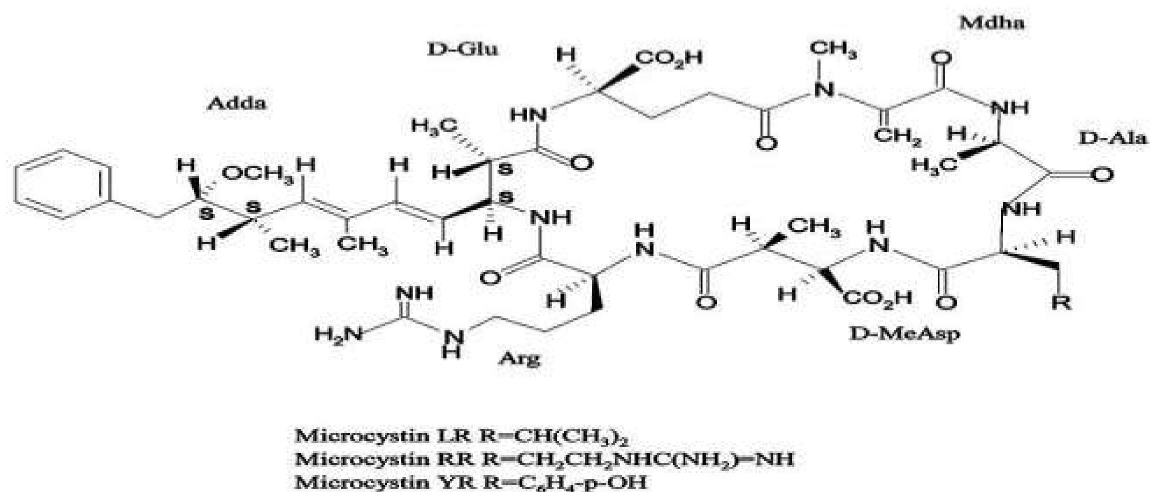

Fig.2.1: General structure of microcystin;adapted from (Campos & Vasconcelos, 2010); In MC-LR X represents L-Leucine; Z L-Arginine; R1 and R2 CH3.

Several research on Real time reverse transcriptase PCR analysis of harmful blue-green algae, involved mostly on identification of species with a potential of possessing mcy genes using both cultured and field samples (Massey *et al.,* 2020). However, for one to reliably interpret mcy gene expression data from environmental samples, it is important to understand how co-occurring cyanobacteria might impact gene expression patterns and MC concentrations. Furthermore, understanding the relationship between mcy transcript levels and actual cellular microcystin concentrations could provide insight on the utility of mcy gene transcript levels in assessing the hazards from microcystins in water produced by blue-green algae blooms. According to Ngwa *et al.,* (2013), the measurement of the levels of MCs synthesizing cynobacteria could permit a more reliable tool for assessing the health risk factor pre-disposed to the riparian communities by microcystin which was the core objective of this study. These levels of MCs can therefore be used to determine the safety of water bodies hence the need to clarify the correlation between mcyE gene expression and MC concentration in water and its effects on human serine-threonine phosphatase serum levels, when microcystin contaminated water is consumed.

Therefore, a detection and quantification method are paramount to ascertain the presence of these genes in tissues, water, and plant. This study was therefore set to quantify microcystin toxicity synthesis (*mcyE*) and degradation (*mlrA*) genes by Real Time PCR (qPCR) as well as determine the relationship between *mcyE* and *mlrA* genes in along Nyanza gulf.

2.4. Primary Liver Cancer and its associated risk factors

2.4.1 Introduction

Primary liver cancer (PLC) is the common prevalent hepatic malignancy in the third world countries (D. Mak & Kramvis, 2021). It is ranked 6th among the neoplasm and the second leading cause of cancer related death cases globally (Bannon *et al.,* 2019). In Africa, the regions with high incidence rates are; sub-Sahara and East Asia (Melaram 2021). In Kenya PLC ranks 6th in males and 5th in females according to Nairobi cancer registry 2011, (Otedo *et al.,* 2018) with a high number of cases (5.2%) located in Nyanza region (Otedo*et al.,* 2018). The incidence rates are estimated to beincreasing by 4.6 % globally per annum (Santana *et al.,* 2018). This is due to lack of sufficient knowledge on the prognosis mechanisms of PLC, therefore early prevention, detection and treatment of the ailment is still a challenge (Sung *et al.,* 2021). Identification of unknown risk factors and their mechanism was therefore important to help in reducing the increasing rate of morbidity and mortality burden in the region that could be associated with microcystin toxicity.

2.4.2 Risk factors associated with PLC development.

PLC risk factors include but not limited to Hepatitis B and C viral infections, exposure to aflatoxin, excessive consumption of alcohol, obesity, Diabetes mellitus as well as genetic predisposition (Akinyemiju *et al*., 2017). HBV & HCV are highly associated to PLC as a major causative agent (Ozakyol *et al.,* 2017). The mechanism of initiating PLC development is through

the genomic integration of HBV into the DNA of hepatocytes cells in human thus inducing genetic instability and interference with the DNA repair hence enhancing the development of PLC (Zhang *et al.,* 2015). While HCV acts directly through immune responses, especially CD8+ T cells responses, against Hepatitis C Viral-infected liver cells, causing hepatic cells injury, fibrosis, and cirrhosis leading to primary liver cancer development. Chronic alcohol consumption is another risk factor which has either direct or indirect mechanisms of action to develop PLC (Ratna and Mandrekar, 2017). The most probable mechanism is through development of liver cirrhosis genetic mutation or altered hepatic metabolism (Jiang *et al.,* 2018). In addition, Diabetes-mellitus (DM) is established as an independent risk factor for liver cancer, since it is linked to overweight, insulin resistance and hepatic steaosis (Arman *et al.,* 2019). According to (Akinyemiju, 2017) primary liver cancer can be managed through; vaccination, aseptic therapeutic measures, well-kept way of living, choices and environmental strategies. In the year 2012, WHO made it an immunization policy that infants should be vaccinated against HBV in order to reduce the rate of incidence cases on the highly risk regions. However cross drug reactions of respective antiviral therapies could play a role in alleviating incidences of re-current infection among those with subtle illness (Karakasiliotisand Mavromara, 2015). Nevertheless, all these mitigating factors have not been implored in third world countries because of curtailed and insufficient resources, lack of adaptability of management programs by respective health practitioners and inappropriate scientific information to support respective policy implementation (Mak *et al.,*2020). Furthermore, quantification and estimations of the incidence rates and the specific sites of high prevalence of PLC in developing countries has been a challenge due to insufficient resources and infrastructure to maintain cancer registries (Fitzmaurice *et al.,* 2015) thus creating a knowledge gap on cancer management that needs to be unraveled. Demographic data, knowledge and attitude towards cancer prognosis and

management is lagging with no single or multiple causative agents underpinned (SingalandEl-Serag, 2015). Environmental factors like microsystin levels in the population are not known as contributors to PLC. Thus, this study endeavored to unravel the intricacies surrounding the epidemiology of PLC within populations living around the Lake Victoria regions in Kenya.

2.5. Molecular mechanism of microcystin toxicity in animals and human health.

Human Protein phosphatases (PPs) are enzymes that regulate the role of kinases by adjourning the addition of phosphate group on the binding sites of regulatory molecules, hence controlling the activation of cell signaling pathways ((Wera &Hemmingst, 1995). They are classified into two: serine-threonine phosphatase and tyrosine phosphatases basing on the amino acid residues they de-phosphorylate (Wang *et al.,* 2019). Serine-threonine phosphatase is a form of phosphoprotein phosphatase that regulates the activated serine/threonine amino acid residues. The incorporation and elimination of phosphate groups controls many cellular pathways in mammalian cells such as cell proliferation, apoptosis, embryonic development, cell differentiation, signal transduction and gene expression (Mclellan & Manderville, 2017). Therefore, inhibition of serine-threonine PPs generates a subsequent counterbalance on cellular functions.

PP1 and PP2A are the major phosphatase in eukaryotic cells that dephosphorylate serine-threonine residues (Wera & Hemmingst, 1995) and are reported to be equally inhibited by the potent microcystin toxin (Massey *et al.,* 2018)through binding to these enzymes. These leads to an increase level of serine-threonine proteins and to deformation of cytoskeleton, interference with shape of the cells with subsequent destructions of hepatic cells leading to liver dysfunction (Mclellan & Manderville, 2017) as shown in **Fig 2.2**. Their shipping and intake into the liver through the bile acid transport system is facilitated by organic anion-transporting polypeptides (OATPS) that are expressed in liver (Campos & Vasconcelos, 2010). Nevertheless,

OATPs are revealed to be found in other digestive organs such as the stomach, small and large intestines, kidney and brain, implying that MCs could also affect other body tissues (Massey *et al.,* 2018). They are also described to be answerable for the elevated levels of free radicals in the cells eventually leading to activated caspase-mediated cell death (Mclellan & Manderville, 2017). According to Liang *et al*., (2011), hyper-phosphorylation of serine-threonine PP2A/PP1 by microcystin induces a cascade of negative effects on cellular functions such as de-regulation of phosphoproteins (P53,DNA-PK,&MAPK) resulting into tumor promotion and apoptosis among others. This is seen through mitogen-activated protein kinases (MAPKs) that regulate the expression of proto-oncogenes such as (c-Jun, c-Fos, and c-Myc) which are responsible in regulation of the transcription of genes involved in the growth and differentiation (Mclellan & Manderville, 2017). Expression of MAPKs is mediated by PP2A (Campos & Vasconcelos, 2010) and therefore inhibition of PP2A by MC-LR will result to activation of MAPK which will activate proto-oncogenes responsible for initiating transcription of growth and differentiation genes as presented in **Fig.2.2**. These suggest that microcystin molecules has the potential to promote liver tumorigenesis (Melaram, 2021). However, adequate information regarding involvement of MCs in PLC is lacking due to limited studies that has been under taken in humans with liver cancer condition (Massey *et al.,* 2018) hence this study.

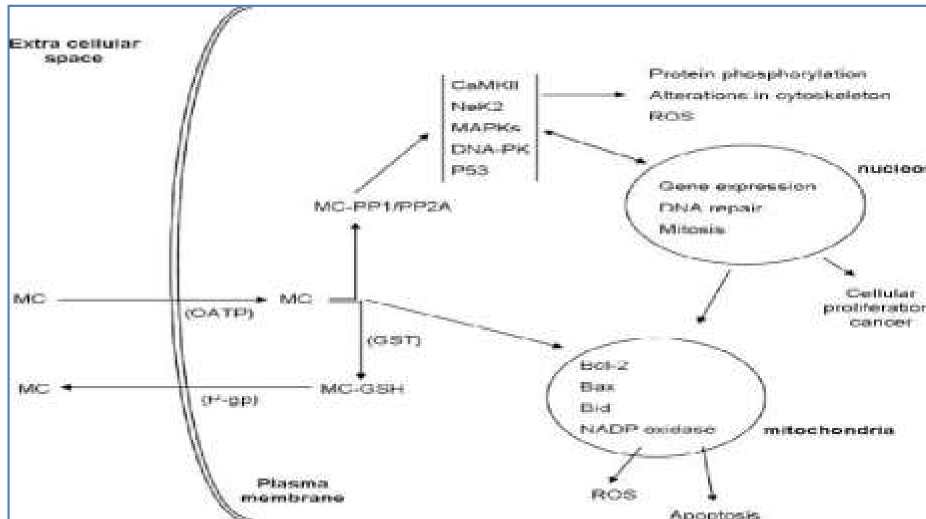

Fig.2.2: Identified routes for MC intake, bio-processing, lethality and elimination in animal cells. (Adapted from Vasconcelus *et al.,* 2016)

Several research cases on animal models has confirmed that long-term exposure in dose-ratio of Microcystin interfere with liver enzymes levels an indicator of liver impairment (Carmichael, 2017). For instance, in Caruaru, Brazil, 76 clients lose life because of microcystin-contaminated water used for haemodialysis (Carmichael, 2001). Ueno *et al.,* (1996), demonstrated that increase number of Primary Liver cancer cases in Southeast China was directly associated with microcystin contamination in drinking water. On the other hand (Chen *et al.,* 2009), confirmed the presence of microcystin in the serum of highly exposed Brazilian fishermen as well as their impact on liver cells. However, scarce information has been reported on variations of total serine-threonine proteins in primary liver cancer patients who have had an exposure to microcystin toxin. As a result, the study aimed to find out the effects of serum microcystin on the total levels of serine-threonine protein phosphates among liver cancer patients attending Jaramogi Oginga Odinga teaching and referral hospital in Kisumu , Kenya.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study methodology

3.2 Study Area

This study was carried out in two different sites. Nyanza gulf region and Jaramogi Oginga Odinga Teaching and Referral Hospital (JOOTRH).

Water sampling was done from Nyanza gulf bay at three different sites: allocated as NG1 (Dunga), 2 (Homa/ Bay), 3 (Mbita) as shown in appendix I, fig 3.1, during dry season (Nov-Dec 2020). Nyanza Gulf is situated close to the equator at the Northeastern corner of Lake Victoria. The area has an approximate area of 1,400km^2, with an average depth 7metres, and irregular shoreline (Sitoki & Kurmayer, 2012). The gulf is river-fed by multiple rivers largely arising from the Kenya highlands on East and Southeast. Nyanza Gulf borders five counties namely Busia, Siaya, Kisumu, Homabay and Migori. The main economic activities among the riparian communities are fishing, farming, business and formal employment. Nyanza Gulf of Lake Victoria was considered for this study due to the large size of livelihood it supports.

Sampling sites were selected basing on their proximity anthropogenic activities such as fishing, boating, which result in high nutrient enrichment, phytoplankton composition and the concentration of hepatotoxic microcystin.

Collection of blood samples from study participants were carried out at JOOTRH, having been the major referral hospital with well-established comprehensive liver cancer units among the five counties bordering Nyanza gulf. PLC cases came from the medical wards, outpatient clinic and lower levelhealth facilities and hospitals lacking the infrastructure to take care of Liver diseases and therefore referred to JOOTRH.

3.3 Study Design

This was a cross-sectional study design, conducted between June to Dec2020. During this period study participants were reviewed by a qualified study clinicians and blood sample collected from those confirmed positive for PLC and those who tested negative but had risk factors for PLC. Oral interview were conducted to get demographic data based on age, place of residence and the main source of water for their domestic use. Water sampling was done along Nyanza gulf at different 3 sites as shown in Appendix I. The study was approved by Ethics Committee of Maseno University (MSU/DRPI/MUERC/00753/19), JOOTRH (IERC/JOOTRH/179/20) and NACOSTI (NACOSTI/P/20/4122).

3.4 Target Population.

The study populations were patients of 15 to about 60 years of age found to be undergoing PLC treatment at JOOTRH. A total of 40 participants were enrolled into the study: 20 PLC patients and 20 controls. To obtain patients demographic characteristics, questionnaires' were used (Appendix III).

3.5 Inclusion and Exclusion Criteria:

3.5.1 Inclusion Criteria:

The people enrolled for this study were patients who were confirmed to be positively diagnosed of PLC at JOOTRH and lived within Nyanza region for at least five years. Those who were able to consent to the study and were willing to sign the provided informed consent regarding appendix II and the willingness to undergo study procedure. The controls enrolled to the study had risk factors for PLC and were referred to the liver clinic for medical management of the risk factors of PLC, but they did not have PLC. The controls had risk factors for PLC, such as HBV/HCC infection, Diabetes and alcohol use but no PLC. They were recruited from blood transfusion services, medical outpatient department, liver clinic and medical wards. The hall mark of development of HCC is inflammation of the liver, hence, the

Controls with risk factors were the best group since they had a factor which can cause liver inflammation.

3.5.2 Exclusion Criteria:

The people, who were not enrolled to this study, included: those who did not consent to the study; those that had other types of tumors; those who decline to fill the questionnaire; and those who had severe digestive system diseases (such as liver cirrhosis).

3.6 Sampling Design

3.6.1 Sample size determination.

The prevalence of primary liver cancer in Nyanza is known to be 5.2% according to Kisumu cancer registry 2011-2014 data. Basing on these the study sample size was determined using the formula by Fisher's *et al*., (1998):

$$N = \frac{Z^2 * p * q}{d^2}$$

Where:

N=the desired sample size (if the target population is greater than 10,000)

Z = the standard normal deviate 1.96 at 95% confidence interval

P = assuming the prevalence of primary liver cancer is 5.2%

q = 1 –0.052 = 0.948

d = level of statistical significances at 0.05.

Therefore;

$$N = \frac{(1.96)^2(0.052)(0.948)}{0.05*0.05}$$

= 75.75

A further 10% of 76, which is, approximately 8 were added to cover for unresponsive subjects, bringing the total sample size to 84 subjects. However, we couldn’t manage to get 84 study participants, due to quick and untimely deaths of PLC patients, since majority of the reported cases were at late stages of the diagnosis and therefore chances of survival were minimal. Therefore, we opted to use non-propabilistic purposive sampling technique method which gave us 40 study participants (20 cases: 20 controls)

3.7 Variables

3.7.1 Independent variable

Concentration of microcystin

3.7.2 Dependent variable

Expression of mlrA gene

Levels of serine-threonine protein phosphatase PP1 and 2A liver enzymes.

3.8 Sample collection

3.8.1 Water sampling

A total of 12 surface water samples were collected using 1.5 litre environmental sterile plastic sampling bottles from 3 different sites each 4 samples ;(Dunga, Homa/ Bay and Mbita) along Nyanza gulf as shown in appendix I for cyanobacteria extraction and total RNA isolation and analysis. These stations were chosen based on their proximity to areas that support important economic and recreational activities (summer resorts, aquatic sports and fisheries) and the water intake for drinking. The collected water sample are stored in a cooler box with icepacks (4°C) for transportation to the KMFRI laboratory Kisumu branch for storage and subsequent analysis.

Depth integrated water samples for nutrients analysis were taken, specifically for Total Nitrogen (TN) and Total Phosphorus (TP). Water samples were contained in the bottles well labeled, filled, without controlled preservation and were stored in cooler box at temperatures of about 4^{0}C, for further laboratory analysis using photometric methods

3.8.2 Blood sample collections

About five (5) mls of blood sample were collected from each study participant by a trained phlebotomist and aliquoted into anticoagulant free vacutainer tubes. They were then transported to laboratory at Maseno University Zoology laboratory, for processing within 6 hours of collection. Upon arrival, centrifugation was done at 8000 rpm for 2 minutes, leaving out serum as supernatant. The serum was aliquoted into cryovials tubes and stored at -20°C until required for further analysis.

3.9 Sample Analysis

The following experiments were carried out in the laboratory for the blood and water samples collected.

3.9.1 Microcystin toxin analysis

Polyclonal ELISA kits were used for microcystin analysis and measured calorimetrically in an ELISA plate reader at 450 nm. 6 sampling stations were used to quantify the levels of microcystin along the Nyanza gulf. Antibody solution were mixed with samples to be analyzed in ratio of 100:100 and were pipetted into a 96-well plate, in duplicates. The contents were covered by parafilm and vortexed. After 90 minutes incubation at room temperature (25°C), and the plate contents were vigorously shaken and discarded before washing three times with buffer solution. Thereafter, a particular enzyme conjugate (e.g., microcystin-HRP conjugate) solution were added to the wells, covered and vortexed once again and incubated for 30 minutes at room temperature. After incubation, the covering was removed, contents vortexed and decanted. The strips were then washed three times using buffer solution. A substrate solution was added and incubated for 30 minutes at room temperature. Finally, a stop solution was added to the wells before reading the absorbance at 450 nm using ELISA reader.

3.9.2 Phytoplankton Sampling, Identification and Nutrients analysis

Environmental water samples for phytoplankton and nutrient analysis were collected with a 3-litre Van Dorn sampler for integrated and sub-surface waters. A portion of the water sample (25 ml) was preserved in acidic Lugol's solution. A 2 ml phytoplankton sub-sample was placed in an Utermöhl sedimentation chamber and left to settle for at least three hours. Phytoplankton species identification and enumeration were done using a Zeiss Axioinvert 35 inverted microscope at 400x magnification. Phytoplankton taxa were identified using the methods of Huber –Pestalozzi (1968). Phytoplanktons were estimated by counting all the individuals whether these organisms were single cells, colonies or filamentous. Nutrients analyses were carried out in the laboratory using photometric methods.

3.9.2.1 Nutrients analysis

3.9.2.1.1: Determination of phosphate ion.

100mls of each water sample and the standard solutions were pipetted into a 500 cm^3 volumetric flask per sample, 10mls of Ammonium molybdate solution and 6mls of ascorbic acid were added with swirling, the mixtures were diluted to the mark with deionised water and were allowed to stand for 30 minutes for maximum colour development, the absorbance were then read at 660nm including the blank.

3.9.2.1.2: Determination of Nitrate ion

20mls of the water samples and standard solutions were pipetted into a 50 cm^3 volumetric flask. 20mls of 13N sulphuric acid were added and mixed with swirling, the flask was allowed to come to a thermal equilibrium in cold water bath (0-10) ^oC. 1ml of brocine-sulfanilic acid were added to each sample and diluted to the mark with deionised water, the solution was then placed on the 50^oC hot water bath for about 25 minutes for maximum colour development, the flask was then cooled to room temperature. The absorbance were read at 410nm including the blank.

3.9.2.2 Isolation and analysis of Total RNA from phytoplanktons

A total of 12 surface water samples were collected using 1.5 litre environmental sterile sampling bottles from 3 different sites along Nyanza gulf: Dunga (NG1), Homabay (NG2) and Mbita (NG3) for cyanobacteria analysis.

3.9.2.2.1 Total RNA isolation

The preserved wet phytoplanktons for RNA isolation were placed in ice and ground using a pestle and mortar. Total RNA was extracted using RNeasy-Minikit (Qiagen Inc. Germany) following manufactures instructions. 1.5mls of grounded wet phytoplankton was placed in a 2 ml tube. 450µl of buffer RLT was added into tube and vortexed vigorously. The lysate was

transferred into the QIAshredder spin column (Qiagen, Gmbh-Germany) placed in a 2ml collection tube, then centrifuged at 15000rpm for 2 minutes at room temperature (25ºC). After removing the supernatant, the pellet was transferred to afresh microcentrifuge tube. Six hundred microliters (600µl) of ethanol (96%) were added into the tube and pipetted gently. Six hundred and fifty microliters (650µl) of mixed solution were transferred into RNeasy spin column (Qiagen, Gmbh-Germany). In a 2ml collection tube centrifugation was then done at 1000rpm for 15 seconds at room temperature. The flow through was discarded. A total of 700 µl of buffer RW1 was added to RNeasy spin column and centrifuged at 1000 rpm for 15 seconds at room temperature. The filtrate was then discarded. 500µl of buffer RPE were added to RNeasy spin column, and then centrifuged at 1000 rpm for 2minutes at room temperature (25ºC). The RNeasy spin column used in the procedure above was put in a new1.5-ml micro centrifuge tube. 30µl of sterile distilled water was added into RNeasy spin column, then centrifuged at 14000 rpm for 1 minute at room temperature (25ºC). The RNeasy spin column was then removed from the tube. The eluted RNA solution was preserved at -20˚C. RNA concentration and integrity was assessed using a Nano-Drop ND 2000 UV-VIS spectrophotometer (US/Canada) (A260/280).

3.9.2.2.1 Reverse transcriptions (RT) of isolated Total-RNA into c-DNA

Prior to RT, residual g-DNA co-extracted with RNA was destroyed using g-DNA wipeout buffer at 42ºC for 5 minutes. A total of 2µl purified RNA was reverse transcribed using the RT^2Pre AMP-c-DNA synthesis kit (Qiagen Inc., Canada,):10µl of reverse transcription master mix made up of (c-DNA synthesis Enzyme Mix, 5X Buffer BC3, control P2, RNase Inhibitors and RNase free water) were added into each tube containing 2µl of purified RNA molecule. The mixture was then incubated at 42°C for 30 minutes to allow for reaction to take place. Their action was then stopped by incubating at 95ºC for 5 minutes and the cDNA kept at-20°C for subsequent analysis of mcyE and mlrA gene expression using RT-qPCR processes.

3.9.2.2.2 Analysis of mcyE and mlrA gene expression using RT-qPCR.

Microcystin synthetase E (mcyE) and Microcystin methylopeptidase A (mlrA) gene transcript levels were estimated by the relative quantification approach described by (Schmittgen & Livak, 2008) method. The PCR assays were performed on two target genes (mcyE and mlrA) and reference genes (16S rRNA) using the primer pairs shown in Table 1. All samples were amplified in 2Plex-HRM rotor gene Real-time PCR machine using 2X QuantiFast ® SYBR® Green master mix (Qiagen Inc., Canada) as per the manufacturers' recommendations. Amplifications were performed in 25 µL reaction mixtures containing 300 nM and 200 nM of forward and reverse primers for mcyE and mlrA gene respectively, 2x QuantiFast®SYBR® Green I mastermix (with ROX dye), and 2µL cDNA template. Amplification of reference genes on the other hand was performed in 25µl reactions containing 300nm/l of each 16S rRNA primer and 500nm/l rpoC1 forward and reverse primers respectively, 2 x quantifast SYBR green master mix with (ROX dye) and 2µl of c-DNA template. The PCR thermal cycle consisted of a hot start cycle at 95ºC for 5 min, followed by 40 cycles comprising denaturation at 95ºC for 10 sec, and a combined annealing/extension step at 60ºC for 30s. All fluorescence data were collected at 60ºCand CT values determined using Rotor-gene Q software.

Table 3.1: Types of primers used in the current study.

Target gene	Primer name	Primer sequence (5'-3')	Melting temp°C	Reference
Microcystin synthetase E	MicmcyE-415F1	CCTGCACTCCCTGAGAG AGAAC	60	(Ngwa, 2013)
	MicmcyE-581R	AATGACCGCCAATTTCAAAG	60	(Ngwa, 2013)
Microcystinmethylo peptidase A	QmlrAf	AGCCCKGGCCCRCTGC	60	(Stelzer *et al.*, 2013)
	QmlrAr	ATGCCARGCCACCACAT		
16S ribosomal RNA	16srRNA-RTF2	CTGAAGATGGGCTCGCGT	61.7	(Rantala *et al.,*2013)
	16srRNA-RTR	CGTATTACCGCGGCTGCT	61.3	

[1]The MicmcyE and QmlrA primer pairs are specific for microcystis mcyE and mlrA genes respectively

[2]The 16S rRNA primer pairs is reference genes used to normalize the mcyE and mlrA gene expression data.

3.9.2.2.3. Quantification of microcystis mcyE and mlrA gene expression

To quantify the expression levels of the MC synthetase (mcyE) and degradation (mlrA) gene in the collected phytoplankton samples from Lake Victoria, relative comparative method of quantification was used. The individual RT-PCR was performed in triplicate and analyzed using the following equation $2^{-\Delta\Delta CT}$ according to (Livak *et al.,* 2008). The Ct values were collected from Rotor gene software for each of the target gene and reference gene then imported into Excel spreadsheet for further analysis. The mean, ± standard deviation was determined from the triplicate samples for each respective gene at specific site. The CT values were then inputted into the equation below: The gene expression ratio was calculated by the $2^{-\Delta\Delta CT}$ method according to Livak *et al.,* 2008, (ΔΔCT= (CT, target gene-CT, 16S rRNA) stress-(CT, target gene- CT, 16S

rRNA) control).

Where: Ct $_{GOI}$ = CT value of gene of interest,

CTref = CT value of reference gene and

$\Delta CT_{calibrator}$ = is the CT value of laboratory control gene of the study.

The data obtained were expressed as fold change in gene expressions normalized to reference genes (16S rRNA) and relative to calibrator. The calibrator for $2^{-\Delta\Delta CT}$ method values to 1 and can be treated sample, or sample obtained for the experimental samples with higher change value in ΔCT among the samples, which is the case in this study.

3.9.3. Serum sample analysis on the total levels of serine-threonine protein phosphatase 1 and 2A among PLC cases and controls using Sensolyt p-NPP phosphatase assay Kit

3.9.3.1 Preparation of working solutions

pNPP alkaline phosphatase substrate kit (comp. A) was ready for use. Alkaline phosphatase dilution buffer was prepared for protein phosphatase using the following components :(20mM Tris HCL, pH=7.5,5 mM $MgCl_2$, 1mMEDTA, 0.02% DTT[3]). Alkaline phosphatase standards (10µg/ml-comp E) were then diluted to 0.2µg/ml (1:50) using dilution buffer to make 2-fold serial dilutions of the following concentration: 100, 50, 25, 12.5, 6.2, 3.1 and 0ng/mL.

3.9.3.1.1 Assay procedure

Serum sample from each participant was picked and diluted with dilution buffer in a ratio of 1:100and added into each well (clear 96-well plate) in duplicates. A 100µl of each serially diluted standard concentration were also added into their specific wells in duplicates. A non-phosphates-containing sample was included as a negative control. 50µl of para-Nitrophenylphosphate (p-NPP) substrate solution were added into each well. The reagents were mixed gently by shaking the plate for 30 seconds. Their action mix was incubated at room

temperature (25ºC) for 30 minutes. After incubation50 µL of stop solution were added into each well (96-well plate), the plates were then shaken on a plate shaker for 1min before the reading. The absorbance was then measured at 405 nm using ELISA reader.

3.10 Data Management/ Data Analysis

Data was stored in both source records and computer databases accessible only to the authorized persons throughout the study. Data management was performed using Statistical Package for Social Sciences (SPSS) version (17.0) installed in personal computer (PC). Microcystin toxin and nutrients levels were analyzed using Excel Microsoft installed. The levels of serine-threonine protein phosphatase liver enzymes between the PLC cases and normal individuals were analyzed using independent t-test to determine the significance in variation levels. Pearson correlation was used to test the correlation between *mcyE* and *mIrA* genes expression.

3.11 Ethical considerations and Informed consent

Ethical approvals were obtained from Maseno University Ethical Review Committee (MUERC) and Jaramogi Oginga Odinga Teaching and Referral Hospital Ethical Review Committee and NACOSTI. A written informed consent was obtained from the guardians and parents of children; also, child assent (Appendix II) was mandatory before sample collection was done. There were no extra charges for the participants and participation was entirely voluntary. Strict confidentiality was maintained, and personal identifiers removed from data during data analysis. Codes were used instead of individual identity. Restriction to data access both in its electronic and physical form was done using passwords and lockable lockers respectively. The potential risks such as pain experienced when the needle goes into your arm for blood to be drawn and a small risk of bruising or infection at that site together with the benefits of participation were explained in a language understandable to the participants during the process of informed

consent (Appendix II). The guardian or parents were informed that any withdrawal from the study was allowed

CHAPTER FOUR

RESULTS

4.1 Levels of extracellular Microcystin toxin and Nutrients along Nyanza gulf.

All the sampling stations detected microcystin levels beyond the recommended WHO guidelines of 1.0µg/l for quality water safety. Highest MCs levels were detected in Homa/Bay Sewage discharge (B) with MCs levels of 21.4µg/l which corresponded to high nitrogen and phosphorus levels within the same station (Table 4.2.1).This was followed by Mbita A (13.1µg/l), Homa/Bay A (7.9µg/l), as well as Dunga B (7.1µg/l) and Mbita B (5.9µg/l). However, Dunga site A had the lowest level of toxin detection of about 2.2µg/l, despite having high levels of nitrogen

Table: 4.1 Sampling sites for toxins and total dissolved phosphorus and nitrogen levels.

SITE	Extracellular MCs toxin(ug/l)	TP (ug/l)	TN (ug/l)
Dunga A	2.2	71.14	247.58
Dunga B	7.1	84.71	308.37
Homabay A	7.9	100	395.21
Homabay Sewage Discharge (B)	21.4	136.15	498.64
Mbita A	13.1	46.14	327.32
Mbita B	5.9	59	330.47

Legend: Determined concentrations of microcystin toxins,dissolved phosphorus and nitrogen from the different sampled sites (measured in µg/l).

4.2 Quantification of *mcyE* and *mlrA* gene expressions

RNA was successfully extracted from 12 environmental water samples and was measured using a nano-drop machine as shown in appendix VI, (table 4.2). Out of the 12 samples, 2 samples could not produce any peak from the synthesized cDNA, and all fluorescence data generated

from such samples were always below the detection threshold. Relative quantification of gene expression levels of the MC synthetase (mcyE) and degradation (mlrA) gene were analyzed using $2^{-\Delta\Delta CT}$ method according to (Livak *et al.,* 2008) method.The data obtained were normalized to 16S rRNA reference genes relative to calibrator. The Threshold Cycle (CT) values from triplicate samples were imported to excel spreadsheet and the mean, and ±SD were determined for each respective gene of interest and reference genes at specific site as shown in (Table 4.2 *mcyE*; Table 4.3 *mlrA;*Table 4.4, ref gene 16S rRNA.> appendix VII).

4.2.1 Expression of ***mcyE*** and ***mlr A*** genes

Analysis of gene expression of the extracted RNA was done using RT-PCR Rotor gene Q machine (Qiagen; Germany). Microcystis *mcyE* and *mlrA* genes were expressed in (10) ten samples from various stations with different transcript levels. Expression of Microcystis mlrA genes were generally higher in some stations like Mbita A, C, H/bay B, and Dunga as compared to mcyE genes collected from similar stations, which had lower fold change in expression in the samples collected as shown in table 4.2 and 4.3. Samples from Mbita A, Dunga B and Dunga A showed a higher fold change in expression of mlrA genes of (22.47; 22.32 and 20.39) as compared to mcyE genes from the same samples stations which gave a fold change expression of (9.58; 9.78 and 8.00) respectively. Contrastingly, samples from Mbita B, H/bay C, D station showed a different observation by having a higher fold change in expression of mcyE genes of 8.88, 3.86 and 6.11, as compared to mlrA genes which expressed itself with the lowest fold change of 2.14, 3.58 and 2.57.

Table 4.2: Mean Ct values (±S.E.M) for mcyE and their level of gene expressions

Site	CT1	CT2	CT3	Mean (GOI)	SD	Mean Exp.Ref	ΔCT	ΔΔCT	2^-ΔΔCT
MbitaA	1.73	1.78	2.83	2.11	0.62	1.91	0.2	-3.26	9.58±0.6
MbitaB	1.79	2.24	2.43	2.15	0.33	1.84	0.31	-3.15	8.88±0.3
MbitaC	1.81	2.01	1.95	1.92	0.10	1.89	0.03	-3.43	10.78±0.1
Callibrator	4.62	4.56	6.39	5.19	1.04	1.73	3.46	0	1±1
H/bayB	1.55	1.46	1.66	1.56	0.10	1.91	-0.35	-3.81	14.03±0.1
H/bayC	3.26	2.48	3.47	3.07	0.52	1.56	1.51	-1.95	3.86±0.5
H/bayD	2.4	1.68	3.04	2.37	0.68	1.52	0.85	-2.61	6.11±0.7
DungaA	2.72	1.69	2.28	2.23	0.52	1.77	0.46	-3	8±0.5
DungaB	2.68	1.79	1.67	2.05	0.55	1.88	0.17	-3.29	9.78±0.6
DungaC	2.46	3.01	2.89	2.79	0.29	1.43	1.36	-2.1	4.29±0.3

Legend: Amount of Fold change gene expression of microcystin synthetase E.(mcyE) from sampling sites**:** CT; threshold cycle, GOI; gene of interest, SD; standard deviation, ΔCT; delta threshold cycle, ΔΔCT; delta delta threshold cycle, S.E.M=Standard error of mean; Exp.Ref :reference gene expression values used in the experiment. Normalization of mcyE gene expression was performed using 16 ribosomal RNA (appendix VII).

Table 4.3. Mean Ct values (± S.E.M) for (mlrA) and their level of gene expressions

Site	CT1	CT2	CT3	Mean (GOI)	SD	Mean Exp.Ref	ΔCT	ΔΔCT	2^-ΔΔCT
MbitaA	1.71	1.82	1.75	1.76	0.056	1.91	-0.15	-4.49	22.47±0.1
MbitaB	4.88	5.25	5.12	5.08	0.19	1.84	3.24	-1.1	2.14±0.2
MbitaC	2.05	2.54	1.98	2.19	0.31	1.89	0.3	-4.04	16.45±0.3
Callibrator	6.73	5.48	6.01	6.07	0.63	1.73	4.34	0	1±0.6
H/bayB	2.22	2.26	2.03	2.17	0.13	1.91	0.26	-4.08	16.91±0.1
H/bayC	4.28	3.89	4.01	4.06	0.20	1.56	2.5	-1.84	3.58±0.2
H/bayD	5.4	3.21	4.89	4.5	1.15	1.52	2.98	-1.36	2.57±1.2
DungaA	1.72	1.76	1.81	1.76	0.05	1.77	-.01	-4.35	20.39±0.1
DungaB	1.73	1.86	1.62	1.74	0.12	1.88	-0.14	-4.48	22.32±0.1
DungaC	2.03	1.89	2.05	1.99	0.09	1.43	0.56	-3.78	13.74±0.1

Legend: Amount of Fold change gene expression of Microcystin methylopeptidase A gene E.(mlrA) from sampling sites: CT; threshold cycle, GOI; gene of interest, SD; standard deviation, ΔCT; delta threshold cycle, ΔΔCT; delta delta threshold cycle, S.E.M=Standard error of mean; Exp.Ref: reference gene expression values used in the experiment. Normalization of mlrA gene expression was performed using 16 ribosomal RNA (appendix VII).

4.2.2 Correlation analysis

To determine the strength of relationship between mcyE and mlrA gene expression in thisstudy,Pearson correlation coefficients was used. The results of these analyses were presented in Fig 4.1. Pearson analysis revealed a moderate positive correlation between *mcyE* and *mlrA* genes, (r=0.625, P(two-tailed) =0.053). There was no statistically significant differences in the relative expression between the two genes: *mcyE* and *mlrA* among cyanobacteria samples (p>0.05) collected from various sites in Lake Victoria, Kenya.

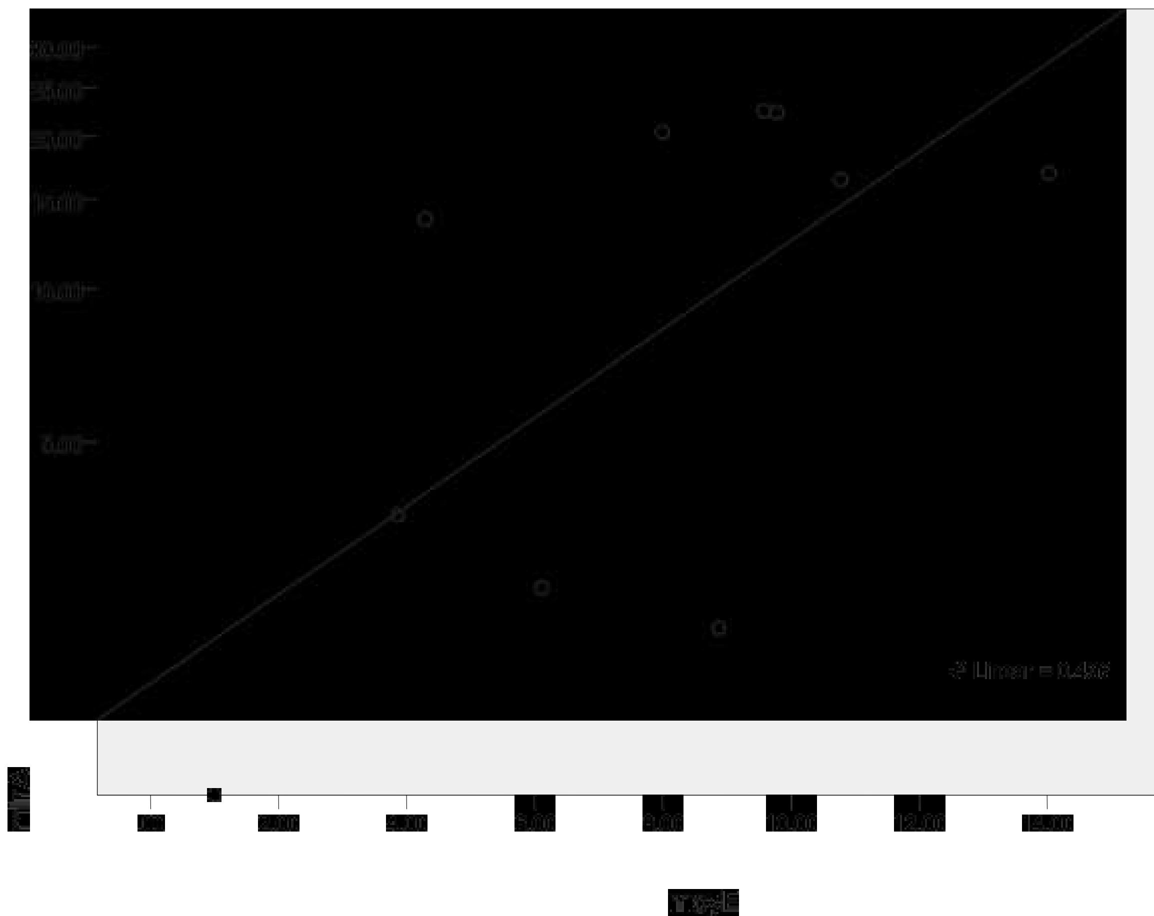

Fig4.1: Correlation analysis between mcyE and mlrA gene expression levels. Y-axis represent relative expression of MC-biodegrading bacteria (mlrA) vs the X-axis representing relative expression of MC-producing bacteria (mcyE). Pearson correlation analysis: (r=0.625, P(two-

tailed) =0.053). The relative expression was statistically insignificant (P>0.05).

4.3.0 Socio-demographic characteristics of the study participants.

Table 4.4 Socio-demographic characteristics

Characteristic		Total	Plc Cases N(%)	Controls N(%)
Participants		40	20	20
Gender	Male	23 (57.5%)	14 (70%)	9 (45%)
	Female	17 (42.5%)	6 (30%)	11 (55%)
Age group	15-30yrs	11 (27.5%)	5 (25%)	6 (30%)
	31-49yrs	17 (42.5%)	8 (40%)	9 (45%)
	>50yrs	12 (30%)	7 (35%)	5 (25%)
Source of drinking water	Lake	17 (42.5%)	8 (40%)	9 (45%)
	Tap Water	11 (27.5%)	5 (25%)	6 (30%)
	Well	12 (30%)	7 (35%)	5 (25%)

A total of 40 participants were enrolled into the study, 20 PLC cases and 20 controls. Out of these 23(57.5%) were male and 17 (42.5%) were female. Male presented a high case of PLC of about 14 (70%), whereas female were few in number 6 (30%). Majority of participant especially those with PLC, were within the age of (30-49yrs); 17(42.5%). Through questioner (appendix III) we were able to infer that most of them came in contact with the Lake Victoria water in their lifetime table 4.5

4.3.1 Total serum levels of Serine-threonine protein phosphatases among PLC cases and Controls

In this study, patient serum serine/threonine levels were determined using direct ELISA technique and the outcomes read at wavelength of 405 nm. The results showed that the serine-threonine protein phophatase activities in liver cancer patients were highly expressed beyond the normal range of a healthy individual (20-140U/L) as compared to controls suggesting that presence of MC could be the cause of this increase (Table 4.6; Fig. 4.2). This because other con-

founding risk factors were clinically under control**.** There was a statistically significant increase ($P<0.05$; $t_{38}=4.298$;) in the mean concentration of serum levels of total serine-threonine proteins phosphatases (PP1, PP2A) among the Liver cancer patients(103.9 ±17.83µg/ml) than controls (25.91±3.342µg/ml).

Table 4.5: Summary of total levels of serine-threonine protein phosphatases among cases/controls

Total levels of serine–threonine protein phosphatase in serum sample			
Conditions/Levels	**LOW (below 20µg/ml)**	**NORMAL (20-125 ug/ml)**	**HIGH(above125 ug/ml)**
PLC Cases(n=20)	3 (15%)	8 (40%)	9 (45%)
Controls(N-PLC)(n=20)	5 (25%)	15 (75%)	0

Legend: A summary of the data obtained from the total serine-threonine protein phosphatase of PLC cases and controls PLC > Primary Liver cancer; N-PLC > controls, Negative Primary Liver cancer; n=sample size; Normal range of serine-threonine protein phosphates (20-125 µg/ml).

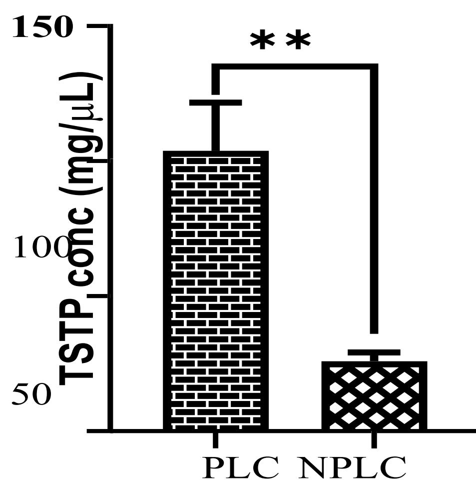

Fig4.2. A bar-graph representation of the mean concentration variation of Serine-Threonine protein phosphatase among the study participants. The mean concentration variation was statistically significant;**p-value = 0.0001; Y-axis represent. TSTP>Total threonine protein phosphatase whereas the X-axis represent the condition of study participants; PLC>Primary liver cancer cases andcontrols (non-primary liver cancer patients- N-PLC).

CHAPTER FIVE

DISCUSSION

5.1 Determination of microcystin concentration and nutrients levels within Nyanza gulf.

MCs are toxins usually stored in the cyanobacterial cells and are abundantly released into the environment due to cell lysis or external environmental stress (Melaram, 2021). In the present study the presence of microcystin toxins along Nyanza gulf was observed in all the sample sites selected. All the samples stations recorded microcystin toxins levels beyond the recommended WHO standards value of 1.0µg/l for quality water safety, thus providing evidence of highest possible health risk to riparian communities. Homabay Sewage discharge (B) is reported to have the highest levels of MCs toxins (21.4µg/l) which corresponds to high levels of nutrients (TP = 136.15µg/L; TN =498.64µg/L) recorded within the same stations as shown in table 4.1. This was followed by Mbita A (13.1µg/l), Homa/BayA (7.9µg/l) (TP = 100µg/L; TN = 395.21µg/L), as well as Dunga B (7.1µg/l); (TP = 84.71µg/L; TN = 308.37µg/L) and Mbita B (5.9µg/l); (TP = 59µg/L; TN = 330.47µg/L). However, Dunga site A recorded the lowest level of toxin detection of about 2.2µg/l, despite having high levels of nitrogen (TP = 71.14µg/L; TN = 247.58µf/L). This may be attributed to the shallow mean depth along the gulf, which is strongly influenced by extremely variable seasonal wind patterns and runoff from the city of Kisumu and adjacent agricultural farmlands, industrial effluents and recreational activities (Okely *et al.* 2010). All these factors together with the river inflows negatively affect the development of algal blooms and the production of algal toxins hence low levels detected.

Microcystin production is reported to be influenced by high nutrients enrichment particularly nitrogen and phosphates (Babu *et al.* 2021). This is in support to the results obtained from Homabay sewage (B) which recorded highest levels of microcystin corresponding to Nutrients

levels. The results are further like those reported by Sitoki *et al.,*(2012),who reported high abundance of cyanobacteria made up of Microcystis species in the Lake which led to temporary shut-down of drinking water supply from L. Victoria in Kenya in 2004 and in Uganda in 2008. In addition, all the sampling sites recorded high levels of Nitrogen compared to phosphorus levels; suggesting that Nitrogen could be having the highest impact in the growth of blue-green algae. This observation is similar to that reported by (Babu *et al.,*2021), where total nitrogen and total nitrite concentrations were high, and a rich community of cyanophytes algae (Microcystis spp.; anabaena spp.; Merismopedia spp.; and Planktolyngbya spp.) were reported to be prominent along Kisumu Bay. Blue-green algae composition are highly enriched in nitrogen compounds because of their high protein (which accounts for much of the N) and lipid contents Mwamburi *et al*.,2020. Thus, the high nitrogen content in the water column is mainly organic nitrogen derived from algae hence the high levels of microcystin detected that depends on it for protein synthesis.

The increased levels of microcystin in the lake, has exerted an already existing pressures resulting in reduced fish species recovery rates and further fish deaths like those recorded in the 1990s; as well as exposing health risk to communities who rely on Lake water for domestic chores (Onyango *et al.,*2020). Moreover, fish consumption can also be a key and sometimes dominant microcystin exposure route on humans especially when the day-to-day consumption levels recommended by WHO is exceeded (Simiyu *et al.,*2018). Upon consumption, MC may not suddenly cause a health threat to human beings, but over time it may bring about long-term negative implications such as liver cancer as reported in previous studies (Massey *et al.,* 2020).Therefore, the high level depicted along Nyanza gulf need to be mitigated and riparian communities to be enlighten on the dangers of using the lake water directly with no treatment.

5.2 Correlation between mcyE and mlrA gene expression in cyanobacteria.

mcyE is a gene encoding for microcystin synthetas E, an enzyme responsible to produce microcystin toxin whereas mlrA encodes for microcystinmethylopeptidase A, an enzyme responsible for biodegrading microcystin molecule. In this study mcyE and mlrA were all expressed with different variations from the phytoplanktons collected from the sampling sites. This indicates the presence of active toxin-producing cyanobacterial species and co-existing microcystin biodegrading bacteria in the water column along Nyanza gulf. This was arrived at after the levels of mcyE and mlrA was determined by use of RT-PCR software to obtained the CT values and analyzed using the equation (2-ΔΔCT) according to Livak *et al.,(*2008). From the results mlrA genes were found to be generally higher with a fold change of 22.47; 22.32, 20.39 and 16.91 compared to mcyE genes with a lower-fold change in expression of 9.58; 9.78, 8 and 14.03 in the samples collected from MbitaA, Dunga B, Dunga A and Homabay B respectively. Contrastingly, samples from Mbita B; Homabay D, Homabay C station showed a different observation by having a higher fold change in expression of mcyE genes of 8.88, 6.11,3.86 as compared to mlrA genes which expressed itself with the lowest fold change of 2.14, 2.57 ,3.58 respectively. The variation of the expression of mcyE and mlrA gene transcript levels in different sites of the Nyanza Gulfs may be due to the growth processes of various cyanobacteria strains as well as algae bloom concentration (Mchau *et al.,* 2019) which varied during spatial and temporal sampling seasons. This could also be linked to change in surrounding parameters like exogenous nutrients, temperature, light, pH and bacterial composition as observed in the recent studies (Lezcano *et al*.,2016; Ngwa *et al*.,2014b). According to Zhu *et al.,* (2014), MCs levels and mlrA genes have both positive and negative correlation. Moreover the presence of MC-degrading bacteria(mlrA),alters cyanobacteria population from harmful to harmless blue-green algae. The results of this study were in conformity to those observed by Zhu *et al.,*(2014), in which a direct

correlation was reported between mlrA, and mcyE gene expression and the levels of MCs in water. The results were further in consensus with (Wang *et al.,* 2019), who reported that exposure to MCs induces the up-regulation of mlrA gene expression-suggesting that presence of MCs has the potential to activate the mlrA gene expression. However, the correspondence of the increase in mlrA gene transcript levels with the decrease in the mcyE gene transcript levels and vice versa, during the period of study, may outline an indirect relationship among the bacterial population. The expression of the mcyE gene is affected by the levels of carbon and nitrogen concentration in the environment which were low in these sites during sampling (unpublished data). Generally, high MC concentrations are observed in association with high N concentrations in culture and field studies (Lezcano *et al*.,2016). However, the opposite has also been proposed and is largely supported by the observation that NtcA, the global nitrogen regulator,can bind to the promoter sequences for MC production (Kimambo *et al.,* 2019). This implies that in times of high N availability, NtcA production would increase and block the transcription of MC genes as depicted in our findings. The surrounding parameters for instance nutrient conditions, oxygen conditions ,temperature and levels of MCs and chlorophyll;(Mwamburi *et al.,* 2020; Vadeboncoeur *et al.,* 2021) in water were observed to influence the biotransformation rate of MC structure. Irrespective of MC structure reported to be resistant to both physical and biochemical process(Edwards & Lawton, 2014) recent studies have identified some natural occurring biodegrading bacteria which are reported to effectively degrade the harmful MCs structure into less toxic components (Massey *et al.,*, 2020). These degrading bacteria's co-exist with the cyanobacteria in the same aquatic environment and they happen to influence each other both directly and indirectly (Lezcano *et al.,* 2016).However, it is not clear whether the degradation rate of MCs depends on the number of biodegrading bacterial populations, or existence of functional gene expression of mlrA or both is required, hence needed for further study.

Correlation analysis revealed an insignificant moderate positive relationship between mcyE and mlrA gene expression transcript level (P=0.053;r=0.0.625). This is supported in the previous studies by (Guo *et al.,* 2015), who reported a correlation of $r^2=0.54$ between mlrA gene copy number and MCs biodegradation rate. These suggest that MCs degradations are partially explained by mlrA possessing MCs-degrading bacteria but are other alternative MC-degradation pathways for the overall degradation of MCs in the environment (Lezcano *et al.,* 2016). However, this could also be attributed to limited number of samples used in the study and variation in nutrients concentrations conditions required for the expression of the genes. On the other hand, the increase in the mlrA gene transcript levels coincide with the decrease in the toxic cyanobacteria indicating a possible indirect relationship among the populations that needs further research.

5.3 Total serine-threonine protein phosphatase (PP1andPP2A) enzyme levels in serum samples of primary Liver cancer patient and controls

This study hypothesized an increase level of serine-threonine protein phosphates among liver cancer patients regarding inhibition effects of MC that was present in serum sample of PLC clients as reported from previous studies. The results therefore, showed that the serine-threonine protein phosphatase activities in liver cancer patients were highly expressed above the normal range of a healthy individual (20-140U/L) as compared to controls. In addition, we further report that, there was a significant increase ($t_{38}=4.295; p<0.05$;) in the mean concentration of serum levels of total serine-threonine proteins phosphatases (PP1, PP2A) among the Liver cancer patients (103.9 ±17.83µg/ml)compared to controls (25.91± 3.342µg/ml). This is an indication of existence of a factor that directly affects the physiological function of serine-threonine protein phosphatases and their modes of action given that the other clinical factors were under medical control. Therefore, it is extrapolated that the increase in the levels of serine-threonine PP1/2A in

these patients could be attributed to the presence of microcystin in the blood of these patients. These results are in consistent with those of (Liang *et al.,* 2011)who reported increase in PP2A activities in human amnion FL cells treated with low-dose treatment of MCLR for a period of 6 h ,whereas high-dose treatment of MCLR for 24 h decreased the activity of PP2A.Moreover,studies by (Guo *et al.,* 2015; He *et al.,* 2018) where exposure to MC-LR and bloom extract increased the protein levels of the A subunit of PP2A in vivo and in vitro, supports our findings. Hence, supporting a direct correlation of serum microcystin presence to activities of PP1/2 that serves to regulate cell haemostasis accomplished through phosphorylation of cell proteins for cell proliferation, metabolism and cell death (Mclellan & Manderville,2017). According to (Liang *et al*., 2011),hyper-phosphorylation of serine-threonine PP2A/PP1 by microcystin induces a cascade of negative effects on cellular functions such as de-regulation of phosphoproteins (P53,DNA-PK,&MAPK) resulting into tumor promotion and apoptosis among others. In this regard, inhibition activities of PP1/2 by MC as is reported by this study, is known to promote tumor-activities especially Primary liver cancer is appreciated (Guo *et al.,* 2015;Liang *et al.,* 2011; Ueno *et al.,* 1996).MCs exert their toxicity through inhibition of eukaryotic serine/threonine protein phosphatases 1 and 2A, thereby increasing the overall level of phosphorylation in hepatocytes (D.Mak & Kramvis, 2021;Massey *et al*.,2018).Hyper-phosphorylation of cytoskeletal proteins results in alteration of cytoskeleton, loss of cell shape ,induction of hepatocyte deformation and trigger of apoptosis due to oxidative stress which may result into primary liver cancer development (Massey *et al*.,2018). Therefore, cellular toxicity in response to acute and chronic MC exposure can be assessed by monitoring the activities of PP2A/PP1(Campos&Vasconcelos, 2010) as observed in this study, which is the main target of MC-mechanism of toxicity once it is ingested into the body.This is seen through Mitogen-activated protein kinases (MAPKs) that regulates the expression of proto-oncogenes such as c-

Jun, c-Fos, and c-Myc which are responsible in regulation of the transcription of genes involved in the growth and differentiation (Mclellan & Manderville, 2017).Expression of MAPKs is mediated by PP2A (Campos & Vasconcelos, 2010) and therefore inhibition of PP2A by MC-LR will result to activation of MAPK which will activate proto-oncogenes responsible for initiating transcription of growth and differentiation genes as presented in Fig 2.1.

5.4 Conclusion

In conclusion this study found that:

1. MCs levels recorded along Nyanza gulf exceeded recommended WHO guide line levels of about 1µg/l.
2. Microcystin synthesizing gene (mcyE) andMicrocystin biodegrading gene(mlrA) genes, were expressed, thus release of the toxin to water bodies, hence posing danger to riparian communities.
3. The increased levels of serine-threonine PP1/2A in PLC patients could be attributed to the presence of microcystin in the Lake water being used by these patients.

5.5 Recommendations for Further Studies

1. Ways of getting rid of the microcystin levels detected need to be developed. This should include the removal of both intracellular and extracellure toxins
2. There should be sensitization of the riparian communities about the health risk that comes with consuming water contaminated with cyanobacteria and cyanotoxins. The county governments should carry out advocacy sessions in the riparian communities regarding the health risk of cyanotoxins from drinking water directly from the lake.

3. Further research needs to be carried out to find out the possible presence of synergistic, potentiation, antagonistic or additive effects of the co-existing bacteria; Mc-producing and MC-biodegrading.

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APPENDICES

Appendix I; Map of Lake Victoria, Nyanza gulf showing Sampling Stations.

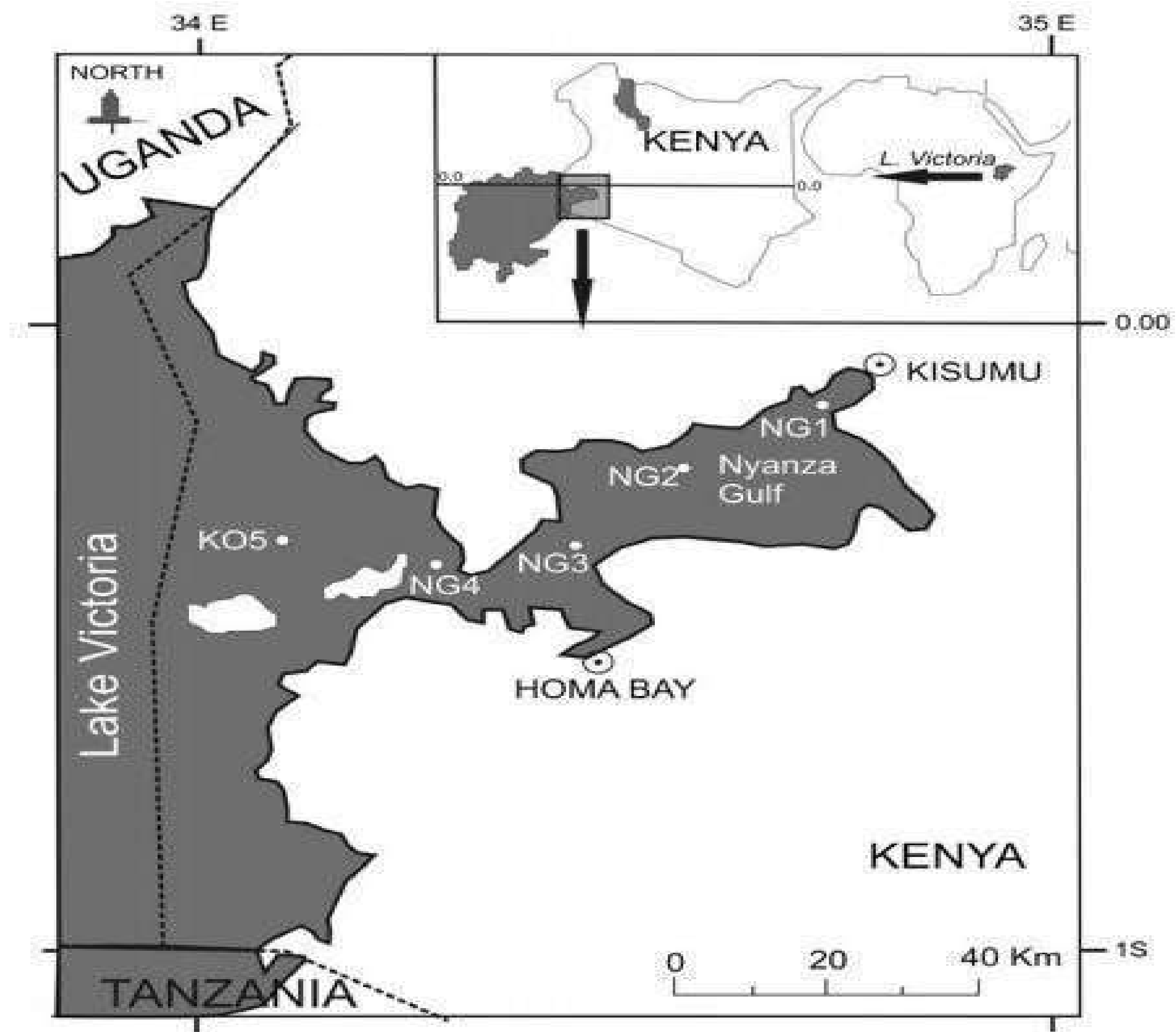

Fig3.1. Map of Lake Victoria Kenya sector, showing Nyanza gulf and the locations of the three sampling stations (NG1, NG2, NG3)

Appendix II: Letter of Consent.

RELATIONSHIP BETWEEN THE CONCENTRATION OF MYCROCYSTIN IN SERUM AND PRIMARY LIVER CANCER IN PATIENTS ATTENDING JOOTRH, JARAMOGI OGINGA ODINGA TEACHING AND REFERAL HOSPITAL, KISUMU KENYA.

This is a study being conducted by Chemutai Evaline of Maseno University to assess the association between the presence of microcystin in blood serum of a primary liver patient and the pathogenesis of primary liver cancer in liver cancer patients attending the Jaramogi Oginga Odinga Teaching and Referral Hospital in Kisumu County. The clients aged between 15-60 years old and are confirmed to be primary liver cancer positive are requested to participate in the study. This participation is entirely voluntary and you have a choice to withdraw at any stage during the study. This won't affect your health status in any way. It is entirely dependent on your will to consent to this study. Once you consent for participation, I will take your blood sample and medical history.To ensure confidentiality,your personal identity will not be included in the records. Relevant findings from this exercise will be provided to your current health care provider to facilitate your healthcare management. If you have understood what l have explained to you, l ask you to confirm and sign up for your participation in the study. Incaseyoudonotgiveconsentforparticipationtherewillbenopenaltyandnormalcare will proceed.

In case of need for further information about this study, please contact; **Chemutai Evaline Tel; 0724420565 or The secretary Maseno University Ethics Review Committee Tel. Numbers; 057-51622, 0722203411, 0721543976 or 0733230878.**

DECLARATION:

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any question that l have asked to have been answered to my satisfaction.

I have been explained the purpose of the study and l do understand the risk and benefits of this study. I hereby consent voluntarily on behalf of the subject to participate as a participant in this study. You are making decision whether to participate. Your signature indicates that you have decided to participate, having understood the information provided above.

Signature……………….....Date……………………Time……………………..

Relationship to the Subject………………………...

Appendix III: Study Questionnaire

QUESTIONNAIRE

My name is Evaline Chemutai.Iam a student from Maseno University and Iam carrying out research on association of Microcystin toxin found in blood sample and the occurrence of Primary Liver Cancer. I will appreciate your participation in this survey.

The information that you will provide will help the government and other stakeholders to plan,implement, monitor and evaluate programs/strategies on the ways to provide treatment and monitor the management of this toxin in Lake Victoria water.

(Kindly request the respondent to answer this questionnaire while you indicate his/her honest response either by ticking his/her option or by filling in the blanks giving as many details as possible.)

1. Where do you get your household water from?

LakeVictoria

Other (excluded from study)

(Research Assistant to obtain informed consent before proceeding to the next part.)

SECTION A: BACKGROUND INFORMATION (RA to give description)

i. Village…………………………………………

ii. Questionnaire no……………………………….

SECTIONB: SOCIO-DEMOGRAPHICINFORMATION

1. Gender……… (not to be asked, to be identified by research asst.)

Male

Female

2. What is your marital status?

Single

Married/Cohabit

Divorced/Widowed

3. What is the occupation of the household head?

Fisherman

Businessman

Farmer

Employed (government officers, NGOs)

Others. Please specify……………………….

4. How old are you? ....

SECTIONC: WATERUSEANDTREATMENT

1. Do you use the lake water for cooking?

Yes

No

2. Do you use the lake water for drinking?

Yes (go to question iii,)

No (go to question vi)

3. If you use Lake Water for drinking, do you treat it?

Yes (go to question iv,)

No (go to question v)

4. If you treat, how do you treat it?

Filtration

Boiling

Filtration and boiling

Chlorination (Waterguard, Aquaguard, Pur etc.)

Others. Please specify ……………………….

We have come to the end of this discussion.

Thank you once more for participating in this survey

Appendix IV: Research Approvals

MUERC

JOOTRH

NACOSTI

Appendix V: Cyanobacteria species viewed in water samples collected.

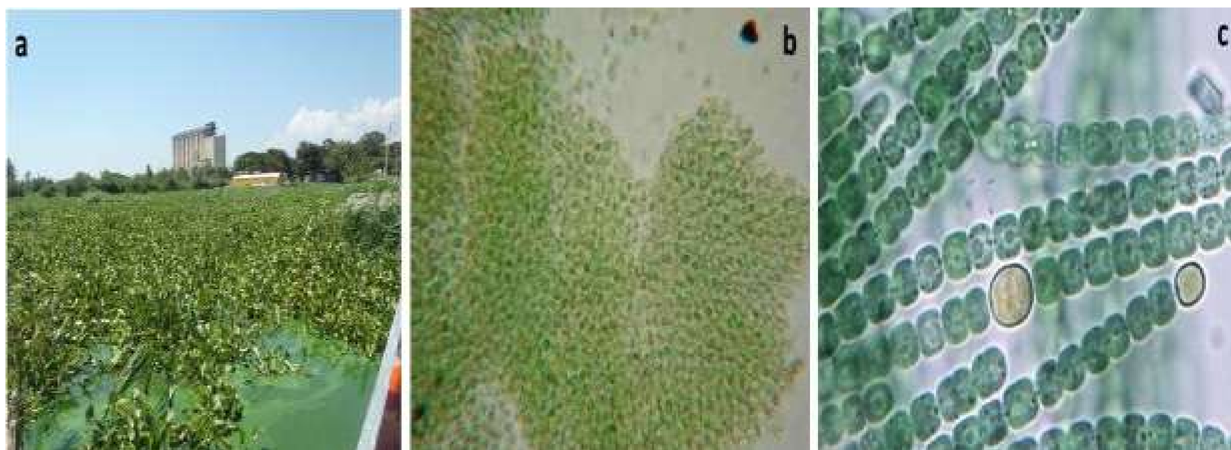

Fig. 4.1: Picture showing toxic cyanobacteria and water hyacinth clogging the KMFRI -Kisumu Pier; b) Microcystis aeruginosa as seen under a microscope c) Anabeana flosaquae as seen under a microscope (Picture courtesy of Onyango *et al*., 2018).

Appendix VI: Amount of Total RNA extracted from phytoplankton measured using Nanodrop.

Site	NucleicAcid ng/ul)	260/280	260/230	Sampletype
MbitaC	26.2	1.86	0.57	RNA
MbitaB	23.8	2.54	0.09	RNA
MbitaA	28.3	1.98	0.14	RNA
H/bayC	4	1.51	0.06	RNA
H/bayD	10.9	1.79	0.47	RNA
H/bayA	27.6	2.05	0.45	RNA
H/bayB	23.5	2.36	0.66	RNA
DungaA	22.3	2.54	0.09	RNA
DungaB	15.9	2.24	0.18	RNA
DungaC	2.6	1.83	0.1	RNA

Table 4.2: Total RNA extracted from each sample sites, represented in the column of OD ratio 260/280.

Appendix VII: Table 4.5; MeanCt values for 16sRNA

Site	CT1	CT2	CT3	Mean(RefG)	SD
MbitaA	2.01	1.73	1.95	1.90	0.15
MbitaB	2.09	1.23	2.19	1.84	0.53
MbitaC	2.11	1.56	2.01	1.89	0.29
H/bay A	1.62	1.81	1.77	1.73	0.10
H/bayB	1.41	1.72	2.6	1.91	0.62
H/bayC	1.51	1.6	1.58	1.56	0.047
H/bayD	1.48	1.51	1.56	1.52	0.04
DungaA	1.81	1.77	1.73	1.77	0.04
DungaB	1.05	2.73	1.86	1.88	0.84
DungaC	1.62	1.42	1.24	1.43	0.19

Appendix VIII: Total serine-threonine protein phosphates concentration PLC patients attending JOOTRH

Cases Sample	MeanOD (450)	STDEV	Absorbance	TotalSerine-threonine
1-PLC	1.27	0.254558	1.178	144.125
2-PLC	1.895	0.33234	1.803	222.25
3-PLC	1.7445	0.357089	1.6525	203.4375
4-PLC	1.2525	0.205768	1.1605	141.9375
5-PLC	1.291	0.312541	1.199	146.75
6-PLC	1.305	0.189505	1.213	148.5
7-PLC	0.346	0.009899	0.254	28.625
8-PLC	1.3645	0.287792	1.2725	155.9375
9-PLC	0.3985	0.036062	0.3065	35.1875
10-PLC	1.4255	0.314663	1.3335	163.5625
11-PLC	0.517	0.113137	0.425	50
12-PLC	0.5945	0.071418	0.5025	59.6875
13-PLC	2.4065	0.171827	2.3145	286.1875
14-PLC	0.209	0.046669	0.117	11.5
15-PLC	0.99	0.082024	0.898	109.125
16–PLC	0.222	0.096167	0.13	13.125
17-PLC	0.394	0.033941	0.302	34.625
18-PLC	0.6875	0.030406	0.5955	71.3125
19-PLC	0.216	0.043841	0.124	12.375

20-PLC	0.43	0.10748	0.338	39.125
Controls				
21-NPLC	0.3985	0.002121	0.3065	35.1875
22-NPLC	0.2535	0.095459	0.1615	17.0625
23-NPLC	0.74	0.118794	0.648	77.875
24-NPLC	0.397	0.038184	0.305	35
25-NPLC	0.2575	0.126572	0.1655	17.5625
26-NPLC	0.2675	0.082731	0.1755	18.8125
27-NPLC	0.38	0.019799	0.288	32.875
28-NPLC	0.5725	0.065761	0.4805	56.9375
29-NPLC	0.9025	0.566393	0.8105	98.1875
30-NPLC	0.2965	0.061518	0.2045	22.4375
31-NPLC	0.2715	0.140714	0.1795	19.3125
32-NPLC	0.333	0.02687	0.241	27
33-NPLC	0.2635	0.012021	0.1715	18.3125
34-NPLC	0.432	0.156978	0.34	39.375
35-NPLC	0.426	0.271529	0.334	38.625
36-NPLC	0.436	0.258801	0.344	39.875
37-NPLC	0.516	0.062225	0.424	49.875
38-NPLC	0.306	0.055154	0.214	23.625
39-NPLC	0.4995	0.091217	0.4075	47.8125
40-NPLC	0.3575	0.143543	0.2655	30.0625

Legend: PLC- Primary Liver Cancer; NPLC= Negative Primary Liver Cancer (Controls)