Antibiotic Resistance Profiling and Evaluation of Multiple Antibiotic Resistance (MAR) Index of Bacterial Isolates from Surface Water of Lake Lanao, Philippines

Nourshamsia C. Barosa
Department of Science and Technology VI, Magsaysay Village, La Paz, Iloilo City, Philippines
Email: barosanourshamsia@gmail.com

Beverly B. Amparado, Mariam C. Kabirun, and Anabella G. Villarino
Biology Department, CNSM, Mindanao State University, Marawi City, Philippines
Email: {amparadobevs, bellegerospe}@yahoo.com, mariamkabirun@gmail.com

***Abstract*—Antibiotic resistance was associated with clinical bacterial pathogens but not to environmental isolates. Thus, this study aimed to isolate, characterize, and determine the antibiotic resistance property of the bacteria obtained from Lake Lanao surface water using Filter paper disc diffusion method. A total of 99 distinct bacteria were isolated and screened for antibiotic resistance. Results showed that 54 were resistant to Cefuroxime, 36 to Cefalexin, 19 to Cotrimoxazole, 9 to CoAmoxiclav and none to Streptomycin. Intermediate resistance to Cotrimoxazole was 17, 11 to CoAmoxiclav, 9 to Cefuroxime, and 8 of the isolates to Cefalexin. Susceptibility was recorded to all bacterial isolates to Streptomycin, 79 to CoAmoxiclav, 63 to Cotrimoxazole, 55 to Cefalexin, and 36 to Cefuroxime. Evaluation of Multiple Antibiotic resistance (MAR) index of the isolates showed that 59.59% of the isolates have MAR index ≥ 0.2 which may indicate high risk sources of contamination such as human wastes and commercial poultry farms where antibiotics are often used while 40.41% have MAR index ≤ 0.2 indicating that the strain originated from animals in which antibiotics were seldom or never used. MAR index analysis of the four sampling sites however revealed low MAR value. The findings of this study can serve as a baseline information to monitor antibiotic resistance trends in the future and the need for controlled use of antibiotics and pollution monitoring programs around Lake Lanao.**

***Index Terms*—medical microbiology, antibiotic resistance, bacteria, Lake Lanao, MAR index**

I. Introduction

Man transforms nature. Likewise, man influences microbial community such as Lake Lanao ecosystem, an oligotrophic second largest lake in the Philippines. Lake Lanao is very important source of livelihood for most of the dwellers around the lake such as for domestic, agricultural, hydro-power generation, contact recreation, boating, laundry, bathing, ritual use, water sports, fishes for domestic consumption and livelihood.

In the previous study of Omar (2014), selected sampling stations in Lake Lanao were found to be contaminated with faecal coliforms such as *E. coli* and *Salmonella spp*. indicating the possible presence of pathogenic bacteria. Contamination of this water sources with faecal bacteria like *E. coli* is a serious problem due to its ability to transmit diseases and the risk associated with these bacteria may further increases if they are antibiotic resistant [1] which is a form of biotic pollution.

This present study aimed to investigate the presence of antibiotic resistant bacteria in Lake Lanao and the Multiple Antibiotic Resistance (MAR) index to differentiate bacteria from different sources using antibiotics that are commonly used for human therapy. MAR index provides useful information for the evaluation of health risk [2]. Determination of the multiple antibiotic resistance properties not only helps better understand the epidemiological aspects of antibiotic resistance related diseases, but also aids to decrease the risk of new disease outbreaks [2].

II. Methodology

A. Locale of the Study

Lake Lanao is located in the province of Lanao del Sur, in the heart of Mindanao at 701.35 meters above sea level. It has an area of 354.60 square kilometers and a mean depth of 60 meters, with the deepest part at 112 meters. The said lake lies between 8^{o} N Latitude and 124^{o} East Longitude [3].

B. Collection of Water Samples

Water samples were collected from the littoral zone of Lake Lanao along Wato, Bacolod-Kalawi, Tamparan and Taraka, Lanao del Sur. The coordinates of each sampling site were obtained using Global Positioning System (GPS) (GPS map 60 CSx, Garmin Company) and plotted in

Manuscript received December 5, 2019; revised March 29, 2020.

doi: 10.18178/ijpmbs.9.2.75-80

Google GPS visualizer.com/map to view the exact map and location of sampling sites (Fig. 1).

Figure 1. Map of the Philippines showing Lanao del Sur region where Lake Lanao and the four study sites is located. (http://www.gpsvisualizer.com/map?output_google. Accessed 16 December 2017).

One transect line was established for each sampling site from the shoreline towards the middle part of the lake and three stations were employed five meters away from each other using a calibrated rope. Using a 100 mL sterile bottle, three water sub-samples were collected from every station within one-meter depth from water surface and then mixed as a composite sample and immediately transported to the Microbiology laboratory, Biology Department, Mindanao State University-Marawi for immediate processing.

C. *Isolation of Bacterial Colonies*

Spread plate method was used to isolate bacterial colonies. One mL of collected water samples was diluted in 9 ml 0.9% saline solution using sterile pipette and was shaken to make 10^{-1} dilution. 0.1 mL of the 10^{-1} dilution was then inoculated to the previously prepared sterile Nutrient Broth Yeast Extract (NBYE) plate. Sterile L rod was used to spread the water sample evenly. Finally, plates were inverted and wrapped with paper and were then incubated at 28°C for 24 - 48 hrs.

D. *Isolates Characterization*

Isolates were characterized according to colony morphology, opacity, color, texture, luster, motility, Gram stain reaction, cell shape and cell arrangement, form of growth in nutrient broth and the form of growth in nutrient agar slant and were described according to Bensons and Brown (2001) and Mabuhay-Omar (2012) Microbiology Laboratory Manual.

E. *Antibiotic Resistance Test*

Filter paper disc diffusion method was used to determine the antibiotic resistance isolates. Twenty four-hour broth culture of the isolates were standardized for antibiotic susceptibility test. The turbidity of the broth culture was adjusted using sterile nutrient broth to match 0.5 McFarland standards [ca 10^8 cfu/ml^{-1}) (CLSI, 2015). Five test antibiotics were used to test the antibiotic resistance character of the isolates namely: Cefuroxime, Cotrimoxazole, Cefalexin, Co-Amoxiclav, and Streptomycin. Ten microliters of this test antibiotic were impregnated into the sterile 6 mm disc using sterile micropipette tips and were placed unto the previously swabbed MHA plate prepared in triplicates. After incubation at 28°C for 18-24 hours, the zone of inhibition was measured using a metric ruler. [4]. The isolates were then classified as resistant, intermediate or susceptible based on the Zone of Inhibition (ZOI) and the recommendation of the 2015 Clinical and Laboratory Standards Institute M02-A12 (CLSI, 2015) (Table I).

TABLE I. ZONE DIAMETER INTERPRETIVE CRITERIA (IN MM) FOR THE FIVE TEST ANTIBIOTICS

Test antibiotics	Sensitive	Intermediate	Resistant
Co-Amoxiclav	≥18	14-17	≤13
Cefuroxime	≥ 23	15-22	≤ 14
Streptomycin	≥ 15	12-14	≤ 11
Cotrimoxazole	≥ 26	23-25	≤ 22
Cefalexin	≥ 26	23-25	≤ 22

Adapted in part from the CLSI document M100-S23 (MO2-A11).

F. *Determination of the Multi Drug Resistance (MDR) Character of the Isolates*

Multi-Drug Resistance is defined as non-susceptibility or resistance to at least one agent in three or more antimicrobial categories [5]. The MDR character of the isolates was therefore identified by observing the resistance pattern of the isolates against the five antibiotics used. In the present study, three classes of antimicrobials were used: β-lactams represented by Cefuroxime, Cephalexin and Co-Amoxiclav; Aminoglycosides represented by Streptomycin, and Sulfonamides represented by Cotrimoxazole. Hence, resistance to the three classes of antibiotics used will be classified as Multi-drug Resistant (MDR) isolates.

G. *Determination of the Multiple Antibiotics Resistance (MAR) Index of the Isolates and the Sampling Sites*

The MAR index of an isolate is defined as a/b, where *a* represents the number of antibiotics to which the isolate was resistant, and *b* represents the number of antibiotics to which the isolate was subjected [6]. For all the isolates, MAR index values will be calculated as a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the number of antibiotics the isolate was tested against. MAR index of the site however will be calculated by dividing the average resistance index of all isolates from the site and the product of the number of antibiotics tested to the number of isolates from the site [6].

III. RESULTS AND DISCUSSIONS

A total of 99 distinct bacterial colonies were isolated from the surface water of Lake Lanao along four sampling sites. Twenty-five bacterial isolates were obtained from Wato, 27 from Bacolod-Kalawi, 24 from Tamparan, and 23 from Taraka, Lanao del Sur.

Microscopic examinations of the isolates showed that 45.45% were Gram negative while 54.54% were Gram positive, from these, 60.60% of the isolates were bacilli whereas 28.28% were cocci. There were Gram- coccobacilli, Gram+ diplobacilli, Gram+ diplococci, Gram- streptobacilli, Gram+ streptobacilli, Gram+ sheathed, and Gram- filamentous (Fig. 2).

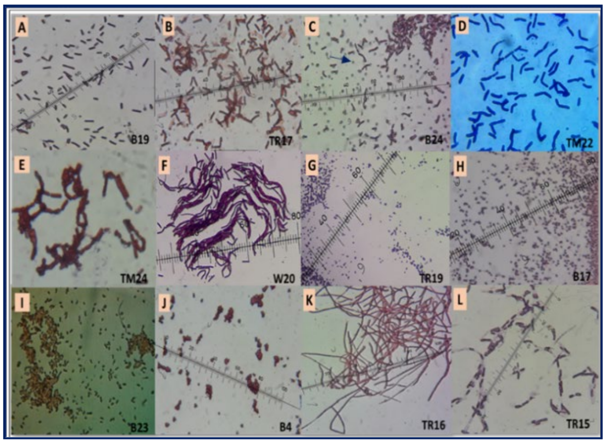

Figure 2. Different microscopic features of the isolates observed showing: A. Gram+ bacilli, B. Gram- bacilli, C. Gram-bacilli with circular endospores D. Gram+ diplobacilli, E. Gram- streptobacilli, F. Gram+ streptobacilli. G. Gram+ cocci, H. Gram- cocci, I. Gram- coccobacilli, J. Gram- cocci (solitary and in clusters), K. Gram- filamentous, and L. Gram+ sheathed viewed under 1000X magnification.

A. *The Antibiotic Susceptibility Profile of the Isolates*

Wato Bacterial Isolates. Seven of the 25 isolates were observed to be resistant to Cefalexin and Cefuroxime. On the other hand, the isolates were mostly susceptible to Streptomycin, Cotrimoxazole and Co-Amoxiclav. None of the isolates showed resistance to Streptomycin. The resistance pattern CFXCF were the most common and was exhibited by seven isolates. One isolate exhibited resistance to three different antibiotics with resistance pattern of CFXCFCOA. Two isolates were resistant to Cefalexin and Cotrimoxazole with resistance pattern of CFCOT.

Bacolod-kalawi Bacterial Isolates. Most of the isolates were observed to be resistant to Cefuroxime. On the other hand, the isolates were most susceptible to Streptomycin, Co-Amoxiclav, Cotrimoxazole and to Cefalexin. For two antibiotics, three exhibited resistance to Cefuroxime and Cotrimoxazole with resistance pattern of CFXCOT, two were resistant to Cefuroxime and Cephalexin having resistance pattern of CFXCF and one isolate was resistant to Cefuroxime and Co-Amoxiclav having resistance pattern of CFCOA. The resistance pattern CFX was the most common.

Tamparan Bacterial Isolates. Most of the isolates were observed to be resistant to Cefuroxime and Cefalexin. On the other hand, the isolates were most susceptible to Streptomycin and Co-Amoxiclav. Resistance to four antibiotics with resistance pattern of CFXCFCOTCOA was observed for one isolate. Six isolates were observed to have resistance to three antibiotics, with the resistance pattern of CFXCFCOA exhibited by four isolates and CFXCFCOT exhibited by the two isolates. Seven isolates exhibited resistance for two antibiotics, of which four exhibited resistance to Cefuroxime and Cefalexin with resistance pattern of CFXCF, two were resistant to Cefuroxime and Cotrimoxazole having resistance pattern of CFXCOT and one were resistant to Cefalexin and Cotrimoxazole having resistance pattern of CFCOT. The resistance pattern CFXCF was the most common.

Taraka Bacterial Isolates. Most of the isolates were observed to be resistant to Cefuroxime while most of the isolates were susceptible to Streptomycin, Co-Amoxiclav and lastly to Cefalexin. All of the isolates were observed to be susceptible to Streptomycin. Resistance to three antibiotics with resistance pattern of CFXCFCOA and CFXCFCOT was observed for two isolates. Six isolates exhibited resistance for two antibiotics, of which two exhibited resistance to Cefuroxime and Cotrimoxazole with resistance pattern of CFXCOT, two were resistant to Cefuroxime and Cefalexin having resistance pattern of CFXCF, and two were resistant to Cefalexin having resistance pattern of CFCOT. The resistance pattern CFXCF were the most common.

About 40-70.83% of the isolated bacteria from the four sampling sites were resistant to Cefuroxime followed by Cefalexin (22.22-50%), then Cotrimoxazole (8-25%) and lastly to Co-Amoxiclav (4-20.83%). None showed resistance towards Streptomycin. Tamparan sampling site had the highest number of resistant isolates followed by Taraka, then Bacolod-Kalawi and lastly in Wato (Fig. 3).

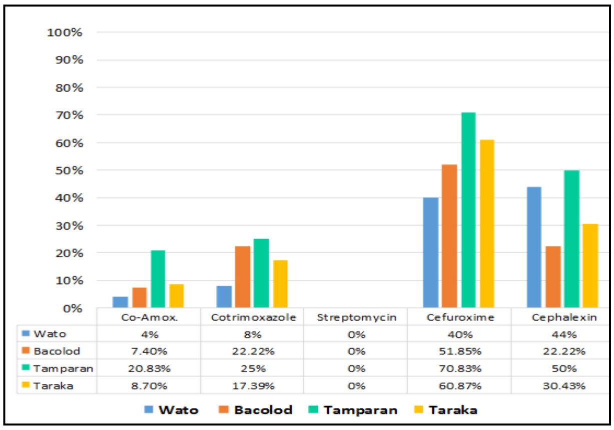

Figure 3. Comparison of the percentage resistant isolates to the Five test antibiotics and in the four sampling sites.

According to Col and O'Connor (1987), antibiotic resistance in aquatic environments is caused by the introduction of antibiotics into the aquatic environments via medical therapy, agriculture and animal husbandry and has resulted in selective pressure on bacterial populations. The use of antibiotics in the treatment of diseases and as growth promoters in farm animals, and the use of antibiotics for treating humans and other factors have also been linked to the development of resistant microorganisms [1], [7].

In the study of Mudryk (2002) in Lake Gardno, the highest bacterial resistance was noted in the cases of ampicillin, clindamicin, cloxacillin, penicillin, sulfamethoxazole, tetracycline, and trimethoprim, while at the same time the bacteria were most sensitive to gentamycin, neomycin, rimfapicin and streptomycin. Also, in other water bodies, both freshwater [7] and marine water [8], a high level of bacterial resistance to ampicillin, penicillin and tetracycline was noted with a simultaneous high sensitivity to gentamycin and streptomycin. Comparatively, results of this study also revealed that bacterial isolates in Lake Lanao were all sensitive to Streptomycin and some of the isolates were resistant to Cotrimoxazole.

B. Multi-drug Resistance (MDR) Character of the Isolates

Results showed that majority of the isolates were resistant to β-lactams group represented by Cefuroxime, Cephalexin, ad Co-Amoxiclav whereas no isolates from the four sampling sites showed resistance to Aminoglycosides represented by Streptomycin. Resistance to Sulfonamides represented by Cotrimoxazole was also observed (Table II).

TABLE II. CLASSIFICATION OF ANTIBIOTICS USED, ITS MECHANISM OF ACTION AND THE PERCENTAGE OF RESISTANT ISOLATES AT THE FOUR SAMPLING SITES

Antibiotic	Class	Mechanism of action	Resistant isolates
Cotrimoxazole	**Sulfonamides**	Inhibitor of folate synthesis	**19 (19.19%)**
Cefuroxime	**β-lactams**	Inhibitor of cell wall synthesis	**54 (54.54%)**
Cephalexin	**β-lactams**	Inhibitor of cell wall synthesis	**36 (36.36%)**
Co-Amoxiclav	**β-lactams**	Inhibitor of cell wall synthesis	**9 (9.09%)**
Streptomycin	**Aminoglycosides**	Inhibitor of protein synthesis	**0 (0%)**

Resistance to β -lactams in many bacteria is usually due to the hydrolysis of the antibiotic by a β-lactamase or the modification of PBPs or cellular permeability. The mechanisms of β-lactam resistance include inaccessibility of the antibiotics to their target enzymes, modifications of target enzymes, and/or direct deactivation of the antibiotics by β-lactamases [5].

In this study, resistance to β-lactams and Cotrimoxazole was observed, however, all of isolates from the four sampling sites did not exhibit resistance towards Streptomycin as representative of Aminoglycoside group. Since this study was limited only to three classes of antimicrobials (due to unavailability of other antibiotics that would represent the other class or categories) and resistance to Streptomycin as representative of Aminoglycoside group was not observed, hence, Multi Drug Resistance (MDR) character of the isolates was not confirmed.

C. The Multiple Antibiotic Resistance (MAR) Index of the Isolates in the Four Sampling Sites

The MAR index of the four sampling sites was calculated to assess the level of health risk associated with the resistant bacteria that were isolated from the four selected sampling sites. The results and implications of the MAR indices of the four sampling sites were discussed in the succeeding sections:

*Wato***.** Evaluation of Multiple Antibiotic Resistance (MAR) index of the isolates showed MAR value ranging from 0-0.6 with average index of 0.184. Twelve isolates (48%) has MAR index < 0.2, suggesting the origin of this strain was from animals in which antibiotics are seldom used. On the other hand, 13 (52%) of the isolates has MAR index of ≥0.2, which may suggest that these strains come from an environment where several antibiotics are often used. Wato (Fig. 4a) having a population of 29,180 people and a District Hospital as well as home cattle and carabao and other livestock products for grazing and pasture (https://en.wikipedia.org/wiki/Balindong_Lanao_del_Sur. Accessed, 10 March 2015) may have contributed to the presence of antibiotic resistant bacteria in Lake Lanao along this area. MAR index of the site was however calculated to be low with MAR value of 0.00147.

Bacolod-kalawi. Computed MAR index of the isolates from Bacolod-Kalawi ranged from 0-0.4 with average index of 0.184. Twenty-six (66.67%) of the bacterial isolates has MAR index of ≥0.2, while 33.33% of the isolates have MAR index less than 0.2. The computed MAR index of the site however was low with MAR value of 0.0014. Low MAR index of Bacolod-Kalawi (Fig. 4b) could be attributed to less anthropogenic activities in the area with the absence of residential houses in the sampling site.

Figure 4. The four sampling sites showing: (a) Wato sampling area (b) Bacolod-kalawi sampling area (c). Taraka sampling area and (d). Tamparan sampling area.

Tamparan. Computed MAR index of the isolates from Tamparan ranged from 0-0.8. Large percentage of the isolates (83.33%) have MAR value of ≥0.2 while 16.67% of the isolates have MAR index less than 0.2. The average index was also high with computed value of 0.35. High MAR index value of bacterial isolates from Tamparan (Fig. 4c) could be attributed to the presence of residential houses within and around the sampling area as well as different anthropogenic activities observed. Another factor could be the presence of the district hospital and poultry farm in the municipality. (http://tamparan.weebly.com/profile.html. Accessed date, March 10, 2017). The computed MAR index of the site was however low with 0.00292 MAR value.

Taraka. Computed MAR index of the isolates from Taraka ranged from 0-0.6. Sixteen (69.56%) of the isolates have MAR value of ≥0.2, while seven (30.43%) of the isolates have MAR index less than 0.2. Computed MAR index of the site was however low with 0.00204 MAR value. The low value of MAR index in Taraka (Fig. 4d) may be due to less anthropogenic activities observed in the area.

In the present study, the calculated MAR index from the four sampling sites were all low, hence, there is no significant antibiotic pollution or contamination in the four sampling areas. Comparatively, higher MAR index value for isolates and site was observed in Tamparan compared to the other three sites. It may be due to the presence of the district hospital and poultry farm in the municipality as well as uncontrolled and improper waste disposal activities compared to the other sites.

Notably, MAR indices value observed at Taraka and Tamparan (situated on eastern part of the Lake Lanao) were higher compared to Wato and Bacolod kalawi (situated in the western part of the Lake). It may be because the sampling area in these two sites were exposed to many residential houses and consequently, higher anthropogenic activities and pollution were made. Further, the river tributaries in the eastern side of the lake may have contributed in the differences of the computed MAR indexes of the four sites.

This study agrees with the result of Omar (2014) where it was shown that the selected sampling stations in Lake Lanao were already contaminated with fecal coliforms such as *E. coli* and *Salmonella* spp. Tambekar *et al.* (2007) also reported high MAR index due to human and non-human faecal contamination, of surface, ground and public supply water sites in Akola and Buldhana inn Maharashtra, India. Chatterjee *et al.* (2012) noted that drinking water sources of Uttarakh, India were contaminated with high MAR index *E. coli* originating from potential risk sources.

IV. Conclusion and Recommendations

Isolated bacteria from Lake Lanao exhibited antibiotic resistance and were mostly resistant to Cefuroxime and Cefalexin under Beta Lactams class. High resistance was observed for Cefuroxime, Cefalexin, and Cotrimoxazole while high susceptibility was observed for Streptomycin and Co-Amoxiclav. Multiple antibiotic resistance index ranged from 0.0-0.8 (that is resistant to 1-5 different antibiotics). Majority of the isolates were resistant to one and two antibiotics, but the resistance pattern of Cfx, CfxCf, and CfxCfCot were the most common. The findings of this study can serve as baseline information on antibiotic resistance of bacteria in Lake Lanao to monitor trends in the future. Further studies are necessary to establish the role of antibiotic substances in the control of lake bacterial populations.

Evaluation of the Multiple Antibiotic Resistance (MAR) index of the bacterial isolates from the four sampling sites revealed resistant strains with MAR value of >0.2 which suggest that these strains must have originated from high risk sources of contamination such as commercial poultry farms, cattle and swine where antibiotics are often used and that the contamination also originated from human wastes. Evaluation on the MAR index of the four sampling sites however revealed that MAR value was low (<0.2). This suggests that the four sampling sites were not significantly exposed to frequent antibiotic use and the associated health risk is low. Tamparan had relatively the highest MAR index followed by Taraka, then Wato and lastly by Bacolod-kalawi bacterial isolates. The findings of this study can serve as a baseline information to monitor antibiotic resistance trends in the future and the need for controlled use of antibiotics and pollution monitoring programs around Lake Lanao.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Ms. Nourshamsia C. Barosa and Ms. Mariam Kabirun conducted the research; All of the authors contributed in the writing of the final paper and all authors had approved the final version.

Acknowledgement

The authors would like to thank Dr. Cesar C. dela Seña, Dr. Fema M. Abamo and Dr. Carmelita G. Hansel, for the insights and discernments in revising the paper and the Biology Department, CNSM, Mindanao State University-Marawi for the equipment and technical assistance. Special thanks to Prof. Camar P. Ameril, Maria Lina B. Gelonga, and Maria Bernarica B. Fusin for the support. This work was supported in part by a grant from National Research Council of the Philippines (NRCP).

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Nourshamsia C. Barosa was born in Isian Norte Leon, Iloilo Philippines on January 15, 1990. She finished her Master of Science major in Biology at Mindanao State University, Marawi City, Philippines and was awarded as Best in Thesis, Lake Lanao Research Advocate Awardee, and Distinguished Student in the Field of Research. She is currently working as Science Research Analyst at the Department of Science and Technology Region VI where she conducts researches under the DOST Halal S&T Program. She was a member of Philippine Society of Microbiology (PSM) and National Research Council of the Philippines (NRCP). Her research interest includes food and medical microbiology, ecology, and biotechnology.

Beverly B. Amparado was born in Poblacion, Aurora Zamboanga del Sur, Philippines on November 11, 1967. She finished her Masters and PhD Major in Biology at MSU-Iligan Institute of Technology, Iligan City. She is currently working as Professor IV at the Biology Department, College of Natural Sciences and Mathematics (CNSM), Mindanao State University, Marawi City. She is an associate member of the National Research Council of the Philippines (NRCP), member of Philippine Society for Microbiology (PSM), Federation for Institutions for Marine and Freshwater Sciences (FIMFS), and Biology Teachers Association (BIOTA).

Mariam C. Kabirun was born in Pangutaran, Jolo, Sulu Philippines on December 15, 1985. She finished her Master of Science major in Biology at Mindanao State University, Marawi City Philippines where she was currently working as Biology Instructor. She was a member of Philippine Society of Microbiology (PSM) and Mycological Society of the Philippines (MSP). Her research interest includes microbiology, ecology, and biotechnology.