A PRELIMINARY INVESTIGATION ON THE EMERGENCE OF ANTIBIOTIC RESISTANT BACTERIA RESULTING FROM INAPPROPRIATE USE OF ANTIBIOTICS IN THE PURIFICATION OF ALGAL CULTURES

Peekate, L. P.[1]*, and ***Abu,*** *G. O.*[2]

[1]*Department of Applied and Environmental Biology, Rivers State University of Science and Technology, P. M. B. 5080, Port Harcourt, Nigeria.*

[2]*Department of Microbiology, University of Port Harcourt, P. M. B. 5323, Port Harcourt, Nigeria.*

Phone no.: 08063353116.

Abstract: Cultures of algal species are often needed for research purposes. These cultures are frequently accompanied by bacteria that are picked up from the environment together with the algal cells, thus the practice of purifying algal cultures using antibiotics. Water sample from fish pond having algal bloom was inoculated into BG-II medium supplemented with 62.5 ug.mF Chloramphenicol and 100 ug.ml-[1] Nystatin. The total heterotrophic bacterial count before the addition of the antibiotics was $3.8xl0^4$cfu.ml[1]. The bacterial population decreased to an average count of $3.5xl0^2$ cfu.ml[1], after a period of 24 hours. This population was made up of three distinct colonies. Colonial morphology and preliminary tests based on physicochemical characteristics gave a probable identification of the isolates as *Bacillus, Proteus,* and *Pseudomonas.* The *Bacillus* showed resistance to Chloramphenicol and Flucloxacillin; *Proteus* showed resistance to Chloramphenicol, Flucloxacillin, Lincomycin, Erythromycin, Ampicilin, Ampiclox, and Septrin; *Pseudomonas* showed resistance to all antibiotics used in the investigation with exception to Ciprofloxacin, Streptomycin, and Rifampin, which were effective on the three isolates. The results indicate that certain bacteria that are present in algal cultures can become resistant to several antibiotics as a result of purification of algal cultures using antibiotics at sub-lethal dose. It is suggested that screening for effective antibiotic(s) and determination of their minimum inhibitory concentration or minimum lethal concentration, as the case may be, against identified bacteria contaminants be part of the procedure for setting up pure cultures of algae.

Key words: Antibiotics, *Bacillus, Proteus, Pseudomonas,* Purifying algal cultures, Resistance to Chloramphenicol,

INTRODUCTION

Algal species occur in the environment alongside other organisms and microorganisms. Some have been shown to be associated with specific bacteria in their natural environment (Sapp *et al.*, 2004).

*Corresponding author:
peekatepedro@gmail.com ***Peekate, L. P.1****,

Algal cultures are thus frequently accompanied by bacteria that are picked up from the environment. A mixture of bacteria and algal cells in an algal culture may be beneficial or harmful to the algal population. The bacteria may be harmful by attacking the algal cells directly or indirectly by using up the available nutrients. *Pseudomonas fluorescens, Bacillus cereus,* and fungi such as *Chytridium polysiphoniae, Pontisma lagenidioides,* and *Labyrinthula* sp. have been shown to attack

freshwater and marine algae directly (Raghukumar, 1987a; Raghukumar, 1987b; Kim *et al.*, 2007; Kataev *et al.*, 2012). Contamination of algal cultures with these biological agents will lead to a decrease in algal productivity and an increase in the cost of maintaining the algal cultures. It is thus sometimes necessary to control the bacterial population in algal cultures. Elimination of bacteria in algal cultures is usually carried out using antibiotics (Droop, 1967; Kooistra *et al.*, 1991; Cottrell and Suttle, 1993; Coutteau, 1996; Anaga and Abu, 1996; Wang *et al.*, 2004). Broad spectrum antibiotics and a wide range of narrow spectrum antibiotics have been used at differing concentrations. Droop (1967) suggested the use of a mixture of specific amounts of three to four broad spectrum antibiotics, and a twofold dilution procedure whereby the final effective concentration of the antibiotic mixture against the total bacterial population will be determined. Kooistra *et al.*, (1991) used Cefotaxime, a narrow spectrum antibiotic. The minimum concentration of the antibiotic against the total bacterial population, which allowed for healthy growth of the alga studied, was determined to be 100 $\mu g.ml^{-1}$. Cottrell and Suttle (1993) used Penicillin G, Neomycin, Gentamician, and kanamycin at bactericidal concentrations worked out for identified bacteria contaminants. Anaga and Abu (1996) used Chloramphenicol (62.5 $\mu g.ml^{-1}$), Nystatin (100 $\mu g.ml^{-1}$), and Cycloheximide (100 $\mu g.ml^{-1}$) to successfully grow an algal species free of bacteria contaminants. Wang *et al.*, (2004) used a mixture of Penicillin G and Streptomycin. The minimum concentration of the antibiotic mixture that inhibited the total bacterial population in their algal cultures and allowed for healthy growth of the algal cells was determined to be 100 $unit.ml^{-1}$. The use of antibiotics to purify algal cultures can lead to the emergence of antibiotic resistant bacteria, just as has occurred in fish aquacultures where antibiotics are used for prophylactic and growth promotion purposes (McPhearson *et al.*, 1991; Spanggaard *et al.*, 1993; Serrano, 2005; Hoa *et al.*, 2011). Antibiotic resistant bacteria may arise as a result of using antibiotics which are not effective in eliminating the total bacterial population or the use of effective antibiotics at sub-lethal concentrations. This practise will in the long run pose a health risk to humans.

This study was carried out as a preliminary investigation on the emergence of antibiotic resistant bacteria resulting from the purification of algal cultures using sub-lethal dose of antibiotics.

MATERIALS AND METHODS

Antibiotic stock solutions

An 80 ml stock solution of 6.25 $mg.ml^{-1}$ Chloramphenicol and 100 ml stock solution of 10 $mg.ml^{-1}$ Nystatin was prepared. The Chloramphenicol stock solution was prepared by weighing 0.5 g of Chloramphenicol, in powdered form, into 80 ml sterile distilled water. The Nystatin stock solution was prepared by weighing 1.0 g of ground Nystatin tablets into 100 ml sterile distilled water. Both stock solutions were stored at 4 ^0C prior to use.

Culture medium

BG-II medium (Kirrolia *et al.*, 2012) supplemented with 62.5 $\mu g.ml^{-1}$ Chloramphenicol and 100 $\mu g.ml^{-1}$

Nystatin was used as the algal culture medium. Nutrient agar supplemented with 62.5 $\mu g.ml^{-1}$ Chloramphenicol and 100 $\mu g.ml^{-1}$ Nystatin was used in the detection and isolation of antibiotic resistant bacteria intimately associated with the algal cultures. Isolated antibiotic resistant bacteria were then propagated using nutrient agar free of antibiotics.

Sample collection

Water sample was collected from a fish pond experiencing algal bloom. The fish pond is among the several ponds located at the African Regional Aquaculture Centre (ARAC) in Aluu, near the University of Port Harcourt, Nigeria. The sample was collected from the pond surface (top 0.0 – 0.3 m), with the aid of sterile glass water bottles.

Experimental setup

About 20 ml of the water sample was added to 176 ml of sterile BG II medium in a 250 ml conical flask, followed by addition of 2 ml each of the antibiotic stock solutions. The total volume which amounted to 200 ml thus contained Chloramphenicol and Nystatin at a concentration of 62.5 $\mu g.ml^{-1}$ and 100 $\mu g.ml^{-1}$ respectively. The flask was plugged with cotton wool, and air bubbled into its content at the rate of 150 bubbles per minute with the aid of an aquarium pump. Three flasks of this setup were prepared and incubated for about 24 hrs in a light chamber at ambient temperature. The light chamber contained two white fluorescent tubes, 2 ft in length, stationed at about 2 ft high from the work surface. The chamber also had openings for ventilation.

Enumeration of bacterial population

The total heterotrophic bacterial population of the water sample was enumerated using the spread plate count method. The count obtained was used to calculate the cfu/ml of culture broth, resulting from the addition of the inoculum and the antibiotic solutions into the BG-II medium.

After 24 hours of putting up the algal culture setup, the total heterotrophic bacterial population in them was enumerated.

Isolation of antibiotic resistant bacteria

Bacterial colonies from the algal culture setup that developed on agar plates enumerated after a period of 24 hours were transferred onto nutrient agar plates containing 62.5 $\mu g.ml^{-1}$ Chloramphenicol and 100 $\mu g.ml^{-1}$ Nystatin. The plates were incubated for 24 hrs at 35 ^{0}C. Colonies that developed were transferred to nutrient agar plates free of antibiotics. The colonies were described and identified using conventional microbiological methods.

Antibiotic sensitivity testing of resistant bacteria

The resistant bacteria isolates were subjected to antibiotic sensitivity testing using the disc method. Broad spectrum and narrow spectrum antibiotics which include Ciprofloxacin, Gentamycin, Rifampin, Ofloxacin, Peflacine, Ceporex, Chloramphenicol, Lincomycin, Streptomycin, Flucloxacillin, Erythromycin, Norfloxacin, Ampiclox, Augmentin, Nalidixic acid, Septrin, and Ampicilin were used for the sensitivity testing.

RESULTS

The water sample collected from the fish pond was light greenish in colour. This feature suggested bloom of green algae (*Chlorophyta*).

The average total heterotrophic bacterial count of the water sample was 3.8×10^5 cfu.ml^{-1}. Using this count to calculate the count that would be present in the algal culture setup as a result of adding 20 ml water sample (inoculum) and the antibiotic solutions (2 ml each) into the BG-II medium (176 ml) resulted in a count of 3.8×10^4 cfu.ml^{-1}. This count thus represented the total heterotrophic bacterial count of the algal culture setup on day 1.

Calculation: Bacterial population in the water sample = 3.8×10^5 cfu.ml^{-1}. Thus bacterial population in the algal culture setup which is made up of 20 ml of the water sample, 176 ml BG-II medium, and 2 ml each of the antibiotics solution totalling a volume of 200 ml = (3.8×10^5 cfu.ml^{-1} x 20 ml) / 200 ml = 3.8×10^4 cfu.ml^{-1}.

On day 2, the total heterotrophic bacterial count of the algal culture setup dropped to an average of 3.5×10^2 cfu.ml^{-1}.

The enumerated bacterial population of the algal broth culture supplemented with the antibiotics was made up of three distinct colonies. These colonies were able to grow on nutrient agar plates containing 62.5 µg.ml^{-1} Chloramphenicol and 100 µg.ml^{-1} Nystatin. The colonial morphology and the results of preliminary biochemical and physicochemical tests carried out on them indicated that they belonged to the genera; *Bacillus*, *Proteus*, and *Pseudomonas*.

Preliminary physicochemical tests carried out on the colonies suspected to be *Pseudomonas* revealed that they are catalase and oxidase positive, and Gram negative motile rods. The colonies produced a yellowish-green to bluish-green pigment which diffused into the agar medium. This has been cited to be a distinctive feature of *Pseudomonas aeruginosa* (Stanier *et al.*, 1977), and the pigment is known as pyocyanin. The results of the tests and the production of pyocyanin by the colonies is a plausible indication that they are actually *Pseudomonas*.

The colonies suspected to be *Bacillus* were Gram positive motile rods that reacted positive to catalase and oxidase test. Microscopic view of a colony, from a week old culture plate, stained with malachite green and safranin revealed rod shaped cells having endospores.

Preliminary physicochemical tests carried out on the colonies suspected to be *Proteus* revealed that they are catalase positive, oxidase negative, and Gram negative motile rods. The colonies appeared as ripples spreading out on the surface of water. This has been cited to be a characteristic feature of colonies of *Proteus* (Stanier *et al.*, 1977). The results of the tests and this colonial characteristic are a plausible indication that the colonies belong to *Proteus* sp.

The result obtained from the antibiotic sensitivity testing on the resistant isolates is presented in Figure 1. From the Figure it can be seen that *Bacillus* showed resistance to Chloramphenicol and Flucloxacillin; *Proteus* showed resistance to Chloramphenicol, Flucloxacillin,

Lincomycin, Erythromycin, Ampicilin, Ampiclox, and Septrin; *Pseudomonas* showed resistance to all antibiotics used in the investigation with exception to Ciprofloxacin, Streptomycin, and Rifampin, which were effective on the three isolates.

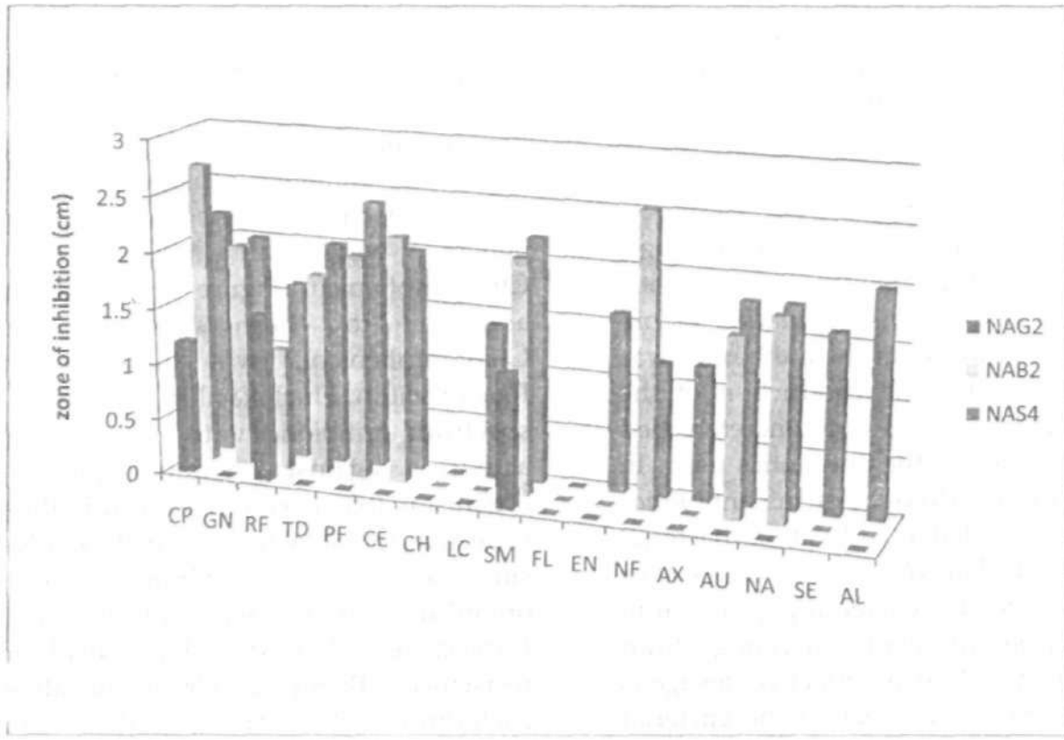

Fig. 1: Antibiotic sensitivity of the resistant bacteria. CP – Ciprofloxacin (10 µg), GN – Gentamycin (10 µg), RF – Rifampin (10 µg), TD – Tarivid (Ofloxacin; 10 µg), PF – Peflacine (10 µg), CE – Ceporex (10 µg), CH – Chloramphenicol (20 µg), LC – Lincomycin (30 µg), SM – Streptomycin (30 µg), FL – Floxapen (Flucloxacillin; 30 µg), EN – Erythromycin (30 µg), NF – Norfloxacin (30 µg), AX – Ampiclox (30 µg), AU – Augmentin (30 µg), NA – Nalidixic acid (30 µg), SE – Septrin (30 µg), AL – Ampicilin (30 µg), NAG2 – *Pseudomonas*, NAB2 – *Proteus*, NAS4 – *Bacillus*.

DISCUSSION

The use of antibiotics at sub-lethal dose will invariably eliminate some members of the targeted bacterial population. The remnant of the population that survives will develop resistance to that antibiotic (Prescott *et al.*, 1999), and may also develop resistance to other antibiotics as well. Sometimes in biological research where antibiotics are needed to eliminate a particular population or detect a mutant, the antibiotics may be used at concentrations considered to be effective so as to evade the cumbersome work involved in antibiotic sensitivity testing and determination of the minimum lethal concentration. This practise has

been observed in research with algal cultures where the need arises to eliminate other microorganisms (Anaga and Abu, 1996; Wang *et al.*, 2004). The use of antibiotics at sub-lethal dose in the purification of algal cultures could result in the emergence of antibiotic resistant bacteria.

The concentration of the antibiotics (62.5 $\mu g.ml^{-1}$ Chloramphenicol and 100 $\mu g.ml^{-1}$ Nystatin) used in this study did not eliminate the entire bacterial population, but reduced the population considerably. Thus these concentrations could be considered a sub-lethal dose. In the work of Anaga and Abu (1996), these concentrations were used to produce axenic cultures of an alga. However, an assessment of the total bacterial and fungal population before and after addition of the antibiotics was not cited. Thus the production of the axenic cultures was not concretely tied to the dosage of the antibiotics used. In this work, the dosages of the antibiotics used are not lethal to the total bacterial population. This observation is however relative since the bacterial population in a given environment can change from time to time. Also, an effective dosage is proportional to the size of the bacterial population.

The Bacteria isolated in this study includes *Proteus* and *Pseudomonas* which belongs to the Proteobacteria, and *Bacillus* which belongs to the Low G + C Gram positive bacteria. They were able to grow in the algal culture medium supplemented with antibiotics. The works of Gonzalez *et al.* (2000), and Goecke *et al.* (2013) showed that bacterial species associated with algae cultured from marine and freshwater environments are distributed among the following bacterial phyla; Actinobacteria, Bacteroidetes, Cyanobacteria, Firmicutes, Planctomycetes, Proteobacteria, and Verrucomicrobia. Two of the bacteria (*Proteus* and *Pseudomonas*) isolated in this study are thus in agreement with the works of other researchers. The presence of *Bacillus* and *Pseudomonas* in algal cultures has been shown to be detrimental to some algal species (Kim *et al.*, 2007; Kataev *et al.*, 2012). There is thus need to eliminate this bacteria group in algal cultures. This can be conveniently achieved through the use of antibiotics.

The antibiotic sensitivity testing showed that the three isolated bacteria genera were all resistant to Chloramphenicol (Figure 1). *Bacillus,* in addition to been resistant to Chloramphenicol, was resistant to Flucloxacillin. Flucloxacillin is a narrow spectrum antibiotic with considerable activity against Gram positive organisms (Saene *et al.*, 1998). It is thus expected to be active against *Bacillus* since *Bacillus* is a Gram positive organism, but this was not the case. *Pseudomonas* showed a high level of resistance. Being a Gram negative bacterium, it was resistant to Gentamycin, Ofloxacin, Peflacine, Ceporex, Erythromycin, Norfloxacin, Ampiclox, Augmentin, Nalidixic acid, and Septrin. Gentamycin is a narrow spectrum antibiotic active against Gram negative bacteria, while Ofloxacin, Peflacine, Ceporex, Erythromycin, Norfloxacin, Ampiclox, Augmentin, Nalidixic acid, and Septrin (Co-Trimoxazole) are all broad spectrum antibiotics (Prescott *et al.*, 1999; Ugbogu *et al.*, 2007; Kigigha and Zige, 2013; White *et al.*, 2004; Geddes *et al.*, 1979).

Pseudomonas is thus expected to be sensitive to Gentamycin and the broad spectrum antibiotics; also this was not the case. *Proteus* being a Gram negative bacterium was resistant to Erythromycin, Ampiclox, and Septrin. These antibiotics which all have broad spectrum of activity were expected to show some level of inhibition against *Proteus*; this also didn't happen. The use of Chloramphenicol at sub-lethal dose, which may have resulted in all three bacteria been resistant to it, could have also led to these entire outcomes. This suggestion is in agreement with results of investigations carried out by Harkaway *et al.* (1992), and Gullberg *et al.* (2014). Harkaway *et al.* (1992) observed that *Staphylococcus epidermidis* became resistant to Erythromycin, Clindamycin and Tetracycline, as a result of treatment with only Erythromycin. The results of the investigations of Gullberg *et al.* (2014) showed that low levels of single antibiotics can select for plasmids that carries resistance to several antibiotics. They pointed out that the spread of these plasmids among the local bacterial population will lead to the emergence of multi-antibiotic-resistant bacteria.

Looking at the result of the antibiotic sensitivity test (Figure 1) from another point of view, it can be seen that all three bacteria genera were sensitive to Ciprofloxacin, Streptomycin, and Rifampin. These antibiotics, including Chloramphenicol, are all broad spectrum antibiotics (Prescott *et al.*, 1999); they are thus expected to inhibit the growth of most bacteria group. However, the use of any of these antibiotics at sub-lethal concentration can lead to the generation of antibiotic resistant strains, as was observed with Chloramphenicol in this study. Exposure to sub-lethal dose of any antibiotic can cause mutations in bacteria thereby giving rise to resistant mutants which may be resistant to several antibiotics. There is a possibility that the resistant mutants can pass on their abilities to other bacteria group through plasmids, thus making the entire bacterial population resistant.

The results of this study indicates that certain bacteria that are present in algal cultures can become resistant to several antibiotics as a result of purification of the algal cultures using antibiotics at sub-lethal concentrations. These bacteria can cause a possible decline in the algal population by directly attacking the algae, as has been observed by Kim *et al.* (2007) and Kataev *et al.* (2012), or indirectly by using up the available nutrients. Incorporating a screening procedure for selecting effective antibiotic(s) against identified bacteria contaminants as part of the procedure for setting up pure algal cultures will go a long way in preventing the emergence and spread of antibiotic resistant bacteria. Determination of the minimum lethal concentration (MLC) or minimum inhibitory concentration (MIC) of such antibiotic(s) against the identified bacteria contaminants should also be part of the screening procedure.

ACKNOWLEDGMENTS

We thank the staffs of the African Regional Aquaculture Centre, Aluu, Rivers State, Nigeria, especially Mr. Ezenwa who helped us in locating and gaining access to the fish pond having algal bloom.

REFERENCES

Anaga, A. and Abu, G. O. (1996). A laboratory-scale cultivation of *Chlorella* and *Spirulina* using waste effluent from Fertilizer Company in Nigeria. *Bioresour. Technol.*, 58: 93-95.

Cottrell, M. T., and Suttle, C. A. (1993). Production of axenic cultures of *Micromonas pusilla* (Prasinophyceae) using antibiotic. *J. Phycology*, 29: 385-387.

Coutteau, P. (1996). Micro-algae. In Lavens P. & Sorgeloos P., Manual on the production and use of live food for aquaculture, FAO Fisheries technical paper 361, (pp. 7-48). Food and Agriculture Organization (FAO) of the United Nations, Rome.

Droop, M. R. (1967). A procedure for routine purification of algal cultures with antibiotics. *Br. Phycological Bull.*, 3 (2): 295-297.

Geddes, A. M., Ball, A. P., and Farrell, I. D. (1979). Co-trimoxazole for the treatment of serious infections. *J. Antimicrob. Chemother.*, 5: 221-230.

Goecke, F., Thiel, V., Wiese, J., Labes, A., and Imhoff, J. F. (2013). Algae as an important environment for bacteria – phylogenetic relationships among new bacterial species isolated from algae. *Phycologia*, 52 (1): 14-24.

Gonzalez, J. M., Simo, R., Massana, R., Covert, J. S., Casamayor, E. O., S-Alio, C. P., and Morani, M. A. (2000). Bacterial Community Structure Associated with a Dimethylsulfoniopropionate-Producing North Atlantic Algal Bloom. *Appl. Environ. Microbiol.*, 66 (10): 4237-4246.

Gullberg, E., Albrecht, L. M., Karlsson, C., Sandegren, L., and Andersson D. I. (2014). Selection of a Multidrug Resistance Plasmid by Sublethal Levels of Antibiotics and Heavy Metals. *mBio* 5 (5): e01918-14. Retrieved from http://mbio.asm.org/content/5/5/e01918-14.full.pdf+html

Harkaway, K. S., McGinley, K. J., Foglia, A. N., Lee, W.-L., Fried, F., Shalita, A. R., and Leyden, J. J. (1992). Antibiotic resistance patterns in coagulase-negative staphylococci after treatment with topical erythromycin, benzoyl peroxide, and combination therapy. *Br. J. Dermatol.*, 126: 586-590.

Hoa, P. T., Managaki, S., Nakada, N., Takada, H., Shimizu, A., Anh, D. H., ... Suzuki, S. (2011). Antibiotic contamination and occurrence of antibiotic-resistant bacteria in aquatic environments of northern Vietnam. *Sci. Total Environ.*, 409 (15): 2894-2901.

Kataev, A. A., Andreeva-Kovalevskaya, Z. I., Solonin, A. S., and Ternovsky, V. I. (2012). Bacillus cereus can attack the cell membranes of the alga Chara corallina by means of HlyII. *Biochimica et Biophysica Acta*, 1818 (5): 1235-1241.

Kigigha, L. T. and Zige, D. V. (2013). Activity of *Chromolaena Odorata* on enteric and superficial etiologic bacterial agents. *Am. J. Res. Commun.*, 1 (11): 266-276.

Kim, J.-D., Kim, B., Lee, C.-G. (2007). Alga-lytic activity of Pseudomonas fluorescens against the red tide causing marine alga Heterosigma akashiwo (Raphidophyceae). *Biol. Control*, 41: 296-303.

Kirroliaa, A., Bishnoia, N. R., and Singh, R. (2012). Effect of shaking, incubation temperature, salinity and media composition on growth traits of green microalgae *Chlorococcum* sp. *J. Algal Biomass Utilization*, 3 (3): 46-53.

Kooistra, W. H. C. F., Boele-Bos, S. A., and Stam, W. T. (1991). A method of obtaining axenic cultures using the antibiotic Cefotaxime with emphasis on *Cladophoropsis membranacea*

(Chlorophyta). *J. Phycology*, 27: 656-658.

McPhearson, R. M., DePaola, A., Zywno, S. R., Motes Jr., M. L., and Guarino, A. M. (1991). Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds. *Aquaculture*, 99 (3-4): 203-211.

Prescott, L. M., Harley, J. P., & Klein, D. A. (1999). Microbiology, 4th edition. New York: WCB/McGraw-Hill.

Raghukumar, C. (1987a). Fungal parasites of marine algae from Mandapam (South India). *Diseases Aquat. Org.*, 3: 137-145.

Raghukumar, C. (1987b). Fungal parasites of the green alga *Chaetomorpha Media*. *Diseases Aquat. Org.*, 3: 147-150.

Sadava, D., Hillis, D. M., Heller, H. C., and Berenbaum, M. (2009). Life: The Science of Biology, 9th Edition. New York: W. H. Freeman.

Saene, R., Fairclough, S., and Petros, A. (1998). Broad- and narrow-spectrum antibiotics: a different approach. *Clinical Microbiology and Infection*, 4: 56-57.

Sapp, M., Schwaderer, A., Wiltshire, K. H., Hoppe, H. G., Wichels, A., and Gerdts, G. (2004). Diversity and succession of bacterial populations in microalgae cultures. 10th International symposium on Microbial Ecology "Microbial Planet: SubSurface to space", 22 – 27 Aug., 2004, Cancun, Mexico.

Serrano, H. P. (2005). Responsible use of antibiotics in aquaculture. *FAO Fisheries Technical Paper*. No. 469. Food and Agricultural Organization of the United Nations, Rome.

Spanggaard, B., Jorgensen, F., Gram, L., Huss, H. H. (1993). Antibiotic resistance in bacteria isolated from three freshwater fish farms and an unpolluted stream in Denmark. *Aquaculture*, 115 (3–4): 195-207.

Stanier, R. Y., Adelberg, E. A., and Ingraham, J. L. (1977). General Microbiology, 4th edition. London: The Macmillan press Ltd.

Ugbogu, O. C., Nwaugo, V. O., Orji, A., and Ihuoma, N. (2007). Quinolone Resistant *Staphylococcus aureus* in Okigwe, Imo State Nigeria. *J. Biol. Sci.*, 7: 697-700.

Wang, C.-H., Ho, A. Y. T., Qian, P.-Y., Wong, P.-K., Hsieh, D. P. H. (2004). Antibiotic treatment enhances C2 toxin production by *Alexandrium tamarense* in batch cultures. *Harmful Algae*, 3: 21-28.

White, A. R, Kaye, C., Poupard, J., Pypstra, R., Woodnutt, G., and Wynne, B. (2004). Augmentin (amoxicillin /clavulanate) in the treatment of community-acquired respiratory tract infection: a review of the continuing development of an innovative antimicrobial agent. *J. Antimicrob. Chemother.*, 53 (1): 13-20.