2012

Possible Effects of the Presence of Common Household Chemicals in the Environment: The Growth of an Aquatic Bacterial Species on High Concentrations of Caffeine

Adrienne M. Gibson
Grand Valley State University

Roderick M. Morgan
Grand Valley State University, morganr@gvsu.edu

Nick MacDonald
Grand Valley State University

Alexey G. Nikitin
Grand Valley State University, nikitin@gvsu.edu

Possible effects of the presence of common household chemicals in the environment: the growth of an aquatic bacterial species on high concentrations of caffeine

Adrienne M. Gibson*, Roderick M. Morgan, Nick MacDonald, Alexey G. Nikitin

Biology Department, Grand Valley State University, Allendale, MI 49401, USA.

Caffeine is a prevalent chemical in the environment, often being found in aquatic ecosystems. Past studies have shown that some bacterial species can metabolize caffeine, but little research has been done to study the effect of different caffeine concentrations on the growth of the bacteria. The goal of the current study is to gain a better understanding of how aquatic bacteria, which have been selected for growth on caffeine, utilize caffeine as a source of carbon. To study the effect of caffeine concentrations on bacterial growth, we isolated a bacterium from an aquarium that had been exposed to caffeine. The organism was able to grow on both solid and liquid media containing only caffeine and potassium phosphate buffer. Colonies formed on caffeine concentrations as low as 300 mg/L and up to 20,000 mg/L. However, caffeine concentrations at 20,000 mg/L began to inhibit the growth of the organism. The DNA sequence analysis of the 16S rRNA gene indicated the organism belongs to the Pseudomonas putida bacterial group. Our results indicate that aquatic microbiota can effectively utilize a wide range of environmental concentrations of caffeine as a nitrogen and carbon source.

* Corresponding author: Adrienne M. Gibson, 253 Padnos, 1 Campus Dr., Allendale, MI 49401, USA. E-mail: amagibson@gmail.com

Introduction

The availability of caffeine-containing beverages and other caffeine-containing items has lead to caffeine becoming one of the most widely consumed chemicals in the United States and Europe with one in three Americans consuming approximately 200 mg of caffeine each day [1]. The most common sources for caffeine are coffee, tea, soft drinks and chocolate with approximately 80% of daily caffeine consumption coming from coffee. The amount of caffeine in various products depends on the serving size, type of product and plant variety, as well as preparation method. Despite the US Food and Drug Administration's placement of caffeine in the category Generally Recognized as Safe (GRAS) in 1958 and the American Cancer Society's Guidelines on Diet, Nutrition, and Cancer stating that drinking coffee is not a risk factor for cancer, there is still some controversy regarding the health effects of caffeine [2].

Caffeine is an alkaloid found in many plant species. It belongs to the group of compounds known as methylxanthines which include theobromine (cocoa) and theophylline (tea). When ingested, caffeine is absorbed by the gastrointestinal tract and distributed throughout tissues and organs where it has been shown to induce several physiological responses which can vary considerably depending on the amount taken, the sensitivity, metabolism, and physical conditions of the individual as well as the frequency of use. Caffeine has been shown to affect energy metabolism, mood and sleep patterns, cause nausea, headaches, muscle tremors and at higher doses induce hypertension or arrhythmias [1]. At moderate amounts caffeine has been shown to increase mental alertness, reasoning abilities and visual reaction time and in a recent study coffee and caffeine intake was associated with a lower incidence of Parkinson's

disease, although a causal relationship has not been established [2].

Once ingested, caffeine is rapidly absorbed through the stomach and small intestine into the bloodstream where it takes 15 to 45 minutes to reach its highest levels depending on how much was ingested as well as on the source of the caffeine. It is then metabolized primarily by the liver where it is converted to two metabolites. These metabolites are excreted mainly by the kidney, although they can also be found in various other bodily excretions. The time necessary for the body to eliminate half of the amount of caffeine consumed can range from hours to days but on average takes from 2.5 to 7.5 hours [1]. This clearance rate of caffeine can be decreased by factors including liver disease, smoking or medications.

The feeling of increased energy many experience from caffeine is likely due to a decreased perception of fatigue caused by caffeine's action as a central nervous system stimulant [2]. Caffeine and some related methylxanthines are believed to act as psychostimulants by inhibiting phosphodiesterase thus enhancing the effects of epinephrine while at the same time acting as an antagonist of adenosine receptors [1]. Adenosine is a vasodepressor and caffeine may reduce cardiovascular response to adenosine leading to elevated blood pressure at higher doses (6 cups of coffee) of caffeine. Adenosine receptors are involved in the regulation of dopamine, inhibition of the release of norepinephrine and increased catecholamine release, which are other mechanisms by which caffeine may stimulate the central nervous system [3].

Experiments with different cell types have suggested various explanations for the potentiation of mutagenic effects. In earlier studies the increased mutations found in organisms such as *E. coli* were generally attributed to the direct induction of mutations by caffeine, but it is now believed to be the result of caffeine altering various repair processes of the cell [4]. Caffeine may modify the response of p53 to DNA damage or it may modify cell cycle checkpoint function by inhibiting phosphodiesterases and protein kinases involved in regulating cell cycle transitions [5]. It has been suggested that caffeine may also affect the cell cycle through alteration of cAMP levels. The level of cAMP within a cell shows a correlation with the timing of the onset of mitosis which could affect the functioning of cellular repair systems. These effects, however, have only been demonstrated in lower organisms and at very high doses so the possibility that caffeine at normal doses could induce mutagenesis seems unlikely [4]. These mutagenic and antimutagenic effects also can vary depending on the carcinogen it is used with, the type of host cell and the stage of cell cycle in which it is introduced. Currently, no causal relationship between caffeine and any type of human cancer has been found [6].

Experimental studies on the effect of caffeine on Drosophila have shown that it can affect lifespan and mortality. A significant reduction in lifespan of male *D. melanogaster* has been reported in flies kept on 1.25 and 2.5 mg/ml of caffeine [7]. Legator & Zimmering determined the mortality rates of different strains of Drosophila that fed on a 1% caffeine solution for two days and found that different strains have different sensitivities to caffeine and that in most strains tested the mortality effects of caffeine were greater in males than in females [4]. In a study where *D. prosaltans* were treated with various concentrations of caffeine over the course of ten generations, lifespan was significantly reduced in flies treated with 2.5 mg/ml while longevity was unaffected in those given lower doses. Significant differences in mortality rate between male and female flies were also found for caffeine concentrations of 0.05, 0.10, 2.0, and 2.5 mg/ml of culture medium [8].

Graf and Wurgler fed adult male *D. melanogaster* coffee solutions for a period of 3

days as well as to larvae during larval development. Instant coffee at a concentration of 4% was toxic to 75% of flies while home-brew coffee at 3% was toxic to 90% of the flies. This difference in toxicity can most likely be attributed to different levels of caffeine in the coffee preparations with a caffeine level of 3.46% for the instant coffee and 5.78% for the home-brew coffee. Neither coffee solution had an effect on larval deaths [9].

The results of others have shown that lifespan may be affected by the genetic damage of somatic cells due to external or internal factors such as treating the cells with X-rays or altering repair processes of the cells [10]. Heat shock proteins have also been found to play a role in lifespan determination of Drosophila. Zhao et al. analyzed RNA levels for two heat shock protein genes in a long-lived and a short-lived Drosophila line and found that the long-lived line exhibited higher RNA levels for both heat shock protein genes suggesting a correlation between heat shock protein gene expression and lifespan in *D. melanogaster* [11]. These findings detailing numerous environmental and genetic influences demonstrate the impact of genetic background in determining the lifespan of Drosophila as well as suggest mechanisms by which a substance such as caffeine may affect the metazoan lifespan.

The above review of caffeine health effects suggests that the environmental presence of this substance may have health-related significance. An evaluation of 139 stream sites in the U.S. for the occurrence of organic wastewater contaminants (OWCs) found that caffeine was the fourth most frequently detected chemical, and occurred in 70% of the samples [12].

Because of its excellent solubility and slow rate of degradation, caffeine can persist in aquatic environments [13] and has the potential to biomagnify through the food chain. Thus, even a small amount of dissolved caffeine in aquatic environment can concentrate over time. The fact that caffeine is one of the most common compounds found in sewage has been known for over 20 years [14], but the nature of its effect on the aquatic environment still remains unclear. These effects need to be studied so the appropriate agencies in charge of environmental protection can create steps to manage caffeine presence in the environment. In the current study, we set out to investigate the effect of the caffeine on the growth of the bacteria present in an aquarium biofilter.

Materials and methods

Liquid Media

The bacterial organism used in this study was isolated from an aquarium populated by goldfish (*Carassius auratus*). The water in the aquarium was composed of deionized water and sodium bicarbonate (0.15 g/L). A goldfish was used to inoculate each aquarium for a 24 hour period prior to administering caffeine. 10 mg/L of caffeine powder was dissolved directly in the beakers every 48 hours. Water samples were taken from the tank and plated on Muller-Hinton agar plates (Difco, BD, Sparks, MD). These plates were kept at room temperature, and inspected everyday for growth. Once the bacteria grew, individual culture samples were taken from the predominant colony-type and re-plated to create the original stock plates of the bacteria. A 0.5 McFarland standard (approximately $1\text{-}5x10^6$ cells/ml) was made from a fresh overnight stock plate of the bacteria. Approximately $1x10^6$ cells were added to Nephelo flasks. Each flask contained a 0-5000 mg/L final concentration of caffeine (Fisher Scientific, Pittsburg, PA) and 1.5 g/L K_2HPO_4 (JT Baker Chemical Co. Phillipsburg, NJ). All Nephelo flasks were standardized to a volume of 45.0 ml. To limit the effects of light on caffeine, the flasks were covered at all times. Absorbance was measured at 12 hour intervals for 149 hours, using a Spectrophotometer 20 at 605 nm. Ammonia levels were determined using standard aquarium ammonia test kits (Mars Fishcare, Chalfont, PA).

Solid Agar Media

Solid media consisted of 0.5 g of K_2HPO_4 (Fisher Scientific, Pittsburg, PA), caffeine concentrations ranging from 300-20,000 mg/L, and 1.5% Agar (Difco, BD, Sparks, MD). Control plates consisted of standard Nutrient Agar (Difco, BD, Sparks, MD). To inoculate the plates, a 0.5 McFarland standard (approximately 1-5x10^6 cells/ml) was made from a fresh overnight stock plate of the bacteria. The number of recoverable cells was enumerated by plating 100 µl appropriate diluents. The plates were incubated at 30^{o}C for 4 days and the number of colonies (representing the number of recovered bacteria) was counted every 24 hours.

Identification of the Bacteria

Thermocycler PCR amplification and DNA sequence analysis of the 16S rRNA gene was used to identify the genus and species. Whole cells of each culture were added to the reaction as a suspension made by vortexing a loop-full of bacteria in 0.5 ml sterile dH_2O. The 16S rRNA gene of each bacterium was amplified by PCR, using the universal primers 27F (5'-AGAGT TTGAT CCTGG CTCAG-3') and 1492R (5'-TACGG TTACC TTGTT ACGAC TT-3'). The PCR reagents were from the PCR Core System I kit from Promega (Madison, WI). Final concentrations for the reactions were: 1X Mg-free reaction buffer; 1.5 mM Mg^{2+}; 0.2 mM each of dATP, dCTP, dGTP, and dTTP; 1 pmol/µl each of 27F and 1492R; and 0.24 mM of Tergitol Type NP-40. To each reaction, 2.5 units of Taq DNA polymerase were added after an initial incubation at 95^{o}C for five minutes. Total reaction volume was 50 µl. Amplifications were then carried out for 30 cycles of 30 seconds of denaturation at 95^{o}C, 30 seconds of annealing at 52^{o}C, and 60 seconds of extension at 72^{o}C. After a final extension at 72^{o}C for five minutes, the products were stored at 4^{o}C until analysis by agarose gel electrophoresis (within 24 hours). PCR products were separated by agarose gel electrophoresis (1.5% agarose in TAE buffer) and visualized by ethidium bromide staining. Successful amplifications manifested as bands at approximately 1.4K bp in length. These bands were excised from the agarose gel and purified using the Geneclean Spin Kit (Q-Bio Gene, Irvine, CA). The sequences of the PCR DNA products were determined by the University of Michigan DNA sequencing core facility, and the results were used to identify the genera of the bacteria by comparison with known sequences in the Ribosomal Database Project.

Results

Identification of Organism

From the aquaria caffeine enrichment experiments, we were able to isolate and purify the predominant bacteria from plating the water samples on Mueller Hinton agar. This organism was able to grow on high concentrations for caffeine on both solid and liquid media. We identified this organism as *Pseudomonas putida*, based on the optimum growth temperature of 30^{o}C, Gram negative, fluorescence under UV light, and the 16S rRNA sequence matching at greater than 98% in the ribosomal database [15].

Liquid Media

As we grew *P. putida spp*. on increasing concentration of caffeine in liquid media, the maximum growth of the bacteria also increased. The Nephelo flasks that contained the 2000 or 5000 mg/L concentrations of caffeine had the highest growth rates (Figure 1). These flasks continued to grow throughout the time of the trial, with the growth appearing to level off near the end of the experiment.

Solid Agar Media

With the use of the solid agar media, the bacteria were exposed to higher concentrations of caffeine, ranging from 0 to 20,000 mg/L. Caffeine concentrations from 300-20,000 mg/L supported growth of the bacteria (Figure 2), However, even though bacterial growth occurred in the 20,000 mg/L concentration, not as many organisms were recovered as the other

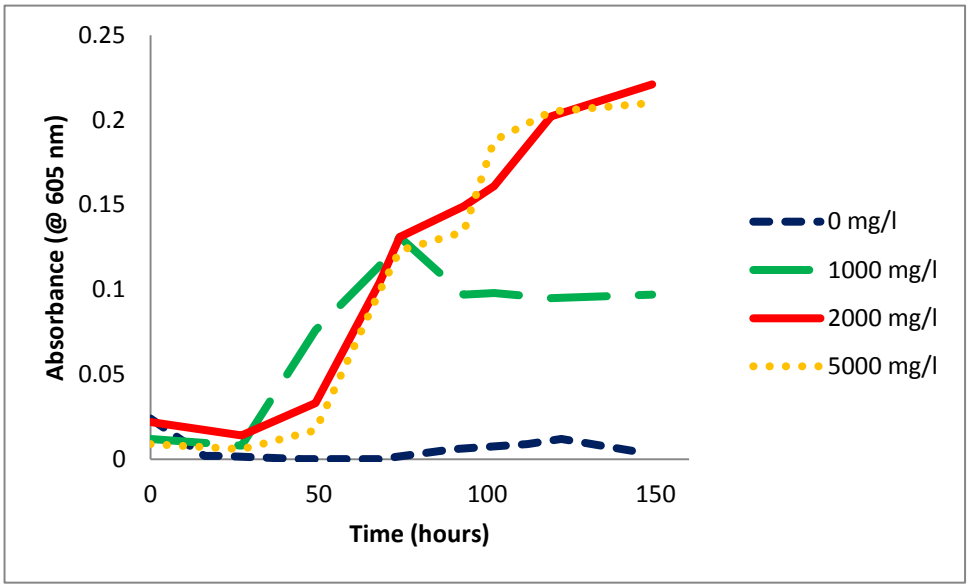

Figure 1. Growth of *Pseudomonas putida spp*. in Nephelo flasks at 30°C with concentrations of of 1000, 2000, or 5000 mg/L of caffeine. The cultured bacteria used the caffeine as a source of carbon and energy for growth.

concentrations, indicating that a concentration of 20,000 mg/L caffeine is inhibitory to the growth of the bacteria (Figure 2).

Discussion and Conclusions

Our results show that culturing aquatic bacteria on caffeine as the sole source of carbon can isolate a specific bacterial species from an aquatic environment. In our case, the bacterial species was *Pseudomonas putida*. In both liquid and solid media there was a clear increase in the growth of these bacteria between cultures that were supplied with the caffeine carbon source and bacteria that was not supplied with the caffeine. Understanding the minimum and maximum concentrations of caffeine the bacteria could utilize was also a major component of this study. This provided information indicating the minimum concentration of caffeine that could support bacterial growth and also the concentration of caffeine that inhibited the growth of the organism. We found that concentrations of caffeine lower than 10,000 mg/L had no negative impact on the growth of our *P. putida* isolate, which is consistent with other findings on caffeine toxicity to bacteria [16]. Furthermore, caffeine concentrations as low as 300 mg/L were able to support colony formation of the organism on a solid medium. Water quality tests used after the completion of the trials showed that flasks with caffeine concentrations above 250 mg/L contained ammonia levels that were approximately 20 mg/L. It is possible that the increased level of ammonia, which is released as a byproduct from the growth of the bacteria, caused the bacteria's slowed growth nearing the end of the experiment. The Nephelo flask containing 1000 mg/L of caffeine also grew, but any growth increase plateaued approximately 74 hours into the experiment. It is likely that carbon was a limiting factor in the growth of this culture of bacteria, causing the growth to level off earlier than the other cultures, which were exposed to more caffeine and therefore had a greater source of carbon. The control flask showed no bacterial growth (Figure 1). It appears that the maximum cell density of a growing culture of the microorganism is dependent on caffeine concentration, implying that caffeine is a suitable carbon source for the cultured bacteria

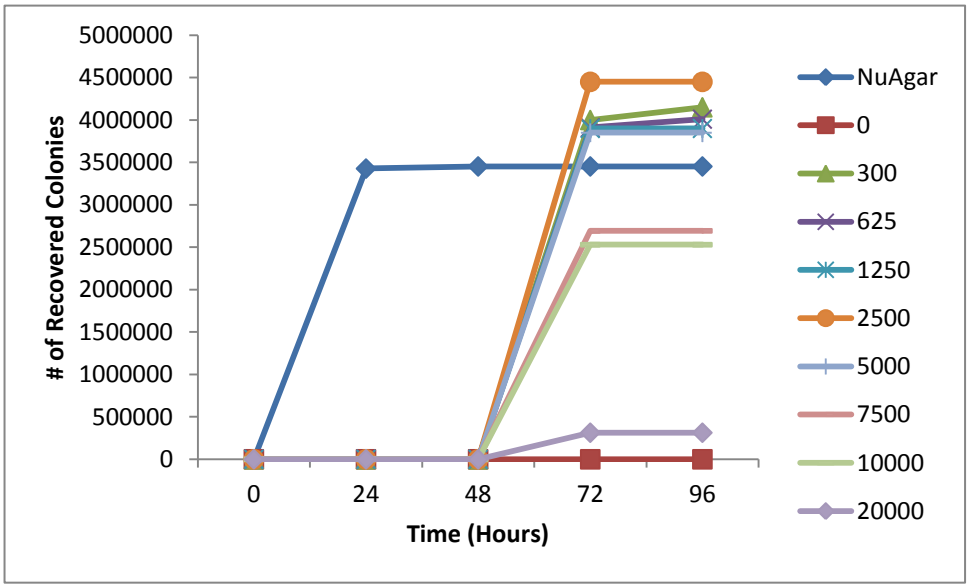

Figure 2. Growth of *Pseudomonas putida spp*. in on solid agar plates with concentrations of caffeine ranging from 0-20,000 mg/L. The cultured bacteria used the caffeine as a source of carbon and energy for growth into distinct colonies. The different concentrations of caffeine show different effects on the growth rates of the cultured bacteria, ultimately with the 20,000 mg/L caffeine concentration inhibiting bacterial growth.

present in an aquatic environment devoid of other carbon sources. This study represents the first published report of the growth of a bacterial species in a defined media that contained only caffeine and a phosphate source as well as measuring the wide range of caffeine concentrations that can support bacterial growth.

Caffeine is becoming a common occurrence in aquatic habitats and is a known anthropogenic marker of wastewater contamination of surface water [17]. With the increased amounts of chemicals being discarded in the aquatic environment it is important to understand the impact they may have on the ecosystem. There have been few studies performed on the interaction of the environment with chemicals such as caffeine, but as the results of this study indicate, there is a need for further research as this chemical becomes more abundant in aquatic environments.

The detection of *P. putida's* ability to use caffeine as a carbon source is only the first step in understanding the effects that the addition of caffeine and other chemicals may have on the aquatic environment. Bacteria from the *Pseudomonas* genus are common in aquatic habitats, and there has been abundant research regarding these organisms in this habitat. From this research, it is known that these organisms respond to anthropogenic changes in the environment. One example of anthropogenic changes affecting *Pseudomonas* would be the discovery of the ability of these organisms, in sewage water, to acquire and transfer antibacterial resistance plasmids [18]. Pseudomonas has been found to be in possession of the ability to carry antibiotic resistance [19]. Currently, the main concern in this area is regarding the spread of drug resistance to pathogens. While the selective promotion of *P. putida* growth by exposure to caffeine will not directly cause bacterial drug resistance to spread, it could promote higher concentrations of the organisms in areas that have been noted for high gene transfer rates. An example of a high bacterial gene transfer area is in wastewater treatment facilities, where it has been predicted that there is a high level of antibacterial resistance genes in the

bacteria growing at the facilities [20]. Waste water treatment facilities are also predicted to be an area of increased caffeine concentration, which would promote the growth of *P. putida* and inadvertently increasing the likelihood of antibiotic resistance gene transfer. Further research is needed to validate this hypothesis.

The release of ammonia as a by-product of caffeine metabolism by *P. putida* could have potential negative implications for the aquatic environment [21]. In our study, we found that ammonia concentrations increased dramatically with the addition of caffeine (data not shown). For example, after bacterial growth in a flask containing a caffeine concentration of 250 mg/L, the ammonia concentration was a staggering 20 mg/L. It is also likely that the build-up of ammonia inhibited the growth of the bacteria during the liquid media portion of the study (Figure 1). Ammonia has a negative effect on the weight of fish [22] and can cause damage to the gill structure, produce convulsions and ultimately, death [23]. Studies have shown that certain levels of ammonia can be toxic to both *Nitrosomonas* and *Nitrobacter* [24] which, like *Pseudomonas,* are part of the nitrogen cycle. By using solid media, we were able to reduce the negative effects of the ammonia on bacterial growth and therefore see a greater utilization of caffeine as a carbon source by the bacteria. The finding of *P. putida* being able to use caffeine as a carbon source opens the door for many potential questions regarding the effects of common household chemicals on the environment.

Reference

1. Leonard TK, Watson RR, Mohs ME. 1987. The effects of caffeine on various body systems: A review. J Am Diet Assoc. 87(8): 1048-1053.
2. Porta M, Vioque J, Ayude D, Alguacil J, Jaroid M, Ruiz L, Murillo JA. 2003. Coffee drinking: The rationale for treating it as a potential effect modifier of carcinogenic exposures. Eur J Epidemiol. 18(4): 289-298.
3. Hogan EH, Hornick BA, Bouchoux A B. 2002. Communicating the message: clarifying the controversies about caffeine. Nutr Today. 37(1): 28-35.
4. Legator MS, Zimmering S. 1979. Review of the genetic effects of caffeine. J Environ. Sci Health. C13(2): 135-188.
5. Nehlig A, Debry G. 1994. Potential genotoxic, mutagenic and antimutagenic effects of coffee: A review. Mutat Res. 317(2): 145-162.
6. Pozniak PC. 1985. The carcinogenicity of caffeine and coffee: A review. J Am Diet Assoc. 85(9): 1127-1133.
7. Nikitin AG, Navitskas S, Gordon LAN. 2008. Effect of varying doses of caffeine on lifespan of *Drosophila melanogaster*. J Gerontol A Biol Sci Med Sci. 63A(2): 149-150.
8. Itoyama MM, Bicudo HE, Manzato AJ. 1998. The development of resistance to caffeine in *Drosophila prosaltans*: productivity and longevity after ten generations of treatment. Cytobios. 96(382): 81-93.
9. Graf U, Wurgler FE. 1986. Investigation of coffee in Drosophila genotoxicity tests. Food Chem Toxicol. 24(8): 835-842.
10. Woodruff RC, Nikitin AG. 1995. P DNA element movement in somatic cells reduces lifespan in *Drosophila melanogaster*: evidence in support of the somatic mutation theory of aging. Mut Res. 338: 35-42.
11. Zhao Y, Sun H, Lu J. Xiaoxue L. Chen X. Tao D. Huang W. Huang B. 2005. Lifespan extension and elevated hsp gene expression in Drosophila caused by histone deacetylase inhibitors. J Exp Biol. 208(4): 697-705.
12. Kolpin DW, Furlong ET, Meyer MT. Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in United States streams, 1999-2000: A national reconnaissance. Environ Sci Technol. 36: 1202-1211.
13. Seiler RL, Zaugg SD, Thomas JM, Howcroft DL. 2005. Caffeine and pharmaceuticals as indicators of waste water contamination in wells. Ground Water. 37(3): 405-410.
14. Garrison AW, Pope JD, Allen FR. From GC/MS analysis of organic compounds in domestic wastewaters. In: Identification and analysis of organic pollutants in water. Edited by Keith LH. Ann Arbor: Ann Arbor Science Publishers Inc; 1976: 517–556.
15. Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM. 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucl Acids Res. 37: 141-145.
16. Ramanavičienė A, Mostovojus V, Bachmatova I, Ramanavičius A. 2003. Anti-bacterial effect of caffeine on *Escherichia coli* and *Pseudomonas fluorensis*. Acta Medica Lituanica. 10(4): 185-188.
17. Buerge IJ, Poiger T, Muller MD, Buser HR. 2003. Caffeine, an anthropogenic marker for wastewater contamination of surface waters. Environ. Sci. Technol. 37(4): 691-700.
18. Witte W. 2000. Ecological impact of antibiotic use in animals on different complex microflora: environment. Int J Antimicro Ag. 14: 321-325.
19. Isken S, Santons PMAC, and de Bont JAM. 1997. Effect of solvent adaptation on the antibiotic resistance in

Psuedomonas putida S12. Appl Microbiol Biotechnol 48: 642-647.

20. Jones OA, Voulvoulis N, Lester JN. 2004. Potential ecological and human health risks associated with the presence of pharmaceutically active compounds in the aquatic environment. Crit Rev Toxicol. 34(4): 335-350.
21. Dash SS, Gummadi SN. 2006. Catabolic pathways and biotechnological applications of microbial caffeine degradation. Biotechnol Let. 28(24): 1993-2002.
22. Foss A, Evensen TH, Vollen T, Oiestad V. 2003. Effects of chronic ammonia exposure on growth and food conversion efficiency in juvenile spotted wolfish. Aquaculture. 228: 215-224.
23. Russo RC. From Ammonia, nitrate, and nitrate. In: Fundamentals of aquatic toxicology. Edited by Rand, G.M., Petrocelli, S.R. Washington D.C: Hemisphere Publishing Corporation; 1985: 455-471.
24. Camargo J, Alonso A. 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environ Int. 32: 832-849.