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Abstract

Caffeine is becoming a common chemical found in the environment but, little research has been done to understand the environmental effects of caffeine, including dissolved caffeine in aquatic systems. The goal of this research study was to begin to understand how caffeine may interact with aquatic environment, using the bacteria of the genus *Pseudomonas*, commonly found in the aquatic habitat. We found that *Pseudomonas* shows an increase in growth when exposed to caffeine, which results in a change in spectrophotometric absorbance, increase in bacterial colony count in the presence of caffeine, and in bio-film like sheen appearing on the glass of the experimental aquarium. Along with the increased bacterial growth, a dramatic increase in ammonia concentrations was observed. Ammonia is toxic to fish, and can be correlated with the metabolic activity of the *Pseudomonas* bacteria, making the caffeinated environment toxic for aquatic life.

Caffeine is a common chemical found in the environment. 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 (Kolpin et al.,2002). Caffeine is one of the most commonly consumed chemicals in the United States and Europe, with one in three Americans consuming approximately 200mg of caffeine each day (Leonard et al., 1987).

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). Because of its excellent solubility and slow rate of degradation, caffeine can persist in aquatic environments (Seiler et al., 2005) 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 (Garrison et al., 1976), 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 take steps to manage caffeine presence in the environment.

In the complicated biocycle of caffeine (Dash and Gummadi, 2006), it is broken down by such bacterial species as *Pseudomonas* and the by-products of their metabolism such as ammonia are further processed by nitrifying microorganisms to nitrites and, ultimately, nitrates.

The genus *Pseudomonas* is present in a wide range of environments (Ramanaviciene et al., 2003), including aquatic sediments where a significant amount of bioprocessing and detoxification takes place (Stickney et al., 2009). Thus, *Pseudomonas* are likely to comprise the bulk of an aquarium biofilter, which represents a sophisticated form of activated biosludge used by wastewater treatment plants.

Methods

Initial Aquarium and Fish Analysis:

The aquarium set up for this experiment included two sets of glass tanks, a 30 gallon and a 20 gallon set. Each set consisted of a control and an experimental tank. The 30-gallon set was equipped with Cascade 150 filters (Penn-Plax, New York). The carbon padding in these filters was removed to make sure the caffeine was not interacting with it. All of the aquariums were equipped with heaters that kept the water temperature at 26˚C. Each tank contained two angelfish (*Pterophyllum scalare*). The experimental tanks in both sets were given caffeine at 10 mg/l once a day for two days. Water was removed from the tank, mixed with the caffeine and then poured back in. Throughout the whole experiment water quality tests were done to document the increase in ammonia and nitrite. Once the concentration of caffeine in the experimental tanks reached 20 mg/l, a 100 µl sample was removed from both the 20 gallon control and the 20 gallon experimental tank for water quality analysis. The water samples were not used from the 30 gallon control and experimental tanks. The water samples were plated on both Mueller-Hinton agar and Nutrient agar. Swabs were taken at this time from the side of the control and experimental tanks in the 20 gallon tank set, with the goal to understand the composition of the substance that lined the sides of the experimental tanks. These swabs were also plated on Mueller-Hinton agar and Nutrient agar. The plates were allowed to incubate at room temperature for one night and then were observed and documented the following afternoon.

Small Scale Batch Culture Analysis:

For this segment, 2-liter glass beakers were used, instead of the aquariums. There was a control beaker and an experimental beaker, each filled with 1800 ml of distilled water. A small amount of bicarbonate was also added to each beaker, to minimize the any changes in pH that could have potentially occurred when the caffeine was added. A goldfish was used to inoculate each beaker for a 24 hour period. After this time the fish was removed and the caffeine was administered. 10 mg/l of caffeine powder was dissolved directly in the beakers every 48 hours.

Ten-fold serial dilutions were performed before the next dose of caffeine was dissolved in the beakers. These dilutions were plated on Muller-Hinton plates, and left at room temperature to incubate for 24 hours. After this time, the number of colonies of the bacteria were counted and recorded.

Nephelo Flask Analysis:

This segment of the project employed a spectrophotometric method of quantification of bacterial growth, instead of plate counts. Autoclaved nephelo flasks were used in this segment. The media consisted of deionized water with 1.5 g/L K_2HPO_4 and 1.5 g/L $MgSO_4$. These concentrations were recommended by HiMedia Laboratories in their recipe for Pseudomonas agar, and were used in the media

to promote the growth of the bacteria. A 0.5 Macfarland of *Pseudomonas fluorescens,* which was isolated from the small scale batch segment, was added to the flasks to achieve a final concentration of 5%. Each flask contained a final concentration of caffeine 0-5000 mg/L. To limit the effects of light on the caffeine, the flasks were covered at all times.

The growth of the bacteria was monitored using a spectrophotometer at 605nm.

Results and Discussion

Initial Aquarium and Fish Analysis (Figure 1):

Water quality tests, which were run throughout the project, showed that there were drastic increases in the ammonia concentration when caffeine was administered to the tank. These ammonia concentrations quickly rose to levels that would have made the environment unhealthy for the fish. In our experiment, the ammonia increased from 0 to .25 ppm at a concentration of 10 mg/l of caffeine. When another 10 mg/l was added the next day, the ammonia levels had risen to 4 ppm. Since the only change in tank environment was the addition of caffeine, and caffeine cannot turn into ammonia on its own, we concluded that ammonia is being produced by some of the bacteria present in the tank environment, as a by-product of caffeine metabolism. With the ammonia at 4 ppm, being toxic to the fish, the fish were removed. While this process was taking place, an opaque sheen was observed on the side of the tank, making the glass and water appear cloudy.

100 µl of water from each tank was plated, and a swab was taken and plated from the side of both tanks. Bacterial colonies were observed after plates were allowed to incubate at room temperature for one day. From these plates, it was easy to observe a visibly higher number of bacterial colonies from the caffeinated tanks.

With the swab plates showing high levels of bacteria, it suggests that the composition of the sheen is a bacterial. Both the bacteria in the swab plates and water plates appear to belong to the genus *Pseudomonas,* based on morphology analysis. Upon further observation, a difference was seen between tanks with or without a filter.

Tanks without a filter were seen to have more bacteria colonies when compared to tanks that did have a filter. Another trial was subsequently run to quantify the change in bacterial colonies over time. The tanks were brought up to a caffeine concentration of 30 mg/l, using the same method as the rest of the trials. The tanks were then allowed to run for 4 days without the addition of caffeine. After 4 days, the bacteria were plated, using the same method as above. The only change in method was that the bacteria were only plated on Mueller-Hinton agar, since the bacteria seemed to grow better on Mueller-Hinton rather than Nutrient agar. Upon observation of the plates, there was a substantial difference in the amount of bacteria between aquariums that had filters and aquariums that did not have filters. More bacteria colonies were found on the plates from the tanks that did not have a filter, and the plates looked similar to the plates that have been seen throughout the experiment. The plates from the tanks with filters showed hardly any bacteria growth. To go along with this observation, the experimental tank that had a filter was starting to lose its bio-film layer. We can speculate that most of caffeine was being trapped within the fibers of the filter, thus providing nutrients for the bacteria in the

immediate environment and not allowing it to get to the bacteria in the water column.

Figure 1: An example of the Bio-film that developed on the side of the aquarium (right) with the addition of caffeine.

Small Scale Batch Culture Analysis (Figure 2):

Water taken from the control and experimental aquariums when plated, showed a dramatic difference in the amount of bacteria. Upon exposing the plates to UV light, the predominant bacteria were found to be *P. fluorescens*. Therefore, it appears that *P. fluorescens* is utilizing caffeine as the carbon source to sustain growth. *P. fluorescens* releases ammonia as a waste by products (Dash and Gummadi, 2006).This would also account for the increase in ammonia levels present in experimental beakers, in comparison to the control beakers.

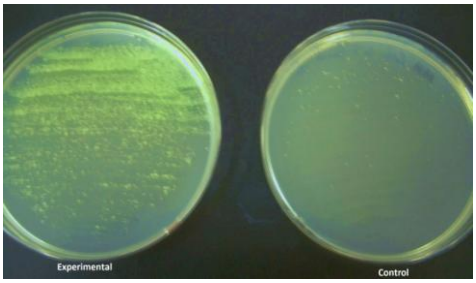

Figure 2: The growth of bacteria, on plates inoculated with samples of water from the aquariums.

Nephelo Flask Analysis (Figure 3 and 4):

As the caffeine concentrations increased, the growth of the bacteria also increased. The 2000 and 5000 mg/L batches had the highest growth rate increase, and the 1000 mg/L batch showed an intermediate increase in growth. The control flask showed no increase in bacterial growth. Like in the previous procedures, the experimental flasks showed an increase in ammonia, in comparison to the control flasks. Thus, it appears that bacterial growth is directly proportional to caffeine concentration, implying that caffeine is a suitable carbon source for *P. fluorescens* in an aquatic environment devoid of other carbon sources.

Figure 3: Flasks containing *Pseudomonas fluorescens* in liquid media

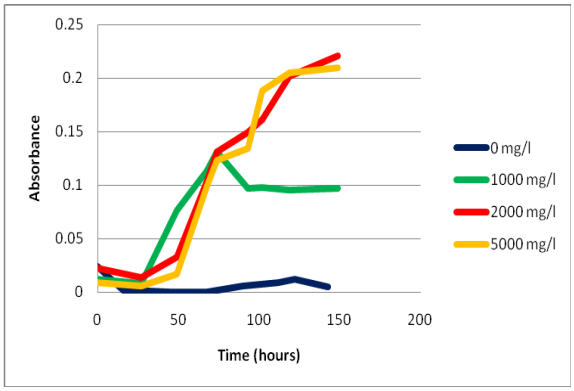

Figure 4: Absorbance data, measured from flasks containing 1000, 2000, & 5000 mg/L of caffeine.

Conclusions and Further Studies

Since there is no additional carbon source being added to the media, the bacteria must be able to utilize the caffeine as a usable carbon source. The addition of caffeine increases the growth of *P. fluorescens.* Due to the increase of *P. fluorescens,* which releases ammonia as a byproduct, there is an increase in ammonia concentrations in the caffeinated environment. Since a high ammonia level is toxic to fish, living in a caffeinated environment may potentially have an effect on fish and, potentially, other aquatic organisms. We are continuing to address the questions of caffeine concentration limits on bacterial growth.

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