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Effects of Protein Concentration in Fish Feed on Physical and Chemical Water Pollution

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ABSTRACT

Nitrates are linked to higher rates of fish mortality. Fish feces discolors water, reducing clarity and affecting the metabolism of organisms. Food eaten by fish is the main source of nitrates and feces. This work studies how protein concentration in fish feed affects the production of wastes that pollute water. It is hypothesized that high protein fish foods lead to more nitrates, less clear water in addition to an increased amounts of trace amino acids entering the water column. Three experiments were performed to determine which food at a 28%, 36%, or 42% protein concentration contributed most to poor water quality. These tests were performed on feces collected from three species of fish, the freshwater angelfish, koi and the tamasaba goldfish. The tests determined that increasing the protein concentration in fish food did in fact increase nitrate levels in aquariums while water clarity and residual amino acid concentration did not necessarily increase. This is significant because it allows for greater insights into fish feeding which are important for the traditional aquarium hobby and aquaculture farms.

Introduction

Fish are aquatic life that are found across the globe and are crucial for a functioning global ecosystem. Fish like every other organism produce waste. Metabolic waste from fish comes in two forms: physical feces and chemical ammonia. Ammonia (NH_3) which is mainly released from a fish's gills, is incredibly toxic to fish (Randall & Wright, 1987). This natural production of ammonia is not too severe of a problem in the wild since oceans, lakes and rivers are massive having hundreds of thousands of gallons of water. In closed environments like aquaria or ponds however, this ammonia builds up and causes chemical burns on fish gills leading to their deaths. In addition, ammonia severely weakens the fish's immune system, increasing the likelihood of secondary bacterial, viral, or parasitic infections (Miramontes et al., 2020). Over time, bacteria like *Nitrosomonas* and *Nitrobacter* will grow in the aquaria and process this ammonia through a nitrogenous pathway. This ammonia is oxidized by *Nitrosomanas* into nitrite (NO_2^-) and then the less toxic nitrate by *Nitrobacter* (Sedlacek et al., 2020; Zhang et al., 2018).

Nitrate (NO_3^-) can only be removed by changing the water in the aquarium and replacing the nitrate-replete water with nitrate-free freshwater. Although nitrate, does not pose as large of a threat to aquarium pets as ammonia and nitrite, it can still have negative consequences at high enough concentrations. High nitrate levels or sustained exposure to nitrates will decrease the oxygen levels in a fish's body and will make the fish more prone to diseases and infections (Camargo et al., 2005). High nitrate levels have been shown to interfere with the endocrine system, weaking fish reproductive health and increasing mortality (Kellock et al., 2018). Therefore, steps must be taken to reduce nitrate levels as high nitrate levels will harm fish health impacting ecosystems and the fish farming industry. This is especially important in the aquaculture industry as aquaculture contributes to 43% of all aquatic food consumed by people (Bostock et al., 2010). In addition to nitrate derived from ammonia, which are both chemical pollutants, fish also produce feces, a physical pollutant. Fish feces can lead to poor water clarity which will prevent fish from finding food and can prevent sunlight from reaching the plants in the aquarium or

aquaculture environment. Thus, fecal pollution is another major issue in aquatic biomes as it affects the metabolic processes of organisms. Water quality is important because it affects the health of fish and the health of the ecosystem. The main source of both nitrate and feces is the food the fish eat. More specifically, the protein found in fish food. In commercial fisheries, the diet of the fish is incredibly important as these fish need to be nutritious for human consumption. In aquaria, the diet of fish is important for the fish's overall health, longevity, and vibrancy. Knowing all this, commercial fisheries and home fish keepers would want to feed their fish protein-rich food. However, the chemical and physical pollution caused by these foods is often neglected, leading to wastes building up in the water. What's currently unknown is whether foods with high protein concentration pollute the water more than foods with less protein. What's also unknown is whether feeding higher protein foods will lead to more trace amino acids in the fish feces. In other words, how does the protein concentration in a fish's diet affect the concentration of amino acids it releases back into the environment. These amino acids can be considered as another nitrogenous pollutant. This work aimed to study whether there was a relationship between the protein concentration in a fish's diet and how chemically and physically the water gets polluted by looking at water clarity, nitrate concentration, and also tryptophan and tyrosine concentration. The hypothesis is that increasing the protein concentration in fish food would lead to increases in the nitrate concentration, fecal pollution, and amino acids due to more nitrogen from the protein entering the water column. The experiments test how protein concentration in fish feed is correlated to water pollution.

Materials and Methods

Animals:

Three species of fish were studied in this project: freshwater angelfish (*Pterophyllum scalare*), Tamasaba Goldfish (*Carassius auratus*), Koi (*Cyprinus rubrofuscus*). The kois and goldfish were purchased locally and raised domestically. The angelfish, whose parents were also purchased at a local pet store, were raised from birth at home. The 50 sibling angelfishes live in an indoor 125-gallon aquarium. The three tamasaba goldfish live in an outdoor 100-gallon pond. The thirteen koi live in an outdoor 430-gallon pond separate from the goldfish.

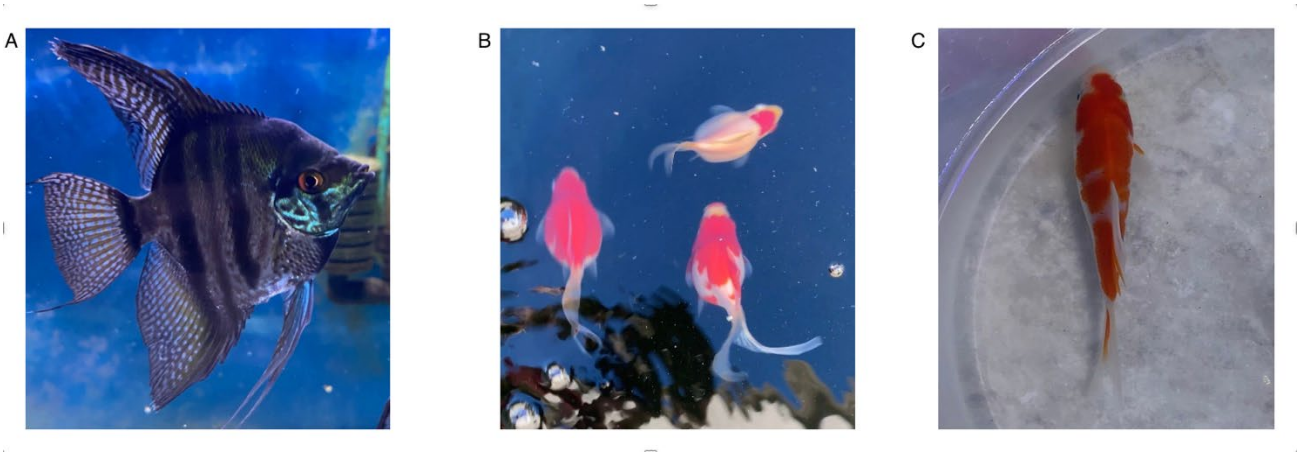

Figure 1: The figure above shows all the fish studied in this work. Figure 1A on the left depicts one of the many angelfish. Figure 1B in the middle depicts the three Tamasaba goldfish. Figure 1C on the right depicts one of the koi in the outdoor pond.

Fish Feed:

Three varieties of protein concentration foods were used in this project: 28%, 36%, and 42% (Figure 2). The feeds were purchased from an aquatic foods distributor. The fish were fed 5 times over the course of three days. Two feedings the first day, two feedings the second day, and one feeding on the third day. Each fish in aquaria and pond were fed 0.032g of food per gallon of water to standardize the feeding.

Figure 2: The figure above shows the three types of protein feed fed to the fish. Figure 2A depicts the food with 28% protein concentration. Figure 2B in the middle depicts the food with 36% protein concentration by weight. Figure 2C on the right depicts the food with 42% protein concentration.

The brand of the 28% protein food was Half Off Ponds All Season Formula purchased from Amazon. The brand of the 36% protein food was Aquamaster Wheat Germ. The brand of the 42% protein food was the Aquamaster Growth food. These last two foods were purchased from Champion Nishikigoi, a local pet store. A total of three tests were performed to determine the water pollution based on protein concentration: nitrate test, water clarity test, and tryptophan/tyrosine concentration test.

Nitrate Test:

First, a 75% water change was conducted on each of the three aquatic environments in which the three species resided. After giving 12 hours for the aquarium parameters to stabilize without feedings in the meantime, a nitrate reading was taken in each of the aquarium and ponds. The liquid nitrate test required 5mL of aquarium water and 10 drops of API nitrate solution #1 (*Liquid Nitrate Test Solution #1*, 2018) composed of hydrochloric Acid and 10 drops of nitrate solution #2 (*Liquid Nitrate Test Solution #2*, 2018) composed of polyethylene glycol and sulfanilamide. Solution 2 was vigorously shaken for 1 minute before adding. Both solutions were from the API freshwater master test kit. The solution of the tank water and the two test solutions was then capped, and the test tube inverted to mix. After 5 minutes, the color of the water sample sufficiently changed to reflect the concentration of the nitrate. Figure 3 shows how the color changed depending on the nitrate concentration in the water. For this kit, a redder color indicated a greater concentration of nitrate.

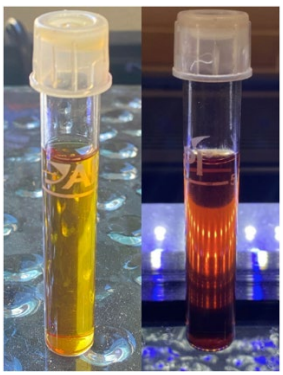

Figure 3: The figure above shows the color variation in the nitrate test depending on the concentration of nitrate present in the aquarium above. When the nitrate test solutions are added to a 5 mL sample of aquarium water, the water changes color to reflect the concentration of nitrate. The left tube shows a nitrate concentration between 0-5 ppm when compared to the reference sheet (figure 4). The right tube shows a nitrate concentration between 20-40 ppm when compared to the reference sheet.

This colored water was compared to a reference sheet (Figure 4) to determine the concentration of nitrate. The column farthest to the left on the reference sheet is the nitrate color chart. The colored water typically did not specifically match with any color on the chart, so a range in which the reading likely fell was taken and the two endpoints of the range were averaged to get the reading. This nitrate reading was the initial reading.

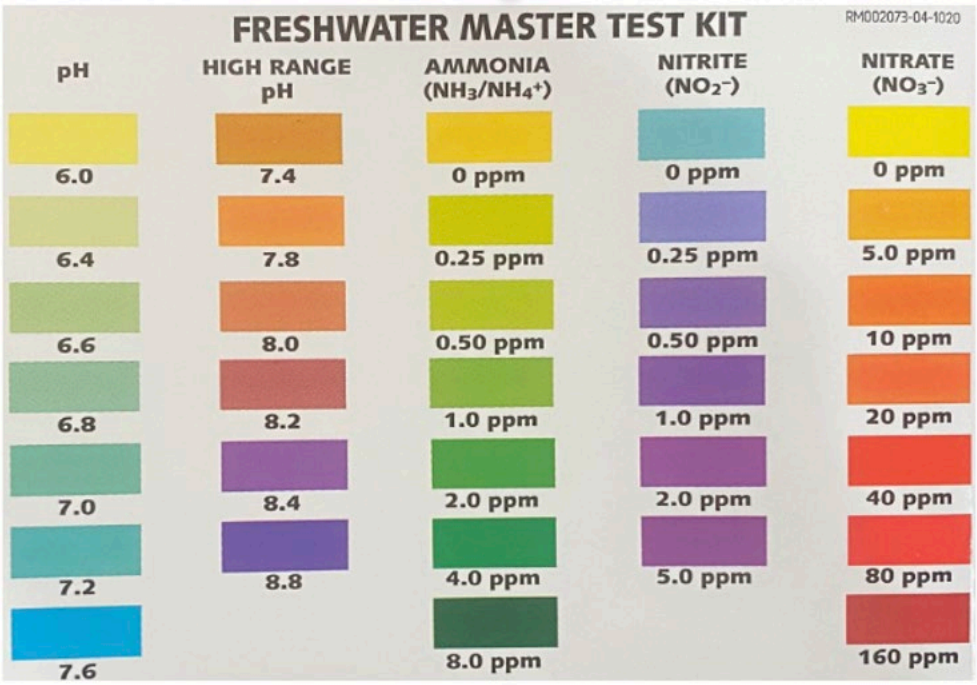

Figure 4: The rightmost column in the reference sheet above shows the standards the nitrate test was compared to.

Then the fish were fed five times total every twelve hours over the course of three days with the food type varying depending on which food was being tested. At the end of this period, another nitrate test was taken to see the

change in nitrate concentration in the aquaria. This is the final nitrate reading. After comparing this new reading to the reference sheet, another 75% water change was done and the steps above were repeated for a different protein concentration of food. This test would help determine which food and which species of fish produced the most nitrates and chemically polluted their habitat the most. The entirety nitrate test was repeated to ensure the repeatability of the results.

Water Clarity Test:

Before changing the aquarium water, the feces produced by the fish was collected with a pipette. Feces was extracted from the aquarium and pond filters and placed in a collection tube. It was then sterilized in a 70% ethanol solution for fifteen minutes and then filtered out with Whatman filter paper. The aquarium filters and the tank were then cleaned so the feces from one type of food did not get mixed up with feces from the next food being tested. The feces was diluted in a 1:2 solution of distilled water. One-part feces (0.75 ml) and two-part water (1.5 mL).

This solution was then vortexed for 15 seconds. After the feces settled out, the supernatant was extracted and placed in a separate container. One milliliter of this supernatant was pipetted into a cuvette and run through the UV-Vis program on the Denovix DS-11 Spectrophotometer (Figure 5). The spectrophotometer provided an absorption curve that detailed what wavelengths of light (220-760 nm) were being absorbed the most by the supernatant. The higher the absorbance, the more light that was absorbed at that wavelength and the less clear the water was due to the type of food being fed. Each sample was tested 5-10 times and then averaged for the absorption curve. The solutions were blanked with distilled water.

Tyrosine and Tryptophan Test:

For this test, a new microliter of supernatant was run through the A280 Protein program on the Denovix DS-11 Spectrophotometer (Figure 5). After entering the molecular weight and extinction coefficient of Tyrosine into the Spectrophotometer, the machine analyzed a 1 microliter sample of the supernatant and determined how many mg/mL of protein was present. Then the molecular weight and extinction coefficient were adjusted to the values of tryptophan and the test was repeated. This test was conducted on the feces of each fish from every protein concentration food. Tryptophan and tyrosine are some common amino acids in feces (Gauthankar et al., 2021). Each sample was tested 5 times and then averaged. The molecular weight of tyrosine is 181.19 amu and the extinction coefficient is 1280. The molecular weight of tryptophan is 204.09 amu and the extinction coefficient is 5690.

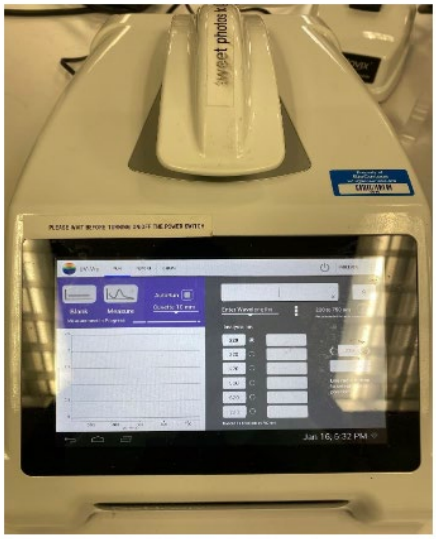

Figure 5: The figure shows the Denovix Spectrophotometer with the UV-Vis spectrophotometry software loaded.

The importance of the nitrate test is that it will indicate which food led to the most chemical pollution of the water. The water clarity test is important because it indicates how physically polluted and unclear the water had become due to the feedings. The tryptophan/tyrosine test indicates how well the protein is being absorbed by and what concentration is just ending up back into the environment.

Results and Discussion:

A few trends can be pointed out by simply looking at the data. For the nitrate test (Table 1), the change column was calculated by subtracting the initial reading average (n=6) from the final reading (n=6) of each fish and each food. Increasing the protein concentration, generally increased the nitrate levels in the goldfish, angelfish, and koi aquarium/ponds. This is shown by the change column where as the concentration of protein in the food increased, more nitrate was produced by the fish leading to a larger difference between the initial and final readings. This can be seen by comparing the change column between the foods for each fish. One thing to note is that for the koi and goldfish, the nitrate change for the 42% protein food was identical to the change of the 36% protein food as the upper and lower limit of the test kit had been reached.

Table 1: The table above shows the results of the nitrate test.

	28% Protein			**36% Protein**			**42% Protein**			**Con-trol**
	Initial	**Final**	**Change**	**Initial**	**Final**	**Change**	**Initial**	**Final**	**Change**	
Angel-fish	10.5 ppm	10.5 ppm	0 ppm	15 ppm	30 ppm	15 ppm	2.5 ppm	30 ppm	27.5 ppm	0 ppm
Koi	7.5 ppm	60 ppm	52.5 ppm	15 ppm	120 ppm	105 ppm	15 ppm	120 ppm	105 ppm	0 ppm
Goldfish	2.5 ppm	2.5 ppm	0 ppm	2.5 ppm	7.5 ppm	5 ppm	2.5 ppm	7.5 ppm	5 ppm	0 ppm

The type of fish is on the left while the food the fish was fed is on the top. The table itself then contains the initial and final nitrate concentration of the tank when the respective fish were fed the food. The change in nitrate was calculated

by subtracting the initial from the final nitrate readings. The control was the nitrate test being run on distilled water. The change tended to increase as protein concentration increased.

In terms of water clarity, increasing the protein concentration did not necessarily decrease the clarity of the water. The absorbance curve of the 42% protein food was not always the highest and the absorbance curve for the 28% curve was not always the lowest (Figure 6, Figure 7 and Figure 8) for each of the fish. What this proves is that there is no relationship between the protein concentration in the food and how much the water clarity was affected. For example, in Figure 7, the absorbance curve for the 28% protein food on average was the greatest, then the 42% curve and then the 36% curve at the bottom of the graph. This indicates that the 28% food polluted the water clarity the most as it led to more turbidity and a greater absorbance. This contrasts with the nitrate test where feeding fish 28% protein least changed the nitrate concentration (Table 1).

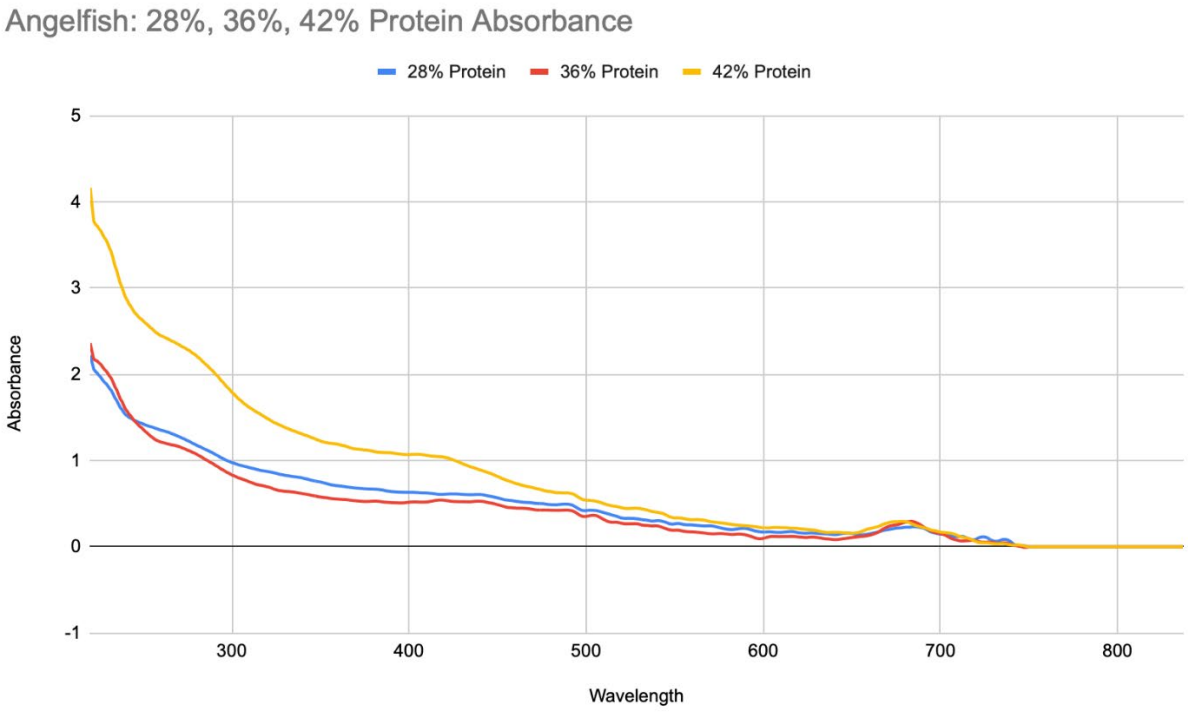

Figure 6: The table above shows the average absorbance at different wavelengths of light for angelfish for the water clarity test. The blue line represents the absorbance curve of the 28% protein food, the red line the 36% protein food and the yellow line the 42% protein curve.

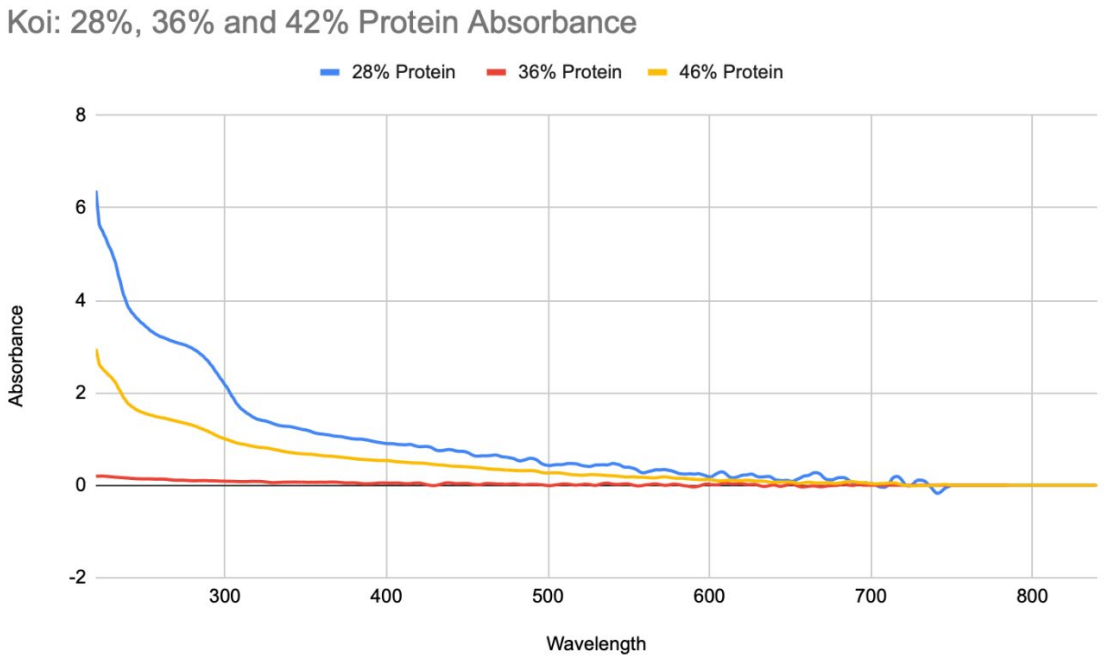

Figure 7: The table above shows the average absorbance at different wavelengths of light for koi for the water clarity test. The blue line represents the absorbance curve of the 28% protein food, the red line the 36% protein food and the yellow line the 42% protein curve.

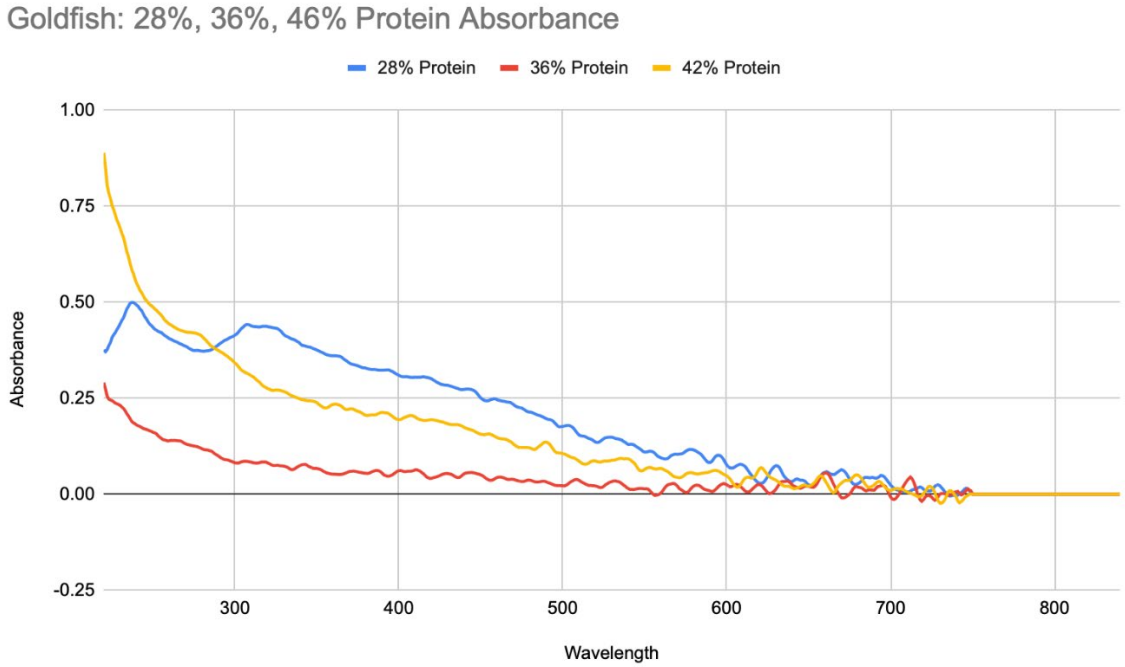

Figure 8: The table above shows the average absorbance at different wavelengths of light for goldfish for the water clarity test. The blue line represents the absorbance curve of the 28% protein food, the red line the 36% protein food and the yellow line the 42% protein curve.

For tyrosine (n=6), there was a direct relationship between increasing protein concentration and the presence of tyrosine for the goldfish and angelfish, but there was no relationship for the koi. (Table 2)

Table 2: Tyrosine concentrations for each fish when fed with varying protein concentrations of food. The type of fish is labelled on the top and the food the fish were fed is on the left.

	Angelfish	**Goldfish**	**Koi**
28% Protein	0.021 mg/mL	0.0008 mg/mL	0.15 mg/mL
36% Protein	0.047 mg/mL	0.0031 mg/mL	0.0054 mg/mL
42%Protein	0.060 mg/mL	0.045 mg/mL	0.037 mg/mL

For tryptophan (n=6), (Table 3) there was a direct relationship between increasing protein concentration and the presence of tryptophan for the goldfish and somewhat for the angelfish, but no relationship for the koi. What all this means is that while trace amino acids were found in feces, they were not necessarily always increasing with protein concentration.

Table 3: Tryptophan concentrations for each fish when fed with varying protein concentrations of food. The type of fish is labelled on the top and the food the fish were fed is on the left.

	Angelfish	**Goldfish**	**Koi**
28% Protein	0.00711 mg/mL	0.00033 mg/mL	0.03 mg/mL
36% Protein	0.0172 mg/mL	0.0016 mg/mL	0.002 mg/mL
42% Protein	0.016 mg/mL	0.0061mg/mL	0.0115 mg/mL

Conclusions:

From this preliminary data, the hypothesis is only partially supported. It was hypothesized that increasing the protein concentration in a fish's diet would increase the nitrates produced by the fish. This was generally true for the goldfish, angelfish, and koi. It was also hypothesized that increasing the protein concentration would increase the absorbance and decrease the water clarity. This only held true for the 36% and 42% protein foods as the 28% protein food led to considerably more water turbidity and reduced clarity. Finally, it was hypothesized increasing the protein concentration would increase the presence of amino acids in the feces. This did not hold true for all the fish and protein concentrations but was mostly true. Therefore, in conclusion, foods with higher protein concentrations did in fact chemically pollute the environment with more nitrate but did not necessarily affect the clarity of the environment or lead to more trace amino acids in the feces. This finding can be used by aquarium hobbyists or aquaculture farmers for the optimal growth and health of their aquatic life. Now, fish keepers will be more aware of the effects of feeding protein rich foods to their fish.

Future Expansions

In the future, this test will be run during different seasons, with different other species of fish or, with different protein concentrations. One thing to note is that the three foods used in this work did not all come from the same manufacturer. The 28% food came from a different provider, and this may explain why it was consistently higher than the other two foods on the absorbance table. Food produced by the same manufacturers will be used next time.

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