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Article

Increased survival of aquatic life through algal pretreatment of nitrate-contaminated runoff

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SUMMARY

Industrial discharges, agricultural runoff, and waste from livestock farms contain high concentrations of nitrates which contaminate groundwater, rivers, lakes, and oceans. Accumulation of nitrates in water bodies has been shown to have significant negative effects, from physiological harm to fish and invertebrates to ecosystem-level impacts and food web-wide effects. Elevated nitrate levels also contribute to harmful algal blooms and eutrophication, creating dead zones that further threaten aquatic life. Humans may develop several health complications such as methemoglobinemia in infants, increased risks of certain cancers, thyroid dysfunction, and other health problems from consuming aquatic animals with high levels of nitrate. This research explores methods for reducing the toxicity of nitrate on aquatic life. We hypothesized that treating nitrate-contaminated river water with algae (algal pretreatment) will reduce nitrate levels and allow fish introduced into the treated water to survive longer than they would in untreated water. We tested our hypothesis by adding a range of nitrate concentrations to river water, performing or withholding algal pretreatment, and then adding fish. We found that algal pretreatment extended fish survival by up to 50%, supporting the hypothesis that algal pretreatment can effectively mitigate the harmful effects of nitrate runoff on aquatic life. We believe the ability of algae to consume nitrate makes them an effective tool to protect aquatic life. Further studies are needed to confirm that the reduction in nitrate by algae was the cause of the increase in fish survival.

INTRODUCTION

Nitrogen is an essential element for all living organisms. It is a key building block of DNA, proteins, and chlorophyll, which makes it vital for plant growth (1). Because of this, nitrogen-based compounds, especially nitrates, are common ingredients in fertilizers used in agriculture around the world. However, while nitrogen is necessary in small amounts, too much of it in the environment can disrupt ecosystems (2).

Nitrate (NO_3^-) is a compound found naturally in the environment, but human activities have resulted in elevated and toxic nitrate levels in water bodies (3, 4). Major sources of nitrate release include discharge from wastewater treatment plants due to insufficient nitrogen removal processes, industrial plant waste, runoff from farms sprayed with synthetic fertilizers, and livestock effluents (5–7).

Nitrogen and algae themselves are not inherently toxic to the environment; it is their overabundance that disrupts the natural equilibrium of ecosystems (8). Nitrate toxicity disrupts the balance of aquatic ecosystems through a process where elevated nitrate levels contribute to the growth of algal blooms (8,9). These blooms deplete oxygen in the water, leading to hypoxic dead zones that inhibit aquatic life (7, 8). Such dead zones are prevalent in the areas of the Gulf of Mexico and Lake Erie (11–13). Some algal species such as cyanobacteria can also produce harmful toxins that affect both humans and aquatic life (14).

Elevated nitrate concentrations can harm both humans and aquatic organisms (15, 16). For humans, the U.S. Environmental Protection Agency and the World Health Organization recommend that drinking water contain no more than about 10 mg/L nitrate-nitrogen (approximately 45–50 mg/L nitrate) (10, 15). Aquatic organisms are also affected by nitrate pollution, though sensitivity varies by species and life stage. Studies have shown that juvenile fish can experience stress and developmental effects at nitrate-nitrogen concentrations as low as 6–25 mg/L, while adult fish typically tolerate higher levels before acute effects occur (16).

Nitrate pollution disrupts food webs and reduces biodiversity, resulting in cascading negative impacts on aquatic ecosystems, livestock populations, and human health (17). High nitrate levels have been shown to be toxic to fish and invertebrates, leading to abnormal behaviors such as respiratory distress, erratic swimming, reduced feeding, impaired growth and reproduction, and increased mortality rates (18). Nitrate concentrations as low as 20–30 mg/L can cause physiological stress and behavioral changes in some fish species, while concentrations above 90 mg/L can lead to acute toxicity and death (16). In humans, ingestion of nitrate-contaminated food or water has been linked to methemoglobinemia or "blue baby syndrome," certain types of cancers, and thyroid disorders (19, 20). Overall, nitrate pollution is a serious environmental and health concern, requiring proactive measures to limit excess nitrates in water and prevent their harmful effects on ecosystems.

In some extreme cases, nitrate pollution has reached concentrations within the hundreds or even thousands of mg/L. Such cases usually involve intense pollution sources or industrial accidents (21, 22). Nitrate-rich effluent from explosives factories (e.g., those using ammonium nitrate) can reach extraordinarily high concentrations. Recorded values include about 3000 mg/L (as nitrate) in wastewaters from munitions plants in Poland (21). In areas of heavy fertilizer use and arid climates, nitrates can accumulate in soil and leach into groundwater at extreme levels. Groundwater under intensively farmed land in Pakistan was reported at 1610 mg/L nitrate, and about 700 mg/L was found in a farming region of Namibia (22, 23). Nitrate contamination approaching four

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figures has also been observed in areas with poor sewage disposal (24). Even in the United States, isolated wells have recorded extremely high nitrate concentrations in agricultural regions. For instance, an EPA survey in Runnels County, Texas, reported a well with nitrate levels reaching 3584 mg/L, one of the highest ever recorded in a U.S. groundwater source (25).

A number of methods have been proposed for the reduction of nitrate pollution in water. Constructed wetlands use plants and microbial communities to remove nitrate (26). Denitrification walls involve burying organic carbon material in the ground to promote microbial denitrification (27). These techniques, however, require significant land area and release potent greenhouse gases. Other methods, such as adsorption with activated carbon or biochar or microbial conversion of nitrates to nitrogen gas are efficient but have limited capacity, disposal challenges, and require careful monitoring (28, 29).

Recent work has shown that algae can reduce the nitrate concentrations in wastewater by assimilating nitrates for growth, effectively removing them from the water as they incorporate nitrogen into their biomass (30). There is an increasing focus towards cultivating algal biomass in nutrient-rich environments which, in addition to effectively lowering nitrate levels, can produce biomass for food, fertilizer, and biodiesel production (31, 32). These studies highlight algae's dual role in wastewater treatment and in biological manufacturing.

In this study, we conducted experiments to test whether algal pretreatment could reduce the toxicity of nitrate-contaminated runoff water. Specifically, we grew algae in nitrate-amended river water to allow them to assimilate and remove nitrate before introducing fish. We hypothesized that pretreating water with algae would lower nitrate concentrations and thereby increase fish survival compared to water without algal pretreatment. Consistent with this hypothesis, our results showed that fish survived about 50% longer in pretreated water, indicating that algal pretreatment can be an effective strategy for improving water quality and supporting aquatic life. This study also captures our vision for how the dual benefits of algal pretreatment could be utilized - reducing nitrate contamination while producing biofuel sustainably (**Figure 1**).

Although the term "algae" is used throughout this paper, our study organism is likely a mixed population that includes cyanobacteria, commonly referred to as blue-green algae. While cyanobacteria are technically bacteria and not true algae, they perform photosynthesis and play similar ecological roles, especially in nutrient-rich waters. In the context of this study, we use the term "algal pretreatment" to describe the effect of photosynthetic microbial growth, regardless of precise taxonomy, on nitrate levels and aquatic toxicity.

RESULTS

We hypothesized that pretreating nitrate-contaminated water with algae would reduce nitrate toxicity and improve fish survival. To test this, we simulated nitrate-contaminated river water and evaluated whether algal pretreatment lowered toxicity. To simulate the broad range of nitrate levels observed in the real world, two sets of four 1-liter jars of river water were prepared. In each of those two sets, nitrate was added

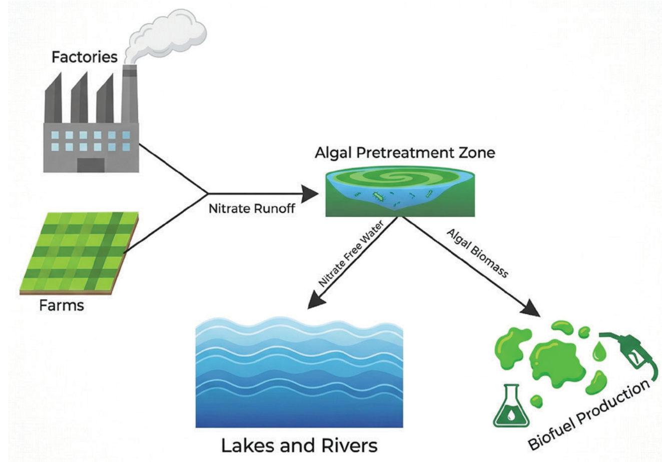

Figure 1: A schematic representation of a potential solution to use algal pretreatment for wastewater cleanup and biofuel production. Nitrate runoff is directed to an algal pretreatment zone where algae absorb the excess nutrients. The process results in nitrate-free water being returned to lakes and rivers, while the harvested algal biomass can be used for biofuel production. This dual-purpose approach addresses both water pollution and sustainable energy generation.

to achieve concentrations of 0, 133, 400, or 1200 ppm. Algae were also introduced to all jars. To test the effect of algal pretreatment, half of the jars were exposed to light for nine days, promoting algal growth (pretreatment) (**Figure 2**). The other half were kept in the dark (no-pretreatment control) (**Figure 3**). The jars exposed to light showed clear algal growth, while no visible algal growth was observed in the jars kept in the dark (**Figure 2, 3**). After the nine-day period, the algae were filtered out from both sets of jars using a standard paper coffee filter to prevent blooms. Fathead minnows (*Pimephales promelas*), a freshwater species native to the river were introduced into each jar, and fish survival time was monitored as an indicator of nitrate toxicity. This design choice allowed us to isolate the effect of algal pretreatment (i.e., active growth) rather than algae presence alone.

We observed that the fish in the jars with higher concentrations of nitrate died much sooner than those in jars with lower nitrate concentrations. Specifically, fish lifespan decreased by approximately 4.7 hours per 100 ppm of added nitrate in pretreated jars, and by approximately 3.1 hours per 100 ppm in untreated jars (**Figure 4**). At the lowest nitrate level (0 ppm added), fish survived approximately 184 hours (~7.7 days) with pretreatment versus 119 hours (~5.0 days) without, a difference of 65 hours. At the highest nitrate level tested (1200 ppm added), survival dropped to approximately 129 hours (~5.4 days) with pretreatment versus 82 hours (~3.4 days) without pretreatment - a difference of 47 hours (**Figure 4**). Across all conditions, fish survived an average of 56 +/- 2.5% longer in pretreated jars than in untreated jars (**Figure 4**). Linear regression of fish survival against nitrate concentration showed strong fits for both treatment groups (pretreatment: $R^2 = 0.999$, $p = 0.023$; no pretreatment: $R^2 = 0.945$, $p = 0.028$). The results supported our hypothesis that algal pretreatment of nitrate-polluted water can improve aquatic life.

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Figure 2: Jars with added algal pretreatment in a light-rich environment. Four jars were kept under daylight bulbs (40-watt each) to promote algal growth. The jars contained river water with nitrate added at the following concentrations, from left to right: 0, 133, 400, and 1200 ppm. Each jar included algal biomass consisting mainly of blue-green algae (likely cyanobacteria such as *Anabaena* or *Microcystis spp.*), though no formal identification was performed. This photo was taken on Day 9, immediately before the algae were filtered out and fish were introduced.

DISCUSSION

We constructed a simple system in which river water was amended with varying levels of extra nitrates and subsequently mixed with a fixed amount of algal biomass. One set of jars received algal pretreatment by being exposed to light for 9 days, while a parallel control set was kept in the dark. We found that algal pretreatment increased fish survival length, while higher nitrate concentrations were toxic to the fish (**Figure 4**).

We did not include a control group with nitrate-amended water that lacked both algae and light exposure. Instead, our control jars received algae but were kept in the dark to prevent algal activity. It is worth noting that the water itself was not sterile. We added algae, but even a control without the added algae would have developed biomass if exposed to light. Since this was an imperfect control, we chose to use the presence or absence of light as a clearer way to represent industrial algae treatment in this scaled-down model. However, we acknowledge that including a no-algae nitrate control would have helped determine whether the presence of algae (independent of light) had any effect on fish survival.

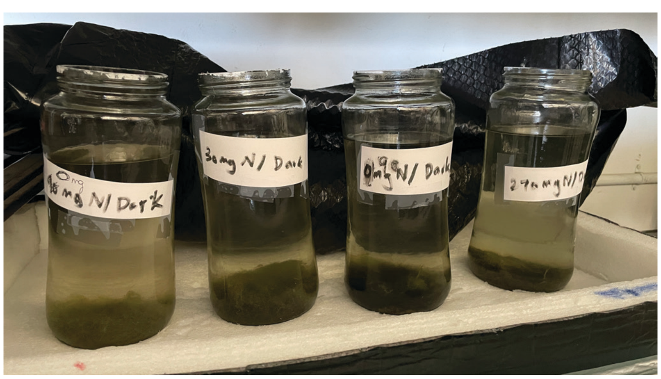

Figure 3: Jars with no algal pretreatment in a dark environment. Four jars containing river water with nitrate added at the following concentrations, from left to right: 0, 133, 400, and 1200 ppm, were kept in complete darkness to inhibit algal growth. The same algal biomass shown in **Figure 2** (primarily blue-green algae, likely cyanobacteria such as *Anabaena* or *Microcystis* spp., though not taxonomically confirmed) was added to each jar. This photo was taken on Day 9, immediately before the algae were filtered out and fish were introduced. The jars were kept under identical conditions except for light exposure; no light source was provided in this setup.

It was unexpected that the pretreatment had its greatest effect when there was no added nitrate. The mechanism by which pretreatment is assumed to act is to remove the added nitrate, so the pretreatment effect is expected to be the smallest at a 0 ppm nitrate concentration. A possible explanation for this result is that there could be preexisting nitrates in the Guadalupe River water used in our experiment. We did not measure the baseline nitrate concentration of the river water prior to the experiment. However, the USGS water quality survey of Santa Clara Valley streams reports detectable nitrate levels in the Guadalupe River at San Jose, with total nitrite plus nitrate concentrations ranging from approximately 0.9 to 1.7 mg/L as N (equivalent to roughly 4 to 7.5 mg/L as NO3) during the study period (37). The removal of these preexisting nitrates by algal pretreatment may have contributed to the increased fish survival observed in the 0 ppm group. In future experiments, baseline nitrate concentration should be measured before adding any amendments, using a quantitative method such as a colorimetric assay or ion-selective electrode, which would provide more precise measurements than the indicator strips used in this study. Additionally, the algal pretreatment may have consumed other toxic compounds not accounted for in our experimental setup, such as organic pollutants, which may have also contributed to the increased fish survival observed in the 0 ppm group. In future experiments, weighing the mass of algae removed from each jar could allow us to infer whether higher nitrate

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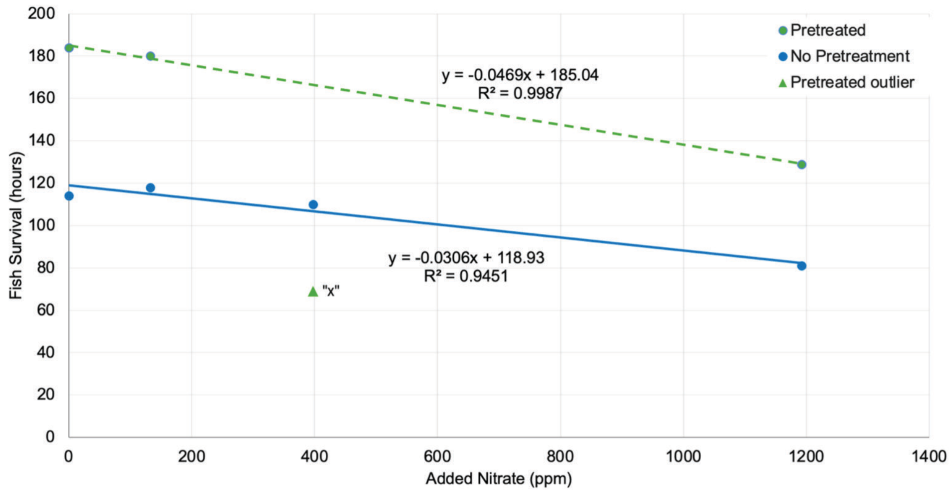

Figure 4: Algal pretreatment improves fish survival rates, while increasing nitrate concentrations decrease fish survival rates. Scatter plot of survival time versus nitrate amendment, with line fits for pretreated (green) and not-pretreated (blue) conditions. The green line (pretreatment) has an R^2 value of 0.999 and *p*-value of 0.023, and blue line (no pretreatment) has an R^2 value of 0.945 and *p*-value of 0.028. Each point represents the survival time of a single fish in one jar per condition. The green triangle ("x") indicates an outlier data point at 400 ppm in the pretreated group, where a fish survived for a significantly shorter time than expected based on the regression trend.

concentrations led to greater algal growth and thus greater removal of potentially toxic compounds.

It was also surprising that the decrease in lifespan due to nitrate, represented by the slope of the line fit, is greater with pretreatment (0.047 hours/ppm vs. 0.031 hours/ppm) (**Figure 4**). A possible explanation is that the algal pretreatment was capable of removing only a certain fraction of the nitrate present in the water and thus had a greater impact on jars with the lower added concentrations. High nitrate concentrations were still quite toxic with or without pretreatment. This result could be clarified by repeating the experiment with more quantitative nitrate measurements.

One data point was a clear outlier to the trend established by the rest and was omitted from the analysis (**Figure 4**, "x"). This fish in the 400 ppm nitrate pretreated jar died a day before the fish in the untreated jar at the highest concentration of added nitrate. Since there was only one fish in each jar and only one jar at each condition, this jar has been treated as an outlier and omitted from our analysis. In future experiments, it would be desirable to avoid such potential bias by having multiple fish per jar and multiple jars per condition.

While this study showed a clear association between pretreatment, nitrate concentration, and lifespan, we could not achieve a more subtle understanding of the direct relationship between nitrate concentration and lifespan. We attempted to measure nitrate concentration, but the assay performance of indicator strips — even on the initial, untreated water — was too inconsistent and qualitative to be included in our report of results or the subsequent analysis. We recommend more precise and quantitative nitrate quantitation methods be used in future studies.

The algae obtained for this experiment were not specifically filtered by species. We collected algae from a pond originating in natural springs. Although we made sure to collect blue-green colored algae which is a characteristic trait of cyanobacteria, it could have been composed of other organisms. Blending these algae into a homogeneous solution might have allowed for the algal species to be more evenly distributed. In the real-world setting, aquatic life would be exposed to sunlight and darkness alternatively. However, in our experiments, we did not follow a day-night schedule and instead kept the lights on 24 hours for the pretreated jars, which may have caused algal strain.

A similar challenge in consistency was the process of obtaining freshwater minnows native to the area. It was difficult to obtain fish of similar age and size, and this could have led to discrepancies in our data. Future experiments will use fish hatched at approximately the same time to reduce variability.

Administering the correct concentration of the nitrate was also a challenge. The fertilizer came in the form of pellets, and the entire package had a composition of 10.87% nitrogen in the form of nitrate (NO_3^-). Since nitrogen makes up only a fraction of the mass of nitrate, we used stoichiometric conversion to calculate that this corresponds to 48.13% nitrate (NO_3^-) by mass. We assumed that each pellet contained 48.13% nitrate and accordingly administered the specific number of pellets for the desired total concentration. To more carefully repeat the experiment, we would mix a specific mass of nitrate pellets with a specific volume of water, calculate the concentration of the resulting solution, prepare dilutions of that solution, and then use precise liquid-transfer methods to amend all eight jar conditions with the same volume of the appropriate nitrate solutions.

The level of algal pretreatment required for real-world implementation depends on the extent of nitrate contamination and local environmental conditions. We reviewed literature for regions with high fertilizer use, such as agricultural farms in the Central United States, and areas with heavy livestock farming, such as Minnesota (33, 34). In both situations, specific runoff studies show nitrate levels as high as 25 mg/L, especially in periods following heavy rainfall or irrigation events (16, 35). In

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extreme conditions, nitrate concentrations can exceed 1,000 mg/L, and in some instances, exceed 3,000 mg/L (21, 22, 25). Our experiment was designed to account for this broad range of nitrate pollution typically observed in agricultural and industrial runoff. The observations in this study allow us to estimate that even the simple algal pretreatment we used would decrease the loss of aquatic life by around 50%.

In this study, we observed that the lifespan of fish decreased significantly with increasing nitrate concentrations, even after algal pretreatment (**Figure 4**). This indicates that while algal pretreatment can mitigate nitrate toxicity to some extent, its effectiveness diminishes at higher nitrate levels. One possible reason is that the same amount of algae was added to all jars regardless of nitrate concentration, leading to insufficient nitrate removal in jars with higher contamination. In future experiments, we will test whether increasing the algal biomass or extending the pretreatment duration improves nitrate removal efficiency. These results suggest that algal pretreatment could serve as a potential method for reducing nitrate levels in contaminated water, particularly when appropriately scaled to match nitrate concentration.

In addition to cleaning up agricultural runoff, algal biomass can be used to produce a green alternative to traditional fuels. A recent study showed that compressing or heating algae can convert the internal fats and sugars into fuel which is readily separated out during the process (36).

The algae used in the pretreatment step can be harvested before they can overgrow, preventing the excessive buildup that leads to algal blooms. This captures our vision for how the dual benefits of algal pretreatment could be utilized - reducing nitrate contamination while producing biofuel sustainably (**Figure 1**).

Our experiments highlight that algal pretreatment has the potential to support aquatic life and we believe that algal pretreatment is a promising solution for mitigating nitrate pollution in water. As expected, adding nitrate to river water resulted in a decrease in fish lifespan. Pretreating the nitrate-amended river water with 9 days of algae growth extended fish lifespan by 50% (**Figure 4**). A mechanistic understanding of the interaction of nitrate levels, algal pretreatment, and lifespan requires more experiments, but this study demonstrates that algae could be a viable option within broader strategies aimed at reducing the impact of nitrates from agricultural and industrial runoff. As algal biomass has its own economic value, such pretreatment processes may have a very favorable cost-to-benefit comparison.

MATERIALS AND METHODS

Water Collection

We collected the water used for the experiments from the Guadalupe River in California. The river water contained leaves, plants, microbes and insects that are typical of the composition of water in runoff. According to the USGS water quality survey of Santa Clara Valley streams, the Guadalupe River at San Jose had total nitrite plus nitrate concentrations ranging from 0.29 to 1.7 mg/L as N, median total phosphorus of 0.22 mg/L as P, median dissolved oxygen of 7.4 mg/L during non-storm periods, and median dissolved organic carbon of 9.0 mg/L (37). We collected the water during the daytime and in the fall season.

Nitrate Source

We used the fertilizer Better-Gro Orchid Plus Water Soluble Orchid Food as the source of nitrate for the experiments. The packet of 0.45 kg contains nitrates as the main element (20% nitrogen in the form of nitrogen from nitrate and nitrogen from ammonia), with other elements such as hydrogen, phosphorus, copper, and iron making up the remaining composition.

Algae Collection

In the attempt to obtain cyanobacteria, we scooped out blue-green algae floating atop Sunnyvale Baylands Park, Santa Clara County into two clear jars, which we later transferred to a platter kept under two direct daylight 40-watt bulbs. The algae were in a clumpy, concentrated form. The collected sample appeared as blue-green clumps floating at the pond surface, a visual trait often associated with cyanobacteria. Although we refer to the biomass as algae throughout this paper, we did not perform a taxonomic identification. Therefore, it is likely that the sample contained a mix of photosynthetic organisms, including cyanobacteria and possibly eukaryotic algae. Our use of the term "algae" refers broadly to this mixed biomass.

Fish Selection

We used fathead minnows (*Pimephales promelas*), a freshwater species native to the area, from a local Petco store. Minnows were chosen for this study because they are resilient and widely found in freshwater environments.

Administering Nitrate

We filled two groups of four 1-liter jars each with river water. In both groups, we administered 0 ppm, 133 ppm, 400 ppm, and 1200 ppm of nitrates respectively. To precisely calculate the nitrate concentration, we used the following method.

We noted that 1 liter of water weighs 1 million mg. The fertilizer used came in the form of pellets and the entire package had 10.87% of nitrate nitrogen. Using a stoichiometric conversion, we calculated that this corresponds to 48.13% nitrate (NO_3^-) concentration, as shown below:

$$\frac{10.87\text{ g N (from NO}_3)}{100\text{ g fertilizer}} \text{ x } \frac{1\text{ mol N}}{14\text{ g N}} \text{ x } \frac{1\text{ mol NO}_3}{1\text{ mol N}} \text{ x } \frac{62\text{ g NO}_3}{1\text{ mol NO}_3} = \frac{48.13\text{ g NO}_3}{100\text{ g fertilizer}} = 48.13\% \quad \textbf{(1)}$$

For each target nitrate concentration, we first converted nitrate ppm to nitrate-nitrogen ppm using the molar mass ratio of nitrogen to nitrate (14/62). We then used ~9.2 x[desired nitrate-nitrogen ppm] mg of the fertilizer (since it is composed of 10.87% nitrate nitrogen) per liter of water, to obtain the desired nitrate concentration.

Algal Pretreatment Setup

We added two tablespoons of algae to each of the eight jars. We kept the jars designated for algal pretreatment in a light-rich environment with one daylight 40-watt bulb dangling above each jar to promote algal growth (**Figure 2**). We kept the other group of jars in an enclosed, dark environment, not giving the algae light to thrive (**Figure 3**). Together, these figures show the two environmental conditions used to test the effect of algal pretreatment. We kept these jars in this setting, undisturbed, for nine days. On the tenth day, we filtered out the algae from both sets of jars using a standard paper coffee filter.

Fish Introduction and Observation Protocol

We added one minnow to each jar and every day we fed the minnows a pinch of fish food (Aqueon GoldFish Flakes) per jar. The jars were left open to air, allowing for natural oxygen diffusion. No additional oxygenation was introduced, as the goal was to simulate stagnant or slow-moving water typical of nitrate runoff environments. We checked the viability of the fish three times (morning, noon and night) each day and created a photographic record. We continued recording until the last fish had died, which was on the 7th day. The health and time of death of the fish were observed and recorded.

Analysis of Fish Lifespan Data

To analyze the effect of nitrate concentration and algal pretreatment on fish lifespan, we used linear regression models. Fish survival duration (in hours) was plotted against nitrate concentration for each treatment group (pretreated and untreated). A separate linear trendline was fitted for each group to quantify the relationship between nitrate level and lifespan. The slope of each line reflects the change in survival time per ppm increase in nitrate, and the intercept represents the estimated lifespan at 0 ppm.

Regression analysis yielded the following lifespan trends:

- Lifespan with pretreatment = 185 hours − 0.047 hours/ppm x (nitrate in ppm)
- Lifespan without pretreatment = 119 hours − 0.031 hours/ppm × (nitrate in ppm)

These regression equations were used to compare the impact of nitrate on fish survival across treatments. Due to the small sample size (one fish per jar), results should be interpreted with caution. Linear regression was performed to quantify the relationship between nitrate concentration and fish survival, yielding p-values of 0.023 and 0.028 for the pretreated and untreated groups respectively.

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