HIGH SCHOOL EDITION
Journal of Student Research

Volume 12 Issue 3 (2023)

Effects of Nitrate Pollution on Cellular Regeneration and Survivability in Planarian Flatworms

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ABSTRACT

Increased nitrogen runoff from commercial fertilizer use has raised issues regarding hypoxia in freshwater habitats. One species noted as a water indicator is planarians, known for their pluripotent stem cell regeneration. This study tested the effects of various nitrate concentrations on planarian specimens with ablated heads, to ascertain the effects of the nitrate on regeneration and survivability. The tests found that there is a negative correlation between nitrate concentration and planarian survival rates, proving that nitrogen runoff can indeed adversely affect similar organisms in a freshwater habitat.

Introduction

In recent years, there has been a growing concern regarding the presence of nitrogen-based fertilisers in agricultural soil runoff. Though naturally occurring, an overabundance of nitrogen compounds can render freshwater sources uninhabitable. Researchers have conducted various studies on how nitrogen affects freshwater organisms, such as dragonflies and minnows (Howarth, 2008). However, few studies have covered the impact of nitrogen on planarians— an important indicator species.

Planarians have long been treated as model organisms in the study of stem cell tissue regeneration. This is due to their ability to regenerate in their entirety through an abundance of neoblasts (adult stem cells). Planarian regeneration is highly sensitive to their surrounding environments, also making them a good water quality indicator (Rink, 2013).

Review of Literature

Although nitrogen is a naturally-occurring element and the nitrogen cycle is key to a healthy ecosystem, an overabundance of nitrogen compounds can render freshwater sources uninhabitable. Researchers have conducted various studies on how nitrogen affects freshwater organisms, such as dragonflies and minnows (Howarth, 2008). However, few studies have covered the impact of nitrogen on planarians— an important indicator species.

Planarians have long been treated as model organisms in the study of stem cell tissue regeneration. This is due to their ability to regenerate in their entirety even from tissue fragments and showing no apparent signs of physiological ageing (Rink, 2013). These traits originate from an abundance of adult stem cells, also known as neoblasts, in the planarians. Neoblasts make up almost 30% of all cells in a planarian, and are proven to be pluripotent (able to differentiate into all zygotic cell types) or even totipotent (capable of regenerating an entire organism), based on an experiment which showed that a “lethally irradiated worm can be restored to perfect condition by transplanting a single Neoblast from a healthy donor into it” (Rink, 2013).

The time required for planarian tissue regeneration depends greatly on the number of tissues lost and the location of the injury, as well as environmental factors such as temperature and pH. Dean and Duncan establish that the optimal protocol for cultivating planarians is to house them in low-salt water solutions at 18-22°C and neutral pH ranging from 6.9-8.1 (2020). Using these parameters, Deochand et al. tested the time required for regenerating from various injuries in the planarian species *Schmidtea mediterranea* (2020). Deochand et al. performed single-eye, double-eye, and entire head ablations on planarian specimens before recording the specimen morphology over the course of 28 days.

The results of their experiment showed that the planarians were in all cases able to regenerate fully functional eyes and even their brain structures 14 days after the ablation. However, the regenerated organs were smaller than the original specimen, and the planarians were only able to grow back to their original size 28 days after ablation (Deochand et al.,2020). Another observation made during the research was that for the eye ablations, "wound sizes at 1h post-ablation revealed that the wound site contracted to an average of 47.83% of the original eye size prior to ablation and this size was not statistically different from the overall eye size later on day 14" (Deochand et al.,2020). This suggests that the planarians regenerate by reallocating existing neoblasts to the injury site instead of creating new tissues. These findings set a basis for other studies involving the temporal and morphological aspects of planarian regeneration, especially when foreign elements are introduced to test the effects they have on planarian regenerative capabilities.

One such study conducted by Calevro et al. focused on determining the effects of heavy metal exposure on regenerating planarians (1998). The research revolved around three heavy metals: aluminium, cadmium, and chromium, which were each introduced at varying concentrations into the habitat solutions housing decapitated planarian specimens. The results showed that "Al^{3+} and Cr^{3+} were lethal at 1.5 mM concentration", while Cd^{2+} was lethal at concentrations over 1.8 mM (Calevro et al., 1998). More importantly, the study displayed the various sublethal responses to heavy metal exposure at lower concentrations between 0.25 mM and 0.5 mM. In most cases, despite the non-lethal dose, specimens showed blastemas (mass of neoblasts at the site of injury) that were either abnormally shaped or of reduced size. Furthermore, "50% of the regenerants degenerated the blastema within 6 days and the resulting stumps were not able to start regeneration again" (Calevro et al., 1998). At the end of the test, most of the remaining planarians could only manage to regenerate a small and abnormal head. Calevro and his associates 'results showcased the physiological responses of planarians when they are exposed to pollutants, which led to other similar experiments conducted with different variables in mind.

Ding et al. conducted a study similar to Calevro et al., opting to use a much more common heavy metal—Fe^{3+} (2019). Ding's study also went in a slightly different direction and focused on observing the rate of heavy metal poisoning when the planarians were subjected to different temperatures ranging from 15°C to 25°C. The results yielded showed that the specimens died the quickest at 20°C and slowest at 15°C, meaning that "low temperature could slow down the effect of Fe^{3+} on the planarian toxicity, and at a suitable temperature, the toxic effects of Fe^{3+} on the planarian can be accelerated, which leads to the increased planarian mortality" (Ding et al., 2019). This study also gave some insight into how the planarian's metabolic rate affects the speed at which toxins are able to be absorbed and take effect.

Cao et al. built upon Ding's research and carried out various tests on how planarians react to extreme environmental stress (2020). Other than altering temperature, Cao et al. also induced environments of extreme gravity, magnetic fields, and oxygen concentration. Both intact and regenerating worms were then observed. The results on extreme temperature fluctuations corroborated the results of Ding et al., while the other experimental groups each displayed abnormal physiological responses. Most notable was the effect of inhibiting reactive oxygen species (ROS) on regenerating planarians. ROS are "natural byproducts of cellular oxidative metabolism and play important roles in the modulation of cell survival, cell death, differentiation, cell signalling, and inflammation-related factor production" (Abdal Dayem et al., 2017). As such, reducing the surrounding oxygen concentration would also inhibit ROS production. This caused the planarians to fail at regenerating lost parts and also restricted neoblast differentiation. The effect of limited oxygen is especially pronounced in planarians since it inhibits neoblast differentiation and not stem cell proliferation, and as proven by Deochand et al., planarians mostly regenerate by reallocating existing neoblasts to the site of injury (2016).

Despite the countless studies done on planarians and their regenerative capabilities, there are still many potential variables and experiments that still need to be addressed. The effect of inorganic nitrogen compounds in particular has never been tested on planarian regeneration. Nitrogen compounds can cause hypoxia due to nitrite ions that convert oxygen-carrying pigments into forms that are incapable of carrying oxygen, thereby inhibiting oxidative metabolism (Carmago & Alonso, 2006). Due to their extreme dependence on oxygen reactions for regeneration, planarians should be affected by high nitrite concentrations as well, since ecess nitrate poisoning is known to cause anoxia (extreme hypoxia) in animals (Thompson, 2021). This basis leads to the development of my fundamental research question: How does nitrite pollution alter cellular regeneration in planarian flatworms?

Methods

Materials

This study was carried out on brown planarian (*Dugesia tigrina*) specimens (Carolina Biological Supply Company). The specimens were housed in 15mm x 60 mm Petri dishes containing 15 ml of Poland spring water. The dishes were maintained at room temperature (20-25°C), with a pH of 7-8. For the experiment, sodium nitrite crystals (Shor International Corp.) were mixed with spring water to create a stock solution of 2000 ppm. The solution was then administered at different concentrations through pipettes. Nitrate testing slips (SJ Wave) and freshwater pH testing kits (SJ Wave) were used to maintain suitable water conditions. A tweezer and scalpel were used to perform the head ablation, which took place in a petri dish.

Procedure

The planarians were split into four equal groups (9 in each group), each housed in a separate Petri dish with equal volumes (15 ml) of liquid. One dish served as the control setup containing no nitrates. The sodium nitrate solution (2000 ppm) was then diluted with the Poland spring water into each of the three remaining dishes to create nitrate concentrations of 5 ppm, 10 ppm, and 25 ppm. Each planarian specimen was then ablated right below the auricles with a scalpel, removing its head and brain structure (refer to Fig 1).

Fig 1: Black line shows the site of the ablation

The planarians then remained in their respective dishes for a period of 16 days. The water in each dish was tested for nitrate and pH concentration and replaced every 2-3 days to maintain stable nitrate concentrations. The experiment was then repeated with increased nitrate concentrations (50ppm, 100ppm, 250ppm, 500ppm, 1000ppm, and 2000ppm).

Design and Analysis

Throughout the experiment, photos and observations of the planarians 'regenerative process were taken every 2-3 days via a microscope and phone camera.

Observed physiological traits for specimens in each of the four dishes were recorded. These traits included the overall appearance of the planarian (colouration, presence of cellular degradation and lesions, reaction to physical stimuli) and the development of the ablation site (amount of tissue regenerated, colouration,).

At the end of the experimental period, the final state of the specimens was recorded (number of dead and surviving planarians, number of successfully regenerated heads). The presence of complete optic receptors, intact auricles, and complete replenishment of lost internal and external tissues defines a fully regenerated head. Heads lacking any one of these aspects were considered partially regenerated, and other mutations such as excess tissue were considered abnormally regenerated. Instances where the specimen population increased due to reproduction were also recorded.

After all the data had been collected, the effects of different nitrate levels on Planaria survival and tissue regeneration were compared through graphs, and the survival probability of each nitrate concentration setup was calculated using the Kaplan-Meier Estimate, which is the preferred method of measuring the percentage of living subjects over an experimental period(Goel et al., 2010). At any specific time period, the survival probability is calculated with the following equation:

$$S_t = \frac{\text{Number of subjects living at the start - Number of subjects died}}{\text{Number of subjects living at the start}}$$

Results

The purpose of this experiment was to determine if the presence of nitrate at varying levels in planarian habitat water affects their regeneration process and survival rates. In each petri dish, the number of living planarians with fully regenerated heads was recorded along with the number of planarians with partially/abnormally regenerated heads and the number of deceased planarians after a period of 14 days (time taken for a planarian head to fully regenerate).

Initially, the experiment was carried out with nitrate concentrations of 5, 15, and 25 ppm. However, all specimens were able to completely regenerate their heads with no visible differences. Further testing was conducted with concentrations of 50, 100, and 250 ppm, which revealed that the planarians were not affected by concentrations under 250 ppm (refer to appendices A-C for raw data). The experiment that yielded the most varied results was the final one, with concentrations of 0, 500, 1000, and 2000 ppm. Furthermore, all planaria that survived had fully regenerated heads and there were no instances of incomplete or abnormal regeneration amongst all specimens.

Table 1. Planarian specimen data after 14 days (starting with 9 specimens each)

Nitrate Concentration (ppm)	Living planarians (fully regenerated heads)	Living planarians (partially/abnormal regenerated heads)	Deceased planarians
0	16 (number increased due to reproduction)	0	0
500	3	0	6
1000	0	0	9
2000	0	0	9

Figure 2 shows the percentage increase/decrease of living planarians after the test period. The number of living specimens alive at various points during the experiment was also recorded and graphed in Figure 3. Out of the 4 Petri Dishes, specimens in the 0 ppm concentration dish were able to split and reproduce, increasing in number.

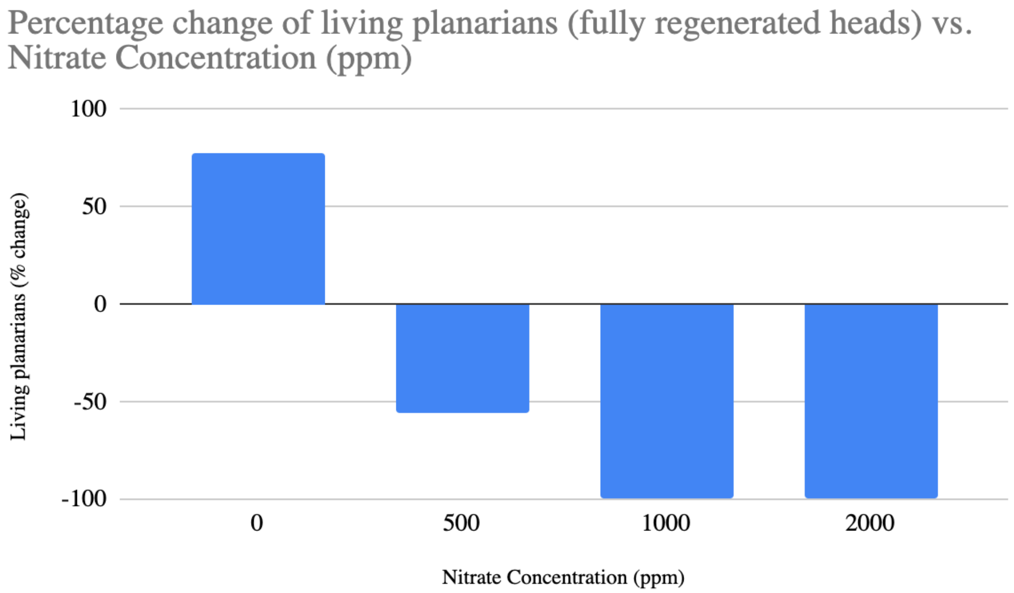

Fig 2. Percentage increase/decrease of living planarians after the test period

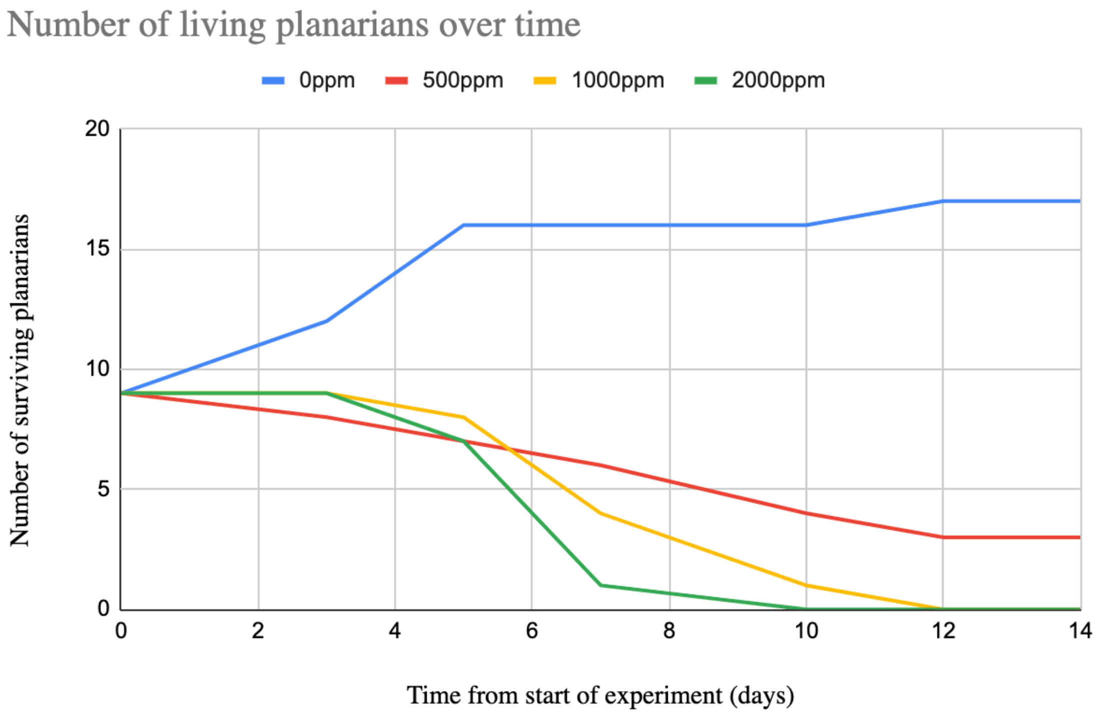

Fig 3. Comparison of living planarians over experimental period

The survival probability of each nitrate concentration was calculated using the Kaplan-Meier estimate.

Table 2: Kaplan-Meier estimate for specimens in 500ppm solution (similar calculations were performed for 1000 and 2000ppm)

Days from the start of the experiment	No. of specimens died(d)	No. of specimens alive at start of day(a)	Estimated survival probability (1- d/a)	Survival Probability at end of time (500ppm)
3	1	9	0.889	0.889
5	1	8	0.875	0.889 x 0.875 = 0.778
7	1	7	0.857	0.778 x 0.857= 0.667
10	2	6	0.667	0.667 x 0.667 = 0.444
12	1	4	0.750	0.444 x 0.750 = 0.333
14	0	3	1.000	0.333 x 1.000 = 0.333

Table 3. Comparison of Kaplan-Meier survival probability between different nitrate concentrations

Time from the start of the experiment (days)	Survival Probability at end of time (500ppm)	Survival Probability at end of time (1000ppm)	Survival Probability at end of time (2000ppm)
3	0.889	1	1
5	0.778	0.889	0.778
7	0.667	0.444	0.111
10	0.444	0.111	0
12	0.333	0	0
14	0.333	0	0

The data shows that as time goes on, the survival probability of both the 1000 and 2000 ppm specimens decreases at a much faster rate than that of the 500 ppm setup.

Discussion

The results of the experiments proved that at concentrations greater than 500 ppm, increased nitrate concentration results in decreased planarian survival rate. While 8 out of 9 specimens in the 250 ppm solution were able to survive and completely regenerate their heads, none of the specimens subjected to concentrations of 500 ppm and above were able to survive or regenerate successfully. Furthermore, when compared to the planarians in 500 ppm nitrate solutions that had a linearly decreasing survival rate, the specimens in 1000 ppm and 2000 ppm concentrations had an exponentially decreasing probability of survival.

These results are in line with previous research restricting oxygen in planarian specimens (Cao et al., 2020). Due to the planarians having a high reliance on stem cell regeneration for survival, nitrogen poisoning is detrimental to their overall survival and regeneration. The nitrate compounds hijack the stem cell's ability to bind to oxygen, resulting in hypoxia that hinders tissue regeneration and eventually leads to cell death. This also means that soil runoff containing nitrogen-based fertilizers will have a detrimental effect on planarian survivability.

One unexpected outcome of the first few experiments was that the planarians were unaffected by concentrations ranging from 10-200 ppm. The CDC states that water containing less than 10 ppm of nitrates is safe for all consumption purposes. Concentrations higher than 10 ppm could harm vulnerable individuals such as infants (Centres). This led to my original hypothesis that planarians would also be affected at concentrations greater than 10 ppm. However, the specimens proved to be relatively unaffected by the lower concentrations throughout the experimental period. This could be due to the nitrate compounds at lower concentrations being unable to hinder oxygen transport within the two-week period.

One of the research limitations was the relatively small sample sizes for each experiment and that the specimens were not able to be checked on a daily basis. This hindered the process of accurately determining the exact time when the planarian populations decreased, which decreases the accuracy of the calculated survival probabilities. The small sample sizes decreased the reliability of the experiment, as the survival rate could have been altered by irregular factors such as the size and prior health of each planarian.

Future research prospects could include research on how alternative fertilizers affect planarian survival. Some examples include ammonia and urea-based fertilizers. Since nitrogen exists in more complex forms in these compounds, the toxicity is lessened in such variants (Cregg, 2018). This experiment could be repeated using the two compounds to determine if they are detrimental to planarian survival.

In conclusion, high nitrate concentrations (>250 ppm) result in increased planarian mortality, which may be a reflection of the impact of increased nitrate concentration in freshwater habitats.

Conclusion

Nitrate pollution is one of the most common forms of contamination in freshwater habitats. The effects of nitrate toxicity on the regeneration and survival of key indicator species like the planarian has profound implications for its impact on freshwater ecosystems. The results from this study can also be applied to other animals capable of and reliant upon pluripotent stem cell regeneration. Before these experiments, many heavy metal toxins had been tested on planarian specimens (Calevro et al., 1998), but the effects of nitrate hypoxia were never tested on soft-bodied invertebrates.

The negative correlation between planarian survivability, regenerative ability, and water nitrate concentration proves that high nitrate levels could also cause cellular degeneration in other soft-bodied organisms. This highlights the dangers of nitrate runoff from over usage of commercial fertilizer, as well as the harm they could cause to freshwater invertebrates. To prevent the hypoxia and eutrophication of marine life, it would be beneficial to adopt the usage of alternative fertilizers, such as urea or ammonium-based fertilizers, for future agricultural purposes.

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