The Effects of Glyphosate on the Growth and Viability of *Azotobacter vinelandii*

Ethan Feild

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Lab Supervisor: Dr. Malhotra- (805)-404-4120

Thousand Oaks High School

Expert Advisor: Dennis Fenton- (805)-231-9590

Fenton and Associates

Location of Experiments: Room E8 laboratory at Thousand Oaks High School

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Abstract:

With an overall increase in the use of pesticides around the world, it is important to understand their effects on organisms that are not necessarily the desired target of their effects. This study focused on how the commonly used herbicide glyphosate affects the growth of *Azotobacter vinelandii*, a soil living, nitrogen fixing bacteria that acts well as a model organism for many other soil dwelling species. The bacteria were exposed to glyphosate in varying concentrations and the growth was measured over the course of a week, along with the pH of the cultures. What was found was that the glyphosate treated cultures had a much lower rate of growth than the ones without, averaging at around 71% less growth in the treated cultures. From this it can be concluded that the glyphosate did inhibit the bacteria's ability to grow and negatively impacted their rate of reproduction. It is likely that other bacteria in the soil are susceptible to having their growth inhibited as well, due to the fact that *Azotobacter vinelandii* is a model species.

Purpose:

To better understand how glyphosate affects important soil dwelling organisms, in this case the nitrogen fixing bacteria *Azotobacter vinelandii*.

Introduction:

Pesticides are a modern miracle to the agricultural industry, having increased yield and reduced labor costs by removing pests that would otherwise harm or damage crops (Ghimire & Woodward, 2013). Herbicides kill off weeds that would compete with the crop species,

insecticides kill insects that would otherwise consume the crop and kill it, and fungicides wipe out fungal infections in roots. Ever since the 1950s pesticide use has grown by 50%, whilst continued development has made them more potent as well (Jin et al., 2014). This trend of growing use in conjunction with the increased toxicity has led to some unprecedented and unwanted side effects. It should come to as no surprise that exposure to these toxic chemicals can cause complications to a wide variety of organisms, including humans. One well-known incident of pesticides harming wildlife is the case of dichlorodiphenyltrichloroethane, better known as DDT. DDT was a popular insecticide banned in 1972 that was found to weaken the egg shells of birds and had been the cause of decline in population in many species, sometimes to the point of near extinction (Yang et al., 2012). Additionally, it was found that exposure to DDT led to osteomalacia (softening of the bones) and a vitamin D deficiency in humans, even in those who were never exposed to the insecticide directly. Another study conducted in France found a connection between exposure to pesticides in pregnant women and a higher chance of a low birth weight, increased likelihood of premature birth, a smaller head circumference, and other complications (Mayhoub et al., 2014). Likely a cause of cell communication disruptions during development. Additionally, a group of researchers were able to link exposure to pesticides with blood cancers such as acute myeloid leukemia (Jin et al., 2014).

Chemicals like these are generally used in very controlled amounts over specific areas, but it is near impossible to prevent the chemicals from entering into the environment after being introduced to the fields. A study done in China found around 29.7 parts per trillion of organochlorine pesticide particles in precipitation from around the country (Yang et al., 2012).

This, in of itself, is a very small concentration, but it shows that pesticides can retain their form even when evaporating into the atmosphere.

Many species of bacteria play a key role in soil preservation, processing nitrates and carbon for plants to absorb, while also secreting auxins and cytokinins, hormones that encourage plant growth and development (Raynaud & Nunan, 2014)(Huhe et al., 2014). But perhaps one of the most important functions of soil bacteria is nitrogen fixation. Nitrogen is second only to water in its importance to plant growth, being one of the major components of chlorophyll and proteins (Huhe et al., 2014). Without nitrogen the plant cannot photosynthesize and produce the sugars it needs, causing it to wither up and die. Even the nitrogen source matters, with one study showing that nitrogen from a bacterial source is more effective at promoting plant growth than a chemical fertilizer (Esmailpour, Hassanzadehdelouei, & Madani, 2012).

One of the most common bacteria involved in nitrogen fixation in soil is *Azotobacter vinelandii*. This organism is considered a model organism for nitrogen fixing bacteria due to its worldwide range and the ability to adapt its metabolism based on its environment (Sebutal et al., 2009). These bacteria have been used for many years in the field of biochemistry for studying the process of nitrogen fixation. Unlike other bacteria that can fix nitrogen, *A. vinelandii* is unique in that it has three separate enzymes with which it can fix nitrogen, known as nitrogenases: molybdenum nitrogenase, vanadium nitrogenase, and a purely iron nitrogenase. Under aerobic conditions, those with oxygen in them, the bacteria will take nitrogen from the air and convert it into ammonia, which is then secreted into the surrounding area. The ammonia is then converted by other bacteria into nitrates and then taken up into the plant for use. Additionally, these bacteria are free living, meaning that they do not reside in special nodes in the roots like many

other nitrogen-fixing genera, but rather dwell in the soil itself (Huhe et al., 2014). Said bacteria aren't immune to the toxicity of pesticides, as seen by an experiment that investigated how pesticides affect soil bacterial diversity (Feld et al., 2015). What the researchers found was that the pesticides lowered the diversity of bacteria and impeded nitrate production.

An article from the National Center for Biotechnology Information even reveals that organochlorine pesticides have the known effect of suppressing nitrogen-fixation in soil, which harms the ability of vegetation to grow in it (Potera, 2007). Whilst it doesn't specify which genera are affected, *A. vinelandii* fits into that wide category of bacteria, making more reasonable to use this model organism for the experiments.

The chemical being tested, glyphosate is an organophosphate pesticide, unlike DDT. It is the active ingredient in the weed killer Roundup, which is used to kill a wide array of invasive plants in lawns, fields, and gardens (Henderson, Geravis, Luukinen, Buhl Stone, 2010). While glyphosate has a relatively low toxicity to humans and other large, multicellular animals, it's known to kill off bacteria and other microorganisms in addition to plants (Henderson, Geravis, Luukinen, Buhl Stone, 2010; WHO, 1996).

Glyphosate targets the enzyme EPSP-synthase, which is found in fungi, plants, and many microorganisms. It is also damaging to cell membranes and walls, though to a lesser extent (Wang, Lin, Li, and Lin, 2016; Schönbrunn et al., 2001). EPSP-synthase is part of the amino acid synthetic pathway that is vital in the creation of the aromatic amino acids tyrosine, tryptophan, and phenylalanine. Glyphosate interrupts this pathway by competitively inhibiting the active site of the enzyme where phosphoenol pyruvate, simple a sugar, would normally bind and renders the entire enzyme complex inactive. Without the ability to synthesize these amino acids many

organisms that rely on creating their own are eventually unable to synthesize the proteins that require them and the cell dies off.

This experiment investigated whether glyphosate had any effect on the growth, survival, and reproduction of *Azotobacter vinelandii* when it is in the optimal environment for its growth. By studying how this commonly used herbicide interacts with an equally common soil bacterium we can better understand the effects humans have on the soil that they use. This better understanding of these interactions can aid in improving agricultural techniques as well as aid in environmental protection.

Hypothesis:

The glyphosate will inhibit *A. vinelandii*'s growth in an agitated liquid media, with higher concentrations showing a more significantly slowed rate of growth as compared to the controls.

Null Hypothesis:

The pesticides will not inhibit bacterial growth and reproduction and the bacteria will divide and grow in a manner similar to the controls.

Safety:

The bacteria were almost entirely harmless, being classified as a biosafety level one bacteria, which only requires sterilization of the work area after use and thorough hand washing. However, the pesticides were treated as potentially carcinogenic and toxic, and were handled with the utmost care to minimize exposure and release into the environment. Solid glyphosate

was handled in small quantities, milligram amounts at the greatest and was always handled inside the chemical fume hood to ensure none was able to contaminate the air of the lab. Relatively low concentrations of dissolved glyphosate were handled carefully to prevent spills and absorbed with activated carbon after use. Contaminated tools were cleaned with a solution activated carbon before being disposed of or washed.

Materials:

- *Azotobacter vinelandii* culture (Ward Science)
- Ashby's Sucrose Broth (Recipe from Himedia, materials from TOHS Lab)
    - Sucrose
    - Dipotassium phosphate
    - Magnesium sulfate
    - Sodium chloride
    - Potassium sulfate
    - Calcium carbonate
- Glyphosate (Sigma-Aldrich)
- Micropipettes (From TOHS Lab)
- Micropipette tips (From TOHS Lab)
- Incubator (Boekel from TOHS Lab)
- Fume Hood (Fisher Hamilton from TOHS Lab)
- Activated Carbon (Sigma-Aldrich)
- Litmus paper (TOHS Lab)

- Spectrophotometer (Metash Model V-5000 from TOHS Lab)
- Spectrophotometer cuvettes (From TOHS Lab)
- Autoclave (Bioclave from TOHS Lab)
- Erlenmeyer flask (From TOHS Lab)
- Aluminum foil (From TOHS Lab)
- Masking tape (From TOHS Lab)
- Analytical balance (From TOHS Lab)

(See page 10 for MSDS and back of packet for hard copies if printed out.)

Methods:

Broth Preparation

Before the cultures could be grown a broth for *Azotobacter vinelandii* was made with 10.0 grams of sucrose, 0.100 grams of dipotassium phosphate, 0.100 grams of magnesium sulfate, 0.100 grams of sodium chloride, 0.050 grams of potassium phosphate, and 2.500 grams of calcium carbonate were measured out on an analytical balance. The solids were then placed into a 1 L Erlenmeyer flask. 500 mL of DI water was then poured into a graduated cylinder before being added to the flask with the other ingredients. After ensuring the broth was properly mixed, the flask was set on a hotplate and the top was covered with aluminum foil before being allowed to just reach boiling before being removed. The broth was then split equally into two 500 mL flasks and placed in the autoclave at 134°C and then stored in the refrigerator until needed.

Azotobacter vinelandii Culturing

To ensure a uniform starting bacteria density a standing culture of *Azotobacter vinelandii* was created by using an inoculation loop to remove some bacteria out of the solid streak tube that it was shipped in and placed into 50 mL of the previously prepared liquid media described above. It was then incubated for a few days under the same conditions as the other cultures detailed below. Inoculation of the cultures consisted of mixing 4 mL of inoculant from the standing culture and 46 mL of sterile broth.

Experimental Design

During the experiment there were six different culture groups, with duplicates for each. The first two cultures were controls groups, with one pair of replicates consisting of just 50 mL of liquid media for a negative control and another of 50 mL of media inoculated with *A. vinelandii* but not exposed to any of the glyphosate for a positive control. The four experimental groups contained 50 mL of the liquid media and were inoculated with *A. vinelandii*, but also contained either 5, 10, 50, or 100 parts per million of glyphosate.

Glyphosate Stock Preparation

In order to create the glyphosate concentrations a 5,000 ppm stock of glyphosate was made in water, and then the calculated amounts necessary to reach the target concentrations were added to the cultures. Creation of the stock was performed under the fume hood to prevent any glyphosate dust particles from making it into the air and any disposable items such as weighing dishes and aluminum foil was disposed of into a sealable plastic bag for later hazardous waste disposal.

Culture Incubation

The cultures were next placed into an incubator set at 25°C, the optimal temperature for *Azotobacter vinelandii* growth, which ensured that the temperature remained constant during the treatments. An orbital shaker was placed inside the incubator and set to 100 rpm to keep the cultures agitated. This ensured that the calcium carbonate remained suspended in the media while also making sure enough oxygen and nitrogen were able dissolve to allow for the bacteria to grow.

Culture Sampling

After inoculation 0, 20, 24, 48, and 120 hour samples of 2 milliliters were taken by a P-1000 micropipette and placed into a rectangular prism cuvette. However, due to the calcium carbonate in the media, a white precipitate remained suspended that was crucial to bacterial growth. Therefore, the cultures were allowed to sit out for approximately 10 minutes to allow the precipitate to settle before the samples were taken from near the top of the culture.

In order to measure the growth of the bacteria, a spectrophotometer was used. The instrument was set to a wavelength of 600 nm and blanked against DI water. Then the cuvette with the sample inside it was placed into the spectrophotometer and the optical density at 600 nm was measured. Each sample was then removed from the instrument and set aside for obtaining pH measurements.

Since the sample volume was small, the size of a pH probe meant that it would be too large to measure changes in the solution's pH. Instead the sample was pH was measured by soaking it in calibrated litmus paper. This test determined whether or not a change in pH was the potential cause of the bacteria's poor growth death and not the glyphosate as *A. vinelandii* prefers a neutral to slightly basic environment for optimal growth.

All equipment and glassware were washed thoroughly before and after use and had activated carbon applied to them in order to absorb the glyphosate. While bleach or 70% alcohol was used to destroy the *A. vinelandii* cultures. Contaminants were then disposed of into the appropriate waste disposal.

Results:

The sterile control culture showed very little growth, ending with an optical density of 0.084 after 120 hours of incubation, while the glyphosate negative control bacteria culture ended with an OD of 1.49 after 120 hours of incubation. Compared to the bacterial control at 120 hours the 5 ppm glyphosate culture was inhibited by 69.8% (P=0.003), the 10 ppm glyphosate culture was inhibited by 67.2% (P=0.014), the 50 ppm glyphosate culture was inhibited by 62.3% (P=0.003), and the 100 ppm glyphosate culture was inhibited by 85.3% (P=0.018). The average percent inhibition for all glyphosate treatments at 120 hours as related to the OD was 71.2%. There was no significant deviation in the measured pH throughout the trial, which remained at 7.0 for all 120 hours in each culture.

Table 1 Averages of the optical density for each culture in order to measure how well the bacteria were growing in their respective media.

Cultures	0 hours	20 hours	24 hours	48 hours	120 hours
Sterile control	0.011	0.017	0.024	0.019	0.084
Control bacteria	0.086	0.337	0.311	0.627	1.490
5 ppm	0.273	0.188	0.205	0.185	0.450

glyphosate					
10 ppm glyphosate	0.248	0.239	0.234	0.174	0.489
50 ppm glyphosate	0.318	0.263	0.271	0.267	0.561
100 ppm glyphosate	0.077	0.114	0.113	0.191	0.219

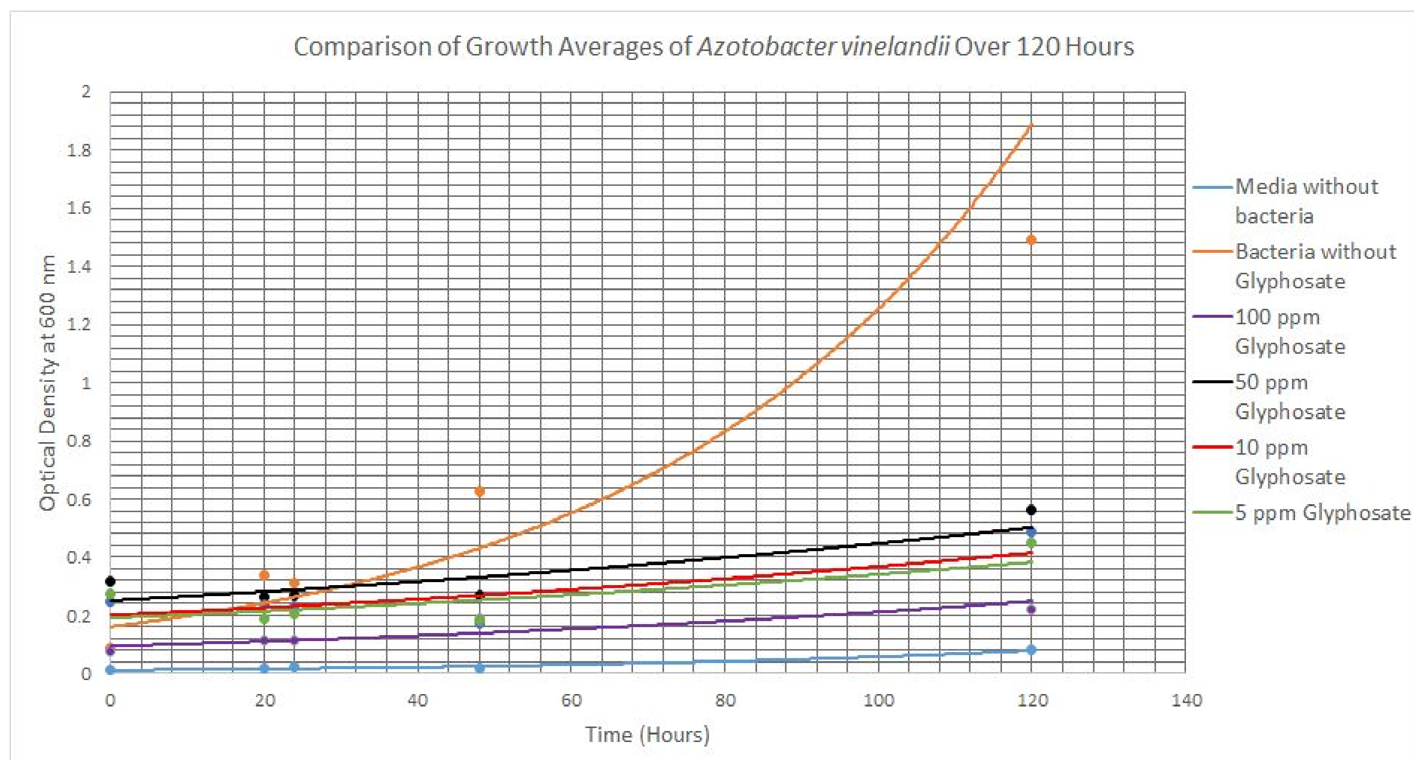

Fig. 1. Graphed averages of the cultures' average optical densities to better visualize the growth curves

Discussion:

This experiment was able to determine that glyphosate did inhibit the growth of *A. vinelandii* when grown in an agitated liquid media, even at fairly low concentrations. As shown in **Fig. 1.**, all of the cultures containing glyphosate remained at around the same optical density with very little apparent growth while the control bacteria culture continued to grow at a typical exponential rate. The miniscule increase in optical density in the sterile control is most likely due to contamination from repeated samples being removed from the flask, exposing the media to the open air for long enough for microorganisms to contaminate it without making a large overall difference. While there was some variation in the optical density between the different glyphosate containing cultures the differences do not appear to be significant enough to indicate a trend of increasing inhibition between the three lowest concentrations of glyphosate. This is most likely because the difference in glyphosate concentration is not large enough to show a change in the inhibition of the bacteria's growth. However, the 100 ppm cultures showed slightly less growth as compared to the more dilute cultures, meaning there is a probable connection between the concentration of glyphosate and the inhibition of the bacterial growth when there is a larger difference.

Throughout the course of the experiment the pH of the media remained stable, staying within at most 0.5 of 7.0 (Ashby's Sucrose broth has a pH of 7.4±0.2). Since this was the case throughout all of the cultures, it can be assumed that the bacteria were unable to change enough atmospheric nitrogen into ammonia to cause a rise in pH within the span of 120 hours, even in higher concentrations like the control culture. As such, a change in pH can be eliminated as the cause for the bacteria's slower growth in the experiment.

The most likely reason glyphosate was able to inhibit the growth of *A. vinelandii* is because it could no longer produce aromatic amino acids and thus, proteins and enzymes that required any of the three in their polypeptide sequences were no longer possible to synthesize. No longer able to produce essential proteins the metabolisms of the bacteria slow and they are unable to reproduce. As the proteins synthesized prior to glyphosate exposure degrade to the point of being unusable the cell eventually dies off due to the inability to perform basic cellular functions.

Additionally, the concentrations of glyphosate used were much smaller than that found in products made for industrial and personal use. Areas surrounding where the pesticide is sprayed are likely to experience inhibition, even if it was not subject to direct application, similar to those seen in the experiment. Higher doses of glyphosate have the potential to inhibit the growth of *A. vinelandii* even further, possibly even to the point of entirely sterilizing the culture, though that would have to be tested further before a definite conclusion can be drawn.

Because *A. vinelandii* showed signs of greatly hindered reproduction it is likely that other soil dwelling microorganisms are just as vulnerable to the growth inhibiting effects of glyphosate. Bacteria that use EPSP-synthase, which is common in many species, for the creation of aromatic amino acids are the most likely to be harmfully affected by glyphosate. However, glyphosate can also interrupt cell wall and cell membrane functions which could further impair the cell’s ability to take in nutrients and by extension its ability to reproduce.

With a lack of beneficial bacteria in the soil plants will face a shortage of usable nitrogen, as well as a lack of beneficial auxins and cytokinins. Auxins are a plant hormone that encourages the elongation of plant cells, increasing the stem’s height and helping it reach up higher than

surrounding competitors. They also contribute to the formation of leaves, elongation of roots, and the effect of phototropism, where plants grow towards a light source. Cytokinins help with many of the same processes, such as the growth of new leaves and roots while also encouraging branching outward from the stem, but do so instead through the promotion of cell division. While plants can produce these hormones on their own, the extra created by microbial life leads to further growth, as the plants themselves do not need to allocate resources to the synthesis of the hormones. A lack of these helpful chemicals can lead to stunted growth or in some cases could cause the soil to become barren of plant life until the microorganisms are able to repopulate and increase the nitrogen content of the soil. In order to make up for the lack of nitrogen, agricultural farming companies as well as homeowners would have to use fertilizer, some of which can contain their own pesticides that can be more harmful to larger organisms as well as microorganisms and plants. The cycle of fertilizer and pesticides creates an undesirable positive feedback loop, suppressing the growth of natural rhizobacteria and encouraging the further use of harmful chemicals for plant cultivation. Not only does this end up costing money for agriculture, but it also harms the nearby environment in the long run should these chemicals manage to leach out into the surrounding areas.

One way environmental damage can be reduced or possibly prevented is through the use of more selective herbicides and pesticides. By using chemicals that target specific pests or unwanted weeds rather than an entire kingdom of organisms the chances of these chemicals causing adverse effects in other nontarget species is lessened. However, this would mean that agricultural industries would have to rely on a wider range of pesticides to keep crops properly protected, as well as likely leading to an overall increase in their use. Another, more efficient and

long term solution would be to look for other ways to prevent pests from attacking crops. Genetically Modified Organisms (GMOs) that create chemicals to discourage attacks by pests already exist, and could possibly prove to be a much better replacement for the widespread use of pesticides. However, many people are opposed to the use of GMOs and there are many controversial issues surrounding them, meaning that implementing them probably won't come into effect for some time.

Along with this experiment came several possible sources of error, the first of which being that some of the optical density readings were thrown off by the calcium carbonate precipitate. Despite letting the cultures settle prior to samples being taken, it is still possible that movement of the flasks during the sampling process was enough to mix up the media and therefore cause the sample to read higher than it should due to the particles causing the light to scatter. Another potential problem could have been with the litmus paper. It may have been too old for use and gave false readings, though it is unlikely.

There is a multitude of directions in which this research could be furthered. The first and most obvious of the next experiments would be to test a wider range glyphosate concentrations, exposing *A. vinelandii* to concentrations closer to those of the solution used in herbicide products to see whether the glyphosate is capable of producing a lethal effect rather than an inhibitory one. RoundUp and other similar products use anywhere from 1-2% in their lower concentration sprays, to up to 50% for the super concentrated herbicides, and testing a higher concentration the lab would be a better simulation of the products. Similarly, the growth could be measured over a longer period of time in order to observe whether or not the bacteria stay at a lower rate of growth for longer than the period of a week. Seeing as glyphosate can remain active

for weeks or even for years depending on the conditions, a longer monitoring of the bacteria growth would show how long the glyphosate could continue to inhibit them. Another test would be to streak plate the samples in order to get an accurate cell count and a clearer measurement of how many live cells are present per milliliter of media which could not be done in this experiment due to time constraints and a lack of space. In order to monitor the bacteria's activity as well, nitrogen fixation could be measured by using the HPLC or through gas chromatography. Doing so would metabolic activity to be measured in a relatively easy way whilst also allowing to investigate any effect that glyphosate may have on nitrogen fixation. Lastly, other pesticides could be tested in order to see how they interact with soil bacteria and other rhizobium dwelling microorganisms as well since glyphosate is not the only pesticide commonly used at both the industrial level and the private level.

Conclusion:

Measurements of the growth of *Azotobacter vinelandii* in glyphosate treated cultures has shown that glyphosate does inhibit the growth of *A. vinelandii*. The smaller concentrations did not show a correlation between concentration and inhibition but at higher doses there is a sign that the bacteria can be inhibited further. Since *A. vinelandii* is a model organism it is likely that glyphosate adversely affects other soil dwelling microbial life as well, interfering with their ability to reproduce and thus making it harder for plant life to grow.

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SDS:

Glyphosate- https://goo.gl/EN2Du5+-

Ashby’s Sucrose Broth- https://goo.gl/diw2dn

Activated carbon- https://goo.gl/4hqdB1

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