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**Title**

Lettuce not be salty*:* An update of a common secondary education experiment measuring seed germination under salt-stressed conditions

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## Abstract

Assessing the ecological impact of deicing roadways is a well-documented high school and undergraduate toxicology laboratory experiment. Most commonly, this experiment has been done using different concentrations of rock salt (NaCl) as the toxicant/deicer and buttercrunch lettuce seeds for the bioassay. However, as we are becoming a more environmentally conscious society, people, businesses, and city governments have explored substituting NaCl as a deicer for more eco-friendly alternatives, although the impact of these rock salt alternatives have not been well explored in the literature or in the classroom. Thus, the aim of this article is to update the common deicer experiment by having students compare the toxicity of rock salt to that of two different rock salt alternatives (beet salt and calcium chloride, $CaCl_2$). The students then have to provide a recommendation for which deicer to use under different scenarios (for a homeowner, for a business, and for a city) and defend their choices based on an extensive literature search. By designing this experiment to have the students defend their recommendations, they will be utilizing higher-level thinking per Bloom's Taxonomy, and they will be getting additional hands-on laboratory training in making solutions and performing serial dilutions by examining three deicers rather than just one. Thus, we believe that our update to this laboratory exercise should be considered for faculty interested in having their students conduct toxicology-based laboratory experiments.

**Keywords**

Laboratory instruction, High school/introductory chemistry, biotechnology, applications of chemistry, toxicology, water chemistry

## Introduction

In cold climates, rock salt (NaCl) is commonly applied to roadways and sidewalks as a deicer due to its low cost and widespread availability[1–5]. In areas where the application of large quantities of salt is required, there is a major concern about the impact of the salt on the ecosystem, particularly for aquatic organisms and plant life[1–4]. While plant seeds already contain the nutrients necessary to begin life, being exposed to saline solutions can induce stress in various plant species and affect their ability to germinate under high salinity conditions. Many research studies have been conducted to assess the effects of salt toxicity on plant growth; these research studies have shown that increased salinity can lead to a slowing or inhibition of their development[6–14]. These studies have found that there is an inverse relationship between salt concentration and seed germination and seed length in the progeny.

To study this topic in the classroom, there is a commonly utilized laboratory exercise that exists in which students measure how different concentrations of salt impact the growth and germination of lettuce seeds[15–17]. Lettuce seeds are a commonly used bioassay to assess the toxicity of pure compounds and complex mixtures. In addition, they are readily available, economical, require no special treatment, are easy to use, and have high germination rates[18]. A limitation of this experiment, however, is that these procedures do not take into account new and emerging chemicals that are being used as substitutes for rock salt.

One of the most popular rock salt alternatives is $CaCl_2$. $CaCl_2$ is advertised as being more pet-friendly because it is more effective at disrupting the chemical bonds within ice and snow, lowering the freezing point of the ice and requiring less salt to melt more ice[1]. Additionally, $CaCl_2$ has a van't Hoff factor of three and a high water solubility, making it more effective as a salt deicer than NaCl, which has a van't Hoff factor of two and a lower water solubility. $CaCl_2$ begins to melt ice immediately down to -29 °C[2], whereas traditional NaCl road salt requires almost 20 minutes to begin the melting process at a higher temperature of -9 °C[2]. $CaCl_2$ can be purchased at many pet stores, as well as online. Another, more novel salt deicer alternative is beet salt, in which beet juice is added to NaCl as a supplement. Beet salt is advertised as being less harmful to the environment because it contains beet juice, a natural ingredient, to assist in melting the ice; beet salt is also enhanced with calcium magnesium acetate, which does not contain harmful chlorides that might impact seed growth and toxicity[3]. Similar to $CaCl_2$, beet salt is more effective at lowering the freezing point of ice down to -30 °C[19], which is lower than both NaCl and $CaCl_2$. Interestingly, Syracuse, NY – one of the snowiest big cities in the United States – launched a pilot program in 2010 to use beet salt on one of its major highways to supplement common rock salt as a deicer in an effort to be more environmentally friendly[20]. In addition, in 2018, Syracuse then started using beet salt on the city's sidewalks to clear the walkways of ice[21]. Other cities, including Washington, D.C., have begun to develop plans to replace rock salt with beet salt[19], suggesting that its use may become more common as cities move towards more environmentally-friendly salt alternatives compared to NaCl.

Thus, to bring this experiment into the 21st century, we have updated the common deicer experiment. To do this, students will assess the toxicity of rock salt and a variety of rock salt alternatives. The rock salt alternatives suggested in this work include both those that are widely used (such as $CaCl_2$) in addition to a new, environmentally friendly rock salt alternative (beet salt). Using our experimental design, students are using higher order thinking by comparing the results obtained from the three different deicing chemicals and making well-informed and researched recommendations as to which salt type they think should be utilized for personal use and by businesses and city governments. This procedure can be easily replicated in both a high school and college classroom.

**Learning Objectives**

The learning objectives for this laboratory experiment are to:

1. Compare how different concentrations of salt deicers can influence the germination and seed growth of lettuce seeds
2. Recommend the best salt deicer for clearing ice from the roadways, keeping in mind their results, cost, large-scale availability, ecological impacts, and geographical area (i.e. home, business, city)
3. Organize and record large sets of data in both a laboratory notebook and Excel
4. Gain experience creating solutions and performing serial dilutions

**Experimental Procedure**

*Day 1 – Salt solution preparation and application*

All the materials for the laboratory experiment were purchased commercially. First, a 10%$_{w/v}$ solution of each salt deicer (NaCl, $CaCl_2$, and beet salt) was made in 100 mL pre-labeled volumetric flasks. Five additional concentrations were prepared using 1:10 serial dilutions, creating 1%, 0.1%, 0.01%, 0.001%, and 0.0001% solutions of each salt to be tested. A water control (0%) was utilized as a blank.

Buttercrunch lettuce seeds were used for this experiment to represent the ecosystem, and this type of seed has also been used in prior research and education-based deicing experiments[9,14–16]. The seeds were germinated in paper towels placed into pre-labeled plastic baggies. For each solution, one paper towel was folded into thirds and placed inside a pre-labeled plastic bag. Then, 10 mL of the salt solution was added into the plastic baggie while making sure the paper towel was thoroughly saturated. Once the entire paper towel was wet, it was removed, unfolded, and 10 seeds were evenly dispersed throughout the paper towel. The paper towel was refolded, placed inside the baggie, and sealed while pushing the air out from the inside. There was total of 19 baggies to cover each salt type and concentration as well as the water blank. Once the baggies were prepared, they were stored on a laboratory benchtop for the remainder of the experiment.

*Days 2-5 – Data collection*

The extent of seed growth (in inches) was measured once per day for five days using a ruler. To do this, the paper towel was removed from the baggie, unfolded, and placed on a clean surface to avoid cross-contamination. First, the number of seeds (out of 10) that had germinated was counted and recorded in the laboratory notebook. Then, the extent of seed growth (in inches) was measured using the ruler and each length was recorded. Only the seeds that had germinated were measured.

*Data analysis*

The number of germinated seeds in each baggie were reported as a whole number out of 10. The extent of seed growth data was reported as the average ± the standard deviation of all seed sprout lengths in the baggie on a given day. If a seed had not germinated, its length was reported as zero, and it was included in the average and standard deviation calculations. To assess for statistical differences in our data, t-tests were run between the water control and salt solution for the average seed growths on each of the five days. Statistical significance was set to $p < 0.05$.

**Results**

Each day when conducting this experiment, the number of sprouted seeds were counted, and the average radicle length for each seed was measured. Table 1 shows the number of germinated lettuce seeds for each salt solution. After five days of growth, the highest number of seeds germinated in the NaCl solutions (75%, 45 out of 60), followed by the beet salt (68%, 41 out of 60) and $CaCl_2$ (58%, 35 out of 60). No seeds germinated in any of the 10% salt solutions, and only one seed germinated at the second highest concentration tested (1.0%), suggesting that these concentrations were too high for this experiment. For the other concentrations studied (0.0001%, 0.001%, 0.01%, 0.1%), the number of germinated seeds were relatively similar for each salt type. Thus, the different salt types did not influence seed germination for concentrations ranging from 0.0001-0.1%. In the water control, 9 seeds germinated after 5 days.

Table 1. Number of germinated buttercrunch lettuce seeds (out of 10) in each salt solution after five days of growth. Salt solution concentrations are reported in percent by weight.

| | **0%** | **0.0001%** | **0.001%** | **0.01%** | **0.1%** | **1.0%** | **10%** | **Total seeds** |
|---|---|---|---|---|---|---|---|---|
| NaCl | 9 | 9 | 8 | 10 | 8 | 0 | 0 | 45 |
| $CaCl_2$ | 9 | 9 | 8 | 8 | 10 | 0 | 0 | 35 |
| Beet salt | 9 | 10 | 10 | 10 | 10 | 1 | 0 | 41 |

Although the same trend of radicle length was observed for the remaining concentrations, the 0.1% salt solution will be discussed in greater detail for clarity below. By the end of the five-day period, 9 seeds in the water control, 8 seeds in the NaCl, and 10 seeds in both $CaCl_2$ and beet salt had grown to average lengths of $0.59 \pm 0.34$, $0.50 \pm 0.34$, $0.62 \pm 0.23$, and $0.80 \pm 0.16$ inches, respectively (Figure 1). The lettuce seeds grown in the presence of the beet salt grew the longest, even surpassing that of the water control. The seeds grown in NaCl grew slightly shorter than the water control, and comparable radicle lengths were seen between the water control and $CaCl_2$. The error bars for these results were large, suggesting that there was a lot of variability in the radicle lengths. Overall, there was no significant difference between seed growth in the control and the salt solutions, except on Day 2 in the $CaCl_2$ solution ($p = 0.01$).

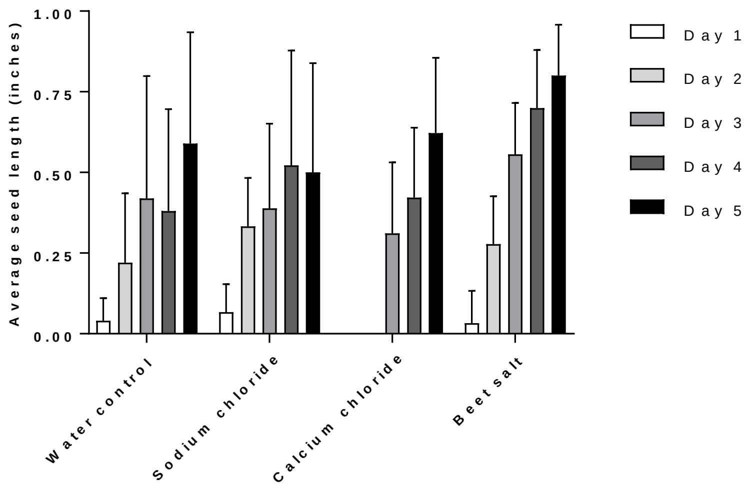


Figure 1. Lettuce seed germination over time in 0.1% salt solutions and a water control. Seed length is reported as the average length (± standard deviation) of all 10 seeds in the baggie on each day, including those that did not germinate.

**Discussion**

While the traditional deicer experiment explored the toxic effects of rock salt on lettuce seed growth and germination, this updated laboratory experiment expands upon that work to compare the toxicity of more environmentally friendly deicer alternatives to that of NaCl. We have shown that there was no clear difference in the number of seeds germinated between the three salt types tested (NaCl, $CaCl_2$, beet salt) for the lower concentrations, but using 10% salt concentrations was too high for this experiment. We also found that seeds grown in the beet salt grew the best (even better than those grown in the water blank), and the seeds grown in the NaCl solution were the most stunted, suggesting that beet salt might be environmentally beneficial.

We found that the preparation of the salt solutions was one of the most time-consuming steps of the procedure. If time in the classroom is limited, some of these solutions could be prepared by the instructor ahead of time. However, having the students gain additional experience making solutions and performing serial dilutions was a learning objective for our version of this experiment, but it does not have to be a major focus depending on the course this experiment is to be run in. If time is a concern, fewer solutions could be made or student groups could pool or share their data. In addition, about 20 minutes per day was devoted to measuring the sprout lengths for all 18 solutions and the water blank (~190 radicle measurements daily). If time is a concern or the students cannot get into the lab every day to take measurements, multiple students could work together on measuring radicle lengths from the different baggies, fewer days could be used, or the students can take the baggies home and do their measurements there.

After conducting this experiment, we found a lot of variability in our radicle length measurements (Figure 1). This variability can actually be an advantage for this experiment. As the results are not so clear-cut, the variability of the radicle lengths opens up the discussion about whether using the beet salt, which is more costly and harder to buy in bulk, is worth it for the small change in plant growth it can have. Additionally, beet juice has been found to stain clothing and other materials, which should be a consideration when comparing options for salt usage on roadways based on potential impacts for societal perception[21]. In our version of this experiment, after the experiment is complete, rather than write a traditional laboratory report or complete a short handout, students can write well-researched proposals for a homeowner, small business, and/or city government as to their recommendation for the best salt deicer to use. This recommendation should, at a minimum, take into account the eco-friendlier behavior of the beet salt and $CaCl_2$ as evidenced by their results, and weigh that with the higher cost of these salts and their limited availability on a large scale. For this, the students would need to be well versed on the pros and cons of the lettuce seed bioassay they used and the salts they tested. In addition, the students will most likely need to look up the local city land area, number of major roadways, number of sidewalks each business needs to clear, the local and projected weather patterns taking into account climate change, and how close the major roadways are to lakes or waterways, amongst other things.

The variability in radicle length measurements could also be a disadvantage for this experiment, especially if few seeds germinate in a baggie on a given day. We chose to complete our calculations of average radicle length including the non-germinated seeds; however, students could also analyze the data without the non-germinated seeds and eliminate all zeroes in their calculations. When we analyzed our data with the non-germinated seed lengths excluded, the results did not greatly differ. Although the results were similar for both methods, it would be interesting to give the students the option to complete their analyses either way, with the caveat that they must defend their method in the post-laboratory questions.

While our experiment introduced a new twist to a well-established lab, there are additional variables that could be investigated depending upon classroom needs or student desires. In this procedure, all seeds were placed on a laboratory benchtop and were not exposed to any direct sunlight. If classrooms were interested in the effects of light on seed germination, two experimental sets could be prepared with one placed in darkness (such as in a drawer) and the other placed in direct sunlight (such as in front of a window). Additionally, different salt solution concentrations could be prepared and applied to the seeds. Seeds were still able to grow in the 1% solutions, but no growth was observed in the 10% solutions. If students wanted to determine the maximum concentration in which seeds could grow, additional concentrations between 1% and 10% could be prepared and tested to calculate an $EC_{50}$. Variation in germination rates for different seed types, such as radish or cabbage seeds, could also be studied to assess whether their findings hold for other plant types, and aquatic bioassays including daphnia or zebrafish could be utilized as well (or in addition to the lettuce seeds). Lastly, because seed germination was successful using the paper towel setup, the seeds could also be transferred to potted soil at the completion of the experiment to analyze how germination in high-salt environments affected future plant growth. If time allotted, the students could design their own future experiments to transform this experiment into a multi-week inquiry-guided exercise, as after doing their literature searches and writing their recommendations they should be better prepared to design their own prospective research.

**Conclusion**

We were successfully able to update a well-established and documented toxicology experiment measuring the toxicity of deicing solutions on a common bioassay (i.e. lettuce seeds). This update involved having students compare various deicing solutions – two which are touted as more environmentally friendly and one which has already been utilized by a city government – and writing literature-based recommendations for homeowners, businesses, and city governments as to the best deicer to use for each stakeholder. We also proposed different ways to maximize class time, as well as offer additional experiments that can expand upon the exercise outlined here or that students can explore in more detail on their own as an inquiry-guided research project. This update involves higher-level thinking compared to the prior experimental design.

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**Conflict of Interest Disclosure**

The authors declare no competing financial interest.

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