What is the effect of ammonium sulfate on the heart rate of *Daphnia magnia*?

Qin Xiang Ng

Ammonium sulfate [$(NH_4)_2SO_4$] is one of the most widely used nitrogen-based fertilizer in agriculture, and has been produced for over 150 years. However, limited research has been done to investigate the eco-toxic effects of ammonium sulfate, commonly present in surface runoff. This study therefore aimed to investigate the effects of varying ammonium sulfate concentrations on the normal physiology of *Daphnia magna* through a modified acute toxicity testing. Concentrations of ammonium sulfate solutions at 0M, 0.05M, 0.10M, 0.15M, 0.20M, 0.25M, and 0.30M were prepared and tested on 10 *D. magna* for each concentration of ammonium sulfate solution. The bioassay test was done by observing the effects of different concentrations of ammonium sulfate solution on the heart rate of *D. magna.* The percentage increase in average heart rate of *D. magna* after exposure to the respective concentrations of ammonium sulfate solution were calculated and a relationship between varying concentrations of ammonium sulfate concentration and the heart rate of *D. magna* was illustrated by plotting a graph using the respective data points obtained. Results indicated that increasing concentrations of ammonium sulfate solution resulted in an increase in the heart rate of *D. magna* per minute, up till 0.20M concentration. Increasing concentrations of ammonium sulfate solution beyond 0.20M resulted in a decrease in the heart rate of *D. magna* per minute. It was also discovered that specifically, the ammonium ions present when ammonium sulfate dissociates in water, is responsibility for toxicity, and not the sulfate ions. It is reasonable to conclude that ammonium sulfate poses significant eco-toxic effects as *D. magna* is a common primary consumer in many freshwater aquatic ecosystems, any change in its population quality or quantity can cause irreparable effects to the populations of other aquatic organisms.

What is the effect of ammonium sulfate on the heart rate of *Daphnia magnia*?

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PeerJ PrePrints | https://doi.org/10.7287/peerj.preprints.1581v1 | CC-BY 4.0 Open Access | rec: 11 Dec 2015, publ: 11 Dec 2015

INTRODUCTION

Eutrophication is a process where water bodies receive excess organic nutrients which are used by aquatic plants such as algae, resulting in rapid plant growth. The excessive organic nutrients are a result of increased leaching of essential soil nutrients into water bodies due to the increase in rainfall in certain parts of the world such as the Pacific region, resulting in eutrophication.

Eutrophication, if not controlled, increases the biochemical oxygen demand (BOD) in water bodies, decreasing the total concentration of dissolved oxygen in the water, hence, making it more difficult to support marine life. Other than BOD, some of the chemicals involved in eutrophication may have toxic effects on aquatic life and animals. Together, these have detrimental effects on the ecological balance, diversity and fitness of the aquatic ecosystem.

The fundamental nutrients responsible for eutrophication are nitrates and phosphates compounds in soil. Nitrates are essential for amino acids and protein synthesis, which promotes stem and leaf growth while phosphates are required to synthesize nucleic acids, promote root growth, and strengthen the stem of plants. Agriculture fertilizers that contain large amount of nitrates and phosphates are frequently used to supplement soil to increase crop yield. Ammonium sulfate is present in high concentrations in these agricultural fertilizers.

Ammonium sulfate [$(NH_4)_2SO_4$] is one of the most widely used nitrogen-based fertilizer in agriculture, and has been produced for over 150 years. Ammonium sulfate is toxic to water organisms such as juvenile *Salmo gairdneri* and water algae (OECD, 2004). However, there is a lack of research on the eco-toxicity and lethal concentration of ammonium sulfate. This report thus aimed to investigate the effects of ammonium sulfate on the physiology of *Daphnia magna*. *Daphnia magna*, a freshwater filter-feeding crustacean, is one of the most sensitive organisms used in eco-toxicity experiments, as described in the standard protocols of the U.S. Environmental Protection Agency (EPA), Organization for Economic Cooperation and Development (OECD), and International Standards Organization (ISO). Furthermore, since *D. magna* is a primary consumer in freshwater aquatic ecosystems, any change in its population quality or quantity can cause significant effects to the populations of other aquatic organisms, resulting in loss of biodiversity and disruption of ecological balance.

Further studies have also showed that *D. magna* showed similar pharmacological reactions as humans when exposed to cardiac glycosides (Grant, 2000). This is significant as the reactions of *D. magna* to the test substances might be an indication that there is a possibility of a similar response by humans when exposed to the test substance. Hence, this makes *D. magna* an ideal test organism to determine toxicity. In this study, the heart rate of *D. magna* was monitored to investigate its cardiac reaction to various concentrations of ammonium sulfate.

MATERIALS AND METHODS

A healthy stock of *Daphnia magna* Straus (Crustacea, Cladocera) was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). In this study, the *Daphnia magna* culture media used for all experiments was made by first separately dissolving salts (11.76 g of $CaCl_2.2H_2O$, 4.93 g of $MgSO_4.7H_2O$, 2.59 g of $NaHCO_3$ and 0.25 g of KCl) in 1 L of Millipore ultrapure water to make stock solutions. Next, 25 mL of each salt stock solution was aliquoted into a clean 1 L screw cap bottle and made up to 1 L volume using Millipore ultrapure water to obtain the final culture media.

The test solution to be used in this experiment is ammonium sulfate solution. This was prepared by disssolving 13.21g of ammonium sulfate salt (Sigma A-4418, ≥99.0%) in $100cm^3$ of culture media to make up a 1M stock solution. Various concentrations of test solutions (0.05M, 0.10M, 0.15M, 0.20M, 0.25M, and 0.30M) were prepared and stored in labelled test tubes. 0M solution (culture media) was also used as a control solution as a confirmation that any change in heart rate of *D. magna* was due to the introduction of ammonium sulfate solution only.

D. magna specimens were removed from the container individually using a plastic dropper and placed on a cavity slide. It was then left untouched for 2 minutes in order for the heart beats of *D. magna* to stabilise back to normal so as to reduce involuntary changes in heart rate which could be due to agitation when transferring the specimens. After 2 minutes, the excess solution was then removed from the cavity slide using some tissue to reduce mobility of *D. magna*. 50 µl of 0.05M ammonium sulfate solution was then transferred using a micropipette from the test tube to the cavity slide to expose the specimen to ammonium sulfate solution. The specimen was left in the test solution for 2 minutes. Excess test solution was removed from the cavity slide using tissue paper and the heartbeat of the specimen was observed using a light microscope under 10x magnification. Data was collected by recording 1 minute video clips of the heartbeats of the specimen by attaching a digital camera to the eyepiece of the micropscope. The procedure was then repeated for the remaining concentrations of ammonium sulfate solution.

Altogether, 10 *D. magna* specimens were exposed to each concentration of ammonium sulfate solution. The mean percentage change in heart rate was calculated to obtain a general trend across the various concentrations.

RESULTS AND DISCUSSION

One *D. magna* was placed on the cavity slide and the heart rate was measured by observing the specimen under the microscope. The original water sample containing *D. magna* was blotted dry before *D. magna* was exposed to respective concentrations of ammonium sulfate solution for 2 minutes before the heart rate was measured.

Table 1: Heart rate of *D. magna* in culture media (control) in 1 minute

	Specimen number									
	1	2	3	4	5	6	7	8	9	10
Number of heart beats in 1 min	152	185	175	158	176	173	161	169	148	157

Table 2: Heart rate of *D. magna* in 0.05moldm^{-3} ammonium sulfate solution in 1 minute

		Specimen number									
		1	2	3	4	5	6	7	8	9	10
Number of heart beats in 1 min	Before addition of test solution	174	163	157	182	170	153	144	172	164	180
	With 0.05 moldm^{-3} test solution	214	201	173	197	204	181	179	193	186	212

Table 3: Heart rate of *D. magna* in 0.1 moldm^{-3} ammonium sulfate solution in 1 minute

		Specimen number									
		1	2	3	4	5	6	7	8	9	10
Number of heart beats in 1 min	Before addition of test solution	166	155	183	175	175	168	158	170	156	147
	With 0.10 moldm^{-3} test solution	224	190	233	238	239	213	197	226	215	206

Table 4: Heart rate of *D. magna* in 0.15 moldm^{-3} ammonium sulfate solution in 1 minute

		Specimen number									
		1	2	3	4	5	6	7	8	9	10
Number of heart beats in 1 min	Before addition of test solution	178	182	162	175	169	173	149	171	167	154
	With 0.15 moldm^{-3} test solution	289	281	245	270	261	253	219	275	273	232

Table 5: Heart rate of *D. magna* in 0.2 moldm^{-3} ammonium sulfate solution in 1 minute

		Specimen number									
		1	2	3	4	5	6	7	8	9	10
Number of heart beats in 1 min	Before addition of test solution	186	174	166	165	182	159	172	170	149	164
	With 0.20 moldm^{-3} test solution	329	312	289	316	318	291	302	316	284	301

Table 6: Heart rate of *D. magna* in 0.25 moldm^{-3} ammonium sulfate solution in 1 minute

		Specimen number									
		1	2	3	4	5	6	7	8	9	10
Number of heart beats in 1 min	Before addition of test solution	178	182	162	175	169	165	170	156	177	176
	With 0.25 moldm^{-3} test solution	310	314	298	283	311	300	318	282	289	273

Table 7: Heart rate of *D. magna* in 0.30 moldm^{-3} ammonium sulfate solution in 1 minute

		Specimen number									
		1	2	3	4	5	6	7	8	9	10
Number of heart beats in 1 min	Before addition of test solution	159	168	154	189	179	147	177	158	163	168
	With 0.30 moldm^{-3} test solution	93 [1]	262	102 [2]	148 [3]	243	75 [4]	266	122 [5]	107 [6]	110 [7]

The average heart rate of *D. magna* per minute exposed to each respective concentrations of ammonium sulfate solution was calculated as shown:

$$\text{Average heart rate} = \frac{\sum heart\ beat\ of\ all\ 10\ specimens}{10}$$

Table 8: Average heart rate of *D. magna* per minute exposed to respective concentrations of ammonium sulfate solution

D. magna in respective concentrations of ammonium sulfate solutions (moldm^{-3})	Average heartbeat per min for all five specimens exposed to respective concentrations of ammonium sulfate solutions (b) (rounded off to the nearest whole number)	
	Before addition of ammonium sulfate solution	After addition of ammonium sulfate solution
0 (control)	166	N.A.
0.05	166	194
0.10	165	218
0.15	168	260
0.20	169	306
0.25	171	298
0.30	166	257*

***Only 3 values were used in calculating mean heartbeat for 0.30moldm^{-3} solutions as the heart beat of the other 7 specimens stopped before the 1 minute mark. Hence, they were not taken into consideration, as they would reflect a negative decrease in mean heart rate.**

[1] Heart beat stopped after 21s
[2] Heart beat stopped after 39s
[3] Heart beat stopped after 32s
[4] Heart beat stopped after 16s
[5] Heart beat stopped after 27s
[6] Heart beat stopped after 35s
[7] Heart beat stopped after 16s

Calculation for average heart rate of 10 specimens after exposure to 0.05M ammonium sulfate solution:

$$\text{Average heart rate} = \frac{214 + 201 + 173 + 197 + 204 + 181 + 179 + 193 + 186 + 212}{10}$$

$$= 194$$

Calculation for average heart rate of 10 specimens after exposure to 0.3M ammonium sulfate solution:

$$\text{Average heart rate} = \frac{262+243+266}{3}$$

$$= 257$$

The overall percentage change in the heart rate of *D. magna* in the respective concentrations of ammonium sulfate solutions is calculated as shown:

Percentage change in heart rate of *D. magna*

$$= \frac{mean\ heartbeat\ after\ exposure\ to\ test\ solution - mean\ heartbeat\ before\ exposure\ to\ solution}{mean\ heartbeat\ before\ exposure\ to\ test\ solution} \times 100\%$$

Calculation of percentage change in heart rate for specimens exposed to 0.05M solution:

$$\frac{194-166}{166} \times 100 = 16.7\%$$

Table 9: Overall percentage change in heart rate of *D. magna* in different concentrations of test solution

		Concentration of Ammonium Sulfate stock solution ($moldm^{-3}$)					
		0.05	0.10	0.15	0.20	0.25	0.30
Daphnia heart rate (beats per minute)	Before addition of test solution	166	165	168	169	171	166
	After addition of test solution	194	218	260	306	298	257
	Percentage change(%) (to 1 d.p.)	+ 16.7	+ 32.1	+ 54.8	+ 81.1	+ 74.3	+ 54.8*

*** Selective data used in calculating percentage change**

For calculation of percentage change in heart rate per minute for *D. magna* in 0.30M solution, only 3 out of 10 set of raw data values were used in the calculation of mean heart beats in 1 minute. This is because there were inconsistency in the other 7 values

observed. It was observed that the heart beat of the other 7 specimens stopped before the stipulated 1 minute observation time. If these values were taken into consideration in calculating the mean percentage change in heart rate, it would register an overall negative increase in percentage change in the heart beat of *D. magna* in 1 minute, which is an inaccurate reflection of the results for that specific concentration as the 3 of the 10 specimens did show an increase in heart rate. However, it has to be taken into consideration that there is a possibility of the other 7 readings being more accurate reflections of the actual affect of ammonium sulfate at 0.30M concentration on *D. magna*. Hence, analysis of data was only focused on concentrations up till 0.25M and the data collected for 0.30M concentration was classified as anomaly.

Bioassay of *D. magna* on concentrations above 0.30M (i.e. 0.35M and 0.40M solution) were done and experimental observations showed that upon addition of the respective concentrations of ammonium sulfate solution, *D. magna* specimens showed an extremely rapid increase in heartbeat initially. However, the heart beat of the *D. magna* specimens stopped before the stipulated 1 minute observation time. Hence, the results from those concentrations were not reflected.

Table 10: Effect of various concentrations of ammonium sulfate solution concentration on heart rate of *D. magna*

		Before/after addition of test solution	Heart beats per minute (bpm)											
			Daphnia specimen no.										Average heart rate	Percentage Change (%) (to 1 d.p.)
			1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*		
Concentration of ammonium sulfate test solution ($moldm^{-3}$)	**0.05**	***Before***	174	163	157	182	170	153	144	172	164	180	166	+ 16.7
		After	214	201	173	197	204	181	179	193	186	212	194	
	0.10	***Before***	166	155	183	175	175	168	158	170	156	147	165	+ 32.1
		After	224	190	233	238	239	213	197	226	215	206	218	
	0.15	***Before***	178	182	162	175	169	173	149	171	167	154	168	+ 54.8
		After	289	281	245	270	261	253	219	275	273	232	260	
	0.20	***Before***	186	174	166	165	182	159	172	170	149	164	169	+ 81.1
		After	329	312	289	316	318	291	302	316	284	301	306	
	0.25	***Before***	178	182	162	175	169	165	170	156	177	176	171	+ 74.3
		After	310	314	298	283	311	300	318	282	289	273	298	
	0.30	***Before***	159	168	154	189	179	147	177	158	163	168	166	+ 54.8
		After	93	262	102	148	243	75	266	122	107	110	257	
Control setup			152	185	175	158	176	173	161	169	148	157	166	N.A.

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Figure 1: Heart rate of *D. magna* before and after exposure to varying concentraions of test solution (n=10)

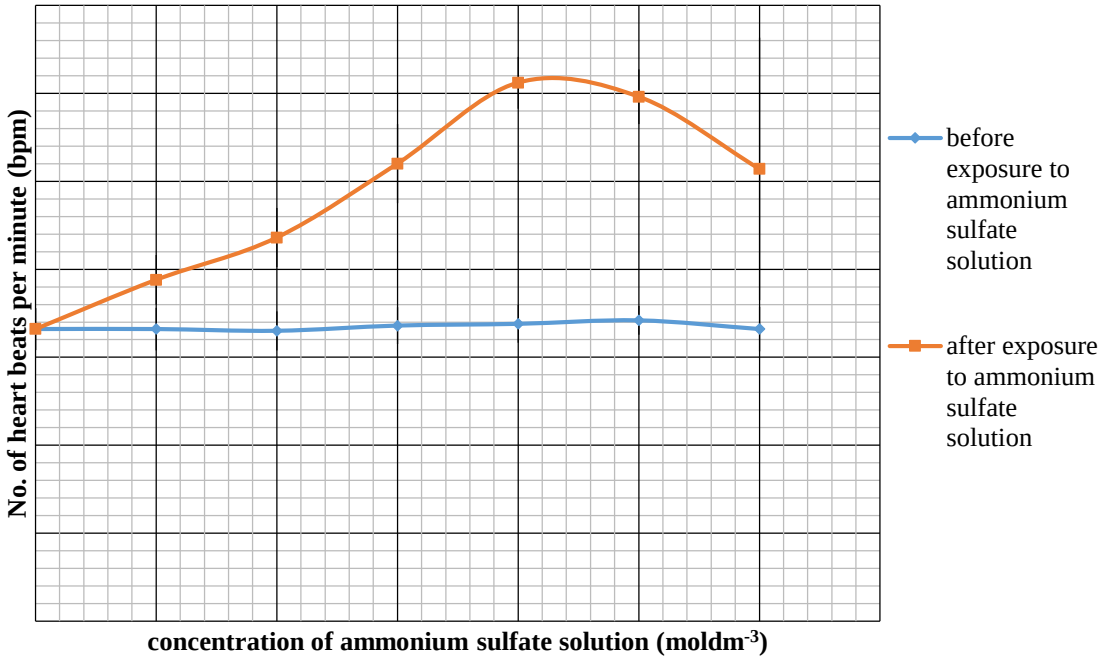

Figure 2: Relationship between varying concentrations of ammonium sulphate test solutions and percentage change in heart rate of *D. magna* (n=10)

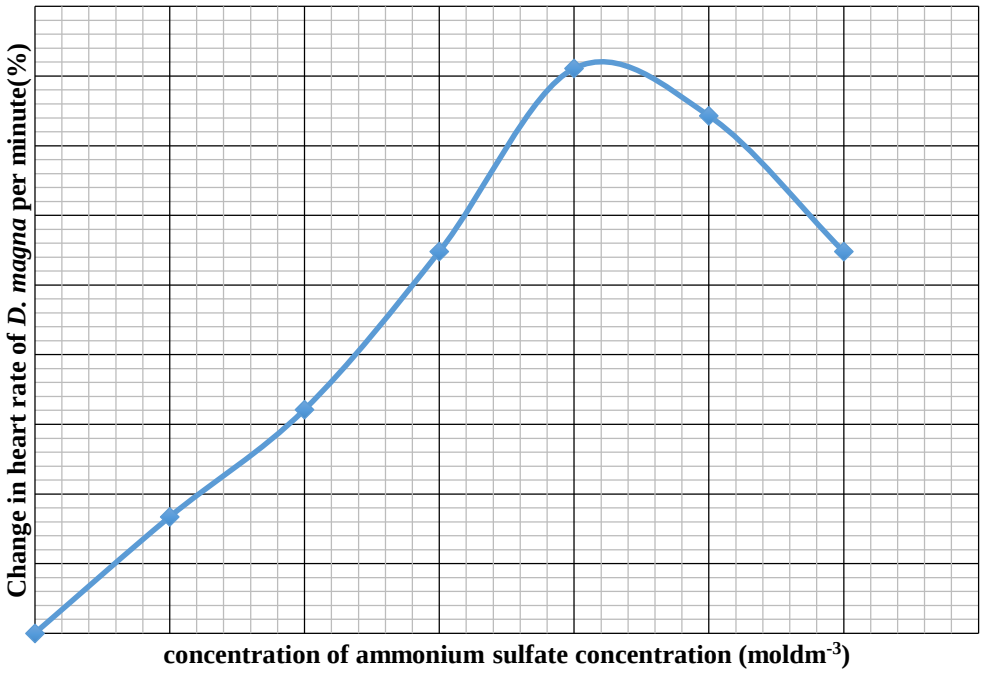

Table 11: Calculated standard deviation for the mean heart rate for each respective concentrations

Concentration of ammonium sulfate solution ($moldm^{-3}$)	Mean heart rate per minute (bpm)		Standard deviation (to 5 s.f.)	
	Before exposure to test solution	*After exposure to test solution*	*Series 1: before exposure to test solution*	*Series 2: after exposure to test solution*
0 (control)	166	166	11.955	11.955
0.05	166	194	12.087	14.071
0.10	165	218	11.116	16.895
0.15	168	260	10.403	22.399
0.20	169	306	10.761	14.711
0.25	171	298	8.1240	15.491
0.30	166	257	12.709	74.525

*Calculated using Microsoft Excel spreadsheet

Table 12: Calculated 95% confidence interval values

Concentration of ammonium sulfate solution ($moldm^{-3}$)	95% confidence interval value (to 5 s.f.)	
	Series 1: before exposure to test solution	*Series 2: after exposure to test solution*
0 (control)	7.4099	7.4099
0.05	7.4916	8.7213
0.10	6.8897	10.471
0.15	6.4477	13.883
0.20	6.6693	9.1175
0.25	5.0352	9.6009
0.30	7.8768	46.190

*Calculated using Microsoft Excel spreadsheet

A larger standard deviation means that the data points are further from the mean and hence, the wider the spread of data and vice versa. Hence, from Table 11, it can be seen that the standard deviation for the mean heart rates before and after exposure to each concentration of ammonium sulfate solution is relatively large due to the physiological nature of the experiment.

From Table 12, it shows that the confidence interval for all mean heart rates for all concentration (with the exception of 0.30M ammonium sulfate solution) is small relative to the mean, which shows that the mean values are reliable. However in the case of the mean values for 0.30M ammonium sulfate solution, the confidence interval is relatively larger as compared to the confidence interval for the other concentrations, this shows that there are anomalies in the results obtained.

The general trend of the graph shows that as the concentration of ammonium sulfate solution increased from 0M to 0.20M, the average heart rate of *D. magna* increases. This increasing trend is consistent from 0M concentration to 0.2M concentration. At concentrations above 0.20M, increasing concentrations of ammonium sulfate test solutions resulted in a decreasing percentage change in heart rate of *D. magna* per minute as shown in Figure 2. It was also shown that 0.35M solution killed all 10 *D. magna* specimens (i.e. there was no heart beat observed upon addition of 0.35M ammonium sulfate solution and *D. magna* specimens appeared to be immobile); hence, no heart beats for the *D. magna* were recorded.

Only 3 out of 10 *D. magna* specimens survived in 0.30M solution. The heartbeats of the other 7 *D. magna* stopped after 21s, 39s, 32s, 16s, 27s, 35s and 16s respectively. This might be due to the fact that the concentrations of ammonium sulfate solutions being tested were lethal to *D. magna*. However, the exact lethal concentration could not be determined as the intervals of ammonium solution concentrations tested were too large to extrapolate the lethal concentration from the data.

The heart rate decreased gradually as the concentration of ammonium sulfate solution increased beyond 0.20M.

From the data collected, it can be concluded that the experimental observations showed that as ammonium sulfate solution concentration increases, the heart rate of *D. magna* would increase. This increasing trend was observed until a specific lethal concentration that killed the *D. magna*.

CONCLUSION

As the experiment was aimed at determining the effects of increasing concentrations of ammonium sulfate on *D. magna*, one limitation of this experiment is that it was unable to ascertain specifically the physiological mechanism of *D. magna* that was affected by ammonium sulfate. In this experiment, only changes in heart rate were investigated. However, ammonium sulfate could have also affected other aspects of *D. magna* metabolism such as circulatory system or the respiratory system.

Ammonium sulfate is a weak alkali due to the presence of ammonia (NH_3) in aqueous solution. When ammonium sulfate compound dissociates, it produces ammonia ions and sulfate ions, which results in fluctuations in pH of the solutions, and hence, amount of un-ionized NH_3 present in the solution. Changes in pH might be a confounding factor resulting in the deaths of *D. magna* specimens.

Fluctuations in pH might have altered the metabolic pathway of *D. magna.* Extreme pH may interfere with numerous metabolic processes of the specimens such as osmoregulation, oxygen consumption and aerobic metabolism in different crustacean species (Jose, Chatelain, & Dufresne, 2009). In the case of *D. magna,* uptake of oxygen is severely depressed at high pH values while it is not influenced by low pH values (Jose, Chatelain, & Dufresne, 2009). Higher concentrations of ammonium sulfate solution result in more un-ionized NH_3 present in the solution, which results in higher alkalinity and higher pH. Hence, at higher concentrations of ammonium sulfate solution, the heart rate of *D. magna* would increase so as to increase the amount of oxygenated blood pumped through the body in order to compensate the decrease in oxygen uptake at higher pH.

Since oxidative phosphorylation is an enzyme dependent process, an increase in alkalinity of solution might have therefore decreased the efficiency of respiratory enzymes in carrying out their functions in ATP synthesis. This is due to the fact that enzymes being globular proteins with a specific active site have a specific three-dimensional conformation that is held in position by specific chemical groups and bonds (Campbell & Reece, 2005). Fluctuations in pH might disrupt the bonds, hence altering the conformation of the active site of the enzyme. This reduces the ability of the enzyme to function optimally by inhibiting its ability to form substrate-enzyme complex, hence decreasing rate of ATP production. Since ATP production is greatly

impaired, *D. magna* has to increase its heart rate to increase the uptake of oxygen for oxidative phosphorylation and thus the amount of ATP produced.

Further acute toxicity tests could be carried out to determine the LC_{50} and LD_{50} of ammonium sulfate solution on *D. magna* so as to provide a more comparable set of data. In addition, the lethal concentration of ammonium sulfate that is required to kill half the population of *D. magna* specimens of a fixed sample size could be accurately determined.

There is a possibility that it might not be the toxicity of ammonium sulfate that resulted in the eventual death of *D. magna* at higher concentrations but it might be the individual effect of either ammonia ion (NH_3) or sulfate ions (SO_4^{2-}) when ammonium sulfate dissociates in aqueous solution. It was found that ammonia toxicity is due to the un-ionized form of NH_3 given that the highest toxicity of ammonium sulfate is at pH 8 (Clement & Merlin, 1995).

A similar experiment could be carried out by testing other salt solutions containing either ammonia or sulfate compounds. For example, the similar experiment can be carried out using ammonium chloride, which will provide the ammonia ion, and magnesium sulfate, which will provide the sulfate ion. A comparison can then be done to determine if salts containing ammonia or sulfate ions have the same effect on *D. magna*. This information can then be used to determine if the change in heart rate of *D. magna* specimens were due to the ammonia ions, the sulfate ions or both.

In the ecological context, ammonium sulfate would not be the sole contaminant in water bodies. Its interactions with other compounds, such as heavy metals might have more detrimental effects on *D. magna* as compared to ammonium sulfate itself. Hence, further recommendations to monitoring of the ecosystem could be extended by testing combinations of compounds with ammonium sulfate on *D. magna.*

ACKNOWLEDGEMENTS

No conflict of interest to declare. This project was done with assistance from the National University of Singapore.

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