The response of *Scenedesmus quadricauda* and *Selenastrum capricornutum* to glyphosate toxicity (Roundup® formulation) with cellular growth and chlorophyll-a synthesis as endpoints

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Abstract

Glyphosate is a commonly-used agricultural herbicide which enters freshwater sources and risks affecting non-target aquatic organisms, including algae. In this study, lab cultures of *Scenedesmus quadricauda* and *Selenastrum capricornutum* were inoculated with glyphosate (Roundup® formulation) to determine its impact on cellular growth and chlorophyll-a (Ch-a) synthesis. A concentration of 10 mg/L of glyphosate significantly inhibited growth and Ch-a synthesis in *S. quadricauda* and *S. capricornutum*. Moreover, concentrations ranging from 0 to 3 mg/L of glyphosate did not affect cellular growth or Ch-a synthesis in either species. Finally, a concentration of 6 mg/L of glyphosate did initially reduce the growth of *S. quadricauda*, but growth recovered and Ch-a concentrations were high. In the case of *S. capricornutum*, growth and Ch-a synthesis were low, and pheophytin concentrations were significantly elevated relative to the control at 6 mg/L of glyphosate. Based on these results, *S. capricornutum* was more sensitive to glyphosate than *S. quadricauda*; this was likely due to differences in the surface area to volume ratios between the species. In the future, glyphosate toxicity should be studied in greater detail by conducting mesocosm studies within the natural aquatic environment.

Keywords: Glyphosate, green algae, Roundup®, cellular growth, chlorophyll

Introduction

Glyphosate (N-phosphonomethylglycine) is one of the world's most used and fastest growing agricultural herbicide (Baylis, 2000). It targets grasses, sedges, and broad-leaved weeds (Goldsborough & Brown, 1988); therefore, water bodies surrounded by agricultural activity are at risk of glyphosate contamination through wind drift, surface run-off, or direct overspray (Peterson et al., 1994). Roundup® is a common commercial herbicide consisting of glyphosate, formulated as isopropylamine salt (IPA salt), and polyethoxylated tallow amine (POEA) as surfactant (Tsui & Chu, 2003). Initial absorption of glyphosate depends on the surfactant to facilitate entry into the plant (Cranmer & Linscott, 1991). Within a cell, glyphosate disrupts aromatic acid biosynthesis, which reduces protein synthesis and causes cellular death (Vera et al., 2010). High glyphosate concentrations also cause chlorosis, which is the insufficient production of Ch-a (Shikha & Singh, 2004); lack of Ch-a reduces photosynthesis, causing death of the organism.

Glyphosate is reaching aquatic sources within Manitoba and is potentially harming algae and other organisms that are dependent on the algae for nutrient cycling, oxygen production, and food (Beck, 1987; Kent & Caux, 1995). In this study, the green algal species, *Scenedesmus quadricauda* and *Selenastrum capricornutum,* were chosen to evaluate the toxicity of glyphosate. Algae are important bio-indicator species due to their sensitivity and high occurrence within the aquatic ecosystem (Tsui & Chu, 2003). Algal death resulting from glyphosate toxicity could affect the entire aquatic ecosystem due to their significance as a food source to organisms at higher trophic levels (Kent & Caux, 1995).

Previous studies have found that high glyphosate concentrations inhibit cellular growth and Ch-a synthesis in green algal species (Peterson et al., 1994; Saenz, DiMarzio, Alberdi, & Tortorelli, 1997; Wong, 2000; Shikha & Singh, 2004; Vendrell, de Barreda Ferraz, Sabater, & Carrasco, 2009). Wong (2000) found that 2 mg/L or more significantly inhibited Ch-a synthesis and cellular growth. Other studies have also found that algal species vary in sensitivity to glyphosate (Peterson et al., 1994; Perez

et al., 2007; Vendrell et al., 2009; Vera et al., 2010). Peterson et al. (1994) tested the effects of glyphosate on *S. quadricauda* and *S. capricornutum* and found that *S. capricornutum* exhibited greater percent inhibition (18%) compared to *S. quadricauda* (3%). In another study, Rojickova-Padrtova and Marsalek (1999) found that *S. capricornutum* was more sensitive to toxic contaminants compared to *S. quadricauda*, and suggested that these findings were the result of differences in the surface area: volume (SA:V) ratios between the two species.

The objective of this study was to determine toxicity and sensitivity differences between *S. quadricauda* and *S. capricornutum* by measuring cellular growth rate and final Ch-a concentrations as endpoints. We hypothesized that glyphosate would affect cellular growth and Ch-a synthesis in both algal species. Similar to Wong (2000), we predicted that glyphosate would cause inhibition at high concentrations (>2 mg/L). We also hypothesized that there would be sensitivity differences between the green algal species; specifically, *S. capricornutum* would be more sensitive to glyphosate than *S. quadricauda.*

Results

A concentration of 10 mg/L of glyphosate inhibited cellular growth and Ch-a synthesis in *S. quadricauda* (Table 1; Fig. 2A). Initially, the growth rate was negative subsequent to glyphosate inoculation at 3 and 6 mg/L, but the effect of glyphosate lessened and living cells continued to grow and reproduce at a positive rate.

Based on these results, cellular growth rates between glyphosate treatments were statistically different ($F_{4,15}$: 16.32; p = 0.0001). Cellular growth at 0, 1, 3, and 6 mg/L of glyphosate was significantly different from growth at 10 mg/L ($p < 0.025$), and growth at 3 mg/L of glyphosate was statistically different from growth at 6 mg/L (p = 0.006).

Unlike *S. quadricauda*, the growth rate of *S. capricornutum* was negative at 6 mg/L of glyphosate (-55417 cells/mL/day), and at 10 mg/L of glyphosate, cells continued to die at a negative rate (-329167 cells/mL/day) until day nine, at which point growth was completely inhibited (Fig 2B). Based on these results, growth rates between glyphosate treatments were statistically different ($F_{4,15}$: 44.97; p = 0.0001). Mean growth at 0, 1, 3, and 6 mg/L of glyphosate was significantly different from growth at 10 mg/L (p = 0.0001), and growth at 1 mg/L of glyphosate was different from growth at 6 mg/L (p = 0.0490).

Ch-a concentrations of *S. quadricauda* were highest at 6 mg/L of glyphosate (194 µg/L) (Fig. 3A). At 1 and 3 mg/L of glyphosate, Ch-a levels were greater (141 and 121 µg/L) compared to respective pheophytin concentrations (72 and 93 µg/L), and at 10 mg/L, only pheophytin was present (173 µg/L). Despite these differences, chlorophyll and pheophytin concentrations were not statistically different between glyphosate treatments.

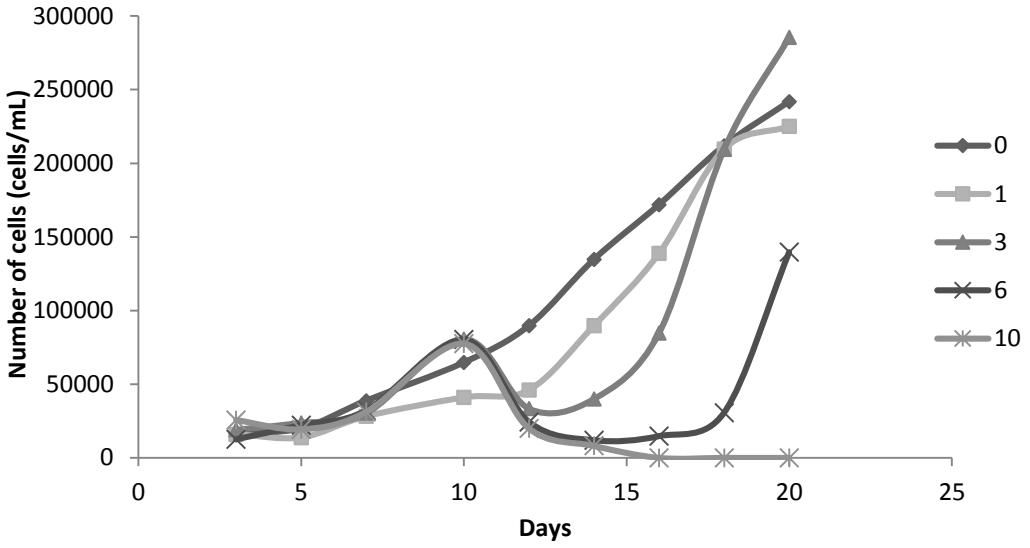
A

Table 1. Mean cellular growth (cells/mL/day ± SE, n = 20) of S*. quadricauda* and *S. capricornutum*, as affected by glyphosate (A).

Glyphosate concentration (mg/L)	Mean cellular growth (cells/mL/day)*	
	Scenedesmus quadricauda	***Selenastrum capricornutum***
Control (0)	19079 ± 4243^{ab}	22292 ± 23002^{ab}
1	23907 ± 4021^{ab}	47292 ± 30468^{a}
3	33669 ± 3301^{a}	30208± 10100^{ab}
6	12469 ± 3701^{b}	-55417 ± 12677^{b}
10	-5000 ± 2437^{c}	-329167± 32050^{c}

* Concentrations marked by the same letter are not statistically different.

A



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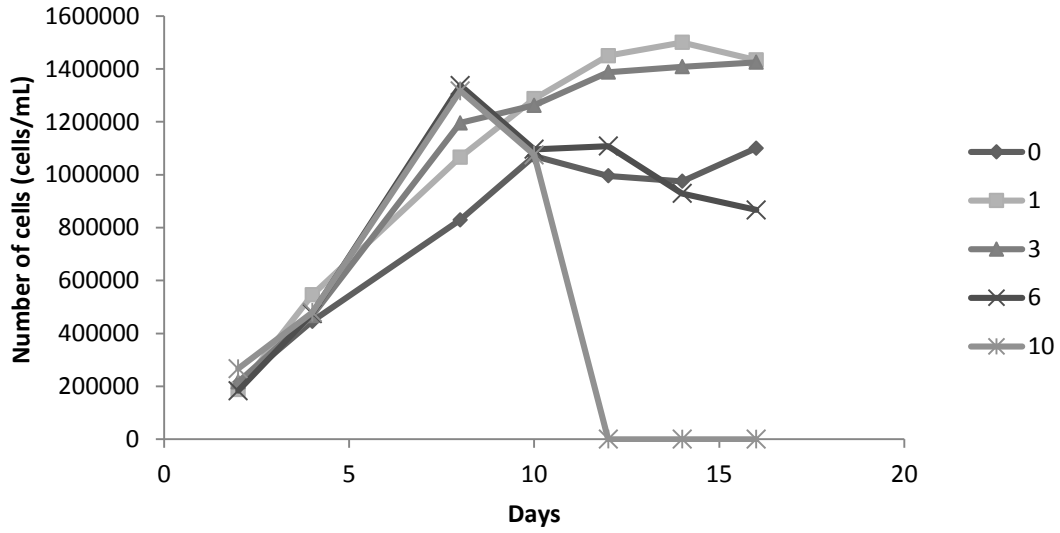

Figure 2. Number of algal cells through time (cells/ mL, n = 20). Glyphosate (0, 1, 3, 6, and 10 mg/L) was added at day twelve for *S. quadricauda* (A) and day eight for *S. capricornutum* (B). Standard error bars were removed for clarity.

At 0, 1, and 3 mg/L of glyphosate, Ch-a levels of *S. capricornutum* were greater (281, 362, and 241 µg/L) compared to respective pheophytin concentrations (11, 29, and 35 µg/L) (Fig. 3B). At 6 mg/L of glyphosate, pheophytin concentrations were greater (128 µg/L) than Ch-a (18 µg/L), and at 10 mg/L of glyphosate, only pheophytin was present (96 µg/L). After analyses, we found that Ch-a ($F\text{-ratio}_{4,15} = 9.63$; p

= 0.0005) and pheophytin ($F_{4,15}$= 13.07; P = 0.0001) concentrations were statistically different between glyphosate treatments; Ch-a concentrations at 0, 1, and 3 mg/L of glyphosate were significantly different from Ch-a at 10 mg/L ($p < 0.0366$), and Ch-a at 1 mg/L of glyphosate differed from Ch-a at 6 mg/L ($p = 0.0025$). Pheophytin concentrations at 0, 1, and 3 mg/L of glyphosate were significantly different from pheophytin at 6 mg/L ($P < 0.0027$), and pheophytin at 0 and 1 mg/L of glyphosate differed from pheophytin at 10 mg/L ($P < 0.0129$).

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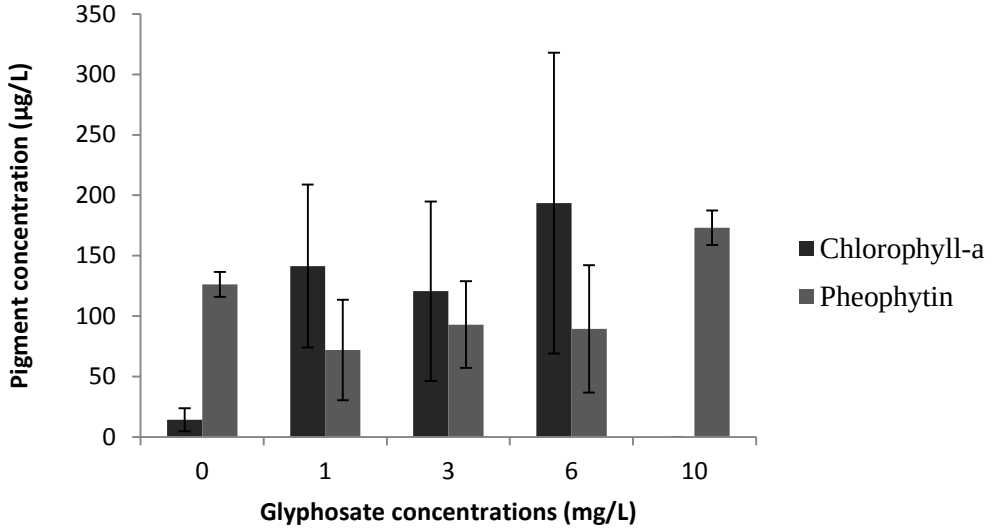

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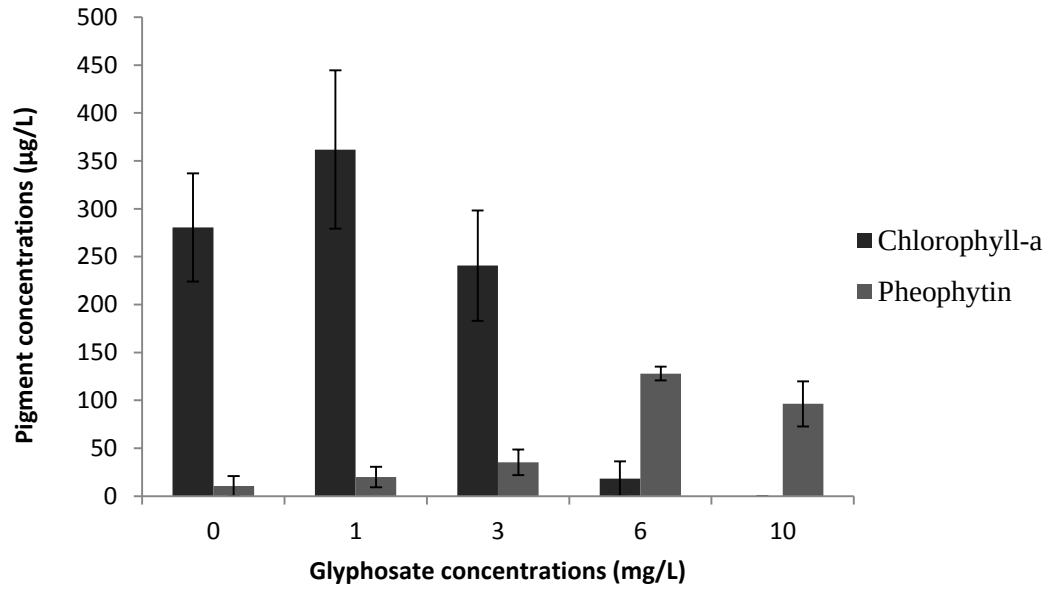

Figure 3. Chlorophyll-a and pheophytin concentrations (µg/L ± SE, n = 20) of *S. quadricauda* (A) and *S. capricornutum* (B). Ch-a was determined when growth approached stationary phase (maximum cellular yield).

Discussion and Conclusion

We had predicted that a concentration greater than 2 mg/L of glyphosate would inhibit cellular growth and Ch-a synthesis in both species. Concentrations of 1 and 3 mg/L of glyphosate did not affect growth or Ch-a synthesis in either species. In a similar study, Peterson et al. (1994) also found that glyphosate was relatively non-toxic at the expected environmental concentration (EEC) of 2.848 mg/L.

At 6 mg/L of glyphosate, cellular growth was affected in both algal species (Fig. 2). In *S. quadricauda*, cellular growth was less subsequent to glyphosate inoculation, but once glyphosate molecules broke-down and toxicity weakened, living cells grew and reproduced at a positive rate (Fig. 2A). This phenomenon could be attributed to the properties and half-life of glyphosate. In water, the half-life of glyphosate is 4.2 days, after which it is degraded by bacteria or incorporated into algal biomass (Vera et al., 2010). A short half-life could justify the initial and abrupt toxicity of glyphosate and explain why Ch-a concentrations were high at final yield (Fig. 3A). For *S. capricornutum*, growth was reduced and pheophytin levels were high relative to the control at 6 mg/L of glyphosate (Fig. 2B and 3B). These results support our second hypothesis; we found that *S. capricornutum* was more sensitive to glyphosate than *S. quadricauda*. Previous studies have found that sensitivity will vary based on the SA:V ratios of the organisms (Pirszel, Pawlik, & Skowronski, 1995; Lei, Hu, Wong, & Tam, 2007). Lei et al. (2007) measured a SA:V ratio of 0.89 μm^{-1} for *S. quadricauda* and 1.82 μm^{-1} for *S. capricornutum*; the greater the SA:V ratio of an organism, the greater its exposure to the surrounding environment (Pirszel et al., 1995). Dvorakova, Rojickova-Padrtova, and Marsalek (1999) also suggested that sensitivity differences could be attributed to the morphology (cell size and shape, colony formation, etc.), cytology (cell wall and intracellular structure), physiology (growth, nutrient uptake, metabolic rate, etc.) or genetics of the organisms.

Finally, we found that a concentration of 10 mg/L of glyphosate significantly inhibited cellular growth and Ch-a synthesis in both species. In similar studies, Saenz et al. (1997) found that concentrations of 12.5 and 25 mg/ L of glyphosate significantly reduced cellular growth in *S. quadricauda*, and Wong (2000) found that a concentration of 20 mg/L of glyphosate completely inhibited cellular growth in *S. quadricauda*. In another study, Tsui and Chu (2003) found that a concentration of 5.81 mg/L of glyphosate partially inhibited (50%) cellular growth in *S. capricornutum*, suggesting that a concentration near 10 mg/L of glyphosate would completely inhibit growth. It has been suggested that glyphosate causes growth inhibition by means of the shikimate pathway enzyme, 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSPS) (Tesfamariam, Bott, Cakmak, Roemheld, & Neumann, 2009). The inhibition of EPSPS stops aromatic and amino biosynthesis, reducing protein synthesis and the overall growth of the algae (Vera et al., 2010).

Ch-a synthesis of *S. quadricauda* and *S. capricornutum* was also inhibited at 10 mg/L of glyphosate (Fig. 3). These results were comparable with the study of Wong (2000), who found that 2 mg/L of glyphosate affected Ch-a synthesis, whereas a concentration of 20 mg/L of glyphosate completely inhibited Ch-a synthesis in *S. quadricauda*. In another study, Bozeman, Koopman, and Bitton (1989) found that 7.8 mg/L of glyphosate partially inhibited (50%) Ch-a synthesis in *S. capricornutum*, suggesting that a concentration near 10 mg/L of glyphosate would completely inhibit Ch-a synthesis. The inhibition of Ch-a synthesis reduces photosynthesis, resulting in cellular death (Shikha & Singh, 2004).

Throughout our study, we encountered issues with variability between the treatment replicates. Dvorakova et al. (1999) proposed that the formation of 2-, 4- or 8-celled coenobia of *S. quadricauda* causes lack of uniformity and results in counting variability. Dvorakova et al. (1999) also stated that *S. quadricauda* has the tendency to stick to flask walls. In contrast, Blaise (1993) considered *S. capricornutum* a good test species because it is unicellular and non-motile. Skulberg (1967) also reasoned that *S. capricornutum* is ideal to test because it has little morphological variability. However, we found that *S. capricornutum* was difficult to count because of its small size, which made it difficult to determine whether cells were living or dead under the microscope. Based on these issues, we suggest using methods other than cell counts to determine algal biomass. The American Water Works Association (2010) stated that cell aggregations increase the variability between replicate samples and every subsample taken from the original flask increases the probability of error. Alternative methods, such as CO_2 uptake and ash-free dry mass might be more appropriate indicators of algal biomass (American Water Works Association, 2010).

Other studies have detected no greater than 1 mg/L of glyphosate in Manitoba freshwater systems (Beck, 1987; Goldsborough & Brown, 1993); however, direct spray or concentrated spills of glyphosate could

affect algal species and influence organisms that rely on algae as food. To further understand the toxicity of glyphosate, outdoor mesocosm studies should be implemented since the effects of glyphosate could be influenced by abiotic factors such as light availability and temperature associated with seasonal change (Pesce et al., 2009). More studies should also focus on the toxicological effects of POEA and other surfactants that are combined with glyphosate.

Materials and Methods

Treatments

We purchased isolated cultures of *S. quadricauda* and *S. capricornutum* from Carolina Biological Supply (Burlington, NC, USA). These species were selected based on their common use in batch culture studies and their varying sensitivity to glyphosate toxicity (Peterson et al., 1994; Saenz et al., 1997; Wong, 2000). We also purchased commercial Roundup® manufactured by Monsanto Chemical Co. (St. Louis, MO, USA) (540 g/L of glyphosate as active ingredient) from Ag Advantage Ltd. (Marquette, MB, Canada). For a previous experiment, we applied treatments ranging from 0, 0.01, 0.1, 1, and 10 mg/ L of glyphosate, but found that most of these concentrations did not affect *S. quadricauda* and *S. capricornutum*. Therefore, we chose additional treatment levels to determine the threshold concentration at which growth and Ch-a synthesis were inhibited: 0 (control), 1, 3, 6 and 10 mg/L. All treatment levels were replicated four times to account for variability.

Culturing techniques

A batch culture medium provides a finite source of nutrients to growing algae (Andersen, 2005). The medium of choice for this study was Chu no.10 (40 g/L Ca $(NO_3)_2$, 5 g/L K_2HPO_4, 25 g/L $MgSO_4 \cdot 7H_2O$, 20 g/L Na_2CO_3, 20 g/L Na_2SiO_3, and 0.8 g/L $FeCl_3$) because it is often used to culture freshwater green algae (Chu, 1942; Stein, 1973). We added hydrochloric acid (HCl) to acidify the medium to a suitable pH (7.00-7.50) for algal growth (Chu, 1942). We then dispensed 100 mL of the medium into 20 autoclaved Erlenmeyer flasks (250 ml) and added the appropriate algal solution (3 ml).

The cultured algae were stored in a growth chamber to ensure that light and temperature were kept consistent throughout the course of the experiment. The particular chamber maintained a regular temperature of 22 ± 0.3° C and a light-dark cycle of 16 to 8 hours. Typically, algae are light-saturated at an intensity of 250 µmol/m^2/s (i.e. represents the point at which light is no longer limiting the rate of photosynthesis) (Andersen, 2005); therefore, we maintained a light intensity of approximately 250-300 µmol/m^2/s. We also randomized the placement of flasks within the chamber between density counts so that each flask received approximately equal exposures of light.

Cell count procedures and growth rate analysis

Before counting a sample, we shook the flasks to ensure the algal cultures were homogenized. We then pipetted a small algal sample to fill both counting chambers of a Neubauer Improved haemocytometer (C.A. Hausser and Son, Philadelphia, PA, U.S.A) and used a phase-contrast compound microscope (Leitz Diaplan) to count the cells within the grid regions of the counting chambers. Once cell count reached exponential phase (Fig. 1), we inoculated the cultured algae with glyphosate. After inoculation, we continued counting until cellular growth approached stationary phase (maximum cellular yield).

To determine glyphosate toxicity after inoculation, we calculated the slope of exponential growth by linear regression using JMP® 10.0.1 (SAS Institute Inc., Cary, NC, U.S.A.). This was accomplished by plotting day versus cell count for each treatment. All the assumptions of a linear regression were tested prior to analysis. If the data did not follow parametric assumptions, we still considered the linear regression to be robust enough to account for deviations, which were likely the result of a small sample size.

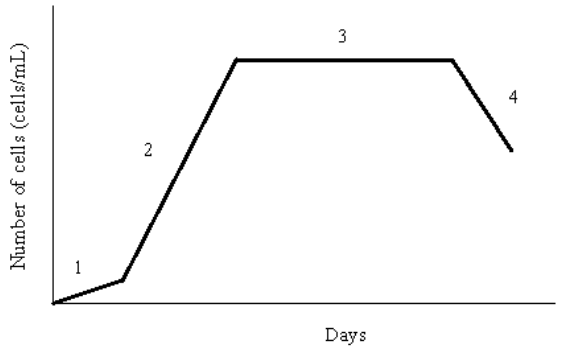

Figure. 1. Growth curve representing algal growth through time (days). The curve comprises of the lag phase (1), log or exponential phase (2), the stationary phase (3), and the death phase (4).

Chlorophyll-a analysis

Ch-a concentrations (µg/L) were analyzed when cellular growth approached stationary phase. We passed the culture medium of each flask through a vacuum filter system using Whatman GF/C filter papers. We used these particular filter papers because they were a suitable size (1.2 µm) for collecting our algal species. After filtration, we pipetted the algal samples into plastic cuvettes and loaded them into a spectrophotometer, which read the absorbance values of all pigment samples. Absorbance was measured at both 665 and 750 nm for all samples. A wavelength of 665 nm represented the peak absorbance of Ch-a and a wavelength of 750 nm accounted for contaminants outside the typical wavelength of Ch-a. We then added (0.1 mL) diluted hydrochloric acid (0.02 M) to the cuvettes in order to destroy all Ch-a within the samples. After one hour, we measured absorbance readings again at 665 and 750 nm to determine the amount of pheophytin (break-down product of Ch-a) within the samples. Finally, we calculated Ch-a and pheophytin concentrations based on the formulae described by Marker, Crowther, and Gunn (1980).

Statistical Analysis

The significance between glyphosate treatments and mean cellular growth rates, final Ch-a, and pheophytin concentrations was tested in JMP® using a one-way analysis of variance test (ANOVA) followed by a post-hoc Tukey test (p-value ≤ α = 0.05). We tested the assumptions of the ANOVA prior to analysis. If the data did not follow parametric assumptions, we still considered the ANOVA to be robust enough to account for deviations, which were likely the result of a small sample size.

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