Acidus Photosynthetica: pH dependence of growth and oxygen evolution in the unicellular green alga *Eremosphaera viridis*[1].

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OBJECTIVE

To determine the pH dependence of growth and oxygen evolution in the unicellular model algal cell *Eremosphaera viridis* (De Bary).

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INTRODUCTION

Photosynthesis is a highly regulated process that includes the harvest of solar energy, transfer of excitation energy, energy conversion, electron transfer from water to $NADP^+$, ATP generation and a series of enzymatic reactions that assimilate carbon dioxide and synthesize carbohydrate (Tanaka and Makino 2009). Two major players in photosynthesis are photosystem I and photosystem II. Together with the light-absorbing green pigment chlorophyll, they transform sunlight into electrochemical potential (Figure 1). The process begins when light energy is used to drive the oxidation of water to oxygen by photosystem II and ends with the production of ATP and NADPH that are used to fuel the 'dark reactions' to make sugars and the other molecules of life (Allen and Martin 2007).

In plants, algae and certain types of bacteria, the photosynthetic process results in the release of molecular oxygen as a by-product and the removal of carbon dioxide from the atmosphere (Singhal et al 1999). Light energy is used to oxidize water and molecular oxygen is yielded in the process as a by-product:

$$2H_2O + \text{light} \rightarrow 4H^+ + 4e^- + O_2$$

Oxygen released in the presence of light by a plant is a direct measure photosynthetic activity (Hall and Rao, 1999). Oxygen production is usually measured with a Clark-type oxygen electrode, a technique we used in our research. The organism we selected for our research was the unicellular green alga *Eremosphaera viridis*.

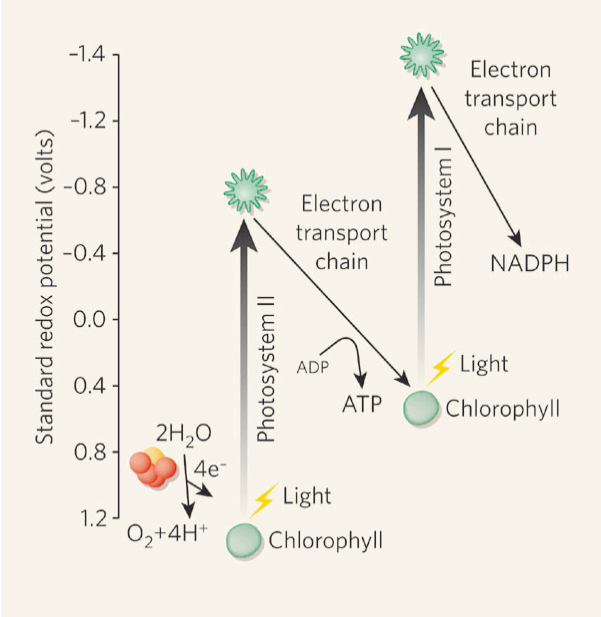

Figure 1. The two photosystems in photosynthesis.
The figure (by John F. Allen & William Martin) was obtained from http://www.nature.com/nature/journal/v445/n7128/fig_tab/445610a_F1.html

Eremosphaera viridis De Bary is a large, unicellular microalga, spherical and 90 – 120 μm in diameter. The species was originally described by Anton De Bary in 1858 in his Untersuchungen über die Familie der Conjugaten. The spherical cells of *E. viridis* have numerous chloroplasts that radiate from a large centrally placed nucleus on strands of cytoplasm and line the inside of the cell wall (Figure 2 shows an example of a dividing cell).

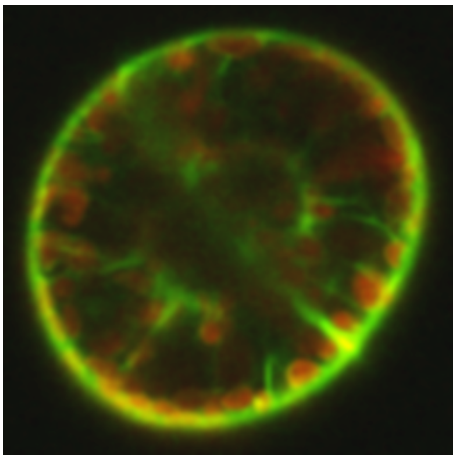

Figure 2. Three-dimensional Z-profile of the *Eremosphaera viridis* cell. Reconstructed from z-sectioned confocal fluorescence image slices. In this example, the cell is dividing, so two nuclei can be observed on either side of the developing cell plate. Both membranes (green) and chloroplasts are (red) were imaged. The diameter of the cell is approximately 100 microns.

E. viridis is an ideal model organism due in part to their large size and simple spherical geometry, which make them amenable to microscopic quantification, observation techniques and micro-manipulation. They grow quickly on a minimal maintenance media and concentrated suspensions of cells are easy to prepare.

E. viridis are known to inhabit acidic lakes. The strain we used was isolated from Plastic Lake, which had a pH of about of 5.8 in 1981 and continued to acidify in subsequent years (at a rate of 0.035 pH units a year) (Dillon et al, 1987). They have also been reported to grow in acidic bogs. Therefore, they are considered acidophilic alga. The cytosol of the alga has a large buffering capacity of 90 ± 26 mM-W/pH (Plieth et al. 1997), enhancing its ability to adapt to its normally acidic habitat.

In this report, we confirm the acidophilic nature of the alga with respect to growth, and measure the effect of external pH on oxygen evolution. Initially, the effects of cell density and bicarbonate concentration on photosynthetic oxygen evolution were measured to determine the optimal conditions to measure photosynthetic rates *versus* pH.

MATERIALS AND METHODS

Strain. The alga *E. viridis* De Bary strain (CPCC 127) was obtained from the Canadian Phycological Culture Centre (University of Waterloo, Waterloo, Canada). The strain is designated LB (live bacteria). Sterile techniques were used in the transferring of all solutions and algae. This included working in a sterile laminar flow hood and flaming the lips of the flasks and pipettes used for transfers.

The cultures were grown at room temperature (21–27ºC) (Figure 8) under 50 μmol m^{-2} s^{-1} photon flux from T8 fluorescence lamps on an orbital shaker (125 r.p.m.) in 125 mL Ehrlenmeyer flasks containing 25 mL of Bold's basal medium (BBM) supplemented with vitamins (Appendix A). The major ions in BBM are Na^+ (5.96 meq), K^+ (2.26 meq), Mg^{2+} (0.30 meq), Ca^{2+} (0.17 meq), Cl^- (3.17 meq), NO_3^- (2.94 meq), PO_4^{3-} (1.72 meg) and SO_4^{2-} (0.32 meq). The media pH is *ca* 5.8. Samples or transfers were taken from cultures that were one to two weeks old, which is during log phase growth.

pH Dependence of Growth. Cultures were grown at pH 3, 4, 5, 6, 7 and 8 in three replicate 125 mL Ehrlenmeyer flasks containing 25 mL of Bold's basal medium (BBM) supplemented with vitamins (Appendix A) plus 50 mM of buffer (Table 1) under T8 fluorescence lamps on a orbital shaker (125 r.p.m.) (Figure 3).

Figure 3. *E. viridis* cells at pH 3, 4, 5, 6, 7 and 8 in three replicate 125 mL Ehrlenmeyer flasks on day one of the experiment.

Buffers having a pK_a within +/- 1.5 pH units of the final desired pH of the culture medium were selected. The pH of the BBM-buffer solutions were adjusted with 5N NaOH as required. 25 mL of the adjusted BBM-buffer solution were filtered sterilized and added to each of the three flasks. Each flask was inoculated with 4 mL of *E. viridis* cells taken from a two week old culture. The cells tend to settle to the bottom of the flasks quickly due to their large size. Therefore, the stock flask was swirled vigorously prior to removing the 4 mL aliquot.

Table 1. Buffers for Growth Experiments

pH	Buffer	pKa	Molecular Weight (g/mol)	50mM (g/L)	50mM (g/80mL)
3	Citric acid	3.13	210.14	10.51	0.84
4	Citric acid	3.13	210.14	10.51	0.84
5	MES	6.15	195.23	9.76	0.78
6	MES	6.15	195.23	9.76	0.78
7	MOPS	7.20	293.30	14.67	0.84
8	TES	7.50	229.25	11.46	0.92

Cell Counting Protocol. A spectrophotometer or haemocytometer could not be used to determine the cell density because the cells rapidly settle due to their large size. Therefore, to determine the cell density of the algae cells in a flask, samples were removed using a Drummond 10µL positive displacement pipette. A volume of either 10µL or 5µL was selected to obtain an optimal number of cells for counting. Each flask was swirled vigorously to ensure the cells were in suspension and well mixed before the sample was taken. The samples were placed on the lids of culture dishes (35 × 10 mm), and the cells in each droplet were counted using a ×10 objective. The number of cells per droplet was divided by the volume of the droplet to calculate the cell density (cells/mL). Three samples were taken from each flask for counting. Cell count measurements were performed twice daily.

Culture Preparation for Oxygen Electrode Experiments. An aliquot of 4 mL of cells from a 1–2 week old culture were transferred to a 15 mL plastic tube and bubbled with N_2 for 5 minutes (to deplete oxygen in preparation for oxygen evolution measurements). Three mL of the cells ware transferred to the oxygen electrode chamber with a magnetic flea in place and the stirrer turned on. Once oxygen evolution appeared to achieve a steady rate, 150μL of 100 mM $NaHCO_3$ in Bold's medium were added to the cell suspension in the oxygen electrode chamber in order to stimulate oxygen production by supplying the CO_2 for photosynthesis. During the run, three aliquots (2.5, 5, or 10 μl) of the cells suspension were removed using a Drummond 10µL positive displacement micropipette to obtain the cell density. The oxygen electrode chamber was draped with a light-tight cloth for dark treatments.

Oxygen Electrode Experiments. Photosynthetic rates were measured as oxygen evolution in a Clark-type oxygen electrode from Hansatech (DW1 oxygen electrode chamber and CB1-D3 electrode control unit; Hansatech Instruments Ltd., Norfolk, England). The electrode was calibrated twice before and twice after each experimental run with either dH_2O or Bold's medium at 'zero' and air-saturated oxygen levels (270 μM at 23 ºC). During the calibrations, air-saturated oxygen was depleted from the oxygen electrode chamber solution using sodium dithionate ($Na_2O_4S_2$). The temperature for all measurements was maintained constant at 23ºC using a circulating water bath. Output was recorded on a Kipp and Zonen chart recorder (model BD 11E; Kipp & Zonen, Delft, Netherlands). Light was provided from a 50 W tungsten-halogen lamp powered by a variable DC power supply to control light intensity. The lamp output was focused onto the oxygen electrode chamber with a collimating lens. A 3.5% (w/v) cupric sulfate ($CuSO_4$•$5H_2O$) filter (1.4 cm path-length) was placed between the collector lens and chamber (Figure 4). The filter was used to selectively irradiate the oxygen electrode chamber with photosynthetically active light, blocking UV and infrared light. Photon flux was between about 450 and 630 nm (maximal flux at 560 nm).

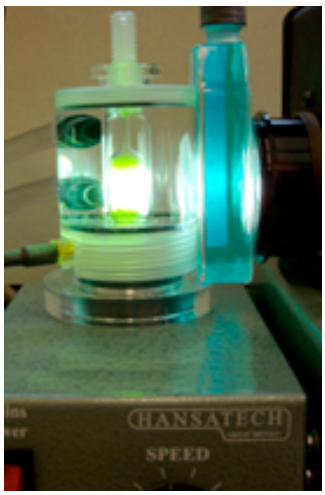

Figure 4. Oxygen electrode set-up. Light is filtered through a cupric sulfate solution to remove UV and infrared light.

The light intensity was measured with a radiometric probe (Model 268R with an internal radiometric filter [maximal light responsivity between 400 and 1000 nm] attached to a Model S471 portable optometer; UDT Instruments, San Diego, California, USA). The probe was placed on the side of the oxygen electrode chamber opposite from the tungsten-halogen lamp. The light intensity of the lamp was measured a few seconds after the lamp was turned on.

Culture Preparation for pH Dependence vs. Oxygen Evolution Experiments. An aliquot of 5 mL of cells from a 1–2 week old culture were transferred to a 15 mL plastic tube. The cells were allowed to settle in the test tube placed vertically in a stationary position. Due to the weight and size of the cells, they settled to the bottom in a couple minutes. The supernatant was removed and 6 mL of a 100 mM buffer solution in ddH_2O was added to the cells. Buffers were selected on the basis of having a pK_a near the desired pH for the solution (Table 2). The pH of the buffer solutions was adjusted using 5 N NaOH. After addition of the buffer solution, the cells were allowed to settle to the bottom, the supernatant decanted, and 6 mL of the same buffer solution were added to the cells. When the cells settled to the bottom, the supernatant was removed. Then, 6 mL of the buffer solution were added to the cells and the solution was bubbled with N_2 (to deplete oxygen in preparation for oxygen evolution measurements) for 5 minutes. An aliquot of 3 mL of cells was transferred to the oxygen electrode chamber, and stirred with a magnetic flea.

Table 2. Buffers for pH Dependence Experiments

Buffer	pH	pKa	Molecular Weight (g/mol)	100 mM (g/100 mL)	Volume of 5N NaOH added to adjust pH (µL)
Citric acid	3	3.13	210.14	2.1014	1000
Citric acid	4	3.13	210.14	2.1014	1700
MES	5	6.15	213.20	2.1320	100
MES	6	6.15	213.20	2.1320	300
MOPS	7	7.20	209.26	2.0926	1150
TES	8	7.50	229.25	2.2925	1500

Oxygen Electrode pH Dependence Experiments. The set up and procedure for these experiments were the same as the protocol followed for the oxygen electrode experiments above. However, during these experiments the glass stopper of the electrode chamber was replaced with a pH probe in order to constantly monitor to pH of the cell suspension (since the addition of $NaHCO_3$ caused the pH to become more alkaline). Three pH readings were recorded. Results are plotted versus the second pH reading.

RESULTS

The Effects of Cell Density and Bicarbonate Concentration on Oxygen Evolution. Oxygen evolution is half-maximal at a cell density of 12,000 cells/mL, and maximal at cell densities greater than about 40,000 cells/mL (Figure 5A). Oxygen evolution is dependent on the availability of carbon dioxide (added as the bicarbonate ion), so we determined the rate of photosynthetic oxygen evolution *versus* bicarbonate concentration at a cell density of about 10,000/mL. The photosynthetic rate is maximal at a Na_2CO_3 concentration of 5 mM (Figure 5B).

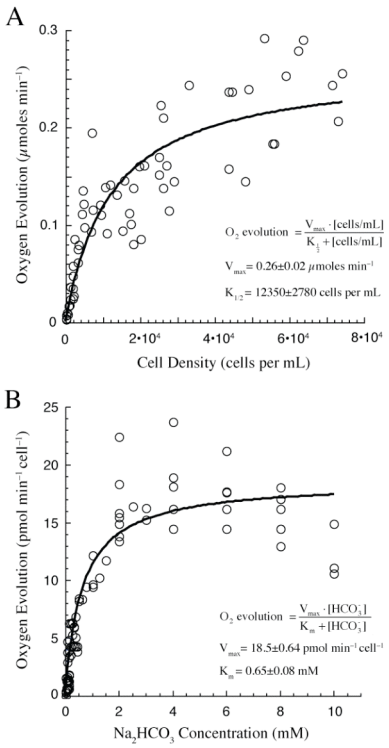

Figure 5. The effects of cell density (A) and bicarbonate concentration (B) on oxygen evolution. The curves are best fits to the Michaelis-Menten equation:

$$Rate = \frac{V_{max}[X]}{K_{1/2} + [X]}$$

Best fits (± standard error) of V_{max} and $K_{1/2}$ are shown.

The Effects of Buffer Concentration on Oxygen Evolution and Changes in pH. Because pH will change when adding Na_2CO_3 to the chamber, I examined the effect of buffer concentration on the pH change and the rate of photosynthetic activity (Figure 6). These experiments were done at pH 7 using MOPS buffer. Buffer concentrations up to 100 mM had very little effect on photosynthetic oxygen evolution (Figure 6A). Changes in pH caused by bicarbonate addition were very slight above a buffer concentration of 60 mM (Figure 6B). So, an optimal buffer concentration of 80 mM was selected.

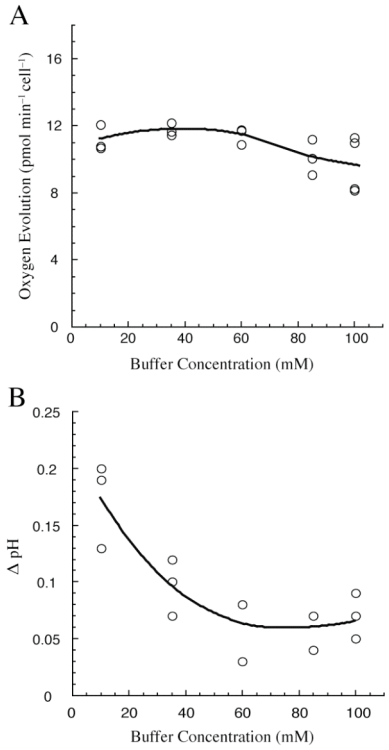

Figure 6. The effects of buffer concentration on oxygen evolution (A) and the effects of buffer concentration on changes in pH (B).

Rate of Oxygen Evolution and Phthalic Acid Buffer. The initial oxygen evolution experiments for pH 3 and pH 4 used the buffer phthalic acid. The effect of the phthalic acid on photosynthetic oxygen evolution were unusual. The cells were unable to produce oxygen (Figure 10B) and appeared unhealthy, changing to a brown colour after a short period of time (Figure 7). Based on these observations, I used citric acid to buffer the cells at pH 3 and pH 4 for both oxygen evolution experiments and growth experiments.

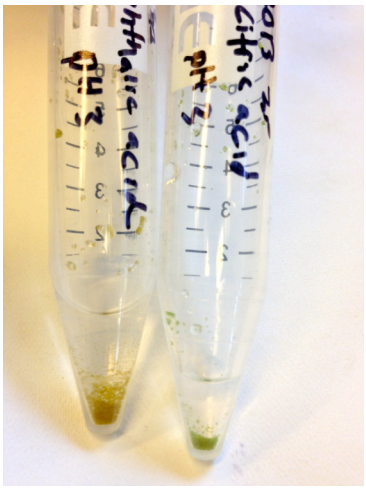

Figure 7. *E. viridis* cells buffered with phthalic acid (left) and citric acid (right) after 18 hours.

pH Dependence of Growth. An example of the counting method is shown in Table 3. The cell density for each flask was averaged over three samples and the cell density for each pH was averaged over the three flasks.

Table 3. Example of Cell Count and Density Calculation

Time (Hrs)	pH	Flask	Sample 1 (# of cells)	Sample 2 (# of cells)	Sample 3 (# of cells)	Sample Average	Sample Size (mL)	Density (Cells/mL)	Mean Density
0	3	1	10	4	15	10	0.01	967	822
		2	8	10	6	8	0.01	800	
		3	5	7	9	7	0.01	700	
19	3	1	8	7	5	7	0.01	667	756
		2	8	9	6	8	0.01	767	
		3	9	10	6	8	0.01	833	

The mean cell density *versus* time at each pH (Figure 8) was fit to a logistic growth curve:

$$N = \frac{K \cdot N_0 \cdot e^{T/g}}{K + N_0 \cdot (e^{T/g} - 1)}$$

where N is the cell density (dependent variable), K is the carrying capacity (maximum number of cells), N_0 is the initial cell density, T is the time in hours (independent variable), and g is the generation time (time for cell density to double).

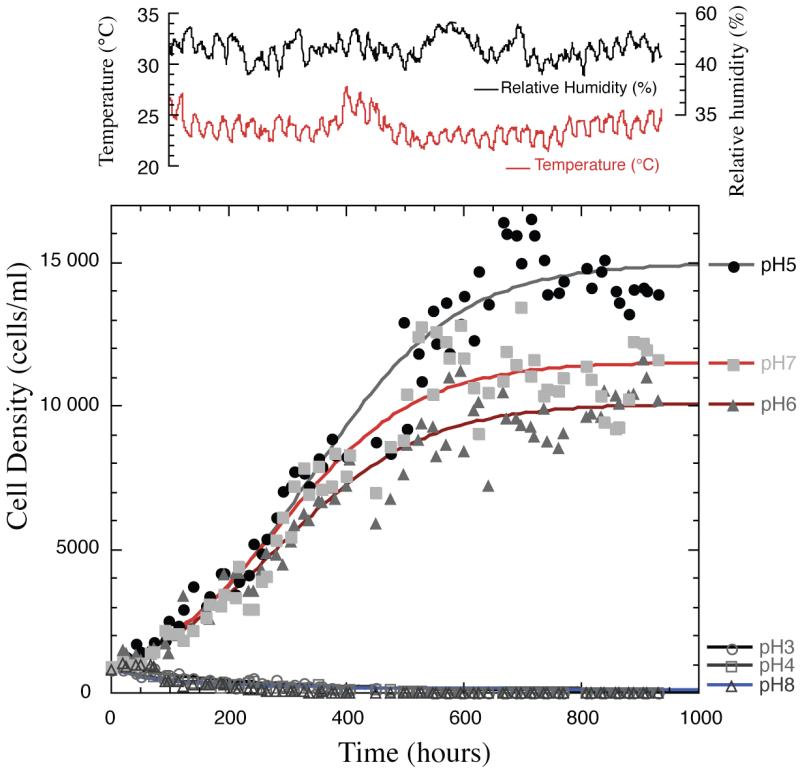

Figure 8. *E. viridis* growth curve data for pH 3 - 8. The top panel shows the temperature and relative humidity during most of the growth experiment. The cyclic pattern is due to the day/night cycle. The bottom panel shows the mean cell densities for each pH. The curves are best fits to a logistic growth curve equation

$$N = \frac{K \cdot N_0 \cdot e^{T/g}}{K + N_0 \cdot (e^{T/g} - 1)}$$

as described in the results section

The effects of pH on carrying capacity and generation time are shown in Table 4. Generation time is independent of pH, but pH does have a large effect on the carrying capacity.

Sustained growth was only observed at pH 5 – 7. All the cells at pH 8 died off by day 15 and all the cells at pH 3 and pH 4 died off by day 20 (Figure 9). Recorded live cells were those that appeared under the microscope to have green chloroplasts that had not retracted from the cell wall, and with no dark coalesced regions.

Table 4. pH versus Carrying Capacity and Generation Time

pH	Carrying Capacity (cells/mL)	Generation Time (hours)
3	"0"	No growth
4	"0"	No growth
5	14924 ± 250	125 ± 3
6	10027 ± 171	124 ± 4
7	11469 ± 231	118 ± 4
8	"0"	No growth

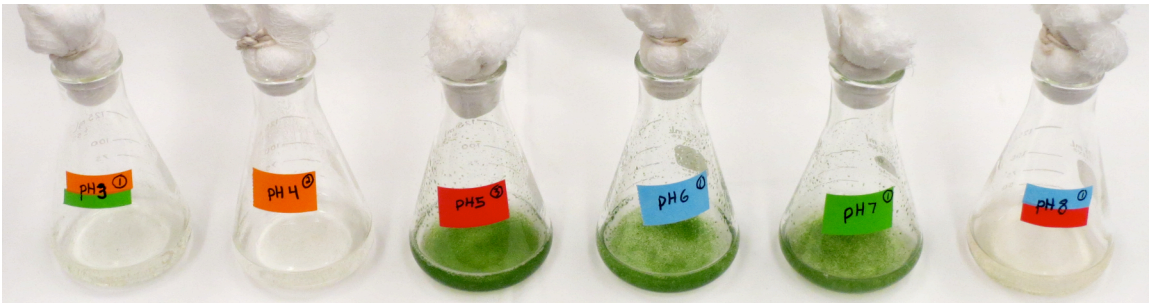

Figure 9. *E. viridis* cells in order of increasing pH (3 to 8) on day 21.

The Optimal pH Dependence of Growth and Rate of Oxygen Evolution are Different. The cells sustained optimal growth at pH 5 (Figure 10A and Figure 11). In contrast, the cells experienced the highest rate of oxygen evolution at a pH of 7 (Figure 9B) declining at more acid (pH 5) and alkaline (pH 8.4) conditions.

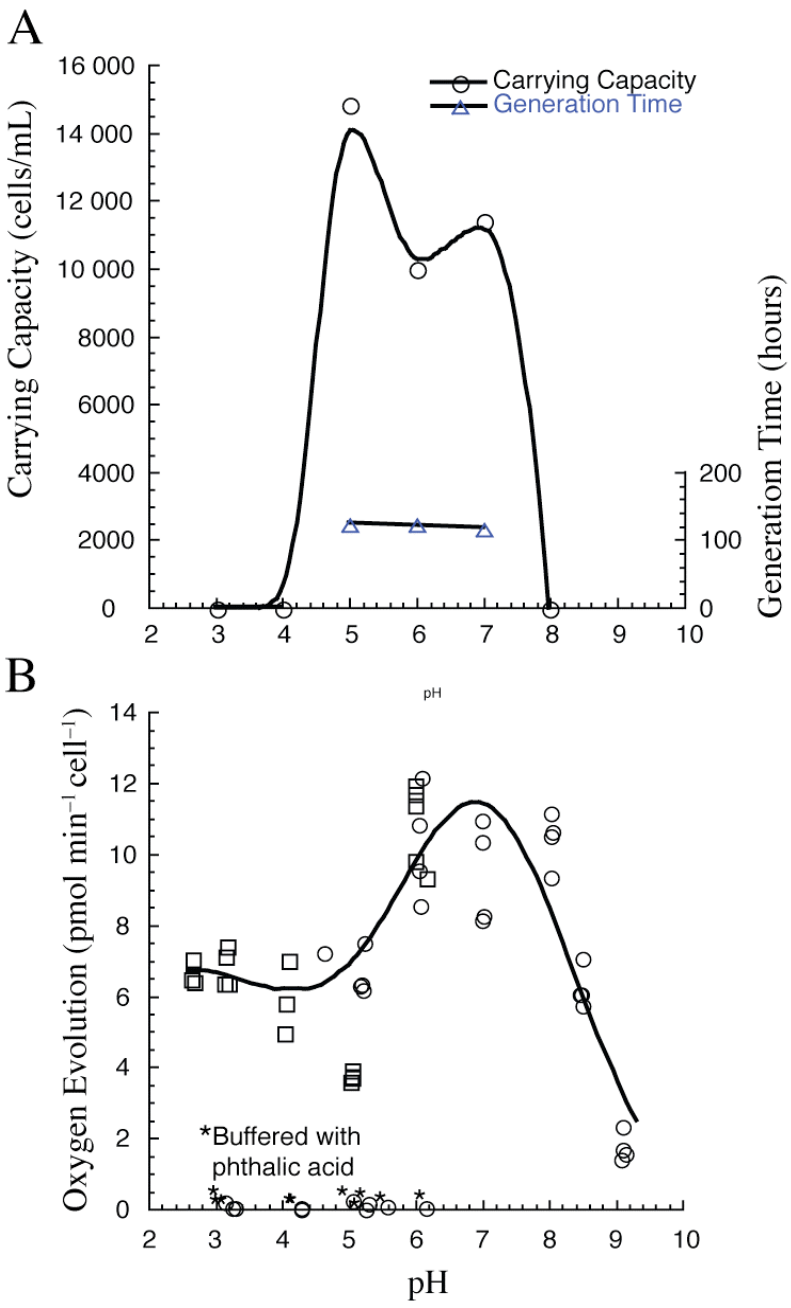

Figure 10. The effect of pH on growth (A) and the rate of oxygen evolution (B). Note that the maxima do not match. Growth is optimal at pH 5, while photosynthesis is optimal at pH 7. In the growth experiments, cells at pH 5 had a healthier appearance (Figure 11).

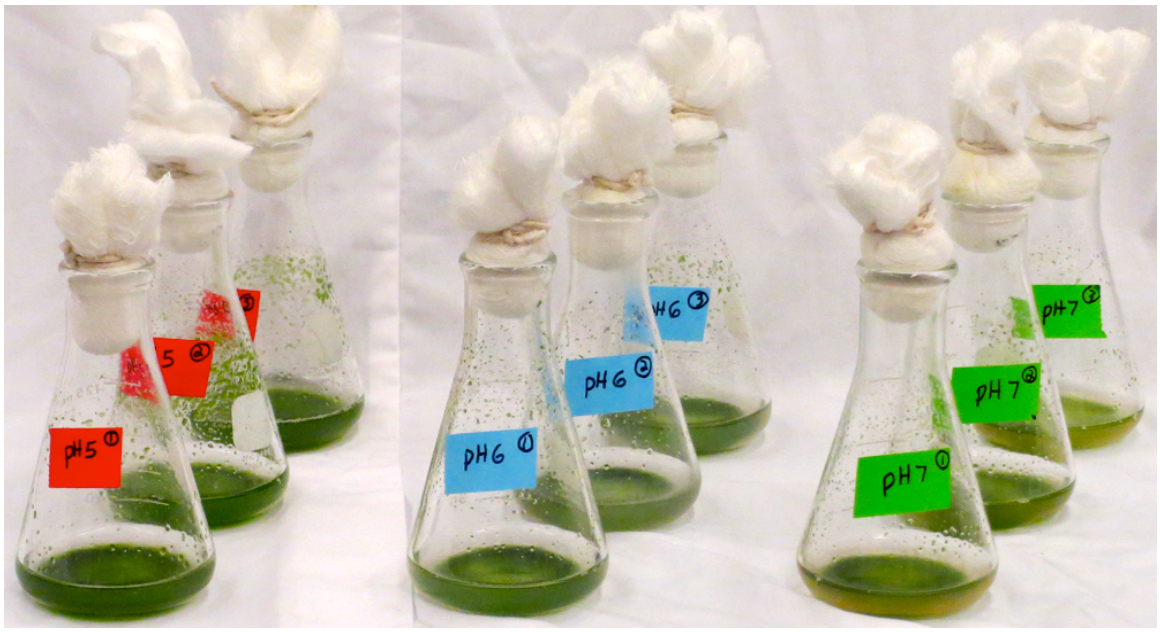

Figure 11. *E. viridis* on the last day of growth experiments (day 40). The cells had reached maximum cell density. Note the shift in green coloration from 5 to 7. By visual inspection, the cells at pH 5 were much healthier with few cells exhibiting a damaged appearance (chloroplasts retracted from the cell perimeter).

DISCUSSION

The pH growth experiments show that *Eremosphaera viridis* is an acidophile, with maximal growth at pH 5. There was a sharp decline in carrying capacity to no growth at pH 4, and a more gradual decline at pH 6 to pH 7. The doubling times across pH values of observable growth (5, 6, and 7) were essentially the same, ranging from 3.3 to 4.8 days. These effects are not due to changes in the pH of the growth medium, which was fairly constant, changing to slightly alkaline (about 0.2 units) by the end of the growth experiments. The pH dependence of growth was very similar to a previous report by Khine and Lew (2010): Optimal at pH 5 with less growth at pH 6 and 7, and no growth at acid (4 and 5, using phthalic acid to buffer the growth media) or alkaline (8 and 9) pH.

In contrast, the pH dependence of photosynthetic activity was more alkaline: it was optimal at pH 7 and lower at pH 5–6. One would expect that growth would be determined by the capacity for photosynthesis in a one-to-one correspondence, but this is clearly not the case for *E. viridis*. Previous research has shown that *E. viridis* is capable of concentrating CO_2 inside the cell. It lacks external carbonic anhydrase (to catalyze the inter-conversion of CO_2 and HCO_3^-). This means that the cells rely only upon CO_2, not HCO_3^- (Rotatore et al., 1992). So, the additional supply of inorganic carbon at pH 7 (due to higher [HCO_3^-] is not the cause of the higher photosynthetic activity at neutral pH.

The pH-insensitive doubling time and the different pH dependencies of growth and photosynthetic activity indicate that photosynthesis is not the sole determinant of cellular growth. Since all other environmental and nutrient components are the same for all the cells, it must be related to the external pH, but separate from a direct effect on photosynthesis. Photosynthesis is known to cause alkalinization of the extracellular medium (Lew, 2010), but the H^+ fluxes are much lower than the buffering capacity of the cell, and would reverse during the dark period when respiration dominates.

In summary, the alga *Eremosphaera viridis* is an acidophile, but not on the basis of a direct correspondence with the pH dependence of photosynthetic activity. Other factors must play a role in its growth in acidic environments.

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http://www.yorku.ca/planters/student_reports/viridis_pH.pdf (accessed 25 July 2012).

APPENDIX A

BOLD'S BASAL MEDIUM (BBM) Modified from original[3]
Reference: Stein, J. (ED.) Handbook of Phycological methods. Culture methods and growth measurements. Cambridge University Press. 448 pp.

This medium is highly enriched and is used for many of the green algae.

STOCK	STOCK SOLUTION	ml/Litre	ml/500 ml	ml/250 ml
KH_2PO_4	1.75 g /100 ml	10 ml	5 ml	2.5 ml
$CaCl_2 \cdot 2H_2O$	0.25 g /100 ml	10 ml	5 ml	2.5 ml
$MgSO_4 \cdot 7H_2O$	0.75 g /100 ml	10 ml	5 ml	2.5 ml
$NaNO_3$	2.50 g /100 ml	10 ml	5 ml	2.5 ml
K_2HPO_4	0.75 g /100 ml	10 ml	5 ml	2.5 ml
NaCl	0.25 g /100 ml	10 ml	5 ml	2.5 ml
Na_2EDTA KOH	1.00 g /100 ml 0.62 g /100 ml	1 ml	0.5 ml	0.25 ml
$FeSO_4 \cdot 7H_2O$ H_2SO_4 (conc.)	0.498 g /100 ml 0.1 ml /100 ml	1 ml	0.5 ml	0.25 ml
Trace Metal Solution	See below*	1 ml	0.5 ml	0.25 ml
H_3BO_3	1.15 g /100 ml	0.7 ml	0.35 ml	0.175 ml
Vitamin Stock	See below[1]	0.05 ml	0.025 ml	0.0125 ml
d H_2O		936 ml	468 ml	234 ml

***Trace Metal Solution:**

Substance	g/100 ml
H_3BO_3	0.286 g
$MnCl_2 \cdot 4H_2O$	0.181 g
$ZnSO_4 \cdot 7H_2O$	0.0222 g
Na $MoO_4 \cdot 5H_2O$ (used $Na_2MoO_4 \cdot 2H_2O$)	0.0390 g
$CuSO_4 \cdot 5H_2O$	0.0079 g
$Co(NO_3)_2 \cdot 6H_2O$	0.00494 g

*Dissolve each of the above substances for the Trace Metal solution separately, prior to adding the next on the list.

Adjust the pH of the medium to 6.8 with NaOH or HCL and filter sterilize. ***Nota bene*** **pH without adjustment is 5.8, suitable for *E.viridis* growth.**
OPTIONS: As this is a highly enriched medium it can be diluted to 10% of the above concentrations and used successfully when fast and dense growth is not required. For 10% BBM, use 100 ml of 100% BBM/Litre of distilled water. The addition of 5 ml/L of soil extract is also beneficial to some algae particularly when problems with poor growth or morphology occur.

[3] From UTCC: University of Toronto Culture Collection of Algae and Cyanobacteria. Website: http://www.botany.utoronto.ca/utcc/Culture%20Media.html. Revision Date 28oct2010
Revised: 27 May 2008 to include vitamin supplement: STOCK (100X) Thiamine-HCl 0.1 gm/10 ml; Vitamin B_{12} 0.002 gm/10 ml; Biotin 0.001 gm/10 ml. Store frozen (–20ºC).

BOLD'S BASAL MEDIUM (BBM) Modified from original
Reference: Stein, J. (ED.) Handbook of Phycological methods. Culture methods and growth measurements. Cambridge University Press. 448 pp.

This medium is highly enriched and is used for many of the green algae.

STOCK	FINAL CONCENTRATION
KH_2PO_4	1.29 mM
$CaCl_2 \cdot 2H_2O$	0.17 mM
$MgSO_4 \cdot 7H_2O$	0.30 mM
$NaNO_3$	2.94 mM
K_2HPO_4	0.43 mM
NaCl	2.99 mM
Na_2EDTA KOH	0.027 mM 0.11 mM
$FeSO_4 \cdot 7H_2O$ H_2SO_4 (conc.)	0.018 mM 0.00018 mM
Trace Metal Solution	See below*
H_3BO_3	0.012 mM

***Trace Metal Solution:**

Substance	FINAL CONCENTRATION
H_3BO_3	42.0 μM
$MnCl_2 \cdot 4H_2O$	9.1 μM
$ZnSO_4 \cdot 7H_2O$	0.77 μM
Na $MoO_4 \cdot 5H_2O$ (used $Na_2MoO_4 \cdot 2H_2O$)	1.6 μM
$CuSO_4 \cdot 5H_2O$	0.31 μM
$Co(NO_3)_2 \cdot 6H_2O$	0.17 μM

Na^+	5.96 meq
K^+	2.26 meq
Mg^{2+}	0.30 meq
Ca^{2+}	0.17 meq
sum	*8.69 meq*

Cl^-	3.17 meq
NO_3^-	2.94 meq
PO_4^{3-}	1.72 meq
SO_4^{2-}	0.32 meq
sum	*8.15 meq*