Advances in Environmental Biology, 10(7) July 2016, Pages: 46-52

AENSI Journals

Advances in Environmental Biology

ISSN-1995-0756 EISSN-1998-1066

Journal home page: http://www.aensiweb.com/AEB/

Effects of Short-Term Exposure of Liquid-cultured Green Algae *Desmococcus* sp. and *Trentepohlia* sp. to Chemicals Simulating Atmospheric Pollutants

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Received 22 May 2016; Accepted 18 July 2016; Available online 8 August 2016

ABSTRACT

Algae are known for its potential to occupy vast range of habitats due to algal ability to withstand various kind of environmental stress, including tolerating the pollutants in ambient atmosphere. This research is aimed to study the response of algal culture *Desmococcus* and *Trentepohlia* towards the time exposure of sulphur dioxide, ammonia and nitrogen dioxide. The algae were collected from oak and beech trees and were cultured in a controlled temperature room. Each algae species were then exposed to six different treatments which are 0.2 mM or 2 mM sodium nitrate, 0.2 mM or 2 mM ammonium chloride, bisulphite and deionized water (control). The cultures were treated by adding 1ml of the treatment stock every week. At the end of every week, 1ml of the algal culture was pipetted out and counted with Scepter Automated Cell Counter. The results showed that *Desmococcus* showed a steady pattern for Day 0 to Day 14, and its number of cells intensified on Day 28 in all treatments. However, samples treated with Sodium Nitrate on *Desmococcus* extracted from beech tree showed a declination of 45%. Meanwhile, *Trentepohlia* showed an unstable pattern with a notable drop for the first two weeks, but its number of cell improved after Day 14. *Trentepohlia* also showed that this species reacted negatively towards the liquid pollutants. Most changes were minor and statistical analysis showed that the main effect of treatment was not significant at P=0.001. However, for both algae, the period of exposure was highly significant (2-way ANOVA, $F_{5,3} = 142.76$, $p < 0.0001$). This current work concluded that there are no significant effect for short term exposure of these atmospheric pollutants towards both *Desmococcus* and *Trentepohlia*, meaning that the short-term exposures of atmospheric pollutants at these concentrations were not toxic. In comparison for both species, *Desmococcus* appears to be more robust and may have specific mechanisms to remove or utilize atmospheric pollutants.

KEYWORDS: Algae, air pollution, atmospheric pollution simulation, algal culture

INTRODUCTION

It is generally believed that some species of algae inhabit diverse habitats and often occur in extremely hostile environments, achieving this by their remarkable ability to tolerate various kinds of stress. Some of the stresses are naturally occurring such as various concentrations of chemical elements in the atmosphere, different availability of light, temperature, pressure, pH and many more. In addition, further sources of stress result from human activities such as agricultural activities, sewage treatment and transportation systems. These activities lead to an increase in atmospheric pollutants and subsequent exposure of epiphytic terrestrial algae. In this study, we examine pollutants as a stress factor potentially limiting growth, the surmise being that either algal

cell are able to tolerate the stress or if not this will contribute to lower productivity. The current work examines the effects of raised N and S levels, provided as inorganic salts, on the growth of selected algae under defined environmental conditions. Nitrate, ammonium and bisulphite dissolved in rain water are among the stress factors influencing algal growth in the sub-aerial environment, which are considered here.

Among the many eukaryotic algal groups, the Chlorophyceae are among the most resilient which explains their success colonizing extreme terrestrial habitats [1]. They possess a variety of adaptations enabling them to survive factors such as desiccation, low temperatures and high light intensity [2]. To the best of our knowledge, no specific study has been conducted to see the response of *Desmococcus* or *Trentepohlia* towards any stress factor. Almost all recent studies relating to the effect of stress on algae focus on their contribution for bioremediation and as a source of biofuels [3,4,5]: one such example being the effect of nitrogen and phosphate starvation on the accumulation of lipid in algae [6, 7]. Other studies have examined the factors limiting algal growth. Some have shown that nitrogen is the most nutrient-limiting factor for algal growth while others have reported that it is phosphorus [7]. A study showed the optimum ratio of N: P for microalgae growth is 10:1, where a ratio higher than 10 signifies phosphorus-limited factor while ratio less than 10 signifies nitrogen-limited factor [8]. In temperate zones phosphorus is usually the limiting factor for algal productivity [9], but when phosphorus is freely available nitrogen becomes the limiting factor. The N:P ratio is believed to influence the outcome of inter-species competition and can influence species diversity [10]. Another study reported that N and P concentrations in the cytoplasm of algae vary in response to changes in the environment and their cellular conditions [11].

For epiphytic terrestrial algae that inhabit the tree trunk as their substratum, nitrogen depletion is faster than for soil or freshwater algae. Epiphytic algae have adapted to survive in a dilute nutrient solution. Whether they respond positively or negatively to enhanced inputs, is the subject of the current study. The aim of this study is to investigate the effects of sulphur dioxide, ammonia and nitrogen dioxide on the growth rate of *Desmococcus* sp. and *Trentepohlia* sp. cultured from field populations growing on oak and beech trees. The specific objectives are as follows:

i) To determine which of the two algae, *Desmococcus* or *Trentepohlia*, is more tolerant of short-term application of dissolved atmospheric pollutants.
ii) To provide information on whether short-term exposure of dissolved sulphur affected algal survival and growth.
iii) To test whether time of exposure played an important role in any effects seen above.

MATERIALS AND METHOD

Algal preparations and culture medium:

This short-term physiological experiment was conducted in a controlled temperature room at Imperial College, Silwood Park campus, England. *Desmococcus* and *Trentepohlia* were collected from the trunks of oak and beech trees. The isolates were named DO, DB, TO and TB to represent *Desmococcus* on oak, *Desmococcus* on beech, *Trentepohlia* on oak and *Trentepohlia* on beech respectively.

These isolates were cultured on Bold Basal Medium (BBM) with 3 fold nitrogen and vitamins. Stock solutions were obtained from the UK Culture Collection of Algae and Protozoa (CCAP). The final medium contained the following salts in the amounts shown made up to a final volume of 1 litre: (1) 25.0 g $NaNO_3$ 30 ml, (2) 2.5 g $CaCl_2.2H_2O$ 10 ml, (3) 7.5 g $MgSO_4.7H_2O$ 10 ml, (4) 7.5 g $K_2HPO_4.3H_2O$ 10 ml, (5) 17.5 g KH_2PO_4 10 ml, (6) 2.5 g NaCl 10 ml, (7) trace element solution 6 ml, (8) vitamin B1 1 ml, (9) vitamin B12 1 ml. 1 litre stock medium was made up by adding distilled water. The stock medium was autoclaved at 15 psi for 15 minutes. Trace element solution (7) was prepared by adding 1000 ml of distilled water to 0.75 g Na_2EDTA and then adding minerals in the following sequence and amounts: $FeCl_3.6H_2O$ 97 mg, $MnCl_2.4H_2O$ 41 mg, $ZnCl_2$ 5 mg, $CoCl_2.6H_2O$ 2 mg, and $Na_2MoO_4.2H_2O$ 4 mg. Vitamin B1 (8) was obtained by adding 0.12 g thiamine hydrochloride to 100 ml distilled water. Vitamin B12 (9) was prepared by adding 0.1 g cyanocobalamin to 100 ml distilled water. 1 ml of this solution was added to 99 ml distilled water and filter sterilised. 20 ml of freshly made culture medium was dispensed into 40 ml polycarbonate culture flasks with membrane layer tops, using an automatic dispenser. The caps of the flasks were loosened to allow an exchange of air through the membrane layer of the cap. After inoculation, contents were mixed by shaking the flasks. During incubation cultures were shaken regularly to prevent cells from clumping.

Controlled temperature room and algal treatments:

Culture flasks were maintained in a controlled temperature room at 18^{o}C with a photon flux density of 25-30 µmol $m^{-2}s^{-1}$, and a day length of 14 hr obtained using white fluorescent tubes supplemented by tungsten bulbs. A total of 36 culture flasks was inoculated with each algal species at an initial cell concentration of 67,490 cells/ml for DO, 43,120 cells/ml for DB, 45,3800 cells/ml for TO and 27,4070 cells/ml for TB. Flasks were subjected to one of the following six treatments in triplicate: 0.2 mM or 2mM sodium nitrate (to represent

an oxidised form of N), 0.2mM or 2mM ammonium chloride (to represent a reduced form of N), 2mM bisulphite (to simulate sulphur pollution) and deionized water (as a control). Culture flasks were treated weekly (top up treatments) by adding a further 1ml of the stock solutions described below once a week for 4 weeks (**Figure 1**). Algal in culture flasks was checked regularly under light microscope to ensure that no contamination has occurred. To avoid confusion, the chemical additives were labelled using syncronyms as follows:

LoSo = 0.2 mM sodium nitrate, HiSo = 2 mM sodium nitrate, LoAm = 0.2 mM ammonium chloride, HiAm = 2 mM ammonium chloride, Bis = 1.2 mM sodium bisulphite, Con = Control, using deionized water only

Fig. 1: Arrangement of culture flasks containing algae randomised within the growth chamber.

Determination of algal cells:

At the end of each week, 1 ml of algal culture was pipetted from the culture flask into a 1.5 ml centrifuge tube. The samples were then mixed with 0.5 ml phosphate buffer solution, shaken and ready for count. An automatic handheld counter (Scepter, Millipore UK) was used to count the algal cells. The readings were made by dipping the Scepter tip into the centrifuge tube for a few seconds until an audible indication that a valid reading had been taken. Data were then displayed on the LCD monitor and transferred to the computer using Scepter Application software. Readings were taken weekly apart from a two week interval before the final counts. The mean of three replicates was calculated: data were recorded as cells per ml.

RESULTS

General pattern of changes in number of algal cells:

In this controlled short-term experiment, the number of algal cells of *Desmococcus* and *Trentepohlia* varied according to treatment and also fluctuated for the duration of the study. *Desmococcus* populations, regardless of source, remained stable for the first two weeks of incubation and then, with one exception showed a period of growth over the final two weeks. *Trentepohlia*, on the other hand showed a fluctuating pattern with a significant decline in cell counts between days 7 and 14, followed by a recovery which was more marked in the case of the population from beech trees. Overall, most changes were minor and statistical analysis showed that the main effect of treatment was not significant at P=0.001. However, for both algae the period of exposure was highly significant (2-way ANOVA, $F_{5,3} = 142.76$, $p < 0.0001$).

The decline in *Trentepohlia* showed that this species reacted negatively towards the liquid pollutants. The Scepter automatic cell counter measures live cells in the flasks so the decline in counts over the first 14 days must represent mortality suggesting that this alga reacts adversely to the transfer to liquid medium. One interesting finding was that after 14 days of given treatments, algal cell density increased substantially in all samples of both *Desmococcus* and *Trentepohlia*, regardless of the origin of the isolation (either from oak or beech). A reasonable interpretation could be that after a period of adaptation to the new environment the algae show a recovery phase where the number of cells begins to increase rapidly after slow growth for the early period after inoculation.

Specific pattern of changes in number of algal cells:

Figure 2 until Figure 5 shows the changes in number of algal cells in culture flasks in relation to time over period of 28 days. Points in line graph representing the mean number of cells in the flasks. Syncronyms in label is used as follows: LoSo = 0.2 mM sodium nitrate, HiSo = 2 mM sodium nitrate, LoAm = 0.2 mM ammonium chloride, HiAm = 2 mM ammonium chloride, Bis = 1.2 mM sodium bisulphite, Con = Control, using deionized water only.

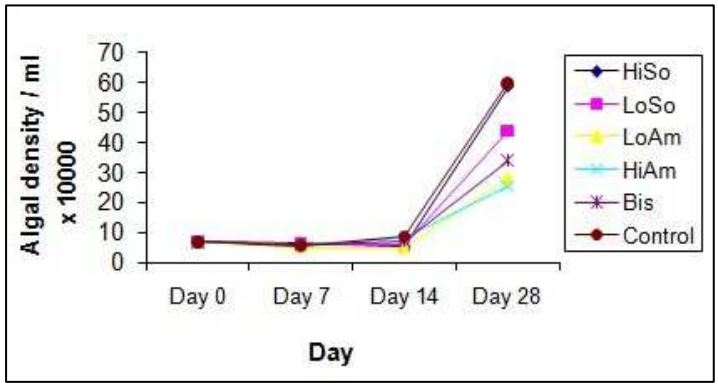

Fig. 2: Graph of *Desmococcus* isolated from oak.

i) Desmococcus from oak (DO):

No obvious changes in terms of number of algal cells was recorded for DO until after 14 days, whereafter cell number proliferated rapidly showing a six-fold increase in both controls and the high nitrate treatments. The same pattern was true to a lesser extent for all treatments.

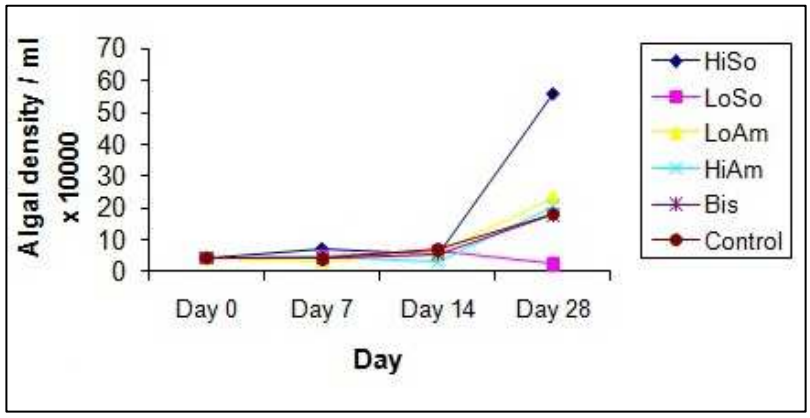

Fig. 3: Graph of *Desmococcus* isolated from beech.

ii) Desmococcus from beech (DB):

Number of algal cells of DB exhibited the same pattern as DO where treatments have no prominent effect until after 14 days. By day 28 most cultures had made considerable growth with the exception of the 0.2 mM sodium nitrate (labelled as LoSo on graph) treatment where there was growth inhibition to the extent of 45 %.

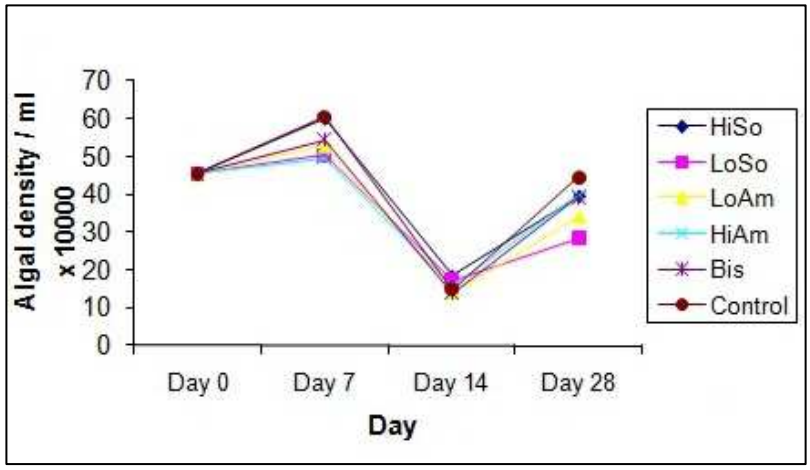

Fig. 4: Graph of *Trentepohlia* isolated from oak.

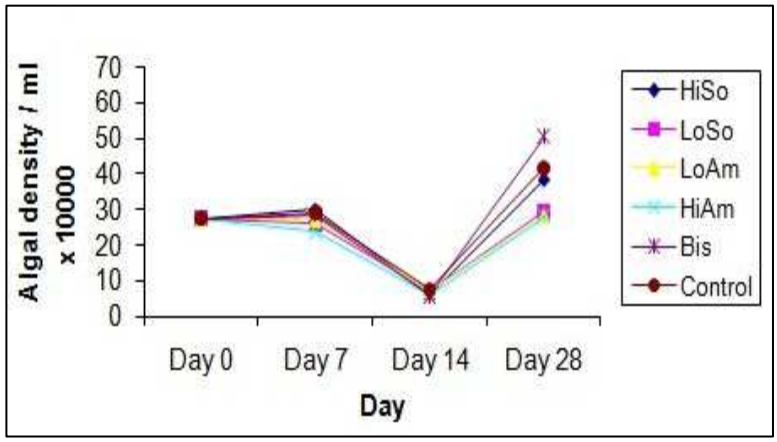

Fig. 5: Graph of *Trentepohlia* isolated from beech.

iii) Trentepohlia from oak (TO) and from beech (TB):

Trentepohlia populations seemed to be more easily affected by the simulated atmospheric pollutants than *Desmococcus.* Regardless of source tree, populations showed a fluctuating pattern typically with a slight initial increase followed by a steep decline and a final recovery phase which was most marked in the case of TB (Figure 5). In all cases cultures with additions of simulated pollutants behaved in a similar fashion to the controls in respect of changes with time.

DISCUSSION

Effects of simulated atmospheric pollutants on number of algal cells:

Algal cells of *Desmococcus* and *Trentepohlia* responded to the various dissolved pollutants by increasing or declining in number in the culture flasks, but for the most part changes were minor and statistical analysis showed that the main effect of treatment was not significant at P=0.001. However, for both algae the period of exposure was highly significant (2-way ANOVA, $F_{5,3}$ = 142.76, p< 0.0001) confirming that the population fluctuations seen were real. Based on these results, it is impossible to pinpoint main effects of pollutants, there being no clear cut trend. Clearly at these concentrations none of the chemical additives are toxic. There is some suggestion of nitrate N being stimulatory and ammonium N being inhibitory but it is not possible to eliminate this being an effect of the different counter ions used.

Toxicity of short-term exposure to dissolved atmospheric pollutants:

Short-term application of N and S sources as simulated atmospheric pollutants had no significant effect on populations of *Desmococcus* and *Trentepohlia* growing in liquid media. Populations fluctuated over time, and this effect was shown to be statistically significant. Even though there are some nutrients already present in the growth medium such as sodium nitrate and sulphur, these nutrients are very small in concentration thus did not affect the overall concentration when added with liquid pollutants.

We conclude that short-term exposures of atmospheric pollutants at these concentrations were not toxic. Since epiphytic algae are known to be very robust and tolerant, this factor might explain the apparent lack of ill-effects in this experiment. To the best of our knowledge, there have been no physiological studies on either *Desmococcus* or *Trentepohlia* so far. However, other green algae such as *Chlorella vulgaris* and *Scenedesmus quadricauda* are known to have the ability to eliminate toxins [12]. Furthermore, green algae have been reported to have variable responses to a variety of toxicants [13]. This supports the initial thought that different algal species respond differently towards external disturbances.

Statistical analyses has proven that time of exposure was highly significant in influencing the algal cell numbers. This is in agreement with our long-term field experiment where the dissolved atmospheric pollutants were applied to the algae for over 18 months, and again time had a significant effect towards the outcome. If the current experiment had been continued for longer treatment effects might have become apparent at a later stage, but equally conducting the experiment with a wider range of concentrations could help differentiate between nutritional and toxicity effects of the additives, especially given the known ability of algae especially green algae to eliminate toxins such as ammonia and excess nitrogen from their cells [14]. Following the result, it is quite difficult to determine which of the treatments caused the most or least damage to the algal cells. Since there is no significant effect of treatments on the cultures, we can only describe the response of the algae towards treatments in terms of their general trends. As an example, a low concentration of sodium nitrate (0.2 mM) was found to be most damaging to *Desmococcus* isolated from beech. On day 28 of the experiment, *Desmococcus* numbers had declined by 45% compared with both controls and other treatments (Figure 3). On the other hand, a higher concentration of sodium nitrate (2 mM) was beneficial to *Desmococcus* numbers, presumably for nutritional reasons, but it is unclear why there is a reversal of the effect with concentration (Figure 3).

When comparing the effect of reduced (ammonium) and oxidized (nitrate) N on the algae, it is clear that the oxidized form of N had a greater growth-promoting effect compared to the reduced form. However, since there seems to be a recovery phase at the end of the experiment it is possible that this trend may change in the long run. Recycling of nitrogen in old cultures is an established phenomenon in all microorganisms as cells die and proteins are broken down. In such a closed environment the form in which nitrogen is found during autolysis will bear little resemblance to the form in which it was originally supplied

Which algal species is most affected by atmospheric pollutants?:

Desmococcus appears to be more tolerant than *Trentepohlia* because it shows no notable response in terms of number of algal cells until after day 14 of the experiment. This is supported by other research where *Desmococcus* sp. was found dominating the sampling sites in any condition of environment [15]. On the contrary, *Trentepohlia* responded quite quickly to treatments, with some effects appearing within days of application (Figure 4 and 5). Even though both species under study here are green algae, *Desmococcus* is more robust and may have specific mechanisms to remove or utilize atmospheric pollutants for its own benefit. *Desmococcus* has a reputation as pollutant-tolerant because at the time when no other epiphytes could survive due to high concentrations of SO_2, *Desmococcus* was the only epiphyte that could thrive in this environment. There has been no conclusive work on the mechanisms of *Desmococcus* that makes the cells pollutant-tolerant. However, we hypothesize that the protective outer layer of the cells in *Desmococcus* helps to prevent the cytoplasm from the toxic effect of pollutants. This cell wall stores reserve water in a mucilaginous sheath, for use when the environment becomes extreme [16].

Trentepohlia, on the other hand is more pollutant-sensitive. Any changes in water chemistry will be absorbed by their surface cells and will directly influence the growth of *Trentepohlia* cells. This algal species was reported to decrease in abundance with enhanced nitrate concentrations but increase when treated with ammonium. Carotenoid pigments called haematochromes in *Trentepohlia* ,that give them the reddish-orange colour and functions to protect the cell against external influences such as high light and UV rays, seems not to be enough to shield the cell from all stresses. This might be due to the fact that these pigments work at an optimum level when there is long day/short night with high light intensity.

One further tentative conclusion from this in-vitro experiment also points to the relative resilience of *Desmococcus* over *Trentepohlia*. Over the 28 day period of culture *Desmococcus* did in almost all treatments increase its population size well above starting level. On the other hand, *Trentepohlia* populations decreased rapidly during the first 14 days of culture and even after the recovery phase, were in most cases still lower (*Trentepohlia* on oak) or only slightly higher than the initial number of cells (*Trentepohlia* on beech).

CONCLUSION

This current research conclude that short-term exposure of dissolved atmospheric pollutants has neither toxic nor beneficial effects on populations of either of *Desmococcus* or *Trentepohlia* isolated from oak and beech trees. There is no evidence that the populations from oak and beech differed materially. In general,

oxidized N was more beneficial to algal growth than the reduced form. The green algae, *Desmococcus* sp. was found to be more tolerant to pollutant than *Trentepohlia* sp.

ACKNOWLEDGEMENTS

Authors would like to thank Imperial College London for providing the facilities and technical support, staffs of Silwood Park Campus for their assistance and guidance upon completing this project. This work was funded by Ministry of Education, Malaysia and Universiti Teknologi MARA under SLAI Scheme, Research Acculturation Grant Scheme (RAGS), Ministry of Education, Malaysia and Universiti Teknologi MARA, Shah Alam, Malaysia 600-RMI/RAGS 5/3 (8/2013).

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