Prymnesium parvum Growth Studies Using the Dunkard Creek Isolate (WANA Strain)

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Introduction

The golden alga *Prymnesium parum* bloomed in Dunkard Creek (WV-PA) in September and October 2009 resulting in devastating fish, mussel, and salamander kills over a 30 mile stretch of the stream. Preliminary investigations led to the hypothesis that increased salinities resulting from high saline discharges by local mining activity were conducive to the bloom. Study with strains of *P. parvum* isolated from Texas and Oklahoma, in waters of relatively high salinities, suggest that *P. parvum* growth rates are depressed at lower salinities. It is further hypothesized growth rates of the Dunkard Creek strain of *P. parvum* might also be reduced at lower salinities. If so, a possible management action aimed at Dunkard Creek salinity reduction is warranted. The purpose of this study was to analyze growth rates of the Dunkard Creek *P. parvum* strain at different salinities.

Methods

The strain of *P. parvum* that was found in the Dunkard Creek Watershed and identified as the proximate cause of fish and other aquatic life kills in September and October 2009 was used to establish laboratory cultures at University of Oklahoma Biological Station (UOBS). Water from Dunkard Creek was shipped to UOBS for establishment of non-axenic cultures in modified COMBO medium (Kilham et al. 1998) with high salinity (6 or 15 g Instant Ocean L^{-1}; equivalent to ~10,000 and 23,000 µS cm^{-1}, respectively) and high nutrients (800 and 50 µ moles L^{-1} N and P). Cultures used in experiments reported here (WANA 576 and WANA 578; different cell lineages isolated from the original water sample) were >99% pure, with unidentified green unicells and diatoms present in extremely low abundances.

We performed two replicate 6-day experiments (experiments 1 and 2) and one 14-day experiment (experiment 3) to track golden algae growth rates (absolute and relative to other Dunkard Creek algae present in cultures) across gradients of salinity. In experiments 1 and 2, salinity treatments were created to mimic the 4:1 sulfate and chloride concentrations in Dunkard Creek water in the area of the coal mine discharge (2 g sulfate, as calcium sulfate, and 0.5 g chloride, as sodium chloride, L^{-1}; i.e., full-strength or 1× mine pool water) and multiple dilutions of full-strength mine discharge water (i.e., at 0.5×, 0.25×, 0.125×, 0.06×, and 0× mine pool water). All salinity treatments were replicated 5 times. Experiments were conducted in 250- (experiment 1) and 125-mL (experiment 2) Erlenmeyer flasks at room temperature and on a 12-hr light:12-hr dark schedule. Following inoculation of experimental flasks, golden algal densities were tracked using flow cytometry-based enumeration of cell densities initially and every second day. Both experiments were terminated after 6 days due to high incidence of contamination in experimental cultures. Experiment 3 was set up in a similar manner, but using Instant Ocean to establish the salinity gradient (0, 2, 4, 6, 10, and 15 g Instant Ocean L^{-1}, three replicates each) and was run for 14 days to measure both, initial growth rates of golden algae, but also to quantify golden algae's growth response to different salinities relative to other algae in the cultures.

Experiment 1 was initiated from a WANA 576 culture containing 7,600 cells mL^{-1}, by adding 30-mL aliquots to 1-L flasks containing COMBO, 80 µ mole N and 5 µ mole P L^{-1}, and variable salinities. Each liter was then divided evenly among five 250-mL Erlenmeyer flasks, 150 mL each, with starting densities of golden algae ~228 cells mL^{-1} in each flask. Experiment 2 was initiated from a WANA 576 culture containing 10,800 cells mL^{-1}, by adding 15-mL aliquots to 500-mL flasks containing COMBO and variable salinities as above. Each liter was then divided evenly among five 125-mL Erlenmeyer flasks, 75 mL each, with starting densities of golden algae ~324 cells mL^{-1} in each flask. Experiment 3 was initiated from WANA 578 culture containing 2,020,000 cells mL^{-1}, by adding 3.5-mL aliquots to 500-mL flasks

containing COMBO, 80 µ mole N and 5 µ mole P L^{-1}, and variable salinities. Starting densities of golden algae in each flask were ~15,000 cells mL^{-1}.

Salinity for each sample was measured as conductivity (Hach HQ40d meter) at 22.4 C and recorded in µS cm^{-1}. pH was measured using a Fisher Accumet pH Meter Model 915. Flasks were swirled daily. Initially, and every second day, a 500 µL sample was analyzed on a BD FACSCaliber flow cytometer to determine golden algal cell densities. For Experiment 3, densities of contaminant algae were also recorded. Additional samples from all experiments were preserved in Lugol's solution and used to verify flow cytometer counts.

In all sulfate-chloride salinity treatments of experiments 1 and 2, the sudden change in culture medium from COMBO with Instant Ocean to COMBO with sulfates and chlorides only as the source of salts resulted in high mortality of golden algae (mean = 37%). Similar initial mortality, or shock, has been observed previously when transferring golden algae to new culture medium conditions. After two days, all cultures had recovered and were growing well, except the highest sulfate-chloride treatments, which are not considered in the analyses below. Maximum growth rates in each treatment were calculated as the slope of the exponential regression of cell density and time (Fig. 1). Maximum growth rates of *P. parvum* in experiment 3 were calculated using data from day 0 to day 7.

Results

Both experiments 1 and 2 revealed similar responses of WANA 576 to changes in salinities using sulfates and chlorides and have been combined for analysis. Growth rates of *P. parvum* between day 2 and day 6 were positive, but declined with declining salinities, especially below 1000 µS cm^{-1} (Fig. 2). Experiments were terminated after day 6 because of relatively high contamination (data not shown). Experiment 3 revealed that the decline in *P. parvum* growth rates with declining salinity, as well as the high level of contamination over time was not an artifact of using sulfate and chloride as sources of salinity in the cultures. Growth rates in the lowest salinity treatment were more than 50% lower than in the highest salinity treatment (Fig. 3). Although all treatments were eventually highly contaminated over time, the level of contamination increased with decreasing salinity (Fig. 4). The contaminants, a small diatom and unidentified green unicell (~4 µm diameter), both presumably from the original Dunkard Creek water and present in all cultures at extremely low abundances, had highest growth rates in low salinity treatments and declining growth rates with increasing salinity (Fig. 5).

Discussion and Conclusions

All experiments revealed a relatively strong relationship between *P. parvum* and culture salinities. Patterns observed for isolates from Dunkard Creek were similar to patterns observed previously in other *P. parvum* isolates (Baker et al. 2007). In general, positive growth rates can be maintained by *P. parvum* across a broad range of salinities (note that Expt 3 salinities covered a much greater range of salinities – up to 15 g L^{-1} Instant Ocean, maximum conductivities >20,000 µS cm^{-1}), but growth rates are substantially lower at salinities equivalent to those observed in most fresh waters (i.e., < 1000 µS cm^{-1}). Moreover, our experiments revealed that not only are *P. parvum* growth rates reduced at lower salinities, but that growth rates of other, presumably native, algae are enhanced at lower salinities. Thus reduced salinities shift the competitive edge from *P. parvum* to other algae.

Reasons behind the lack of *P. parvum* growth in the highest sulfate-chloride treatment are not known. The maximum conductivity obtained with the addition of 2 g of sulfate and 0.5 g of chloride was 4,275 µS cm^{-1}, although the actual amount of sulfate in solution was less than 100%. Compared with Instant Ocean, our standard salinity source of *P. parvum* cultures, this amount of sulfate is high. At 6.6% sulfate by weight, our highest salinity cultures (i.e., 15 g Instant Ocean L^{-1}) contain 1.0 g sulfate L^{-1}, or half the

amount added in the high treatments of experiments 1 and 2. Studies have shown that high sulfates can interfere with nitrogenases in phytoplankton, particularly those associated with nitrogen fixation in cyanobacteria (Marino et al. 1990). But it is also possible that other nitrogenases, such as those used in nitrate assimilation, might also be negatively affected.

High calcium concentrations could be another factor involved with lack of *P. parvum* growth in the high sulfate-chloride treatments and overall low growth rates in all sulfate-chloride treatments (experiments 1 and 2) relative to Instant Ocean treatments (experiment 3) (c.f. Figs. 2 and 3). Sulfates were added as calcium sulfate, in which there is 466 mg of calcium for every 1 g of sulfate. Instant Ocean contains only 1.02% calcium by weight. Thus a 15 g Instant Ocean L^{-1} culture contains only 153 mg calcium L^{-1}. Studies have demonstrated that calcium ions can act as cofactors to *P. parvum* toxins, increasing their toxicity substantially (Shilo 1981). As such, it is conceivable that our use of calcium sulfate inadvertently created conditions of higher toxicity, which may have negatively affected growth or increased mortality via self-toxicity (Olli and Trunov 2007).

Nevertheless, further research could add substantially to our understanding of specific factors involved in the 2009 Dunkard Creek *P. parvum* bloom. In particular, it is recommended that further monitoring and analysis of the chemical composition of the mine water discharges be conducted in order to enhance understanding of the roles of high sulfates and other ions in *P. parvum* population growth and toxicity. Further experimentation also will be required to confidently assess the relative roles of sulfates, calcium, or other ions, in *P. parvum* growth and toxicity in general, but also with respect to the potential for future Dunkard Creek *P. parvum* blooms. While our experiments were conducted in the laboratory with artificially nutrient replete culture media, and there remains uncertainty with respect to sulfates and calcium as described above, our results corroborate the general understanding of *P. parvum* populations, blooms, and fish kills globally – high nutrients and high salinities are major requisites for *P. parvum* domination of algal communities, and especially for *P. parvum* blooms.

Acknowledgments

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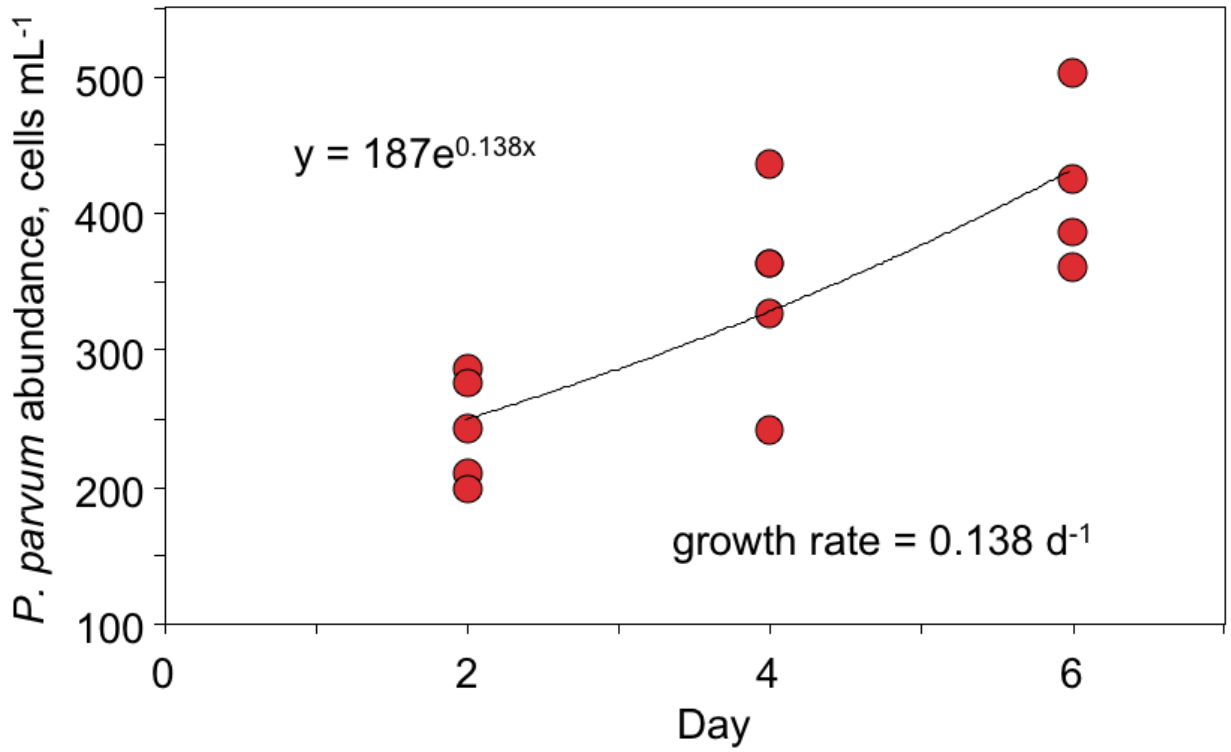

Figure 1. Representative example of growth rate calculation. Points represent *P. parvum* cell densities in experimental flasks (in this case, the 0× treatment of experiment 1) on days 2, 4, and 6. The slope (i.e., the exponent) of an exponential regression through these points is a measure of the instantaneous growth rate in units of per day.

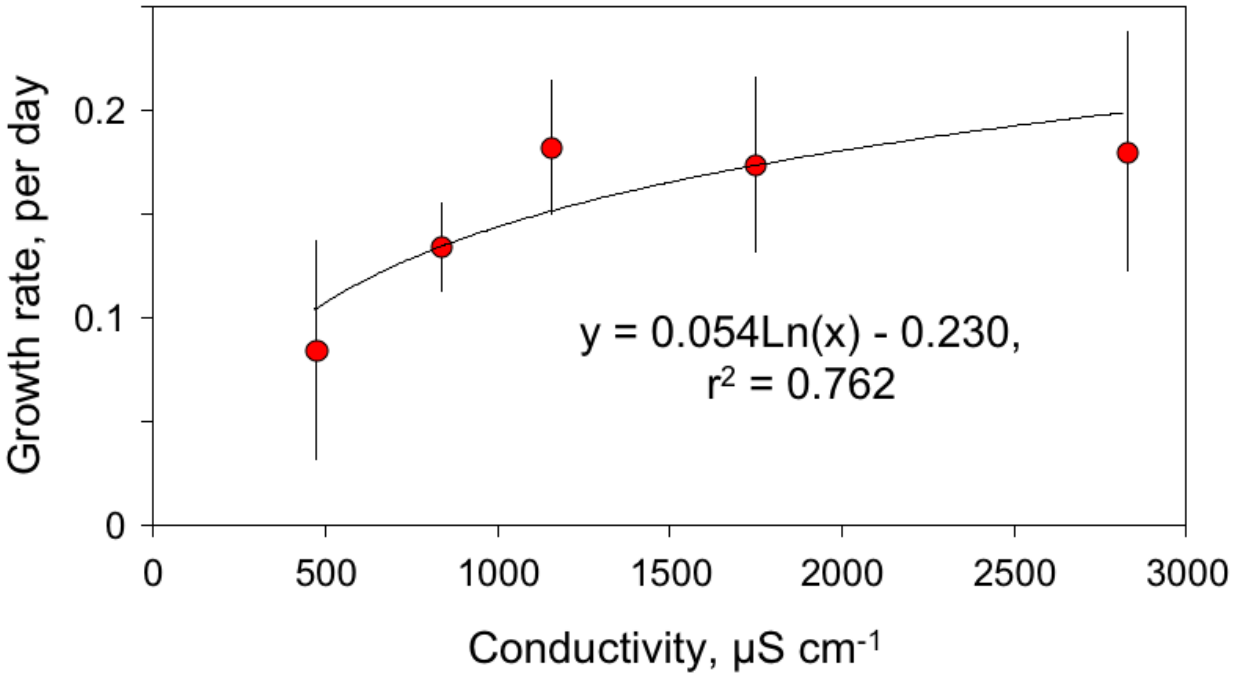

Figure 2. Growth rate of *P. parvum* as a function of sulfate and chloride concentrations (here indicated by conductivity (µS cm^{-1}). Treatments, each replicated 5-fold per experiment and from highest to lowest conductivity, are 1000 mg sulfate and 250 mg chloride, 500 mg sulfate and 125 mg chloride, 250 mg sulfate and 62.5 mg chloride, 125 mg sulfate and 31.3 mg chloride, and 0 mg sulfate and 0 mg chloride. Points represent mean (±SE) values generated separately from experiments 1 and 2 using *P. parvum* cell densities from day 2 to day 6. The highest salinity treatment (2000 mg sulfate and 1000 mg chloride, ~4,082 µS cm^{-1}) was not conducive to *P. parvum* growth and has been omitted here.

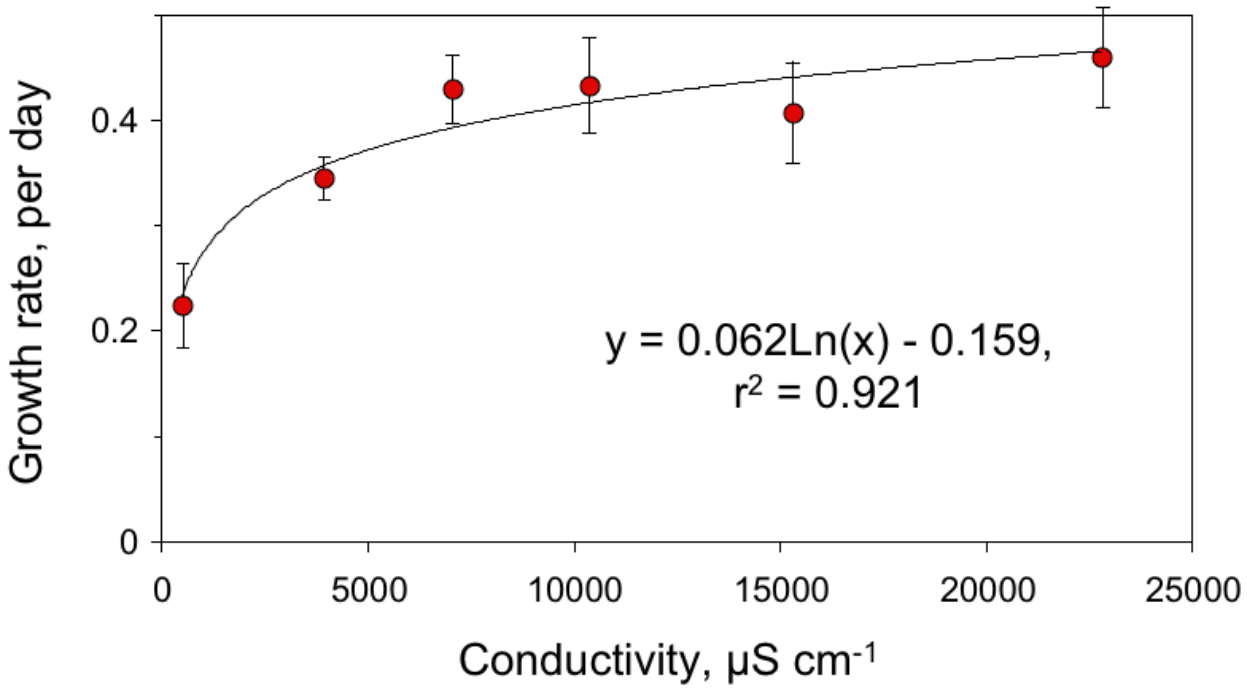

Figure 3. Growth rate of *P. parvum* as a function of Instant Ocean concentrations (here indicated by conductivity (µS cm^{-1}). Treatments, from highest to lowest conductivity, are 15, 10, 6, 4, 2, and 0 g Instant Ocean L^{-1}. Points represent mean (±SE) values generated from day 0 to day 7 growth of *P. parvum* in each treatment from Experiment 3.

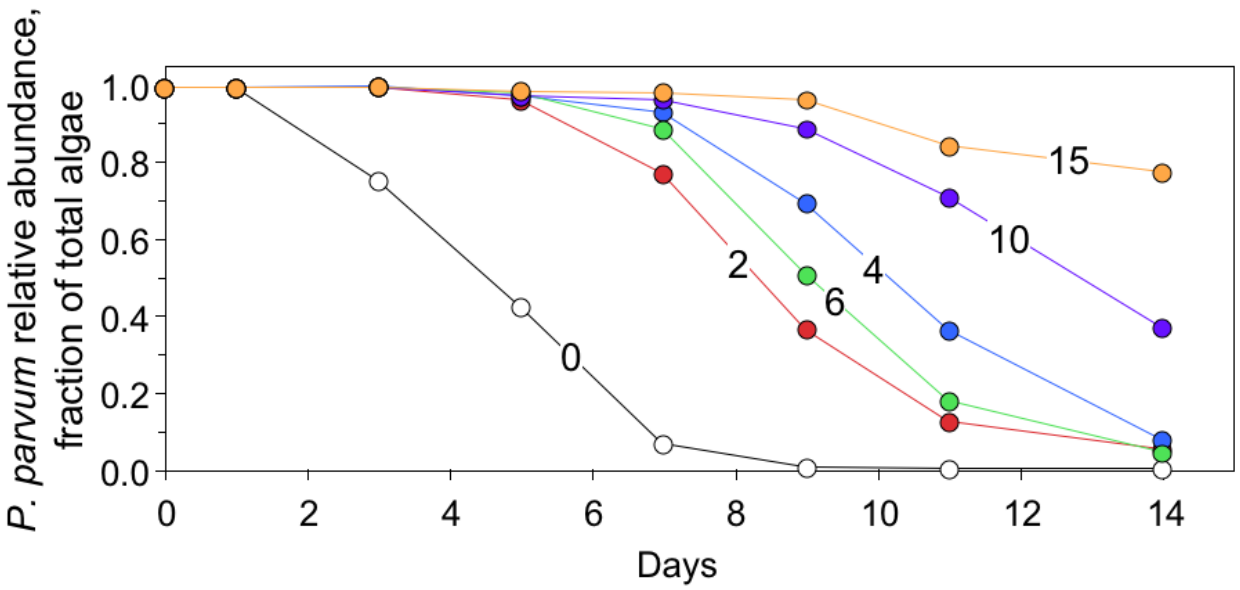

Figure 4. Relative abundance of *P. parvum* (fraction of total algae) in salinity treatments over time in experiment 3. Treatments (i.e., g Instant Ocean L^{-1}) are indicated.

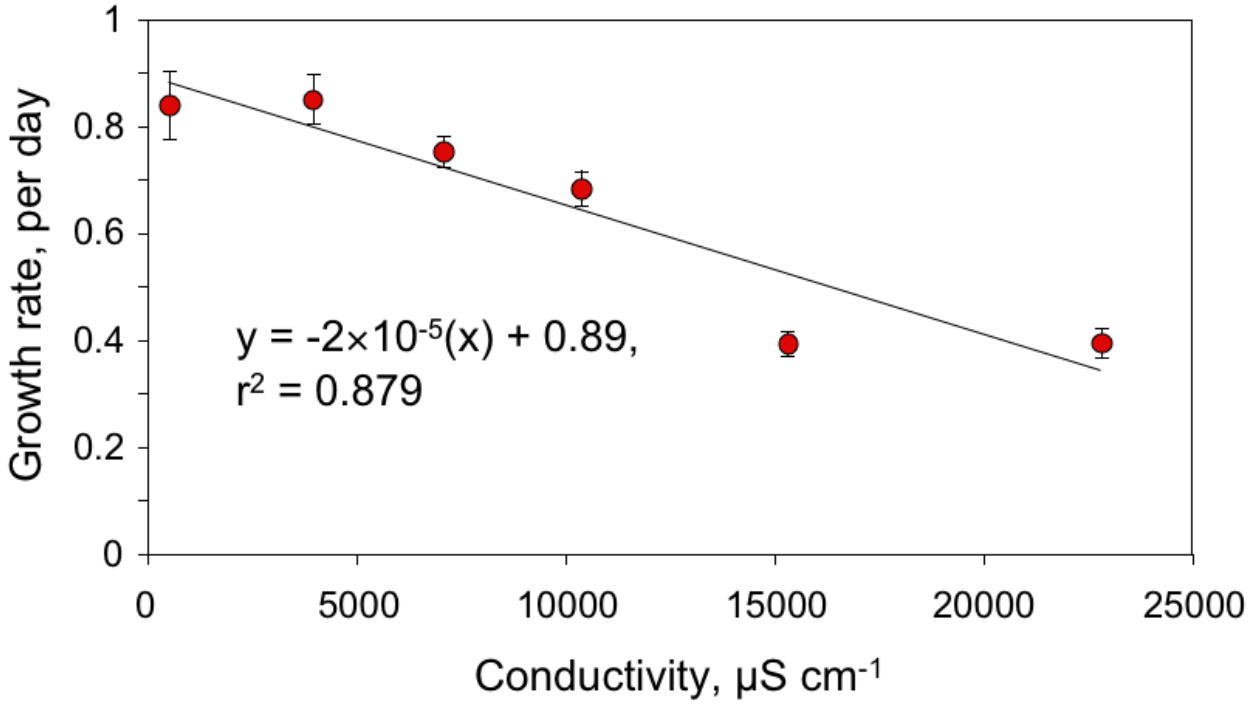

Figure 5. Growth rate of contaminant algae (small unidentified diatom and green unicell) in experiment 3 as a function of Instant Ocean concentrations (here indicated by conductivity (µS cm^{-1}). Rates were calculated from cell densities in days 3 through 14.