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COâ„ effects on marine phytoplankton : inhibition of photosynthetic processes

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CO_2 EFFECTS ON MARINE PHYTOPLANKTON: INHIBITION OF PHOTOSYNTHETIC PROCESSES

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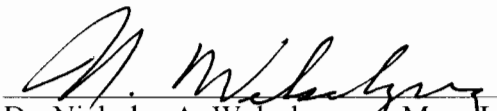

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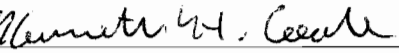

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ABSTRACT

CO_2 EFFECTS ON MARINE PHYTOPLANKTON:

INHIBITION OF PHOTOSYNTHETIC PROCESSES

By Matthew P. Huber

It is shown here that carbon dioxide gas has dramatic inhibitory effects on photosynthesis in representative species from six divisions of marine phytoplankton and two natural sites. Oxygenic photosynthesis was inhibited as a function of increasing CO_2 concentration in the seawater media. The cellular content of ATP also decreased after treatment with CO_2. Photosystem II variable-fluorescence parameters were altered under high-concentrations of CO_2; F_v/F_m decreased and non-photochemical quench (NPQ) increased. Increases in the concentration of CO_2 gas promoted xanthophyll cycle pigment alteration to what is believed a photoprotective state. The effect of CO_2 was reversible in all metrics by returning the media to air saturation. The CO_2 effect was also light dependent and was induced far below the light compensation intensity for photosynthesis. It is suggested that the CO_2 effects described here were not due pH per se.

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My fellow lab mates, Lawrence Younan and Chris Scianni, thank you for your help and support. May your outliers be few, your p-value high, and never ever mix ethanol and corn oil.

Thank you all! I wish you the best of luck in your work and relationships.

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INTRODUCTION

The effect of CO_2 on photoautotrophs has been the focus of much attention, as atmospheric CO_2 levels are known to be increasing (IPCC , 2001). Reports on the response by marine microalgae to changes in CO_2 have not been consistent. Some researchers have shown that varying the CO_2 concentration will effect photosynthetic rates, both in negative and positive fashion, while others report that photosynthetic rates will not be altered (Demidov et al. 2000; Gilmore 1999; Hein and Sand-Jenson 1997; Riebesell 1993).

The long held assumption that inorganic carbon (C_i) does not significantly effect the growth of photoautotrophs in marine systems has been well described (Goldman 1999; Raven 1991). The basis of these arguments depends upon two facts: 1) there is an abundance of inorganic carbon in seawater, and 2) most phytoplankters are thought to be very efficient at acquiring and utilizing two of the three dissolved inorganic carbon species. The dissolved inorganic carbon pool (DIC) in seawater exists in four interconvertible species; CO_2, H_2CO^{3-} (carbonic acid), HCO_3^- (bicarbonate), and $CO_3^=$ (carbonate). While total DIC is large (2.3mM) the concentration of CO_2 is relatively small (10-15 uM) and accounts for less than 1% of the total DIC pool (Millero 1995). This relatively small concentration of CO_2 is not large enough to saturate the enzyme ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) (Badger et al. 1998) which is responsible for the fixation of carbon into carbohydrates. This carbon shortfall is believed to be remedied by carbon concentrating mechanisms (CCM) (Raven 1997), as it has been shown that many phytoplankton can incorporate both CO_2 and HCO_3^- (Burkhardt et al. 1999; Cassar et al. 2002; Rost et al. 2003). CCMs in phytoplankton fill similar roles as CAM or C4 types of photosynthesis in terrestrial environments (Raven 1991). Despite these observations,

recent evidence has shown that rates of photosynthesis can be dependent upon the concentration of CO_2 in seawater (Demidov et al. 2000; Hein and Sand-Jenson 1997; Riebesell 1993).

Extremely-low CO_2 concentrations in seawater have been shown to decrease marine diatom growth rates (Chen and Durbin 1994; Riebesell 1993). Riebesell et al. (1993) showed that three marine diatoms (*Ditylum brightwellii*, *Thalassiosira punctigera*, and *Rhizosolenia alata*) slowed their growth rates as CO_2 concentrations decreased from equilibrium with air. To explain their findings, they developed a mathematical model that incorporated molecular diffusion and inorganic carbon speciation. Using that model they compared the flux rates of carbon, nitrogen, and phosphorus to the cell's surface. They concluded that the net diffusive carbon flux was low relative to the required Redfield ratio and thus should have the greatest effect on growth rates. Chen and Durbin (1994) modified seawater media using CO_2, HCl, NaOH and found that diatoms (*Thalassiosira pseudonana* and *T. Oceanica*) decreased their growth rate as pH rose above 8.8. They suggested that the changes in pH correlated with free CO_2, which indicates a possible limitation of inorganic carbon at high pH. However, they did not observe a corresponding increase in growth rate as pH was lowered below ambient.

In the central Atlantic Ocean, Hein and Sand-Jenson (1997), manipulated the pH of natural seawater using titrations of HCl and NaOH to show changes in photosynthetic ^{14}C-incorporation. A 15-19% increase in carbon-fixation was found when pH was reduced to levels coinciding with CO_2 increases to 36 and 91 μM; in some samples a two fold increase in ^{14}C-fixation was observed.

The studies above suggest that photosynthetic rates are proportional to carbon availability until saturation is reached. However, earlier studies showed that ultra-high concentrations of

CO_2 (5%) resulted in deleterious effects to photosynthesis in green algae. (Nielsen 1955; Osterlind 1948). Since then little attention has been paid to the responses of algae to CO_2 concentrations that are in great excess of atmospheric CO_2 levels (>>0.04% CO_2). More recently, Kodama et al. (1993) have described an ecotype of the marine green microalga, *Chlorococcum littorale*, which can grow under extremely high CO_2 conditions corresponding to that found in industrial exhaust which contains 10-20% CO_2. *C.littorale* shows decreases in photosynthetic oxygen production (Demidov et al. 2000; Iwasaki et al. 1996) , decreases in variable fluorescence, F_v/F_m (Demidov et al. 2000; Iwasaki et al. 1996; Satoh et al. 2001; Satoh et al. 2002), and a shift in energy balance from PSII to PSI (Demidov et al. 2000; Pesheva et al. 1994; Satoh et al. 2002) when exposed to high concentrations of CO_2. Demidov et al. 2000 also suggested that xanthophyll cycling pigments may be involved in photoprotection after high CO_2 exposure.

The microalgal photosynthetic response to changes in the dissolved CO_2 concentrations has resulted in disagreement in the literature. On the one hand, studies have shown that rates of photosynthesis are proportional to carbon availability, until saturation. On the other hand, when CO_2 was increased to levels far in excess of air saturated levels a small group of studies showed negative impacts to photosynthetic performance. This work aims to elucidate the relationship between marine phytoplankton and CO_2. Described below is the CO_2-induced inhibition of photosynthesis and associated effects on photoprotective response shown by marine phytoplankton in response to high levels of CO_2.

METHODS

Phytoplankton

Marine phytoplankton genera; *Phaeodactylum, Dunaliella, Cryptomonas, Porphorydium, Isochrysis and Synechococcus* were used in this study. Cultures were grown in 2 L polystyrene and 1 L polycarbonate culture flasks in the f/2 enriched seawater media, (Guillard & Ryther 1962, Guillard 1975), but without addition of HCl. The cultures were grown on a 12 hour light/dark cycle at approximately 100μE $m^{-2}s^{-1}$. All cultures and experiments were maintained at 20°C. Whole water samples from Elkhorn Slough at Kirby Park and near shore in Monterey Bay were also assessed (Fig. 1)

Dissolved CO_2 Concentration

Alterations of the free CO_2 concentration of the media were done by addition of CO_2 gas, or by titration with acids. The pH change resulting from the addition of CO_2 and acid treatments were measured with an Orion 710 pH meter, calibrated to Fisher Scientific buffering solutions (pH 4.00, 7.00, 10.00). The concentration of total dissolved CO_2 was determined using a UIC Coulometer (Model CM5012) with a manual acidification module. The empirical relationship between dissolved CO_2 and pH of seawater was determined over the range of pH used in this study (Figure 2). Throughout this work, pH was used as a proxy to estimate total dissolved CO_2 concentrations; the corresponding free CO_2 concentration was computed from the CO_2 SYS software routine available online (Louis and Wallace 1998).

CO_2 was added to seawater by bubbling 100% CO_2 gas (Airgas) through a glass gas diffuser. To achieve different concentrations of CO_2 in the media, the volume of the gas

injected was varied by bubbling rate and exposure time; empirical determinations of pH were made after each CO_2 manipulation to determine the CO_2 concentration achieved. Initial pH of cultures varied from 8.0 to 8.35 and saturation of CO_2 gas resulted in a final pH of 4.9; this represented the full pH range achieved from CO_2 perturbations. Alternative changes in pH were made by direct acidification; in this case, acids (Fisher Scientific) were diluted with filtered seawater media to final pHs between 3.0 to 8.35. The volumes of the acids added at no times exceeded 0.1% of the total volume of the media. After the desired pH was reached, the media was sealed to prevent gas exchange with the atmosphere.

Variable Fluorescence

A Water-PAM fluorometer (Walz, Germany) was used to assess variable fluorescent properties of the cultures. The Water-PAM uses the saturation pulse method (Schreiber et al. 1994) to determine fluorescent components. Variable fluorescence measurements are described here using the terminology of Kooten and Snel (1990). The two primary fluorescence parameters, as adapted from Genty (1989) are 1) the quantum efficiency of photosystem II, F_v/F_m and 2) non-photochemical quench (NPQ).

(1) Eq 1. $F_v/F_m = [F_m'-F'] / F_m'$

(2) Eq 2. $NPQ = [F_m-F_m'] / F_m'$, where

F'= fluorescence under ambient light, weak excitation energy

F_m=maximal fluorescence, under dark-adapted conditions, saturation pulse intensity

F_m'=maximal fluorescence, under ambient light, saturation pulse intensity

The measurement F_v/F_m has been shown to reflect the quantum yield of non-cyclic electron transport from photosystem II (Schrieber, et al. 1995). This fluorescent metric produces values ranging from 0.0 to 0.7 (Genty 1989). Exponentially growing phytoplankton usually exhibit dark adapted maximum F_v/F_m values from 0.55-0.70. Decreases from the highest possible values of F_v/F_m have been used to indicate stress to photoautotrophs (Parkhill et al. 2001; Behrenfield et al. 1996).

Non-photochemical fluorescence quench (NPQ) represents that portion of absorbed photons dissipated by non-radiative processes; also termed thermal heat dissipation (Bilger and Bjorkman 1990). Values for NPQ range from 0.0 to infinity. NPQ has been found to be linked to the epoxidation state of xanthophyll cycling pigment thermal energy dissipation (Demmig-Adams and Adams 1996; Govindjee and Seufferheld 2002). A crucial part of determining accurate measurements of NPQ include a dark adaptation period long enough to relax photoprotective processes, thus establishing the condition of 'zero' non-photochemical quench. In all experiments reported here, the dark adapted period prior to determination of NPQ was between 30-180 minutes. Samples were processed in the PAM fluorometer by pipetting suspended phytoplankton into a quartz cuvette which was then sealed with a seawater-wetted microscope cover-slip with thus preventing gas exchange. In all temporal investigations using the PAM fluorometer, the actinic light was provided by LED lighting within instrument.

Oxygen Production Measurements

Photosynthetic rate, based upon the production of oxygen, was determined by measuring changes in dissolved oxygen over time normalized to the chlorophyll-a concentration of the sample. Oxygen concentrations were determined using a Clark-type oxygen electrode (YSI 5300 amplifier and YSI 5331 probe) in 5mL of sample. The media was temperature controlled at 20ºC. Rates of oxygen production were determined in darkness and under a flux of 160 $\mu E\ m^{-2}\ s^{-1}$ generated by a halogen light source. Determinations of oxygenic photosynthesis were made when the rate of production was stable for three minutes. The voltage signal generated by the electrode was recorded over time using a Hewlett Packard 7045a XY chart recorder. Changes in O_2 over time were normalized to chlorophyll-a concentrations and expressed as $gO_2\ gChl\text{-}a^{-1}h^{-1}$. Because high densities of cells were necessary to result in measurable changes in oxygen, cultures were assessed when chlorophyll-a concentrations were above 150 µg/L. Natural samples were centrifuged at 5,000rpm for 5 minutes to concentrate cells prior to oxygen evolution experiments.

ATP

Measurements of phytoplankton ATP were made using a luciferin-luciferase kit (Promega) based upon the photometric assays from Karl (1980). Samples were collected on GF/F filters, extracted into 20 mM Tris buffer (pH 7.75), boiled for five minutes, and then frozen at -20°C until the time of analysis. Luminescence was determined on a Turner Designs TD 20/20 luminometer.

Pigment Analysis

Media containing microalgae was filtered onto 25mm Whatman GF/Filter (0.7 μm pore size). The cells isolated on the pad were extracted for at least 24 hours in 90% acetone 10% water at -20°C. The extracted solutions were analyzed by HPLC (High Pressure Liquid Chromatograph; Zapata et al 2000) and or filter-fluorometric technique (Welschmeyer, 1994).

The HPLC protocol of Zapata et al (2000) employed a binary pumping system of pyridine-containing mobile phase; the column used here was a Microsorb Dynamax (Varian) C8 column, 25cm length, 4.6mm ID, 5μm particles with 100 angstrom pore size. Two detectors were used to analyze eluting pigments, a Linear UV/VIS 200 Spectrophotometer with wavelength set at 440nm for carotenoids/chlorophylls and a Kratos FS 950 Fluoromat for chlorophylls. The HPLC fluorometer was fit with standard blue and red optical filters (Corning 5-60 and 2-64) and a Daylight lamp for chlorophyll analysis (Baker, 1983), thus providing response to all chlorophylls and their degradation products. Eluting pigments were quantified using a computer- based data acquisition system.

Two classes of pigments were of interest, chlorophyll-a and xanthophylls. Chlorophyll-a was used as a proxy for biomass normalization. Epoxide-containing xanthophylls have been shown to be involved with photoprotection in higher photoautotrophs and algae (Review Demmig-Adams and Adams 1996). In the current study, epoxy-xanthophylls were used to estimate pigment-based photoprotection. In Bacillariophytes the xanthophyll cycle involves diadinoxanthin and diatoxanthin. These two pigments are enzymatically cycled from the non-photoprotective form, diadinoxanthin (Dd), to the photoprotective form, diatoxanthin (Dt) through the enzyme, diadinoxanthin de-epoxydase. A pigment-based estimate of photoprotection can be made by normalizing the concentration of the photoprotective

xanthophyll (Dt) to the entire pool of the two pigments; as defined as the epoxidation state (EPS) of the cyclic xanthophylls.

$$EPS = Dt/(Dd+Dt) \qquad \text{Eq.3}$$

To examine if CO_2 could act directly on xanthophyll cycling pigments or indirectly through the enzyme responsible for the pigment conversion, an inhibitor was used to prevent enzyme function. Dithiothreitol (DTT) (Sigma Chemicals) has been shown to be a potent inhibitor of diadinoxanthin deepoxidase in *Phaeodactylum tricornutum* (Jacob T. et. al. 1999). DTT (10mM) was dissolved into filtered seawater then added to cultures during dark adaptation, 30 minutes prior to light exposure.

RESULTS

Exposure to carbon dioxide gas caused changes in photosynthetic metrics in representative species from six divisions of marine phytoplankton and two natural sites.

Photosynthetic metrics changed with time after exposing microalgae to saturating concentrations of CO_2. Changes in variable fluorescence parameters, EPS and ATP, as a function of time are shown in Fig. 3 for the diatom *Phaeodactylum* sp. following exposure to saturating CO_2 concentrations (pH 4.9) at an irradiance flux of 40-50$\mu Em^{-2}s^{-1}$. The changes include decreases in F_v/F_m and cellular ATP content and increases in NPQ and EPS.

The photosynthetic responses to saturating CO_2 concentrations were rapid; decreases in F_v/F_m occurred within 30 seconds of CO_2 addition and increases in NPQ and EPS were observed within one minute. Oxygen production appeared to be completely attenuated within 100 seconds, although this could be an overestimate as 100 seconds was the time necessary to measure the production of O_2 reliably. A summary of control and CO_2 saturated oxygenic photosynthesis measurements for *Phaeodactylum, Dunaliella, Cryptomonas, Porphorydium, Isochrysis, Synechococcus* and whole water samples from Elkhorn Slough and the Monterey Bay are shown in Fig. 4. Finally, ATP concentrations dropped within 30 minutes of CO_2 addition (Fig. 3).

The photosynthetic metrics were examined as a function of CO_2 concentration. These tests were done using *Phaeodactylum* and can be seen in Fig. 5. The concentration of dissolved CO_2 was controlled by bubbling varying volumes of gas into the seawater media and measuring pH. Measurements of variable fluoresce and EPS were taken after 20 minutes exposure to a light flux of 40-50$\mu Em^{-2}s^{-1}$. Oxygen production was measured under conditions of saturating light,

$160\mu Em^{-2}s^{-1}$, after 4 minutes of incubation. In this part of the study, changes in the photosynthetic metrics in response to CO_2 were similar to those above; F_v/F_m and decreased O_2 production while NPQ and EPS both increased.

Typically, as dissolved CO_2 concentrations increased, photosynthetic metrics changed as a function of increasing CO_2, but the relationship between CO_2 concentration and photosynthetic response was not constant in *Phaeodactylum, Dunaliella, Cryptomonas, Porphorydium, Isochrysis, Synechococcus* and two, wild, whole water samples from the Monterey Bay and Elkhorn Slough (Fig. 6-7).

To find if the changes in physiological parameters resulting from CO_2 were light dependent, experiments were performed under saturating concentrations of CO_2 gas but with controlled intensities of ambient light (Fig. 8). It was found that in complete darkness there was no difference in oxygen production, F_v/F_m, NPQ or EPS between cells treated with air or CO_2. However, at any flux of light, between 0.3 to 1300 $\mu E\ m^{-2}\ s^{-1}$ the saturating CO_2 treatment resulted in a decrease in F_v/F_m, an increase in NPQ, the formation of photoprotective pigments in the xanthophylls cycle and inhibition of oxygen production.

To determine if CO_2 acted directly on the xanthophyll cycle pigments or indirectly through enzymatic processes, the enzyme responsible for xanthophyll epoxidation/de-epoxidation was inhibited. The formation of the xanthophyll cycling pigments diatoxanthin (Dt) from diadinoxanthin (Dd) can be inhibited using Dithiothretol (DTT) (Demmig et al. 1990). Figure 9 shows that DTT did inhibit the formation of Dt under CO_2 saturating conditions implying that the effect of CO_2 on xanthophyll cycling operates on the epoxidation enzymes and not directly on the pigments. Furthermore, after treatment with DTT the increase in NPQ of cells

treated with saturating CO_2 was only one fourth that of the NPQ increase noted in DTT-free CO_2 treatments. This is consistent with the current physiological models that link NPQ directly to xanthophyll cycling activity (Demmig et al. 1990).

To assess if the effect of CO_2 on Phaeodactylum was permanent, time-series experiments were executed with temporally-cycled conditions of air and CO_2 saturation (Fig. 10). As the media became saturated with CO_2 (pH 4.9) F_v/F_m decreased, as described above, and, as the media was bubbled with air (yielding pH 8.2) F_v/F_m returned to its pre-CO_2 level; the response of NPQ mirrored that of F_v/F_m. The photoprotective epoxidation states of the xanthophyll cycling pigments as well as oxygenic photosynthesis were also both reversible (Fig. 10).

Since CO_2 additions yield coincident reductions in pH it is not clear whether the observations above reflect pH effects or intrinsic CO_2 effects. In order to identify the causative factor, independent manipulations of pH were made using 1) CO_2 (as described above) or 2) direct addition of reagent acids; the experiments were again made with *Phaeodactylum*. In the first set of experiments, fluorescent parameters were examined as a function of pH as altered by hydrochloric acid (pKa: -7), acetic acid (pKa: 4.75), or by CO_2 (carbonic acid pKa: 6.4) (Fig. 11). The pH at which the fluorescent metric F_v/F_m began to decrease was pH 5.5 for acetic acid and CO_2 and 3.5 for HCl. The pH at the minimum F_v/F_m observations were also different; 4.9 for acetic acid and CO_2 and for 3.0 and HCl. NPQ had a similar inflection points and maxima as did F_v/F_m.

Additional experiments were run to observe physiological parameters of *Phaeodactylum* as a function of pKa (Fig. 12). Concentrated acids (pKa); sulfuric (-10), hydrochloric (-7), nitric

(-1.5), phosphoric (2.1), formic (3.75), lactic (3.84), acetic (4.75), carbonic (6.37). were titrated into culture media to a final pH of 4.85 ± 0.10. The fluorescent metrics F_v/F_m and NPQ remained largely unchanged from the control when treated with stronger acids having a pKa less than 3.75, while weaker acids, with a pKa greater than 3.75, caused decreases in Fv/Fm and increases in NPQ. The deepoxidation state of xanthophyll cycle pigments of treated *Phaeodactylum* was similar to NPQ. Acids with pKa less than 3.75 showed no change in cyclic xanthophyll pigments at pH 4.9, while acids with pKa greater than 3.75 induced a change in the xanthophyll cycle pigments.

DISCUSSION

Studies of the availability of CO_2 on photosynthesis of marine phytoplankton have resulted in two repeatable observations; carbon limitation and carbon saturation. Here we describe an observation largely overlooked; CO_2 inhibition of photosynthesis and promotion of photoprotection.

Products of the light reactions of photosynthesis include O_2 gas, ATP, and variable chlorophyll fluorescence. Changes in measurements of these products can indicate changes in the photosynthetic machinery. The data here show that the addition of high concentrations of CO_2 results in the cessation of O_2 production, a drop in ATP concentration, and a decrease in the fluorescence metric F_v/F_m. This pattern of CO_2-induced changes in photosynthetic metrics were observed in six cultured algal species and two wild samples of mixed marine phytoplankton.

All of the photosynthetic metrics measured in this study responded to conditions of high CO_2 as long as the treatments were made under illuminated conditions. Specifically, under high CO_2, F_v/F_m decreased, NPQ increased, ATP decreased and oxygen production was fully inhibited. The response-times, however, were not identical. At a flux of $70\mu Em^{-2}s^{-1}$ the concentration of ATP did not decrease until 5 minutes after the treatment with CO_2. Conversely, O_2 production and F_v/F_m were both found to decrease within 100 and 30 seconds respectively (logistical constraints prevented finer temporal resolution of oxygen evolution rate; thus the actual effect could be faster).

High CO_2 induced a photoprotective state in *Phaeodactylum* as evidenced by changes in relative concentrations of xanthophyll cycling pigments. Two measurements which are believed

to be indicative of photoprotection are the fluorescence-derived NPQ and the epoxidation state of xanthophyll cycling pigments expressed as the ratio (Dt/(Dt+Dd)) (Demmig et al. 1990). High concentrations of CO_2 resulted in NPQ and Dt/(Dt+Dd) increasing at any irradiance flux > 0.3 $\mu Em^{-2}s^{-1}$; high saturating irradiance was not required to trigger photoprotective responses. Interestingly, no CO_2 response in NPQ or EPS could be detected under fully darkened conditions. The increase in these two metrics under high CO_2 at $3\mu Em^{-2}s^{-1}$ were similar to what was found without CO_2 at a flux of light two orders of magnitude greater. Apparently, high CO_2 imparts a photoprotective response that is super-sensitive to ambient irradiance.

Three trends emerged in oxygen production rates as a function of the concentration of dissolved CO_2. The most commonly observed relationship between CO_2 concentration and oxygen production (type I, Fig. 7) was little to no change in O_2 production until a CO_2 threshold or 'inflection point' was reached, usually around 5% CO_2 (pH 6). Any further increase in CO_2 resulted in decreases in oxygen production rates. A second trend (type II, figure 7) was a decrease in oxygen production with an increase in CO_2 above ambient. In the third relationship (Type III, figure 7), an *increase in oxygen production was observed after a small addition of* CO_2 followed by continuous reduction in O_2 production as CO_2 increased. The most dramatic increase was seen in samples from Monterey Bay which responded to increased CO_2 by doubling their photosynthetic rates. These differences in oxygenic photosynthesis to CO_2 could be the result of the chemistry of the seawater or the physiological state of the cells that were gathered. The observations of increased photosynthesis beg the question, are phytoplankton carbon saturated in the ocean?

There were significant differences in the photosynthetic response to CO_2 and other acids at identical pHs, thus pH, per se, did not appear to be the cause of these changes. However, the pKa of the acid did appear to have a relationship with the photosynthetic response (Fig.10)

The inhibitory and photoprotective effects of CO_2 were not permanent in *Phaeodactylum*. All of these measurements return to their pre-CO_2 levels after the media has been returned to equilibrium with air.

The enzyme diadinoxanthindepoxidase is believed to be activated under conditions of high light or environmental stress resulting in the conversion of Dd to Dt (Olaizola et al 1994). The inhibition of the enzyme using dithiothretol (DTT) prevents the pigment Dd from becoming deepoxidized. The use of the inhibitor prevented the formation of Dt from Dd, but it did not completely attenuate the increase in NPQ. These findings suggest that the CO_2 increase did not effect the xanthophyll pigments directly, but indirectly through an enzyme linked mechanism.

The finding that the photosynthetic substrate, CO_2, is a potent inhibitor of photosynthesis is curious. Discussed below is a proposed mechanism of how CO_2 may inhibit this process. Also discussed are the environmental implications and suggestions for interpretation of past data and future study.

CO_2 inhibition of photosynthesis and the bicarbonate effect, a relationship?

It is possible that the location in which CO_2 has been shown to effect photosynthesis is at the oxygen evolving complex of photosystem II (PSII). This hypothesis stems from the work originally proposed by Warburg and Krippahl (1958) which involves the bicarbonate effect; reviewed by Stemler (2002).

The bicarbonate effect suggests that not only H_2O, but also HCO_3^- may be a substrate for the water oxidizing complex of PSII, through reactions involving a PSII-associated enzyme, carbonic anhydrase. Carbonic anhydrase catalyzes the reaction, $HCO_3^- + H^+ \leftrightarrows H_2O+CO_2$. The theory is that HCO_3^- binds to PSII reacting with carbonic anhydrase to release H_2O which is then oxidized producing O_2, H^+, and e^-.

Proposed here is a form of product or feedback inhibition of PSII, by CO_2. The substrate for the PSII-bound carbonic anhydrase is HCO_3^- which is enzymatically converted to CO_2. When, the product, CO_2 saturates the cell, the reaction which normally would form CO_2 may be prevented from occurring, resulting in the loss of carbonic anhydrase function. This loss of function either directly (by stopping the 'splitting of water') or indirectly (acting as an electron flow rate regulator) may prevent the flow of electrons to the pigment bed. Evidence of the loss of electron flow through the non-cyclic PSII can be seen as a decrease in oxygen production, increase of light protective pigments and ultimately a drop in ATP.

The literature has provided support for this hypothesis. A PSII-associated carbonic anhydrase was shown to play a regulatory role in the oxygen evolving complex of the green alga *Chlamydomonas reinhardtii* (Villarejo et al. 2002). Further, it has been suggested that carbonic anhydrase was responsible for the inhibition of oxygenic photosynthesis in the high CO_2 tolerant green algae *Chlorococcum littorale* (Satoh et al. 2001). Also, *C.littorale*, has been used to describe a CO_2 induced shift in the balance of energy from PSII to PSI. In that study cultures of

the green alga grown under 40% CO_2 reduced their PSII function and increased PSI function. Satoh et al. (2002) proposed that non-cyclic e^- transport, which is unique to PSI, in *C.littorale,* may be important to the survival of this species under high CO_2 (Satoh et al. 2002). They suggest that the observed increase in PSI function may be the result of CO_2 rendering PSII function partially inactive.

If CO_2 is negatively affecting the oxygen evolving complex of PSII then all of the other observations described here can be explained. The function of the oxygen evolving complex is the splitting of water, producing O_2, e^-, and H^+; the electrons are used to recharge excited reaction center chlorophyll *a*, P680, which has transferred electrons after excitation by light absorption. If this function is inhibited, then these three materials will not be produced. The termination of oxygen production is easily detected as it diffuses from the cell into the media. Preventing new e- from entering PSII reaction center could be detected by changes in variable fluorescence. Without e^-s to reduce Qa and Qb there should be no photochemical quench for the antenna pigments of PSII. This would increase the residence time of a photon-induced excitation event on the antenna pigments, as PSII has not been described to have a cyclic e- transport system. The remaining quench processes, fluorescence and non-photochemical quench, must dissipate the energy or risk photo damage. This could explain the findings of decreased F_v/F_m, increased NPQ, and a shift in photoprotective xanthophyll cycling pigments.

Normally, the oxidation of water increases the concentration of H^+ in the lumen of the chloroplast. The proton concentration is further increased with the transport of H^+ into the lumen via the electron transport reactions, reactions using e^- originally obtained from the oxygen evolving complex. Inhibition of these processes would result in the attenuation of the H^+

gradient across the thylakoid membrane. The ATPase which is found in the chloroplast is dependent on the H^+ gradient to power the coupling of ADP+P to form ATP. Thus, if the H^+ is removed, ATP would not be generated in the chloroplast.

Effects of strong and weak acids

While CO_2 elicits photoinhibitory and photoprotective changes in physiology, CO_2 itself may not be the origin of the changes. A small body of evidence, generated here, that suggests that weak acids with pKa values that are greater than 3.75 have a similar photophysiological response as does CO_2 (Figure 12). Thus it is possible that neither CO_2, nor H^+ are the molecules that are causing these effects. Instead the effect could be the result of the conjugate base of these acids either directly or indirectly acting on the cell.

Studies which have attempted to alter the CO_2 concentration of seawater using strong acids, such as HCl, may have come to erroneous conclusions. The findings of this study show that photosynthetic rates are not as sensitive to the pH changes due to strong acids, but are more sensitive to weak acids. Some studies on CO_2 have used HCl (Chen and Durbin 1994; Hein and Sand-Jenson 1997) and others did not disclose how seawater CO_2 levels are altered (Riebesell 1993). *Because the mechanism behind pKa having a greater effect than pH is not understood, it is suggested that in future CO_2 studies, the gas CO_2, should be used rather than an acid to shift the carbonate system.*

Application of the findings

While in the euphotic zone of the world ocean the occurrence of 100% CO_2 is unlikely, seawater does vary in CO_2 concentration. Open ocean CO_2 concentrations do not vary

dramatically; pH ranges from 7.9-8.5 (estimated CO_2 of 760-120ppm). Embayments and eutrophic areas can vary to a much larger degree; pH ranges from 6.0-9.5 (estimated CO_2 of 38000-30ppm) (pH extremes gathered from Hinga 2002 and CO_2 estimated using computer software (CO2SYS v1.05 Brookhaven National Laboratories). According to the results of this study these ranges of CO_2 are unlikely to have a dramatic effect on the variable fluorescence parameters, or photoprotective mechanisms, but may show changes in oxygenic photosynthesis. Thus it is possible that in the open ocean and in enclosed bodies of seawater the photosynthetic rate of marine phytoplankton could be altered by naturally and anthropogenically influenced changes in CO_2.

Since CO_2 is readily available in the combustion of all fossil fuels, the finding that it is a potent inhibitor of oxygenic photosynthesis may have application as an algaecide. While the scope of this project has been to describe short term responses by phytoplankton to CO_2, the utility of these findings may be in longer exposures to high CO_2 and light. If the effects do not wear off, prolonged exposure to CO_2 and light could result in the cell being starved for electrons, resulting in photodamage and possibly cell death.

Final thoughts.

The gas, CO_2, is as essential to plants as oxygen is essential to animals. But where 100% oxygen is often beneficial to animals, 100% CO_2 appears to stop the photosynthesis of marine phytoplankton. In the marine environment it is very rare to observe 100% CO_2 saturation (even if the atmosphere were to more than double it's CO_2) therefore the findings here regarding *complete inhibition* of photosynthesis are not likely to significantly effect global photosynthetic estimates. But, the finding that small changes in the concentration of CO_2 can result in

significant changes in rates of photosynthesis is contrary to the 'carbon-saturated marine photosynthesis' beliefs. The 'carbon-saturated marine photosynthesis' beliefs are rooted in the 'old standard' method for shifting carbon species, the addition of a strong acid like HCl. Here we showed that the photosynthetic response to acids and CO_2 were very different. This begs the question, *if our time tested method for manipulating CO_2 in seawater is fundamentally flawed, what will REALLY happen in our estimates of marine photosynthesis as our atmosphere increases in CO_2?*

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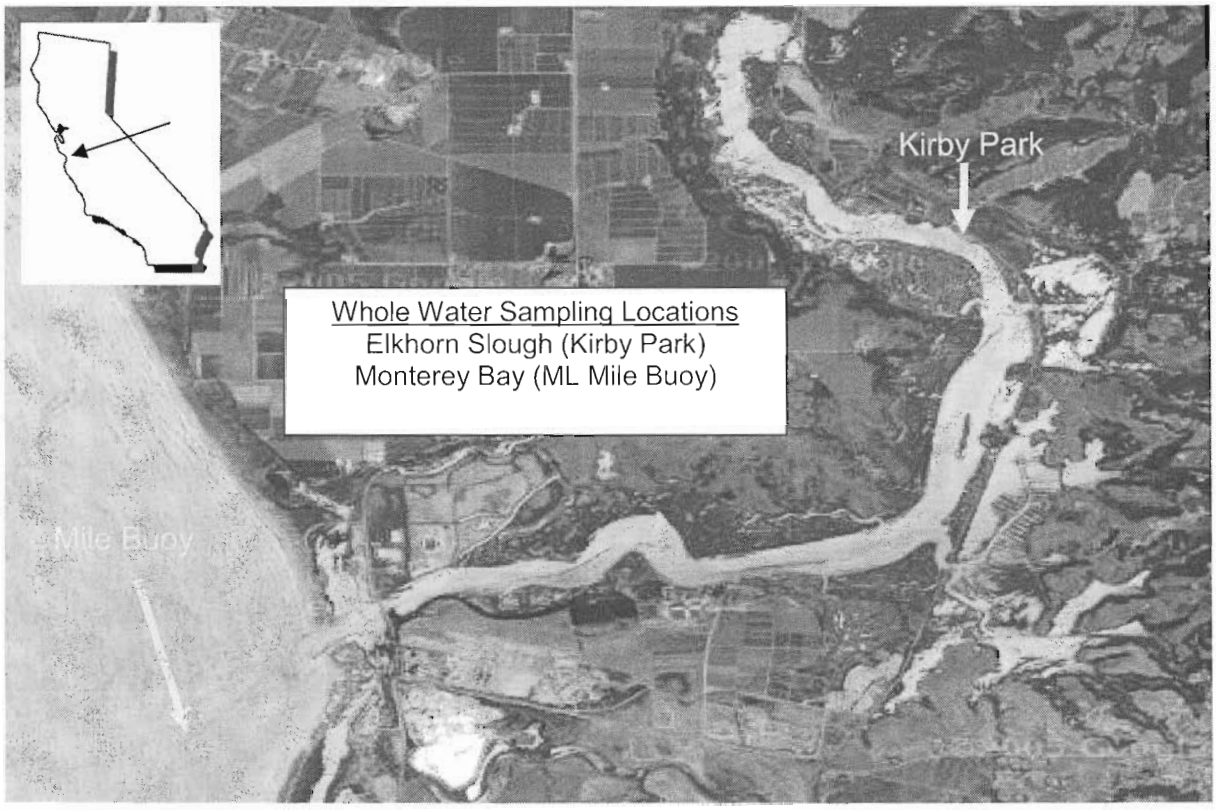


Figure 1
Whole water sampling locations on California's central coast in the Monterey Bay.

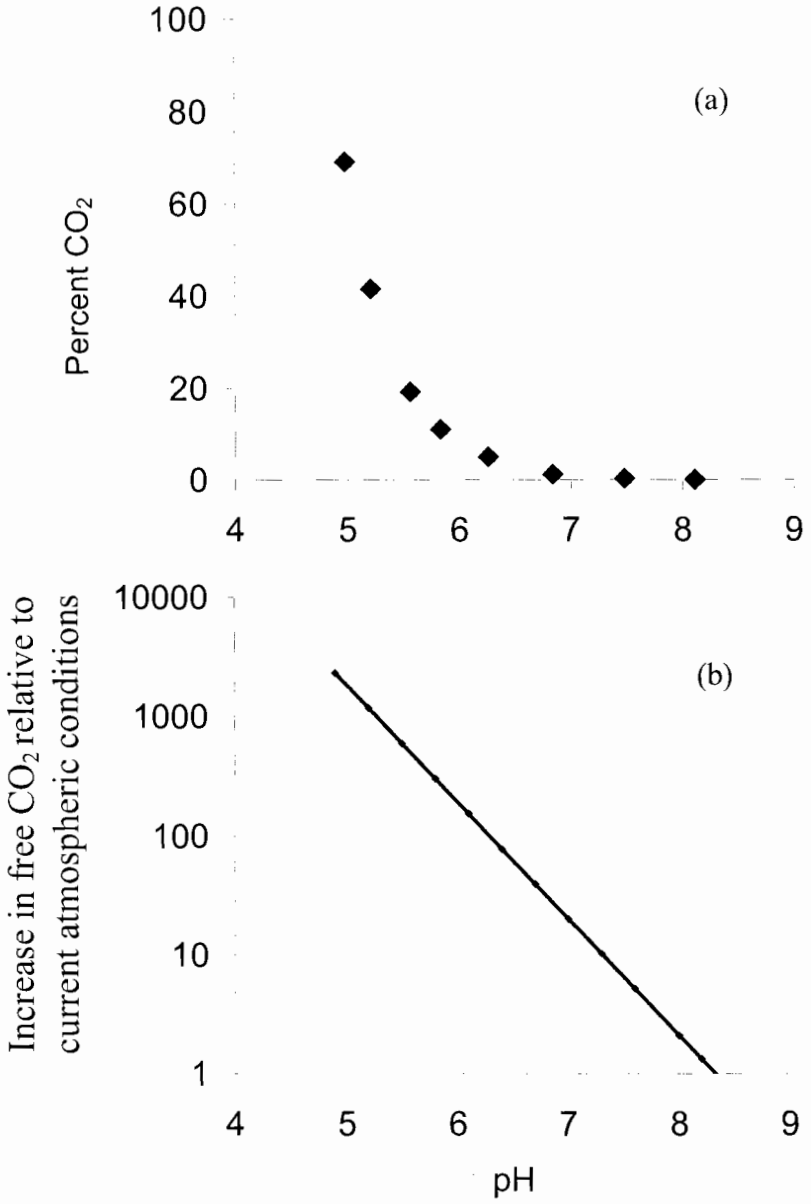


Figure 2:
(a) Dissolved CO_2 as a function of pH, (b) free CO_2 concentrations relative to current atmospheric CO_2 equilibrium as a function of pH.

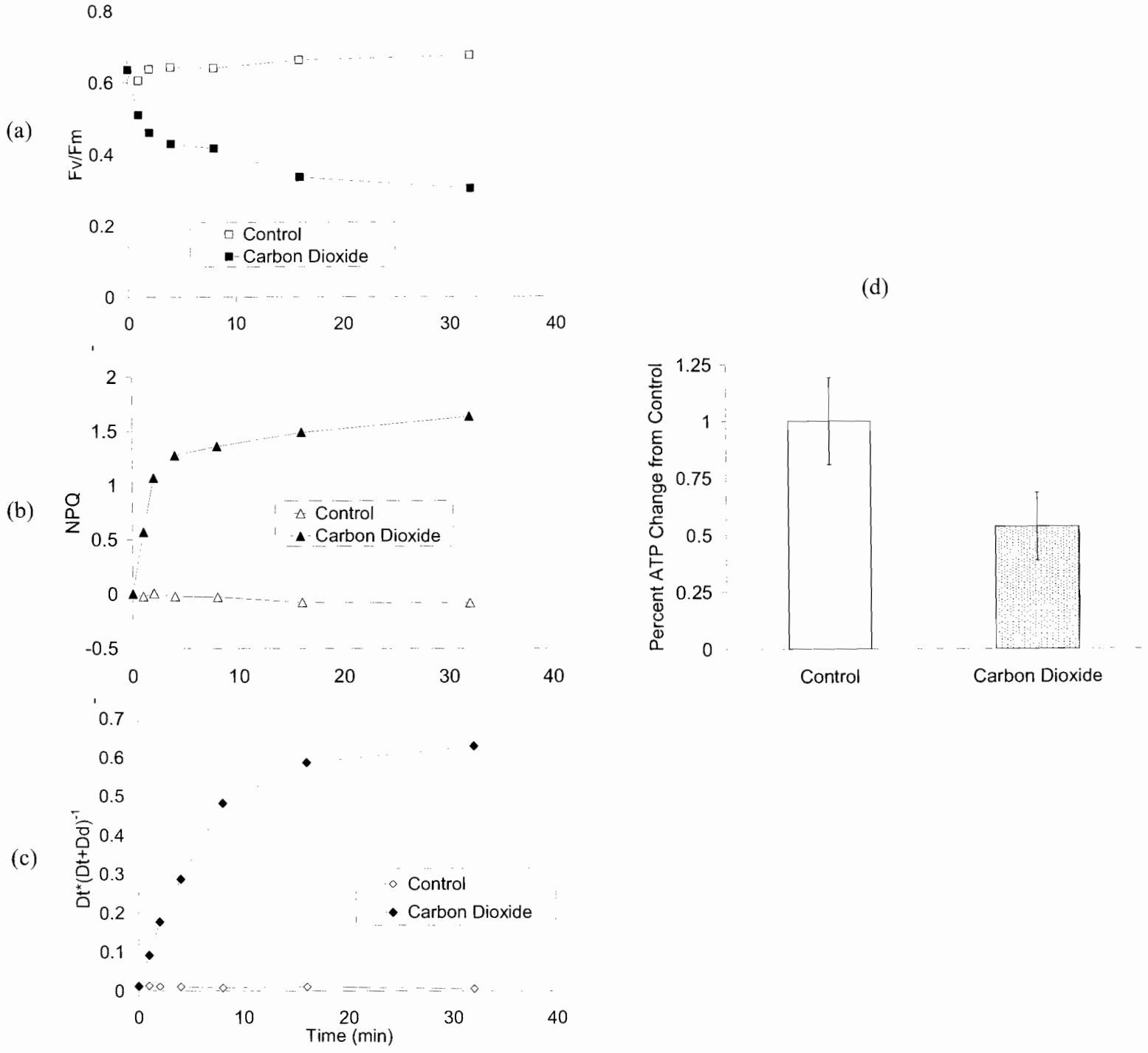


Figure 3:
Change in cellular parameters in the diatom *Phaeodactylum* over time following saturation with 100% CO_2 gas. (a) Photochemical efficiency, F_v/F_m, (b) non photochemical quench NPQ (c) xanthophyll pigment epoxidation state, EPS, $Dt^*(Dt+Dd^{)-1}$ and (d) percent change of ATP after 30 minutes, relative to control samples taken at time zero.

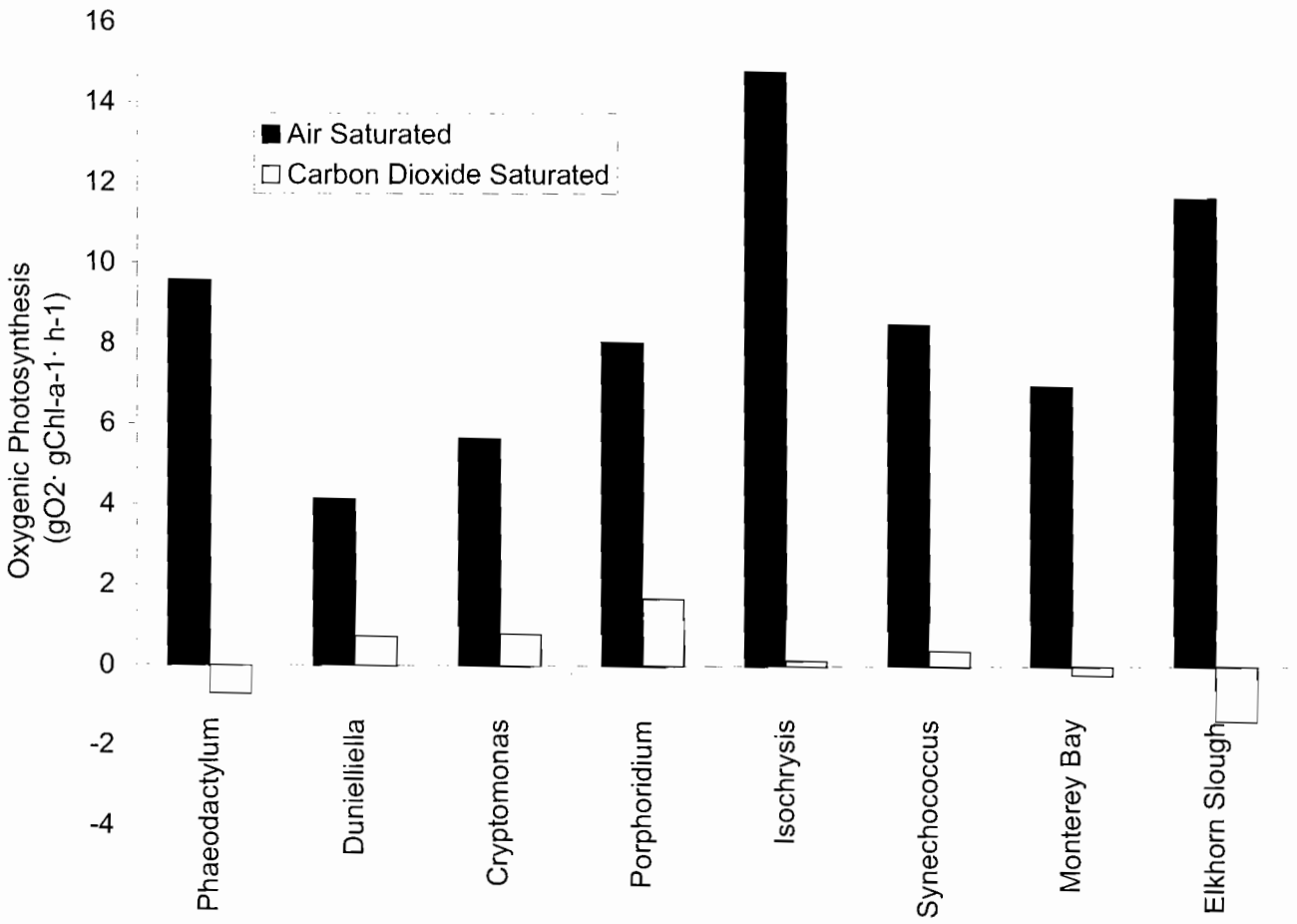


Figure 4
Oxygenic photosynthesis as a function of species. Cultures were air or CO_2 saturated and exposed to 160μEm^{-2}s^{-1} for 20 minutes.

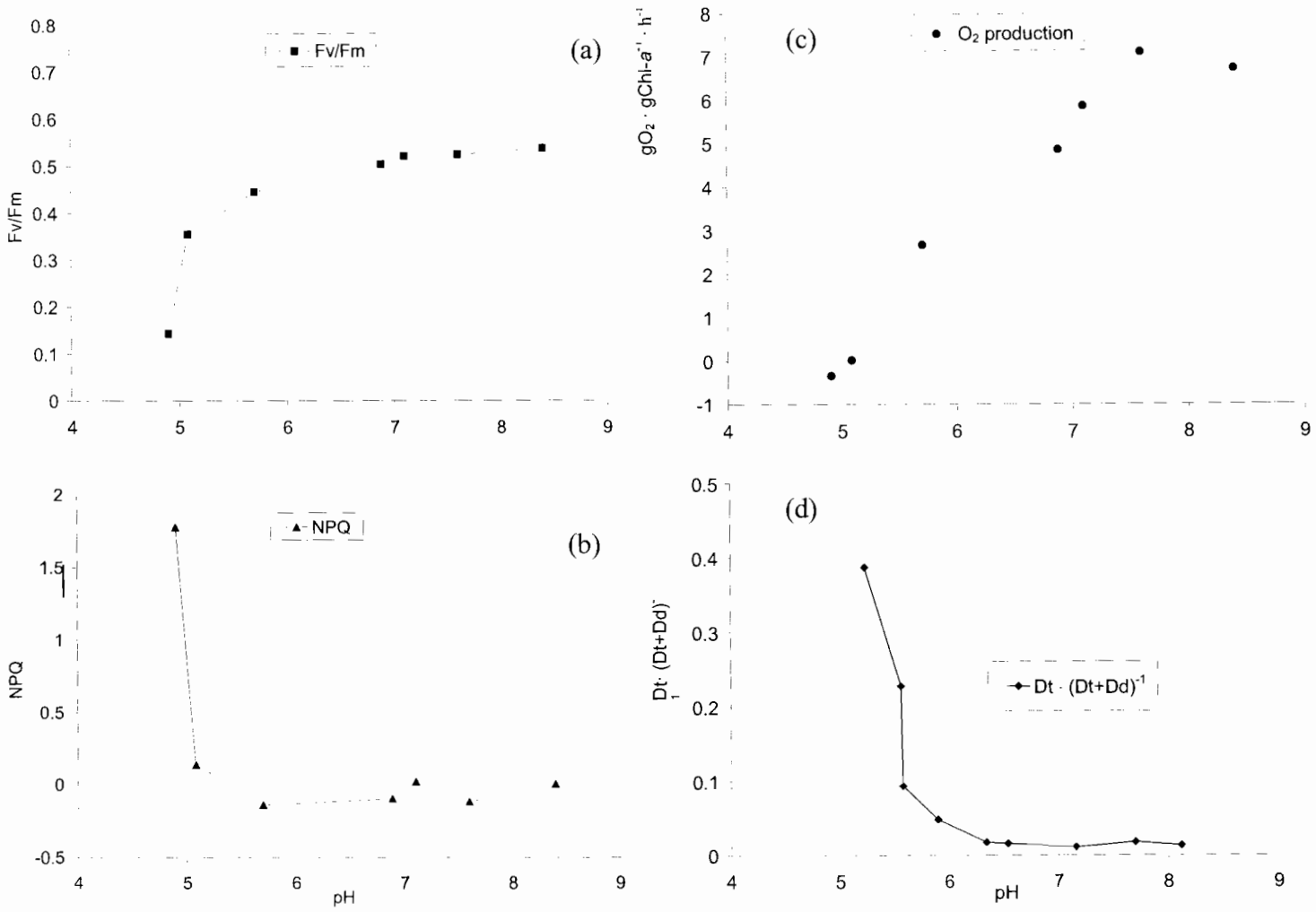


Figure 5:
Changes in F_v/F_m (a), NPQ (b), photosynthetic rate (c) and xanthophyll cycling pigment epoxidation state, $Dt*(Dt+Dd)^{-1}$ (d) as a function of pH as altered by bubbling with 100% CO_2 in the diatom *Phaeodactylum*.

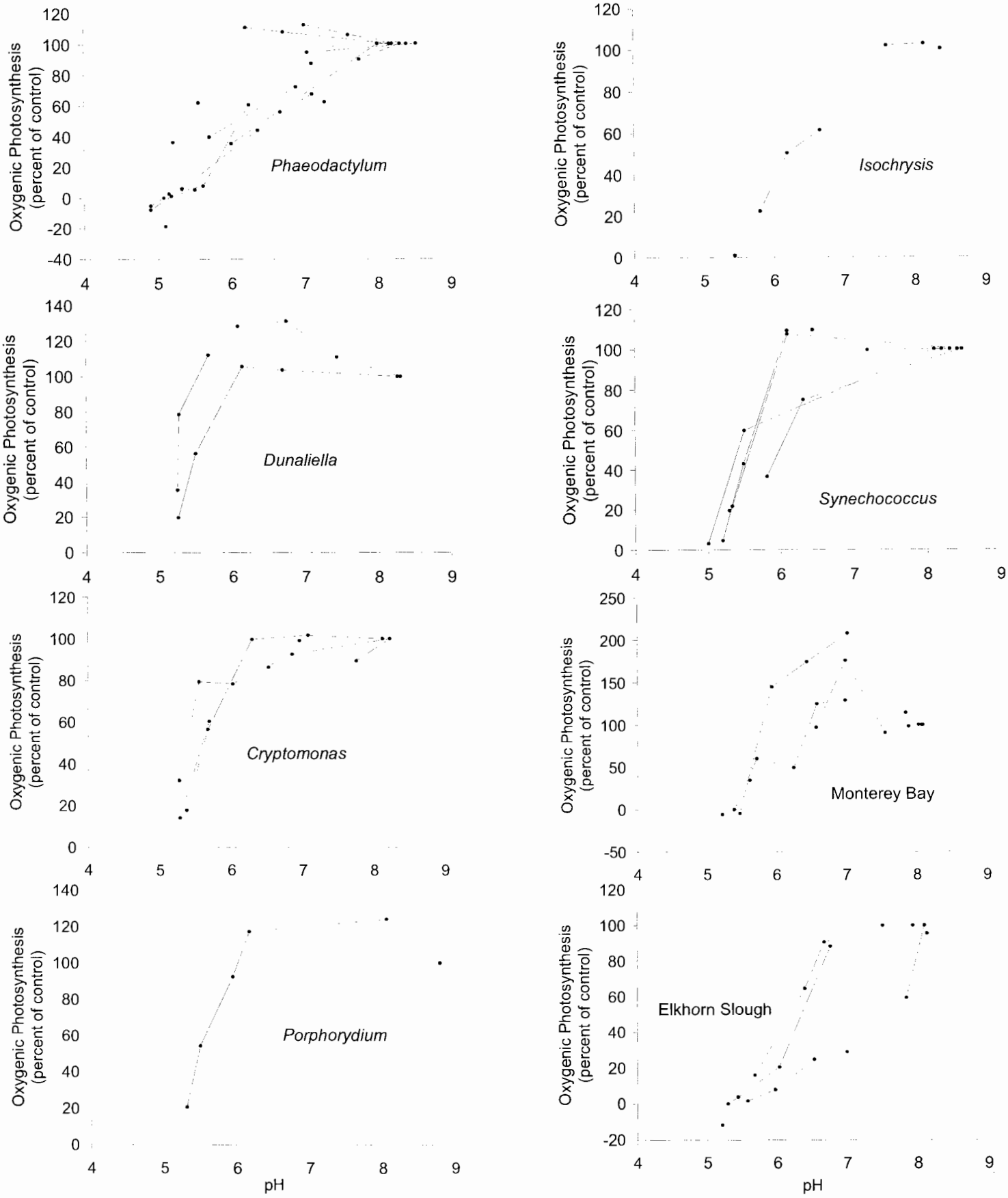


Figure 6
Cultures of *Phaeodactylum, Dunaliella, Cryptomonas, Porphorydium, Isochrysis, Synechococcus* and two, wild, whole water samples from the Monterey Bay and Elkhorn Slough observing relative oxygenic photosynthesis as a function of carbon dioxide concentration, denoted as pH.

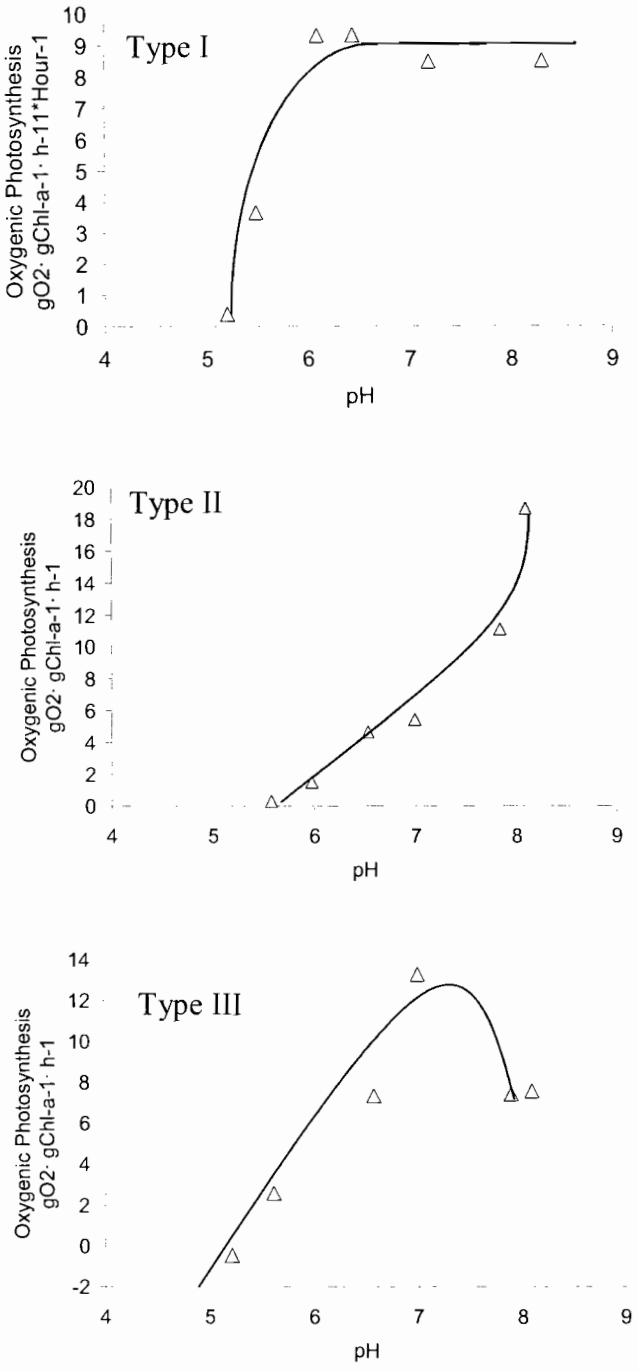


Figure 7:
pH to Photosynthesis Relationships. Type I, as seen in *Phaeodactylum* , Type II, as seen in Elkhorn Slough, and Type III as seen in the Monterey Bay. Lines were drawn by hand thus are not the result of a mathematical equation.

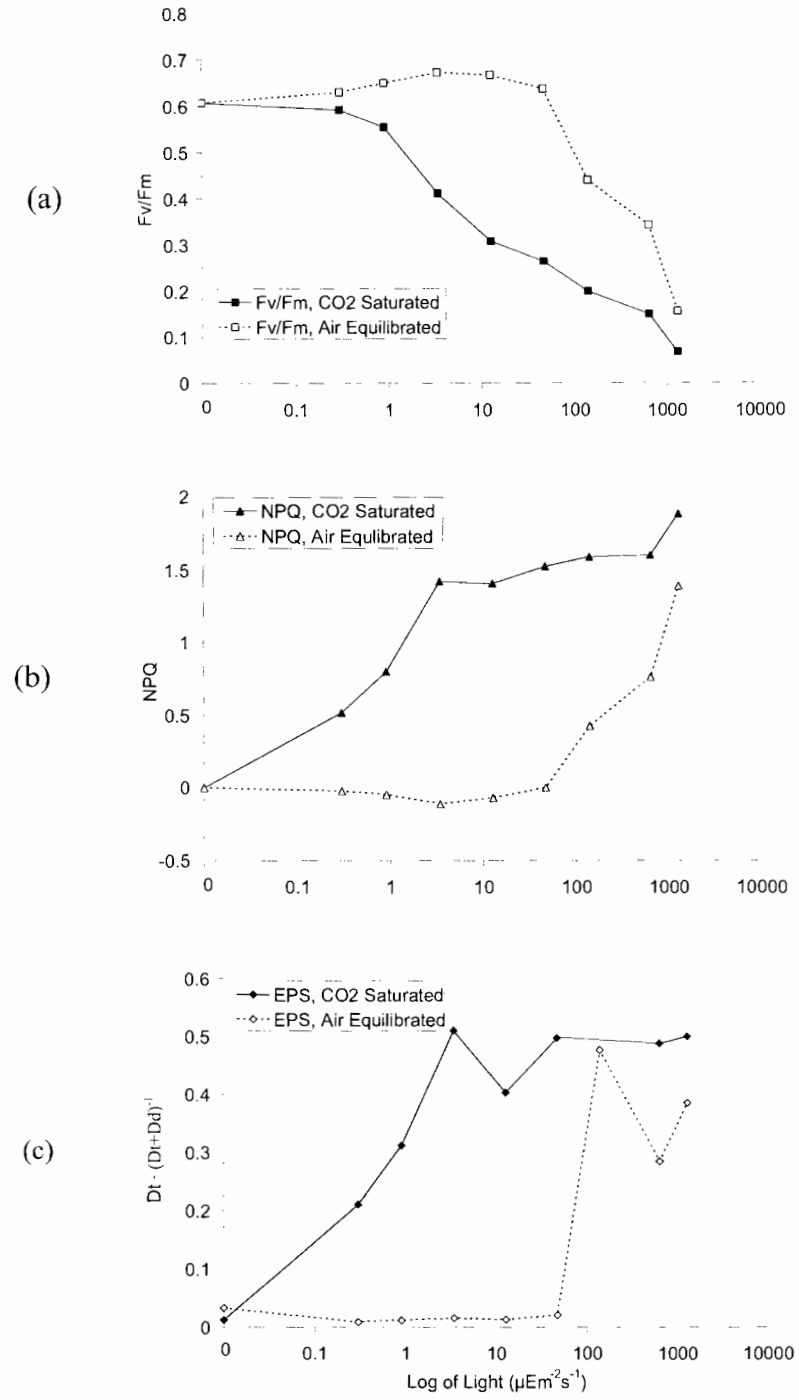
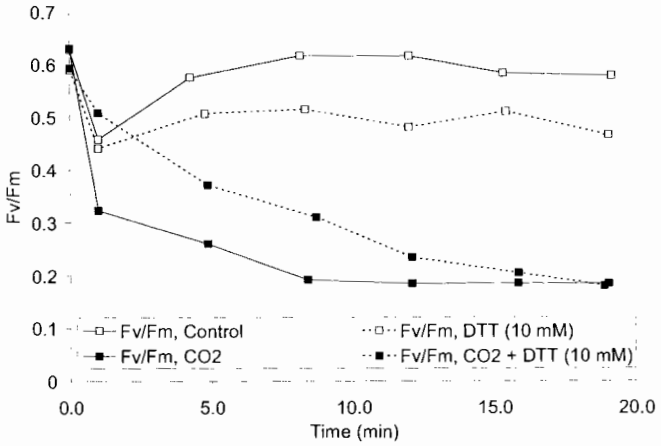
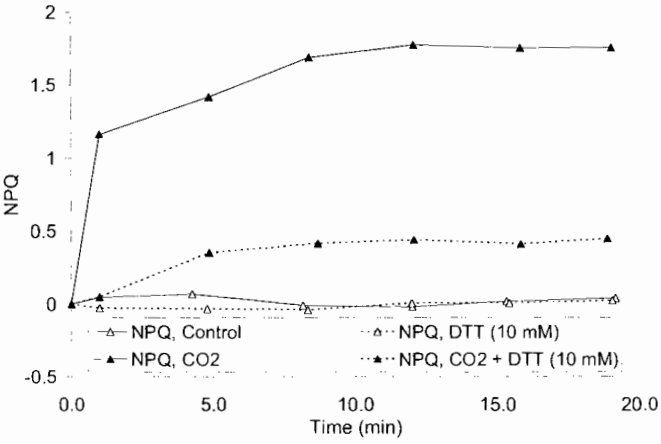


Figure 8:
(a) F_v/F_m, (b) NPQ, and xanthophyll pigment epoxidation state, $Dt \cdot (Dt+Dd)^{-1}$ (c) as a function of light flux after 20 minutes of exposure to 100% CO_2 in the diatom *Phaeodactylum*.

(a)



(b)



(c)

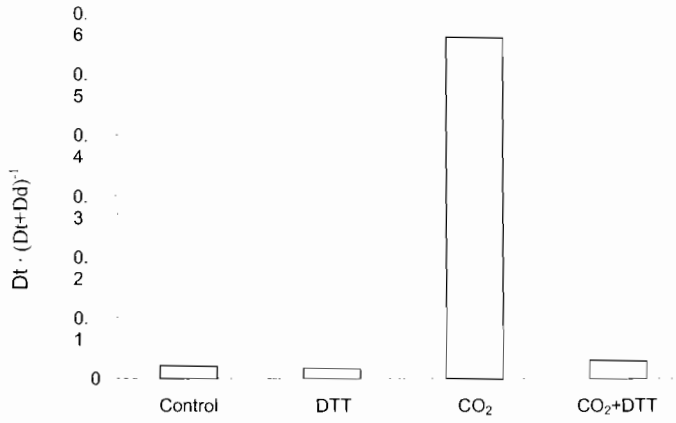


Figure 9:
(a) F_v/F_m (a), and (b) NPQ as a function of time with and without DTT and CO_2 in *Phaeodactylum*. Time=0 is dark adapted, all else at ~60μEm^{-2}s^{-1}. (c) Epoxidation state of xanthophyll cycle pigments, Dt · (Dt+Dd)$^{-1}$, as a function of a twenty minute exposure to saturating CO_2.

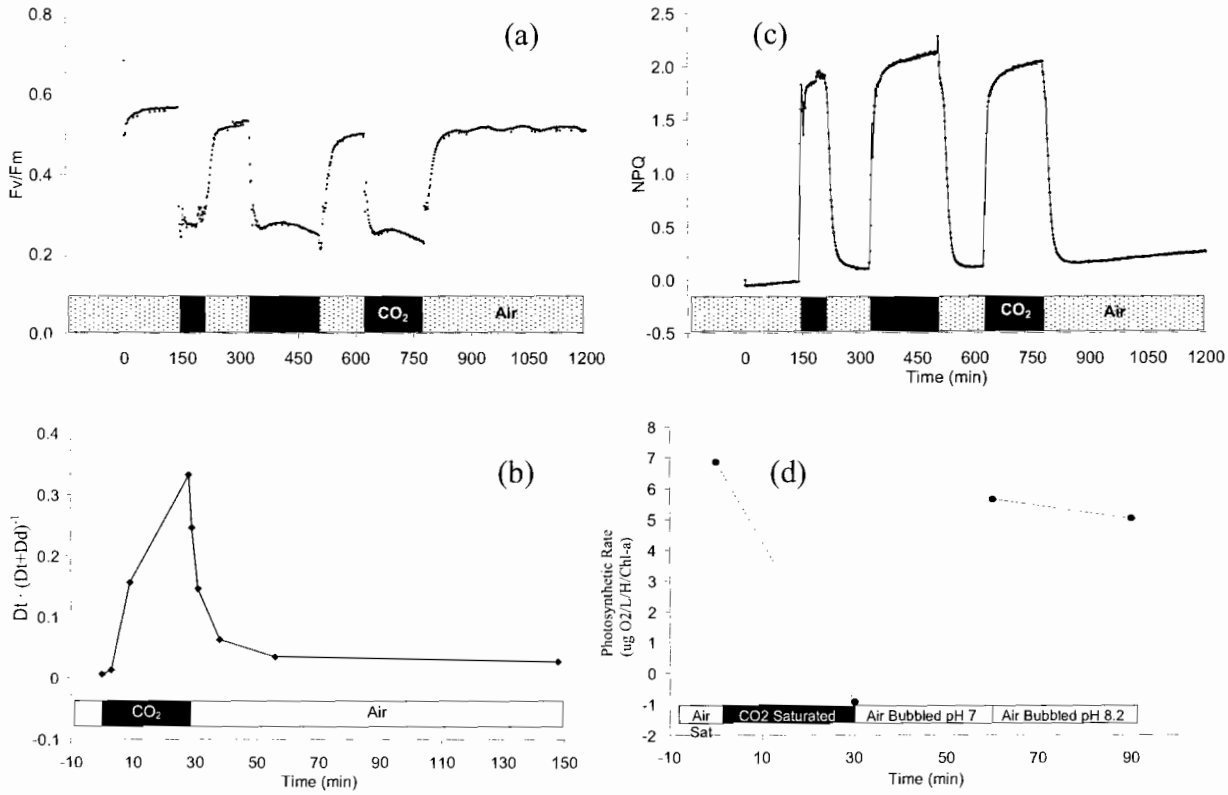


Figure 10:
Change in (a) F_v/F_m, (b) NPQ, (c) epoxidation state of xanthophyll cycle pigments, $Dt \cdot (Dt+Dd)^{-1}$ and (d) oxygen production as a function of time under air equilibration and CO_2 saturated conditions in the diatom *Phaeodactylum*.

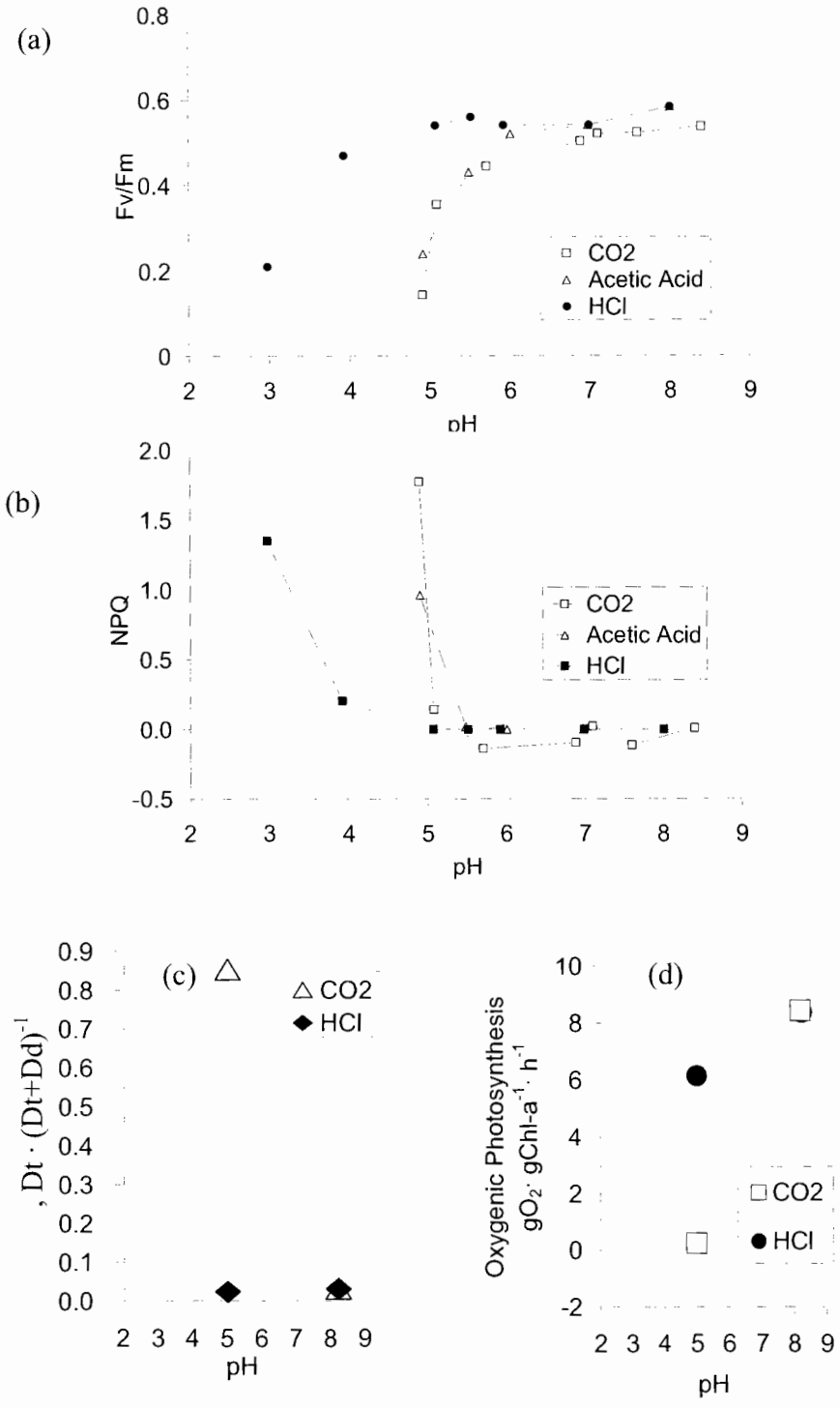


Figure 11:
(a) F_v/F_m and (b) NPQ (c) epoxidation state of xanthophyll cycle pigments, $Dt \cdot (Dt+Dd)^{-1}$ and (d) oxygenic photosynthesis as a function of pH in seawater media as altered by CO_2 acetic acid and hydrochloric acid (HCl). Measurements using the diatom *Phaeodactylum* were taken at each pH after an incubation period of 20 minutes

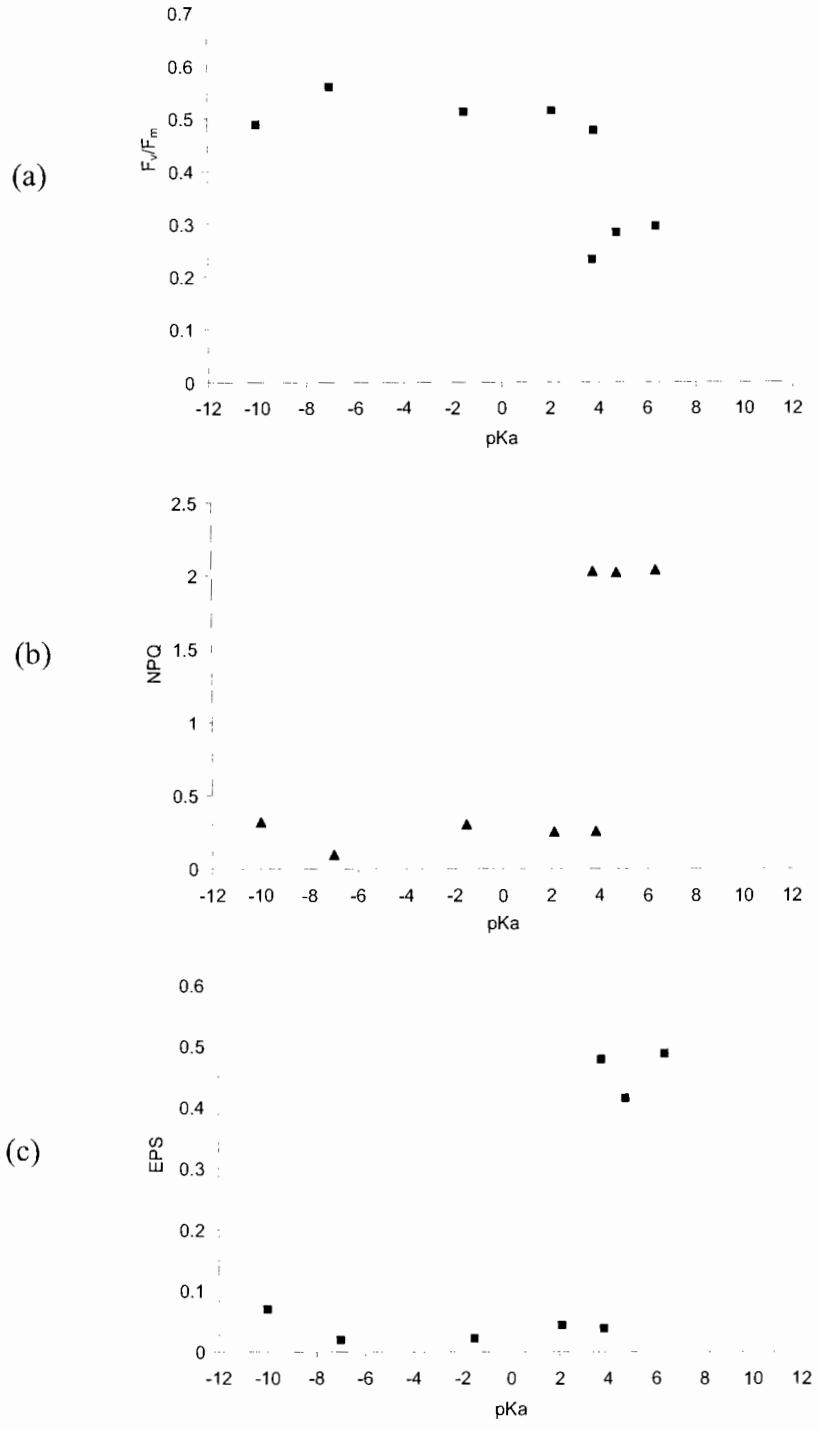


Figure 12:
(a) F_v/F_m, (b) NPQ, and (c) EPS as a function of pKa (pH 4.85 ± 0.1 and PFD 30 μEM^2s^{-1}) in *Phaeodactylum* after treatments with the acids (pKa); sulfuric (-10), hydrochloric (-7), nitric (-1.5), phosphoric (2.1), formic (3.75), lactic (3.84), acetic (4.75), carbonic (6.37).