Incubation Experiments to Determine the Response of a Natural Plankton Community to Treated Sewage Effluent

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ABSTRACT:

The Orange County Sanitation District diverted flow of secondarily-treated effluent from a discharge pipe located 8.0 km offshore at 60 m depth to an outfall located 1.6 km offshore at 16 m depth for three weeks in September of 2012. Two incubation experiments were performed to examine the potential for this effluent to stimulate the growth and impact the structure of natural, coastal plankton communities. The first experiment was initiated a week prior to the diversion of effluent to the nearshore site ('Pre-Diversion'), and the second began a week after the start of the diversion ('Mid-Diversion'). The overall phytoplankton response observed in both experiments following effluent addition was an increase in the abundances of diatoms and photosynthetic picoeukaryotes, and a decrease in picocyanobacteria. A dramatic net increase in heterotrophic bacterial abundances also occurred in both experiments. Additions of a 1:10 dilution of effluent yielded significant increases in chlorophyll *a* concentrations, although this treatment in the Pre-Diversion experiment exhibited a 3 day lag in response to effluent addition, perhaps indicating that the Pre-Diversion community was inhibited by the enhanced chlorination process that was enacted during the diversion. Domoic acid producing diatoms in the genus *Pseudo-nitzschia* were present in the plankton throughout the experiments, but domoic acid production was only detected during the Mid-Diversion experiment. The highest concentration of domoic acid measured, 0.42 ± 0.057 µg/L, coincided with phosphate and silicate concentrations below the detection limit of the method, suggesting limitation by these macronutrients.

1. INTRODUCTION

The impact of anthropogenic activities on marine ecosystems is increasingly apparent in the enhanced nutrient loading of coastal waters (Paerl et al., 2002; Smith et al., 2006; Smith et al., 1999), destruction of coastal habitats by sea level rise (Nicholls et al., 1999; Sahagian et al., 1994), ocean acidification (Fabry et al., 2008; Hoegh-Guldberg et al., 2010) and the expansion of hypoxic and anoxic zones (Chan et al., 2008; Deutsch et al., 2011; Stramma et al., 2010). Southern California is home to a truly 'urban ocean' that includes the largest commercial shipping port in the United States and a coastline that serves as a recreational area to a population of more than 22 million. There are nineteen municipal wastewater treatment facilities in operation that support this large and growing community, and three of the four largest facilities discharge into the coastal ocean off Los Angeles and Orange counties. These discharges are relatively constant while natural nutrient loading, upwelling and storm-water runoff events, are seasonal and stochastic.

Upwelling in the region most commonly occurs during spring, when upwelling favorable winds dominate, while rain events occur from November to March. Previous studies of nutrient loading have focused on upwelling events as the principal process stimulating primary production along the southern California coast (Eppley et al., 1979; Eppley et al., 1978; Schnetzer et al., 2013), but recent research has demonstrated that effluent discharges may contribute significantly to productivity, particularly during periods of upwelling relaxation (Howard et al., 2014). The latter study reported that the magnitude of nitrogen concentrations introduced to the coastal ocean from these two processes is roughly similar when averaged over an annual cycle, but the form of nitrogen differs, with nitrate the primary nitrogen form in upwelled water while the primary nitrogen form in effluent is ammonium. Since nitrogen is considered the primary nutrient limiting primary productivity in southern California waters, and its preferred form can differ among many plankton species (Goldman and Glibert, 1982; Howard et al.,

2007), there is a need to understand how plankton community structure is influenced by the presence of a nutrient source overwhelmingly dominated by ammonium, such as treated effluent. Previous studies have reported both the capacity for growth stimulation (Dunstan and Menzel, 1971; Eppley et al., 1972; Thomas et al., 1974) and inhibition by sewage effluent addition on plankton communities (Dunstan, 1975; MacIsaac et al., 1979; Parker et al., 2012).

Understanding whether secondarily-treated effluent discharged in southern California has the capacity to stimulate harmful algal bloom (HAB) events is of utmost concern. The recent observed global increase in severity and frequency of HAB events has been correlated with human-influenced processes of global climate change (Fu et al., 2012; Moore et al., 2008) and eutrophication (Anderson et al., 2002; Glibert et al., 2006; Paerl, 1997), although not all HAB events can be directly linked to anthropogenic influences (Davidson et al., 2012; Kudela et al., 2008). Diatoms of the genus *Pseudo-nitzschia* are common to southern California waters and HAB events attributed to domoic acid (DA)-producing members of this genus occur frequently during the spring (Caron et al., 2010; Seubert et al., 2013; Seubert et al., 2012). Bloom events in southern California are typically accompanied by illness and often death of marine mammals and birds through DA toxicosis (Fire et al., 2010; Schnetzer et al., 2007; Torres de la Riva et al., 2009). These blooms have been linked to nutrient injection into surface waters and perhaps 'seeding' of blooms with subsurface populations of *Pseudo-nitzschia* during upwelling events in the area (Schnetzer et al., 2013; Seegers et al., 2015; Seubert et al., 2013).

A direct impact of anthropogenic nutrient loading has not been implicated in toxic bloom development in southern California (Lewitus et al., 2012), but the importance of such sources has been postulated for central California (Kudela et al., 2008). Growth rate and DA-production of *Pseudo-nitzschia* have been shown to vary with nitrogen source (Bates et al., 1993; Cochlan et al., 2008; Hillebrand and Sommer, 1996; Howard et al., 2007; Thessen et al., 2009). Additionally, laboratory experiments investigating the effect of varying dilutions of secondarily-treated effluent from a Canadian

treatment plant demonstrated that growth of *Pseudo-nitzschia* was stimulated by effluent additions (Pan and Subba Rao, 1997). Therefore, it might be anticipated that nutrient loading along the coast of southern California due to the discharge of secondarily-treated effluent has the potential to enhance *Pseudo-nitzschia* growth and/or DA-production.

The Orange County Sanitation District (OCSD) in Fountain Valley, CA, USA, averages a discharge of over 380 million liters per day of secondarily-treated effluent. During secondary treatment, much of the organic matter is biologically oxidized and greater than 85 % of the total suspended solids and biochemical oxygen demand is removed. Inorganic nutrients that are a product of the biological oxidation process can stimulate autotrophic growth of plankton when discharged into the coastal ocean. Ocean outfall pipes for effluent discharge are designed to minimize their potential impact by discharging far from shore and below the euphotic zone, and initial dilution of effluent is increased through the use of multiple diffuser ports. The main discharge pipe used by OCSD since 1972 is located 8.0 km from shore at a depth of 56 m. It has a diameter of 3.1 m, and it is estimated to obtain an initial dilution of 1:350 with the surrounding seawater. In September 2012, OCSD diverted their primary effluent discharge from the 8.0 km pipe to an older pipe located 1.6 km from shore in order to perform necessary maintenance and repairs to their 8.0 km pipe. The older pipe discharges at a depth of 16 m and it is estimated to obtain an initial dilution of 1:36. The present study was conducted to experimentally investigate the potential impact of the diversion of secondarily-treated effluent to a shallow, near-shore environment on plankton standing stock and community composition. Toward this end, two incubation experiments were performed to examine the influence of several dilutions of treated effluent on a natural, coastal, plankton community collected one week prior to the planned OCSD diversion, and the second performed a week following the start of the diversion event. The experiments were a part of a large collaborative effort involving researchers from multiple universities

and agencies to understand and document the effect of the OCSD diversion event on the southern California Bight, summarized in Howard et al. (this issue).

2. Materials and Methods

*2.1 Study area and experimental design***.** Incubation experiments were performed in September 2012 employing natural plankton communities collected approximately 2 km off the coast of Orange County, CA, USA (Figure 1). The first experiment ('Pre-Diversion') occurred one week prior to the planned diversion of the OCSD effluent discharge, a second ('Mid-Diversion') occurred one week following the start of the diversion event (Howard et al., this issue). Water was collected in Niskin bottles attached to a rosette that housed conductivity, temperature and depth sensors (CTD; Seabird SBE 911+ CTD, Bellevue, WA, USA) and provided profiles to 100 m. The depth of the subsurface chlorophyll maximum (SCM) was determined utilizing real-time *in situ* fluorometry data from the CTD. Water for the incubation experiments was collected at 5 m or the SCM (see below) in acid-washed (5 % HCl) 20 L carboys directly from the Niskin bottles, taking care to minimize bubbling and agitation during transfer. The carboys were protected from light and kept cool during transport to shore for experimental setup. Incubations were performed in acid-washed, 4-L polycarbonate bottles kept at ambient water temperature, and covered with neutral density screening to approximate 50 % ambient light levels, in Los Angeles Harbor at the Southern California Marine Institute in San Pedro, CA.

The Pre-Diversion experiment was initiated on 6 September 2012, and included an experiment performed using water collected at the surface (5 m) and at the SCM (40 m; 33°30'40" N, 118° 3'32" W; Figure 1). The reason for examining these depths is that the 8.0 km pipe discharges at 56 m depth and could potentially influence the subsurface plankton community, while the 1.6 km pipe discharges at 16 m depth and therefore would presumably influence the community near the surface. In addition, it has

been hypothesized that upwelling events transport offshore subsurface plankton communities to the near coast euphotic zone, exposing them to higher irradiances and possibly effluent discharges (Schnetzer et al., 2013; Seegers et al., 2015; Seubert et al., 2013). The core treatments tested in triplicate were (1) a true control (no additions), (2) a deionized (DI) water control composed of an aliquot of water equivalent to the amount of water added in the effluent dilutions, and OCSD effluent additions representing dilutions of (3) 1:10, (4) 1:100 and (5) 1:1000 of effluent in natural seawater. The volumes of all treatments in each experiment were kept the same, and the amount of freshwater (effluent or DI) reduced the salinity in the treatment bottles from approximately 35 ppt to 32 ppt. The DI control was employed in order to determine any community changes that occurred solely due to the reduction in salinity.

Although the diversion began on 11 September 2012, a significant increase in phytoplankton growth was not observed in the region as originally hypothesized (Caron et al., this issue; Howard et al., this issue), and suggested by the results of the Pre-Diversion experiments conducted as a part of this study. The Mid-Diversion experiment was initiated on 20 September 2012, and focused on the response of a subsurface community to the treatments described above, and additional treatments investigating potential inhibitory substances in the effluent as well as the possibility of micronutrient limitation. Sample water was collected from the SCM at 17 m depth in the same manner as the Pre-Diversion experiment, at a station slightly closer to shore (33°33'38" N, 118° 1'1" W; Figure 1). The presence of inhibitory substances in the effluent was tested by adding an 'effluent mimic'. The effluent mimic was prepared in the laboratory in DI water and contained a final concentration of 2,900 µM ammonium (NH_4), 64 µM phosphate (PO_4), 10 µM silicate (SiO_3), and included micronutrients at the following concentrations - 500 pM of vitamins B_1 and B_7, 5000pM of vitamin B_{12} and a 1:100 dilution of the trace metal solution used in L1 enriched seawater medium (Guillard and Hargraves, 1993). The macronutrient concentrations in the effluent mimic were based on concentrations in natural OCSD effluent measured

in 2010 by Howard et al. (2014). The effluent mimic solution was added to a set of bottles at a 1:10 dilution for comparison, in order to investigate the possibility of substances inhibitory to phytoplankton growth in the 1:10 dilution of OCSD effluent. Additionally, micronutrient limitation was examined in two treatments; (1) a 1:10 dilution of OCSD effluent was supplemented with vitamins and trace metals in concentrations described above in the effluent mimic treatment and (2) addition of only vitamins and trace metals to the natural seawater sample.

2.2 Sample collection and processing. Samples were taken for analysis at the beginning of each experiment, and all bottles were sampled approximately every 24 hours during the first three days of incubation for chlorophyll *a*, particulate DA (pDA), dissolved DA (dDA), flow cytometric counts of microbial populations < 10 µm, cell counts of plankton > 10 µm, and dissolved inorganic nutrient analysis. Samples were taken again at the end of each experiment. The Pre-Diversion experiment was ended after 7 days of incubation, and the Mid-Diversion experiment was ended after 6 days of incubation. Samples for the analysis of dissolved inorganic nutrients were collected from the initial seawater sample, the effluent used in the dilution experiment, and from each treatment bottle during the first three days of incubation. Approximately 20 mL of sample was syringe filtered through a 0.2 µm syringe filter and dispensed into acid-washed plastic scintillation vials and frozen at -20° C until analysis. Nutrient analysis for concentrations of nitrate plus nitrite (NO_3 + NO_2; 0.2 µM limit of detection), NO_2 (0.1 µM limit of detection), NH_4 (0.1 µM limit of detection), PO_4 (0.1 µM limit of detection) and SiO_3 (1.0 µM limit of detection) were performed at the Analytical Lab at the Marine Sciences Institute at the University of California, Santa Barbara on a QuikChem 8000 flow injection analyzer (Lachat Instruments; Loveland, CO) with ± 5 % precision.

Samples for chlorophyll *a* and pDA concentrations were collected in duplicate by vacuum filtration of 10 to 100 mL and 200 mL samples, respectively, onto Grade F glass fiber filters (Sterlitech, Kent, WA) and frozen at -20° C until analysis. The volume filtered for chlorophyll *a* analysis was reduced

as needed from 100 mL when concentrations exceeded the calibrated range of the fluorometer. Filters were extracted in 4 mL of 100 % acetone for 24 hours at -20° C and analyzed on a calibrated Trilogy® laboratory fluorometer with the chlorophyll *a* non-acidification module (7200-046; Turner Designs Inc., Sunnyvale, CA). Filters for pDA were extracted in 3 mL of 10 % methanol and analyzed using the Mercury Science Inc. DA Enzyme-Linked ImmunoSorbent Assay (ELISA; Durham, NC) following the methods described in Seubert et al. (2012) with a 0.02 µg /L limit of detection.

Flow cytometry samples for the abundances of heterotrophic bacteria, picoeukaryotes, *Prochlorococcus* spp. and *Synechococcus* spp. were pre-screened through 80 µm Nitex screening and preserved with a final concentration of 1 % formalin, flash frozen in liquid nitrogen, and stored at -80° C until analysis on a four-color, dual-laser FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Natural, unfiltered seawater samples for plankton community composition were preserved with a final concentration of 1 % formalin, stored in the dark at 4° C and examined by inverted light microscopy at 400x after settling in Utermöhl chambers for 24 hours (Utermöhl, 1958). Organisms greater than 10 µm in size were identified first by major taxonomic group and then to genus and species, when possible. Cells identified as *Pseudo-nitzschia* were divided into size classes based upon frustule width, as conclusive species identification is not possible without electron microscopy (Hasle et al., 1996; Hasle and Syvertsen, 1997) or the use of molecular methods (Hubbard et al., 2008; Scholin et al., 1996). The *P. seriata* size class was defined as cells with frustule widths greater than 3 µm and the *P. delicatissima* size class had frustule widths smaller than 3 µm (Hasle and Syvertsen, 1997).

Statistical analyses and comparisons of the community composition data obtained from the settled cell counts from each experiment were performed using PRIMER v6 (Clarke and Gorley, 2006). Species composition data were log (x+1) transformed prior to Bray-Curtis similarity calculations. The similarity data were plotted using the Cluster and Multi-Dimensional Scaling (MDS) functions available in the PRIMER v6 software (Clarke, 1993). The different treatments from within a single experiment were

compared to each other, and the results from all experiments were also compared directly. The Pre-Diversion and Mid-Diversion results were compared to one another for the first three days of incubation because the three experiments were ended on different days (7 days in the Pre-Diversion, and 6 days in the Mid-Diversion).

3. Results

3.1.1 Pre-Diversion surface water response. Total phytoplankton biomass (i.e. chlorophyll *a* concentration) in the 1:10 effluent treatment (i.e. the highest concentration of effluent) showed no significant response during the first three days of incubation (Figure 2). The values for that treatment were considerably less than chlorophyll values in the 1:100 and 1:1000 treatments during the same period, but increased dramatically to 210 ± 35 µg L^{-1} by Day Seven. The latter value was highest for all treatments and all time points (Figure 2). The increase in chlorophyll *a* concentration in the 1:10 treatment was a > 1,000-fold increase over the initial concentration, 0.20 ± 0.01 µg L^{-1}. Chlorophyll *a* concentrations in the 1:100 and 1:1000 treatments did not exhibit the lag in growth observed in the 1:10 treatment. The 1:100 treatment increased 110-fold over initial concentrations, reaching 22.0 ± 2.4 µg L^{-1} by the end of the experiment. The 1:1000 treatment increased 16-fold in chlorophyll *a* concentrations over the initial value during the first three days of incubation. Chlorophyll *a* concentration decreased in that treatment between Day Three and Day Seven, and the final chlorophyll *a* concentration (0.92 ± 0.18 µg L^{-1}) constituted only a 5-fold increase from the initial concentration.

The phytoplankton community > 10 µm in the surface at the time of collection was dominated by diatoms, and diatoms remained numerically dominant (85 % of community or higher) in all treatments throughout the duration of the experiment. Diatom abundance in the 1:10 treatment of 3.30 ± 1.20 x 10^4 cells mL^{-1} on Day Seven in the 1:10 treatment was the highest measured for all

treatments and time points (Figure 2). The 1:100 and 1:1000 effluent treatments yielded the highest abundances of diatoms during the first three days of the experiment compared to the 1:10 treatment and the controls. Photosynthetic picoeukaryote abundance was approximately 10^6 cells mL^{-1} by the end of the experiment in the 1:10 treatment (Figure 2). The 1:100 and 1:1000 treatments had abundances of picoeukaryotes greater than the 1:10 treatment during the first three days of the incubation, however the former two treatments decreased in picoeukaryote abundance between Day Three and Day Seven.

Abundances of the picocyanobacteria genera, *Prochlorococcus* and *Synechococcus,* did not show the dramatic, positive responses to effluent addition that were observed for the microplanktonic and picoplanktonic eukaryotic phytoplankton (Figure 2). The former populations exhibited only modest increases or even decreases over the entire course of the experiment. In contrast, heterotrophic bacteria responded strongly and positively in the 1:10 effluent treatment throughout the experiment, without the three-day lag observed for chlorophyll *a*, diatoms, picoeukaryotes, and *Prochlorococcus* in this treatment (Figure 2).

Nutrients in the effluent were highly elevated and N:P was markedly different relative to the coastal waters from which the experimental water was collected (Table 1). The surface water used in the incubation experiments had a N:P of 8.2, and NH_4 was the dominant nitrogen source at 1.40 µM. The effluent had a N:P of 97, and NH_4 the dominant nitrogen source at 1,700 µM, although NO_3 and NO_2 were also present in high absolute concentrations of 340 and 100 µM, respectively. Concentrations of macronutrients in the 1:10 and 1:100 treatments remained high and relatively constant throughout the experiment, while NH_4 and PO_4 concentrations decreased slightly after three days in the 1:1000 treatment (Figure 3). NO_3, NO_2 and SiO_3 did not show obvious decreases in concentrations over the first three days of the incubation in the 1:100 treatment. The SiO_3 concentration in the DI control and the

effluent addition treatments that were diluted in DI prior to dosing the experiment bottles (1:100 and 1:1000 treatments) were higher than expected, suggesting the DI water may have contained SiO_3.

3.1.2 Pre-Diversion SCM water response. Chlorophyll *a* concentrations in the 1:10 treatment decreased significantly during the first three days of the Pre-Diversion experiment conducted with the plankton community collected from the subsurface chlorophyll maximum, and then rapidly increased (Figure 4). The highest concentration was measured in this treatment on Day Seven, 175 ± 22 µg L^{-1}, a 270-fold increase over the initial chlorophyll *a* concentration of 0.64 µg L^{-1} (compared to > 1,000-fold increase observed in the 1:10 treatment using surface water). Neither the 1:100 nor 1:1000 treatments experienced significant decreases in total phytoplankton biomass during the first three days of incubation. The 1:100 treatment had the second highest chlorophyll *a* concentration on Day Seven with 54.0 ± 4.8 µg L^{-1}, an 84-fold increase over the initial chlorophyll *a* concentration. The 1:1000 treatment had an 11-fold increase, with a chlorophyll *a* concentration on Day Seven of 7.21 ± 2.18 µg L^{-1}.

Plankton abundance > 10 µm in the SCM at the start of the experiment was dominated by diatoms, comprising approximately 70 % of the community numerically, and increased in relative abundance in all treatments to reach virtually 100 % dominance by Day Six (Figure 4). The 1:10 treatment had the lowest diatom concentration of any treatment during the first three days of the experiment but increased by Day Seven to 1.9 ± 1.1 x 10^4 cells mL^{-1}. The 1:100 treatment had the highest abundance of diatoms on Day Seven at 2.6 ± 0.35 x 10^4 cells mL^{-1}. Photosynthetic picoeukaryote abundances exhibited a trend among treatments similar to the diatoms (Figure 4). Abundances in the 1:10 treatment decreased more than an order of magnitude during the first three days of incubation (Figure 4) but achieved abundances > 10^6 cells mL^{-1} by Day Seven, a value similar to the highest picoeukaryote abundances measured in the same treatment using surface water.

Abundances of *Prochlorococcus* and *Synechococcus* in the SCM water did not exhibit positive responses to the addition of effluent, contrary to the stimulatory results observed in the planktonic

eukaryotes (Figure 4). Abundances of *Prochlorococcus* dropped precipitously (nearly two orders of magnitude) during the first three days of incubation in the 1:10 effluent treatment, and never recovered to initial values. The heterotrophic bacteria assemblage exhibited an overall positive response to effluent addition, although the response was not as substantial as it was in the surface water community (Figure 4). While the 1:10 effluent treatment with a surface water community exceeded 10^7 cells mL^{-1}, none of the effluent addition treatments with the SCM community exceeded 10^6 cells mL^{-1}.

Nutrient concentrations in the unamended SCM water were slightly greater than concentrations in the surface water, but overall nutrient concentrations and N:P of the effluent and SCM were still markedly different (Table 1). The dominant nitrogenous source in the SCM was NH_4 at a concentration of 3.10 µM, followed by NO_3 (2.20 µM) and NO_2 (0.10 µM). Concentrations of PO_4 and SiO_3 were 0.22 and 4.80 µM, respectively, similar to the concentrations measured in the surface water. Concentrations measured during the first three days of the experiment remained relatively consistent without a noticeable decrease (Figure 5) with the exception of the DI controls which appeared to be contaminated with SiO_3.

3.2 Mid-Diversion experimental outcome. Initial chlorophyll *a* concentration in the water collected from the SCM for the Mid-Diversion experiment was greater than values obtained during the Pre-Diversion experiment: 4.10 ± 0.61 µg L^{-1} vs. 0.20 ± 0.01 (surface) and 0.64 ± 0.02 (SCM). Both the true control and DI control decreased in chlorophyll *a* concentration throughout the experiment to final concentrations of 0.67 ± 0.09 and 0.76 ± 0.08 µg L^{-1} in the true and DI controls, respectively (Figure 6). The highest chlorophyll *a* concentration in this experiment (61.2 ± 1.7 µg L^{-1}) was measured on the sixth day of incubation in the 1:10 treatment, a 15-fold increase over the initial concentration. This concentration was substantially lower than the maximal value recorded during the Pre-Diversion experiment performed with surface water, 210 ± 35 µg L^{-1}, and in the relative increase (15-fold increase versus a > 1,000-fold increase).

Diatoms dominated the plankton > 10 µm in the SCM collected for the experiment, comprising nearly 100 % of the community numerically throughout the experiment (Figure 6). Following an initial increase in all treatments through Day Three, diatoms decreased towards the end of the experiment although abundances were still higher in the 1:10 and 1:100 treatments on Day Six relative to initial abundances. Photosynthetic picoeukaryote attained highest abundances in the 1:10 treatment, reaching 8.6 ± 0.91 x 10^4 cells/mL on Day Six (Figure 6). Picoeukaryote abundances in the 1:100 and 1:1000 treatments decreased between Day Three and end of the experiment.

Abundances of *Prochlorococcus* and *Synechococcus* decreased in all treatments throughout the experiment (Figure 6), while heterotrophic bacteria increased in the 1:10 treatment throughout the incubation period, again achieving values > 10^7 ml^{-1} by the end of the experiment (Figure 6). Overall, the negative response by the picocyanobacteria compared to the positive responses observed in the microplanktonic and picoplanktonic eukaryote phytoplankton and bacteria was similar to the results obtained in the Pre-Diversion experiments.

Nutrient concentrations in the SCM water collected for the Mid-Diversion experiment were lower than values in the Pre-Diversion water (Table 1). The dominant form of nitrogen was NH_4 (0.25 µM) while NO_3 and NO_2 were below the method detection limit (0.2 and 0.1 µM, respectively) and the resulting N:P was 2.5. The OCSD effluent collected for the Mid-Diversion experiment had similar PO_4 and SiO_3 concentrations as the sample collected for the Pre-Diversion, but differed most noticeably in the NH_4 and NO_2 concentrations. The NH_4 concentration of 2,200 µM was 30 % higher than the Pre-Diversion effluent and the NO_2 concentration of 59.0 µM was 41 % lower than the Pre-Diversion effluent. The effluent mimic that was prepared in the laboratory based upon measurements made in 2010 was higher in concentration in NH_4 and PO_4 than the Mid-Diversion effluent, but the N:P was significantly lower, 45 versus 122 in the effluent sample.

Macronutrient concentrations decreased in all treatments, in contrast to the relatively stable concentrations measured in the effluent treatments of the Pre-Diversion experiments (Figure 7). The 1:100 treatment had the most remarkable changes in macronutrient concentrations; PO_4 was below detection by the second day of incubation and SiO_3 was below detection by the third day (Figure 7). The decreases in macronutrient concentration in the 1:100 treatment by Day Three ranged from 40 to 85 % of initial values whereas the decreases seen in the surface and SCM 1:100 treatments of the Pre-Diversion experiment ranged from 0 to 42 %.

3.3 Comparisons of community composition and similarity. Cell counts from the > 10 µm plankton were used to assess changes in community composition during the incubations, and in Bray Curtis Similarity calculations and MDS plots comparing the communities in the Pre-Diversion surface, Pre-Diversion SCM, and Mid-Diversion experiments. Comparisons were made within results from each experiment and subsequently between all experiments.

The Pre-Diversion experiment using surface water was dominated by *Pseudo-nitzschia* (*delicatissima* size class) in both the initial sample and throughout the experiment in all treatments except the 1:10 effluent addition. The *P. delicatissima* size class comprised only 16 % of the 1:10 treatment community at its highest abundance, whereas the diatom *Cylindrotheca* spp. was dominant throughout. The *P. seriata* size class was also present throughout the experiment in all treatments, although typically less than 10 % of the community. Samples collected for pDA were analyzed for all treatments and time points but none were above the detection limit of the method (0.02 µg L^{-1}).

The Bray Curtis Similarity comparison of phytoplankton communities in the Pre-Diversion surface treatments revealed that only the 1:10 treatment community sampled after one day of incubation was less than 40 % similar to all other samples collected over the course of the experiment (Figure 8). The communities present in the 1:100 and 1:1000 treatments ranged from 60 to 80 % similar throughout the experiment. Therefore, although the biomass of large phytoplankton changed

dramatically during the experiment, the community structure was not strongly affected by containment or addition of diluted effluent.

The SCM community present at the start of the Pre-Diversion experiment was dominated by the diatom *Navicula* spp. and the *P. delicatissima* size class was below the detection limit of the settled counts (3 cells mL^{-1}). The 1:10 treatment had low abundances of the *P. delicatissima* size class throughout the experiment, ranging from 0 to 13 % of the community. By the end of the experiment, however, the *P. delicatissima* size class was the dominant member of the community in both the true control, DI control and effluent treatments of 1:100 and 1:1000, comprising 70 % or more of the community. The *P. seriata* size class was a minor contributor to the community, akin to the surface community results described above. All samples were below the detection limit for pDA. The community observed in the 1:10 treatment after one and two days of incubation was 60 % similar to the community observed in the initial SCM community, according to the Bray Curtis Similarity analysis (Figure 8). Communities in both controls and the 1:100 and 1:1000 treatment were 60 % similar to one another after Day One and Two of incubation. The communities were 80 % similar in the samples collected on Day Three and at the end of the experiment, again indicating similar trajectories in all treatments of community composition of the plankton > 10 μm.

Diatoms dominated the plankton community collected Mid-Diversion, however none of the individual genera identified comprised more than 25 % of the community. The common diatom genera observed (each constituting 10 to 25 % of total abundance) were *Asterionellopsis*, *Chaetoceros*, *Leptocylindrus* and the *P. delicatissima* and *P. seriata* size classes. The *P. delicatissima* and *P. seriata* size class was present in all treatments and time points, ranging 12 to 48 % and 3 to 28 % of the community, respectively. Measurable pDA concentrations were present in all treatments and time points, although the initial water sample was below detection. The two highest pDA concentrations occurred in the 1:100 effluent treatment samples collected on Day Two and Three, 0.23 ± 0.025 and 0.42 ± 0.057 μg L^{-1},

respectively. Bray Curtis Similarity analysis revealed the communities observed throughout the experiment in the various treatments were 60 % similar (Figure 8). The 1:10 and 1:100 treatment communities present in samples from Day Two and Three of the experiment were 80 % similar.

Direct comparison of the Pre-Diversion surface, Pre-Diversion SCM, and Mid-Diversion experiment community compositions failed to show a strong relationship between the 1:10 effluent communities across experiments (Figure 8). Bray Curtis Similarity analysis revealed the 1:10 effluent communities were less than 40 % similar, regardless of incubation period.

4. Discussion

The addition of effluent at a 1:10 dilution to natural seawater was employed in the present study to represent a 'worst case scenario' that may occur when discharging agencies are required to divert effluent streams to older discharge systems during times of maintenance, repair, or emergency. Designs of modern sewage outfalls rely on rapid and considerable dilution of effluent upon discharge in order to diminish impacts on the marine environment. The 2012 OCSD diversion event diverted effluent flow from a pipe 8.0 km offshore, with an estimated initial dilution of 1:350, to an older pipe located 1.6 km from shore with an estimated initial dilution of 1:36. This substantially lower initial dilution, coupled to the magnitude of the discharge volume, was anticipated to result in a significant positive response of the phytoplankton community as a consequence of increased nutrient loading of surface waters in the vicinity of the outfall.

In support of this expectation, a previous outfall diversion conducted by the Hyperion Water Treatment Plant, in Santa Monica Bay, CA, for three days during 2006 was followed by a phytoplankton bloom of HAB dinoflagellates correlated with environmental parameters indicative of effluent plume water (Reifel et al., 2013). The dilution of the Hyperion effluent plume at their pipe located 1.6 km from

shore was estimated to be 1:13, but observations made during the short diversion determined the initial dilution was actually 1:11 (Reifel et al., 2013). The present study aimed to examine the potential of a HAB event accompanying the OCSD diversion and whether or not increased productivity near the area of diversion could be attributed directly to the stimulation of phytoplankton growth by the presence of effluent.

4.1 Effluent impact on the planktonic community. Chlorophyll *a* concentrations displayed an overall positive response to effluent addition in all experiments and at all effluent concentrations. Chlorophyll *a* concentrations > 10 μg L^{-1} are typically considered 'bloom' events in the southern California region, with major blooms reaching concentrations as high as 40 μg L^{-1} (Kim et al., 2009; Seubert et al., 2013). The 1:10 effluent additions resulted in phytoplankton biomass an order of magnitude above bloom conditions (100 μg L^{-1} chlorophyll). The 1:100 effluent additions also produced chlorophyll *a* concentrations exceeding 10 μg L^{-1}, demonstrating effluent discharges between a 1:10 and a 1:100 dilution in the region have the potential to stimulate blooms. Nonetheless, a massive bloom did not occur during the 2012 OCSD diversion event (Caron et al., this issue; Howard et al., this issue).

OCSD enhanced their chlorination procedures during the diversion, and the byproducts of this process have previously been shown to inhibit photosynthetic performance in the plankton community (Carpenter et al., 1972; Eppley et al., 1976) . The Pre-Diversion experiments in the present study had a three-day lag between effluent addition and an increase in chlorophyll concentration (Figures 2 and 4) which was similar to the three-day inhibition of photosynthetic activity observed in experiments performed by Kudela et al. (this issue) using OCSD chlorination solutions. However the Mid-Diversion experiments did not exhibit a lag in chlorophyll concentration increase, perhaps indicating that chlorination levels were not constant during the diversion, or adaptation of the phytoplankton community as the diversion progressed (Figure 6).

It is also conceivable that the lag in chlorophyll *a* concentration observed during the Pre-Diversion experiments was to some degree a consequence of NH_4 inhibition of NO_3 uptake, although it is unlikely that nitrogen uptake was completely inhibited by NH_4 concentrations attained in coastal waters in this study. Nutrient uptake by plankton is directly influenced by genetics, biochemistry and physiology (Cochlan et al., 1991; Dortch, 1990; Dortch et al., 1984; Kudela et al., 1997; Lomas and Glibert, 2000), and those factors determine the impact that nitrogenous source have on plankton community structure. The dominant nitrogenous source in secondarily treated effluent is NH_4, a reduced form of N that is often preferred over NO_3 by phytoplankton, and is also known to inhibit NO_3 uptake in some species. In San Francisco Bay, for example, high concentrations of NH_4 introduced to the system primarily through effluent discharge, have been implicated in the suppression of NO_3 uptake (Dugdale et al., 2007; Parker et al., 2012; Wilkerson et al., 2006). Observations made in southern Californian waters have demonstrated both NH_4 inhibition of growth (MacIsaac et al., 1979; Thomas et al., 1980; Thomas et al., 1974) as well as growth stimulation by effluent (Allan Hancock Foundation, 1964; Eppley et al., 1972; Thomas et al., 1980; Thomas et al., 1974). The increase in chlorophyll *a* concentrations in the Pre-Diversion experiment treatments occurred after Day Three, before all of the NH_4 had been removed (Figure 2 and 4), and there were no lags in chlorophyll *a* concentration with effluent addition during the Mid-Diversion experiments. These results imply that NH_4 inhibition was not a significant factor retarding initial phytoplankton growth during the OCSD diversion. Micronutrient limitation was examined in the Mid-Diversion experiment, although the addition of vitamins and trace metals did not significantly increase planktonic growth compared to the core treatment results (Figures 5 and Suppl. Figure 1). Chlorination remains the most plausible hypothesis for the three-day lag in response observed in the Pre-Diversion experiments.

Diatoms and photosynthetic picoeukaryotes were the primary phytoplankton groups responsible for increases in chlorophyll *a* concentrations in all experiments (Figures 2, and 4).

Stimulation of diatoms by effluent addition has been previously reported (Oviatt et al., 1989; Pan and Subba Rao, 1997), and some previous work has noted as association between effluent addition and diatom species composition (Dunstan and Tenore, 1972; Thompson and Ho, 1981). In contrast, *Prochlorococcus* and *Synechococcus* had an overall negative response to effluent addition (Figures 2 and 4). This negative response was surprising, and contrary to previous research documenting both positive and neutral responses by *Prochlorococcus* and *Synechococcus* to the presence of effluent (Petrenko et al., 1997; Ritchie et al., 2001; Taslakian and Hardy, 1976). Interestingly, the heterotrophic bacterial community had the most significant response to effluent addition, increasing to concentrations > 5 x 10^7 cells mL^{-1} in some treatments (Figures 2 and 4), presumably indicating the presence of significant amounts of labile organic material in the secondarily-treated effluent, or created via the chlorination process. These dramatic increases parallel the increases in heterotrophic bacterial abundances observed during field monitoring of the nearshore waters throughout the diversion period and discussed in Caron et al. (this issue).

4.2 Stimulation of HAB events. Pseudo-nitzschia from both size classes were observed in all three experiments. The Pre-Diversion experiments did not yield detectable pDA concentrations while the Mid-Diversion experiments had detectable pDA concentrations in all treatments and time points except for the original sample. The highest pDA concentrations occurred in the 1:100 treatment, with a pDA concentration of 0.23 ± 0.025 µg L^{-1} measured on Day Two and 0.42 ± 0.057 µg L^{-1} measured on Day Three. The increase in pDA concentration from Day One (0.064 ± 0.017 µg L^{-1}) to Day Two coincided with a decrease in PO_4 concentration from 0.19 ± 0.015 µM on Day One to below detection by Day Two (0.1 µM detection limit). PO_4 concentration remained below detection on Day Three and the SiO_3 concentration decreased from 2.52 ± 0.48 µM on Day Two to below detection by Day Three. The correspondence between the highest pDA concentration and undetectable PO_4 and SiO_3 concentrations is in agreement with laboratory and field studies that have revealed a relationship between enhanced

pDA production and nutrient limitation by PO_4 and/or SiO_3 (Fehling et al., 2004; Pan et al., 1996a; Pan et al., 1996b; Schnetzer et al., 2007; Seubert et al., 2013; Sun et al., 2011; Tatters et al., 2012). The higher abundances of the *P. seriata* size class observed in the Mid-Diversion treatments presumably had an impact on the detection of DA, as blooms associated with high concentrations of pDA have been attributed mainly to members of this size class, especially in southern California (Busse et al., 2006; Schnetzer et al., 2013; Schnetzer et al., 2007; Seubert et al., 2013).

5. Conclusions

The present study investigated the response of natural plankton communities to several dilutions of OCSD secondarily treated effluent in three separate incubation experiments. The 1:10, 1:100 and 1:1000 effluent additions in the Pre- and Mid-Diversion experiments increased chlorophyll *a* concentrations and the abundances of diatoms, photosynthetic picoeukaryotes and heterotrophic bacteria. The 1:10 treatment, representing a worst case scenario of minimal effluent dilution, revealed that effluent had great potential to stimulate phytoplankton biomass in the coastal receiving waters (3 orders of magnitude in a week). The most dilute effluent tested (1:1000) introduced nutrients at concentrations lower than the magnitude that would be expected during an upwelling event, but higher than the ambient concentrations observed at the start of either experiment. The potential for a major response of the resident plankton community existed during the 2012 diversion, especially for > 10 µm plankton and heterotrophic bacteria.

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Table 1. Nutrient concentrations measured in the initial whole seawater sample and the nutrient concentrations of the effluent sample used in the Pre-Diversion (6 September 2012), and Mid-Diversion (20 September 2012) experiments. N:P is reported by atoms and samples below the method detection limit are reported as "bd".

Date	Source	Type	NH_4 (µM)	NO_3 (µM)	NO_2 (µm)	PO_4 (µM)	SiO_3 (µM)	N:P
6 Sept 2012	Surface	Seawater	1.40	0.23	bd	0.20	4.20	8
	SCM	Seawater	3.10	2.20	0.10	0.22	4.80	25
	OCSD	Effluent	1,700	340	100	22.0	590	97
20 Sept 2012	SCM	Seawater	0.25	bd	bd	0.10	bd	2.5
	OCSD	Effluent	2,200	420	59.0	22.0	590	122
		Effluent Mimic	2,900	-	-	64.0	10	45

Figure 1. Location of the study area in southern California showing the locations where water was collected for the Pre-, and Mid-Diversion effluent incubation experiments.

Figure 2. Chlorophyll *a* concentrations and the abundances of diatoms, photosynthetic picoeukaryotes, *Prochlorococcus* spp., *Synechoccus* spp., and heterotrophic bacteria measured during the Pre-Diversion effluent addition experiment with surface water, plotted separately for each treatment. Results from the true controls are plotted with open circles and dashed lines, DI controls with open circles and dotted lines, 1:10 effluent additions with black circles and solid black lines, 1:100 effluent additions with dark grey circles and solid dark grey lines and 1:1000 effluent additions with light grey circles and solid light grey lines.

Figure 3. Nutrient concentrations of NH_4, NO_3, NO_2, PO_4, and SiO_3 measured during the Pre-Diversion effluent addition experiment with surface water are plotted separately for each treatment. Results from the true controls are plotted with open circles and dashed lines, DI controls with open circles and dotted lines, 1:10 effluent additions with black circles and solid black lines, 1:100 effluent additions with dark grey circles and solid dark grey lines and 1:1000 effluent additions with light grey circles and solid light grey lines. The method detection limit for each nutrient analyte is marked on the graphs with a dotted light grey line.

Figure 4. Chlorophyll *a* concentrations and the abundances of diatoms, photosynthetic picoeukaryotes, *Prochlorococcus* spp., *Synechococcus* spp., and heterotrophic bacteria measured during the Pre-Diversion effluent addition experiment with water from the SCM are plotted separately for each treatment. Results from the true controls are plotted with open circles and dashed lines, DI controls with open circles and dotted lines, 1:10 effluent additions with black circles and solid black lines, 1:100 effluent additions with dark grey circles and solid dark grey lines and 1:1000 effluent additions with light grey circles and solid light grey lines.

Figure 5. Nutrient concentrations of NH_4, NO_3, NO_2, PO_4, and SiO_3 measured during the Pre-Diversion effluent addition experiment with water from the SCM are plotted separately for each treatment. Results from the true controls are plotted with open circles and dashed lines, DI controls with open circles and dotted lines, 1:10 effluent additions with black circles and solid black lines, 1:100 effluent

additions with dark grey circles and solid dark grey lines and 1:1000 effluent additions with light grey circles and solid light grey lines. The method detection limit for each nutrient analyte is marked on the graphs with a dotted light grey line.

Figure 6. Chlorophyll *a* concentrations and the abundances of diatoms, photosynthetic picoeukaryotes, *Prochlorococcus* spp., *Synechococcus* spp., and heterotrophic bacteria measured during the Mid-Diversion effluent addition experiment are plotted separately for each treatment. Results from the true controls are plotted with open circles and dashed lines, DI controls with open circles and dotted lines, 1:10 effluent additions with black circles and solid black lines, 1:100 effluent additions with dark grey circles and solid dark grey lines and 1:1000 effluent additions with light grey circles and solid light grey lines.

Figure 7. Nutrient concentrations of NH_4, NO_3, NO_2, PO_4, and SiO_3 measured during the Mid-Diversion effluent addition experiment are plotted separately for each treatment. Results from the true controls are plotted with open circles and dashed lines, DI controls with open circles and dotted lines, 1:10 effluent additions with black circles and solid black lines, 1:100 effluent additions with dark grey circles and solid dark grey lines and 1:1000 effluent additions with light grey circles and solid light grey lines. The method detection limit for each nutrient analyte is marked on the graphs with a dotted light grey line.

Figure 8. MDS plots using Bray-Curtis Similarities for the plankton community composition of each treatment throughout the Pre-Diversion experiments with surface water and SCM water, the Mid-Diversion experiment, and all three experiments compared to each other. Pre-Diversion surface samples are plotted with upward pointing triangles, SCM samples with downward pointing triangles, the Mid-Diversion samples are plotted with circles. All T0 samples are colored a light grey, true controls are in black, DI controls are black outlined symbols, 1:10 effluent treatments are in red, 1:100 effluent treatments are in yellow and the 1:1000 effluent treatments are in blue. Samples with Bray-Curtis Similarities of 40, 60 and 80 are circled on each plot in green, dark blue and light blue, respectively.

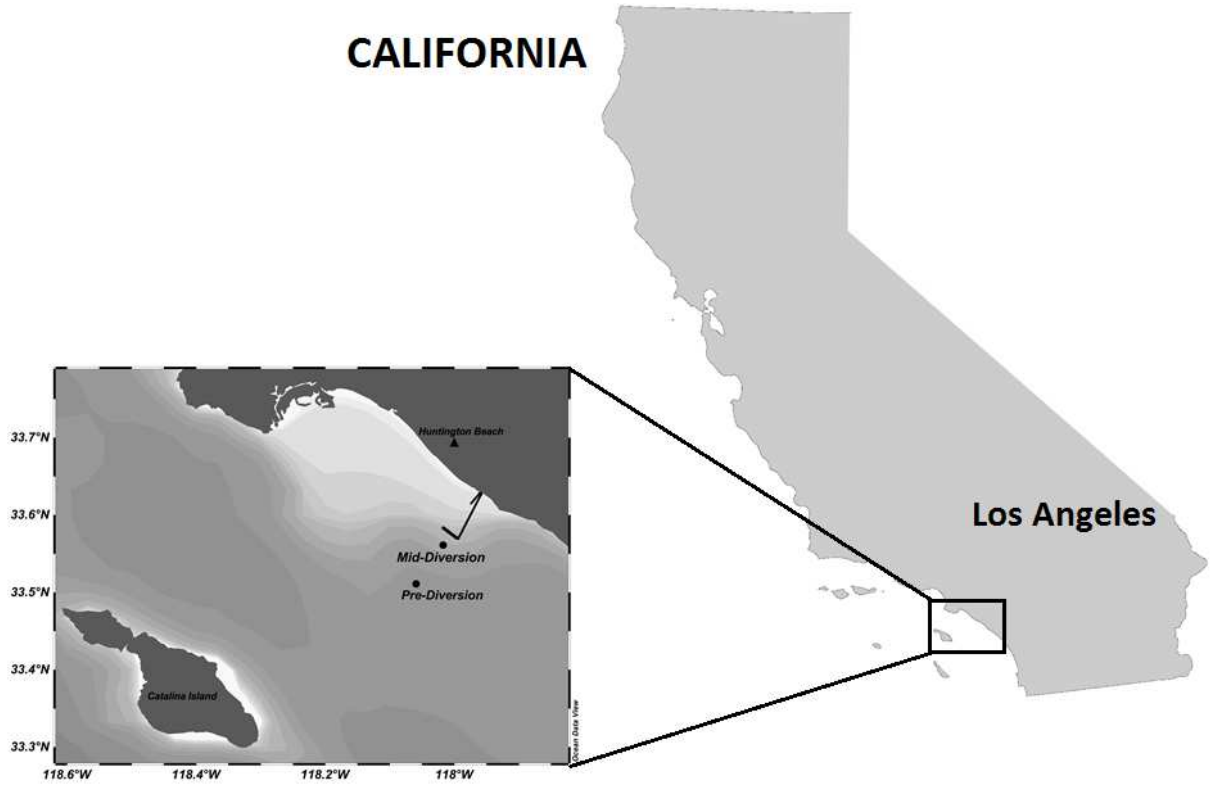
CALIFORNIA
Los Angeles
Huntington Beach
Mid-Diversion
Pre-Diversion
Catalina Island
33.7°N
33.6°N
33.5°N
33.4°N
33.3°N
118.6°W
118.4°W
118.2°W
118°W
Ocean Data View

Figure 1.

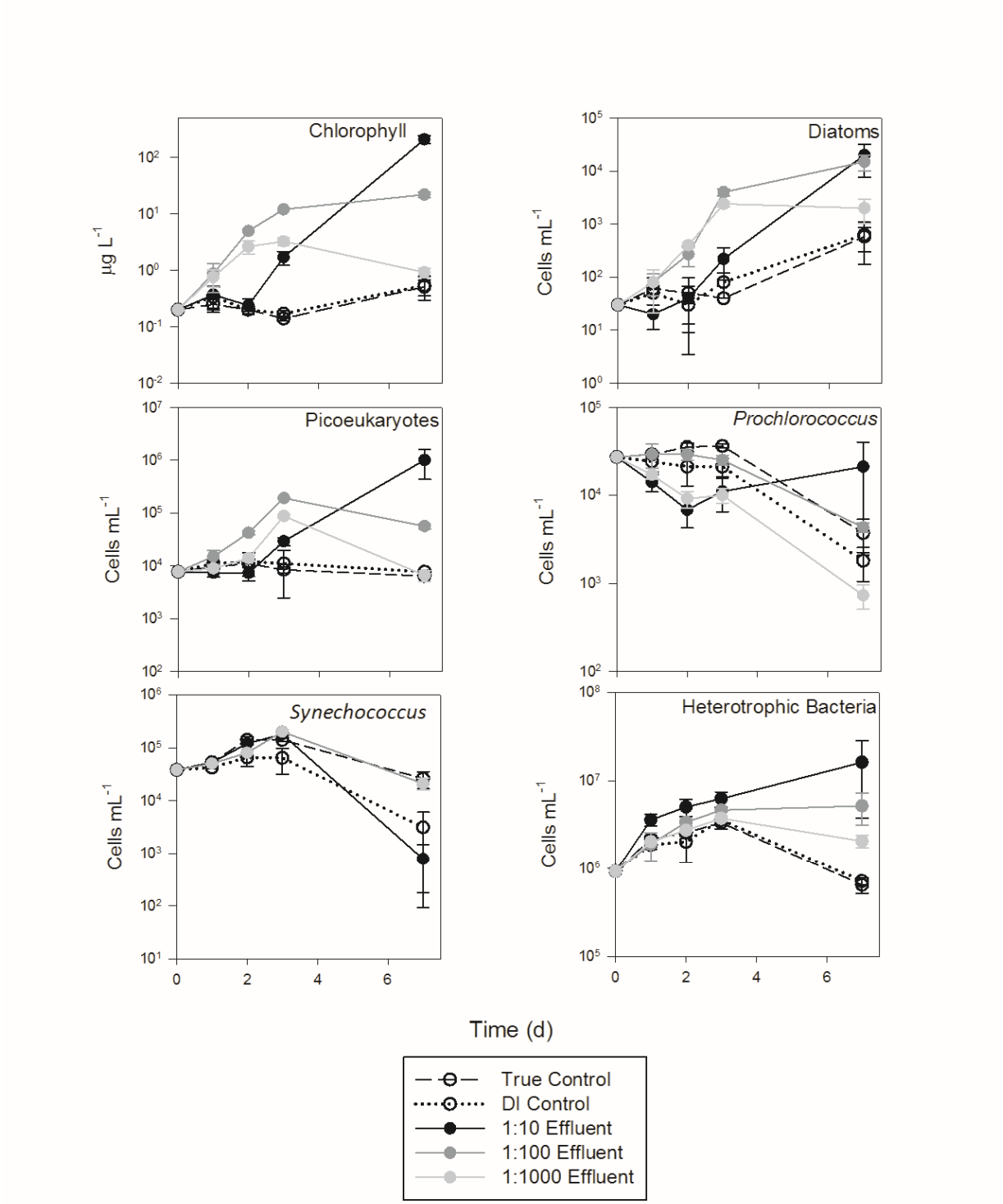
Chlorophyll
µg L-1
Diatoms
Cells mL-1
Picoeukaryotes
Cells mL-1
Prochlorococcus
Cells mL-1
Synechococcus
Cells mL-1
Heterotrophic Bacteria
Cells mL-1
Time (d)
True Control
DI Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent

Figure 2.

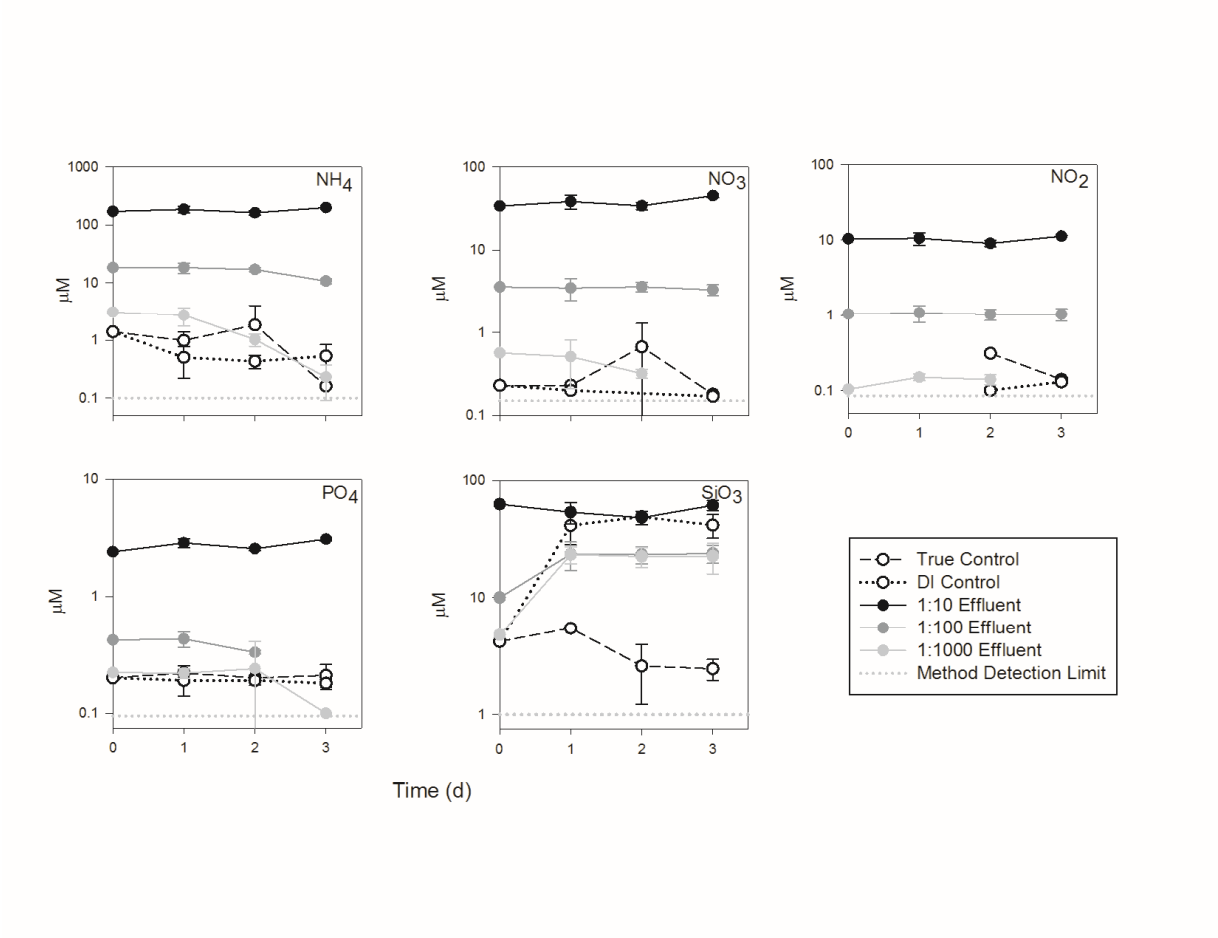
NH4
NO3
NO2
PO4
SiO3
µM
Time (d)
True Control
DI Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent
Method Detection Limit

Figure 3.

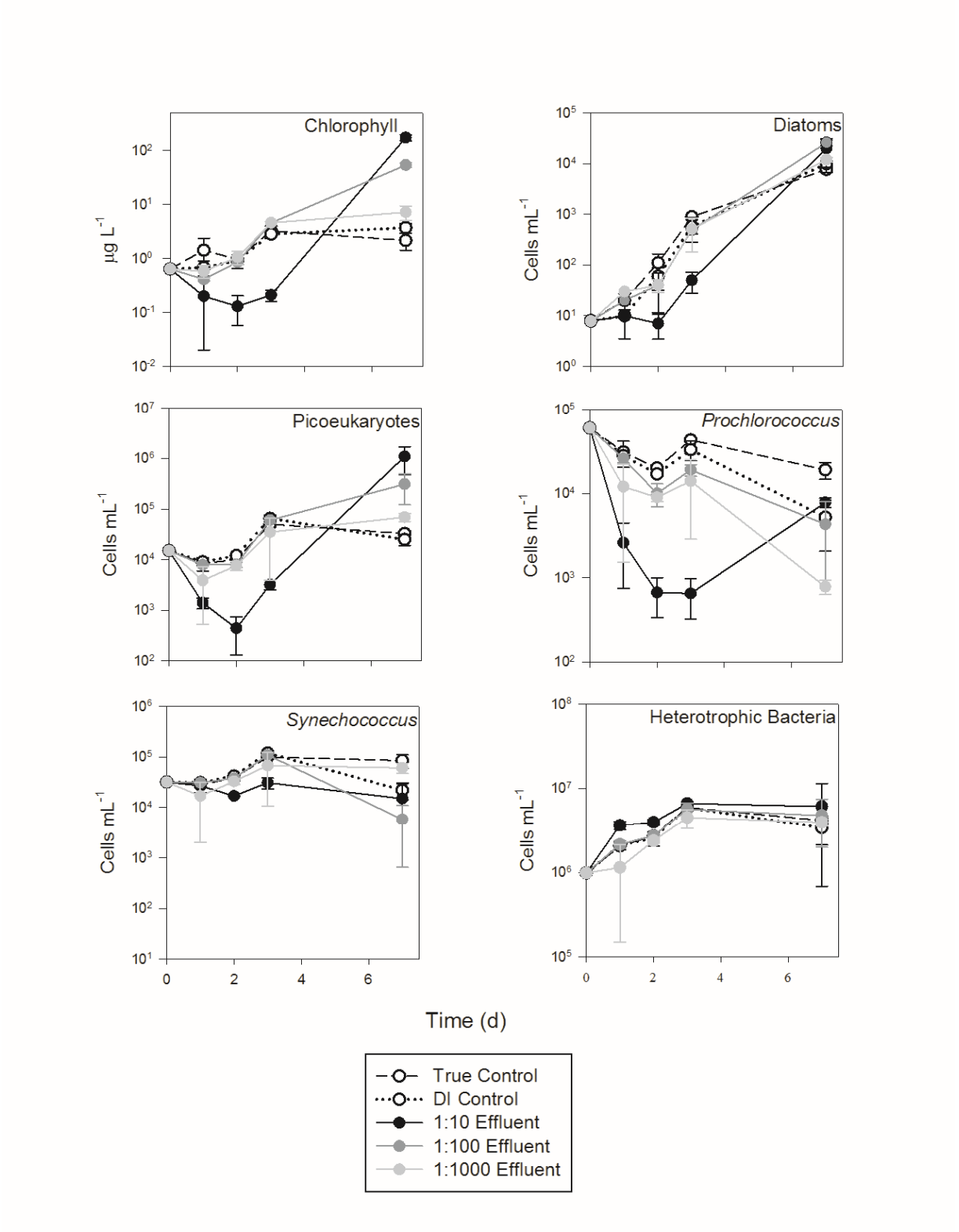
Chlorophyll
µg L-1
Diatoms
Cells mL-1
Picoeukaryotes
Cells mL-1
Prochlorococcus
Cells mL-1
Synechococcus
Cells mL-1
Heterotrophic Bacteria
Cells mL-1
Time (d)
True Control
DI Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent

Figure 4.

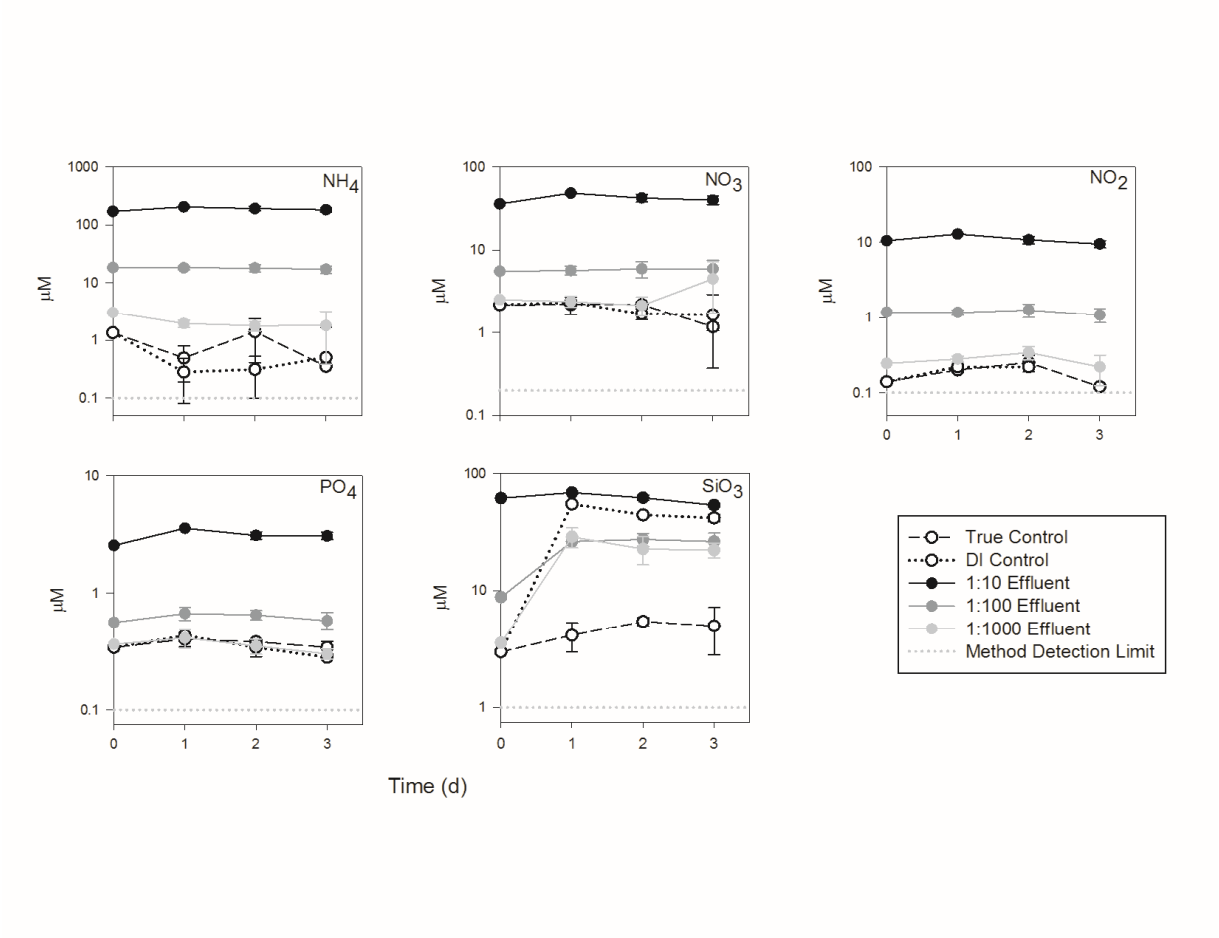
NH4
NO3
NO2
PO4
SiO3
µM
Time (d)
True Control
DI Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent
Method Detection Limit

Figure 5.

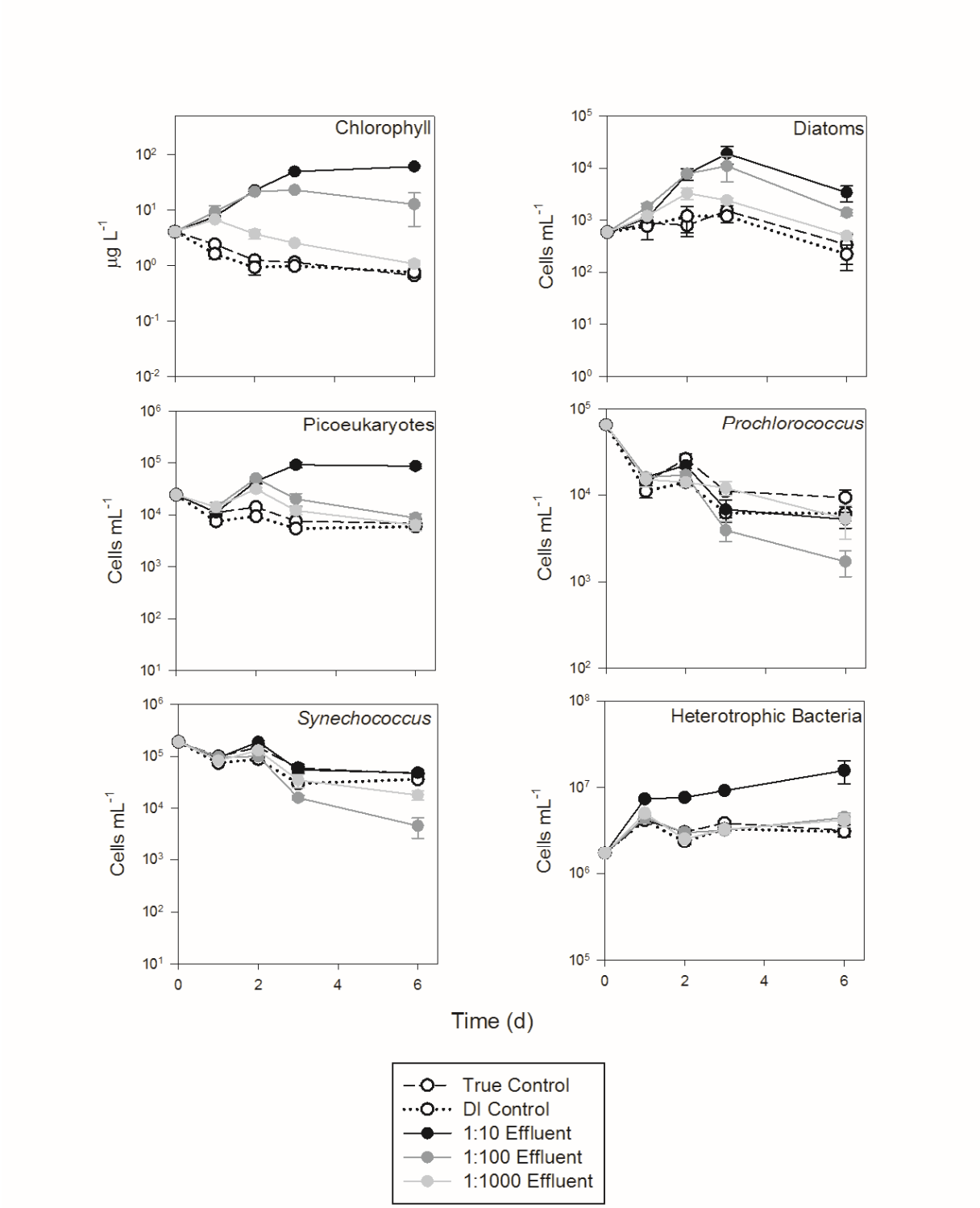
Chlorophyll
µg L-1
Diatoms
Cells mL-1
Picoeukaryotes
Cells mL-1
Prochlorococcus
Cells mL-1
Synechococcus
Cells mL-1
Heterotrophic Bacteria
Cells mL-1
Time (d)
True Control
DI Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent

Figure 6.

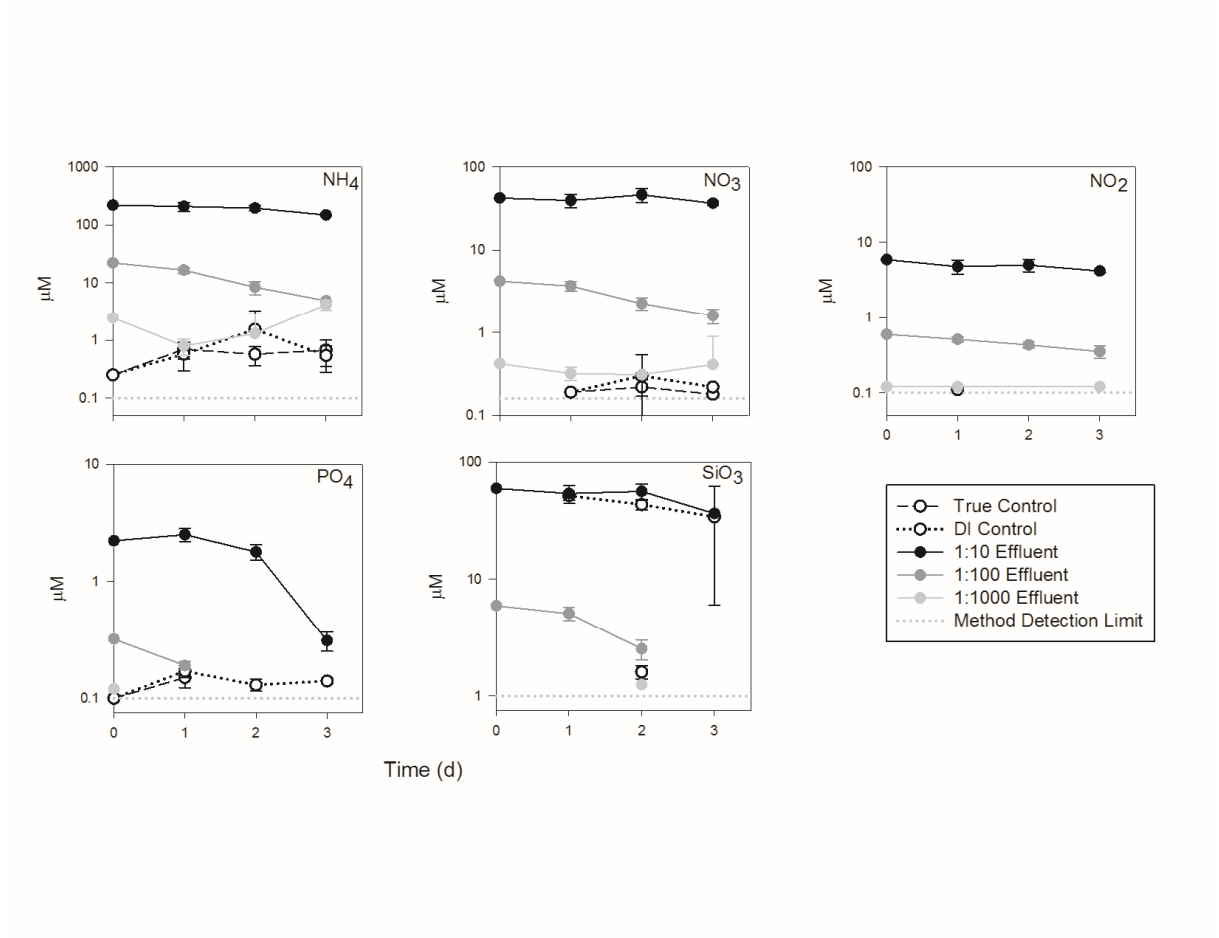
NH4
NO3
NO2
PO4
SiO3
µM
Time (d)
True Control
DI Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent
Method Detection Limit

Figure 7.

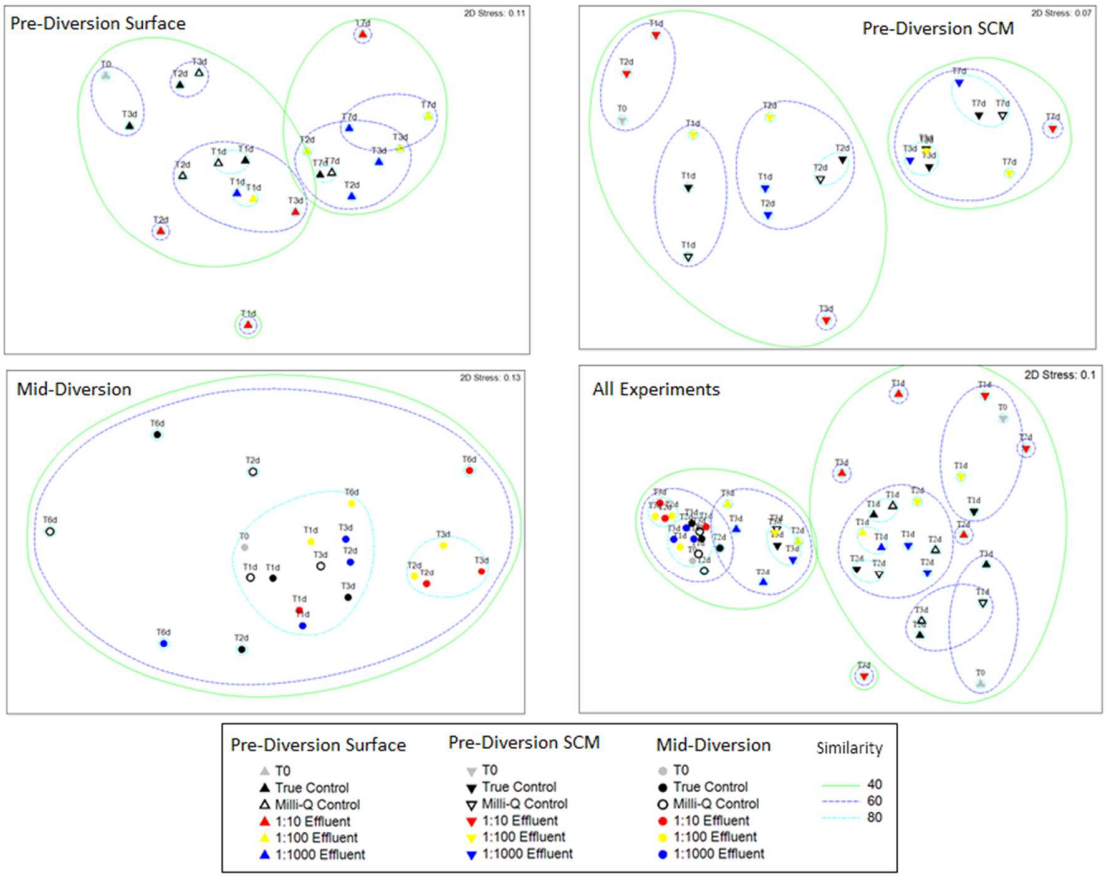
Pre-Diversion Surface
2D Stress: 0.11
Pre-Diversion SCM
2D Stress: 0.07
Mid-Diversion
2D Stress: 0.13
All Experiments
2D Stress: 0.1
Pre-Diversion Surface
T0
True Control
Milli-Q Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent
Pre-Diversion SCM
T0
True Control
Milli-Q Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent
Mid-Diversion
T0
True Control
Milli-Q Control
1:10 Effluent
1:100 Effluent
1:1000 Effluent
Similarity
40
60
80

Figure 8.