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The role of heterotrophic bacteria in mediating hydrogen peroxide concentrations in the Western Basin of Lake Erie

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Abstract
Hydrogen peroxide (H_2O_2) plays a role in numerous environmental processes. Its interactions with biology are complex because microorganisms can be both sources and sinks of H_2O_2, and it is a potent oxidative stressor for all organisms. A newly hypothesized impact of this compound is selection for toxic strains of *Microcystis spp.*, a globally important bloom-forming cyanobacterium. This effect is relevant in the Western Basin of Lake Erie of the Laurentian Great Lakes, where *Microcystis spp*. often dominate cyanobacterial blooms during the warm summer months. Previous measurements in the lake have revealed a dynamic and recurrent trend in H_2O_2 concentration over the bloom season: in mid-July of 2014 and 2015, there was a rapid increase in concentration, followed closely by a sharp decrease. In the past, H_2O_2 production in aquatic ecosystems was thought to occur primarily through abiotic pathways, but a recent study in Lake Erie suggested that biological activity was the primary determining factor in both H_2O_2 production and degradation over the bloom season. The present study sought to elucidate the mechanisms of H_2O_2 production and decay in the Western Basin of Lake Erie by studying bacteria that may influence H_2O_2 concentrations in the lake. Heterotrophic bacteria were isolated from Lake Erie water samples and enrichment cultures of *Microcystis* and other phytoplankton, characterized by 16S rRNA gene sequencing, and evaluated in terms of their environmental abundance. Experiments were performed on selected isolates that quantified their ability to both produce and degrade H_2O_2 in liquid culture. Rates of H_2O_2 production and decay measured in culture under environmentally relevant conditions show that these bacteria could account for the H_2O_2 trend in the Western Basin of Lake Erie. The results support the idea that heterotrophic organisms play an important role in mediating H_2O_2 concentration in cyanobacterial bloom communities and other aquatic ecosystems.

Introduction

The reactive oxygen species hydrogen peroxide (H_2O_2) plays an important role in a wide range of aqueous environmental and biological reactions including the oxidation of organic matter, cellular signal transduction, and the cycling of metals (Apel and Hirt 2004; Moffett and Zika 1987). This molecule can also have a dramatic negative effect on microbial metabolism (Glaeser et al., 2014) and its relatively long lifetime in the environment allows for harmful levels to occur in many systems (Cooper et al., 1988; Cooper and Lean, 1989; Cooper et al., 1989; Xenopoulos et al., 1997; Häkkinen et al., 2004). As a result of these functions and its prevalence in diverse environments, the sources and sinks for this compound have been studied extensively. However, the exact mechanisms for the degradation and production of H_2O_2 in many systems are not fully understood.

Sources and sinks of H_2O_2

Traditionally, the primary source of H_2O_2 in aquatic systems was thought to be the reaction between dissolved organic matter (DOM) and sunlight (Petasne and Zika, 1997; Cooper et al., 1988; Cooper et al., 1994; Obernosterer et al., 2001). The production of H_2O_2 occurs when the UV fraction of sunlight reacts with the chromophoric portion of dissolved organic matter present in the environment (Cooper and Zika, 1983). While the photochemical production of H_2O_2 is significant in the photic zone, recent studies have shown that photochemical production is not sufficient to account for the measured H_2O_2 levels of many environments (Cory et al., 2016; Dixon et al., 2013; Richard et al., 2007; Yuan and Shiller, 2005). Many of these studies have implicated a biological source as a significant contributor to the H_2O_2 concentration in natural waters. For example, Vermilyea et al. (2010) measured significant rates of dark production of H_2O_2, thus identifying a biological source decoupled from photochemical production. H_2O_2 freely diffuses across cell membranes (Seaver and Imlay, 2001) and is a known byproduct of all aerobic metabolisms (Apel and Hirt, 2004), lending support to the possibility that biological generation is an important factor in determining environmental concentrations, as hypothesized based on production rate measurements in these studies. Other work has demonstrated that taxonomically diverse heterotrophic bacteria produce superoxide, the key precursor to H_2O_2 in natural waters (Diaz et al., 2013).

As well as being an important source of H_2O_2, bacterial activity is also thought to be the dominant degradation pathway (sink) of H_2O_2 in natural systems. Aerobic microorganisms produce H_2O_2 as a byproduct of their metabolism, which causes oxidative stress and threatens microbial function. As a strategy to minimize this oxidative stress, aerobic microorganisms have developed various enzymes such as catalase to degrade H_2O_2 to oxygen and water (Zámocký et al., 2012). While catalase is known for mitigating the oxidative stress caused by metabolically-produced H_2O_2, there are also extracellular variants (Gasselhuber et al., 2015) that allow microbes to degrade exogenous H_2O_2 in aqueous solution (Wong et al., 2003; Vermilyea et al., 2010). This function is thought to have a powerful regulatory role in determining environmental H_2O_2 concentrations (Moffett & Zafiriou, 1990; Cooper et al., 1994; Wong et al., 2003; Richard et al. 2007). Heterotrophic bacteria have been shown to be the main contributor to this H_2O_2 sink (Cooper et al., 1994). Taken together with the recent measurements of microbial H_2O_2 production, the role of microbes in shaping overall H_2O_2 concentrations in natural systems remains poorly understood, as the microbial community has now been shown to be both a significant source as well as a sink for H_2O_2.

Effects of H_2O_2 on microbial communities

Further complicating the relationship between microbes and H_2O_2 concentrations are the reciprocal effects that the compound has on these organisms. At a fundamental level, oxidative

stress caused by H_2O_2 has been shown to have a negative effect on bacterial production and the function of bacterial enzymes (Baltar et al., 2013). Specifically, H_2O_2 can oxidatively damage the iron-sulfur clusters of proteins and amino acids containing sulfur and play a role in protein carbonylation and subsequent inactivation (Imlay, 2003).

H_2O_2 may have other indirect effects on bacteria in natural waters because it is a key reactant in the Fenton reaction, in which it reacts with ferrous iron to produce hydroxyl radical. Hydroxyl radical can subsequently oxidize DOM (Page et al., 2014), producing compounds labile to bacteria (Goldstone et al., 2002). In studies that exposed water containing DOM and bacterial populations to UV light (Anesio et al., 2005; Judd et al., 2007) or to H_2O_2 directly (Cory et al., 2010), the initial inhibition of bacterial growth that was observed was attributed to oxidative stress caused by H_2O_2. This initial inhibitory period was followed by a period of increased bacterial respiration (Judd et al., 2007; Cory et al., 2010), consistent with the decrease in H_2O_2 over the course of the experiments (Anesio et al., 2005). These results highlight the dynamic nature of bacterial interaction with H_2O_2, and also lead to uncertainty in the exact mechanism of the eventual drawdown in H_2O_2 concentration. Specifically, do bacterial enzymes simply take time to be constructed and degrade H_2O_2 in solution? Or do higher H_2O_2 concentrations select for populations of bacteria that have the capacity to degrade this compound?

The potential influence of H_2O_2 on toxicity of harmful cyanobacterial blooms

While H_2O_2 is present in all aquatic environments and undoubtedly plays key roles in many biogeochemical processes, there are some systems in which this compound could have a negative impact on water quality. Bodies of water that are subject to cyanobacterial blooms are one such case. Cyanobacterial blooms are a widespread issue, affecting water used by millions of people worldwide (Paerl and Huisman, 2009). While these events are perhaps best known for inducing anoxia in the water column by stimulating aerobic respiration, certain species of cyanobacteria are producers of harmful toxins and in extreme cases can toxify natural waters (Codd et al., 2005; Paerl and Otten, 2013a). The primary drivers of cyanobacterial blooms are known to be nutrient loading and increased temperature, but the controls on bloom toxicity are less completely understood (Heisler et al., 2008; Steffen et al., 2014).

In comparison to other phytoplankton and bacteria, cyanobacteria are highly sensitive to H_2O_2. Laboratory experiments have recorded negative effects on cyanobacterial growth at concentrations of H_2O_2 1-2 orders of magnitude lower than negative effects were observed on green algae or diatoms (Drabkova et al., 2007, Leunert et al., 2013). During a field study in which a small lake was uniformly exposed to 60 μM H_2O_2, the entire cyanobacterial population was selectively eliminated with minimal effects on the phytoplankton or larger organisms in the lake (Matthjis et al., 2012).

Oxidative stress by H_2O_2 was recently implicated as a potential driver of toxicity in *Microcystis spp*., a cyanobacterium that dominates harmful blooms worldwide (Paerl and Otten, 2013b). There is currently not a strong consensus on the physiological function of toxin production, nor a firm understanding of what selects for toxic and non-toxic strains of cyanobacterial species in the environment. Previous research has identified temperature and nutrient availability as potential determining factors (Davis et al., 2009, 2010), but recent studies point to environmental H_2O_2 concentrations as another selective agent. In studies testing exposure of *Microcystis* to H_2O_2, toxin-producing strains were shown to be more resistant to the oxidative stress than non-toxic strains, and one study observed the produced toxin binding to and apparently protecting cyanobacterial proteins (Dziallas and Grossart, 2011a; Zilliges et al., 2011). Based on these results, it has been suggested that toxin production is an important defense for an organism that is otherwise vulnerable to oxidative stress, and furthermore that H_2O_2

concentrations in the environment may play an important role in selecting for toxin-producing strains. While this defense-mechanism hypothesis could explain a toxic cyanobacterial response to high H_2O_2 levels in the environment, it is likely that other bacteria, specifically heterotrophs, also have a role in determining the environmental H_2O_2 concentrations that affect these toxin-producing cyanobacteria.

The associated heterotrophic community and its role in oxidative stress mitigation

While cyanobacterial bloom events are dominated by their characteristic cyanobacterial species, they also support diverse communities of bacteria that use the organic carbon produced by the bloom (Eiler and Bertilsson, 2004). This heterotrophic community can be quite variable, changing in accordance with temperature as well as nutrient and light availability (Dziallias and Grossart, 2011b). In addition to benefitting from the organic matter produced by cyanobacterial blooms (Worm and Søndergaard, 1998), these communities have further, significant relations with the dominant photosynthetic bacteria. Heterotrophic bacteria have been shown to be important in bloom development as well as cyanobacterial colony formation, facilitating the aggregation of cyanobacterial cells in culture (Shen et al., 2011). They have also been implicated in the transfer and storage of phosphorus, a limiting nutrient in many bloom communities (Jiang et al., 2007). These relationships are some of the possible reasons that heterotrophs have also been demonstrated to enhance cyanobacterial growth. Berg et al. (2009) tested the effect on cyanobacterial growth of 184 heterotrophic strains isolated from bodies of water that are characterized by bloom events. Of the 110 strains that affected cyanobacterial growth, 89 had a positive effect. A pair of studies by Morris et al. (2008, 2011) also found that heterotrophs had a positive effect on cyanobacterial growth. The latter of these studies identified a specific relationship that leads to the higher growth rate. This study tested the growth of cyanobacteria exposed to H_2O_2 grown axenically and in the presence of a single heterotrophic species. The results showed that cyanobacterial growth was facilitated by the co-cultured heterotrophic bacteria, and further that this facilitation was directly due to the mitigation of oxidative stress by the heterotroph (Morris et al., 2011).

The present study seeks to determine the role that heterotrophic bacteria play in the H_2O_2 chemistry of Lake Erie, the smallest of the Laurentian Great Lakes. In recent years, this lake has been the site of toxic cyanobacterial blooms dominated by *Microcystis aeruginosa*, a producer of the hepatotoxin microcystin (Steffen et al., 2014). Concurrent measurements of this toxin and H_2O_2 over the bloom season in the Western Basin of Lake Erie have revealed a recurrent trend. In July of 2014 and 2015, H_2O_2 concentrations increased to greater than 500 nM above average concentrations (~300 nM) in the Western Basin, then declined sharply back to average concentrations the rest of the season (Cory et al., 2016). This increase in H_2O_2 correlated with a significant rise in phycocyanin, a cyanobacterial pigment and proxy for bloom density, and was immediately followed by an increase in particulate microcystin in the lake (Cory et al., 2016). No comparable levels of toxicity occurred concurrently with a later increase in bloom density, when H_2O_2 had returned to average concentrations. Because of the elevated toxin levels seen immediately following the increase in H_2O_2, these observations support the hypothesis that one function of toxin production by *Microcystis* could be defense against oxidative stress.

In addition to providing insight into the effect of H_2O_2 on cyanobacterial toxin production, this dynamic H_2O_2 trend in the lake raises questions about the sources and sinks of the compound over the bloom season. Various results indicate that biological processes play an essential role in the dramatic fluctuation observed. Over the 2014 and 2015 bloom seasons, concentrations of H_2O_2 were not higher in surface waters than bottom waters, even at times of the season when vertical mixing was virtually absent (Cory et al., 2016). Furthermore, H_2O_2 concentrations were more closely tied to biological processes such as total water column

respiration than the presence of chromophoric DOM (Cory et al., 2016). These results rule out the notion that the photochemical production of H_2O_2 is the sole controlling factor in the H_2O_2 trend in the lake and support the idea that biology has a strong influence.

While heterotrophic bacteria may produce some of the H_2O_2 that causes the dramatic increase in its concentration, it is difficult to separate their role in this increase from the activity of other bacteria present in the environment, as all aerobic metabolisms produce H_2O_2. What seems more apparent is that the enzymatic activity of heterotrophic bacteria is a probable explanation for the rapid drawdown in H_2O_2 concentration that immediately followed the sharp increase observed in Lake Erie during July of 2014 and 2015. Because there is no rapid decrease in potential sources of H_2O_2 that occurs with this drawdown, it is instead likely that there is an increase in H_2O_2 sink. To better understand these trends of H_2O_2 concentration, this study directly tested the capacity of heterotrophic bacteria isolated from Lake Erie water samples and environmentally derived cultures to both produce and degrade H_2O_2.

Methods

Environmental sample collection

All environmental samples were collected onboard the NOAA Great Lakes Environmental Research Laboratory (GLERL) vessel R4105. Depth integrated (1-5 meters) Lake Erie water samples were collected weekly from the Western Basin of the lake (Figure 1) throughout the 2015 bloom season. In addition to water samples, net tow samples for phytoplankton community enrichment were obtained. A net with a pore size of 100 μm was lowered into the lake to a depth of 1 meter. All resulting water and microorganisms were placed in a culture flask containing a 1:3 mixture of modified BG-11 media and 0.22 μm filtered Lake Erie water.

Isolation of heterotrophs

Heterotrophic bacteria were isolated from June and August Lake Erie water samples as well as from *Microcystis* and phytoplankton enrichment cultures. A series of three 10-fold dilutions in sterile DI water was performed on the water samples in order to select for environmentally relevant organisms. For cultivation and isolation, 100 μl of each dilution was spread on multiple LB agar plates made with unfiltered, autoclaved Lake Erie water. All plates were incubated at room temperature, and individual colonies were isolated using traditional methods.

Isolates were also obtained from two separate phytoplankton enrichment cultures, as well as from the *M. aeruginosa* LE-3 culture (provided by Timothy Davis of NOAA GLERL). These cultures were mixed well, and 100 μl of each was spread on multiple separate BG-11 agar plates supplemented with autoclaved *Microcystis* culture. Subsequent isolation followed the protocol described above.

16S rRNA gene sequencing and determination of environmental abundance

DNA was extracted from cultures isolated from June water samples with a lysis buffer containing 50 mg/mL lysozyme, 10 mg/mL proteinase K, and 10% sodium dodecyl sulfate, with incubation in a 52 °C water bath for 12 hours. A double phenol-chloroform extraction was then performed on the lysed cells. For all August water sample and enrichment culture isolates, the FastDNA® Spin Kit for Soil (MPBio) was used to extract the DNA from single colonies grown on solid media.

PCR amplification of the entire 16S rRNA gene was performed on the DNA of all isolates using the 27F and 1492R primers. Before being submitted to the University of Michigan Sequencing Core for analysis, the PCR products were purified using the UltraClean® PCR Clean-Up Kit (MO BIO).

The DNA sequences obtained were analyzed using the Ribosomal Database Project (RDP) classifier tool (Wang et al., 2007). This software provides a genus-level classification based on 16S rRNA gene sequence. A bootstrap value of 95% was set as the confidence threshold, and each isolate was assigned the lowest taxonomic ranking above the confidence threshold. For isolates that could only be classified to the family level with the selected confidence threshold, the RDP Seqmatch tool was used to obtain classification to the genus level (Cole et al., 2014).

For determination of environmental abundance, isolate 16S rRNA sequences were compared to the 16S rRNA sequences of 362 operational taxonomic units (OTUs) that were defined based on an analysis of the bacterial community in Lake Erie from May to November 2014 (Berry et al., in prep.). This analysis also calculated the relative abundance of each OTU in five different communities based on filter pore size (whole community, 100μm, 53μm, 22μm and 3μm) on a weekly basis.

Isolate cryopreservation

Between all sources, 21 heterotrophic isolates were obtained. In order to preserve these isolates for further study, cells of each isolate were frozen in a medium appropriate for their survival. Isolates were grown to early stationary phase in rich media (1.5 g/L yeast extract, 1.5 g/L peptone). These cultures were spun down to create a pellet of cells, which was subsequently resuspended in a 1:1 mixture of the growth media used and a sterilized 20% glycerol solution. All isolates were frozen at -80°C.

Catalase test

A standard catalase test was performed on 15 isolates. Single colonies obtained from solid media were exposed to 1 M H_2O_2 in sterile test tubes. A catalase-positive result was defined as immediate effervescence (indicating O_2 evolution from H_2O_2 decay) while a negative result was defined as delayed or minimal effervescence (Madigan et al., 2015).

Effect of cultured bacteria on H_2O_2 concentrations

To determine the potential for heterotrophic organisms to mediate the dynamic H_2O_2 trend in Lake Erie, the effect of isolates on H_2O_2 concentrations in liquid culture was tested. For three isolates, an experiment was performed to determine the absolute rate of dark H_2O_2 production and H_2O_2 decay rate coefficient, following the spike-addition method described by Vermilyea et al. (2010). This method allows for the separation of bacterial production and decay of H_2O_2, which are likely occurring simultaneously. Isolates LE-E3, LE-L1, and LE-L6 were chosen based on their abundance in the environment and the results of their catalase tests. Each isolate was grown separately in liquid media (0.2 g/L yeast extract in 0.22 μm filtered Lake Erie water) in the dark and was in the exponential growth or early stationary phase at the time of the experiment. A control experiment in sterile media was also performed.

Each isolate was tested using three replicates each of two treatments: unamended culture, and culture spiked with 750 nM H_2O_2. Samples were taken from both treatments at the time of H_2O_2 addition and at 20 minutes intervals for one hour thereafter. The samples were subsequently analyzed using flow injection analysis on an Felume instrument (Waterville Analytical), which measures the chemiluminescent peak produced by the reaction between acridinium ester and the conjugate base of H_2O_2 (King et al., 2007). Standard additions of 250 nM and 500 nM H_2O_2 were performed on each sample in order to establish a relationship between the H_2O_2 added and the measured chemiluminescent peak, accounting for matrix effects on the chemiluminescent signal. Linear regression analysis was used to determine the H_2O_2 at each time point (Data Analysis Toolpak, Microsoft Excel).

Determination of absolute rates of isolate-induced H_2O_2 dark production and decay was carried out following the Vermilyea, et al. (2010) study, in which the absolute pseudo–first-order rate coefficient ($k_{loss,H2O2}$) and the absolute dark production rate (P_{H2O2}) were assumed to remain constant over time and between spiked and unamended treatments. To calculate these rates, H_2O_2 over time was modeled using the equation

$$[H_2O_2] = \left(\frac{P_{H2O2}}{k_{loss,H2O2}}\right)(1 - Ae^{-k_{loss,H2O2}t}) \quad (1)$$

where the absolute rates of production and decay are given by the equations

$$\text{Rate of production} = P_{H2O2} \quad (2)$$

$$\text{Rate of decay} = -k_{loss,H2O2}[H_2O_2] \quad (3)$$

A is a constant determined separately for each treatment and defined as

$$A = 1 - \left(\frac{k_{loss,H2O2}}{P_{H2O2}}\right)[H_2O_2]_o \quad (4)$$

where $[H_2O_2]_o$ is the initial concentration of H_2O_2 in a certain treatment. Microsoft Excel's Solver function was used to minimize the sum of squares of the differences between the measured H_2O_2 and the modeled H_2O_2. The fitting parameters used were P_{H2O2}, $k_{loss,H2O2}$, and the initial H_2O_2 concentration in each treatment, as these parameters were found to provide the best fit to measured concentrations by Vermilyea et al. (2010).

Results and discussion

Isolate taxonomy

Based on the RDP classification, the 21 isolates are distributed between 3 bacterial phyla and 4 classes (Table 1). The genus-level diversity of the isolates is low, with an average of 2 unique genus classifications within each class. The source of the isolates did not seem to have an effect on their identity, except in the case of the *Microcystis* enrichment culture (isolates ME-1 to ME-5), from which only members of the genus *Ochrobactrum* were isolated. While no previous studies have tested specific heterotrophic organisms for their role in H_2O_2 production or decay in the context of mediating H_2O_2 trends, many of the bacteria isolated in this study have been shown to play a role in cyanobacterial bloom communities. Heterotrophic organisms are essential in colony formation in cyanobacteria (Shen et al., 2011) and nutrient cycling in bloom communities (Buchan et al., 2014). The previously demonstrated functions of bloom-associated heterotrophs could also include a role in protection from oxidative stress caused by H_2O_2 and other ROS (Morris et al., 2011).

Proteobacteria is the most common phylum assigned, these belonging to either the Gammaproteobacteria (5 isolates) or Alphaproteobacteria (6 isolates). These classes are common in most freshwater environments, though not typically as abundant community members (Newton et al., 2011). In terms of their capacity to mediate H_2O_2 concentrations in the environment, members of the Alpha and Gamma classes of Proteobacteria have been shown to exhibit high tolerance for singlet oxygen ($^1O_2$; Glaeser et al., 2014), which is a stronger oxidant than H_2O_2 and is also ubiquitous in surface waters (Cory et al., 2010). A relatively well-studied oxidative stress response has been documented in these classes. A core set of genes that are well conserved across Alpha- and Gammaproteobacteria respond to $^1O_2$ through activation of defense enzymes by a sigma factor (Dufour et al., 2008). Another link between these organisms and mediation of H_2O_2 was reported by Morris, et al., who found that Proteobacteria protected marine cyanobacteria from oxidative stress caused by H_2O_2 (Morris et al., 2011).

Despite being more common in marine systems (Newton et al., 2011) various lines of evidence support the idea that Alphaproteobacteria are commonly associated with cyanobacterial blooms, which indicates relevance of this group to the bloom community in Lake Erie. In culture-based studies of the microbial communities in freshwater lakes, Alphaproteobacteria have been shown to outcompete other groups at low organic carbon and nutrient concentrations (Eiler et al., 2003; Pinhassi and Berman, 2003). This could be related to their demonstrated ability to use relatively less labile organic matter as a carbon source (Hutalle-Schmelzer et al., 2010). There is also strong genomic evidence for symbiotic or parasitic lifestyles in the class in general (Batut et al., 2004). These facts together could indicate that Alphaproteobacteria have an important role in the degradation of cyanobacterial organic matter after the most labile substrates have been utilized.

Investigations into the structure of the bacterial communities of ecosystems dominated by cyanobacteria have established that Alphaproteobacteria make up a significant fraction of the microbes associated with cyanobacteria. In an analysis of the metagenome of a *M. aeruginosa* bloom community, Li, et al. (2011) found that 83.3% of the non-cyanobacterial sequences obtained belonged to Alphaproteobacteria. Another study characterized heterotrophic bacteria isolated from diverse environments that were frequently dominated by cyanobacteria and found that Alphaproteobacteria made up a significant portion of the isolates (Berg et al., 2009). Furthermore, tests of the bacteria isolated in the study revealed that multiple isolates in this class had either a positive or negative effect on *Microcystis* in culture, perhaps indicating a close relationship in the environment. In Lake Erie, Alphaproteobacteria were a highly abundant heterotrophic group over the 2014 bloom season (Berry et al., in prep.).

The Alphaproteobacteria isolated in this study are dominated by the genus *Ochrobactrum* (isolates ME-1 to ME-5), the only genus obtained from the *Microcystis* enrichment culture. Species of this genus isolated from *M. aeruginosa*-dominated environments have algicidal effects on the cyanobacteria that they are apparently associated with (Mu et al., 2009, 2012). Further testing has revealed that members of the genus are capable of degrading microcystins, the hepatotoxin produced by toxic strains of *M. aeruginosa* (Jing et al., 2014). It has been suggested that microcystin-degrading bacteria such as *Ochrobactrum* could be used in the bioremediation of waters toxified by cyanobacteria (Yang et al., 2014). The fact that all of the isolates classified under this genus originated in the *M. aeruginosa*-dominated culture is intriguing. The apparently abundant nature of this genus in association with a toxin-producing *Microcystis* strain could indicate a favorable and protective adaptation of toxin-degrading capacity.

The only other isolate classified as Alphaproteobacteria in this study belongs to the genus *Rhizobium*, a group widely recognized for its nitrogen-fixing symbioses with the roots of plants (Long, 1996). While no research exists linking *Rhizobium* to cyanobacteria, *Microcystis spp.* is not known to fix nitrogen and this nutrient is thought to be limiting in Lake Erie bloom communities (Steffen et al., 2014). The nitrogen-fixing capacity of the *Rhizobium* isolate was not tested, but the high abundance of Alphaproteobacteria in cyanobacterial bloom communities and the known symbioses between this genus and other species suggest that such a relationship may exist between this bacterium and *Microcystis*.

The Gammaproteobacteria isolated in this study make up the most phylogenetically diverse group of isolates. Two of the three genera in this class that are represented by the isolates have been shown to be associated with cyanobacteria in past studies. The genus *Pseudomonas*, represented by isolates LE-E3, LE-E6, and LE-L6 has been identified as a prominent member of the associated community of both algae and cyanobacteria in diverse environments (Wiese et al., 2009; Berg et al.; 2009, Wu et al., 2007). Specific to the environment and community in this study, multiple studies have identified this genus as playing an important role in *M. aeruginosa*

bloom communities. Li et al. (2011) found that *Pseudomonas* constituted the majority of the Gammaproteobacteria sequences associated with *M. aeruginosa* communities from Lake Taihu, China, an environment commonly dominated by toxic *M. aeruginosa* blooms. One study suggested that the associated heterotrophic community, of which *Pseudomonas* was a highly abundant member, worked cooperatively to provide the cyanobacteria with a source of vitamin B12 (Xie et al., 2016). This study also found that *Pseudomonas* was the second-most abundant heterotrophic bacteria in *M. aeruginosa* colonies, at 6.7% relative abundance. Another study directly quantified the phosphorus transfer between *Pseudomonas spp*. and *M. aeruginosa*, and found that this transfer process takes place in both directions, perhaps alluding to a phosphorus storage role of *Pseudomonas spp*. in this context (Jiang et al., 2007).

Gammaproteobacteria of the genus *Stenotrophomonas*, represented by isolate PE-6, have also been shown to be members of *M. aeruginosa* communities (Shi et al., 2009). Two strains of this genus isolated from Lake Taihu have been shown to be capable of degrading microcystins (Chen et al., 2010; Yang et al., 2014). An additional strain isolated from the same environment is known to produce algicidal compounds (Lin et al., 2016).

The second most abundant class of bacteria isolated was Bacilli. All of the isolates in class Bacilli are classified as genus *Bacillus*, which is not known to be particularly abundant in freshwater systems, but is widely distributed in diverse environments. These eight isolates are distributed between just two OTUs, which could be evidence that these organisms represent bacteria easily cultivated in laboratory settings. This is further supported by the fact that an isolate in genus *Bacillus* was isolated from three of the four original sources (June and August Lake Erie water samples and phytoplankton enrichment culture). While not typically thought of as important member of freshwater environments, Bacilli have been identified as members of cyanobacterial communities in past studies (Berg et al., 2009).

Environmental relevance of isolates

Five of the isolates had a 97% or greater 16S rRNA gene sequence match with one of the OTUs defined by Berry et al. (Table 1; in prep.). Using the data collected by Berry et al., the relative abundance of these representative isolates in each community fraction over the 2014 bloom season in Lake Erie was determined. The relative abundance of each isolate is presented as an average between the data collected from the three Western Lake Erie sampling stations. Although the relative abundance of the isolates varies between the three sampling stations in Western Lake Erie, analysis by Berry, et al. demonstrates that bacterial community composition is more dynamic over time than space (in prep.). Additionally, the temporal trends in relative abundance are more significant to the observed H_2O_2 trend and cyanobacterial bloom dynamics in the Western Basin of Lake Erie, and thus to the present study.

In general, the isolates were most abundant in the 100 μm and 53 μm communities, which represents the particle-attached fraction. It is interesting that this is case, as the majority of isolates analyzed for abundance in the environment were obtained from Lake Erie water samples that did not contain any visible organic material. Particle-associated bacteria have been shown to differ substantially in taxonomy when compared with free-living bacteria (Mohit et al., 2014; Crespo et al., 2013), especially in environments rich in organic matter such as Lake Erie during the bloom season (Ortega-Retuerta et al., 2013; Cory et al., 2016). While the representative isolates in this study certainly varied in abundance between the filter fractions sampled, the bacterial community in Lake Erie as a whole has not been analyzed in terms of the phylogenetic contrast between particle-associated and free-living bacteria.

Isolate LE-E3, classified as *Pseudomonas*, represents OTU00122 (Table 1). This organism was most abundant in the 53 μm community, followed closely by the 100 μm fraction (Figure 2). Although this isolate was a significantly abundant member of the community in these

fractions (~1-4% of all OTUs) it was not regularly present at these high relative abundances throughout the bloom season. In the smaller filter fractions, this isolate was less abundant (<1% of all OTUs), but its abundance in these fractions was much more consistent over time (Figure A1).

Isolates LE-E6 and LE-L6, also classified as *Pseudomonas*, represent OTU00127 (Table 1). These isolates follow the same trend as isolate LE-E3, with high but transient relative abundance in the two largest filter fractions, and a more even, however diminished presence in the smaller fraction communities (Figures 3 and 2A).

An isolate from an August Lake Erie water sample, LE-L1, represents OTU00469 and belongs to the genus *Citrobacter* (Table 1). In general, this isolate was more evenly abundant between the five filter fractions. However, similarly to the isolates previously discussed, it was by far the most abundant in the 53 μm fraction (Figure 4b).

Each of these isolates were present in at least one filter fraction and sampling station on all but one sampling date over the 2014 bloom season (date of absence varied). Based on their nearly constant presence in the heterotrophic community over the bloom season, they represent ecologically important organisms. Although their presence was less substantial in terms of fraction of the smaller filter communities, their consistent presence may suggest that these organisms could play a role in the observed H_2O_2 trend in Lake Erie. The fact they are most consistently abundant in the smaller filter fractions supports this conjecture, as Cooper et al. found that the majority of H_2O_2 decay in an estuarine ecosystem was associated with particles in the 12-1 μm range (1994).

Isolate PE-6, isolated from a phytoplankton enrichment culture, is classified as *Stenotrophomonas* and represents OTU00985 (Table 1). While this isolate followed the trend seen in the others and was most abundant in the particle-associated communities, it was much less abundant overall (Figure 5). It also exhibited a much more transient pattern of abundance in the smaller filter fractions (Figure 4A). Despite its lower overall abundance compared to the other isolates, isolate PE-E6 was still present in some filter fraction on 71% of all sampling days over the 2014 bloom season.

The majority of the isolates do not have a sufficiently high 16S rRNA gene sequence match to reliably represent an OTU classified by Berry, et al., over the 2014 bloom season (97% ID threshold). This fact should not be interpreted to mean that these isolates are necessarily not present in the environment, as all of the isolates were obtained from environmental or environmentally-derived samples. One possible explanation for the poor representation of the analyzed OTUs is that the bacteria in this study were isolated from 2015 Lake Erie water samples, while the OTUs were defined based on samples taken over the 2014 bloom season. It has been shown that the heterotrophic community associated with cyanobacterial blooms can vary substantially, even under similar environmental conditions (Eiler and Bertilsson, 2004). Another possibility for the discrepancy is that the isolates that did not match well with the OTU sequences represent organisms with an extremely low abundance in Lake Erie. A well-known phenomenon that could have led to this occurrence is known as culturing bias. This bias arises from the fact that only a miniscule portion of the microorganisms present in the natural world can be grown in a laboratory setting. Thus, species that constitute a minority of the bacterial community in any given environment could be the majority of the community that is isolated from an environmental sample.

While this bias represents a disadvantage of traditional isolation and culturing approaches, these techniques are still useful to understand the roles of microorganisms in natural settings. One case where this is especially true is in the measurement of specific microbial processes that are relevant to understanding observed environmental trends.

Isolate effect on H_2O_2

From H_2O_2 measurements taken from liquid culture containing one of three isolates, the P_{H2O2} and $k_{loss,H2O2}$ for isolates LE-E3 and LE-L1 were calculated using the curve-fitting method described by Vermilyea et al. (2010), and the effect on H_2O_2 in culture of isolate LE-L6 was determined. Variation in the P_{H2O2} and $k_{loss,H2O2}$ were quantified by separately fitting the H_2O_2 concentration of each replicate of the spiked treatment with the average of the concentration in the control replicates, and comparing the resultant values. The average error between replicates for isolates LE-E3 and LE-L1 were 23% and 4%, respectively, for both P_{H2O2} and $k_{loss,H2O2}$. The P_{H2O2} and $k_{loss,H2O2}$ for isolate LE-L6 were not calculated due to an average error of >40% between replicates and also due to the apparent decay of H_2O_2 below detectable levels by the final time point.

Despite the significant variation between replicates of isolate LE-L6, the H_2O_2 measurements indicate that all isolates had a similar effect on H_2O_2 in culture. The high levels of H_2O_2 in the spiked treatments at the time of H_2O_2 addition were rapidly decayed by the isolates to concentrations nearly identical to those seen in the control treatments (Figures 6, 7, and 8). Isolates LE-E3 and LE-L1 are both positive for the enzyme catalase, while isolate LE-E6 is not (Table 1). Based on the results of their respective catalase tests, it can be assumed that LE-E3 and LE-L1 have the ability to degrade H_2O_2 to oxygen and water through enzymatic activity, but it cannot be assumed that LE-E6 does not have a comparable mechanism for H_2O_2 degradation. The similar trend in H_2O_2 between organisms that had contrary catalase test results indicates that this enzyme is not the sole bacterial control on H_2O_2 concentration. As aerobic organisms, the metabolic activity of all three isolates is a source of H_2O_2.

H_2O_2 concentrations in the control treatments remained relatively consistent throughout the experiments. In the sterile media control experiment, there was no definite pattern in H_2O_2 concentrations, and the initial H_2O_2 concentrations in both treatments were significantly higher than in the bacterial cultures (Figure A5). The measurements of bacterial control on H_2O_2 provide insight into the potential role that the heterotrophic community plays in controlling the H_2O_2 concentrations in Lake Erie.

The measured P_{H2O2} for both LE-E3 and LE-L1 are significantly higher than rates previously measured in aquatic environments (Table 2). The $k_{loss,H2O2}$ calculated for isolate LE-E3 is relatively high compared to previous measurements, while the $k_{loss,H2O2}$ for isolate LE-L1 far surpasses any reported value. Absolute rates of dark H_2O_2 production reported in past studies range from 0.9 to 300 nM hr^{-1}, and $k_{loss,H2O2}$ values range from 0.018 to 10 hr^{-1} (Vermilyea et al., 2010; Marsico et al., 2015; Zhang et al., 2016). The high values in this study could be due to the positive correlation that has been reported between rates of biological H_2O_2 production and decay (Vermilyea et al. 2010). In various other studies that have measured biological production and decay of H_2O_2, the measurements were performed on unfiltered environmental water samples. The fact that the bacteria studied here were isolated from the competition in the natural community could have led to high rates of respiration and production of H_2O_2, which in turn could have led to a higher rate of decay.

Despite the high rates of production and decay observed, there are reasons to believe that these results are realistic for the environment in question. The Western Basin of Lake Erie is eutrophic, with high nutrient inputs from terrestrial sources (Steffen et al., 2014). This is especially true during the cyanobacterial bloom season, when the recurrent H_2O_2 trend that this study is concerned with occurs. Over the time period of the H_2O_2 trend, the Western Basin is the site of a cyanobacterial bloom dominated by *Microcystis spp.* (Berry et al., in prep.). *Microcystis* bloom communities are known to harbor heterotrophic bacteria (Shen et al., 2011; Brunberg et al., 1998) and also provide ample quantities of organic carbon, which heterotrophs benefit from (Worm and Søndergaard, 1998). Thus, Lake Erie during the bloom season represents an

environment replete in growth-limiting nutrients and organic carbon, which are the conditions that the isolates experienced during the performed experiments. These conditions are conducive to increased respiration and H_2O_2 production, which in turn lead to higher rates of H_2O_2 decay. Despite this comparison, it is likely that the values calculated here are upper limits of H_2O_2 production and decay, given the isolated culture conditions and relatively rich media.

Additional lines of evidence that point to the environmental relevance of these results concern the measured H_2O_2 trend in Lake Erie. H_2O_2 concentrations in the Western Basin of the lake dramatically increase in mid-to-late July, when water column respiration is increasing and nutrient inputs are high (Cory et al., 2016; Steffen et al., 2014). The high levels of respiration (as evidenced by the calculated P_{H2O2}) in the conducted experiments mirror this trend in the environment. Furthermore, the net rate of biological production measured in the control treatments of isolate LE-E3 (37 nM hr^{-1}) was similar to the rate measured by Cory et al. (2016) in microcosm experiments with unfiltered Lake Erie water (30 +/- 14 nM hr^{-1}). While other aerobic metabolisms most likely contribute to the environmental increase in H_2O_2 levels in Lake Erie, the high rates of heterotrophic H_2O_2 production measured in this study are evidence that could point to the pivotal role of this group in inducing the dramatic rise in H_2O_2 in Lake Erie.

As well as representing relevant environmental conditions in terms of nutrient availability, the experimental conditions were also suited to measuring the response of the heterotrophic community to the high levels of H_2O_2 observed in the environment. At their highest point over the 2014 and 2015 seasons, H_2O_2 concentrations were ~500-1000 nM above normal levels, which ranged from ~200-400 nM depending on the day (Cory et al., 2016). The H_2O_2 concentrations measured at the initial time point in this study mimic this large difference in H_2O_2 over time in Lake Erie, as the average initial H_2O_2 in the spiked treatments of isolates LE-E3 and LE-L1 was 550 nM above the levels measured in the control treatments.

All isolates degraded the added H_2O_2 before the final time point of the experiments, indicating an ability to tightly control H_2O_2 levels. Interestingly, the isolates lowered the H_2O_2 in the spiked treatments to the concentrations measured in the control treatments. While this "background" level of H_2O_2 was different for each isolate (~250nM for LE-E3, ~20nM for LE-L1, <10 nM for LE-L6), the experimental pattern parallels the H_2O_2 trend in Lake Erie, as the H_2O_2 in the lake returns rapidly to the background level after a dramatic increase. While not observed in a bacterial community, the control that the isolates had on H_2O_2 levels in culture could translate to an essential role in regulating environmental H_2O_2 concentration. Furthermore, based on the absolute decay rate coefficient of isolates LE-E3 and LE-L1, which represent up to 3 and 2% (respectively) of the bacterial community in Lake Erie at some time points, and the similar degradation of H_2O_2 caused by isolate LE-L6, which represents up to 10% of the community at some points, these isolates could be capable of degrading H_2O_2 in the environment at a sufficient rate to account for the rapid drawdown that has been observed over the past two bloom seasons.

Conclusion

The heterotrophic bacteria isolated in this study represent an opportunity to better understand the dynamic and ecologically important trend in H_2O_2 in the Western Basin of Lake Erie. This trend may have consequences relating to toxin production by *Microcystis spp.*, a cyanobacterium that has recently endangered drinking water sources in the lake. The results of experiments on three environmentally abundant isolates have revealed that native heterotrophic bacteria are capable of rapid production and decay of H_2O_2, consistent with the hypothesis that heterotrophic bacteria have a powerful influence on patterns of H_2O_2 concentration in the Western Basin of Lake Erie. This outcome lends support to the conclusions of previous studies implicating microbial activity as an important determinant of environmental H_2O_2 concentrations. Further studies should

investigate the potential shifts in bacterial community and levels of biological production and decay associated with the recurrent sharp peak of H_2O_2 observed in the Western Basin of Lake Erie in 2014 and 2015.

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Tables and figures

Isolate Code	Class	Family	Genus	Boot-strap	Seq-match[b]	OTU[c]	OTU %ID	Cata-lase
LE-E1	Bacilli	Bacillaceae	*Bacillus*	98	-	00683	96.1	+
LE-E3	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*[a]	-	0.987	00122	99.2	+
LE-E4	Actinobacteria	Micrococcaceae	*Micrococcus*	100	-	00036	92.5	-
LE-E6	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*[a]	-	0.989	00127	98.0	-
LE-L1	Gammaproteobacteria	Enterobacteriaceae	*Citrobacter*[a]	-	0.994	00469	100	+
LE-L2	Bacilli	Bacillaceae	*Bacillus*	100	-	00276	95.7	+
LE-L3	Bacilli	Bacillaceae	*Bacillus*	100	-	00683	94.5	+
LE-L4	Bacilli	Bacillaceae	*Bacillus*	98	-	00683	94.5	-
LE-L5	Bacilli	Bacillaceae	*Bacillus*	100	-	00683	96.1	-
LE-L6	Gammaproteobacteria	Pseudomonadaceae	*Pseudomonas*	100	-	00127	97.6	-
PE-1	Bacilli	Bacillaceae	*Bacillus*	97	-	00276	94.9	-
PE-2	Alphaproteobacteria	Rhizobiaceae	*Rhizobium*	96	-	00989	92.5	NT[d]
PE-3	Actinobacteria	Micrococcaceae	*Kocuria*	100	-	00385	92.1	+
PE-4	Actinobacteria	Micrococcaceae	*Kocuria*	100	-	00385	92.1	+
PE-5	Bacilli	Bacillaceae	*Bacillus*[a]	-	0.988	00276	94.5	NT
PE-6	Gammaproteobacteria	Xanthomonadaceae	*Stenotrophomonas*	100	-	00985	99.2	+
ME-1	Alphaproteobacteria	Brucellaceae	*Ochrobactrum*	100	-	00106	92.1	NT
ME-2	Alphaproteobacteria	Brucellaceae	*Ochrobactrum*	100	-	00106	92.1	NT
ME-3	Alphaproteobacteria	Brucellaceae	*Ochrobactrum*	100	-	00106	92.1	NT
ME-4	Alphaproteobacteria	Brucellaceae	*Ochrobactrum*	100	-	00106	92.1	NT
ME-5	Alphaproteobacteria	Brucellaceae	*Ochrobactrum*	100	-	00106	92.1	NT

Table 1. Classification of isolates based on RDP classifier tool, confidence threshold set to 95%. Isolate code is based on source of isolate (LE-E: June water sample; LE-L: August water sample; PE: phytoplankton enrichment; ME: *Microcystis* enrichment)

[a] Genus level classification assigned based on RDP Seqmatch tool

[b] Seqmatch score defined as the number of unique 7-base oligomers shared between the isolate sequence and an RDP sequence divided by the lowest number of unique oligomers in either of the two sequences

[c] Most closely related operational taxonomic unit from analysis by Berry, et al. (in prep.)

[d] Not tested

Isolate code	$k_{loss,H2O2}$ (hr^{-1})	P_{H2O2} ($nM\ hr^{-1}$)
LE-E3	4.0	997
LE-L1	76	1760

Table 2. Absolute dark H_2O_2 decay rate constant and production rate for isolates LE-E3 and LE-L1

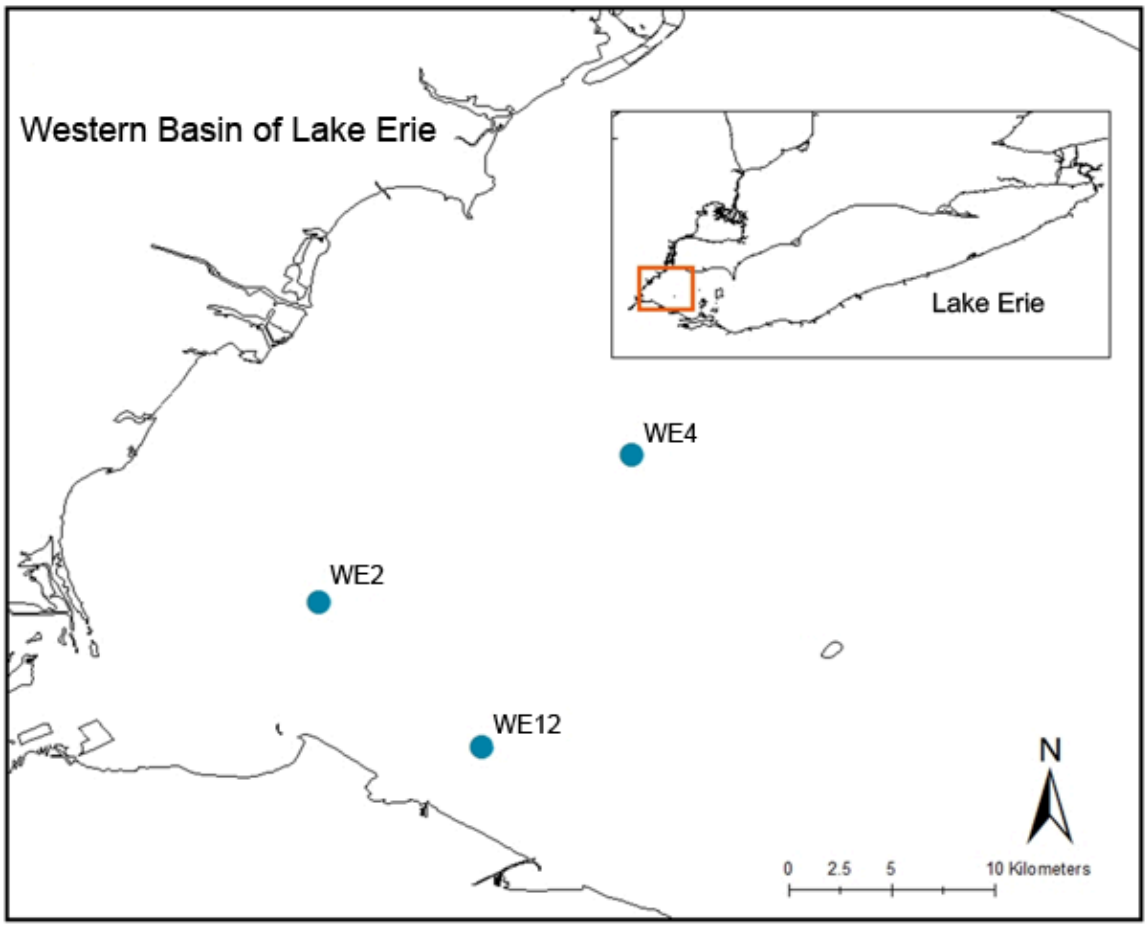

Figure 1. Map showing the Western Basin of Lake Erie and the locations of the three principal sampling stations in the area (WE2, WE4, and WE12). Figure courtesy of Tom Johengen, Cooperative Institute for Limnology and Ecosystems Research.

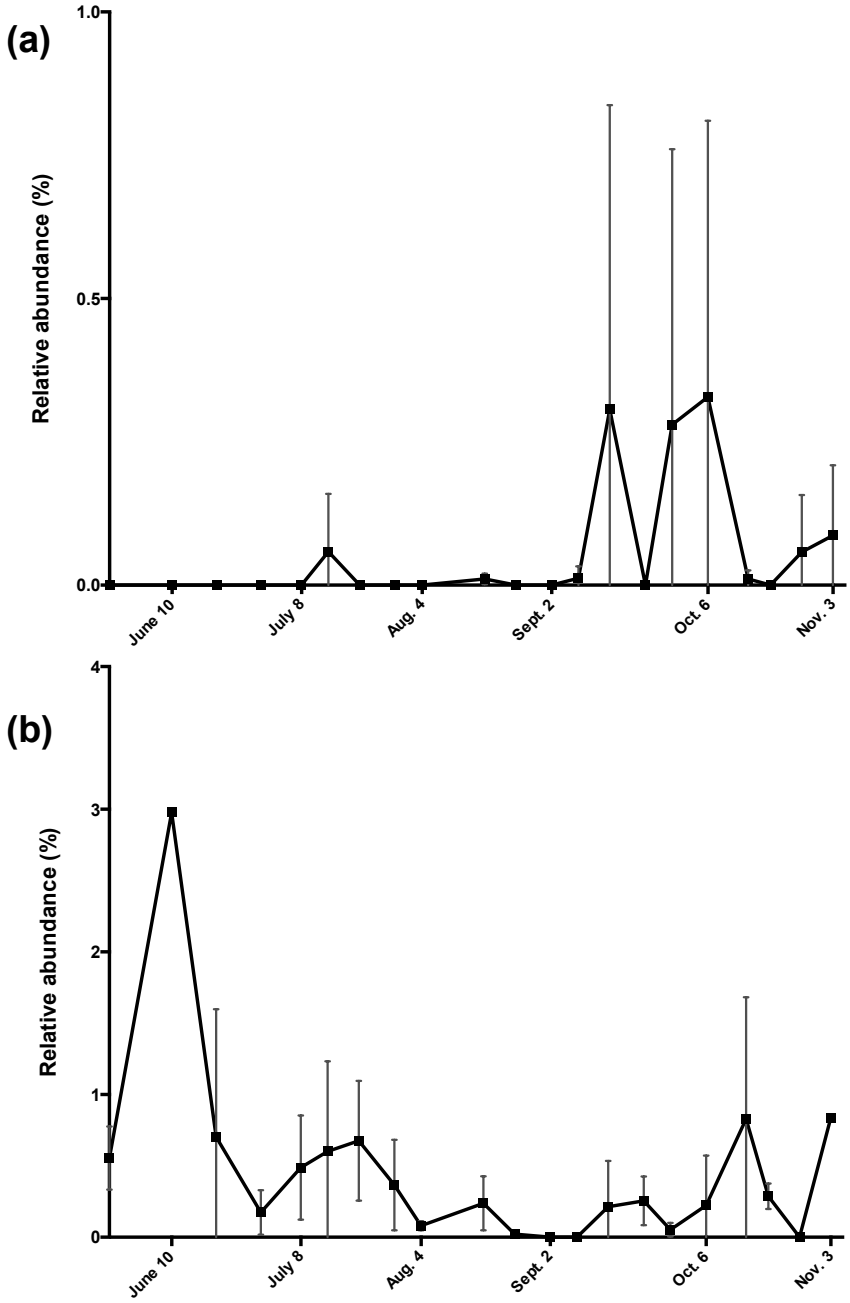

Figure 2. Relative abundance of OTU00122 (isolate LE-E3) in the 100µm (a) and 53µm (b) fractions of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

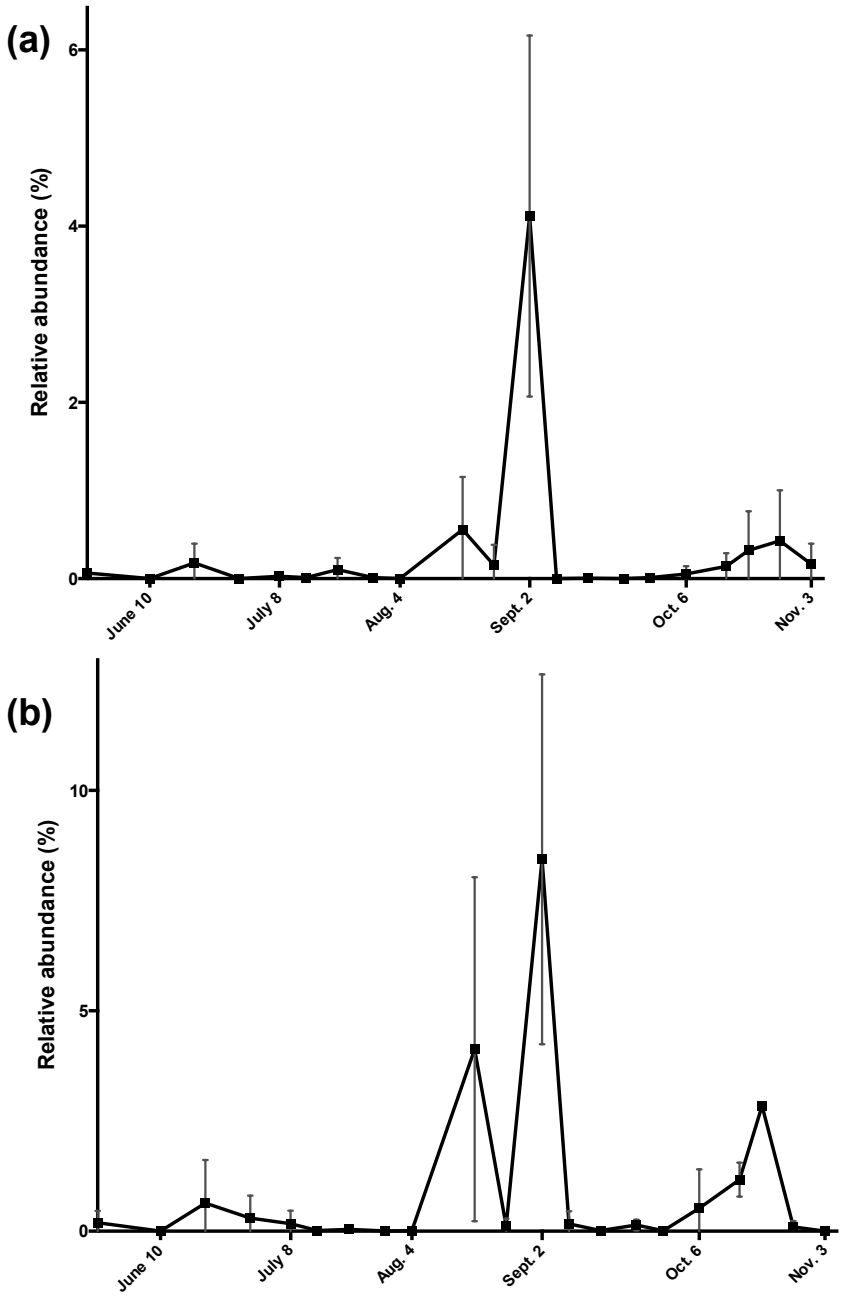

Figure 3. Relative abundance of OTU00127 (isolates LE-E6 and LE-L6) in the 100µm (a) and 53µm (b) fractions of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

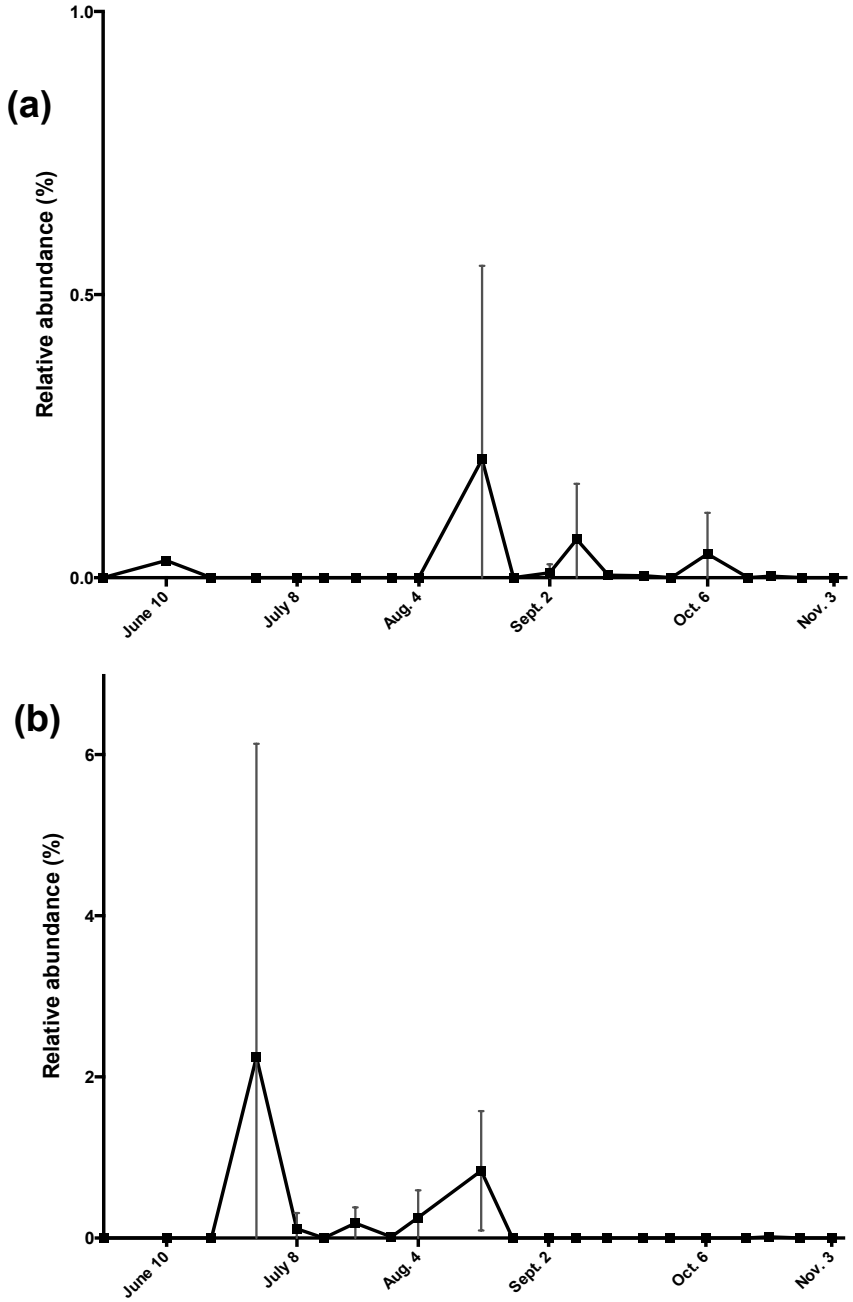

Figure 4. Relative abundance of OTU00469 (isolate LE-L1) in the 100µm (a) and 53µm (b) fractions of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

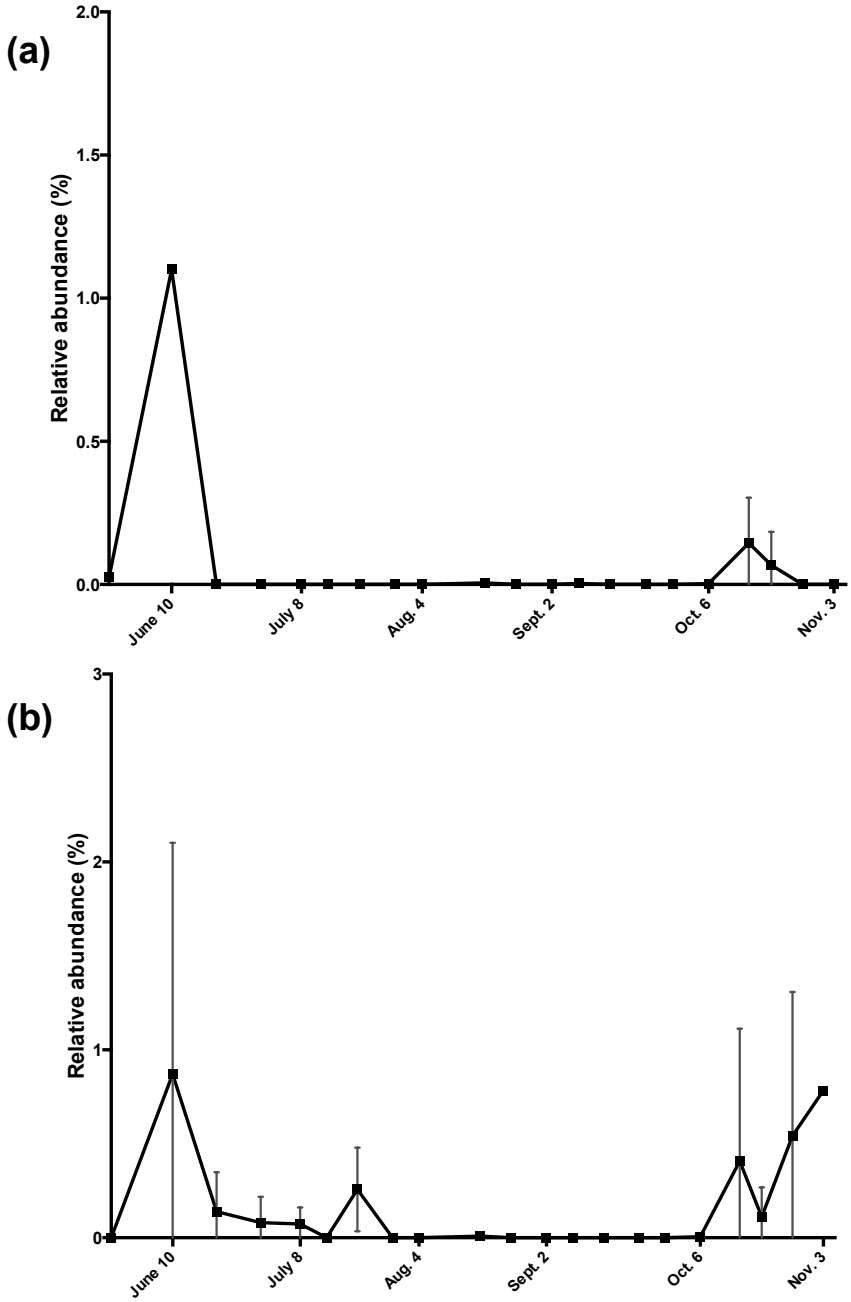

Figure 5. Relative abundance of OTU00985 (isolate PE-6) in the 100µm (a) and 53µm (b) fractions of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

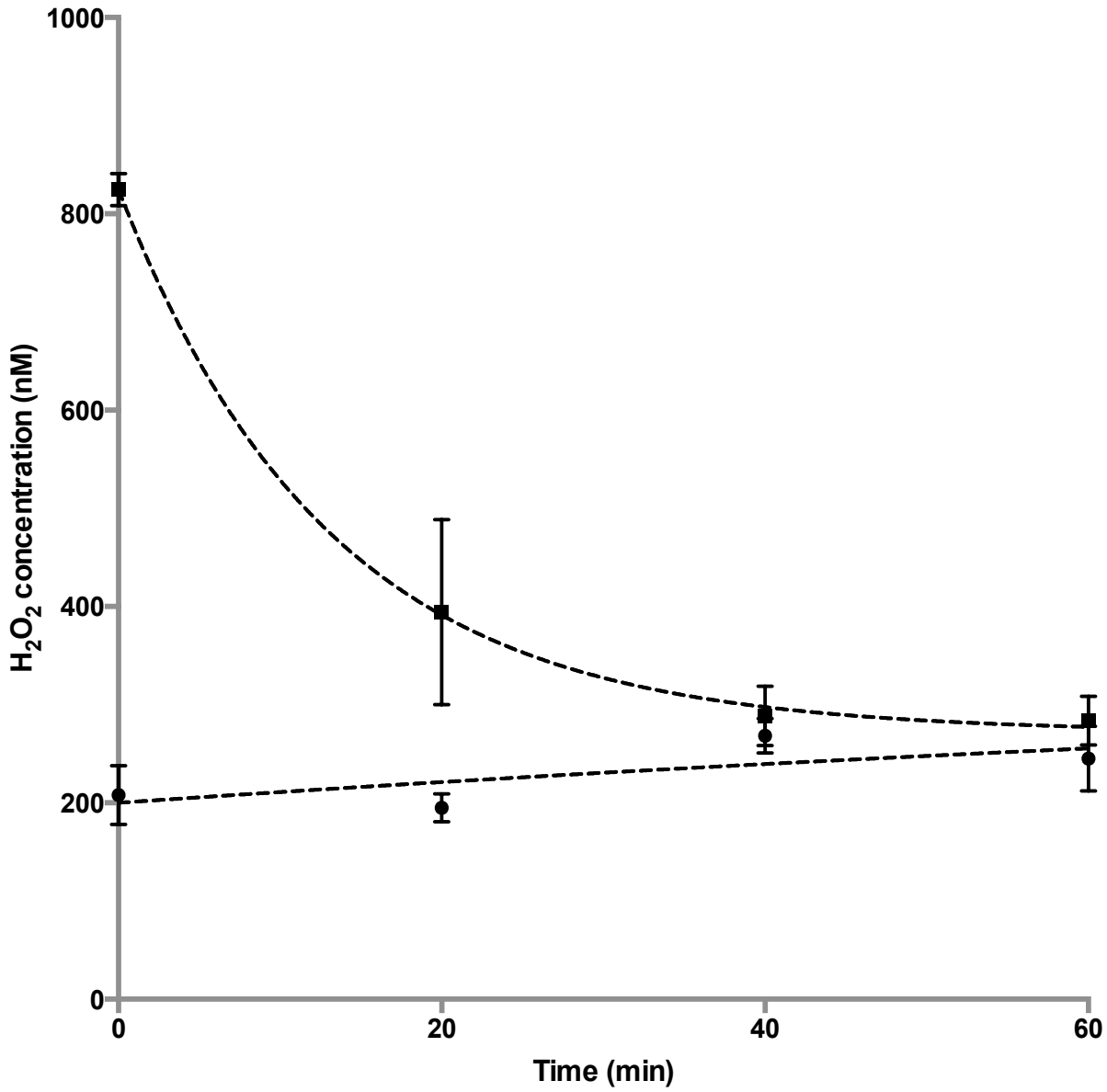

Figure 6. Plot of H_2O_2 over time in the spiked (■) and control (•) treatments testing isolate LE-E3. Dashed lines show curve used to calculate absolute H_2O_2 decay rate coefficient and production rate. Error bars show standard deviation between three replicates of each treatment.

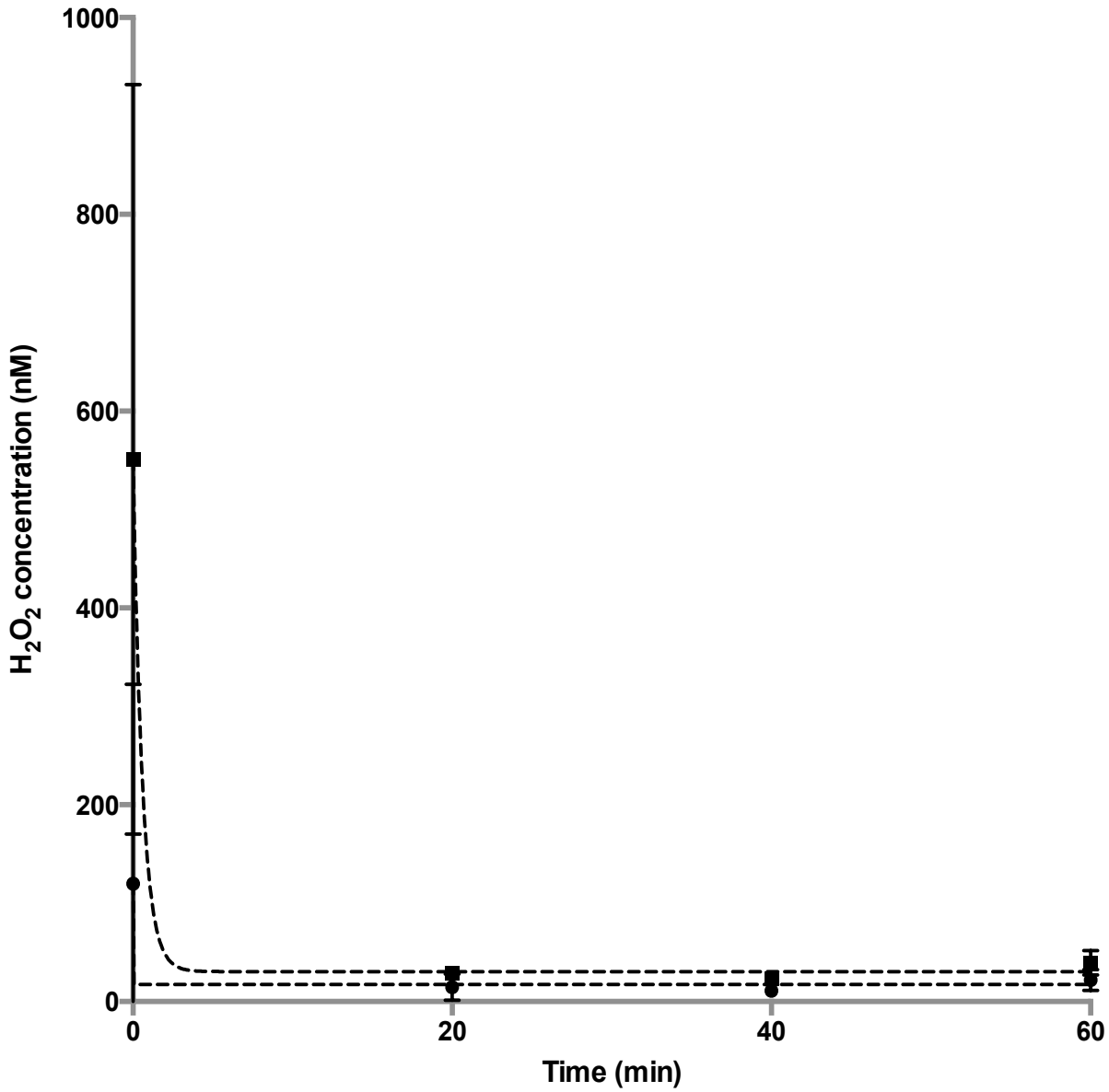

Figure 7. Plot of H_2O_2 over time in the spiked (■) and control (•) treatments testing isolate LE-E1. Dashed lines show curve used to calculate absolute H_2O_2 decay rate coefficient and production rate. Error bars show standard deviation between three replicates of each treatment.

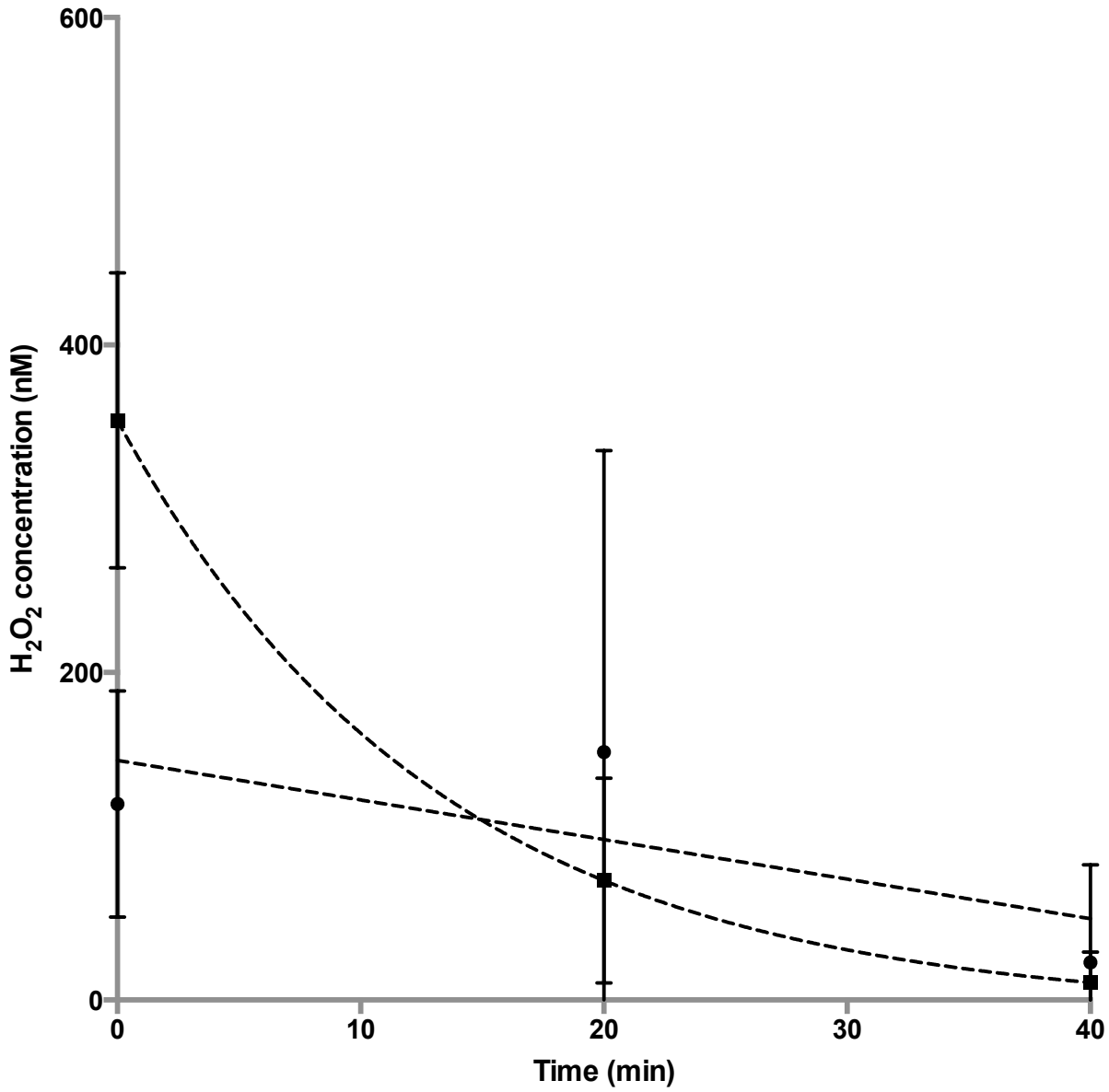

Figure 8. Plot of H_2O_2 over time in the spiked (■) and control (•) treatments testing isolate LE-L6. Dashed lines show curve that would be used to calculate absolute H_2O_2 decay rate coefficient and production rate. Error bars show standard deviation between three replicates of each treatment.

Appendix

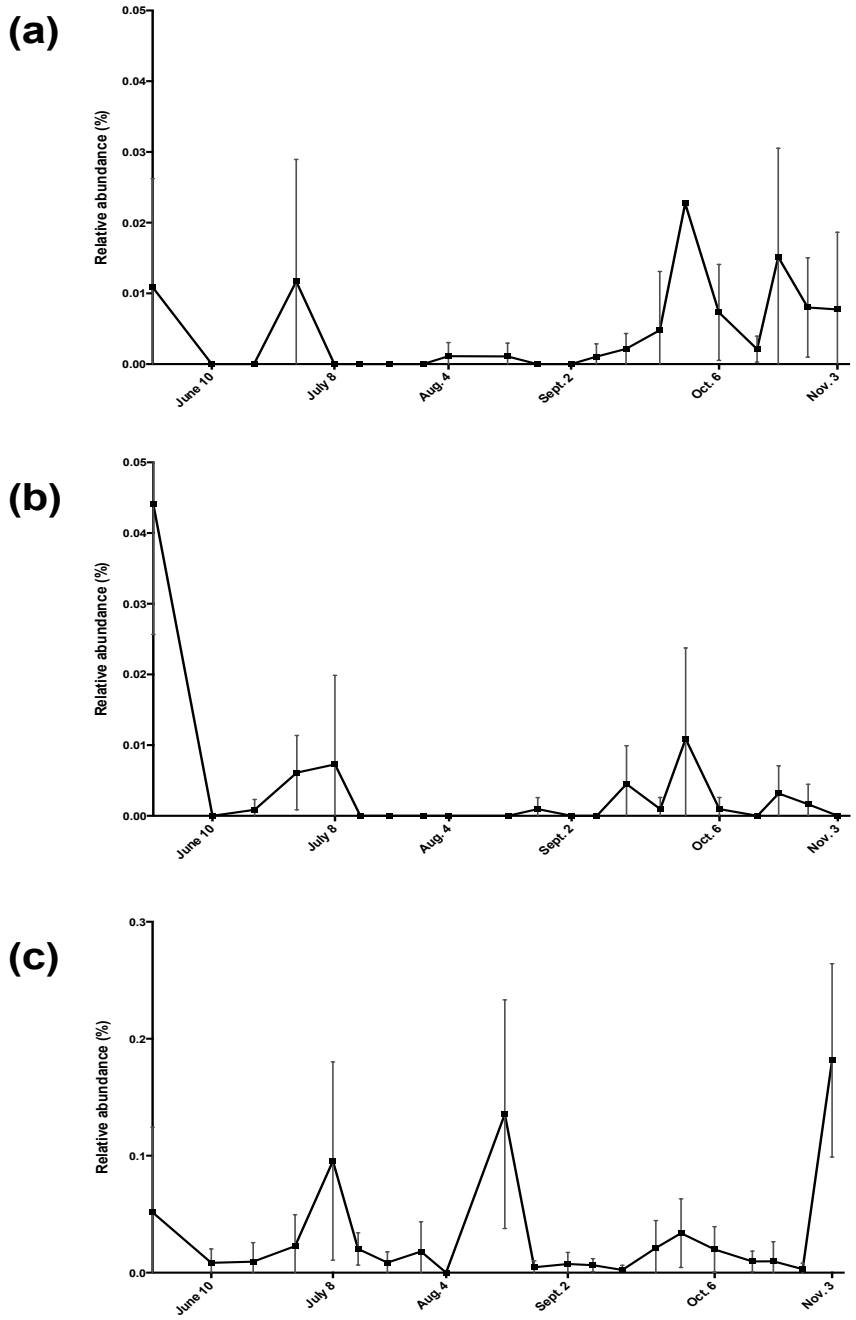

Figure A1. Relative abundance of OTU00122 (isolate LE-E3) in the whole community (a), 22μm (b), and 3μm (c) fractions of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

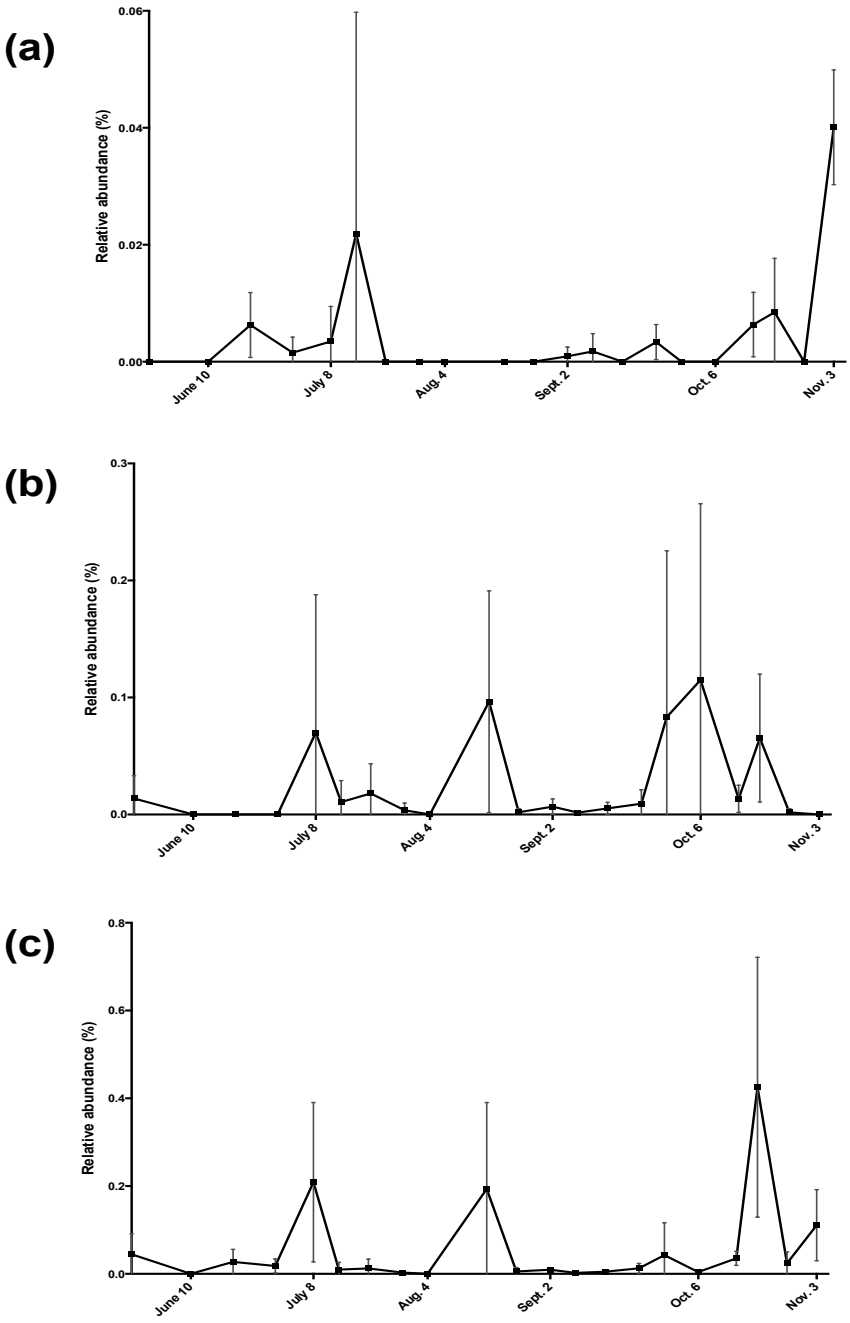

Figure A2. Relative abundance of OTU00127 (isolates LE-E6 and LE-L6) in the whole community (a), 22µm (b), and 3µm (c) fractions of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

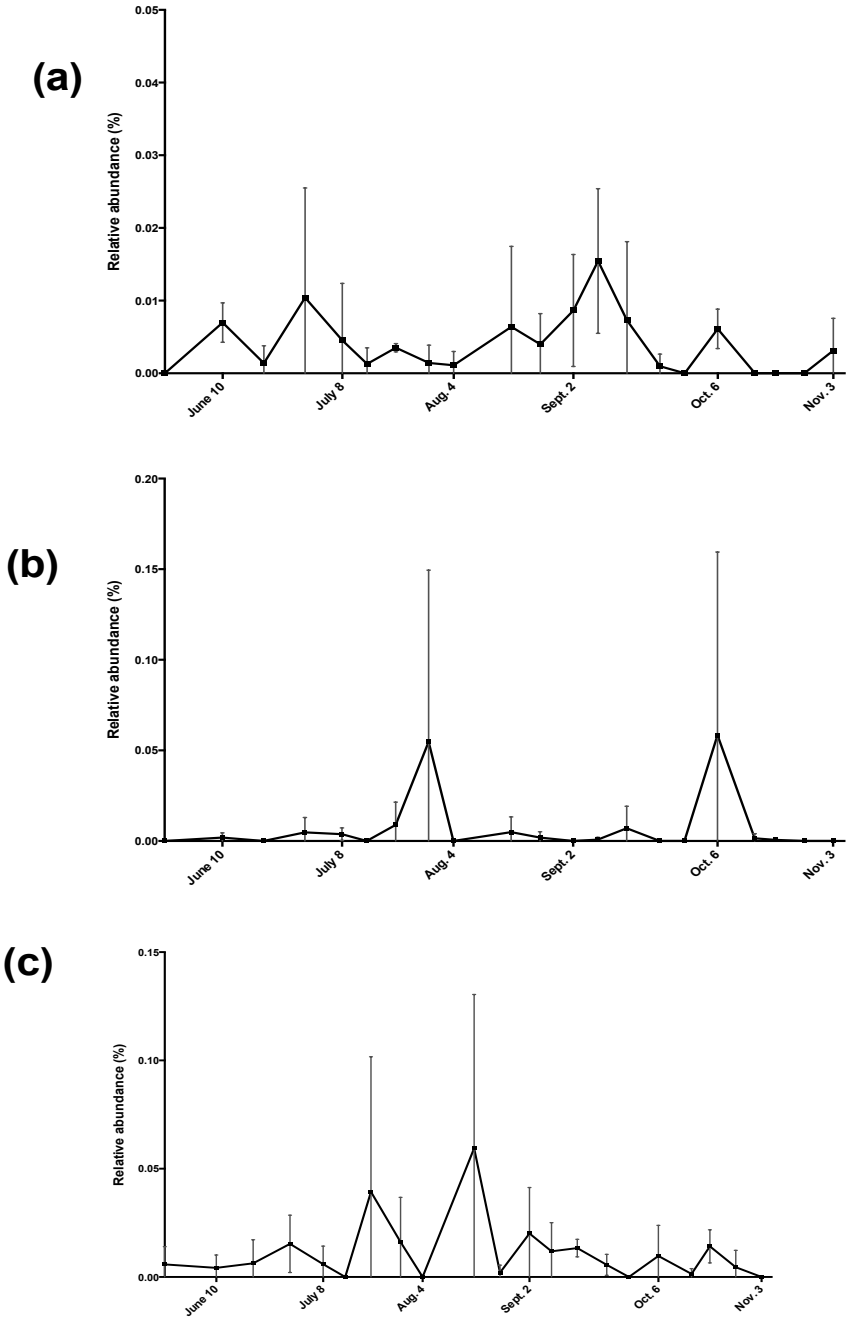

Figure A3. Relative abundance of OTU00469 (isolate LE-L1) in the whole community (a), 22µm (b), and 3µm (c) fractions of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

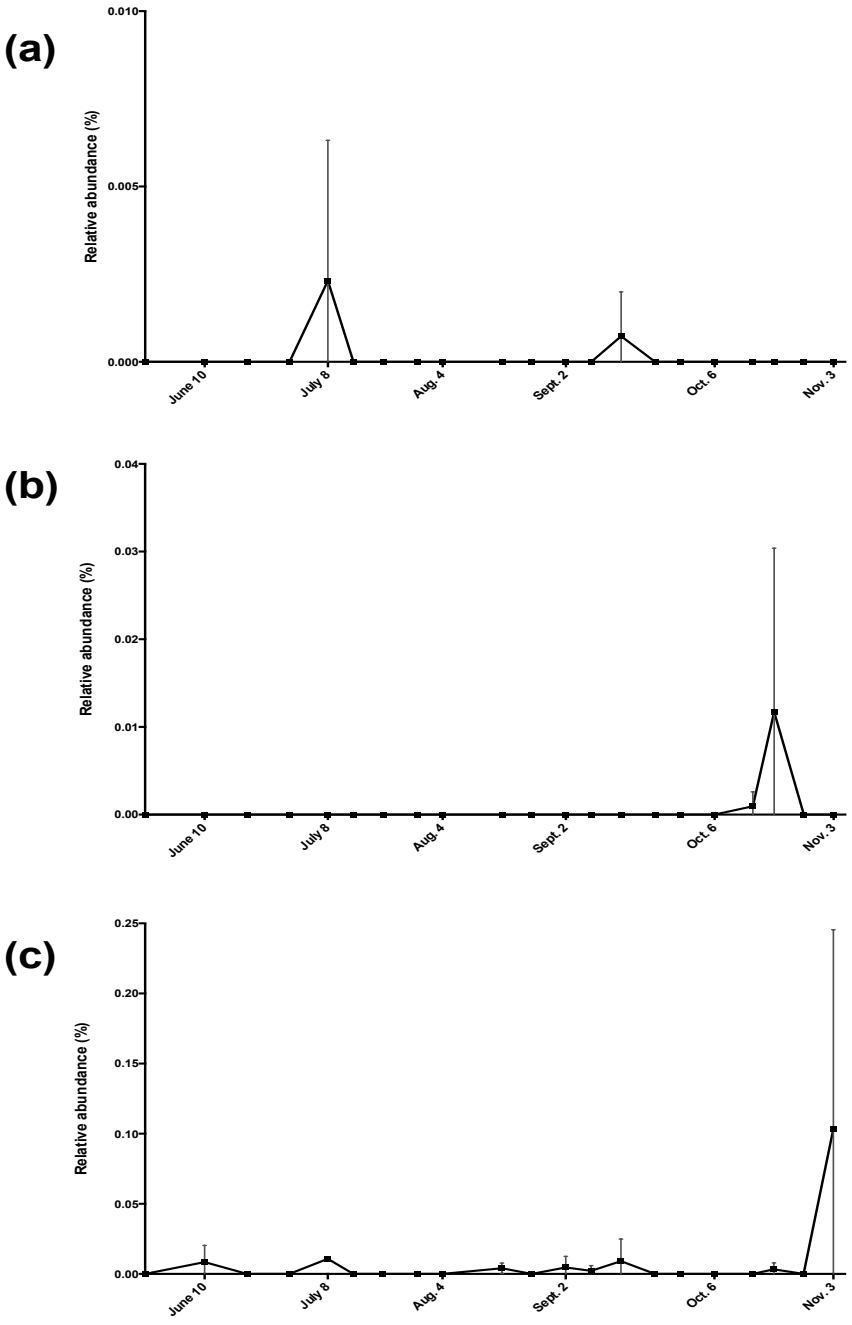

Figure A4. Relative abundance of OTU00985 (isolate PE-6) in the whole community (a), 22µm (b), and 3µm (c) of the bacterial community in the Western Basin of Lake Erie over the cyanobacterial bloom season. Error bars indicate standard deviation of results from three sampling stations.

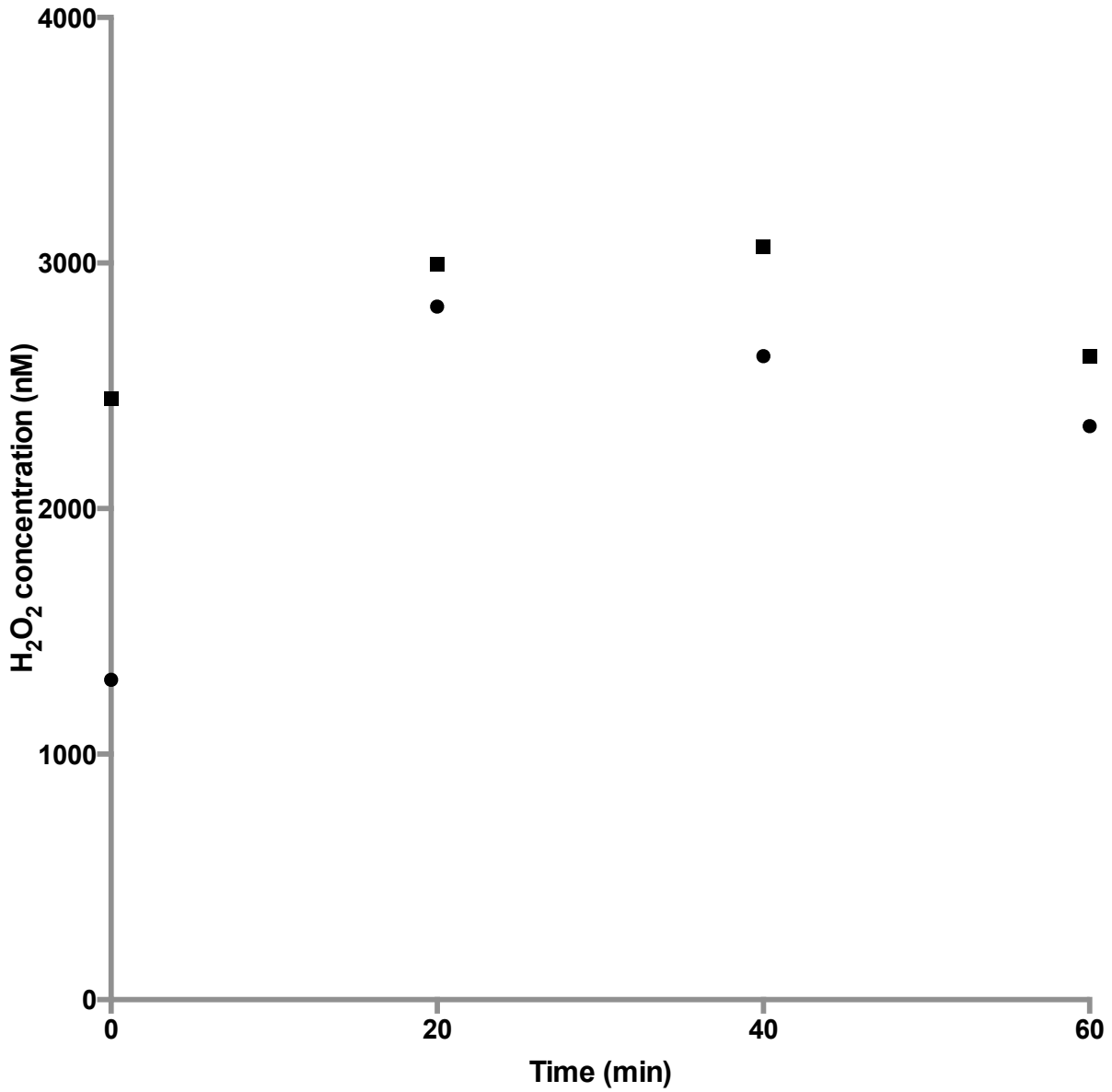

Figure A5. Plot of H_2O_2 over time in the spiked (■) and control (•) treatments in sterile media (0.2 g/L yeast extract in 0.22 μm filtered Lake Erie water).

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