WHERE THE WATER MEETS THE SKY:
THE EFFECTS OF ATMOSPHERIC OZONE POLLUTION
ON AQUATIC ALGAL AND
BACTERIAL COMMUNITIES.

By

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ABSTRACT

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Ozone is a highly reactive oxidant and is the primary constituent of photochemical smog. Since the industrial revolution, ozone levels in the troposphere have been rising and, during warm seasons, regions all over the world reach concentrations that exceed the US EPA’s national health-based standard of 75 ppb. In the most polluted areas, maximum levels have reached over 400 ppb. Numerous studies have shown acute and chronic impacts of ozone pollution on the health of terrestrial plants and animals. Ozone is readily soluble in water and is often gravitationally deposited onto surface waters. This research is the first to examine the potential of tropospheric ozone as an aquatic pollutant by focusing on the effects of atmospheric ozone levels on algal and heterotrophic bacterial assemblages.

Algae and bacteria were grown in three different ozone atmospheres (0, 80, and 250 ppb) and biomass and assemblage composition were measured. Individual experiments focused on 1) the community-level responses of natural, multi-division periphytic algal assemblages to these different ozone levels and the interactive effect of dissolved organic carbon (DOC), 2) the effect of elevated atmospheric ozone levels on heterotrophic bacteria within the above periphyton matrices and in the absence of algal interactions, and 3) the independent responses of diatom and cyanophyte communities to ozone pollution in the absence of interdivisional interactions.
Ozone had both negative direct effects and positive indirect, interactive effects on algal biomass and assemblage composition. Within the natural periphyton assemblages ozone effects varied with algal division and DOC concentration. In the low DOC water, ozone effects were minimal. However in the high DOC water, the interactive effects of ozone and DOC were great. Diatom biomass was maintained at lower levels in ozone treatments but cyanophytes colonies increased by two orders of magnitude. This DOC and ozone interaction therefore led to a shift of assemblage dominance from diatoms to cyanophytes. Heterotrophic bacterial density in these periphyton films was closely correlated with algal biomass.

Responses changed when each group was treated independently. The cyanophyte-only assemblages were directly affected by the oxidative stress created by ozone treated environments and biomass was significantly lower in the ozone treatments. Diatoms, in independent cultures, were unaffected by ozone treatment and heterotrophic bacterial growth was facilitated.

This study indicates the potential of atmospheric ozone to cause ecologically significant changes to aquatic systems and highlights the need to consider direct and indirect effects of any potential ecosystem stressor, species interactions, and effects in different environments. Integrating the results of my experiments indicates that ozone has greater effects on algae and bacteria in high than low DOC waters, and that ozone may cause a shift toward cyanophytes in high DOC waters. I hypothesize this is due to ozone oxidation of DOC and release of organic and inorganic resources that stimulate growth, and diatom mucilages mediated oxidative stress of ozone on bacteria in the periphyton mat. Because ozone effects differed among algal divisions and heterotrophic bacteria, with different roles, atmospheric ozone may change microbial food webs and biochemical cycling within ecosystem, and these effects are likely more important in high than low DOC waters.
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Chapter 1: Introduction to the tropospheric ozone pollution problem and potential effects on aquatic communities

Tropospheric Ozone: Chemistry, Transport and Distribution

Ozone (O₃), the most important constituent of photochemical smog, is a highly reactive oxidant linked to damage of animal and plant tissue as well as numerous inorganic materials. Because of its strong potential to harm living organisms and cost property and crop owners, this trace atmospheric gas has been regulated by the EPA. Being approximately 3000 times stronger a greenhouse gas than CO₂, tropospheric ozone also significantly affects the radiative balance of the atmosphere. The Intergovernmental Panel on Climate Change now considers ozone to be the third most important greenhouse gas after carbon dioxide and methane. It occurs naturally in the troposphere at background levels of 10-20 ppb (Finlayson-Pitts and Pitts 1999) but has increased greatly since the industrial revolution.

Some of this natural ozone is transported from the stratosphere where it is produced, secondarily, from the photodissociation of molecular oxygen. It is also produced directly in the troposphere through the photoreduction of nitrogen dioxide (NO₂) and the subsequent combination of a singlet O atom with molecular oxygen (Fig. 1.1) (Committee on Tropospheric Ozone 1991).

\[
\begin{align*}
\text{NO}_2 + \text{hv} & = \text{NO} + \text{O} \\
\text{O} + \text{O}_2 & = \text{O}_3 \\
\text{O}_3 + \text{NO} & = \text{NO}_2 + \text{O}_2
\end{align*}
\]

Fig. 1.1. O₃ production initialized by the photoreduction of NO₂
However, this ozone is quickly consumed by reacting with nitric oxide (NO) from the initial photochemical reaction, so it does not accumulate to a great extent. Because of the limited intrusion of stratospheric ozone, the quenching of newly produced ozone by NO, and the low levels of naturally occurring NO₂, the pre-industrial troposphere is thought to have contained only 10% of all ozone in the atmosphere and to have varied very little in concentration (Chatfield and Harrison 1977). Since ozone is a trace gas, small changes to ozone precursor molecules may have profound effects on tropospheric concentrations. Since the industrial revolution, near-surface ozone levels have been measured at much greater concentrations than can be accounted for by the above sources. As an example, average pre-industrial tropospheric ozone concentrations in Paris were about 10 ppb, 1988-89 averages in the least polluted parts of Europe were between 20 and 45 ppb (Janach 1989; Volz and Kley 1988). Ambient ozone levels have steadily increased and, today, maxima have been measured above 350 ppb in heavily polluted areas (Kley et al. 1999). In the U.S., the national ambient air quality standard (NAAQS) for ozone, developed to protect human and ecosystem health, is 75 ppb for an eight hour average.

It was determined in the 1950s that this ozone accumulation in the near-surface atmosphere requires volatile organic carbons (VOCs) in addition to NO₂ and NO (the combination of which is represented by “NOₓ”) (Haagensmit et al. 1953). The presence of VOCs causes enhanced conversion from NO to NO₂ and therefore, the accumulation of ozone to concentrations above those found in the background troposphere, through the photolysis of this additional NO₂ (Committee on Tropospheric Ozone 1991). The chemical reactions involved are numerous and complex with over a hundred different chemical species of VOCs, each with varying reactivity and lifespan (Finlayson-Pitts and Pitts 1999). A generalized reaction scheme
is shown in Fig. 1.2, taken from Kley et al. (1999), in which the oxidation of an alkane (RH) is used to illustrate the involvement of VOCs in ozone production and accumulation.

This relationship between NO\textsubscript{X}, VOCs, and ozone is non-linear and the control of ozone production often depends on the specific VOC/NO\textsubscript{X} ratio. In general, for VOC/NO\textsubscript{X} ratios of greater than 8:1\textsuperscript{1}, lowering NO\textsubscript{X} concentrations results in lower peak concentrations of ozone (Committee on Tropospheric Ozone 1991). This is called a “NO\textsubscript{X}–limited” system and is characteristic of less polluted areas downwind of city centers where biogenic or industrial VOC production outpaces NO\textsubscript{X} emissions from power plants and automobile exhaust. In such situations, ozone concentrations are not sensitive to reduction in VOCs if it is not coupled with a reduction in NO\textsubscript{X}. In some highly polluted urban areas with large NO\textsubscript{X} emissions, we see VOC/NO\textsubscript{X} ratios less than 8:1. In these “VOC-limited” atmospheres, decreasing the VOC at constant NO\textsubscript{X} or simultaneously lowering both VOC and NO\textsubscript{X}, proportionately, will result in lower peak ozone concentrations. However, due to the complex photochemistry of this ozone production, if only NO\textsubscript{X} is lowered, peak ozone concentrations will actually increase until the critical ratio is reached. The sources, concentration, and ratio of VOC and NO\textsubscript{X} vary tremendously geographically and temporally, but the underlying photochemistry remains the same worldwide and must be considered when trying to control ozone accumulation.

\textsuperscript{1}The critical ratio may vary with the reactivity of the VOC mixture. Also, Kley et al.,1999 use a VOC/NOx ratio of 4:1 as the ridge line between NO\textsubscript{X}-limited and VOC-limited regimes.
The sources of NO\textsubscript{x} emissions into the troposphere are both natural and anthropogenic. Lightning, naturally-occurring vegetation fires and microbial metabolism in soils contribute the majority of natural emissions. The contribution of lightning varies seasonally and may be more important in the upper troposphere, particularly during summer months. However, globally, these natural emissions contribute less than 20\% of the total NO\textsubscript{x} emissions and in industrialized nations may contribute a much smaller fraction (Zhang et al. 2003). The greater emissions are all anthropogenic in origin and include fossil fuel combustion, biomass burning, and increased microbial action due to fertilizer application (Kley et al. 1999). Estimates for total global emission of NO\textsubscript{x} range from about 30-45 Tg/yr\textsuperscript{2}, but most agree that over half of the total tropospheric inventory of NO\textsubscript{x} is attributable to fossil fuel combustion by coal-fired electricity production and gasoline/diesel use (Fowler et al. 1998). The importance of the two sources (and others) may vary regionally (http://www.epa.gov/air/emissions/), but over the Northern Hemisphere, especially over the mid-latitudes, the majority of these emissions may be attributed to gasoline and diesel engines (Kley et al. 1999).

\textsuperscript{2} Tg=Teragram=1,000,000 metric tons
Fig. 1.2. Generalized reaction scheme illustrating the involvement of VOCs in ozone production and accumulation.

The most recent U.S. EPA inventory (http://www.epa.gov/air/emissions/) lists gasoline and diesel engines as supplying approximately 60% of the national emissions of NO\textsubscript{x}, 35% of that being due to on-road vehicles. Electrical powerplants contributed approximately 20%.
Vehicle traffic has been statistically tied to ozone pollution in European and North American cities (Committee on Tropospheric Ozone 1991; Granier and Brasseur 2003; Ibarra-Berastegi and Madariaga 2003). With automobile and energy use on the rise in the U.S. as well as in emerging economies, such as China and India, we expect to see greater overall emissions. In regions where fossil fuel use is much less, such as the southern hemisphere, the other anthropogenic sources of NO\textsubscript{x} may cause local pollution events. Burning biomass to clear croplands and forests is a common practice in developing countries and has been shown to contribute significantly to ozone production (Fiore et al. 2002; Kondo et al. 2003; Satsangi et al. 2004), especially over southern Africa (Thompson et al. 2002), the South Atlantic (Swap et al. 2003), and South America (Sanhueza et al. 2000). Even soil emissions of NO\textsubscript{x}, from the over-fertilization of croplands, can contribute to local ozone highs in areas with a ready VOC source (Simpson 1993). Due to newly introduced regulations (some voluntary) and demographic shifts, there have been regional reductions in NO\textsubscript{x} emission worldwide but overall, they are on the rise in the Northern Hemisphere, increasing over the US and especially over China and India where energy and automobile use are increasing dramatically from year to year (Butler et al. 2011; Fiore et al. 2002; Meng et al. 2010; Sheel et al. 2010; Vingarzan 2004). In fact, Xing et al. (2011) estimated, based on 2005 emission measurements, that total NO\textsubscript{x} emissions in China will increase 50\% by 2020 if the current regulation and enforcement remains. Also, long range transport of NO\textsubscript{x} emissions from other areas may keep concentrations high even where regional emission reductions have occurred.

Volatile organic compounds are generated both anthropogenically and biogenically. In urban and suburban areas, in the presence of NO\textsubscript{x} pollution, anthropogenic sources such as liquid
fuel combustion and evaporation, industrial use of solvents, and many household organic chemicals, such as those in some paints and cleaners, are the fuel for ozone accumulation. As with nitrogen oxides, the dominant VOC sources vary regionally (http://www.epa.gov/air/emissions/). According to the EPA’s year 2008 measurements, the largest U.S. source of anthropogenic VOCs was fossil fuel combustion by on-road vehicles and non-road equipment. The use of solvents followed closely.

On average, biogenic emission of volatile organic carbons (BVOCs) greatly exceeds anthropogenic sources. In 2005, the EPA estimated that approximately 74% of all VOCs released in the U.S. were biogenic (EPA 2008). Most BVOCs are produced by plants, but bacterial releases can be significant in certain areas. BVOC emission from forests alone is a larger source of VOC than all the anthropogenic sources in the U.S. combined. Forest emissions of BVOCs are important sources of ozone accumulating in rural areas. These compounds, in the presence of NOx pollution downwind from urban or industrial areas, stimulate great ozone accumulation. Although there are many different types of BVOCs with varying chemistry, most are more highly reactive than anthropogenic VOCs. This fact, coupled with their high concentrations, means that BVOCs are often the primary determinant of photochemistry in rural and some suburban locations where vegetation is greatest, although others (Chameides et al. 1988; Chameides et al. 1997) suggest that BVOCs should be considered a potentially significant contributor to the photochemistry above urban areas as well. This large source of biogenic hydrocarbons sometimes leads to higher ozone levels over forested land than over large urban centers (Bell and Ellis 2004). Even considering predictions of fewer trees due to future deforestation, the levels of biogenic VOCs are hypothesized to rise due to increased production by individual trees in response to increases in global CO₂ and warming temperatures.
Because of this uneven emission of ozone precursors, their atmospheric transport, and local weather conditions, the spatial and temporal variability of ozone concentration is great. The levels of ozone recorded in the troposphere currently range from 10 to close to 400 ppb (Eder et al., 1993; Bobbink, 1998; Fast and Heilman, 2003; Vingarzan, 2004). Spatial variability is a function of geographic location, elevation, and meteorology, but more importantly of the extent of anthropogenic emissions of nitrogen oxides as they are often the “limiting factor” in ozone accumulation in rural/suburban areas. Ozone levels are often the highest above urban centers that have both large emissions of nitrogen oxides and VOCs. Mexico City, for example, has reached concentrations greater than 400 ppb (Zambrano and Nash 2000). However, ozone concentrations in the atmosphere above the surrounding areas may often be higher than over the city itself. This may be due to greater emissions of biogenic VOCs or in the following case, a high production of industrial VOCs. Daum et al. (2003) measured ozone in and around Houston, Texas. In this case Houston was measuring 40-90 ppb at the same time that air above a shipping channel outside of the city was reaching 120-200 ppb. Nitrogen oxide concentrations were equivalent but ozone production efficiency was much greater in this far less populated region because of a ready source of industrial hydrocarbons that kept the VOC/NO\textsubscript{x} ratio high.

Rural and remote areas are subject to the same type of phenomenon (Brankov et al. 2003; Chameides et al. 1997; Debaje et al. 2003; Kunhikrishnan et al. 2006; Metcalf et al. 2002; Rosenthal et al. 2003; Swap et al. 2003). Large emissions of biogenic hydrocarbons from vegetation, especially from trees, create NO\textsubscript{x} sensitive zones in these less-populated areas so that the transport of NO\textsubscript{x} to these areas from urban and suburban centers will increase ozone concentrations (Bakwin et al. 1994; Utiyama et al. 2004). From 1993-1995, the air at 30-50% of
measuring stations in the rural eastern U.S. exceeded the national 8-hour standard of 80 ppb and 2-12% exceeded 120 ppb (Fiore et al. 2002). These trends are also seen in rural China (Cheung and Wang 2001). Of primary ecological concern in the U.S. is ozone pollution of national parks. Ozone levels in these protected areas, particularly in the southwest, regularly exceed the 8-hour EPA standard for ground-level ozone (75 ppb) and in summer ozone concentrations often exceed those over urban centers. From April through August of 2011, ozone levels exceeded the NAAQS (75 ppb) 222 times in national parks with ozone monitors (NPS 2011) these maxima are often higher than those in nearby metropolitan areas. Transport from rural areas may influence even more remote areas and threaten some fairly pristine habitats that are largely shielded from non-atmospheric pollutants. For example, ozone concentrations reaching 40 ppb were measured in a remote Venezuelan cloud forest, where pre-industrial levels were 4-8 ppb (Sanhueza et al. 2000).

Local and regional transport is accompanied by long-range, intercontinental transport, which occurs in the upper, free troposphere and contributes to global background ozone (Liu et al. 2003; Thompson et al. 2002). For example ozone over the North Atlantic averages around 25 ppb in the summer and this is attributed to transport from North America (Parrish et al. 1993). The footprint of North American pollution extends to Europe as well. The air over North America is influenced by pollution from China and India (some estimates of influence range from 3-10 ppb) (Liu et al. 2003; Parrish et al. 1993), while African biomass burning may influence ozone production over the Southern Atlantic Ocean and Austral-Asia land masses (Kondo et al. 2003). European emissions have been shown to greatly increase the background pollution over Asia (Parrish et al., 1993). Because of this transport, it has been estimated that global background ozone levels have doubled since the turn of the century (Vingarzan, 2004).
Records of ozone measurements from the late 1800s over North America and Europe indicate 19th century background levels of 5-10 ppb (Vingarzan, 2004). Current estimates indicate background levels of 10-45 ppb (Fiore et al., 2002).

Ozone concentrations also vary temporally on seasonal and diurnal scales. Seasonally, ozone concentrations tend to be highest in the summer when sunlight is most intense and biogenic VOCs are being produced in large quantities. During the day in urban areas, NO\textsubscript{x} and VOCs tend to rise with the increase in industrial and automobile activity and, as a consequence, ozone levels tend to be highest shortly after noon. Areas downwind of urban centers may peak later in the afternoon or early evening. After sundown, photooxidation ceases and existing atmospheric ozone is scavenged by other chemicals or is deposited on land or surface water. Therefore, nighttime ozone levels near the ground can fall to near zero (Kley, et al., 1999)

Local production, regional and longer-distance transport, and high background levels lead to a global ozone pollution problem. Models have estimated a 100-120\% increase in ozone in the northern mid-latitudes over the past 200 years and an increase of 80-100\% in the tropics (Committee on Tropospheric Ozone 1991). All but one scenario proposed by the Intergovernmental Panel on Climate Change (IPCC) projects increases in tropospheric ozone during the 21st century with projected background concentrations of 35-48 ppb by 2040, 38-71 ppb by 2060, and 42-84 by 2100 (Vingarzan, 2004). This means that background concentrations may soon exceed internationally accepted maxima established to protect animal and plant health. The increases are expected to be greatest in the tropics, specifically South Asia, and also in Central America, South America, and southern Africa. However, the northern hemisphere will also see a rise. Since dry deposition of this atmospheric ozone (i.e., deposition not associated with precipitation) is the largest sink (Fowler et al., 1998), the potential for ozone damage to
ecosystems is very real and appears to pose an even bigger threat in the future. Much of this ozone is and will be over surface waters. Because ozone is highly soluble in water some of this atmospheric component may be expected, through dry deposition, to dissolve into and change the oxidative state of the water.

**Tropospheric Ozone: Effects on Terrestrial Organisms**

Ozone, as a highly reactive oxidant, has the potential to react with unsaturated organic chemical bonds, including proteins, lipids, peptidoglycans and enzymes, causing cellular damage or death and inducing defensive responses that will change the chemical composition of cells (Kley et al. 1999; Meehan et al. 2010). This cellular damage results from the oxidative stress caused directly from the ozone molecules but also from other reactive oxygen species (ROS) such as hydrogen peroxide, hydroperoxide, superoxide, hydroxyl, and singlet O. These oxygen species result from ozone speciation, cellular production induced by the initial stress, or oxidation of membranes (Oksanen 2003; Schraudner et al. 1997).

Inhalation is the primary mechanism of internal ozone exposure in humans and other mammals so damage is predominantly to lung tissue. Acute respiratory damage and worsening of asthma may occur at levels as low as 60 ppb and higher concentrations or chronic exposure may lead to persistent effects on lung tissue and function (Lippmann 1991; Tilton 1989). Studies of reptiles and amphibians have indicated that high O₃ exposure may affect core body temperature by changing thermal preferences and, potentially, water balance (Dohm et al. 2001; Mautz and Dohm 2004). Atmospheric ozone may interfere directly with the olfactory senses of insects or indirectly through ozone interaction with the signal chemicals. Several studies have
shown that the searching efficiency of parasitoid wasps and the proportion of hosts parasitized decrease as a consequence of elevated ozone levels (Gate et al. 1995; Mondor et al. 2004).

In addition to the few studies showing direct effects, most studies of the effects of ambient ozone levels on wildlife show indirect effects on herbivores mediated by changes in the chemical composition or availability of their plant food sources (Augustaitis et al. 2007; Ditchkoff et al. 2009). The response varies depending on species. The growth rate and behavior of insects, in particular, have been shown to change in response to ozone-mediated increases in leaf secondary metabolites or other physiological stress responses (Lindroth 2010; Valkama et al. 2007). The growth of many insect species, especially chewers that include potential crop pests, has been positively affected by ozone treatment of their host plants, showing shortened larval development time and greater pupal mass (Jones and Paine 2006). Feeding preference was also affected by ozone treatment but was dependent on herbivore and tree species, however, many insects prefer ozone treated leaves to those raised under ambient conditions (Agrell et al. 2005; Holton et al. 2003). These positive effects have been attributed to the greater concentration of secondary metabolites or the reduction of phenolic glycosides which have been shown to be detrimental to larval performance (Holton et al. 2003; Valkama et al. 2007).

Photosynthetic cells are naturally high in reactive oxygen species (ROS) and therefore are especially susceptible to oxidative overload; therefore, ozone-mediated changes to plants at the individual and community level are common. The physiological changes associated with ozone stress start with the loss of membrane integrity as these chemical species attack unsaturated lipids and proteins leading to internal production of assorted ROS (Skarby et al. 2004). As ROS increase, intracellular damage occurs (Kollner and Krause 2003; Oksanen et al. 2004; Skarby et al. 2004; Yamaji et al. 2003). Organelles, especially chloroplasts, suffer injury (Manning and
Oxidative damage can be mitigated through the production of a multitude of enzymatic and non-enzymatic antioxidants. This appears to be a primary response of photosynthetic cells and serves to quench much of the increased oxidative potential (Schraudner et al., 1997). The type and quantity of antioxidants produced varies depending upon species and may be the basis for different tolerances observed. The metabolic pathways associated with this physiological stress response leads to changes in leaf phytochemistry, specifically an increase in secondary metabolites, such as phenolic acids and flavonoids. At the same time, the production of primary metabolites, such as carbohydrates, is often unaffected or only slightly negatively affected (Holton et al. 2003; Lindroth 2010; Valkama et al. 2007). The response varies depending on species and even genotype within a species ((Furlan et al. 2004; Oksanen et al. 2004)). In terms of the above changes in leaf chemistry, angiosperms have been shown to be much more susceptible to ozone than gymnosperms (Holton et al. 2003).

Primary producers of all types have been shown to be sensitive to elevated levels of atmospheric ozone (Bermejo et al. 2003; Booker et al. 2009; Braun et al. 2004; Fiscus et al. 2005; Fuhrer 2009; Heath 1994; Morgan et al. 2006). This ozone stress causes both acute and chronic effects in plants. Acute responses resulting in foliar injury, pigment loss and premature senescence occur when a plant’s antioxidant response is quickly overwhelmed by a large concentration of ozone (Krupa et al. 1998). For example, necrotic lesions on tobacco plants have developed within 15-72 hours after a single acute ozone dose of 150 ppb (Schraudner et al. 1997). Chronic damage is a cumulative effect of multiple responses to low and high ozone intake by a plant through its life cycle. Responses to chronic exposure vary depending on age, species, and developmental stage but generally include a reduction of photosynthetic rate, growth
rate and decreased root biomass due to efforts to maintain above-ground growth (Chappelka and Samuelson 1998; Schraudner et al. 1997; Valkama et al. 2007).

Due to these effects on plants, tropospheric ozone has been identified as the most damaging atmospheric pollutant to crops and forests (Heagle 1989). In fact, ozone is the only major air pollutant that has been shown to induce plant damage at ambient concentrations (Schraudner et al., 1997). Damage to potato, soybean, wheat, and tobacco crops due to ozone has been reported (Eder et al. 1993; Ishii et al. 2004; Kollner and Krause 2003; Madden and Hogsett 2001; Schraudner et al. 1997). Avnery et al. (2010) estimated “that year 2000 ozone-induced global yield reductions ranged, depending on the metric used, from 8.5-14% for soybean, 3.9-15% for wheat, and 2.2-5.5% for maize. Global crop production losses totaled 79-121 million metric tons, worth $11-18 billion annually.” They and others (Bender and Weigel 2011) predict even greater losses in the future.

In addition to reductions in crop yield, damage to natural communities has been documented, particularly to forest trees (Broadmeadow 1998; Chappelka and Samuelson 1998; Eder et al. 1993; Oksanen et al. 2004) and grassland species (Franzaring et al. 2000), but also to wetland plants such as *Sphagnum* mosses (Niemi et al. 2002). It is estimated that approximately 25% of the global forests are currently at risk during the growing season from ozone concentrations above 60 ppb and estimates indicate that this will rise to 50% by 2100 (Oksanen, 2003). These damage responses are species dependent (Bobbink 1998; Franzaring et al. 2000; Novak et al. 2003) and this differential species response may lead to compositional shifts in natural communities, even if community biomass is not affected.

The study of the response of terrestrial algal cells to atmospheric ozone has been limited to lichen cells and symbiotic cyanophytes. Nitrogen-fixing cyanophyte symbionts were shown
to have inhibited growth, nitrogen fixation, and heterocyst formation when exposed to ozone concentrations as low as 30 ppb for one week (Hur and Wellburn 1994). Lichens have long been used as indicators of air pollution and studies have indicated that a response to ozone stress has led to precipitous declines in lichen species richness and cover in areas outside of Mexico City and in coniferous forests in California (Canas and Pignata 2000; Garcia et al. 2000; Scheidegger and Schroeter 1995; Sigal and Johnston 1986; Zambrano and Nash 2000; Zambrano et al. 1999). This decline has been attributed to a loss of chlorophyll $a$ by the algal symbiont and the subsequent reduction of photosynthetic rate, a response similar to that of plants. Also, a primary response of lichen algae appears to be an increase in the antioxidant, superoxide dismutase (Calatayud et al. 2000).

**Tropospheric Ozone: Hypothesized Effects on Aquatic Ecosystems**

There has been almost no research on the effects of atmospheric ozone on aquatic communities, but we may hypothesize similar effects. Changes to olfactory chemicals in the water, behavioral changes as a response to oxidative stress (i.e. drift), and changing algal community composition or chemistry may influence the abundance and types of invertebrates in the affected area. Studies of wetland plants have indicated a greater sensitivity to ozone than plants not associated with water-logged soils (Franzaring et al. 2000; Power and Ashmore 2002; Williamson et al. 2010). This is attributed to the higher rates of growth, stomatal conductance, and leaf area associated with wetland plants (Power and Ashmore 2002). Given the high surface-to-volume ratio and thin cuticle of most submerged angiosperms and mosses we may hypothesize a greater potential for response as well. Ozone effects on aquatic algae have been studied only in regards to wastewater treatment. At sufficiently high ozone concentrations, algal
cells are “oxidized to death” and even their byproducts may be destroyed (e.g. cyanotoxins) (Yun et al. 1997). There are differences in the way that certain algae respond to ozone, and different concentrations and exposures times are necessary to eliminate different species (Hoeger et al. 2002; Lai et al. 2002; Paralkar and Edzwald 1996; Widrig et al. 1996). Although photosynthetic rates of algae after ozonation were severely retarded, even by short ozone contact (Yun et al., 1997), studies of the longer-term sub-lethal effects of ozone are few.

Studies of heavy metal and hydrogen peroxide (H$_2$O$_2$) induced oxidative stress may serve as models of potential ozone damage. The oxidative stress created by these factors works much the same way that ozone-induced stress does in terrestrial plants; a direct oxidation of lipids and proteins and increased cellular production of reactive oxygen species which may lead to a saturation of antioxidant capacity (Pinto et al. 2003). Algal cells show an immediate increase in both enzymatic and non-enzymatic antioxidants, superoxide dismutase being primary in most species (Leitao et al. 2003; Pinto et al. 2003). Where reactive oxygen species production overwhelms the antioxidants, membrane damage (especially in chloroplasts), pigment loss, and even cell death have occurred (Mallick and Mohn 2000). Algal species differ in their response to H$_2$O$_2$-induced stress (Barroin and Feuillade 1986; Kay et al. 1984). Drabkova et al. (2007) found that photosynthesis was inhibited by ozone to a greater extent in five cyanophyte species than three green algal species and one diatom species. For example, *Microcystis aeruginosa*, a cyanobacterium, was negatively affected at ten times lower H$_2$O$_2$ concentrations than that of the green alga, *Pseudokirchneriella subcapitata*. Using these responses as a model, we may hypothesize that the oxidative damage and defense response caused by ozone exposure may lead to a reduction of algal photosynthetic rate and change of cellular chemistry. These may in turn
lead to ecologically relevant changes in biomass, community composition, as well as algal nutritional value and palatability to herbivores.

Algal communities also may be indirectly affected by the response of other organisms and/or dissolved substances to the increased oxidant levels (Fig. 1.3). Ozone stress may change inter-species interactions within the biofilm by differentially affecting various algal groups or heterotrophic bacteria, changing community dynamics and potentially changing algal community composition or total biomass. Bacterial, viral, and fungal pathogens are common to all types of algae and may have significant impacts on algal community composition, development, and senescence (Hewson et al. 2001; Peterson et al. 1993; Pollard and Ducklow 2011; Proctor and Fuhrman 1991; Vandonk and Ringelberg 1983; Vanetten et al. 1991). Viruses have been shown to be vulnerable to light (Suttle and Chan 1993) and all three pathogens are inactivated by ozone during wastewater treatment and also by gaseous ozone treatment of surfaces (Goncalves and Gagnon 2011; Hudson et al. 2009; Lenes et al. 2010; Mahfoudh et al. 2010; Tseng and Li 2008; Zhang et al. 2011).

Ozone and its dissolution species react with most organic molecules that they contact and, in natural waters, an important component is dissolved organic carbon (DOC). Up to 20% of terrestrial primary production can be exported as dissolved organic carbon, making it very important to aquatic communities (Lennon 2004; Lennon and Pfaff 2005; Wetzel 2001a). DOC molecules such as amino acids, carbohydrates, simple organic acids, and lipids are very labile and assimilated readily by bacteria, and so have very high turnover rates (Wetzel, 2001a). Their instantaneous concentration in the water column is very low. The remaining DOC, composing as much as 80% of that dissolved in inland waters, is humic in nature; made up of complex organic acids (humic and fulvic), that are mostly terrestrial in origin (Rosenstock et al. 2005). Humic
and fulvic acids are also the primary contributors to the tea-like color in high DOC waters. Due to their complex structure and aromatic carbon rings, humics are more recalcitrant than other DOC and thus are only very slowly assimilated by bacteria. They tend to have long residence times in the water column (Wetzel 2001a; Yu et al. 2002) and will be available for interactions with dissolved oxidants. In fact, they are often the primary sink for ozone in water treatment (Cho et al. 2003). Because of its primary and almost immediate reaction with dissolved oxidants, DOC may serve to protect algal cells from direct oxidative damage by quenching the oxidative potential.

Fig. 1.3. Hypothesized direct (solid line) and indirect (dotted line) effects of atmospheric ozone on aquatic algae.
The oxidative breakdown of humic substances leads to the formation of more labile organic compounds such as aldehydes, polysaccharides, and fatty acids that are more bioavailable to bacterial communities (Fahmi et al. 2003; Freese et al. 1999; Goldstone et al. 2002; Lennon 2004; Levine et al. 2000; Romani et al. 2004; Swietlik and Sikorska 2004; Westerhoff et al. 1999). Oxidation of humic substances can also lead to a release of iron and other trace metals, ammonium, amino acids, and phosphates as they often form complexes with these molecules (Ellis et al. 1999; Engelhaupt et al. 2003; Stewart and Wetzel 1982; Wetzel 2001d). Iron oxide forms a particularly tight bond with fulvic acids and there are data to suggest that this may cause iron limitation in humic waters (Imai et al., 1999; Guildford et al. 1987; Kerndorff and Schnizer 1980; Jackson & Hecky 1980; Giesy 1976).

Liberation of labile DOC and inorganic nutrients through the action of ozone on humic substances should serve to stimulate heterotrophic bacterial growth if direct oxidative stress on cells is not too great. There is ample evidence of an increase in bacterial growth rate after breakdown of recalcitrant molecules in certain systems, via uptake of labile DOC (Anesio et al. 2004; Engelhaupt et al. 2003; Lennon 2004; Middelboe and Lundsgaard 2003; Romani et al. 2004). Natural ultraviolet light has been observed to partially oxidize humic substances generating large amounts of simple fatty acids that will serve as ready metabolic substrates by bacteria (Volk et al. 1997). Many studies have found that DOC exposed to natural UV radiation leads to an immediate and sustained increase of bacterial growth (Lindell et al. 1995; Moran and Zepp 1997; Rosenstock et al. 2005; Stewart and Wetzel 1981; Wetzel et al. 1995). Ozone oxidation may do the same, changing algal/bacterial interactions.

The breakdown of aromatic bonds and depolymerization of molecules also leads to a strong decrease in color and UV absorbance (Arvola 1984; Freese et al. 1999; Graham 1999).
Indeed, my preliminary research, in which algae was grown continuously in one of three ozone atmospheres (0, 80, and 300 ppb) led to an unanticipated difference in water color between ozone treatments, that was visible to the naked eye. The clearing of the water and resulting increase in light penetration may have been the catalyst of an observed change from diatoms to green algae in the ozone treatment. In humic rich systems, ozone may release algae from light limitation through added oxidation of light-absorbing molecules leading to a potential increase in benthic and phytoplankton production or a shift in algal species composition.

The hypothesized and observed effects of humic compounds on microorganisms are complicated and may be positive or negative. The functional groups on many humic substances may react with biomembranes leading to increased permeability (Visser 1985). There are some data to indicate a unimodal response of cell membranes to humic effects. Lower amounts of humics can stimulate growth with the consequent increased permeability to essential molecules, but as concentrations increase, growth inhibition ensues (Steinberg et al. 2003; Wang et al. 1999). There is argument over whether or not humic substances are actually taken into cells; however, smaller fractions of humics have been found inside cells and humic substances < 3.5 kDa are known to pass through plant cell membranes (Nardi et al. 2002; Wang et al. 1999). Once inside, these molecules tend to have negative effects on algae, reducing photosynthetic oxygen release and cell yield. The mechanism of effect has not been well discerned but there are two accepted hypotheses: 1) that humic substances directly quench electrons or 2) that they bind to the bioquinones in photosystem II and thereby block electron transfer. Either way, ozone may affect photosynthetic processes.

Prokaryotic algae appear to be more sensitive to humic substances than other algae. Studies have shown cyanophytes reacting to lower concentrations of humics than other algal
groups (Bahrs and Steinberg 2012; Prokhotskaya and Steinberg 2007). Park et al. (2009) found a 24% reduction of green algal cell density at the same concentration of rice hull extract that reduced *Microcystis* densities by >98%. Prokhotskaya and Steinberg (2007) attribute this greater sensitivity to the simpler cell structure of cyanophytes –lacking membrane-bound organelles may leave internal apparatus susceptible to externally introduced substances-- and a smaller suite of antioxidant enzymes. Other studies have indicated that the differential response to these compounds may be species and molecule specific with the finding that genera within the cyanophytes, green algae, diatoms and euglenoids could be “very susceptible” to humic plant leachates (Martin and Ridge 1999). However, there have been studies that also indicate that humic substances in low concentrations may actually stimulate growth of algae, including some cyanophytes (Fagerberg et al. 2009; Sun et al. 2006). If dissolved humic substances do limit or promote the growth of different algal taxa in natural systems, then ozone breakdown of these substances may change competition dynamics and potentially cause a shift in algal community composition.

### Synthesis

Tropospheric ozone levels over extensive regions of the world are much higher than the natural concentrations expected from stratospheric transfer and oxidation of natural NO\textsubscript{X}. During warm seasons, regions all over the world reach concentrations that exceed the US EPA’s national health-based standard of 80 ppb and maximum levels could reach over 400 ppb. Perhaps more important is the prediction that ambient ozone concentrations will increase in the future, as well as the extent of regions affected by high ozone days. These levels have and will significantly change the oxidative potential of the atmosphere. Considering the relative ease of
dissolution into surface waters, we must turn our attention to the potential of tropospheric ozone as an aquatic pollutant.

The myriad of documented effects of ozone pollution on terrestrial primary producers through damage to membranes and proteins (especially those of the photosynthetic apparatus) and pigment loss, suggest a potential effect on aquatic photosynthesizers and heterotrophic microbes that depend on that production. My preliminary research on periphyton grown in a continuous 300 ppb ozone atmosphere demonstrated a division-level community shift and the many studies showing strong oxidative stress corroborate this hypothesis and indicate the need for research on algal communities. Thus, I hypothesized a shift in algal taxonomic composition and related ecosystem processes as a result of differential tolerances to oxidative stress by ozone.

Ozone and its dissolution species react with most organic molecules that they contact and humic substances, which are not readily taken up by bacteria, are available for oxidative breakdown and are often the primary sink for ozone in water treatment. As discussed, this oxidative breakdown leads to the creation of low molecular weight, bioavailable molecules and a loss of water color and hence reduced light attenuation. The effects of these changes on light penetration and bacterial production and the potential to ameliorate the negative effects of humics may be indirect factors affecting algal communities (Fig. 1.3). Thus I hypothesize dissolved organic carbon will mediate ozone-algal interactions.

The objectives of my dissertation are to manipulate atmospheric ozone concentration and DOC concentrations and determine their interactive effects on algal and bacterial communities. Given the absence of detailed research on ozone effects on aquatic ecosystems in ranges expected for tropospheric ozone pollution, my research will provide the foundation for future studies of the effects of ozone pollution on aquatic and semi-aquatic ecosystems. Specifically,
Chapter 2 focuses on the community-level responses of natural, multi-division periphytic algal assemblages to three different ozone levels and two different dissolved organic carbon concentrations. Chapter 3 is a study of the effect of elevated atmospheric ozone levels on heterotrophic bacteria within the original periphyton matrix and in the absence of algal interactions. Finally, Chapter 4 explores the independent responses of diatom and cyanophyte communities to ozone pollution without interdivisional interactions or resource competition. The potential for further research on atmospheric ozone effects on algae, other aquatic communities, and ecosystem functions is great. Invertebrates, vertebrates, and aquatic plants are all potentially affected either physiologically or behaviorally.

Abstract

Atmospheric ozone concentrations are increasing in many parts of the world. Ozone dissolution into surface waters may directly influence algal growth through oxidative stress or indirectly through changes to water chemistry. I used microcosms to address how atmospheric ozone concentrations, independently and interacting with dissolved organic carbon (DOC, in the form of humic substances) may change benthic algal assemblages. My results clearly indicate that both DOC concentration and atmospheric ozone levels affect algal assemblage composition and biomass. DOC concentration had a large independent effect on total algal biovolume but the direction of effect was dependent on algal taxonomic division. Ozone effects were significant but also varied with algal division and DOC treatment. I suggest that atmospheric ozone is a potential aquatic pollutant, but that the degree to which it affects benthic algae will depend on initial assemblage composition and the amount and quality of DOC.

Introduction

Ground-level concentrations of the oxidizing pollutant, ozone, are on the rise as fossil fuel consumption and vehicle use increase worldwide (Granier and Brasseur 2003; Ibarra-Berastegi and Madariaga 2003). The levels of ozone recorded in the troposphere currently range from 10 to close to 400 ppb (Bobbink 1998; Eder et al. 1993; Fast and Heilman 2003; Vingarzan 2004; Zambrano and Nash 2000). During the warm seasons, regions all over the world regularly reach tropospheric concentrations that exceed the US National Ambient Air Quality Standard.
(NAAQS) of 75 ppb. Perhaps more important is the prediction that background ozone concentrations and the extent of regions having high ozone days will continue to increase in the future. In fact, some models predict that summer background levels, generally defined as the concentration attributable to long-range transport, resuspension of previous emissions, and nonanthropogenic sources, will regularly exceed the NAAQS standard within 100 years (Vingarzan 2004).

Due to the long-range transport of ozone precursors from their source and the nature of the chemical reactions that create ozone in the troposphere, emissions in one area may change ozone levels long distances away (Kondo et al. 2003; Liu et al. 2003; Parrish et al. 1993; Thompson et al. 2002) and concentrations may be as high or higher in rural areas downwind of precursor sources than in urban areas (Chameides et al. 1997; Debaje et al. 2003; Metcalfe et al. 2002). In fact, higher ozone levels are routinely recorded in national parks downwind of urban areas than within urban areas (N.P.S. 2011).

Considerable research has been done showing the negative effects of ozone pollution on terrestrial systems, which can be great (Bermejo et al. 2003; Braun et al.; Heath 1994; Wonisch et al. 2004). Photosynthetic cells are naturally high in reactive oxygen species (ROS) and very sensitive to oxidative overload, thereby making them a sensitive receptor of ozone stress. Primary producers of all types, in agricultural as well as natural settings, are sensitive to elevated levels of atmospheric ozone, resulting in foliar injury, pigment loss, premature senescence, and decreased photosynthetic/growth rates (Bermejo et al. 2003; Braun et al. 2004; Heath 1994; Wonisch et al. 2003). In fact, ozone is the only major air pollutant that has been shown to induce plant damage at commonly measured concentrations (Schraudner et al. 1997). These negative
effects are obvious at the organism scale but have also been shown to affect overall crop yield and forest function (Avnery et al. 2010).

The study of the response of algae to atmospheric ozone has been limited to lichen cells and symbiotic cyanobacteria. Lichens have long been used as indicators of air pollution. Recent studies have indicated that a response to ozone stress led to precipitous declines in lichen species richness and cover in areas outside of Mexico City and in coniferous forests in California (Avnery et al. 2010; Canas and Pignata 2000; Garcia et al. 2000; Scheidegger and Schroeter 1995; Sigal and Johnston; Zambrano and Nash 2000; Zambrano et al. 1999). This decline has been attributed to effects on the algal component due to a loss of chlorophyll \(a\) and the subsequent reduction of photosynthetic rate. Nitrogen-fixing cyanobacterial symbionts have shown inhibited growth, nitrogen fixation, and heterocyst formation when exposed to ozone concentrations characterized as being “below ambient” for semi-urban conditions (Hur and Wellburn 1994).

These negative responses of terrestrial algae to ozone pollution suggest a potential impact on aquatic algae, as ozone may readily dissolve into surface waters. Dissolved ozone may directly react with ecosystem constituents or the effect may be through secondary oxygen species created during dissolution, such as hydrogen peroxide and the hydroxyl radical. Algal communities may be directly affected by the oxidative stress caused by these ROS. The response of aquatic algae to ozone has only been studied in regards to wastewater treatment. These studies pumped ozone, in very large quantities, directly into the water as a means of disinfection. At sufficient levels, algal cells are oxidized to death and even their byproducts may be destroyed (e.g. cyanotoxins) (Yun et al. 1997). There are documented differences in algal response to ozone and different concentrations and exposures times are necessary to
eliminate different species (Hoeger et al.; Lai et al. 2006; Lai et al. 2002; Paralkar and Edzwald 1996; Widrig et al. 1996). Although it has been noted that photosynthetic rates of algae after ozonation were severely inhibited even by short ozone contact (Yun et al. 1997), studies of the sub-lethal effects are virtually non-existent.

Algal communities may also be indirectly affected by the response of other organisms and/or dissolved substances to the increased oxidant levels. Ozone stress may change interspecies interactions within the biofilm by differentially affecting various algal groups, heterotrophic bacteria, and algal pathogens. Also, ozone and its dissolution species react with most molecules that they contact and humic substances, which are not readily taken up by bacteria, are available for oxidative breakdown and are often the primary sink for ozone in water treatment (Cho et al. 2003). This breakdown of larger compounds of DOC changes the light regime by reducing light attenuation, produces more labile carbon compounds and potentially liberates inorganic nutrients that may be utilized by algal cells (Freese et al. 1999; Lindell et al. 1995; Tranvik 1988; Wetzel 2001a).

This study is the first to investigate the potential of atmospheric ozone as an aquatic pollutant and the potential mitigating effects of humic substances. The experimental approach consisted of a three (0, 80, 250 ppb ozone) by two (high and low DOC) factorial design testing the independent and interactive effects of atmospheric ozone level and initial DOC concentration on periphyton assemblages. My objectives were 1) to look for evidence of ozone or DOC effects on total algal biomass and algal assemblage composition, and 2) to determine if ozone effects were mediated by DOC concentration in the water column.
Methods

Overall Experimental Set-up

Three ten-gallon glass aquaria were used to create treatment chambers (Fig. 2.1), each having a different atmospheric ozone concentration: a control treatment with 0 ppb O₃; a medium treatment with about 80 ppb O₃; and a high treatment with about 250 ppb O₃. Aquarium lids were sealed with weather-stripping and lined with PTFE sheeting to maintain the treatment atmospheres. Chemically non-reactive PTFE and PFA plastics were required for this experiment to resist corrosion by the large ozone concentrations pumped into the experimental chambers. Ozone was created and measured using a Thermo Scientific Model 49™ O₃ analyzer. The air used to generate the ozone was ambient air filtered through a carbon filtration unit prior to entering the generator. PFA tubing conducted the ozone from the generator into two of the aquaria and back from all three to the sample analyzer. Flow into each aquarium was controlled using a PTFE ball valve and manipulated until the target ozone concentration was measured in the chamber air. The control treatment was supplied with air from an aquarium bubbler filtered through another carbon filtration device. Ozone in each chamber was monitored and manipulated daily to insure an atmospheric concentration above the microcosms that was consistently ± 10 ppb of the target concentration. Ozone levels in the control never measured above 0 ppb. All three chambers were placed on an Eberbach EL600™ Orbital-Reciprocal Shaker Table to create water motion, set to 96 rotations per minute.

Light was provided by fluorescent, natural-spectrum bulbs mounted above the chambers, which generated 63.65 ± 2.16 µE/cm²/sec. To simulate natural, summer, diurnal cycles, ozone generation and light were on a timer, producing from 7:00 a.m. to 9:00 p.m. daily. All three
chambers were placed on an Eberbach EL600™ Orbital-Reciprocal Shaker Table to create water motion, set to 96 rotations per minute.

Water, for use in the experiment, was taken from Vermillion Creek, Rose Lake Wildlife Area, Clinton County, MI and an adjacent, partially formed oxbow wetland. The two water sources are separated by sediment on one arm of the oxbow and a beaver dam on the other.
When water levels are low, exchange between the two sources is limited. Each source constituted a different “DOC water treatment.” However, each source differed greatly in the concentrations of dissolved organic carbon (DOC). Other primary drivers of algal growth, such as pH and temperature, did not differ greatly between the two. Macronutrients (silica, nitrogen, and phosphorus) were directly measured and addressed. The creek water served as the growth medium for the “low doc water” treatment and the wetland water served as growth medium for the “high doc water” treatment. Initial DOC concentration for the high DOC water was 25.1 mg C/L and that for the low doc water was 14.152 mg C/L, measured as nonpurgable organic carbon (NPOC), and the high- and low-DOC sites had pH values of 7.4 and 8.2, respectively. These DOC concentrations are both high by global standards but represent common levels for rivers in our area. The high DOC water was noticeably stained with humic substances from leaf decomposition, and the low DOC water was visibly clear.

Benthic and epiphytic algae were collected by scrubbing biofilms from numerous substrates in each habitat. These samples were then combined into one suspension that was used as the seed population for both DOC water treatments. Half of this composite algae sample was added to the low DOC source water and the other half to the high DOC source water before commencement of the experiment. Therefore the seed populations for both treatments were very much the same, differing only in the suspended algal cells that may have been present during initial water sampling.

Eight 4-inch diameter ceramic dishes were used as microcosms, each containing 150 mL of source water and an inoculum of algae from the composite sample. Each dish contained five 2.5 x 2.5 cm unglazed ceramic tiles. Four of the eight microcosms in each of the three ozone chambers (0, 80 ppb, and 250 ppb) were designated for either low or high doc water treatments.
Fresh source water was added to the microcosms on the $7^{th}$, $14^{th}$, and $21^{st}$ days of the growth period, to refresh nutrients and keep the water level constant. Water temperature stayed at a constant 23-24°C for the four-week duration of the experiment.

Sample Collection

The first sampling date was determined by the appearance of a visible algal film on the tiles. Periphyton mats and water samples were collected after two and four weeks of treatment. Two tiles were sampled from each replicate during the first sampling period and the remaining three tiles were sampled at week 4. Periphyton was removed from each tile using a toothbrush and deionized (DI) water from a squirt bottle. Periphyton samples from individual tiles were pooled by replicate and date. Subsamples were taken from the homogenized, composite sample for chlorophyll $a$ measurement, algal biovolume estimate, and bacterial counts. Algal samples were preserved with gluteraldehyde (2%).

Water samples were filtered through Whatman™ glass fiber filters, type GF/F, for analysis of dissolved organic carbon, total dissolved nitrogen (TDN), and soluble reactive phosphorus (SRP). All water samples were frozen until analysis.

Laboratory Analyses

Soft algal cells were identified and counted in a Palmer-Maloney counting cell at 400X until 400 natural units were counted. This is a modification of a standard method for assessing taxonomic composition and cell density of algal samples used in national ecological assessments (Lowe and Laliberte 1996). I modified the counting method by increasing the number of natural units counted to increase precision of taxonomic and cell density measurements. Natural units are defined as the normal growth form of the alga, such as single cells, filaments, or colonies. The number of cells composing each natural unit was also recorded. Measurements were made
of each taxon during identification for calculating total biovolume as an estimate of algal biomass (Hillebrand 1999).

Diatom frustules were cleaned using nitric acid for cytoplasm digestion. A ratio of one part sample to two parts nitric acid was added to a beaker and simmered on a hot plate for one hour. Samples were rinsed several times with distilled water until the pH was neutral by allowing frustules to settle for 24 hours, siphoning off the supernatant, and adding more water. Neutral samples were concentrated, and then subsamples were dried onto coverslips and subsequently mounted on microscope slides using Naphrax™ mounting medium. Diatom identifications were made on clean frustules at 100X on a Leica compound microscope to a total of 600 valves.

Chlorophyll a (chl a) concentrations were also used as a measure of total algal biomass. Algae from each chlorophyll subsample were filtered onto Whatman™ GF/C glass fiber filters, extracted overnight in ethanol, diluted if necessary, and analyzed fluorometrically before and after acidification to correct for pheopigments (APHA 1998).

Total dissolved carbon (DOC), measured as non-purgeable organic carbon, and total nitrogen concentrations were measured in filtered water samples by Dr. Stephen Hamilton’s lab at Michigan State University using a Shimadzu TOC-VCPH™ Carbon Analyzer with a total nitrogen module (TNM-1) and an ASI-V Autosampler. Because the water samples were filtered prior to analyses, the TN readings did not include particulate nitrogen and were a measure of total dissolved nitrogen (TDN).

Soluble reactive phosphorus (SRP) was analyzed on samples that had been frozen. Due to a freezer malfunction, filtered water samples from week 2 were lost; therefore, SRP
measurements for the week 2 samples were done on a set of samples filtered after freezing. SRP concentration was measured manually following the ascorbic acid method (APHA. 1998) on a Spectronic® Genesys™ 2 Spectrophotometer by Spectronic Instruments.

Statistical Analysis

To test whether DOC, ozone, and sampling date (week) had an effect on total algal biovolume, chl $a$, or division-level biovolume, I conducted three-way ANOVAs using the GLM function in SYSTAT Version 12.0. To better understand the relationships between independent and dependent variables, in the case of a significant three-way interaction, two-way ANOVAs were conducted on the ozone/DOC interaction at each week. Two-way ANOVAs on ozone and week at each DOC treatment were also done to indicate significant changes from week 2 to week 4 and to identify any significant relationships among the dependent and independent variables that were not found in the former two-way ANOVA. All two-way ANOVA results may be found in Appendix 1. Tukey’s HSD tests were used for pair-wise comparisons of independent and interaction effects when ozone was a significant variable. All continuous variables were log-transformed prior to analysis. Because of potential issues with non-normality and some unequal variances, the ANOVA results were corroborated by the non-parametric Kruskal-Wallis One-way Analysis of Variance (KW). Results did not significantly differ between the parametric and nonparametric tests.

To determine if nutrient concentration differed as a function of DOC, ozone, week, and resulting responses of algae, I once again used a three-way ANOVA with subsequent Tukey’s HSD pairwise comparisons. Correlations served to identify significant bivariate relationships between measured nutrient concentrations and biomass. Nutrient data were log-transformed.
Results

Dissolved Organic Carbon

DOC treatment, ozone, and week each independently affected measured DOC concentration ($r^2=0.869$) (Fig. 2.2). The concentration of total DOC increased from week 2 to week 4 in all samples ($F_{1,34}=29.133; p=0.000$), however, it was higher in the high DOC than in the low DOC source water at both sampling dates ($F_{1,34}=170.498; p=0.000$).

![Graph showing measured DOC concentration by ozone and week, for each DOC treatment.](image)

Fig. 2.2. Measured DOC concentration by ozone and week, for each DOC treatment. Lower-cased letters indicate significant differences between ozone treatments at Week 2. Upper-case letters, differences at Week 4. An asterisk denotes a significant change from Week 2 to Week 4. Error bars represent one standard error above and below the mean.

*For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.

Ozone had a negative effect on total DOC as DOC was lower in both medium and high ozone treatments than in the zero ozone treatment ($F_{2,34}=8.099; p=0.002$ and $p=0.008$, respectively).
These differences were also visible in the water color. Over the first two weeks of the experiment, in the high DOC water, water color decreased greatly in the high and medium ozone treatments but not in the zero ozone treatment.

*Total Algal Biomass*

Ozone, DOC, and week complexly affected total algal biomass, with all interaction effects being significant (Table 2.1). Subsequent two-way ANOVAS on the interaction between DOC and ozone at each week indicated that DOC effects were dependent on ozone treatment and this effect varied with week.

Table 2.1. ANOVA table for log-transformed values of total biomass (as measured by biovolume and chlorophyll *a*) as a function of DOC, ozone, and week.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean sqr</th>
<th>F-ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total Biovolume (<em>r</em>²=0.782)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ozone</td>
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<tr>
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<td>33.848</td>
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At week 2, the effect of DOC on total biovolume and chl a was dependent on ozone ($r^2=0.883$; $F_{2,18}=3.553$, $p=0.05$ and $r^2=0.907$; $F_{2,18}=4.337$, $p=0.029$, respectively). At week 4, there was once again a significant interaction of DOC and ozone on both total biovolume and chl a ($r^2=0.632$; $F_{2,18}=10.884$, $p=0.001$ and $r^2=0.627$; $F_{2,18}=5.141$, $p=0.018$, respectively).

In the absence of ozone, DOC had a negative effect on total biomass over the entire study period as total biovolume was three times greater at week 2 and five times greater at week 4 ($p=0.002$ for both weeks). Chl a was also significantly greater in low than high DOC water at week 2 ($p=0.004$) (Fig. 2.3). In the medium and high ozone, DOC initially had a negative effect on total algal biomass but a positive effect later. At week 2 in the high DOC water, total biovolume and chlorophyll a were six times lower in high ozone ($p=0.000$ for both) and three times lower in the medium ozone ($p=0.006$ and $p=0.000$, respectively). However, due to a three-fold increase in biomass in the high and medium ozone treatments in the high DOC water but not in the low DOC water, by week 4 biovolume did not statistically differ between high and low DOC water. Chlorophyll a was greater in high DOC water with the high ozone treatment ($p=0.016$).
Fig. 2.3. Measures of total algal biomass by ozone and week, for each DOC treatment. Lower-case letters indicate significant differences between ozone treatments at week 2. Upper-case letters, differences at week 4. An asterisk denotes a significant biomass change from week 2 to week 4. Error bars represent one standard error above and below the mean.

Ozone effect in the low DOC water was negative but limited to the medium ozone treatment and was only significant at the first sampling date (Fig. 2.3). Biovolume was three times lower in medium ozone than in either the zero or high ozone treatments (p=0.004 and p=0.002, respectively) at week 2. There was no significant difference in chl \( a \) concentration at
week 2. Between sampling times, algal biomass decreased in zero and high ozone but not in medium ozone. Biovolume significantly decreased with high ozone over this period (p=0.005) but did not significantly change in the medium or zero ozone treatments. Chl $a$ significantly decreased in both zero and high ozone treatments ($r^2=0.751$; $F_{1,18}= 50.733$, p=0.000 and p=0.001, respectively).

In high DOC water, ozone had a negative effect on total algal biomass early in the study period but a positive effect later (Fig. 2.3). At week 2, total biovolume was greater in zero ozone than in medium ozone (p=0.011) and also high ozone, although not significantly (p=0.129). Chl $a$ was significantly greater in zero ozone than in either high or medium ozone treatments (p=0.001 for both). As mentioned above, from week 2 to week 4, total biovolume increased threefold in the high and medium ozone treatments (p=0.019 and p=0.011, respectively) but decreased in zero ozone (p=0.019). Chl $a$ changed in the same direction but the change was significant only for the high ozone treatment (p=0.001). By week 4, the zero ozone assemblages had less biovolume than those in high and medium ozone (p=0.001 and p=0.041, respectively). Chl $a$ shows the same trend but only the difference between the high and zero ozone treatment was significant (p=0.003).

**Division-level Biomass**

Assemblages were composed of three major divisions of algae: diatoms, cyanophytes and chlorophytes. The DOC and ozone treatments had very different effects on each division. Early assemblages in all treatments were diatom-dominated by biovolume but, by week 4, cyanophytes were dominant in the high and medium ozone in the high DOC water. Chlorophytes were second by biovolume across treatments and sampling date. As with total
algal biomass, all interactions among DOC, ozone, and week significantly affected both diatom and cyanophyte biovolume (Table 2.2).

Table 2.2. ANOVA table for log-transformed values of division-level biovolume as a function of DOC, ozone, and week.

<table>
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<tr>
<th>Source</th>
<th>df</th>
<th>Mean sqr</th>
<th>F-ratio</th>
<th>P</th>
</tr>
</thead>
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</tr>
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<td>6.134</td>
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<tr>
<td>Error</td>
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<td>0.929</td>
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</table>
Subsequent two-way ANOVAs (DOC water and ozone by week) indicated independent effects of DOC treatment and ozone on diatom biovolume at week 2 ($r^2 = 0.894; F_{1,18} = 107.064, p=0.000$ and $F_{2,18} = 19.656, p=0.000$, respectively). In addition, there was a significant interaction effect of ozone and DOC water at week 4 ($r^2 = 0.708; F_{2,18} = 10.446, p=0.001$) (Fig. 2.4). In the absence of ozone, DOC had a negative effect on diatom biomass over the entire study period. Diatom biovolume was lower in high DOC water in zero ozone at both week 2 and week 4 ($p=0.001$ for both). In the medium and high ozone treatments, DOC initially had a negative effect on diatom biomass (high ozone: $p=0.000$ and medium ozone: $p=0.001$) but by week 4, there was no significant difference between DOC treatments.

From week 2 to week 4, diatom biovolume either decreased or remained the same in both high and low DOC water and this response was dependent on ozone ($r^2 = 0.734; F_{2,18} = 15.139, p=0.000$ and $r^2 = 0.814; F_{2,18} = 3.083, p=0.000$, respectively). In the high DOC water, diatom biovolume significantly declined in the zero ozone treatment ($p=0.000$) but was maintained in high and medium ozone treatments. In the low DOC water, diatom biovolume significantly declined in both the zero ($p=0.023$) and high ozone treatments ($p=0.000$) but was maintained with medium ozone. By week 4, the negative effect of DOC was only significant in the zero ozone treatment ($p=0.001$).

Medium ozone had a significantly negative effect on early diatom biovolume ($F_{2,18} = 19.656, p=0.000$) in both high and low DOC water however this difference was greater in the low DOC water. By week 4, in high DOC water, the zero ozone assemblages had approximately a quarter of the diatom biomass that was present in the high or medium ozone
treatments (p=0.013 and p=0.022, respectively). In low DOC water, diatom biomass was 70% lower in the high ozone than in either the medium or zero ozone treatments (p=0.026 and p=0.029, respectively).
Fig. 2.4. Measures of division-level biomass by ozone and week, for each DOC treatment. Lower-case letters indicate significant differences between ozone treatments at week 2. Upper-case letters, differences at week 4. An asterisk denotes a significant biomass change from week 2 to week 4. Error bars represent one standard error above and below the mean.
Cyanophytes were also significantly affected by the interactions of DOC, ozone, and week (Fig. 2.4). Subsequent two-way ANOVAs (ozone and DOC by week) indicated that DOC had a positive effect on cyanophyte biomass at both sampling dates. At week 2, cyanophyte biovolume was 3 times greater in the high DOC water than in the low DOC water ($r^2=0.508; F_{1,18}=10.579, p=0.004$). By week 4, the difference had increased to two orders of magnitude ($r^2=0.731; F_{1,18}=37.976, p=0.000$). This great difference between cyanophyte biovolume in the high and low DOC waters at week 4 can be explained by a cyanophyte “bloom” that occurred only in high DOC water in the high and medium ozone treatments. A two-way ANOVA (ozone and week by DOC) verified the significant interaction of ozone and week on cyanophyte biomass in the high DOC water ($r^2=0.821; F_{2,18}=9.025, p=0.002$). Indeed, cyanophyte biovolume increased by two orders of magnitude between week 2 and week 4 in the high and medium ozone atmospheres (p=0.000 and p=0.002, respectively). Consequently, cyanophyte biovolume at week 4, in the high DOC water, was approximately 10 times greater in the high and medium ozone treatments than in zero ozone (p=0.012 and p=0.026, respectively). There was no significant ozone effect on cyanophyte biomass in the low DOC water.

Chlorophytes were also a major constituent of all assemblages throughout the experimental period. Chlorophytes were unaffected by ozone treatment but were positively affected by DOC at week 4 (Table 2.2). At week 2 there was no difference between chlorophyte biovolume in the low DOC or high DOC waters. Chlorophyte biovolume significantly increased during that time in the low DOC water (p=0.000) but not in the high DOC water. Consequently, at week 4 chlorophyte biomass was significantly greater in low DOC water than in the high DOC water ($r^2=0.481; F_{1,18}=9.999, p=0.003$).
**Nutrients**

DOC, ozone, and week were all independently significant factors affecting total dissolved nitrogen ($r^2=0.696$) (Fig. 2.5). TDN was lower in the low DOC water ($F_{1,34}=21.710, p=0.000$) than in the high DOC water across all ozone treatments. It was also lower in the medium ozone than in the high or zero ozone treatments ($F_{2,34}=13.099, p=0.000$ both) in both the low and high DOC waters. From week 2 to week 4, TDN increased across DOC and ozone treatments ($F_{1,34}=8.377, p=0.007$).

SRP concentrations were independently affected by DOC and ozone treatment but the ozone effects varied with week ($r^2=0.724$) (Fig. 2.5). SRP concentrations were higher in the high DOC water throughout the study ($F_{2,31}=14.96; p=0.001$). At week 2, SRP was higher in the high ozone treatment ($F_{2,17}=5.504; p=0.014$). From week 2 to week 4, SRP in the high and medium ozone treatments decreased significantly ($F_{1,31}=4.580; p=0.001$ and $p=0.006$, respectively) and at week 4 SRP in the zero ozone treatment was higher than in both the high and medium ozone ($p=0.018$ and $p=0.05$, respectively).

Silica concentrations did not differ significantly between the two DOC waters but significantly varied with ozone treatment (Fig. 2.5). Silica was higher in the zero ozone than in the high or medium ozone treatments ($F_{2,30}=8.068; p=0.013$ and $p=0.002$, respectively) and increased significantly from week 2 to week 4 across treatments.
Fig. 2.5. TDN, SRP, and silica concentration (mg/L) by ozone and week, for each DOC treatment. Lower-cased letters indicate significant differences between ozone treatments at week 2; upper-case letters, differences at week 4. An asterisk denotes a significant biovolume change from week 2 to week 4. Error bars represent one standard error above and below the mean.
Total algal biomass (as biovolume or chl a) was not correlated with nutrients across treatments and sampling periods. Cyanophyte biovolume was positively correlated with TDN (r=0.390; p=0.001) and diatom biovolume was negatively correlated with silica concentration (r=-0.341; p=0.027). However, when analyzed separately at each week, there were no correlations between nutrient concentrations and total algal biomass or division-level biomass.

**Discussion**

Our results clearly indicate that both DOC concentration and atmospheric ozone levels affect algal assemblage composition and biomass. This study presents the first evidence that atmospheric ozone, at levels measured in the troposphere and allowed to dissolve naturally into the water column, may cause changes to aquatic assemblages. Our results also indicate that algal divisions (diatoms, cyanophytes, and chlorophytes) respond differently to the independent and interactive effects of DOC concentration and elevated ozone.

After 2 weeks of exposure, total algal biomass, which was dominated by diatoms, was negatively affected by DOC. This appeared to be driven by the diatom response, as DOC had a negative effect on diatom biomass, no effect on chlorophytes, and a positive effect on cyanophytes. This negative effect of DOC on diatoms was mostly likely indirect as few studies have shown direct negative effects of DOC on diatom growth. TDN did vary statistically with DOC treatment but there was no significant correlation with total or diatom biomass at this sampling date. Also nitrogen levels were high enough to not be a limiting factor for diatom growth (Rier and Stevenson 2002). Light attenuation may have limited total diatom growth in the colored, high DOC water, although diatoms have been shown to be very tolerant of low light conditions (Steinman et al. 1989; Pillsbury and Lowe 1999).
Although different source waters were used in the DOC water manipulation, DOC concentration itself, most likely regulated the observed effects of water treatment. The two treatment water sources were from a connected system separated only by sediment and a beaver dam so some water flow was maintained throughout the year and catchment geology was the same. The primary difference between the two areas was retention of leaf litter which led to a higher DOC concentration in the wetland area. pH differed slightly but, based on autecological similarities of the diatom taxa in samples, the difference in pH was most likely not great enough to cause a significant shift in the division level taxonomy. In fact, cyanophytes, which are generally more sensitive to low pH (Wetzel 2001c), were in greater abundance in the lower pH water. Also, any pH differences between the two treatments would most likely have been a secondary effect of DOC concentration since DOC is often a primary source of acidity in aquatic ecosystems. The algal seed populations for experiments were the same for each treatment so initial source water communities should not be the cause of any differences among treatments. During the experiment, microcosms warmed to room temperature and were maintained at that temperature, in both treatments, throughout the experiment. Macronutrient concentrations were measured directly during the experiment and accounted for during analysis. Light attenuation differed in a small amount, initially, between the two treatments but this difference would have been a secondary effect of humic DOC content in the water. Thus I argue experimental effects in DOC water treatments were likely the direct or indirect effects of DOC because the most important factors ecological factors that could affect algal biomass and composition were measured directly for comparison or held constant and do not provide plausible explanations for observed effects of DOC water treatments.
I did not measure iron concentration, but humic substances are known to chelate iron, potentially reducing its availability to photosynthesizers (Imai et al. 1999; Wetzel 2001a). Although speculative, iron limitation would explain both the lower diatom and greater cyanophyte biomass at week 2 in the high DOC water. Many cyanophyte species will produce soluble, iron-chelating compounds (siderophores) in response to iron limitation which have been shown to give them a competitive advantage over other algal groups (Murphy 1976; Mahasneh and Tiwari 1992; Imai et al. 1999). Simultaneously, iron limitation of diatom biomass would reduce competition for other nutrients, particularly phosphorus, explaining the greater cyanophyte biomass in the high DOC than in the low DOC water. However, cyanophytes have been shown to be inhibited by the light attenuating effects of humic acids (Jackson and Hecky 1980; Sun et al. 2006). Although the water depth was quite shallow, the intensity of water color at the beginning of the experiment may influence the light regime enough to give diatoms the overall competitive advantage at week 2.

Ozone effects at week 2 were influenced by DOC concentrations. In the high DOC water there was more total algal biomass with zero ozone, indicating a direct negative effect on algal growth. However, in the low DOC water, only the medium ozone treatment showed a statistically significant growth effect. This may have been an anomaly, however, but evidence indicates it was not. If we look at the division level results, diatoms were the only algal group significantly affected by ozone at this sampling date and only medium ozone had a negative effect on their growth. It is possible that the oxidative stress caused by medium ozone levels was enough to slow diatom growth but was not a strong enough stressor to induce cellular defense mechanisms that would then ameliorate the negative effects at higher levels of ozone.
Alternatively, there was an indirect effect caused at medium ozone that was mitigated at high ozone levels.

Algal assemblage changes from week 2 to week 4 appear to show both senescence and succession. By week 4, the high biomass diatom assemblages (high and zero ozone treatments in the low DOC water and zero ozone in the high DOC water) precipitously declined while the lower biomass diatom assemblages were maintained at close to the initial biomass. These diatom assemblages most likely reached peak biomass and were experiencing density-dependent stressors that led to autogenic community succession. Density-dependent periphyton senescence is common and may be precipitated by low nutrient levels, toxin build-up within the algal film, or an increase of pathogen/cell interactions (Borchardt 1996; Hewson et al. 2001; Peterson et al. 1993; Wetzel 2001c). The diatom communities that did not reach that “peak” did not crash and were maintained at close to their original biomass.

From week 2 to week 4, in the high DOC water only, there was an ozone correlated succession from diatom dominance to cyanophyte dominance. Chlorophyte and diatom biomass did not significantly change, while cyanophyte biomass increased greatly in ozone-treated microcosms. That this is a direct stimulation of cyanophyte growth by ozone or humic DOC is unlikely, since there was no such stimulation at week 2 and since previous studies indicate negative direct effects of humic substances on cyanophyte growth (Bahrs and Steinberg 2012; Kosakowska 2007; Pflugmacher 2001).

A clearly visible reduction of water color coincided with the cyanophyte increase. This loss of water color can be considered indicative of the breakdown of DOC as many studies have shown the strong relationship between color units and dissolved organic matter (Wetzel 2001b). Since color is produced by chromophoric compounds, which are comprised primarily of high to
medium molecular-weight dissolved humic and fulvic acids (Wetzel 2001b), we may assume that an oxidative breakdown of complex humic substances took place in the ozone treatments during the course of the experiment. It is this breakdown of humic substances that may have been the indirect mechanism of ozone effect on the cyanophyte populations in the high DOC water.

The color reduction in the ozone-treated water would have increased available light and may have released cyanophytes from light limitation. At the same time, this breakdown of humic substances may have released chelated iron, increasing bioavailability. Cyanophytes have been shown to quickly respond to iron additions by facilitated production of siderophores (Imai et al. 1999) and so the coupling of a more favorable light regime with an increase in nutrients may have been sufficient to stimulate the cyanophyte “bloom” that occurred by week 4. Competition for iron would also explain why chlorophytes did not take off, as well, when light increased.

Finally, humic substances are complex molecules with greatly varying molecular weights and chemical characteristics and studies have shown both stimulation and reduction of algal growth as a response to added humic compounds (Fagerberg et al. 2009; Steinberg et al. 2003; Sun et al. 2006; Wang et al. 1999). The bloom may be indicative of a stimulation of cyanophytes by heterotrophy. Studies have shown that many types of algae exhibit heterotrophic use of photolytic degradation products of recalcitrant humic substances and even aromatics (Larson et al. 2002; Semple et al. 1999; Tittel and Kamjunke 2004) as a survival mechanism in extremely light-limited situations. These organic carbon substrates may be utilized by algae at natural concentrations and in the presence of bacteria, and it has been suggested that heterotrophy may also be an efficient mode of metabolism in systems with high levels of labile
dissolved organic compounds even in the presence of light (Tittel and Kamjunke 2004; Tittel et al. 2009). It is possible, then, that the abundance of labile organic compounds being produced through the oxidation of humic compounds in the ozone treatments may have stimulated facultative heterotrophy in the cyanobacteria.

Chlorophytes appear to be unaffected by ozone treatment and the significantly lower chlorophyte biovolume in the high DOC water at week 4 may have more to do with a reduction of competition with diatoms after the diatom communities declined in the low doc water. This hypothesis is corroborated by the fact that this difference between DOC treatments was only significant in the zero and high ozone treatments, the treatments that witnessed the diatom crash.

These complex responses of algae to ozone and their interactions with DOC indicate many direct, indirect, and interactive causal pathways. In the absence of ozone interactions, assumed humic DOC generally had a negative effect on algal biomass with the exception of cyanophytes that were unique in their positive response to higher DOC concentration. These results support previous research that showed humic control of algal biomass through altering the light and/nutrient regime in aquatic systems. It is difficult to specifically isolate the role of ozone when comparing the effects of ozone in both the high and low DOC waters but its effect was generally greater in high DOC water, negative in the short-term, and positive in the long-term, and was dependent on algal taxonomic division.

In conclusion, my results indicate that ozone will likely have a greater effect in high DOC waters, by breaking down complex molecules which will change the light and nutrient regime and, at least for cyanophytes, this interactive effect appears to be positive. However, the response of phytoplankton assemblages, which are less physically complex, may be very different. The interaction of ozone and DOC will cause changes in algal function in ecosystems
because their effects differ across divisions of algae with varying nutritional content for herbivores, nutrient and light requirements, and roles in biochemical cycling. This study indicates the potential of atmospheric ozone as an agent of change to aquatic algal assemblages, especially in humic waters, and underscores the importance of considering species interactions and other complex indirect responses when determining the potential impact of a given stressor.
Chapter 3: Mediation of atmospheric ozone effects on benthic heterotrophic bacterial biomass by dissolved organic carbon and algae

Abstract

Ozone is an EPA regulated atmospheric pollutant that has been shown to greatly damage human and ecosystem health. Few studies have addressed the potential of atmospheric ozone as an aquatic pollutant through dissolution into surface waters. The objective of this study was to examine the direct and indirect effects of dissolved atmospheric ozone on aquatic heterotrophic bacteria. Ozone effects on bacteria-only assemblages and bacteria/periphyton assemblages were assessed using microcosms. In the absence of algal constituents, heterotrophic bacteria were stimulated by ozone treatment. When bacteria grew in the presence of algae, ozone also had a significant effect on bacterial biomass but these changes were better explained by total algal biomass than ozone treatment. These results indicate an indirect effect of ozone on heterotrophic bacteria in benthic periphyton, likely through a change in the availability of organic carbon and/or altered interspecific interactions.

Introduction

Ozone (O₃) is a primary constituent of photochemical smog and is often considered a pollutant of serious concern because of the great potential for damage to human and ecosystem health. During summer, regions all over the world reach concentrations that exceed the U.S. national health standard of 75 ppb, with recorded maximum levels reaching 400 ppb (Vingarzan 2004, Sanchez and Ayala 2008). Tropospheric ozone is spatially and temporally heterogeneous and while maximum levels have decreased in some areas, such as North America and Europe, global background ozone concentrations are increasing steadily. Background O₃, generally
defined as the concentration attributable to long-range transport, resuspension of historical emissions, and non-anthropogenic sources is predicted to increase by 0.5–2% per year over the next century, and some models predict that background concentrations will reach 75 ppb by the end of the this century (Vingarzan 2004).

Ozone damage to terrestrial ecosystems has been extensively studied and damage to crops, forests, and other natural systems is widely reported (Broadmeadow 1998, Madden and Hogsett 2001, Niemi et al. 2002, Oksanen 2003, Avnery et al. 2010). Considering the extent of surface waters globally and that ozone readily dissolves in water, we should consider the potential of atmospheric ozone as an aquatic pollutant.

Heterotrophic bacteria are primary constituents of freshwater plankton and benthos, where they contribute to the base of the food web and interact complexly with algae (Wetzel 2001a). They are integral to the aquatic carbon cycle. Changes in the quantity and health of aquatic bacteria may greatly affect ecological functions from the biofilm to the ecosystem scale. Wastewater treatment and food preservation studies have repeatedly shown the antibacterial potential of high concentrations of dissolved ozone (far exceeding atmospheric levels) to both Gram positive and Gram negative bacteria (Tanner et al. 2004, Voidarou et al. 2007, de Velasquez et al. 2008, Gerrity et al. 2011, Izdebski et al. 2011). Primary damage results from degradation of lipids in the cell wall and membrane, particularly the double bonds of unsaturated lipids, resulting in membrane disruption, leakage of cytoplasm and eventually cell lysis (Guzel-Seydim et al. 2004). However, we have little knowledge of the direct or indirect effects of dissolved ozone on aquatic bacteria in the ranges observed and expected under ambient and elevated atmospheric ozone concentrations, and the degree to which observations from ozonation treatment of water can be extrapolated to lower exposures is unknown.
Dissolved ozone may also cause indirect effects on bacterial biomass if the increased oxidative stress affects other biota in the ecosystem. Heterotrophic bacteria and algae form tight relationships in the plankton and benthos that may be either facilitative or competitive (Wetzel 2001d). Whether the relationship is negative or positive largely depends upon the supply of inorganic nutrients and organic carbon available for bacterial use. Natural bacterial communities are commonly limited by phosphorus and/or organic carbon concentrations and, since they are much better competitors for phosphate than algae, they are expected to outcompete algae in low phosphorus situations leading to a decline in algal biomass as bacterial biomass increases (Jansson et al. 1996). However, algae and bacteria commonly coexist and their relationship is often found to be mutually positive. It is widely accepted that algae provide, through exudates, labile organic carbon for use by bacteria and will stimulate bacterial growth when phosphorus is not limiting (Klug 2005, Rier, 2002). Benthic algal mats may also provide greater physical substrate for bacterial colonization, facilitate internal nutrient cycling (Rier and Stevenson 2002), and act as protection for the bacterial communities. These bacterial/algal interactions will be affected by any environmental variables that alter algal biomass and composition or the availability of organic carbon. My research in Chapter 2 showed a change in both biomass and species composition of algal communities after exposure to high ozone atmospheres.

Dissolved organic carbon (DOC) may also mediate the effect of ozone on bacterial communities. Most DOC in the water column is composed of large, complex, and more recalcitrant substances, such as humic and fulvic acids as well as lignocelluloses (Wetzel 2001a). These are utilized slowly by bacteria and therefore accumulate in the water column; whereas small and simple organic molecules, like amino and fatty acids and simple carbohydrates, have a very high turnover rate because they are preferentially taken up by bacteria (Moran and Hodson...
Oxidation of large, complex DOC molecules by UV-B and ozone result in production of smaller more labile molecules such as aldehydes, ketones, fatty acids, and amino acids (Mann and Wetzel 1995, Moran and Zepp 1997, Volk et al. 1997, Escobar et al. 2001, Judd et al. 2007). An increase in bioavailable molecules may lead to greater bacterial metabolism and biomass. Indeed, many studies have shown the stimulation of bacterial growth after photooxidation of humic substances (Engelhaupt et al. 2003, Middelboe and Lundsgaard 2003, Anesio et al. 2004, Lennon 2004, Romani et al. 2004). Oxidation of humic substances can be an important sink for ozone (Cho et al. 2003). If the concentration of humic substances is sufficiently high, oxidation of humic substances may ameliorate the oxidative potential of ozone and protect aquatic biota from much of the direct oxidative stress.

The objective of this study was to examine the direct and indirect effects of dissolved atmospheric ozone on aquatic heterotrophic bacteria. My study consisted of two experiments, the first being a “bacteria only” study in which bacteria were grown in the absence of algae, in three different ozone atmospheres. This experiment was intended to discern direct effects of ozone concentration. The second was a “periphyton and bacteria experiment” in which natural algal/bacterial assemblages were grown in the same experimental ozone conditions as the previous experiment with the additional variable of DOC concentration. This experiment was meant to simulate a more natural system in which competition with or facilitation by algae may influence bacterial response to ozone treatment. I hypothesized that high and medium ozone levels would reduce bacterial biomass in the absence of algae due to oxidative stress. In the bacterial and algal assemblages, I expect indirect ozone effects, such as a facilitation of internal
organic matter and nutrient recycling through the ozone-mediated breakdown of periphyton mucilage or altered algal/bacterial interactions.

Methods

Bacteria only experiment

Three covered, ten-gallon glass aquaria were used to create the treatment chambers, each having a different atmospheric ozone concentration: a control treatment with 0 ppb O₃; a medium treatment with about 80 ppb O₃; and a high treatment with about 250 ppb O₃ (Fig. 3.1). Each chamber was completely covered in reflective paper to exclude ambient light and thus prevent algal growth. Aquarium lids were sealed with weather-stripping and lined with PTFE sheeting to maintain the treatment atmospheres. Chemically non-reactive PTFE and PFA plastics were required for this experiment to resist corrosion by the large ozone concentrations pumped into the experimental chambers. Ozone was created and measured using a Thermo Scientific Model 49™ O₃ analyzer. The air used to generate the ozone was ambient air filtered through a carbon filtration unit prior to entering the generator. PFA tubing conducted the ozone from the generator into two of the aquaria and back from all three to the sample analyzer. Flow into each aquarium was controlled using a PTFE ball valve and manipulated until the target ozone concentration was measured in the chamber air. The control treatment was supplied with air from an aquarium bubbler filtered through another carbon filtration device. Ozone in each chamber was monitored and manipulated daily to insure an atmospheric concentration above the microcosms that was consistently ± 10 ppb of the target concentration. Ozone levels in the
control never measured above 0 ppb. All three chambers were placed on an Eberbach EL600™ Orbital-Reciprocal Shaker Table to create water motion, set to 96 rotations per minute.

Water samples were taken from Vermillion Creek, Rose Lake Wildlife Area, Clinton County, MI. This supply water served as the growth medium and provided the bacterial seed populations for the ozone experiments. The DOC of the source water was 19.7 mg C/L. Before ozone treatment, the inoculum of bacteria was created by filtering the supply water through a three µm mesh plankton net to remove zooplankton, protozoans, and most of the existing algal community.

Eight 4 inch diameter ceramic dishes were used as microcosms. Each dish contained five clean 2.5 x 2.5 cm unglazed ceramic tiles. 150 mL of the filtered source water were added to each microcosm and bacteria grew for two weeks before sampling. To simulate natural, summer, diurnal shifts, ozone generation was on a timer, producing ozone from 7:00 a.m. to 9:00 p.m. daily. Water temperature stayed at a constant 23-24°C for the duration of the experiment.
Fig. 3.1. Experimental set-up. Rectangles represent individual chambers and circles represent microcosms within each chamber. *The high and low DOC water treatments only pertain to the periphyton/bacteria experiment.

At the end of the experiment, all tiles and the entire water volume were sampled.

Bacteria were removed from each tile using a razor blade and rinsed into 20 mL of DI water.

Samples from individual tiles from a microcosm were pooled. Bacterial films were
homogenized using a Biospec® M 133/1281-0 2-speed biohomogenizer, preserved with gluteraldehyde (2%), and refrigerated until counted.

Water samples were filtered through Whatman™ glass fiber filters, type GF/F, for analysis of dissolved organic carbon, total dissolved nitrogen (TDN), and soluble reactive phosphorus (SRP). All water samples were frozen until analysis.

Non-purgeable organic carbon (NPOC) and total nitrogen concentrations were measured in filtered water samples by Dr. Stephen Hamilton’s lab at Michigan State University using a Shimadzu TOC-VCPH™ Carbon Analyzer with a total nitrogen module (TNM-1) and an ASI-V Autosampler. Because the water samples were filtered prior to analyses, the TN reading did not include particulate nitrogen and were a measure of total dissolved nitrogen (TDN).

Soluble reactive phosphorus (SRP) was analyzed on frozen samples. Due to a freezer malfunction, filtered water samples from week 2 were lost; therefore, SRP measurements for the week 2 samples were done on a set of samples filtered post-freezing. SRP concentration was measured manually following the ascorbic acid method (APHA 1998) on a Spectronic® Genesys™ 2 Spectrophotometer by Spectronic Instruments.

Bacterial abundance was estimated by direct cell counts at 1000x magnification on a Nikon Eclipse™ E800 epifluorescent microscope using 4’, 6’-diamidino-2-phenylindole (DAPI) dye. Samples were variously diluted, to a density of 30-300 bacteria in the viewing grid, in a phosphate buffer solution (PBS) to a final volume of 5 mL. Forty microliters of 0.25 mg/mL DAPI stock solution was then added to each sample/PBS mixture and allowed to stain cell DNA for 6 minutes. Using a Millipore™ filtration system 2 mL of PBS and then 1 mL of stained
sample were filtered onto a 0.2µm black, membrane filter with a white grid. The membrane filter was then mounted on a microscope slide using non-fluorescent immersion oil. All bacteria in a viewing grid were enumerated to a total of 20 grids, dispersed over 5 transects.

Periphyton and Bacteria Experiments

The physical set-up was the same as the previous experiment with the exception that chambers were not covered with reflective paper and light was provided by fluorescent, natural-spectrum bulbs mounted above the chambers. These lights provided 63.65 ± 2.16 µE/cm²/sec, which is similar to common conditions for periphyton in streams. To simulate natural, summer, diurnal shifts, ozone generation and light were on a timer, producing ozone and light from 7:00 a.m. to 9:00 p.m. daily.

Water was taken from Vermillion Creek, Rose Lake Wildlife Area, Clinton County, MI and an adjacent, backwater wetland for use in the experiment. Each source constituted a different “DOC water treatment.” The two water bodies were physically connected by a beaver dam that allowed continuous water exchange. However, each source differed greatly in the concentrations of dissolved organic carbon (DOC). Other primary drivers of algal growth, such as pH and temperature, did not differ greatly between the two. Macronutrients (silica, nitrogen, and phosphorus) were directly measured and addressed. The creek water served as the growth medium for the “low DOC water” treatment and the wetland water served as growth medium for the “high DOC water” treatment. Initial DOC concentration for the high DOC water was 25.1 mg C/L and that for the low DOC water was 14.152 mg C/L as nonpurgable organic carbon (NPOC) with pH 7.4 and 8.2, respectively. The high DOC water was noticeably stained with humic substances from leaf decomposition, and the low DOC water was visibly clear. These DOC concentrations are both high by global standards but represent common levels for rivers in
our area. The DOC treatments of this experiment were approximately 5 mg C/L less or greater than the 19.661 mg C/L in the bacteria only experiment. This difference in DOC between the two experiments was due to seasonal changes in the source water, making direct replication impossible.

Benthic and epiphytic algae were collected by scrubbing biofilms from numerous substrates in each habitat. These samples were then combined into one suspension that was used as the seed population for both DOC water treatments. Half of this composite algae sample was added to the low DOC source water and the other have to the high DOC source water before commencement of the experiment. Therefore the seed populations for both experiments were very much the same, differing only in the suspended algal cells that may have been present during water sampling.

150 mL of each source water and an inoculum of algae and bacteria from the composite sample were added to each microcosm. Four of the eight microcosms in each of the three ozone chambers (0, 80 ppb, and 250 ppb) were designated for either low or high DOC water treatments (Fig. 3.1). Fresh source water was added to the microcosms on the 7th, 14th, and 21st days of the growth period, to refresh nutrients and keep the water level constant. Water temperature was maintained at a constant 23-24°C for the four-week duration of the experiment.

Periphyton and water samples were collected after two and four weeks of treatment. The first sampling date was determined by the appearance of a visible algal film on the tiles. Periphyton mats and water samples were collected after two and four weeks of treatment. Two tiles were sampled from each microcosm during the first sampling period and the remaining three tiles were sampled at week 4. Periphyton were removed from each tile using a toothbrush and deionized (DI) water from a squirt bottle. Periphyton samples from individual tiles were
pooled by microcosm and date. Subsamples were taken from the homogenized, composite sample for algal biovolume estimation and bacterial counts. Algal samples were preserved with gluteraldehyde (2%).

Algal cells were identified and counted in a Palmer-Maloney counting chamber at 400X until 400 natural units were counted, which is a modification of a standard method for assessing taxonomic composition and cell density of algal samples used in national ecological assessments (Lowe and LaLiberte 1996). I modified the counting method by increasing the number of natural units counted to increase precision of taxonomic and cell density measurements. Natural units are defined as the normal growth form of the alga, such as single cells, filaments, or colonies. The number of cells composing each natural unit was also recorded. Measurements were made of each taxon during identification for calculating total biovolume as an estimate of algal biomass.

Bacterial cell enumeration and water sample processing for this experiment were done as described in the previous experiment.

Statistical Analysis

A one-way ANOVA was used to determine if ozone treatment had an effect on the density of the bacteria-only assemblages or measured nutrient concentrations. To test whether DOC, ozone, and sampling date (week) had an effect on total algal biovolume, bacterial density, or the ratio of bacteria biomass to algal biomass in the periphyton/bacteria experiment, I conducted three-way ANOVAs using the GLM function in SYSTAT Version 12.0. To better understand the relationships between independent and dependent variables, two-way ANOVAs were conducted on the ozone/DOC interaction at each week. Two-way ANOVAs on ozone and week at each DOC water treatment were also done to indicate significant changes from week 2 to
week 4 and to identify any significant relationships among the dependent and independent variables that were not found in the former two-way ANOVAs.

Tukey’s HSD tests were used for pair-wise comparisons of independent and interaction effects. All continuous variables were log-transformed prior to analysis. Because of issues with non-normality and some unequal variances, the ANOVA results from the periphyton/bacteria experiment were corroborated by the non-parametric Kruskal-Wallis One-way Analysis of Variance (KW). Results did not significantly differ between the parametric and nonparametric tests.

To determine if nutrient concentrations differed as a function of ozone, DOC water, or week, I once again used a three-way ANOVA with subsequent Tukey’s HSD pairwise comparisons. Nutrient data were log-transformed.

Correlations were used to identify significant bivariate relationships between measured nutrient concentrations and algal or bacterial biomass. Correlations were also used to determine if bacterial density was correlated with algal biomass.

**Results**

*Bacteria only experiment*

Ozone had a positive effect on bacterial biomass in the absence of algae. Bacterial density (cells/cm$^2$) was greater in the high and medium ozone than in the zero ozone treatment ($F_{2,21}=5.367$, $p=0.071$, $p=0.012$ respectively) (Fig. 3.2). Although the mean (20509 cells/cm$^2$) and maximum (36569 cells/cm$^2$) bacterial densities in the high ozone treatment were lower than in the medium ozone treatment (Mean: 26613; Max: 44839 cells/cm$^2$), this difference was not statistically significant.
Bacterial films on many tiles consisted of a strong, rubbery, polysaccharide matrix that maintained its integrity after removal from the tile. Although not statistically verifiable, this thick biofilm appeared to be more prevalent in the ozone treatments than the control. Five of eight microcosms in the high and medium ozone treatments contained this matrix while only three of eight had it in the zero ozone treatment. Also, the number of tiles within each microcosm containing this rubbery biofilm was greater in the high and medium ozone treatments than in the zero ozone treatment.

![Bacterial biomass and DOC](image)

**Fig. 3.2.** Bacterial biomass as represented by cells/cm$^2$ and DOC for each treatment in the bacteria-only experiment. Lower-case letters denote significant differences. Bars are means of eight replicates ± SE.

After two-week exposure to the ozone treatments, total DOC was greater in all three treatments than in the inoculum. However, the DOC concentration was significantly lower in high and medium ozone than in the zero ozone treatment ($F_{2,21}=11.345$, $p=0.000$, $p=0.011$).
respectively) (Fig. 3.2). There was no observable color difference between the treatment water and the control at the end of the experiment.

Soluble reactive phosphorus (control=0.016 ± 0.016; medium=0.012± 0.012; high=0.014 ± 0.007 mg/L) did not statistically differ among treatments. The variance was very high for these measurements. Total dissolved nitrogen was greater in the control than in the high and medium ozone treatments (F_{2,21}=5.147, p=0.018, p=0.055 respectively), although the difference was slight (control: 3.592 ± 0.667 mg/L; medium: 3.023 ± 0.322 mg/L, and high: 2.899 ± 0.300 mg/L). Bacterial abundance was not correlated with either SRP or TDN.

**Periphyton and bacteria experiment**

DOC treatment, ozone, and week each independently affected measured DOC concentration (r^2 =0.869) (Fig. 3.3). The concentration of total DOC increased from week 2 to week 4 in all samples (F_{1,34}=29.133; p=0.000), however, it was higher in the high DOC than in the low DOC source water at both sampling dates (F_{1,34}=170.498; p=0.000). Ozone had a negative effect on total DOC as DOC was lower in both medium and high ozone treatments than in the zero ozone treatment (F_{2,34}=8.099; p=0.002 and p=0.008, respectively). These differences were also visible in the water color. Over the first two weeks of the experiment, in the high DOC water, water color decreased greatly in the high and medium ozone treatments but not in the zero ozone treatment.

Ozone, DOC water, and week complexly affected total algal biomass, with all interaction effects being significant. At week 2 and week 4, the effect of ozone on total biovolume was
dependent on DOC water treatment ($r^2=0.883$; $F_{2,18}= 3.553$, $p=0.05$ and $r^2=0.632$; $F_{2,18}= 10.884$, $p=0.001$, respectively).

Fig. 3.3  Measured DOC concentration by ozone and week, for each DOC water treatment. Lower-cased letters indicate significant differences between ozone treatments at Week 2. Upper-case letters, differences at Week 4. An asterisk denotes a significant change from Week 2 to Week 4. Error bars represent one standard error above and below the mean.

In the absence of ozone, DOC had a negative effect on total algal biomass over the entire study period as total biovolume was three times greater in low DOC water at week 2 and five times greater at week 4 ($p=0.002$ for both weeks) (Fig. 3.4). In the medium and high ozone, DOC initially had a negative effect on total algal biovolume but a positive effect later. At week 2 in the high DOC water, total biovolume was six times lower in high ozone ($p=0.000$) and three times lower in the medium ozone ($p= 0.006$). However, due to a three-fold increase in biomass in the high and medium ozone treatments in the high DOC water but not in the low DOC water, by week 4 biovolume did not statistically differ between high and low DOC water.
Ozone effect on algal biomass in the low DOC water was negative but limited to the medium ozone treatment and was only significant at the first sampling date (Fig. 3.4). Biovolume was three times lower in medium ozone than in either the zero or high ozone treatments (p=0.004 and p=0.002, respectively) at week 2. Between sampling times, algal biomass decreased in zero and high ozone but not in medium ozone. Biovolume significantly decreased with high ozone over this period (p=0.005), but did not significantly change in the medium or zero ozone treatments.

In high DOC water, ozone had a negative effect on total algal biomass early in the study period but a positive effect later (Fig. 3.4). At week 2, total biovolume was greater in zero ozone than in medium ozone (p=0.011) and also high ozone, although not significantly (p=0.129). As mentioned above, from week 2 to week 4, total biovolume increased threefold in the high and medium ozone treatments (p=0.019 and p=0.011, respectively) but decreased in zero ozone (p=0.019). By week 4, the zero ozone assemblages had less biovolume than those in high and medium ozone (p=0.001 and p=0.041, respectively).

Bacterial biomass was not significantly affected by DOC water treatment. Ozone had a significant effect on bacterial biomass, as measured by cell density, but the direction of effect was dependent on sampling date ($r^2=0.502$; $F_{2,18}=9.367, p=0.001$) (Fig. 3.4). In high DOC water, medium ozone initially had a negative effect on bacterial biomass. At week 2, bacterial density in the medium ozone treatment was roughly half that in either the high or zero ozone treatments (p=0.002 and p=0.04, respectively). During the next two weeks, bacteria in the high and medium ozone increased, but only significantly in the medium ozone ($r^2=0.385$; $F_{2,18}=13.844, p=0.014$), and by week 4 bacterial density did not significantly differ among the
ozone treatments. The bacteria:algae ratio was greater in the ozone treatments at week 2
\((F_{2,18}=6.916, p=0.015)\) and lower at week 4 \((F_{2,18}=7.964, p=0.012)\). This decrease in
bacteria:algae ratio coincided with a change in dominant algal taxa from diatoms to cyanophytes
in the ozone treatments (Chapter 2).

In the low DOC water, at week 2, ozone had a positive effect on bacterial biomass as
bacterial density was approximately twice as great in the high ozone treatment than either the
medium or zero ozone treatments \((r^2=0.724; F_{2,18}=29.044, p=0.004\) and \(p=0.000\), respectively). Bacteria numbers then declined precipitously in high ozone \((F_{2,18}=22.357, p=0.002)\), but did not
significantly change in zero or medium ozone. Consequently, at week 4, bacterial biomass did
not statistically differ among treatments and the control. The bacteria:algae ratio in the high and
medium ozone was roughly twice that in the zero ozone treatment at week 2 \((F_{2,18}=3.925,
p=0.05)\) and at 2-3 times greater at week 4 \((F_{2,18}=6.832, p=0.016)\). Total algal biovolume and
bacterial density were significantly positively correlated in both high and low DOC water \((r=0.0.647, p=0.001\) and \(r=0.454, p=0.026\), respectively).
Fig. 3.4 Measures of total algal biovolume, bacterial cell density, and ratio of bacterial density to algal density by ozone and week, for each DOC water treatment. Lower-case letters indicate significant differences between ozone treatments at week 2. Upper-case letters, differences at week 4. An asterisk denotes a significant biomass change from week 2 to week 4. Error bars represent one standard error above and below the mean.
DOC water, ozone, and week were all independently significant factors affecting TDN ($r^2 = 0.696$) (Fig. 3.5). Total dissolved nitrogen was lower in the low DOC water treatment ($F_{1,34} = 21.710, p=0.000$) than in the high DOC across ozone treatments. It was also lower in the medium ozone than in the high or zero ozone treatments ($F_{2,34} = 13.099, p=0.000$ both) across DOC water treatments. From week 2 to week 4, TDN increased across DOC water and ozone treatments ($F_{1,34} = 8.377, p=0.007$).

Soluble reactive phosphorus concentrations were affected by DOC water and ozone treatment but the effects varied with week ($r^2 = 0.724$) (Fig. 3.5). At week 2 there was no statistical difference in SRP concentration among the treatments. However from week 2 to week 4, SRP in the high and medium ozone decreased significantly ($F_{2,31} = 4.580; p=0.001$ and $p=0.006$, respectively) and at week 4 SRP in the zero ozone was higher than both ($p=0.018$ and $p=0.05$, respectively). Also, at week 4, SRP was lower in the high DOC than in the low DOC water ($F_{2,31} = 3.973; p=0.055$).

Total algal biovolume was not correlated with nutrients across treatments and sampling periods. Bacterial density was positively correlated with TDN ($r=0.36; p=0.014$) across treatment variables. However, when examined within DOC treatment, this correlation was not significant.
Fig. 3.5  TDN and SRP concentrations (mg/L) by ozone and week, for each DOC treatment. Lower-cased letters indicate significant differences between ozone treatments at week 2; upper-case letters, differences at week 4. An asterisk denotes a significant biovolume change from week 2 to week 4. Error bars represent one standard error above and below the mean.

**Discussion**

My experimental manipulation of ozone, DOC water, and light indicated that atmospheric ozone, in concentrations measured in nature, has a significant effect on benthic bacterial biomass, but the effect is primarily indirect and that bacterial response may depend on community interactions. I did not see the hypothesized negative effects of oxidative stress. In the dark experiment, in the absence of algae, the ozone treatment stimulated bacterial production,
resulting in higher densities in both the medium and high ozone treatments than the control. No published evidence provides a mechanism for sustained stimulation of bacterial metabolism due to direct oxidative exposure, so I infer that the recorded response is due to indirect effects.

Bacterial production is most often limited by phosphorus, organic carbon, or a combination of the two and less often, by nitrogen (Tranvik 1988, Balogh and Voros 1997, Thomas 1997, Bergstrom and Jansson 2000, Drakare et al. 2003, Lennon 2004). In this study, inorganic nutrients were not likely to be secondary factors leading to the higher bacterial density in the ozone treatments. Nitrogen was not limiting as TDN concentrations were above an accepted limiting value of 0.05-0.06 mg/L (Wetzel 2001e) and TDN was not statistically correlated with bacterial density. Also, the higher concentrations were in the control samples which had the lowest bacterial density. Phosphorus concentrations in this experiment were at or near limiting for bacterial growth. Wetzel (2001e) lists the upper end of phosphorus limitation as < 0.015 mg/L, however, SRP concentrations did not statistically differ among treatments, were not correlated with bacterial density and were greater in the control.

Organic carbon limitation of these bacterial communities is likely. As mentioned in the introduction, the oxidation of humic substances generally leads to the creation of smaller, more labile compounds. This increase in preferred carbon sources may lead to increased bacterial production. The greater numbers of bacteria in the ozone treatments agree with the many studies that have shown a stimulation of carbon uptake by bacteria, leading to a higher growth rate, after UV breakdown of recalcitrant humic molecules (Stewart and Wetzel 1981, Lindell et al. 1995, Moran and Zepp 1997, Engelhaupt et al. 2003, Middelboe and Lundsgaard 2003, Rosenstock and Simon 2003, Anesio et al. 2004, Lennon 2004, Rosenstock et al. 2005). Even though the source water was not particularly rich in humic substances, the total DOC concentration at the end of
the experimental run was lower in the ozone-treated waters, indicating a breakdown and loss of organic carbon. This supports the hypothesis that ozone treatment in our experiment stimulated bacterial growth by oxidizing humic substances to more bioavailable compounds. However, there was no statistical correlation between DOC concentration and bacterial density.

Internal biofilm recycling of phosphorus or carbon may be a more important consideration within these communities. Some of these bacterial communities formed very thick, gelatinous matrices that remained intact even when removed from the tiles. The existence of these thick matrices was generally associated with higher bacterial densities and, although not statistically testable, these biofilms were also thicker in the ozone treatments. It is possible that greater mucilage production per cell was a defensive response to oxidative stress occurring in the treatments (Reynolds 2007). These more tightly-associated communities formed by greater production of extracellular polysaccharides would lead to greater internal recycling of carbon and inorganic nutrients and also provide greater surface area for bacterial growth.

Although not statistically significant, bacterial numbers were lower in the high ozone treatment than the medium ozone treatment. This may suggest a unimodal response of bacterial growth to atmospheric ozone. I predict that as ozone increases past a certain point, some of the benefits of increased production of labile organic carbon are lost. As the concentration of oxidants increases there is a greater potential for direct interaction with bacterial cell walls leading to changes in permeability and reducing cell function.

In the periphyton and bacteria experiment, in both DOC water treatments, bacterial biomass was highly correlated with algal biomass. Bacterial density rose and fell with algal biovolume over the course of the experiments. As mentioned previously, heterotrophic bacteria preferentially use smaller, simpler organic molecules. In benthic and plankton communities,
many of these molecules are produced extracellularly by algae or are released from algal cells due to membrane damage or death. These are ready sources of carbon substrates for bacterial metabolism and are almost immediately assimilated. Indeed, many studies have shown a positive correlation between algal and bacterial production when inorganic nutrients are not limiting. Algae are hypothesized to be a very important source of organic compounds for heterotrophic bacterial metabolism in many aquatic systems (Bird and Kalff 1984, Chrzanowski 1985, Sundh and Bell 1992, Wetzel 2001a, Romani et al. 2004).

The relationship between bacteria and algae was not equal among treatments, however. At week 2, when significant differences in bacterial numbers existed among treatments, the bacteria to algae biomass ratio was significantly greater in the ozone treatments. This differential effect of algae on bacterial growth explains why, in the low DOC water experiment, there was significantly higher bacterial biomass in high ozone than in the control even though algal biomass was not greater and why, in the high DOC water experiment, there was comparable bacterial production in the high ozone and the control even though algal biomass was significantly higher in the control. More bacterial biomass may be supported per volume of algae if each algal cell is contributing more nutrients to the bacterial community. As with bacterial cells, reactive oxygen forms will interact with algal cell wall constituents potentially resulting in increased mucilage production as a defense (Reynolds 2007) or a change of cell permeability (Mallick and Mohn 2000) and leakage of labile organic carbon and other nutrients. Either of these scenarios would provide greater resources for heterotrophic bacterial growth. The greater number of bacteria in high ozone and high DOC water may also be explained by a higher concentration of SRP, although there was no significant correlation between SRP and bacterial density. In the periphyton mat, algal interactions are of greater importance to bacterial
numbers than external stressors and ozone had an indirect effect on bacterial biomass through effects on algal biomass.

Direct negative effects of oxidative stress caused by ozone dissolved from the atmosphere appear to be minimal for benthic, heterotrophic bacteria. In fact, ozone treatments stimulated the growth of bacterial communities whether associated with algae or not. Most ozone effects on benthic bacteria are likely indirect through effects on carbon cycling either by breaking down recalcitrant molecules, making them more bioavailable, or by causing increased autochthonous carbon production which is then used by the heterotrophic bacteria. This carbon mediation was indicated in my previous study of the interactive effects of ozone and DOC water on different algal taxa. Ozone initiated changes to the aquatic carbon cycle may have wide-ranging effects. Further studies are needed to verify the mechanism of ozone effects and whether other aquatic groups, particularly the plankton, will respond differently than the benthos.

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Chapter 4: *The effects of atmospheric ozone concentration on benthic cyanophyte and diatom communities.*

**Abstract.**

High concentrations of ozone have been used to kill algal constituents in water used for drinking and recreation. In such treatments, algal taxa have been shown to respond differentially to ozone treatment. No studies published to date have investigated the effects of atmospheric levels of ozone, allowed to dissolve naturally into the water column, on algal groups. My previous studies have indicated that ozone effects in this situation are highly dependent on algal taxonomic division and complicated by interspecies interactions and water chemistry. To test for direct effects of atmospheric ozone on algal groups, I grew cyanophytes and diatoms, independently, and with an abundance of nutrients, in three atmospheric ozone concentrations. In the absence of interactive effects, cyanophytes were negatively affected by ozone concentration, as indicated by reduced total biomass, and diatoms were unaffected. This study indicates that, in the absence of strong indirect effects, algal assemblages may undergo a shift in composition caused by a greater suppression of cyanobacterial than diatom growth.

**Introduction**

Local production, regional and longer distance transport, and high background levels lead to a global ozone pollution problem (Chameides et al. 1997; Granier and Brasseur 2003). Models have estimated a 100-120% increase in ozone in the northern mid-latitudes over the past 200 years and an increase of 80-100% in the tropics (Committee on Tropospheric Ozone 1991). All but one scenario proposed by the Intergovernmental Panel on Climate Change (IPCC) project increases in tropospheric ozone during the 21st century with projected background
concentrations of 35-48 ppb by 2040, 38-71 ppb by 2060, and 42-84 ppb by 2100 (Vingarzan 2004). These models predict that background concentrations may exceed internationally accepted criteria established to protect animal and plant health.

Increases in atmospheric ozone have led to significant acute and chronic effects on terrestrial primary producers. A chronic response to ozone pollution appears to be a general reduction of growth rate due to a decrease in photosynthesis. The acute, physiological changes associated with ozone stress start with a loss of membrane integrity as unsaturated lipids and proteins are attacked (Skarby et al. 2004). As reactive oxygen species (ROS) increase and overwhelm the cell’s ability to compensate, intracellular damage occurs (Kollner and Krause 2003; Oksanen 2003; Skarby et al. 2004; Yamaji et al. 2003). This cellular damage results from the oxidative stress caused directly by ozone molecules but also from other reactive oxygen species. These oxygen compounds result from ozone speciation into other reactive oxygen molecules, cellular production induced by the initial stress, or are secondary products resulting from the oxidation of membranes (Oksanen et al. 2004; Schraudner et al. 1997). As photosynthetic cells are naturally high in ROS they are especially susceptible to oxidative overload and the photosynthetic apparatus can readily suffer injury (Prozherina et al. 2003).

Aquatic primary producers may be susceptible to similar ozone-induced damage. Numerous wastewater treatment studies have shown the lethal effects of large amounts of dissolved ozone on algal and bacterial cells when pumped directly into the water. At large enough concentrations, no microbial cells survive, but lethal levels vary widely among taxa (Betzer et al. 1980; Gavand et al. 2007; Huang et al. 2006; Widrig et al. 1996; Yun et al. 1997). These studies have all been done with the aim of sanitizing waters and have utilized ozone concentrations in the parts-per-million range. They do little to address atmospheric ozone as an
aquatic pollutant in natural settings. Other reactive oxygen species have been examined at lower concentrations, in ecological and physiological studies (Abd El-Baky et al. 2009; Choo et al. 2005; Gorbi et al. 2006; Ledford and Niyogi 2005; Mallick and Mohn 2000). Research has shown that externally introduced hydrogen peroxide (H$_2$O$_2$), a potential secondary ROS created from dissolved ozone, can cause negative effects on algal populations at natural or near natural levels and different algal taxa show widely varying sensitivities (Barroin and Feuillade 1986; Drabkova et al. 2007; Kay et al. 1984). Growth rates and pigment concentrations of cyanobacterial cells are negatively affected at much lower (up to ten times lower) H$_2$O$_2$ concentrations than other algal groups, particularly green algae (Drabkova et al. 2007b). This may give us insight into the effects of dissolved ozone at natural levels. However, due to the specificity of antioxidant enzymes we cannot assume the same outcome for oxidation by ozone and its breakdown products.

In addition to the potential for direct oxidative stress, there are several possible indirect effects of ozone on algal communities. Changes in competition, disease vectors, light, and nutrients may all be facilitated by ozone. The research done for Chapter 2, studying the effects of atmospheric ozone and DOC concentrations (selected to represent real-world exposures to aquatic ecosystems) on multi-division periphyton assemblages, found complex independent and interactive effects on algal biomass that varied by algal division. These results indicated that inter-taxa interactions and the chemical constituents of the environment may mitigate or change the effects of ozone stress on algal populations.

The following study was devised to resolve some of these complex interactive effects of ozone on algal communities by looking at single division assemblages in the absence of inorganic nutrient limitation and high concentrations of humic substances. Cyanophytes and
diatoms were grown independently in three different ozone atmospheres and high levels of nutrients. The results add to the general body of knowledge on the effects of oxidants on benthic communities by using sub-lethal concentrations of ozone. I hypothesized that, in the absence of these interactions, algal biomass would be lower in the high ozone treatments and that cyanophytes would be more sensitive to the oxidative stress than diatoms.

**Methods**

*Experimental set-up common to both the cyanophyte and diatom experiments*

Three ten-gallon glass aquaria were used to create treatment chambers, each having a different atmospheric ozone concentration: a control treatment with 0 ppb O₃; a medium treatment with about 80 ppb O₃; and a high treatment with about 250 ppb O₃. Aquarium lids were sealed with weather-stripping and lined with PTFE sheeting to maintain the treatment atmospheres. Chemically non-reactive PTFE and PFA plastics were required for this experiment to resist corrosion by the large ozone concentrations pumped into the experimental chambers. Ozone was created and measured using a Thermo Scientific Model 49™ O₃ analyzer. Ozone in each chamber was monitored and manipulated daily to insure an atmospheric concentration above the microcosms that was consistently ± 10 ppb of the target concentration. Ozone levels in the control never measured above 0 ppb.

The air used to generate the ozone was ambient air filtered through a carbon filtration unit prior to entering the generator. PFA tubing conducted the ozone from the generator into two of the aquaria and back from all three to the sample analyzer. Flow into each aquarium was controlled using a PTFE ball valve and manipulated until the target ozone concentration was
measured in the chamber air. The control treatment was supplied with air from an aquarium bubbler filtered through another carbon filtration device. All three chambers were placed on an Eberbach EL600™ Orbital-Reciprocal Shaker Table to create water motion, set to 96 rotations per minute.

Light was provided by fluorescent, natural-spectrum bulbs mounted above the chambers, which generated $63.65 \pm 2.16 \mu E/cm^2/sec$. To simulate natural, summer, diurnal cycles, ozone generation and light were on a timer, producing from 7:00 a.m. to 9:00 p.m. daily. All three chambers were placed on an Eberbach EL600™ Orbital-Reciprocal Shaker Table to create water motion, set to 96 rotations per minute.

Cyanophyte experiment

Replicates were filled with 146 mL of water from Vermillion Creek, Rose Lake Wildlife Area, Clinton County, MI that had been filtered through a 3 µm mesh plankton net to remove most of the algae. Nutrients were supplemented by adding 2 mL of 5 mL/L Bold Modified Basal Freshwater Nutrient Solution. The following cyanophytes, including two nitrogen-fixing taxa, were purchased from Carolina Biological Supply Co.: *Tolypothrix distorta* cultured in a soil-water medium; and *Oscillatoria* sp., *Lyngbya* sp., and *Anabaena affinis* Lemmerman cultured in Alga-Gro® Freshwater Medium. I combined half the volume from each specimen vial into a composite seed population. Each replicate was inoculated with 2 mL of the homogenized seed sample. Algae grew in the treatments for a total of 12 days and additional creek water was added once during the incubation at day six to refresh nutrient supplies and maintain water levels.
**Diatom experiment**

A diatom inoculant was created by sampling from several diatom-dominated communities growing in the lab. In each replicate, two mL of this inoculant was added to 148 mL of a 20 mL/L sterile medium of Bold Modified Basal Nutrient Solution supplemented with 0.06 g/L NaSiO$_3$. A sterile medium was used instead of stream water to decrease the chance of a cyanophyte or chlorophyte bloom during the duration of the study. Also, since diatoms are more tolerant of low-light situations than other algal groups (Pillsbury and Lowe 1999), shade cloth was used to encourage diatom growth over that of other algal groups. The communities developed on the blank tiles for nine days, after which diatom dominance was verified under the microscope, and then the ozone treatment was started. The diatom experiment was run for 11 days.

**Sample Collection**

At the end of each experiment, a significant growth was observed on the bottom of the microcosms as well as on the tiles, so all periphyton and the total water volume from each cup was sampled. Algae were scraped from individual tiles, using a toothbrush and DI water from a squirt bottle, and added into the corresponding water sample which also contained the loose algal biomass that grew off the tiles. Subsamples were taken from this homogenized, composite sample for chlorophyll $a$ measurement and analysis of soft algal and diatom community composition. Algal samples were preserved with gluteraldehyde (2%). Water samples were filtered through Whatman™ glass fiber filters, type GF/F, for analysis of TDN, soluble reactive phosphorous (SRP), and silicate concentration. All water samples were frozen until analysis.
**Analysis of periphyton samples**

Cyanophyte cells were identified and counted in a Palmer-Maloney counting chamber at 400X until 300 natural units were counted (Lowe and Laliberte 1996). Natural units are defined as the normal growth form of the alga, such as single cells, filaments, or colonies. The number of cells composing each natural unit was also recorded. Measurements were made of each taxon during identification for calculating total biovolume as an estimate of algal biomass.

Live and dead diatom species were identified from subsamples mounted on microscope slides using the modification by Stevenson (1984) of Taft’s (1978) glucose mounting technique at 1000X on a Leica compound microscope to a total of 600 live and dead cells.

Chlorophyll \( a \) concentrations were also used as a measure of total algal biomass. Algae from each chlorophyll subsample were filtered onto Whatman™ GF/C glass fiber filters, extracted overnight in ethanol, diluted if necessary, and analyzed fluorometrically before and after acidification to correct for pheopigments (APHA 1998).

Non-purgeable organic carbon (NPOC) and total nitrogen concentrations were measured in filtered water samples by Dr. Stephen Hamilton’s lab at Michigan State University using a Shimadzu TOC-VCPH™ Carbon Analyzer with a total nitrogen module (TNM-1) and an ASI-V Autosampler. Because the water samples were filtered prior to analyses, the TN reading did not include particulate nitrogen and is thus representative of TDN.

Soluble reactive phosphorous (SRP) was analyzed on frozen samples. SRP concentration was measured manually following the ascorbic acid method (APHA 1998) on a Spectronic® Genesys™ 2 Spectrophotometer by Spectronic Instruments. Silicate concentration was measured on a Skalar Auto Analyzer following the molybdosilicate method (APHA 1998).
Statistical Analysis

A one-way between-subjects ANOVA was conducted to compare the effect of ozone treatment on algal biovolumes and DOC or nutrient concentrations. If the effect of ozone was significant, a Tukey’s HSD post-hoc comparison was used to evaluate differences among ozone treatments. All continuous variables were log-transformed prior to analysis. Relative abundance data were square-root transformed. I used an alpha level of 0.05 as the significance criterion for all tests.

Results

Cyanophyte experiment

TDN and silica concentrations did not vary significantly between control and the ozone treatments (Table 4.1). SRP concentrations were significantly lower in the control than in either of the ozone treatments ($F_{2,21}=9.244$, $p=0.002$). TDN and SRP concentrations were very high in all samples due to the growth medium.

Table 4.1. Average nutrient concentrations (mg/L) for the cyanophyte experiment, +/- standard deviation. Letters denote statistically significant differences.

<table>
<thead>
<tr>
<th>Nutrient (mg/L)</th>
<th>Control</th>
<th>Medium Ozone</th>
<th>High Ozone</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDN</td>
<td>9.001 ± 1.097</td>
<td>8.026 ± 0.986</td>
<td>8.039 ± 0.868</td>
</tr>
<tr>
<td>SRP</td>
<td>0.873 ± 0.195$^a$</td>
<td>1.220 ± 0.296$^b$</td>
<td>1.408 ± 0.244$^b$</td>
</tr>
<tr>
<td>SiO$_2$</td>
<td>4.935 ± 0.506</td>
<td>5.073 ± 0.380</td>
<td>4.651 ± 0.224</td>
</tr>
</tbody>
</table>

Ozone treatment had a significantly negative effect on cyanobacterial growth, as represented by both biovolume ($\mu m^3/cm^2$) ($F_{2,21}=7.316$, $p=0.004$) and chlorophyll $a$ (mg/cm$^2$) ($F_{2,21}=7.085$, $p=0.005$) (Fig. 4.1). Tukey’s Post Hoc comparisons indicated that biovolume and
chlorophyll *a* content in the communities exposed to the high ozone treatment were almost half that in either the medium treatment or the control (biovolume: *p*=0.030, *p*=0.004; chlorophyll *a*: *p*=0.013 and *p*=0.002, respectively). The communities in the medium (80 ppb) treatment and control did not differ significantly from each other in either chlorophyll *a* or total algal biovolume although biomass in the medium ozone treatment was lower.

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**Fig. 4.1.** Total biomass by ozone treatment in the cyanophyte experiment. Lowercase letters denote statistically significant differences. Bars are means of eight replicates ± SE.

Biovolume of all cyanophyte genera, except for *Tolypothrix*, which was a limited constituent of all samples, was lower in the high ozone treatment (Fig. 4.2). The biovolumes of *Anabaena* and *Oscillatoria* were lower in the high ozone treatment than in both the medium ozone treatment and the control (*Anabaena*: $F_{2,21}$=8.220, *p*=0.02 and *p*=0.002, respectively; *Oscillatoria*: $F_{2,21}$=7.697, *p*=0.01 and *p*=0.006). *Lyngbya* biovolume in the high ozone treatment was also lower than in the medium ozone treatment and the control, however, only
significantly for the control (F_{2,21}=7.780, p=0.093 and p=0.003, respectively). Biovolume in the medium ozone treatment was lower, though never significantly, than in the control for *Anabaena*, *Lyngbya*, and *Oscillatoria*.

Fig. 4.2 Cyanophyte biovolume by genus as a function of ozone. Lowercase letters denote statistically significant differences. Bars are means of eight replicates ± SE.
While total biovolume of the various cyanophyte genera differed significantly as a result of ozone exposure, relative abundances of these taxa did not, keeping the community composition consistent among ozone levels (Fig. 4.3). *Lyngbya* was the largest constituent of all communities by biovolume, followed by *Oscillatoria*, and *Anabaena*. *Tolypothrix* was less than 0.05% of any sample.

Fig. 4.3. Relative abundance of cyanophyte genera by biovolume for each ozone treatment. *Tolypothrix* was less than 1% of all assemblages. Bars are means of eight replicates.

**Diatom experiment**

TDN and SRP were very high in all samples, due to the growth medium, and did not statistically differ among treatments (Table 4.2). SiO$_2$ was drawn down to moderate levels but did not reach limiting concentrations for diatom growth, and was not statistically different between control and treatments.
Table 4.2  Average nutrient concentrations (mg/L) in the diatom experiment +/- standard deviation.

<table>
<thead>
<tr>
<th>Nutrient (mg/L)</th>
<th>Zero Ozone</th>
<th>Medium Ozone</th>
<th>High Ozone</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDN</td>
<td>76.428 ± 4.699</td>
<td>79.016 ± 10.683</td>
<td>75.755 ± 7.068</td>
</tr>
<tr>
<td>SRP</td>
<td>72.609 ±10.952</td>
<td>65.157 ± 14.379</td>
<td>65.571 ± 11.433</td>
</tr>
<tr>
<td>SiO₂</td>
<td>5.541 ± 0.209</td>
<td>5.686 ± 0.299</td>
<td>5.597 ± 0.314</td>
</tr>
</tbody>
</table>

Diatom biomass did not significantly differ as a function of ozone treatment (Fig. 4.4).

Chlorophyll $a$ ranged from 0.5 and 1.7 mg/cm$^2$ for all samples. Chlorophyll averages for the high, medium and control treatments were, respectively, 1.09, 1.06, and 1.25 µg/cm$^2$. Total diatom biovolume did not statistically change with ozone treatment. Total biovolume ranged from $1.85 \times 10^7$ - $1.02 \times 10^8 \mu m^3/cm^2$.

Fig. 4.4. Total biomass by ozone treatment in the diatom experiment. There are no statistically significant differences among treatments. Bars are means of eight replicates ± SE.
Individual species biovolumes also did not differ among the control and treatments. The ratio of live:dead diatoms did not differ among treatments and control.

Nineteen diatom taxa were observed in the experiment (Table 4.3). No differences were observed in relative or absolute abundances of diatom species. The largest community constituents in all samples, by biovolume, were *D. confervacea* (average 64.2%±126), *F. cf. pelliculosa* (average 11.6%±5.3), *G. parvulum* (average 8.9%±4.8), *P. elginensis* (average 6.2%±5.0), and *P. frequentissimum* (average 3.9%±2.2). The live:dead diatom ratio did not differ among treatments for any individual species.

Table 4.3. Relative abundances of diatom taxa by ozone treatment (percentage of assemblage).

<table>
<thead>
<tr>
<th>Taxon</th>
<th>0 ppb O₃</th>
<th>80 ppb O₃</th>
<th>250 ppb O₃</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Diadesmis confervacea</em> Kützing</td>
<td>63.9 ± 14.5</td>
<td>65.4 ± 14.8</td>
<td>62.5 ± 8.3</td>
</tr>
<tr>
<td><em>Fistulifera cf. pelliculosa</em> (Brebisson ex Kützing) Lange-Bertalot</td>
<td>53.1 ± 4.6</td>
<td>53.8 ± 8.3</td>
<td>59.7 ± 7.9</td>
</tr>
<tr>
<td><em>Planothidium frequentissimum</em> (Lange-Bertalot) Lange-Bertalot</td>
<td>7.0 ± 3.6</td>
<td>6.2 ± 3.3</td>
<td>6.6 ± 1.7</td>
</tr>
<tr>
<td><em>Gomphonema parvulum</em> Kützing</td>
<td>5.0 ± 2.5</td>
<td>4.9 ± 2.3</td>
<td>4.8 ± 2.0</td>
</tr>
<tr>
<td><em>Achnanthidium minutissimum</em> (Kützing) Czarnecki</td>
<td>2.0 ± 0.7</td>
<td>5.0 ± 3.3</td>
<td>3.5 ± 1.7</td>
</tr>
<tr>
<td><em>Nitzschia inconspicua</em> Grunow</td>
<td>2.4 ± 0.9</td>
<td>1.4 ± 0.8</td>
<td>1.7 ± 1.1</td>
</tr>
<tr>
<td><em>Nitzschia palea</em> (Kützing) W. Smith</td>
<td>2.2 ± 1.0</td>
<td>1.3 ± 0.7</td>
<td>1.7 ± 1.4</td>
</tr>
<tr>
<td><em>Achnanthidium exiguum</em> (Grunow) Czarnecki</td>
<td>1.8 ± 0.9</td>
<td>1.5 ± 0.9</td>
<td>1.7 ± 1.2</td>
</tr>
<tr>
<td><em>Nitzschia frustulum</em> (Kützing) Grunow</td>
<td>1.6 ± 1.1</td>
<td>1.3 ± 0.6</td>
<td>1.5 ± 0.9</td>
</tr>
<tr>
<td><em>Placoneis elginensis</em> (Gregory) Ralfs</td>
<td>1.2 ± 0.7</td>
<td>1.0 ± 0.5</td>
<td>1.2 ± 0.7</td>
</tr>
<tr>
<td><em>Amphora pediculus</em> (Kützing) Grunow</td>
<td>&lt; 1</td>
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<tr>
<td><em>Fragilaria capucina</em> Desmazieres</td>
<td>&lt; 1</td>
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<tr>
<td><em>Navicula cryptotenella</em> Krammer &amp; Lange-Bertalot</td>
<td>&lt; 1</td>
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<tr>
<td><em>Nitzschia linearis</em> (Agardh ex W. Smith) W. Smith</td>
<td>&lt; 1</td>
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<tr>
<td><em>Navicula schadei</em> Krasske</td>
<td>&lt; 1</td>
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<tr>
<td><em>Rhoicosphenia abbreviata</em> (Agardh) Lange-Bertalot</td>
<td>&lt; 1</td>
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</tr>
<tr>
<td><em>Staurosira construens</em> (Ehrenberg) Williams &amp; Round</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
<tr>
<td><em>Synedra parasitica var. subconstricta</em> (Grunow) Hustedt</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>
Discussion

Few studies have assessed the effects of ozone at atmospheric concentrations, let alone, the effects of the oxidant entering the water naturally through diffusion from the atmosphere. The current study shows a significant but taxon-selective negative effect of atmospheric ozone on algal communities, in the absence of competing algal groups and high concentration of humic substances.

Cyanophytes were negatively affected by the ozone treatment. Total cyanophyte biovolume and chlorophyll $a$ were significantly lower in the high ozone than in the medium ozone treatment and the control. Community composition did not differ, however, as all major constituents, *Lyngbya*, *Oscillatoria*, and *Anabaena*, responded in the same way. Although statistically not significant, there is an apparent linear cyanophyte biovolume response to ozone in the medium treatment that was intermediate between that in the high ozone and the control for all major community constituents.

Diatom biomass, unlike that of the cyanophytes, was not affected by ozone treatment at any taxonomic level. Total biovolume, chlorophyll $a$, and species biovolumes were statistically the same among both treatments and the control.

The negative cyanophyte response is likely due to the direct oxidative stress caused by the dissolution of atmospheric ozone. Light and temperature did not differ among treatments, no other algal groups were competing for resources, and nutrients were not significant factors in the algal response to treatment because they were above saturating concentrations (Rier and Stevenson, 2002). Phosphorus was the only macro-nutrient that significantly varied with treatment and it was higher in the high ozone treatment, which had the lower cyanophyte biomass. This negative relationship between SRP and biomass indicates that it is biomass that is
influencing the nutrient concentration, and not the other way around. This SRP/biomass relationship, the lack of statistical difference in SiO$_2$ and TDN levels, and the biologically non-limiting concentrations of all three (Bothwell 1988; Horner et al. 1990; Lohman et al. 1991; Rosemarin 1982; Wong and Clark 1976), provide evidence that differences in these inorganic nutrients are likely not an indirect cause of ozone-related biomass differences in this experiment. Heterotrophic bacteria were not enumerated for these samples, but the high level of available nutrients makes competition between algae and bacteria an unlikely indirect factor.

These experiments indicate that cyanophytes are more sensitive to ozone-induced oxidative stress than diatoms, corroborating other studies that show cyanophyte species responding to lower concentrations of oxidants than other algal groups (Apostolova et al.; Drabkova et al. 2007). These different tolerances may be a result of cell morphology. The simple prokaryotic cell structure of cyanobacteria leaves these cells more susceptible to external stress factors. Light-harvesting complexes are on the outside of the thylakoidal membrane and the thylakoidal membrane itself is not contained in a membrane-bound chloroplast. This may cause the photosynthetic apparatus to be more susceptible to molecules entering the cytosol. However, oxidative damage can be mitigated through the production of a multitude of enzymatic and non-enzymatic antioxidants, which appears to be a primary response of photosynthetic cells to ameliorate much of the increased oxidative potential (Schraudner et al. 1997). The type and quantity of antioxidants produced varies depending upon taxon and may be the basis for differing tolerances to oxidative stress (Choo et al. 2004; Contreras et al. 2009; Gorbi et al. 2006; Knauert and Knauer 2008; Sabatini et al. 2009). Taxa within the Cyanophyta typically have a smaller suite of molecules available to decompose reactive oxygen species (Drabkova et al. 2007). The
simpler set of antioxidants and less-protected cellular components may leave prokaryotic cells more susceptible to damage from externally introduced oxidants.

The cyanophyte response in the current research is different than that of cyanophytes in the high DOC water in the Chapter 2 experiment. In that study, cyanophyte growth rate increased significantly in the latter half of the experiments in the ozone treatments but not in the control. In fact, community dominance shifted from diatoms to cyanophytes in the ozone-treated samples. As our current study indicates the cyanophyte sensitivity to ozone-induced oxidative stress, it is unlikely that the cyanophyte bloom seen in the Chapter 2 experiment was due to direct stimulation of cyanophyte cell growth by ozone, but, instead, to ozone effects on other ecological factors. The success of cyanophytes in those systems, in spite of the high ozone load, suggests that other system components may have mitigated negative effects. The higher DOC concentration may have protected algal cells from direct oxidation by reacting with the reactive oxygen molecules before they reacted with the algal cells themselves. It is known that DOC is a sink for ozone in wastewater treatment (Huang et al. 2006; Rositano et al. 2001) and that humic substances, in particular, mitigate the negative effects of other stressors such as herbicides and heavy metals by reacting with the offending molecules (Kulikova et al. 2005; Steinberg et al. 2008; Wetzel 2001b). In addition, the cyanophytes may be insulated by the mucilage layer associated with the periphyton mat. In the Chapter 2 assemblages, a diatom mat was already established before the cyanophyte bloom and may have provided a stable biofilm with associated mucilage that protected cyanophytes from water column oxidants.

The significant results of the current research and, those seen in Chapters and 3, indicate that atmospheric ozone, at levels seen in nature, is a potential aquatic pollutant and illustrates its complex direct and indirect effects. The direct oxidative stress caused by ozone dissolution into
freshwaters may create an environment that differentially affects groups of algae within each community, causing a shift in relative abundance. This study indicates that, in the absence of strong indirect effects, this shift may be caused by a greater suppression of cyanobacterial than diatom growth. However, other dissolved molecules and complex species interactions may alter the effect greatly, as was evidenced by the cyanophyte “bloom” in the ozone treatments in the Chapter 2 experiment.

Ozone levels continue to rise with increasing worldwide industrialization and fossil fuel combustion. When modeling aquatic ecosystem health, it may be important to consider the effects of this pollutant and its oxidative potential on aquatic as well as terrestrial ecosystems. Algal as well as bacterial communities can be changed at a very fundamental level. Further research to untangle the complex, direct and indirect effects of atmospheric ozone on aquatic biota is warranted.
Chapter 5: Summary

Background levels of tropospheric ozone and local maxima have been increasing since the industrial revolution causing ozone pollution problems in both rural and urban areas around the globe. There is a tremendous amount of data that show significant negative effects of ozone pollution on human and ecosystem health. Terrestrial primary producers are particularly susceptible to ozone-mediated oxidative stress and responses to both acute exposure to high concentrations and chronic exposure to lower concentrations may cause tissue damage and slow the growth rate of plants. The results of laboratory experiments in my dissertation research indicate that there is also a potential for atmospheric ozone to act as an aquatic pollutant. My examination of the effects of ozone concentration and DOC on both multi-divisional periphyton assemblages and independent cultures of cyanophytes, diatoms, and heterotrophic bacteria have shown that atmospheric ozone may significantly affect aquatic ecosystems through changes to algae and bacteria in periphyton films. Periphyton responses to elevated atmospheric ozone were complex and varied with algal division, water chemistry, and time. In general the hypothesized negative effects of oxidative stress were minimal in natural periphyton assemblages and occurred early in assemblage development. Indirect effects on biomass, brought about by interactions with other periphyton and water chemistry, were primarily positive and varied with algal division. These effects were seemingly time-dependent as the direct effects appeared to be of greater importance early in film development and indirect effects more important as the assemblage grew and changed and as ozone effects in the water column increased.

The effects of atmospheric ozone and DOC on natural periphyton assemblages, those consisting of several algal divisions and heterotrophic bacteria, indicated many direct, indirect,
and interactive causal pathways. High DOC water, in general, had a negative effect on diatom biomass, a positive effect on cyanophytes, and no effect on chlorophytes and heterotrophic bacteria. Responses of diatoms and cyanophytes to high DOC water seem to be due to DOC concentration rather than other covarying factors. The response of algal biomass to ozone treatment appeared to be primarily indirect and largely influenced by the DOC concentration of the water. Statistical significance of ozone effect was seen in both the low and high DOC waters, but ozone effects were more distinct in the high DOC water with great differences between the cyanophyte and diatom response.

As constituents of these complex periphyton films, cyanophytes were stimulated by the interaction between ozone and DOC. The greatest assemblage change that occurred in this experiment was a cyanophyte bloom in the high DOC water in both the medium and high ozone treatments between weeks 2 and 4. Cyanophyte biomass increased two orders of magnitude in those microcosms. The mechanism of this ozone-DOC interaction effect cannot be directly discerned from the experiment but may be related to the breakdown of complex humic molecules, by ozone and other reactive oxygen species, and the subsequent changes in the light and/or nutrient regime. Cyanophytes were not affected by ozone in the low DOC water.

Diatoms also responded to ozone treatment, but their response was negative and of a lesser magnitude than that of the cyanophytes. This negative response was only statistically significant in the medium ozone treatments in both DOC waters and only at week 2. This may indicate a non-linear effect of ozone on these diatom assemblages, but further studies would be needed to support this hypothesis.

Chlorophyte biomass did not significantly respond to ozone treatment in either DOC water. Ozone effects on heterotrophic bacterial biomass within these periphyton films were
variable and echoed those of total algal biomass. In fact, bacterial density was strongly correlated with algal biovolume, indicating that it was an interaction with these algal constituents that most heavily influenced bacterial growth. The results indicate that ozone will likely have a greater effect in high DOC waters and, at least for cyanophytes, this interactive effect appears to be positive.

Cyanophytes, diatoms and heterotrophic bacteria responded differently to ozone treatment when grown as independent cultures with sufficient nutrients and low humic carbon content. The results indicated differential sensitivity of each taxonomic group to the direct effects of atmospheric ozone. Cyanophytes were the only taxonomic group studied that responded negatively to ozone treatment. Cyanophyte biomass, when grown in the absence of other algal groups and provided with saturating nutrients, was reduced in both the medium and high ozone environments, indicating a direct negative effect of oxidative stress. All cyanophyte genera used responded similarly. Independent diatom assemblages, also grown with saturating nutrients, were not sensitive to ozone treatment. Neither diatom biomass nor assemblage composition was affected by oxidative stress. Heterotrophic bacteria, when grown in the absence of algae, were not negatively affected by ozone treatment indicating no direct effects of ozone treatment. However, positive indirect effects were apparent as bacterial biomass was stimulated by both the medium and high ozone treatments.

The differences in response between the single group experiments and that of the same groups in the mixed periphyton experiment suggest that the role of ozone, whether it stresses or benefits a population, depends on several ecosystem factors. This illustrates the importance of considering inter-taxa interactions and other environmental variables when considering organism responses to stressors. The complex effects of ozone on algae and bacteria reveal a strong
interactive effect of community composition, pre-exposure water chemistry, and oxidative stress. This is not unexpected when considering the intimate relationship between individual algal or bacterial cells with the dissolved substances in the water column and the complex nature of a periphyton community. It is most likely indicative of periphyton responses to other types of stress as well. Within a periphyton film, inter-organismal interactions are great due the proximity of individual cells, making competition and facilitation very important factors affecting growth of the different groups. Secondly, the physical structure of periphyton films creates micro-climates within the mats that may vary in nutrient, chemical, and light regime. Finally, the upper layers of cells may act as a buffer between water column stressors and those assemblage constituents deeper in the film.

In spite of these ambiguous results, it’s clear that the dissolution of atmospheric ozone into surface waters may have an ecologically significant effect on aquatic communities, particularly the interaction of ozone with dissolved organic carbon. The differential responses of algal divisions and the apparent stimulation of heterotrophic bacterial communities may greatly change the functional status of periphyton assemblages, as algal taxa and heterotrophic bacteria differ in their roles in food webs and geochemical cycles and have different metabolic rates and requirements. My results also indicate that systems high in humic carbon will be most likely to be affected by high atmospheric ozone levels.

This research was an initial look into the effects of atmospheric ozone as an aquatic pollutant. The results beg for further research to discern the mechanisms of ozone effects and explore the potential differential responses of other algal assemblages and habitats. For example, phytoplankton communities lack the physical and chemical complexity of periphyton films and species interactions are reduced due to a greater distance between cells. Subaerial
algae on tree bark or wet walls, for example, are less protected than either periphyton or phytoplankton assemblages since they are in direct contact with atmospheric ozone. Ozone effects on these assemblages may be very different than those on algae and bacteria in periphyton films. And certainly, due to the great potential of high DOC waters to mediate ozone effects on algal communities, the study of humic heavy systems such as wetland habitats and black water lakes would be beneficial.

As we were only able to speculate on the potential mechanisms of indirect ozone effects, further detailed studies are warranted to support our current hypotheses or generate new ones. For example, experimental designs with more detailed analysis and direct manipulation of DOC may support the hypothesized role of humic substances in the response of periphyton to ozone concentrations. Fractionation of the DOC samples before and after ozone treatment may be used to verify a breakdown of larger molecules and also identify the production and quantity of algal-derived polysaccharides. Isotope-labeled DOC molecules could be used to test the nutrient addition hypotheses by tracking the breakdown of humic substances, potential release of inorganic minerals, and their subsequent uptake by algae and bacteria.

This dissertation has provided the foundation for a novel area of ecotoxicological research within the field of aquatic ecology. My results indicate that atmospheric ozone pollution may have a significant impact on aquatic communities through functional changes to algal and bacterial communities. These changes may impact carbon and nutrient cycling as well as the quality of algal communities as a food source for zooplankton and invertebrates. In addition, the photo breakdown of DOC molecules, which was shown to indirectly affect periphyton assemblages, may also have direct effects on water chemistry and light penetration.
The ecologically significant effects of these alterations will most likely be greatest in humic waterbodies.
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