Assessing the Impacts of Anthropogenic Acidification on Autotrophic Stream Biofilms Using Pulse Amplitude Modulation Fluorometry

ABSTRACT

Pulse amplitude modulation fluorometry (PAM) has not been extensively studied as a rapid assessment tool for acidification in freshwater biofilms. This project focused on two types of anthropogenic acidification to determine the efficacy of PAM as a rapid health assessment tool for impaired freshwater streams by using algal responses to non-visible stress indicators. Acidification alters algal community composition such that only acid- and metal-tolerant species thrive. Metal and acid impairment affect primary production by blocking electron transport and reducing the amount of NADP and ATP created in nonacidophilic algae, potentially lowering photosynthetic capacity of the overall algal community. PAM fluorometry allows for the rapid assessment of photosynthetic potential in algae and other plants. A Walz MiniPAM II was used to perform rapid light curves and measure F_v/F_m on periphyton in 13 streams throughout eastern Pennsylvania, including unimpaired streams and streams impaired by acid mine drainage or acid deposition. Water chemistry and algal biomass measurements were taken for each stream. The results of this study demonstrate pulse amplitude modulation fluorometry to be an effective tool for assessing algal stress in acid impaired streams. Overall, acidified streams had low biomass and productivity. Based on Akaike Information Criterion (c), log maximum electron transport rate, (a measure of maximum potential photosynthesis), was best described by a model that included pH and log chlorophyll *a*. This was the most parsimonious model

comparing the relationships between the observed PAM parameters and environmental variables.

INTRODUCTION

Anthropogenic acidification is a pervasive problem for aquatic ecosystems. Its two main sources are: 1) atmospheric deposition originating from fossil fuel combustion, and 2) acid mine drainage (AMD), a legacy effect from extensive coal mining (Bott et al. 2012, Wingington et al. 2010). Currently, 8,946 kilometers of Pennsylvania's streams are listed as impaired by AMD (Pennsylvania Department of Environmental Protection, 2020). At least 217 km have been classified as chronically acidified from acid rain (Schmidt, 2002). However, the total amount of stream habitat affected by acid deposition is unknown due to the episodic nature of runoff, where high flow events temporarily increase stream acidity. Episodic acidification can damage the biota but is not easy to detect during routine sampling (Schmidt, 2002). Changes in water quality directly affect aquatic organisms through exposure to H+ ions and through increased levels of dissolved metals such as iron, aluminum, and manganese (Muniz, 1990 and Brake, 2010).

Acid deposition (ACD) is any wet or dry precipitation with acidic components (New Hampshire Department of Environmental Services, 2019). Nitrogen oxides (NOx) and sulfur dioxide (SO2) gasses are released from power plants that burn coal and other types of fossil fuels (Kirby et al. 2008). After combustion, these gasses combine primarily with water in the atmosphere to make secondary products such as nitric (HNO3) and sulfuric (H2SO4) acids. Wind transports the corrosive pollutants far distances from their source as dry particles, either settling out of the air as dust or mixed with water and precipitating in a wet form (rain, snow, sleet, fog, etc.) (New Hampshire Department of Environmental Services, 2019).

ACD impairment affects organisms by introducing aluminum ions and acid into waterways leading to decreased pH and metal toxicity (Kirby et al, 2008). Aluminum is the primary metal leached from the ground when ACD contacts bedrock or soil. Acidic products include nitric acid, nitrates, sulfuric acid, and sulfates (New Hampshire Department of Environmental Services, 2019). Surface deposition in waterways directly lowers pH leading to chronic toxicity in fish and other aquatic animals by compromising gill membranes, thereby impairing respiration (Jennings et al., 2008). Depending on concentration, ACD can also lead to disease and reproductive issues (Kirby et al, 2008).

The region's buffering capacity and the severity of ACD affects the amount of aluminum dissolution (Environmental Protection Agency, 2020). Buffering capacity, or acid-neutralizing capacity, is the potential of a substance (soil, bedrock, etc.) to mitigate the effects of acid and other dissolved ions before they enter waterways (Kirby et al., 2008). The efficacy is dependent on the region's geology, soil, and acid input. Decades of acid rain in areas of bedrock with lower acid resistance or lower carbonate content has eroded the soil and caused the immediate area's buffering capacity to be reduced, allowing more acidity into waterways (Wingington et al., 2010). Not only will existing water chemistry alter the incoming pollution, but soil and water interfaces can also drastically change the impact of acid rain entering a stream. For example, regions of karst topography have carbonate-rich bedrock, which buffers any incoming water before it can reach streams. In contrast, the major geology in Atlantic Canada is granite and shale bedrock.

Acid deposition is low, but surface water acidity is high due to low buffering capacity and sensitivity to episodic changes in pH (Clair et al., 2007). Many naturally acidic streams also are episodically acidic, requiring a rainfall or snowmelt event to temporarily lower pH (Hyer et al., 1995). In areas where the bedrock is not a sufficient buffering agent, the pollutants will lower or eliminate macroinvertebrate and fish populations by stunting reproduction, or degrading the water quality to the point of hypoxic conditions because of damaged, inadequate, or recently non-existent algal communities (Kirby et al., 2008).

AMD originates from flooded boreholes, weathered tailings, and abandoned surface and underground mines (Smucker, 2014). The greatest contributor to acid mine drainage comes from pyrite oxidation (Skousen et al, 2018), where water and air oxidizes FeS2 into soluble iron and sulfuric acid. After oxidation, the new iron precipitate causes a rust color that is typical of AMD streams (Reinhardt, 1999). The sulfuric acid causes the dissolution of metals (Fe, Mn, and Al) from surrounding soil and bedrock (Reinhardt, 1999). Metal precipitation is controlled by both oxygen and pH conditions. Aluminum will precipitate as (Al(OH)3) in the pH 6-8 range. It can stay dissolved outside of this range, which can cause pH to drop. Iron (Fe(OH)3) crusts build up on substrate when Fe+2 is oxidized to Fe+3, which then hydrolyzes with water, leading to an iron crust and lowered pH (Drover, 2018).

AMD has many negative impacts on macroinvertebrates and algae. Acute and chronic toxicity in organisms is caused by both high metal concentrations and acidity, leading to reduced biodiversity in streams (Bott et al, 2012). Bott et al (2012) also found acidification and heavy metal leaching reduced macroinvertebrate density by about 76 to

98%, depending on the type of coal leaching (Drover, 2018). Because macroinvertebrates are especially sensitive to degraded water quality, AMD typically results in a reduction in the diversity and total numbers of macroinvertebrates and large shifts in community structure, favoring pollution tolerant species (Bott et al. 2012). In algae, AMD alters the community composition in favor of only acid- and metal-tolerant species. Examples of acid-tolerant species include the diatom, Eunotia sp. and the euglenoid, Euglena sp. (Brake, 2010). These impairments to non-acidophilic algae include blocking of electron transport and reduction of the overall efficiency of photosystem II (Suresh Kumar et al. 2014).

Because anthropogenic acidification may reduce photosynthetic efficiency in stream algae, pulse amplitude modulated (PAM) fluorometry has tremendous potential as a non-invasive tool for monitoring these impacts. PAM is a technique that uses patterns in chlorophyll a fluorescence to measure photosynthetic efficiency and the overall capacity of photosystem II (PSII) in plants and algae (Coehlo et al 2013). Altered environmental conditions will change chlorophyll a fluorescence, where any damage affecting the photosystem centers would be present in the form of an altered fluorescent response (Suresh Kumar et al., 2014). PAM is used to read these responses by various light modulation patterns, intensities, and durations, creating real-time response curves (Herlory et al 2007). Under normal conditions, light received by a chloroplast takes 1 of 3 paths; absorption for energy production, heat dissipation, or emission as fluorescence. (Maxwell and Johnson, 2000). Under impaired conditions, low pH or metal ion interference disrupts the electron transport chain in ATP synthesis. The interrupted energy production causes a lower photosynthetic capacity in PSII, which causes fewer photons to be emitted in fluorescence (Suresh Kumar et al. 2014). PAM analyzes chlorophyll a fluorescence-yields by directing a specific wavelength (690 nm) of light to a sample and measuring the quantity of light emitted back at the longer wavelengths. The modulation of light (repeatedly turned on and off quickly) isolates the new fluorescence made by the probe's light as the relative fluorescent yield, so that background light does not interfere with the measurements.

Previously, PAM has been applied as a rapid assessment tool for a variety of water quality issues. Cook and Francoeur (2012) found that PSII capability was reduced by simulated winter road salt exposure. Hiriart-Baer, et al. (2008) used PAM to compare Fv/Fm (the measurement of PSII's maximum photosynthetic efficiency) to phosphorus nutrient status across a water quality gradient, noting that light-saturating conditions are needed to find the severity of nutrient limitation. They found that nutrient stress or starvation can result in a decrease in Cladophora sp. photochemistry through degraded reaction centers as well as lowered Fv/Fm. Lastly, in impaired Portuguese streams, Coelho et al. (2013), used rapid light curves (RLCs) to find the effect of AMD on benthic diatom communities. They found seasonal patterns in chlorophyll a fluorescence, with a lower maximum relative electron transport rate (ETRm) in impacted sites. Summer was the period of lower values for both impacted and non-impacted sites due to light damage, while winter was a more productive period.

PAM as an assessment for acidification in freshwater biofilms has not been extensively studied. The purpose of this study was to determine how well PAM can assess the impacts of anthropogenic acidification on algal physiology in selected Pennsylvania algal-dominated stream biofilms. Biofilms in unimpaired streams were compared to streams impacted by acidic atmospheric deposition, and streams impacted by acid mine drainage in central Pennsylvania, USA.

METHODS

Study Sites

This project involved sampling in 13 streams throughout central Pennsylvania (Figure 1) between early September and mid-November 2020. A second round of water quality and pH measurements occurred on July 31, 2021. Sites included reference (REF), acid mine drainage (AMD), and acid deposition (ACD) streams. The interactive GIS program PaGEODE (PA DCNR, 2021) was used to determine which major geologic units were at each site. All streams in this study, regardless of impairment or origin, drain into the Susquehanna River and ultimately into the Chesapeake Bay.

Five atmospherically acidified streams (ACD) were sampled from the northern glacial plateau region (Sullivan County, Pennsylvania, USA). This area contains both continuous and episodically acidic streams from bedrock percolation with some metal runoff (FCWA. 2007). ACD streams included Sullivan Branch (SB), Heberly Run (HB), Painter Run (PR), Oxhorn Run (OR), and Bloody Run (BR). The streams SB, PR, OR, and BR have the Catskill Formation, a mostly sandstone unit with a smaller mix of shale and mudstone, as their primary bedrock unit. The Heberly Run site was in the Huntley Mountain Formation, also primarily sandstone. All ACD streams originate from this unit.

This area with both continual and episodically acidic streams thus had lower overall buffering capacity (PaGEODE, 2021).

The four AMD-impaired streams are further south in the anthracite coal region (Northumberland + Schuylkill Counties, PA). AMD streams included Black Creek (BC), Catawissa Creek (CC), Tomhicken Creek (TC), and Morris Run (MR). Both TC and CC's bedrock unit is the Mauch Chunk Formation, a mainly shale unit. MR is in the Huntley Mountain Formation, a dual sandstone sequence. Black Creek runs mainly through the Llewellyn Formation, a majority sandstone unit with interbedding of sandstone, shale, siltstone, conglomerate, and coal. This region's inclusions of clay, black shale and coal help acidify the streams. Black Creek, a tributary of Nescopeck Creek, has been reported to have high aluminum, iron, manganese, and acid impairment (PA DEP, 2005). Catawissa Creek has acid impairment, with a pH of 4.5 (Environmental Protection Agency, 2003). Both Catawissa Creek and Tomhicken Creek were sampled below their AMD treatment systems.

Reference streams included Fishing Creek (FC), Hallowing Run (HR), Plum Creek (PC), and Mauses Creek (MC). Reference streams for this project were defined as any stream not impaired by acidification for any category. Fishing Creek and Hallowing Run were both listed as unimpaired for aquatic life (Environmental Protection Agency, 2020). Plum Creek is unimpaired for potable water and aquatic life; however, it is listed as impaired for recreation due to pathogens. Mauses Creek is impaired for cold water fishes due to siltation, but not impaired for any other category. Fishing Creek's bedrock unit is the Sherman Creek Member of the Catskill Formation, a majority mudstone unit. The point of sampling for Mauses Creek was in the Wills Creek Formation, a calcareous shale. The stream passes through many units on its way through a syncline. The Hamilton Group, a shale unit, makes up both Heberly Run and Plum Creek's bedrock geology (Mahantango and Marcellus Formations).

Plum Creek (Northumberland County, PA) is a small, perennial tributary of Little Shamokin Creek and does not have any impairments from acid deposition or mine drainage. Less than a mile is impaired by siltation, low dissolved oxygen & organic enrichment from grazing upstream, however the rest remains unimpaired (PA DEP, 2004). Hallowing Run (Northumberland Co., PA) is a small tributary of the Susquehanna. Mauses Creek (Montour Co., PA) is a small tributary of Mahoning Creek. Fishing Creek (Columbia Co., PA) is a medium tributary of the Susquehanna.

PAM Fluorometry

To make sure enough biofilm was present in each stream, we initially checked background chlorophyll *a* fluorescence (F_o) for a value greater than 100. The Walz MiniPAM II was used to measure F_v/F_m and perform sequential rapid light curves (RLCs) on stream periphyton. On site F_v/F_m is found by dividing the variable fluorescence (F_v) by the maximum (F_m), to obtain a normalized ratio that represents what the maximum efficiency of PSII would be if every capable reaction center were open. This is achieved by sending a saturating pulse of light that closes every reaction center to form a sharp peak of fluorescent yield. Singular F_v/F_m scores were found by taking periphyton coated rocks, submerging them in buckets, and dark-adapting them for 20 mins before measuring. The purpose of dark-adapting samples is to standardize readings across different ambient light levels through ensuring that all reaction centers were open (Lu, Zhang, 2000). Typically, a low F_v/F_m indicates an injured, unhealthy, or more dormant plant, while a high F_v/F_m shows a more healthy or active plant (Herlory et al., 2007). Rapid light curves are generated by sequentially acclimating the reaction centers to increasing irradiance levels, with saturating pulses administered to measure F_v/F_m after acclimating to each level (Herlory et al. 2007).

Chemistry

In addition to PAM readings, water chemistry and algal biomass were also measured at each site. I used a Eureka Manta sonde to measure pH, conductivity, dissolved oxygen, and temperature in situ. Water samples were taken to look for metals, alkalinity, and acidity. To collect periphyton, 10 periphyton-coated rocks were scraped with a toothbrush using a 4 cm diameter template. These were combined into a single composite sample for each stream. Each composite sample was centrifuged in 15 mL tubes with a tabletop analytical centrifuge at a low rpm for ~2 minutes, then removed the water with a pipet. The remaining pellet was preserved with 7-8 mL of a 3.7% buffered formaldehyde solution. To obtain ash free dry mass, I first removed the formaldehyde by rinsing with DI water and centrifuging 3 times. I pipetted 5 mL periphyton samples onto GF/F filters and vacuum filtered. The filters were then placed in foil pans, dried in a drying oven at 105° C, then weighed. After a period of at least 24 hours, the pans were placed in a muffle furnace (500° C) for one hour and weighed again. The difference between the dry weight and the ash weight represented the loss on ignition (LOI)(Kreeger, 1995).

Chlorophyll *a* was measured by taking algal scrape samples and freezing them at -80 °C. After thawing the stock samples, the contents were poured into a glass jar, with the remaining space filled with DI water to a known amount (100 to 120 mL depending on the sample). Afterwards, I added a magnet to the sample and set it on a stir plate for about 10-15 minutes. Once suspended, I extracted 5 ml into a microtube, ran it through the microcentrifuge for 30 minutes at 2600 rpm, and froze them until analysis. Thawed aliquots were vacuum filtered through GF/F filters. These were placed into new 15 mL centrifuge tubes and kept in a -20° C freezer overnight. 5 mL of 90% ethanol were added the next day under yellow light before placing the samples in an 80° C water bath for 5 minutes. After covering in foil, the tubes sat in darkness overnight in a refrigerator to extract chl *a*. Using a spectrophotometer (Spectronic Genesys 2, Thermo Fisher Scientific) I measured absorbance of the samples at 750 and 665 nm, then acidified them with 0.1 mL HCl and read absorbance again at those wavelengths to adjust for phaeopigments (degradation products of the chlorophyll). Chlorophyll *a* content was calculated as milligrams per liter by subtracting the phaeopigment concentration from the non-acidified sample.

The initial fall 2020 water samples were thawed, then analyzed, using inductively coupled plasma-optical emissions spectroscopy (Profile Plus High Dispersion ICP, Teledyne Leeman Labs) to determine Fe, Al, and Mn concentrations. In total 3 replicates were run for 3 acid preserved samples with a pH of 2, and 17 non-acid preserved samples (SMEWW, 2018).

Both alkalinity (Hach Method 8203) and acidity (Hach Method 8201/2) were analyzed using the summer 2021 water samples. Both standard methods and complete results can be found in Appendix C.

Statistics

I performed mixed-model regression analyses on the PAM parameters (ETR_m, alpha, F_v/F_m) with pH and chlorophyll *a* included as independent variables. The chlorophyll *a* data was log transformed for all analyses to standardize any outliers. All statistical analyses were performed using the statistical program R. Akaike information criteria (AICc) were compared using the dredge function in the MuMIn package to obtain the most parsimonious models describing the relationships between PAM parameters and environmental variables.

RESULTS

In situ water chemistry differed between the 13 streams (Table 1). REF streams had the highest overall pH (7.75 ± .48), ACD had the lowest average pH (5.89 ± .50), and one AMD stream (Morris Run) had the lowest individual pH of all sites (3.23). Tomhicken, Catawissa, and Black Creeks were all sampled past their treatment systems, and are not included in the average for AMD. Alkalinity was highest at 77.8 mg/L as $CaCO_3$ at Mauses Creek (REF), while Morris Run (AMD) was so low in pH that it had no alkalinity. The lowest measurable alkalinity value was 0.67 mg/L as $CaCO_3$ at Bloody Run (ACD). Morris Run (AMD) had the highest acidity at 33.27 mg/L as $CaCO_3$, while the lowest acidity was in Bloody Run (ACD) at 4.4 mg/L as $CaCO_3$ (Table 1). Complete sonde and water chemistry data from both the fall and summer can be found in Appendix A.

Values for in situ parameters measured with the sonde were similar in pattern in both fall 2020 and summer 2021. ACD streams have the lowest conductivity overall (21.02 ± 5.40 uS/cm). Conductivities for REF streams were highly variable, ranging from 59.9 for Fishing Creek to 431.7 µS/cm. AMD streams had the highest overall conductivities, ranging from 224.2 to 783.6 µS/cm. Manganese, iron and aluminum were detectable in only Catawissa Creek and Morris Run, with Morris Run having the highest concentrations of all three (Table 2).

The best fitting overall AIC model found was log transformed ETR_m, pH, and log chlorophyll *a* with a value of -20.4. Between the AICc models tested, pH had the highest relevance, impact, and influence on algal photosynthetic efficiency compared to other factors (Table 4).

Generally, ETR_m, an indicator of photosynthetic capacity, was higher in the less impaired streams (Figure 3), with ETR_m lower in the AMD streams, and lowest in the ACD streams. The particularly low ETR_m values in MR and the ACD streams, compared to their higher rates of acidification, support the suggestion that impaired or reduced electron transport has a direct negative effect on algal physiology.

DISCUSSION

The evidence found using PAM indicated that anthropogenic acidification negatively impacted the photosynthetic capacity of stream algae. Lower ETR_m, as well as lowered biomass, are two such consequences of introduced acidification. This is consistent with Coelho et al. (2013) where acidification reduced photosynthetic activity. Our study used similar chl *a* parameters (ETR_m and alpha) and observed correlation to acidification levels similar to those that Coelho observed in AMD source-to-peripheral collection sites.

While a macroinvertebrate portion was not included in this study, it has been shown that a lack of grazers leads to greater diatom biomass in impaired streams (Niyogi et al. 2002). Contrary to results reported in prior studies (e.g., Niyogi et al., 2002; Smucker et al., 2014), the acid-impacted stream sites had lower algal biomass than the unimpaired sites, possibly due to a combination of factors such as another drought year, agricultural pollution, and interrupted energy production via heavy metals and low pH.

Algal community composition and genera diversity are important in acidification studies and are useful for determining the severity of acidification (Smucker et al., 2014). However, the focus of this study included productivity and electron transport rates as my main two criteria to distinguish between stream type sites. For example, Fishing Creek had the highest individual and average ETR_m and higher production overall, possibly due to it not being a headwater stream.

Buffering capacity was not as much of a factor as expected because the samples were taken in a majority shale and sandstone bedrock area. As shown in Clair et al., (2007), high shale bedrock (among others) will result in ineffective buffering material. This leaves any watershed in that area prone to acidification, natural or anthropogenic. Unlike Atlantic Canada, this region of Pennsylvania still receives a comparatively larger amount of acid deposition, although it is greatly reduced from the time of Clair et al's initial data (Environmental Protection Agency PSP, 2020). Initially, the widespread shale formations were expected to affect the streams more directly.

However, the results demonstrated the remediated AMD streams (Black Creek, Catawissa Creek, Tomhicken Creek) had high buffering capacity similar to reference streams, due to the introduced limestone. As expected, Morris Run's high acidification, low productivity, and high amounts of dissolved $CaCO_3$ exemplify a typical AMD stream. Tomhicken Creek, Black Creek, and Catawissa Creek were sampled below their AMD treatment systems; likely the reason for their higher pH.

As found in the 2012 study of Cook and Francoeur on salt damage, higher mineral content correlated with reduced PSII capability of the periphyton. Unlike over-salting winter roads documented by Cook and Francoeur, the majority of the pollution in this study came from dissolved atmospheric ions for ACD streams, and dissolved ions from pyrite and clays in coal for AMD streams. In time these inclusions lower the pH of the water and further erode the matrices they come into contact with. The study sites did not have a very high or even moderately high buffering capacity (PaGEODE, 2021), save for certain reference streams. PAM worked as expected in showing those early warning stress signals in anthropogenically acidified streams with low buffering capacity through low ETR_m and low F_v/F_m.

CONCLUSIONS

Our study demonstrates that pulse amplitude modulated fluorometry is an effective method of identifying metal and pH impairments in algal physiology. It is fast to use, takes less time to analyze than typical laboratory tests, and gives an accurate view into how stressed the periphyton is without destroying it. There were some limitations in the chemical analyses in this study. The first sampling period occurred during a drought after a dry summer with sampling continuing until right before the first freeze. The second set of water chemistry and pH samples used were taken the next summer over the course of two days, during much wetter conditions. Three of the acid mine drainage sites (Tomhicken Creek, Catawissa Creek, Black Creek) were sampled past their treatment systems. Despite these differences of time, temperature, remediation, and precipitation levels across two different field seasons, we're confident the data is still representative of the effects that differing acidification has on stream algae.

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