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Arkansas Bulletin of Water Research

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## Arkansas Bulletin of Water Research - Inaugural Issue 2017

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# Arkansas Bulletin of Water Research

Issue 2017  
Inaugural Issue

A publication of the  
Arkansas Water Resources Center



# Arkansas Bulletin of Water Research

A publication of the Arkansas Water Resources Center

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The Arkansas Bulletin of Water Research is a publication of the Arkansas Water Resources Center (AWRC). This bulletin is produced in an effort to share water research relevant to Arkansas water stakeholders in an easily searchable and aesthetically engaging way.

This is the inaugural publication of the bulletin and will be published annually.

The submission of a paper to this bulletin is appropriate for topics at all related to water resources, by anyone conducting water research or investigations. This includes but is not limited to university researchers, consulting firms, watershed groups, and other agencies.

Prospective authors should read the "Introduction to the Arkansas Bulletin of Water Research" contained within this publication and should refer to the AWRC website for additional information.

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*Cover Photo:* Alex Baecher captures a northern map turtle (*Graptemys geographica*) sunning on a rock on the Buffalo National River.



# Arkansas Bulletin of Water Research

A publication of the Arkansas Water Resources Center

## Issue 2017

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<sup>φ</sup> Denotes research that is part of the US Geological Survey's 104B Grant Program

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Image caption: Student researcher analyzes water samples in the lab.

## Introduction to the Arkansas Bulletin of Water Research

Erin E. Scott\* and Brian E. Haggard

Arkansas Water Resources Center, University of Arkansas

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### Introduction

There is a lot of research being done in Arkansas that can provide valuable information to water stakeholders throughout the State. The research itself can come with a multitude of challenges, and sometimes what to do with that information can be even more difficult. But, sharing research results with the public is tantamount to the research itself.

The Arkansas Bulletin of Water Research was developed to provide an outlet for researchers to communicate project findings that might not be published in national or international journals, yet is extremely important to stakeholders in Arkansas. Further, this bulletin is designed to allow research to be disseminated in an easily searchable and aesthetically engaging way. The contents of this bulletin can be used to guide management decisions about water resources in Arkansas and the region.

Articles in this bulletin will inform the reader not only in the context of the research details, but especially in why such research is important to Arkansas. How can the research be used to address water problems for Arkansas? Can the research results be broadened to address water issues important in the region, and even the country?

### Who Should Submit Articles?

The submission of papers to this bulletin is appropriate for topics related to water resources by anyone conducting water research or investigations in Arkansas. This includes but is not limited to university and student researchers, consulting firms, watershed groups, and other agencies.

### Review Procedures

Papers will be reviewed by the editors of the Bulletin. The editors might send papers out for external reviews as needed; external reviews may become standard procedure for all papers in the future. The editors and or external reviewers will determine if the paper should be published with minor revisions, revised and resubmitted, or rejected. The editors will provide a written review with comments. The author will be expected to address comments in the paper and in a response to reviewer comments.

### What Should the Paper Include?

The aim of this bulletin is to communicate applied research findings that people of various specialties can understand. Therefore, papers should be written in a relatively casual

way, like a conversation with the reader.

“The most important rule: write for the busy reader who is easily distracted.” This statement comes from a great reference on scientific writing,

Griffies, S.M., W.A. Perrie, and G. Hull. *Elements of Style for Writing Scientific Journal Articles*. 2013. Elsevier.

Another nice reference on scientific writing is,

Mackay, R.J. *Writing Readable Papers: How to Tell a Good Story*. Reprinted from the *Bulletin of the North American Benthological Society* 12(3):381-388; 1995.

Papers should be less than 2,500 words from the introduction through the conclusions and recommendations (not counting title, abstract, key points, references, or figure and table captions). Refer to the website [arkansas-water-center.uark.edu](http://arkansas-water-center.uark.edu) to see style and formatting guidelines. The following sections should be included in submitted papers.

## **Title**

### **Short Title**

A title of 90 characters or less (including spaces).

### **Author Information**

Include author first and last name, affiliation, and department of affiliation (if applicable). Also, identify the corresponding author if there is more than one author.

### **Abstract**

In 250 words or less, summarize the report. Include the basic problem, why it's important to Arkansas, what's the research question, what's the objective(s) of the research, brief description of methods, specific results, and conclusions or recommendations to water managers.

### **Key Points**

Include 3 to 5 bulleted statements of 25 words or less that concisely describe the overall importance, applicability, or impacts of the research.

### **Introduction**

This is where you really get to capture the reader's attention and set up the story you're about to tell. The introduction should start fairly broadly by describing the general topic and problem. References to the literature should be used to describe what's already known about the topic, but also to show what the knowledge gap is that your research will address.

As you convey the basic facts and importance of the topic, the introduction should start to narrow focus to a more specific problem, location, or mechanism. This should then lead

to specific objectives and hypotheses. This is also a great time to emphasize to the reader how the research can be applied by others...what's the big impact? How might this work be used by water resource specialists in Arkansas and perhaps around the region and country?

The introduction should be 3 to 5 paragraphs, each of 3 to 5 sentences.

## **Methods**

The methods should provide adequate detail about the project such that someone else could repeat it. Include information about the study design, location or site description, sampling procedure, data collection, laboratory analyses, and statistical analyses.

## **Results and Discussion**

What were the major or important findings that help to answer your research question? Be sure to include tables, figures, and statistical results. How do you interpret these findings, and how do they fit or not fit into the existing body of knowledge?

## **Conclusions**

What do you want the reader to take away? What are your recommendations to water resource specialists? What are the benefits to Arkansas; also the region and the country, if applicable? This is the section where you should emphasize how your research can be applied by others to address pressing water problems in Arkansas.

## **Acknowledgements**

This section allows you to recognize funding support and other assistance. It's also a place to include any disclaimers on behalf of your funding support if applicable.

## **References**

## **Advice to Authors**

Some scientists are great communicators, and some scientists struggle with how to convey information to the public. The goal of this bulletin is to provide information that's easy for people to understand who are from a range of disciplines. The writing should be interesting and conversational, and complex jargon should be left out.

This bulletin is designed to be a valuable resource to water specialists who have to make some tough decisions on how to address our most pressing water resource problems. It will also provide valuable reference material for current and future researchers focused on water issues in Arkansas. As you are writing the paper, frequently ask yourself, “how can results of this work help stakeholders in Arkansas.”





Image caption: Eddy covariance tower deployed at a rice field in Arkansas.

# Partitioning Rice Field Evapotranspiration into Evaporation and Transpiration Components

Benjamin R.K. Runkle

Department of Biological and Agricultural Engineering, University of Arkansas

**Abstract:** This project aimed to resolve uncertainties in the evapotranspiration (ET) portion of the water balance as rice farms transition from conventional to alternate wetting-drying (AWD) irrigation strategies. As 64% of regional precipitation is converted to ET, it is a dominant part of the surface water balance, and understanding its behavior is a key priority to determine the state's water resources situation. Our project's research work is performed at several scales. First, we directly monitor ET rates with the eddy covariance method at several rice production fields in Arkansas in concert with biometeorological measurements to detect underlying, predictive mechanisms. We interpret these measurements in a number of ways, including the Food and Agricultural Organization's implementation of the Penman-Monteith equation to partition ET into its transpiration and evaporation components. Here we find that AWD management does not significantly alter the surface water balance due to the high rates of transpiration during the growing season. Second, we have generated a regional network of research scientists focused on ET and related fluxes (e.g., land-atmosphere exchange of  $\text{CO}_2$ , which plays a major, interacting role in controlling plant water use). Further, we have connected to a USGS groundwater modeling team to enhance their representation of ET in their projections. Our local and regional results lay the groundwork for more nuanced experimental research in both ground observations and modeling strategies. The initial results will help to constrain the rate of ET in the region so that USGS-driven models more accurately anticipate changes in the region's water resources.

## Key Points:

- Evapotranspiration (ET) is largely composed of transpiration during the growing season (74% over the season; up to 95% in the mid-summer)
- The transpiration signal is strong such that drying periods do not seem to show significant reductions in ET.
- The project team has expanded its spatial reach by developing a regional network of ET observation sites and will work with a USGS team to help constrain regional groundwater models.



## Introduction

Rice agriculture uses 35% of Arkansas's irrigation water and contributes to the unsustainable depletion of the state's water resources (Reba et al., 2013; ANRC, 2014). A variety of new irrigation methods have been proposed to reduce water use, including alternate wetting and drying (AWD), which floods the soil and then allows a strategic dry down before reflooding to save water, reduce the risk of the straighthead disability on rice, and decrease field methane production. This method reduces greenhouse gas emissions by more than 70% (including from methane, which is produced under water-saturated conditions and is 20-30 times more potent as a greenhouse gas than  $\text{CO}_2$ ) (Rogers et al., 2013; Linquist et al., 2015). Our 2015 project found that total evapotranspiration (ET) from an AWD field is similar or even slightly greater than a comparison, conventionally flooded field. This response may be due to the strong ability of rice roots to pull water from the soil matrix and from the relatively short length of the dry down period (approximately 11 days).

Therefore this project aimed to investigate further the relationships between evaporation and transpiration and to quantify a second growing season of ET rates in Arkansas rice production to test whether the initial results were robust over time. This project also aimed to generate broader interest through the creation of a regional network of measurement sites. While our eddy covariance datasets are still being developed, we have been able to compare initial findings with the Food and Agricultural Organization's Penman-Monteith method of reference ET (known as FAO56; Allen et al., 1998). The FAO56 method is also used to partition the total ET into contributing portions of evaporation and transpiration by applying a dual crop coefficient method.

Additionally, we recognize a need for a more regional perspective, and so sought out strategic partners who both collect and interpret ET observations. We generated the re-

gional Delta-Flux observation network, established ties to South Korean researchers, and have begun working with a USGS team dedicated to improving groundwater modeling of the Mississippi Alluvial Aquifer. These efforts are described in more detail in the Results and Conclusions sections.

## Methods

This research is situated within a larger project aimed to measure year-round land-atmosphere fluxes of energy, water vapor,  $\text{CO}_2$  and  $\text{CH}_4$  from two side-by-side pairs of rice fields near Humnoke and Burdette, AR, respectively (Figure 1). This larger project provides meteorological instrumentation, eddy covariance equipment to measure the fluxes, and associated environmental monitoring devices to capture terms such as the water level and soil temperature. Presented here are the water vapor fluxes measured by the eddy covariance method, for the Humnoke fields in 2015.

Water vapor fluxes are both measured by the eddy covariance method to determine turbulent transport between the surface and atmosphere (Baldocchi, 2003) and they are modeled by the Penman-Monteith equation (Monteith, 1981). The eddy covariance measurements are generated from observations of vertical wind and water vapor recorded 20 Hz by using the EddyPro software, version 6.2 (Li-cor, USA), and are carefully quality controlled following standard protocols and an additional screen for outliers in the scalar statistics. The eddy covariance observations are gap-filled using an artificial neural network approach (Knox et al., 2015, 2016). These models use data equally apportioned into training, testing, and validating groups from natural data clustered identified using a k-means method. The procedure was replicated across 20 resampling runs and the median prediction was used for gap-filling. To estimate conservative uncertainty bounds from this procedure for the seasonal budget, we use the 95% confidence interval from the 20 extractions used to

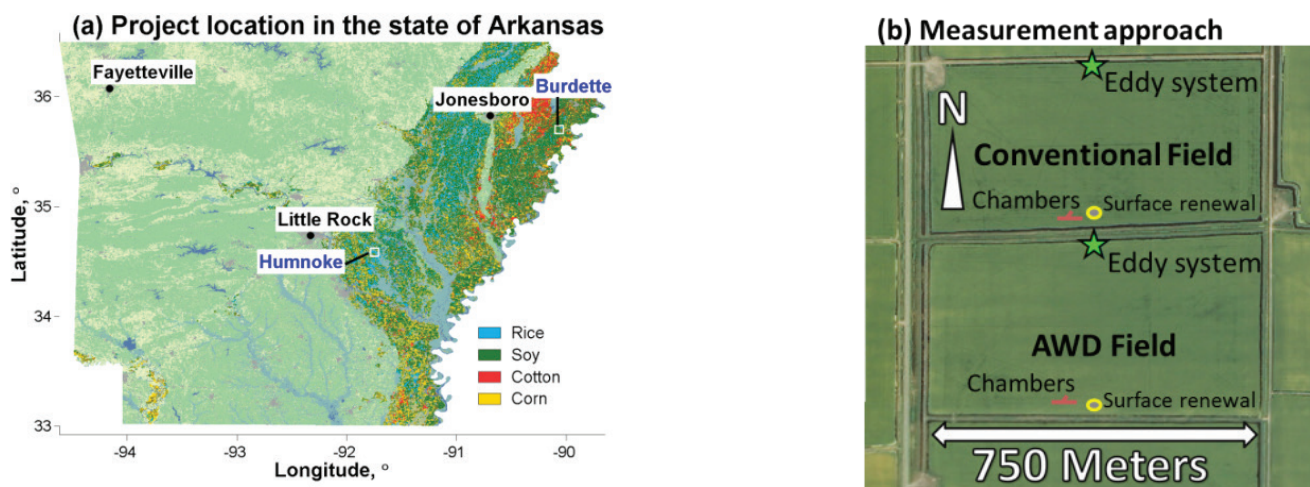


Figure 1: Two project field locations in Humnoke and Burdette, Arkansas, mapped upon a 2013 crop cover dataset (Han et al., 2014) with selected crops in legend. (b) Representative paired field site (Humnoke, AR farm) with measurement sites for the eddy covariance system (which includes soil and biometeorological measurements, closed chambers, and surface renewal system indicated).

fill each gap. The ANN model for ET was created with explanatory variables including decimal day since the start of the study period, leaf area index (LAI) and plant height interpolated using growing degree day, the friction velocity  $u^*$ , air temperature, incoming solar radiation ( $R_g$ ), vapor pressure deficit (VPD), and water table depth. The model also included representations of seasonality (spring, summer, and autumn) and the time of day (morning, afternoon, evening, and night), following the method of Papale and Valentini (2003).

Using observations of ET, meteorology, and assumptions about the roughness length and aerodynamic conductance, the Penman-Monteith equation can be inverted to estimate the canopy conductance  $g_c$ . The model is inverted to create estimates of  $g_c$  based on measured ET. This approach was previously used by the PI to determine canopy controls on ET in a Russian wetland (Runkle et al., 2014). The canopy conductance term is assessed during wet periods for both fields under the hypothesis that it should behave very similarly between fields under similar conditions. In the future, using the photosynthesis estimates derived from the simultaneous  $CO_2$  flux measurements could enable a partition of ET into plant-controlled (transpiration) and water or soil controlled (evaporation) components. During dry down periods the hypothesis is that canopy conductance will become an increasingly important control on ET rates. The transpiration portion of ET should also increase during these periods even if the overall ET rate is similar to wetter periods. The dual crop coefficient method requires biometeorological and phenological inputs in order to calculate two separate crop coefficients used to convert reference evapotranspiration ( $ET_{ref}$ ) into transpiration and evaporation:

$$ET = (K_{trans.} + K_{evap.}) * ET_{ref}$$

where the part modified by  $K_{trans.}$  is the estimated transpiration and the part modified by  $K_{evap.}$  is the estimated evaporation. Each coefficient was calculated separately using guidelines presented in FAO56, including recommendations and considerations for different crops, management practices, and climate. These coefficients are also adjusted for the higher relatively humid conditions present in the US Mid-South. The reference evapotranspiration rate was calculated using methods also outlined in FAO56 as part of the Penman-Monteith method for calculating reference evapotranspiration.

### Site description

Two privately farmed, adjacent rice fields (34° 35' 8.58" N, 91° 44' 51.07" W) located just outside of Humnoke, Arkansas, were used for this research. Each field is approximately 350m wide from north to south and 750m long from east to west (i.e., 26 ha). One field was managed with continuous flooding (CF) during the rice growing season and the other with AWD management practice, facilitating a direct comparison of the two types of systems with minimal

spatial separation. Both sites have been zero-graded and thus have approximately 0% slopes. Although only about 12.3% of total rice in Arkansas is grown on zero-graded land, this practice is growing due to the potential to save water in the fields (Hardke, 2015), to serve as a carbon-offset credit option (ACR, 2014) and to receive credit in the Natural Resources Conservation Service's Environmental Quality Incentives Program (EQIP). The sites are not tilled and are flooded for two months in winter for duck habitat and hunting. The dominant soil mapping unit in this area is a poorly-drained Perry silty clay. In 2015, the fields were drill-seed planted April 7 (AWD) and April 8 (CF), given an irrigation flush on May 3 (CF) and May 4 (AWD), and given a permanent flood on May 16 (CF) and May 18 (AWD). The AWD field dried on June 5 and received 3 more dry periods through the summer.

## Results

### Evapotranspiration observations and partition into evaporation and transpiration

Observed ET in each field in 2015 was similar, regardless of water management (Figure 2). Even during periods when the AWD field had a water table below the surface and the CF field had a standing water table, the daily observed ET was very similar (the AWD field ET was  $1.07 \pm 0.06$  times the CF field ET,  $n=25$  observed days; alternately, when both fields had a standing water table, the slope was  $1.01 \pm 0.03$ ,  $n=63$ ). In 2015 the fields also had similar yields, though the field under AWD treatment had higher peak LAI (approx. 5 vs 4.5). The contributions of modeled evaporation and transpiration to ET – both as observed and as modeled by the FAO56 method – for the entire 2015 growing season can be viewed in Figure 3. Transpiration was the highest contributing portion in both fields, composing 73-75% of total ET. Seasonal totals for each portion as well as eddy covariance observations can be found in Table 1. With these fields the modeled ET tended to overestimate the observed and gap-filled ET. Further work is being performed to test this finding by assessing the eddy covariance data for further corrections, including transducer shadowing on the sonic anemometer (Horst et al., 2015) and other possible causes for the well-known potential under-estimation bias of eddy covariance measurements (Foken et al., 2011).

Our initial investigation of surface conductance, looking at the noon-time value as representative of canopy characteristics, indicates that both fields were similar whether the two fields were under similar, ponded-water conditions or whether the AWD field was dry and the CF field was wet. In these cases the relationship between  $g_c$  of the AWD field and  $g_c$  of the CF field had a slope of  $1.12 \pm 0.01$  ( $n=18$ ) or  $1.17 \pm 0.004$  ( $n=51$ ), respectively (data not shown). Because these relationships look so similar, we cannot yet use surface conductance as a clear indicator of flooded or dried water flux source conditions, nor use it as a clear indicator by which to partition the flux into evaporation or transpiration components.

While we observed a second rice growing season, in 2016,

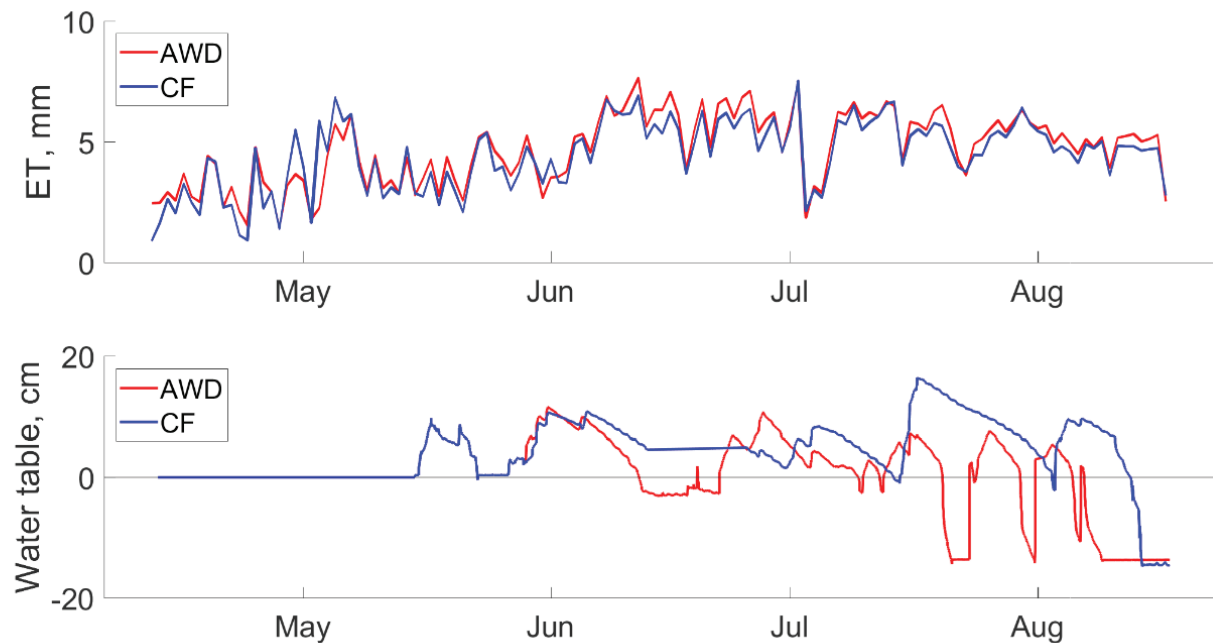


Figure 2: Daily ET estimates for both CF and AWD fields using eddy covariance, gap-filled with a neural network model, and presented with 30-min water table measurements throughout the 2015 growing season.

and expanded our efforts to include measurements near Burdette, Arkansas, those results are not yet ready for release. They are being quality-controlled and checked for accuracy, and they were delayed in part through re-coding for the transducer shadowing effect as described above. An initial look at this data suggests that the findings are consistent with the 2015 growing season. These results will be published as soon as possible and then widely shared through the AmeriFlux website.

### Network generation and project expansion

A major result of this project was an effort to generate several regional networks. Networked research sites are increasingly used to study regional land management impacts on carbon and water fluxes. However, key national networks lack contributions from the Lower Mississippi River Basin (LMRB), whose highly productive agricultural areas have potential for soil carbon sequestration through conservation practices. Therefore, we established the new Delta-Flux network to coordinate efforts to quantify carbon and water budgets and their interactions at seventeen eddy covariance flux tower sites in Arkansas, Mississippi, and Louisiana (Runkle et al., 2017). We are also working with USGS researchers to improve the water budget of the Mississippi Embayment Regional Aquifer System (MERAS) groundwater model (Clark and Hart, 2009) which is being used to provide projections on groundwater supply under various scenarios of climate and land use changes for the MAP. However, this modeling group lacks ground-based observations of ET, and we hope to integrate the MERAS model with the Delta-Flux network.

Beyond these regional networks, we also expanded our

international network to build on work funded through the USGS 104(b) project. We leveraged the 104(b) project to seek funding from the AsiaRice Foundation for a travel grant for project graduate student Colby Reavis. In January, 2017, he visited Youngryel Ryu's research group at Seoul National University in South Korea. There, he learned how to use the Breathing Earth System Simulator (BESS) product, based on remote sensing products and ecophysiological relationships and built by Ryu's group (Ryu et al., 2011; Jiang and Ryu, 2016). The visit to Korea also involved a visit to a rice research site with an eddy covariance tower and discussions about how to better parameterize and clarify the role of rice phenology as an important factor in field ET. Together the site visit and rice phenology discussion highlighted the need to take advantage of cutting edge site-monitoring tools such as drone-based imagery and solar-induced fluorescence.

### Conclusions, Recommendations and Benefits

The project findings that ET is largely composed of transpiration during the peak growing season highlight that water savings from AWD are not derived from reduced ET. They are instead derived from a mixture of reduced over-application of water, AWD's ability to capture mid-summer rainfall that would otherwise have drained off the field edge, and reductions in other end-of-field drainage and soil percolation. The ET rates of the fields in this study are very similar to modeled ET using the Penman-Monteith method. This finding lends confidence to regional modeling initiatives that they can constrain this term's uncertainties and reduce uncertainty in projections of the region's full water balance,



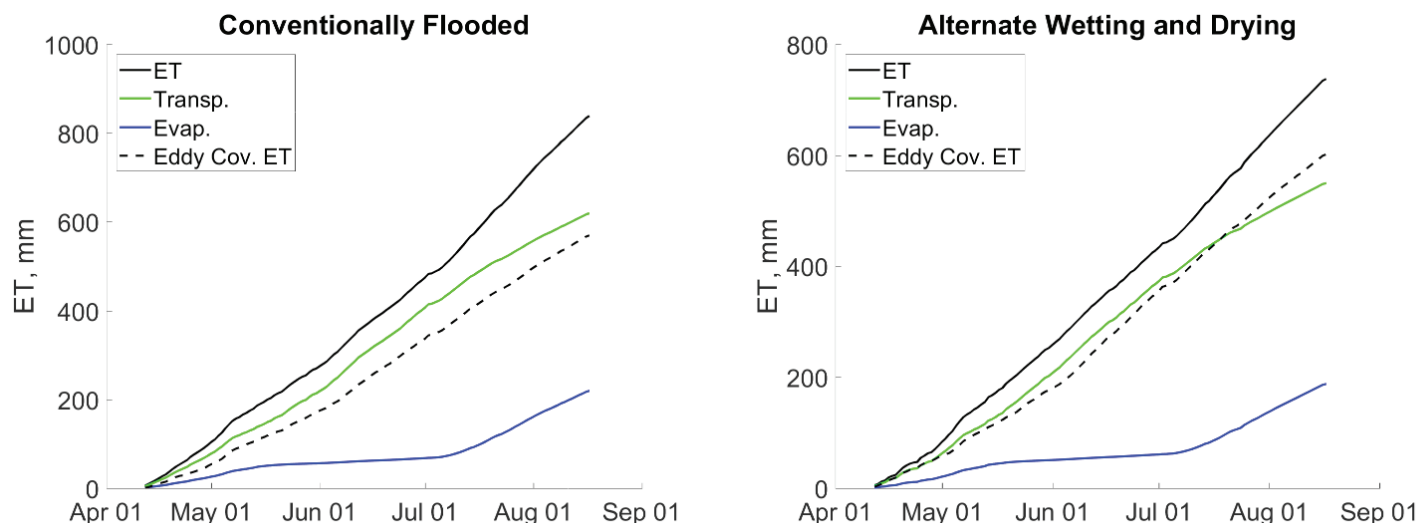


Figure 3. Cumulative transpiration (green) and evaporation (blue) for the 2015 growing season with both portions summing to total evapotranspiration (black) as predicted from the dual crop coefficient model. Eddy covariance observations (dashed) are also included for reference.

including its groundwater levels. To enhance partitioning efforts between evaporation and transpiration, we encourage more field-based techniques such as leaf photosynthesis measurements, analysis of water table fluctuations, or the use of lysimeters or isotopic methods. Coupling an analysis of ET rates with landscape CO<sub>2</sub> exchange may also prove fruitful for helping differentiate the two water flux pathways.

## Local, regional, and national benefits

Local measurements of the ET terms will help in managing water demand and irrigation scheduling. Increased knowledge of how the components of rice field evapotranspiration respond to different weather conditions will enable two types of upscaling: (1) temporally, these relationships can be used to expand and improve on models of crop water use in different future climate scenarios, (2) spatially, changes in weather patterns across the state can generate a mosaic pattern of ET. The project outcome will therefore constrain estimates of groundwater recharge, the regional meteorological energy balance, and downstream water quality. We have begun collaborating

with USGS partners on the MERAS groundwater model to contribute our ET datasets to their regional modeling initiatives. In addition to providing quantitative data on the magnitude of ET we also hope to generate locally-calibrated mechanistic relationships to place within their modeling framework.

## Acknowledgements

This material is based upon work supported by the U.S. Geological Survey under grant agreement No. G16AP00040 and administered by the Arkansas Water Resources Center. The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the opinions or policies of the U.S. Geological Survey. Mention of trade names or commercial products does not constitute endorsement by the U.S. Geological Survey.

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Table 1. Seasonal totals for each contributing portion of evapotranspiration for the 2015 growing season (April 13 to August 17) in Humnoke, Arkansas, based on the dual crop coefficient model.

	Seasonal Total, mm	
	AWD	CF
Transpiration	550	619
Evaporation	188	220
Total ET	738	839

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Image caption: View of Flint Creek in Oklahoma, courtesy of USDA Natural Resources Conservation Service.

## Stoneroller Fish (*Campostoma* spp.) Influence on Dose-Response Relationship Between Nutrients and Algae in Summer 2016 and Winter 2017

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**Abstract:** Elevated nitrogen (N) and phosphorous (P) in streams can cause nutrient pollution leading to instream and downstream problems of excess algal growth which can constrain the recreational use of streams and reduce stream biodiversity (Dodds and Welch, 2000). The United States Environmental Protection Agency (USEPA) provided national numeric nutrient criteria standards based on ecoregion, and states and tribes can adopt these criteria or develop their own standards. The objective of this project was to examine how stonerollers (*Campostoma* spp.) may modify the dose-response relationship between nutrients and algal biomass in wadeable Ozark Highland streams seasonally. Grazers tended to reduce algal biomass measured as chlorophyll *a* (chl *a*) in each stream, but most of the differences between grazer excluded and grazer present treatments were not statistically significant at  $p < 0.05$ ; grazer chl *a* effect sizes tended to be positively related to TP ( $p > 0.05$ ) and were greater in the summer compared to the winter (ANCOVA  $F = 59.85$ ,  $p = 0.0163$ ). This suggests that seasonality plays a role in stoneroller's influence on stream algae and it should be considered when examining dose-response relationships between nutrients and algae.

### Key Points:

- Increased nutrient concentrations can stimulate benthic algal biomass; grazers, like the stoneroller (*Campostoma* spp.), may dampen the effect of nutrients on benthic algal biomass,
- But grazers are often not considered when constructing nutrient-algal relationships for the development of numeric nutrient criteria.
- Grazers appeared to reduce algal biomass measured as chlorophyll *a* (chl *a*) although differences were not significant.
- Grazer chl *a* effect sizes tended to be positively related to TP ( $p > 0.05$ ) and were greater in the summer compared to the winter (ANCOVA  $F = 59.85$ ,  $p = 0.0163$ ).
- Our results suggest that nutrient and grazer effects on benthic algae can be variable and seasonal.



## Introduction

Elevated nitrogen (N) and phosphorous (P) in streams can cause excess algal growth, which can constrain the recreational use of streams and reduce stream biodiversity (Dodds and Welch, 2000). The United States Environmental Protection Agency (USEPA) provided national numeric nutrient criteria standards based on ecoregion and states and tribes can adopt these criteria or develop their own. Therefore, many states have decided to develop regional numeric nutrient criteria standards based on scientific methods, which can include assessment of algal biomass (USEPA, 2017). The Arkansas Department of Environmental Quality (ADEQ) is currently working toward federal TN and TP standards by assessing dose-response relationships between algae (chlorophyll *a* and ash-free dry mass), but does not currently have published federal total nitrogen (TN) or total phosphorus (TP) numeric nutrient criteria in accordance with the EPA (USEPA, 2017). Arkansas currently has algae narrative criteria for all water bodies and TP point source criteria for streams.

Arkansas currently has narrative standards for algae in waterbodies, according to Regulation No. 2 from the Arkansas Pollution Control and Ecology Commission (APCEC), which states that “Materials stimulating algal growth shall not be present in concentrations sufficient to cause objectionable algal densities or other nuisance aquatic vegetation or otherwise impair any designated use of the waterbody” (APCEC, 2015). The state intends to develop numeric nutrient criteria from dose-response relationships between nutrient levels and stream benthic algae; ADEQ is leading that effort. Relationships between nutrient concentrations and algae can be variable in Arkansas and Oklahoma (Stevenson et al., 2012, Haggard, 2013) since other factors in addition to nutrient concentrations can affect benthic algal concentrations. Specifically, some of the variation in the relationship between nutrients and benthic algae may be explained by macrograzer activity (Stevenson et al., 2012).

Seasonal variations in algal density and associated determining factors, such as macrograzer activity, may cause some of the variation in dose-response relationship between nutrients and benthic algal biomass. Thus, these variations in dose-response relationships should be considered when developing numeric nutrient criteria for the Ozark Highland Ecoregion. Most studies examining the relationships between grazers, algae, and nutrients have used snails and caddisflies as the study organism while less is known about the influence of algivorous fish, such as stonerollers on algal biomass responses to nutrient enrichment (Cattaneo and Mousseau, 1995). Stonerollers (*Campostoma* spp.) are minnows that occur in high abundances in Ozark streams, and possess a sub-terminal mouth that makes them well-equipped grazers. *Campostoma* spp. grazing can be an important determining factor on algal biomass and community composition (Steward, 1987; Power et al., 1988) and they are thought to be grazing most actively during the warm season since they are ectotherms. During late summer, the standing stock of algae in pools can be nearly devoid of algae biomass

due to grazing by *Campostoma* spp. (Matthews et al., 1987), but little is known about their potential to affect algal biomass in the winter. Seasonal variation in *Campostoma* spp. grazing could explain variation in algal biomass across seasons and sites in Ozark streams with varying nutrient concentrations.

The proposed study examining the seasonality of *Campostoma* spp. effects on benthic algae across streams with a gradient of total phosphorus concentrations can help the state understand how and why seasonality may result in variation in the relationship between nutrients and algae. The objective of this project is to examine how stonerollers (*Campostoma* spp.) may modify the dose-response relationship between nutrients and algal biomass in Wadeable Ozark Highland streams seasonally. We hypothesized that stonerollers would have a significant negative effect on benthic algae within each stream during the summer (hypothesis 1; H1). Our second hypothesis was that stoneroller effects on algae would increase with total phosphorus (TP; hypothesis 2; H2). Finally, we expected that the stoneroller effect would be greater in the summer than the winter due to greater activity at greater stream temperatures (hypothesis 3; H3).

## Methods

Our experiment was conducted in five Ozark Highland Wadeable streams during the summer of 2016 (18 July- 3 October) and three streams during the winter of 2017 (24 January-6 March). Sites with a gradient of TP were selected (Table 1). Three blocks were set up in runs in the upper, middle, and lower sections of each stream reach (reach  $\geq 200\text{m}$ ) where each block was separated by at least one pool. Each block consisted of one treatment enclosure (stoneroller excluded) and one unelectrified control enclosure (stoneroller present) that were set up side-by side in equal flow conditions. Four unglazed tiles ( $121\text{cm}^2$ ) were zip-tied into each quadrat enclosure (31 X 5cm built from 19mm polyvinyl chloride pipe) to measure benthic algae. Treatment enclosures were set up with a 12 gauge insulated copper wire surrounding tiles and connected to a six volt ParMak solar fence charger (ParMak Precision Kansas City, MO) that sent an electrical pulse into the water deterring large-bodied organisms ( $> 1\text{cm}$ ) which exclude most crayfish and fish (Pringle and Blake, 1994). The charge extends about 10 cm outside the quadrat (Ludlam and Magoulick, 2009). Tiles were inoculated for 14 days in treatment and control conditions before they were collected on days 14, 21, and 28 in the summer and 14, 21, 28, and 35 in the winter. Algae was then measured for chlorophyll *a*, and ash-free dry mass (AFDM) was calculated using slurry from the whole tile. Water samples were taken throughout the experiment at each stream bi-weekly, placed in an iced cooler, and frozen upon returning to the laboratory to measure total phosphorus (TP) and total nitrogen (TN). Total phosphorus was measured in water samples by using a persulfate digestion and colorimetric analysis using the ascorbic acid method (American Public Health Association, 2005). Total

Table 1: Study information and nutrient concentrations, as measured on day 28 of the study in summer of 2016 (Sept 27-Oct 3) and winter of 2017 (15-16 February). Land use data from King et al., 2016. An asterisk (\*) denotes values that were below the detection limit.

Stream	State	Watershed	Summer		Winter		Land use
			TP (mg/L)	TN (mg/L)	TP (mg/L)	TN (mg/L)	
Saline	OK	Eucha	0*	4.2	0*	0.7	60% Forest, 26% Pasture, 8% Grassland
Evansville	OK	Illinois	0.009	2.1	---	---	52% Forest, 40% Pasture, 3% Grassland
Beaty	OK	Eucha	0.027	1.9	0.029	1.7	30% Forest, 61% Pasture, 2% Grassland
Baron Fork	OK	Illinois	0.047	3.7	---	---	45% Forest, 48% Pasture, 2% Grassland
Flint	OK	Eucha	0.06	1.2	0.049	7.3	28% Forest, 58% Pasture, 3% Grassland

nitrogen was measured in water by using a sodium hydroxide digest to convert all nitrogen forms to nitrate and colorimetric (Hach DR 3900) analysis using Hach reagent powder pillows (Hach Permachem® Regant NitroVer© 5 nitrate reagent).

Statistical analysis was conducted in a hierarchical manner to understand the influence of grazers within each stream (H1), nutrients among streams (H2), and season among streams (H3). We addressed the grazing effect on benthic algal chlorophyll *a* and AFDM collected on day 28 within each stream during the summer and winter using a randomized-block analysis of variance (RB-ANOVA). Assumptions of variance, covariance, and normality were assessed visually using histograms and box plots. Interactions between environment and experiment were visually assessed using a line graph. The mean effect size was calculated per stream by averaging the effect size from each block (treatment: control, Grazer-excluded:Grazer-present) to address our second hypothesis. The mean effect size was regressed against nutrient concentrations (TP) to determine whether the grazer effect on benthic algae depended upon stream nutrient concentrations for the summer using all five stream reaches. Assumptions of normality and homogeneity of variance were assessed visually. Last, analysis of covariance (ANCOVA) was used on streams sampled in both winter and summer (Beaty, Saline, and Flint) to understand how effect of stonerollers differs between the two seasons. In the ANCOVA, the mean effect size (ratio Grazer-excluded:Grazer-present chlorophyll *a* and AFDM for each block averaged per stream) was the dependent variable, nutrient concentrations were the independent variable, and season was the covariate. Assumptions of linearity, homogeneity of variance, and relationship dependent and independent variable were assessed.

## Results

As expected, stream TP ranged from below detection to 0.06 mg/L (Table 1). The TN concentration was high at all sites and varied less than TP. Grazers reduced benthic chlorophyll *a* in Saline and Beaty Creek in the summer (Table 2; Figure 1), but not in the winter (Table 3; Figure 2; H1). Grazers reduced

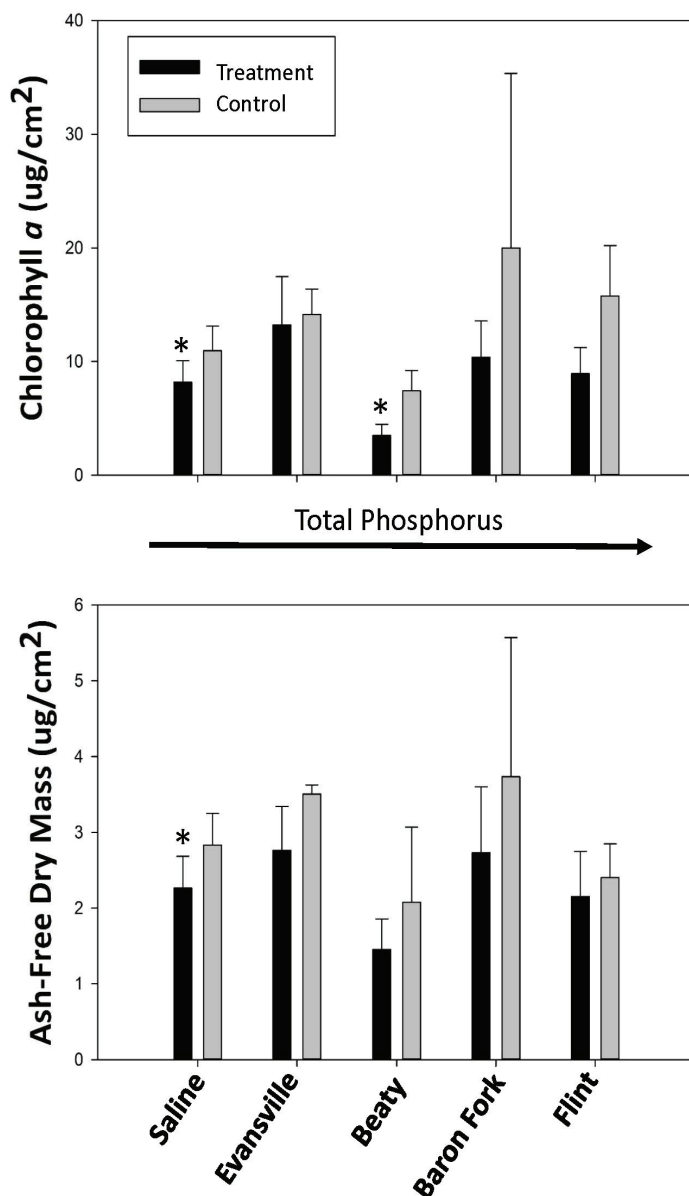


Figure 1: Algae collected from tiles on day 28 in late-summer of 2016, was measured for chlorophyll *a* and ash-free dry mass (AFDM, ug/cm<sup>2</sup>) values under treatment and control conditions. Mean and standard error (SE) were calculated for each stream (n=3). Significant differences are indicated with an asterisk (\*).

Table 2: A Randomized block analysis of variance (ANOVA) was run on each stream to understand the influence on algae that was under grazer excluded or grazer present condition. There was a treatment effect in Saline creek on both chlorophyll *a* and ash-free dry mass (AFDM). Beaty Creek also had a significant treatment effect but only for chlorophyll *a*.

Stream	Variable	Factor	df	F-value	P-value
Saline	Chlorophyll <i>a</i>	Treatment	2	6.497	0.056*
		Block	2	20.952	0.006*
	AFDM	Treatment	2	7.003	0.049*
		Block	2	26.47	0.004*
	Chlorophyll <i>a</i>	Treatment	2	0.75	0.529
		Block	2	0.874	0.484
Evansville	AFDM	Treatment	2	2.668	0.184
		Block	2	0.55	0.615
	Chlorophyll <i>a</i>	Treatment	2	0.885	0.481
		Block	2	2.126	0.235
	AFDM	Treatment	2	0.947	0.461
		Block	2	1.786	0.279
Beaty	Chlorophyll <i>a</i>	Treatment	2	11.545	0.022*
		Block	2	7.365	0.046*
	AFDM	Treatment	2	0.287	0.765
		Block	2	1.404	0.345
	Chlorophyll <i>a</i>	Treatment	2	1.836	0.272
		Block	2	0.017	0.983
Flint	AFDM	Treatment	2	1.012	0.441
		Block	2	0.107	0.901

Table 3: Five streams were sampled on day 28 in winter 2017 (Feb 15-16).

A Randomized block analysis of variance (ANOVA) was run on each stream understand the influence on algae that was under grazer excluded or grazer present conditions. There was no treatment or block effects.

Stream	Variable	Factor	df	F-value	P-value
Saline	Chlorophyll <i>a</i>	Treatment	2	1.98	0.252
		Block	2	0.33	0.735
	AFDM	Treatment	2	1.05	0.429
		Block	2	0.4	0.695
Beaty	Chlorophyll <i>a</i>	Treatment	2	2.96	0.234
		Block	2	3.85	0.117
	AFDM	Treatment	2	2.14	0.234
		Block	2	0.08	0.921
Flint	Chlorophyll <i>a</i>	Treatment	2	0.42	0.681
		Block	2	0.68	0.555
	AFDM	Treatment	2	0.88	0.482
		Block	2	0.22	0.81

tors. Therefore, all six chlorophyll *a* effect sizes were combined into one regression, which was not statistically significant.

### Conclusions, Recommendations and Benefits

Many studies have shown negative effects of stream grazers on benthic algae (Matthews et al., 1987; Steward, 1987; Power et al., 1988). Although grazer-exlosures tended to have greater benthic algal biomass than grazer-present treatments in the present study, these differences were only statistically significant in two streams with low to moderate TP concentrations during the summer (Table 2; Figure 1). A large amount of variation was observed in response variables across sites and increasing the number of replicates would help improve the power to address the interactive effects of grazers and nutrients on benthic algal biomass (Figures 1 and 2). Additionally, electrical exclosures did not exclude smaller macroinvertebrate grazers, like snails, that can negatively affect benthic algal biomass (Steinman et al., 1996). The electrical treatment should not have affected their presence, but the abundance and biomass of smaller benthic macroinvertebrates were not measured in this study and they could have added to the variability in effect sizes.

Our results suggest that macrograzers, such as *Camptostoma* spp., can be more active and effective at grazing in the summer relative to the winter. The mean and variation in grazer chlorophyll *a* effect sizes tended to increase with TP concentrations in the summer, but not in the winter season (Figures 3 and 4). In addition, the mean grazer chlorophyll *a* effect size was greater in the summer than in the winter. *Camptostoma* spp. were not seen during winter months except on a few occasions when the temperature was high in sunny runs. Other studies in Ozark streams suggest that Cam-

benthic AFDM in the summer in Saline Creek only (Table 1; Figure 1). There was no statistically significant difference between treatment and control for either chlorophyll *a* or AFDM in any stream during the winter (Figure 2). *Camptostoma* spp. abundance was measured in summer 2015, but we found that our abundance measurements did not influence the relationship between chlorophyll *a* and TP in this study (104b-Sayre and Evans-White 2016), and this data does not correlate with effect size for data taken in summer 2016 ( $p=0.82$ ).

Chlorophyll *a* effect size and stream TP appeared to have a positive trend in the summer when all five study streams were included, but this trend was not statistically significant (Figure 3). However, there was no relationship between AFDM effect size and stream TP in the summer (Figure 3). The ANCOVA that included the three study sites sampled in both the summer and winter found no interaction between season and TP for either chlorophyll *a* or AFDM (Table 4; Figure 4). There was a season and a TP main effect for chlorophyll *a* (Table 4; Figure 4), but no interaction between those fac-



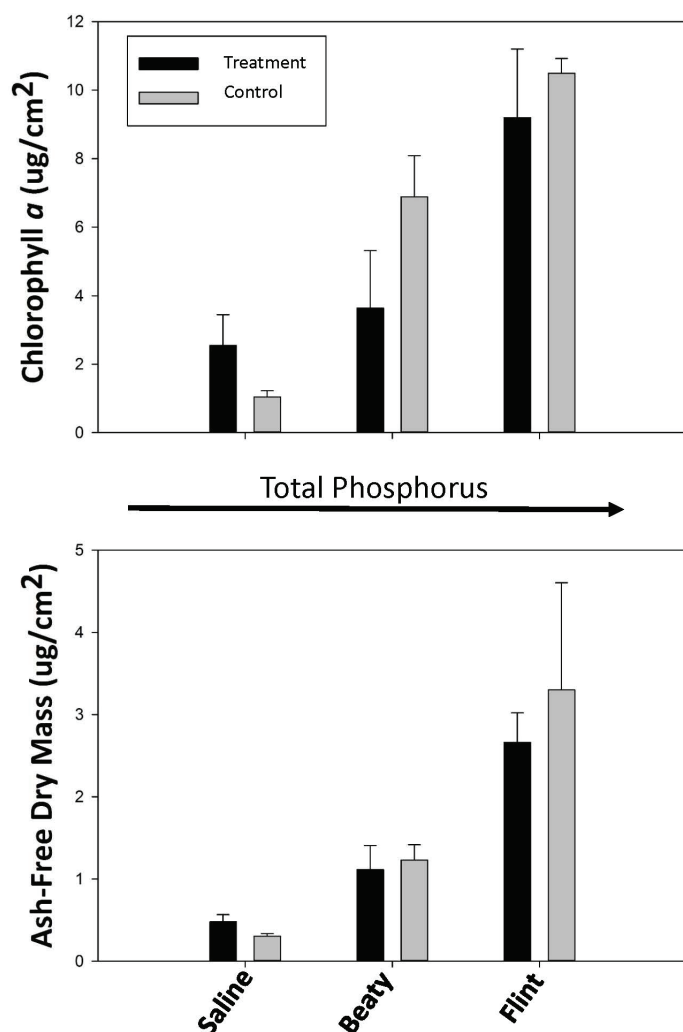


Figure 2: Algae collected from tiles on day 28 in winter of 2017, was measured for chlorophyll *a* and ash-free dry mass (AFDM, g/cm<sup>2</sup>) under treatment and control conditions. Mean and standard error (SE) were calculated for each stream (n=3). \*RB-ANOVA indicated no statistically significant influence of grazer-exclusion for chlorophyll *a* or AFDM.

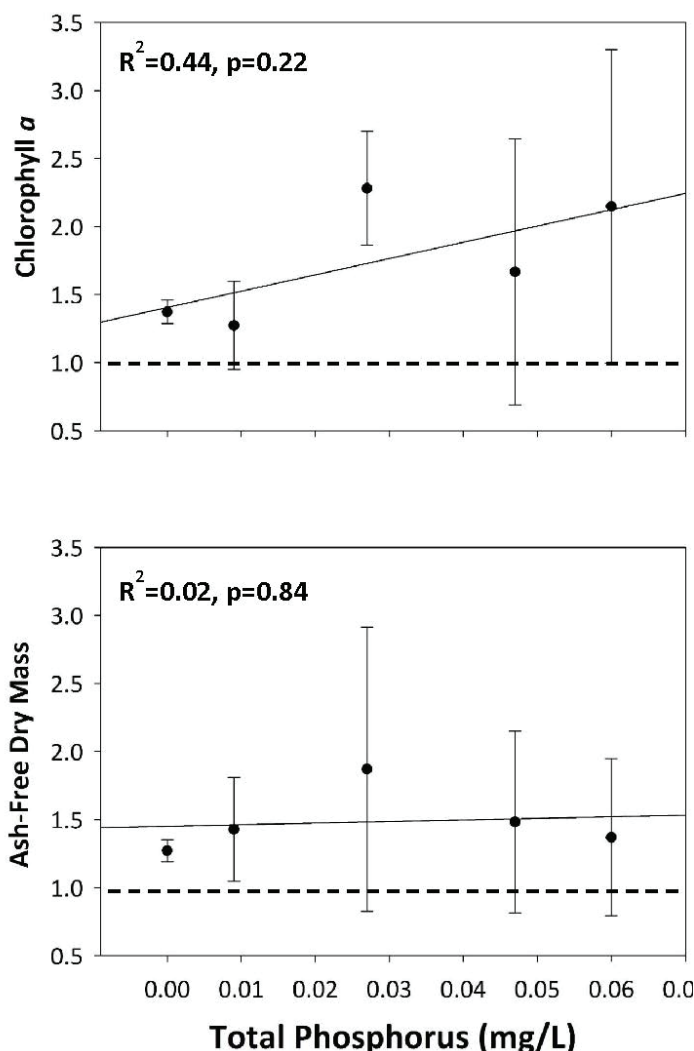


Figure 3: Mean effect size for algae collected from tiles on day 28 in late-summer of 2016, measured for chlorophyll *a* and ash-free dry mass (AFDM, ug/cm<sup>2</sup>) values under treatment and control (grazer-excluded and grazed) conditions. Bars represent the standard error of the effect size, but are not used in calculating regression statistics. The dashed-line indicates the 1:1 ratio at which treatment is equal to control where grazers do not have an influence.

postoma spp. influence can vary spatially and temporally within a single stream (Ludlam and Magoulick, 2009). The influence of grazers in these Ozark streams can depend on the presence of predators, stream conditions (e.g. drying), and depth (Ludlam and Magoulick, 2009) and our study suggests that their effects may also vary across nutrient levels. Grazer chlorophyll *a* and AFDM effect sizes were always greater than one suggesting that grazers tended to reduce benthic algal biomass across the stream TP gradient in the present study. A prior study that manipulated *Campostoma* and streamwater P levels in experimental streams found that stonerollers may stimulate benthic algal chlorophyll *a*, reduce benthic AFDM, and increase the autotrophic index even under P enriched conditions (Taylor et al., 2012). Taylor et al. (2012) focused on grazing effects in pools, and included a greater P enrichment up to 0.1 mg/L, and was completed in outdoor experimental streams in the early spring (March-April). All of these factors could result in the differences

observed between these two studies and future experiments could manipulate temperature as well as nutrient concentrations in experimental streams to get at relative effects.

Dodds et al. (1997) proposed an oligotrophic-mesotrophic boundary at 2.0  $\mu\text{g}/\text{cm}^2$ , and a mesotrophic-eutrophic boundary at 7.0  $\mu\text{g}/\text{cm}^2$  of chlorophyll *a*. Chlorophyll *a* measurements in the present study indicate that all streams were within the oligotrophic to mesotrophic range during the summer months. However, Flint became eutrophic in the winter, with Beaty on the border of eutrophic (Dodds et al., 1997). Therefore, adding in-stream manipulations in reaches with greater TP and benthic algal biomass would improve our understanding of the effects of grazers across nutrient gradients.

Overall, our data suggest the importance of seasonality with respect to macrograzer resource acquisition, macrograzer

Table 4: Three streams were sampled in both summer of 2016 and winter of 2017. Analysis of covariance (ANCOVA) was run where total phosphorus (TP) is the predictor, effect size chlorophyll *a* and ash-free dry mass (AFDM) was the response variables, and season (winter and summer) is the covariate. \* Chlorophyll *a* effect size was significant for both TP and season. There was no interaction between TP and season.

Response	Predictor	df	F-Value	P-value
Effect Size Chlorophyll <i>a</i>	Total Phosphorus	1	47.84	0.0203*
	Season	1	59.85	0.0163*
	TP x Season	1	2.03	0.295
Effect Size AFDM	Total Phosphorus	1	0.1	0.101
	Season	1	0.26	0.258
	TP x Season	1	0.08	0.077
	Residuals	2	0.33	0.164

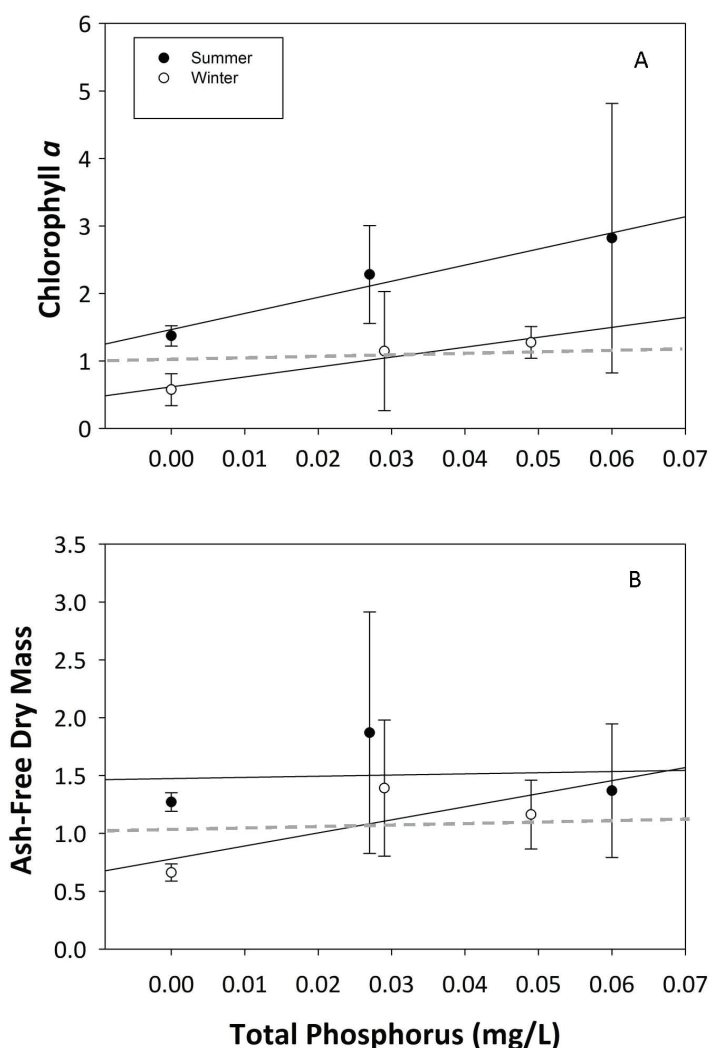


Figure 4: Mean effect size for algae collected from tiles on day 28 (27 September-3 October) in winter of 2017, measured for chlorophyll *a* and ash-free dry mass (AFDM, ug/cm<sup>2</sup>) values under treatment and control (grazer-excluded and grazed) conditions. Bars represent the standard error of the effect size, but are not used in calculating regression statistics. The dashed-line indicates the 1:1 ratio at which treatment is equal to control where grazers do not have an influence. There was a season and a TP main effect for chlorophyll *a*, but not for AFDM.

effect size, and dose-response relationship between nutrients and algae. A prior study in the Illinois River basin found that nutrients explained more variation in benthic algal biomass in the spring compared to the summer (Stevenson, 2012). The present study suggests that grazer effects are also lower in winter season and they may play a role in the observed relationship between nutrients and benthic algae. This seasonality effect on grazer influence should be considered when developing nutrient-algal dose response relationships and developing numeric nutrient criteria for the Ozark Highlands Ecoregion.

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Image caption: Stonefly nymph, an aquatic insect that is sensitive to changes in water quality.

## Biological and Ecological Consequences of Sub-Lethal Ion Concentrations on Microbial and Macroinvertebrate Detritivores

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**Abstract:** Freshwater detritivores are essential to stream productivity, carbon cycling, and subsidies to terrestrial systems. Gradual low-level, sub-lethal increases in ion concentrations such as sodium (Na), chloride (Cl), and bicarbonate ( $\text{HCO}_3^-$ ) are common, but their impacts on freshwater detritivores and stream processes are not well understood. However, these ions may impact leaf litter decomposition in various ways. We tested each of the pathways in stream mesocosms by amending water with one of 3 NaCl and 3  $\text{NaHCO}_3$  treatments: natural (from a local stream), low ( $16 \text{ mgL}^{-1}$  Na added), medium ( $32 \text{ mgL}^{-1}$  Na added), and high ( $64 \text{ mgL}^{-1}$  Na added) and measuring stonefly growth, respiration, and consumption, and fungi and algal growth over 8 weeks. Similarly, we measured the same variables for isopods that were raised in stream water but fed leaf discs amended with Na as above. Salt treatments had little effect on microbial-mediated leaf litter decomposition and the associated fungal and algal community; however, microbial respiration tended to be elevated on the leaves incubated in  $\text{NaHCO}_3$  throughout the 134-day study with the lowest  $\text{NaHCO}_3$  concentration having the greatest stimulatory effect. Further, algal growth also showed a pattern of increase from  $\text{HCO}_3^-$  that may have been an added food resource for macroinvertebrate detritivores in the previous studies but these changes in microbial activity did not change decomposition rates. The stonefly *Amphinemura* increased in biomass and respired more in Na- (both Cl and  $\text{HCO}_3^-$ ) amended water without increased leaf consumption. Na-incubated leaf discs resulted in decreased isopod *Lirceus* growth relative to stream water with little change in respiration and leaf consumption in Na-amended treatments. Together, these results demonstrate that low-level, non-lethal NaCl impacts detritivores both directly and indirectly even at concentrations that are near the chloride reference values for different ecoregions in Arkansas Regulation 2 (ranges from 6 to  $36 \text{ mgL}^{-1}$  depending on the ecoregion). Other ions, like  $\text{HCO}_3^-$ , have a similar effect on detritivores but are not currently considered in State regulations despite their prevalence in the environment from waste water.

### Key Points:

- Anthropogenic activities can cause subtle increases in ion concentrations in freshwaters of Arkansas.
- Sub-lethal increases in ions can cause stress in organisms due to challenges regulating water and salt balances.
- Sub-lethal increases in NaCl and  $\text{NaHCO}_3$  can affect microbial activity, leaf litter quality, and carbon cycling in detrital streams.
- A better understanding of sub-lethal ion concentrations is important when considering water quality standards.

## Introduction

Ion increases in Arkansas streams are from a combination of agriculture, wastewater effluent and development associated with urbanization and resource extraction (Griffith, 2014; Musto, 2013). Small amounts of Na and Cl are essential for animals, bacteria, and fungi to maintain hormone signaling pathways, generate electrical cell potentials and regulate bodily fluids (Kaspari et al., 2009). However, increased Na and Cl concentrations have the potential to alter rates of leaf litter decomposition and subsequent carbon cycling in streams by three pathways: 1) directly altering heterotrophic fungi and bacteria consumption, respiration, and growth that colonize and decompose leaf litter from osmoregulatory changes, 2) directly altering macroinvertebrate detritivore consumption and respiration from osmoregulatory changes or 3) indirectly altering macroinvertebrate detritivore feeding rates via changes in litter quality. Greater fungal and bacterial biomass increases the nutritional value of detritus for macroinvertebrate detritivores and typically results in increased leaf litter decomposition rates. Macro-detritivores both, directly and indirectly, increase leaf litter decomposition rates via leaf consumption and by increasing surface area for microbial colonization. Thus, changes in stream ions can have large impacts on freshwater ecosystems through these direct and indirect effects on detrital processing.

Sodium and chloride ions play a key role in osmoregulatory processes of freshwater organisms, and ion imbalances between organisms and their environment can negatively impact freshwater organisms and ecosystems through increased energy expenditure to maintain osmotic balance. Arkansas streams and rivers have among the lowest natural ion concentrations in the U.S. (Griffith, 2014). However, our past studies have documented small, but increased ion concentrations from sodium (Na: 0.7-7.0 mgL<sup>-1</sup>) and chloride (Cl: 0.8-21.2 mgL<sup>-1</sup>) in 20 Wadeable streams. Additionally, the Arkansas Department of Environmental Quality (ADEQ) has measured a range in Cl concentrations from 0.4 to over 150 mgL<sup>-1</sup> in Arkansas Valley streams (ADEQ database accessed 27Oct15). Sodium bicarbonate (NaHCO<sub>3</sub>) has also increased in streams in the Illinois River Basin (Scott et al., 2016). Our study will inform ecological impacts of rising ions that are below documented toxicity levels but are 1) below-, 2) near- and 3) more than- state-set chloride concentrations and quality standards detailed in Arkansas State Regulation 2 (as low as 6 mgL<sup>-1</sup> depending on the site and ecoregion; APCEC 2014). We aim to investigate how detrital organisms and their associated processes change in response to sub-lethal increases in common ions; specifically, Na, Cl and bicarbonate (HCO<sub>3</sub>). Changes in litter processing rates in combination with altered detritivore growth will support stream ecosystem responses to modified surface water quality.

## Methods

### **Experiment 1 (micro-detritivores):**

We tested low-level NaCl and NaHCO<sub>3</sub> additions on heterotrophic fungal biomass on leaf litter. First, sweet gum

leaves were cut into standard-sized discs, leached, and incubated in one of 3 NaCl and 3 NaHCO<sub>3</sub> treatments: natural (from a local stream), low (16 mgL<sup>-1</sup> Na added), medium (32mgL<sup>-1</sup> Na added), and high (64 mgL<sup>-1</sup> Na added). Each salt treatment was represented by 10 growth chambers, and each chamber had 10 leaf discs (N=70). Conductivity and total dissolved solids increase with mineral concentrations and they were measured and interpreted along with effects from salt additions. Chambers were aerated each day to prevent low oxygen conditions and kept in a greenhouse for normal day-night cycles. Leaf discs were incubated for about 4.5 months to allow for possible microbial adaptation. Respiration was measured at the end of weeks 1, 4, 7, 10, 13, 17, and 19 following at least 2 hours of dark incubation using a Membrane Inlet Mass Spectrophotometer (MIMS; Halvorson et al., 2016). Fungal biomass was measured by solid-phase extraction (SPE) of ergosterol followed by high pressure liquid chromatography (HPLC) (Gessner, 2005). Leaf mass was measured before and after the experiment to estimate amount remaining. Finally, chlorophyll a was estimated after observing growth on leaf discs late in the experiment using ethanol extraction and standard spectrophotometric methods (Steinman, 1996).

### **Experiment 2 (macro-detritivore exposed to salts and fed naturally conditioned leaves):**

We tested if experimental addition of salts reduce macro-detritivore growth and litter consumption from an increase in osmoregulatory stress. We used the same salt concentrations as in experiment 1. The common macro-detritivore, *Amphipemura*, was collected from a local stream that has low stream water conductivity (<50 µS cm<sup>-1</sup>), sorted into size class to the nearest 2 mm, weighed, and placed in one of two salt types and one of the 4 treatments (natural, low, medium, and high). The detritivores were placed in their own growth chamber (10 chambers per treatment; 2 salt types x 3 concentrations +1 stream water x 10 growth chambers, N=70) and fed microbial conditioned leaf litter incubated for 30 days in natural stream water. Leaf discs were replaced each week after 7 days to estimate consumption and to prevent starvation. Detritivores were weighed at the end of 4 weeks. Macro-detritivore growth was expressed as (final-initial mass)/final mass\*100. Initial leaf mass was measured from subsampled leaf discs and final leaf mass was measured after the 7-day exposure to detritivores upon experiment termination. Leaf disc respiration and fungal biomass were measured as described in experiment 1.

### **Experiment 3 (macro-detritivore not exposed to salts but fed salt-incubated leaf discs):**

We measured the effects of long-term, low-level salt additions used in the other two experiments on litter quality and macro-detritivore growth. First, we used the same common macro-detritivore, *Amphipemura*, as in experiment 2, collected from a local stream, separated by size class and placed in natural stream water with no added salts. Unfortunately,



because of an unusually warm winter, the stoneflies emerged after a week into the experiment. We set-up a second experiment with the Isopod, *Lirceus*. The detritivores were then fed sweet gum discs from one of the above 2 salts and 3 salt concentrations after a 30-day incubation period. Detritivores were separated by size class as above and randomly placed in one chamber. Experimental design was as above except 5 isopods were placed in each chamber and their average growth was used as the unit of replication (2 salt types x 3 concentrations + 1 stream water x 10 growth chambers, N=70). A sub-sample of detritivores that did not get placed in chambers were dried and weighed and their size class was recorded. Final detritivore dry mass was measured for all individuals. Macro-detritivore growth was measured as (final-initial mass)/final mass\*100. Leaf mass lost was measured using the same methods as above.

### Statistical Analysis

We used one-way analysis of variance to compare salt treatments effects on response variables (e.g. growth, biomass, leaf mass loss) for each of the proposed experiments and Student's *t* post-hoc pairwise comparison if main model  $\alpha \leq 0.05$ . Repeated measures ANOVA was used to test differences in leaf disc respiration with a Tukey's honest significance test. If data did not follow parametric assumptions, then Wilcoxon test was used with a follow-up Wilcoxon each pair post-hoc test when  $\alpha \leq 0.05$ .

## Results

### Experiment 1 (micro-detritivore; Figures 1-4 & Tables 1-4).

Overall, salt treatments had little effect on leaf litter decomposition and the associated fungal and algal community; however, respiration tended to be greater on the leaves incubated in  $\text{NaHCO}_3$  throughout the 134-day study with the lowest  $\text{NaHCO}_3$  concentration having the greatest stimulatory effect. Both salt treatment and time had significant main effects on microbial respiration ( $p < 0.001, 0.013$ ), but did not interact ( $p > 0.005$ , Table 1). Salt treatment appeared to be the primary

driver of microbial respiration and respiration varied across time (Figure 1). During week 1, low  $\text{NaHCO}_3$  and NaCl treatments elicited greater respiration than moderate and high  $\text{NaHCO}_3$  and high NaCl treatments on discs compared to stream water (SW). Low NaCl also resulted in significantly greater respiration than moderate NaCl on leaf discs. During week 19, low and moderate  $\text{NaHCO}_3$  elicited a significantly greater respiration response than SW, high  $\text{NaHCO}_3$ , and all NaCl treatments; low  $\text{NaHCO}_3$  respiration was significantly greater than moderate  $\text{NaHCO}_3$ . Despite differences in respiration, there were no statistically significant differences in dry mass remaining across salt treatments (Table 2). However, percent dry mass remaining in  $\text{NaHCO}_3$  treatments tended to be greater than in SW and peaked at the medium  $\text{NaHCO}_3$ , suggesting the least amount of microbial activity (Figure 2). Fungal biomass did not differ statistically across treatments either (Table 3), but tended to increase with salt concentrations where it peaked in medium salt treatments and then decreased below fungal biomass on leaves incubated in SW (Figure 3). Algal biomass also did not differ across treatments statistically (Table 4) but NaCl treatments tended to have lower algal biomass than SW (Figure 4). Leaf discs incubated in  $\text{NaHCO}_3$  treatments showed a pattern of increasing algal biomass where it was most variable at the

Figure 1. Mean microbial respiration expressed per unit dry mass over time. Salt treatments were: SW-3=ambient stream water (3mg/L Na);  $\text{HCO}_3$ -16,-32,-64=low, moderate, and high  $\text{NaHCO}_3$  treatments (16, 32, 64mg/L, respectively); Cl-16,-32,-64=low, moderate, and high NaCl treatments (16,32,64mg/L, respectively). Both salt treatment and time had significant main effects on microbial respiration ( $p < 0.001, 0.013$ ), but did not interact ( $p > 0.005$ ). Salt treatment appeared to be the primary driver of microbial respiration responses and respiration varied across time.

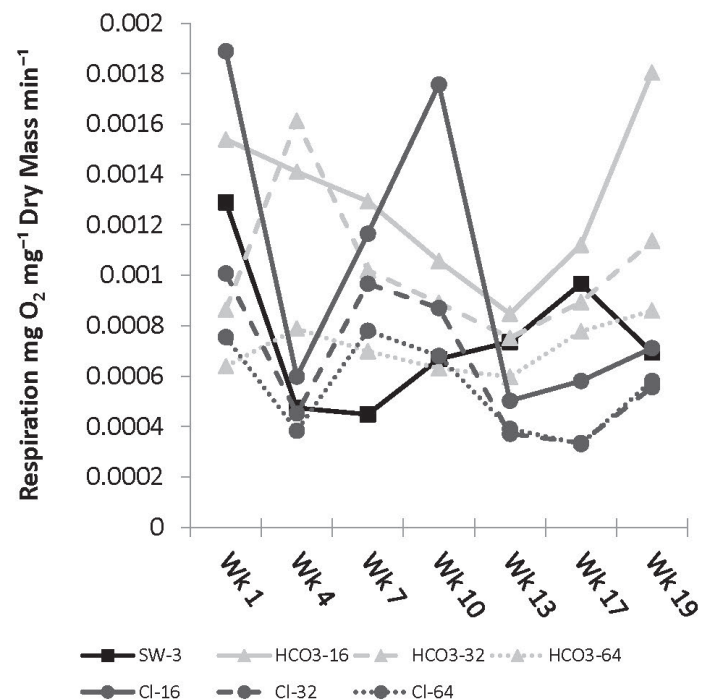


Table 1. One-way repeated measures ANOVA ( $\alpha = 0.05$ ) output for microbial respiration across time. Salt factor includes 7 levels: filtered stream water at ambient salinity (3 mg/L Na); filtered stream water amended to low, medium, and high sodium bicarbonate concentrations (16, 32, and 64 mg/L Na); and filtered stream water amended to low, medium, and high sodium chloride concentrations (16, 32, and 64 mg/L Na). Repeated measures were carried out on weeks 1, 4, 7, 10, 13, 17, and 19.

	Factor	df	F	p
Dry Mass	Salt	6	6.299	<b>&lt;0.001</b>
	Time	6	2.738	<b>0.013</b>
	Salt*Time	36	1.159	0.247
AFDM	Salt	6	2.973	<b>0.007</b>
	Time	6	1.901	0.079
	Salt*Time	36	0.717	0.889



Table 2. One-way ANOVA ( $\alpha=0.05$ ) output for % leaf litter remaining at termination (week 19, day 134).

	df	F	p
Dry Mass	6	1.577	0.169
AFDM	6	0.389	0.884

Table 3. One-way ANOVA ( $\alpha=0.05$ ) output for fungal biomass at termination (week 19, day 134).

	df	F	p
Dry Mass	6	0.517	0.793
AFDM	6	1.115	0.364

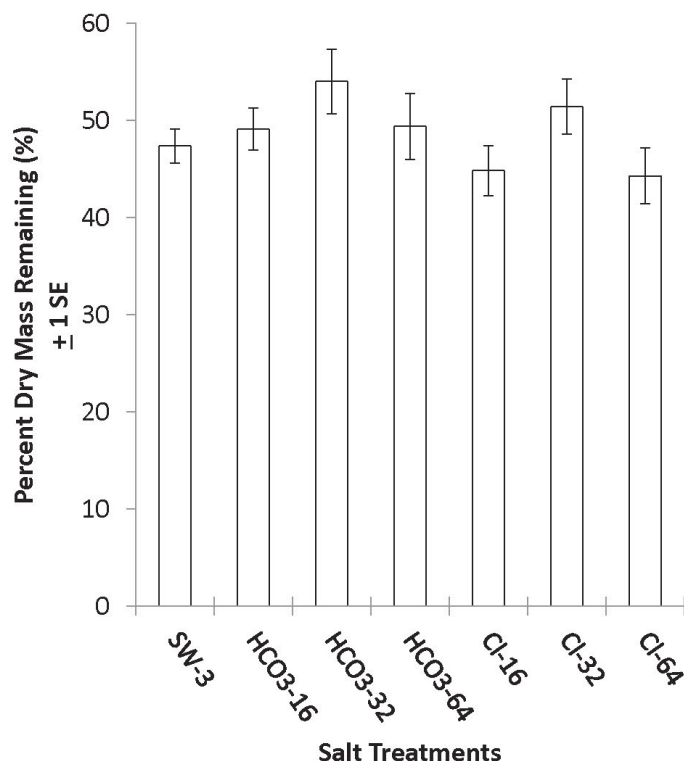


Figure 2. Mean (+1 SE) percent dry mass of litter remaining. There were no statistically significant differences in percent dry mass remaining across salt treatments, although percent dry mass remaining in  $\text{NaHCO}_3$  treatments tended to be greater than in ambient ( $3\text{mg/L}$ ) stream water. Additionally, percent dry mass remaining showed an increasing pattern with increasing salt concentration for  $\text{NaHCO}_3$  treatments until peaking at median salt and then decreasing at the two greatest salt concentrations.

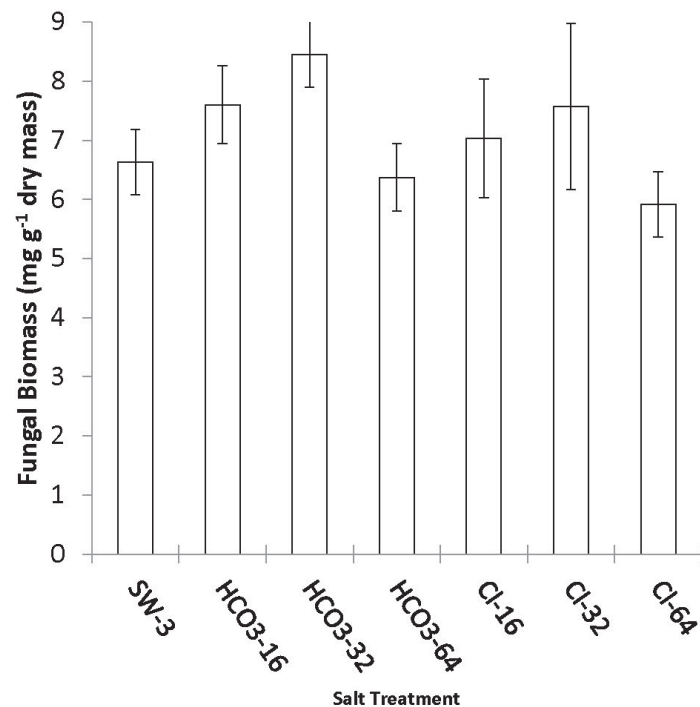


Figure 3. Mean fungal (+1 SE) expressed per unit litter dry mass across salt treatments. Fungal biomass tended to increase with salt concentrations peaking in moderate salt treatments ( $32\text{mg/L}$ ) and then decreasing in the highest salt treatments ( $64\text{mg/L}$ ) to levels below that found in ambient salinity controls for  $\text{NaCl}$  and  $\text{NaCO}_3$  salts ( $p>0.05$ ).

greatest  $\text{NaHCO}_3$  concentration that was likely from the more basic pH that supports optimal algal growth (Brock, 1973).

### Experiment 2 (macro-detritivores exposed to added salts in streamwater and fed naturally conditioned leaves; Figures 5-6).

Overall, salt amendments to SW tended to stimulate stonefly growth, respiration, and fungal biomass on leaf discs. Stoneflies in stream water gained about 50% mass over the month long experiment compared to ~60% increase for stoneflies in low and high  $\text{NaCl}$  and  $\text{NaHCO}_3$  amended water ( $p=0.04$ ). Stoneflies in the medium salt treatments gained about the same mass as those in SW ( $p>0.05$ ). Added low and high salts resulted in ~10% increase in mass (Figure 5A). Stonefly respiration was measured on day 30 of the experiment. Stonefly

respiration in salt-amended water was  $\geq$  stonefly respiration for individuals in SW ( $p=0.02$ ). Stonefly respiration was ~3 times faster for individuals in the highest  $\text{NaHCO}_3$  treatments and the low and medium  $\text{NaCl}$  than for stoneflies in SW (Figure 5B). Leaf litter mass remaining after 7 days in stonefly chambers did not differ across treatments ( $p=0.73$ ). Leaf discs lost 20-30% of their mass over the week-long feeding period (Figure 6A). Leaf discs placed in salt amended water with stoneflies gained fungal biomass particularly in  $\text{NaCl}$  amendments from  $1\text{ mg/g}$  on leaves in SW up to an average of  $9\text{ mgg}^{-1}$  on leaves in the lowest  $\text{NaCl}$  added treatment ( $p=0.04$ , Figure 6B). The increase in fungal biomass on leaves fed to stoneflies incubated in added salt treatments may be from added nutrients provided by stonefly excretion and the overall positive stonefly growth response is probably from this added fungal biomass as a more nutritious food resource (Ferreira et al., 2014).

Table 4. One-way ANOVA ( $\alpha=0.05$ ) output for algal biomass at termination (week 19, day 134).

	df	F	p
Dry Mass	6	1.167	0.336
AFDM	6	1.664	0.145

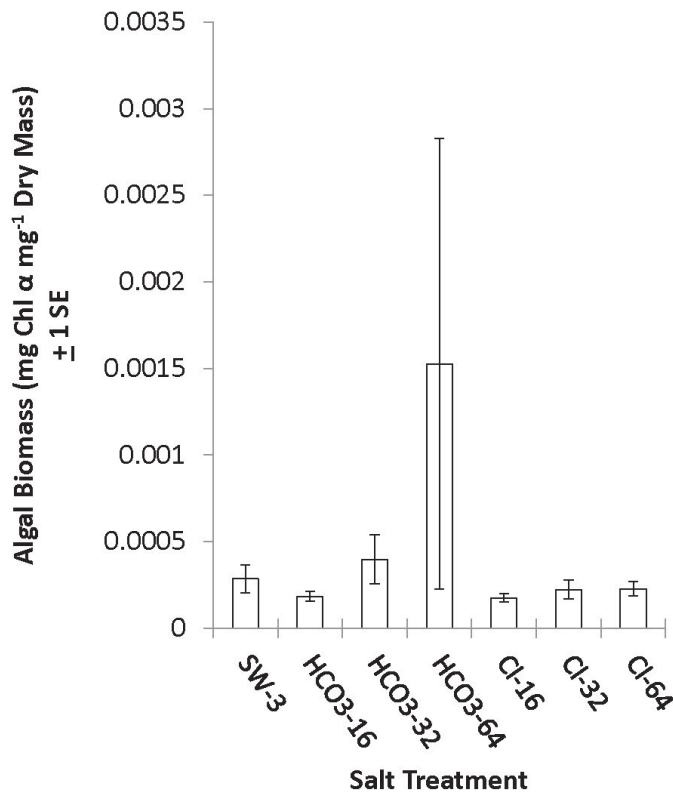


Figure 4. Mean algal biomass measured as chlorophyll a (chl a  $\pm$  1 SE) expressed per unit litter dry mass across salt treatments. NaCl treatments tended to have lower algal biomass than ambient stream water ( $p>0.05$ ). NaHCO<sub>3</sub> treatments had increasing algal biomass with increasing salinity, but only moderate (32mg/L) to high (64mg/L) NaHCO<sub>3</sub> treatments had higher algal biomass than ambient (3mg/L) stream water ( $p>0.05$ ). The greatest variation occurred in high (64mg/L) NaHCO<sub>3</sub> treatments.

### Experiment 3 (macro-detritivore not exposed to added salts in streamwater but fed added salt-incubated leaf discs; Figures 7-8).

Overall, feeding isopods leaves that were incubated in some of the added salt treatments suppressed isopod growth and respiration compared to isopods that were fed leaves incubated in SW alone. Isopods fed leaves incubated in SW increased their mass by 70%. In contrast, isopods fed leaves incubated in medium NaHCO<sub>3</sub> and NaCl grew 20% less. Isopods fed leaves incubated in 32mgL<sup>-1</sup> NaCl amendments grew about 28% less than those fed SW-incubated leaves (Figure 7A). Isopod respiration was equal to or greater than respiration of isopods fed leaves incubated in SW compared to salts. Isopods that were fed leaves from low NaCl incubations respired the least (and gained the least amount of mass) with

nearly 3x lower respiration than isopods fed leaves from SW and medium NaHCO<sub>3</sub> and NaCl ( $p=0.03$ , Figure 7B). There was no measurable difference in leaf mass remaining across salt treatments ( $p=0.13$ ). All leaf discs lost 20-40% of their mass over the week-long feeding period. Although not statistically significant, the trend was more leaf mass was lost in the low NaCl incubated leaf discs where isopod growth and respiration were lowest (Figure 7A&B, 8A). Fungal biomass on discs incubated and then fed to isopods had variable biomass ranging from 2 to 6 mgg<sup>-1</sup> and there was no treatment effect ( $p=0.41$ ).

## Conclusions

These results demonstrate the complexities of nutrient subsidies on stream processes. In spite of the lack of significance for fungal biomass estimates, low level salts, especially NaHCO<sub>3</sub>, appear to stimulate microbial respiration. Considering there were no significant differences in percent dry mass remaining across treatments, higher microbial respiration rates may be indicative of microbial energy diverted toward osmoregulation in the presence of ionic stress instead of growth and consumption. Increased algal biomass and fungal biomass can provide added resources to detrital invertebrates, which may initially help mitigate macro-detritivore osmoregulatory stress from increased ion concentrations. *Amphinemura* increased growth rates and respired more in Na- (both Cl and HCO<sub>3</sub>) amended water without increased leaf consumption. Conservation of mass suggests that stoneflies may be feeding on an alternative resource like fungi or algae when NaCl or NaHCO<sub>3</sub> is present. However, diet switching could have long term effects on resource availability (Brown et al., 2004). In addition to potential osmoregulatory stress caused by water ion concentrations, changes to detritus from salts resulted in decreased *Lirceus* growth relative to stream water with little change in respiration and leaf consumption in salt-amended treatments. This suggests that salts impact the quality of detritus. Although non-lethal, ion increases may impact stream ecosystem processes 1) directly via changes in fungi biomass and respiration, 2) directly by altering macroinvertebrate detritivore consumption, respiration, and growth, and 3) indirectly by altering litter quality. Together, these results demonstrate that low-level, non-lethal NaCl and NaHCO<sub>3</sub> impacts detritivores both directly and indirectly even at concentrations that are near the existing chloride standards in Arkansas. Other ions, like HCO<sub>3</sub>, have a similar effect on detritivores but are not currently considered in state and federal regulatory standards despite their prevalence in the environment from waste water treatment and release (Canedo-Arguelles et al., 2016).

## Acknowledgements

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### Stonefly response to salt-amended stream water

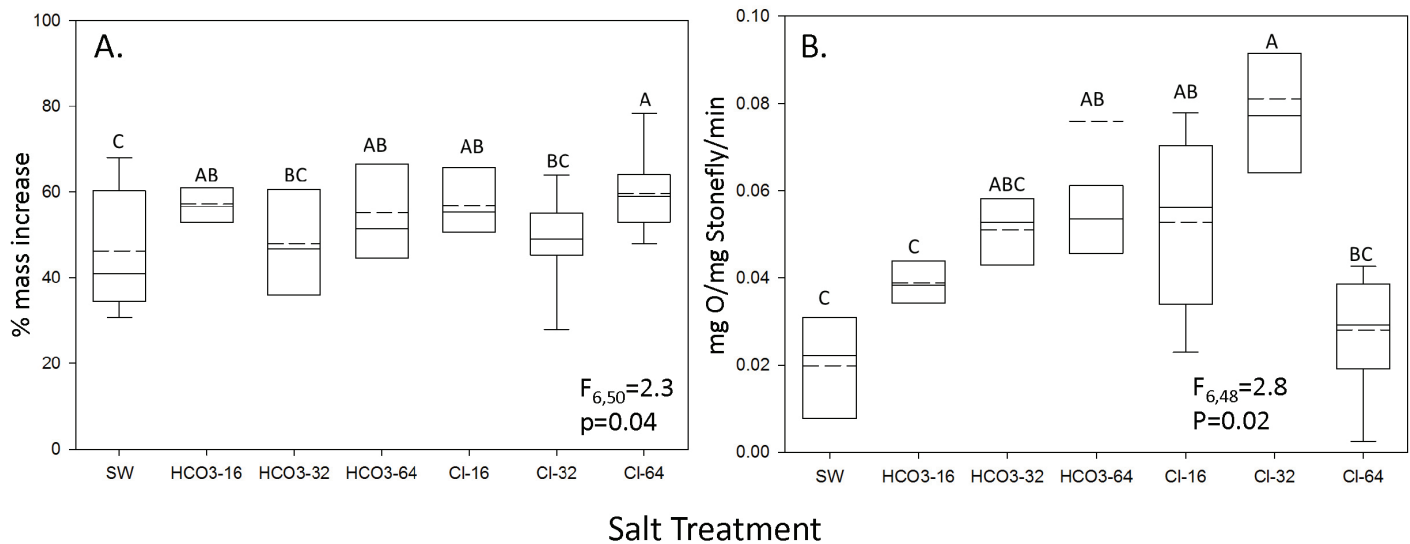


Figure 5. Stoneflies (*Amphinemura* sp.) were fed sweet gum leaves incubated in stream water and reared in chambers with stream water amended with salts. Salt treatments were: SW=ambient stream water (3mg/L Na); HCO<sub>3</sub>-16,-32,-64=low, moderate, and high NaHCO<sub>3</sub> treatments (16, 32, 64mg/L, respectively); Cl-16,-32,-64=low, moderate, and high NaCl treatments (16,32,64mg/L, respectively). Box plots show the upper value as the top whisker that is not an outlier, upper quartile, then a dashed line represents the average and the solid line is the median. Lower box is the lower quartile and the lower whisker is the minimum value excluding outliers. When whiskers are not present it is because they equal the upper and lower quartile, respectively. Panel A. is stonefly growth. Panel B. is stonefly respiration measured on the final day of the experiment. Different letters represent statistical significance at  $\alpha=0.05$ .

### Leaf litter decomposition and fungal biomass following Stonefly feeding

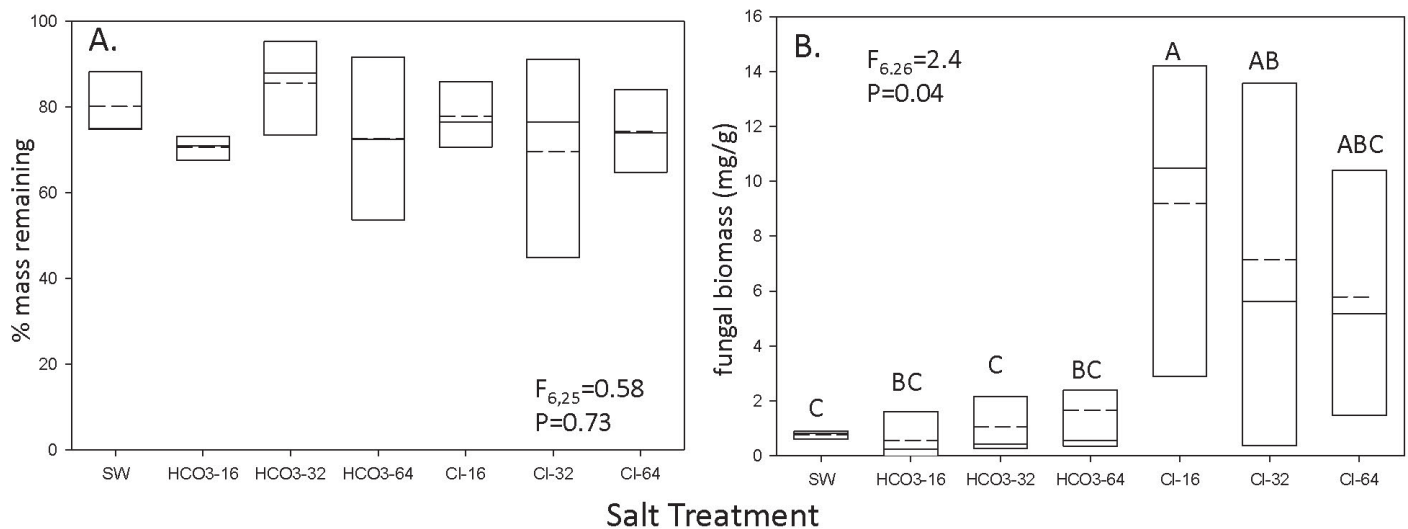


Figure 6. Stoneflies (*Amphinemura* sp.) were fed sweet gum leaves incubated in stream water and reared in chambers with stream water amended with salts. Salt treatments were: SW=3=ambient stream water (3mg/L Na); HCO<sub>3</sub>-16,-32,-64=low, moderate, and high NaHCO<sub>3</sub> treatments (16, 32, 64mg/L, respectively); Cl-16,-32,-64=low, moderate, and high NaCl treatments (16,32,64mg/L, respectively). Box plots show the upper value as the top whisker that is not an outlier, upper quartile, then a dashed line represents the average and the solid line is the median. Lower box is the lower quartile and the lower whisker is the minimum value excluding outliers. When whiskers are not present it is because they equal the upper and lower quartile, respectively. Panel A is leaf disc mass remaining on final discs. Panel B is fungal biomass on leaf discs following the final stonefly feeding period. Different letters represent statistical significance at  $\alpha=0.05$ .



### Isopod response to eating leaf discs incubated in salt-amended stream water

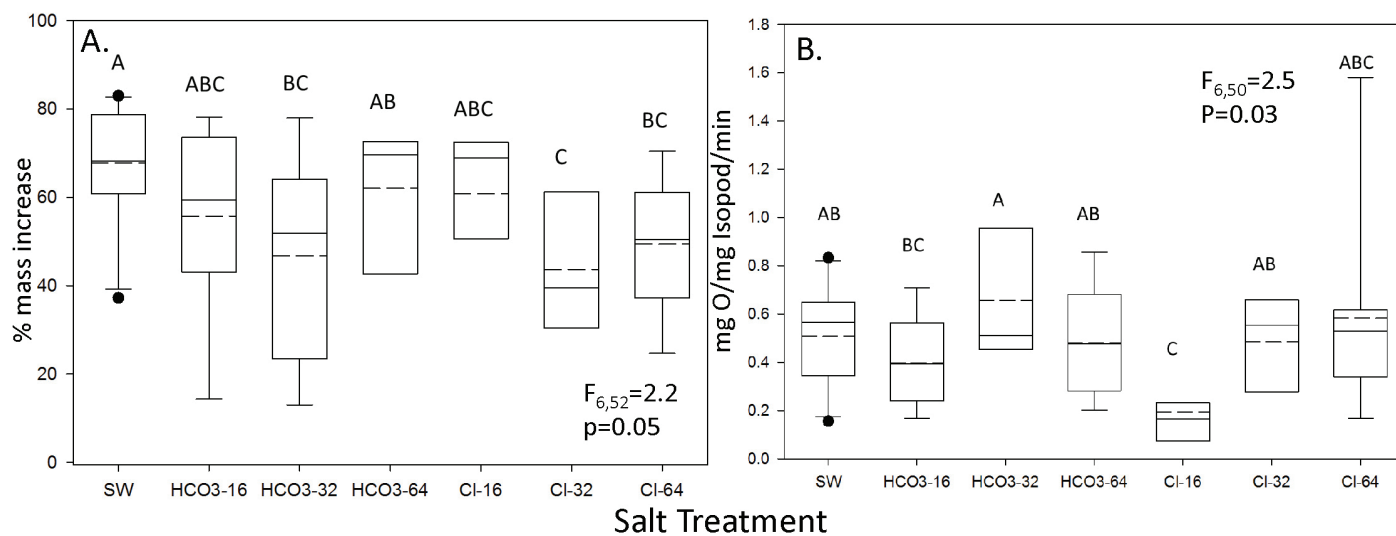


Figure 7. Isopods were fed leaves incubated in stream water amended with salts and chambers had only stream water. Salt treatments that leaves incubated in prior to being offered to isopods were: SW-3=ambient stream water (3mg/L Na); HCO3-16,-32,-64=low, moderate, and high NaHCO3 treatments (16, 32, 64mg/L, respectively); Cl-16,-32,-64=low, moderate, and high NaCl treatments (16,32,64mg/L, respectively). Box plots show black circles as outliers, the upper value as the top whisker that is not an outlier, upper quartile, then a dashed line represents the average and the solid line is the median. Lower box is the lower quartile and the lower whisker is the minimum value excluding outliers. When whiskers are not present it is because they equal the upper and lower quartile, respectively. Panel A is isopod growth about one month after being fed salt-incubated leaves. Panel B is isopod respiration per mg of their body mass (mg). Different letters represent statistical significance at  $\alpha=0.05$ .

### Leaf litter mass remaining and fungal biomass incubated in salt-amended stream water

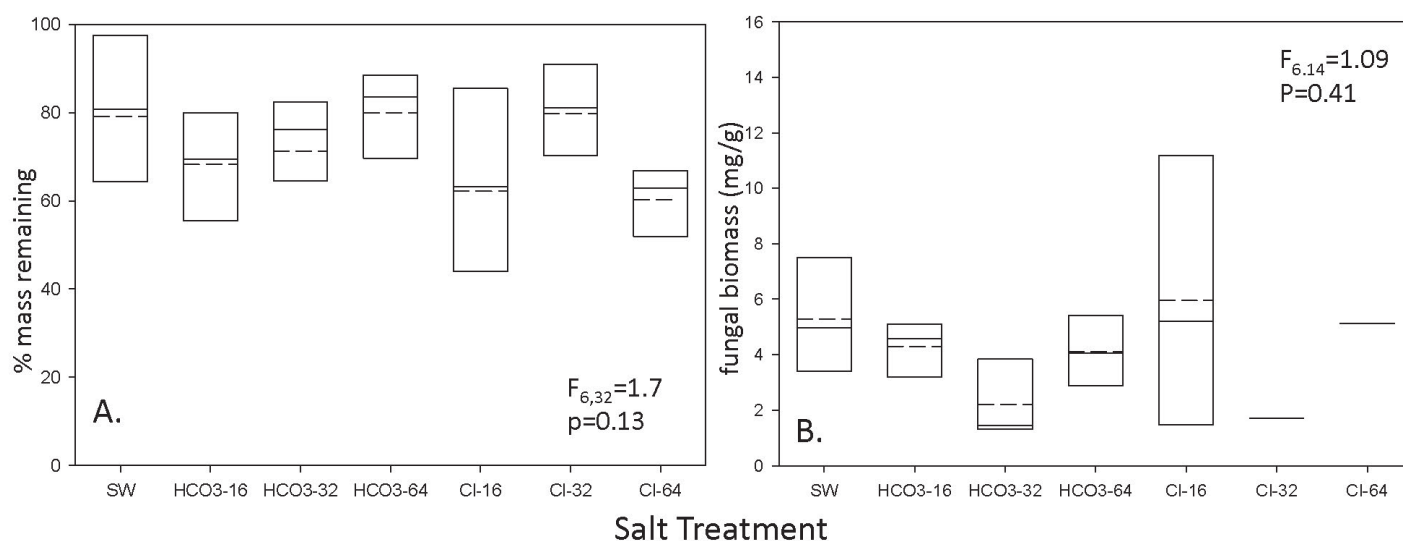


Figure 8. Salt-incubated leaf disc mass remaining and fungal biomass following the last isopod feeding period. Salt treatments that leaves incubated in prior to being offered to isopods were: SW-3=ambient stream water (3mg/L Na); HCO3-16,-32,-64=low, moderate, and high NaHCO3 treatments (16, 32, 64mg/L, respectively); Cl-16,-32,-64=low, moderate, and high NaCl treatments (16,32,64mg/L, respectively). Box plots show black circles as outliers, the upper value as the top whisker that is not an outlier, upper quartile, then a dashed line represents the average and the solid line is the median. Lower box is the lower quartile and the lower whisker is the minimum value excluding outliers. When whiskers are not present it is because they equal the upper and lower quartile, respectively. Panel A. is average leaf disc mass remaining on final discs following isopod feeding. Panel B is fungal biomass on final discs.

the authors and should not be interpreted as representing the opinions or policies of the U.S. Geological Survey. Mention of trade names or commercial products does not constitute endorsement by the U.S. Geological Survey.

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Image caption: Biofilm reactors at a wastewater treatment facility.

## Investigating Fate of Engineered Nanoparticles in Wastewater Biofilms

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**Abstract:** Engineered nanoparticles incorporated into consumer products have shown to negatively impact vital ecosystems once released into the environment. As wastewater reuse practices become increasingly necessary in areas of water scarcity, innovative wastewater treatment applications will be required. Attached growth (i.e. biofilm) processes for wastewater treatment generate less waste and are easier to operate compared to activated sludge. This study examines the interaction between silver nanoparticles (Ag-NPs) and wastewater biofilms. Two bench scale reactors were used to examine the impact of Ag-NPs on model biofilm, as well as the attachment of Ag-NPs to biofilm. The insights provided offer a basis for understanding the removal capabilities of Ag-NPs from wastewater through biofilm processes.

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### Key Points:

- Silver nanoparticles can attach to model wastewater biofilm without significantly impacting biofilm biomass.
  - Wastewater biofilm can become stressed under exposure to  $1 \text{ mgL}^{-1}$  of silver nanoparticles.
  - By applying a mass balance, model biofilm *Comamonas testosteroni* was observed to accumulate  $0.172 \text{ ng mm}^{-2}$  of silver nanoparticles.
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## Introduction

The application of silver nanoparticles (Ag-NPs) has expanded exponentially within manufactured products such as food packaging, cosmetics, and textiles (Boxall et al., 2009). Reuse of treated wastewater for various purposes such as drinking water, irrigation water, and/or cooling water is now a reality and will continue to increase as traditional freshwater sources become progressively stressed. Although Ag-NPs have previously been referred to as emerging contaminants, their presence is now a long-term issue that might have damaged vital microbiological ecosystems (de Faria et al., 2014). By modeling the fate and transport of Ag-NPs, environmentally relevant quantities will vary depending on location type. These concentrations are predicted generally in the range of  $0.003 - 100 \text{ ngL}^{-1}$  (Mitrano et al., 2012). Wastewater treatment plants, an important barrier between consumers and their surroundings, are not designed specifically for the removal of Ag-NPs (Walden and Zhang, 2016). As wastewater influent complexity increases, treatment plants should be re-evaluated for their processing efficiency. Likewise, as competing demands increase upon limited freshwater resources, reuse practices of treated wastewater will increase across the United States, including Arkansas. Consequently, there is a pressing need for economical yet effective regionalized wastewater treatment. Biofilm systems (Figure 1) are easy to maintain and convenient for small communities. Here, we investigated the role of wastewater biofilms in the removal of Ag-NPs from waste streams. The goal of this proposal investigated the following hypotheses: (1) ENPs within wastewater can attach to biofilms without significantly altering nutrient reduction capacity; and (2) under certain steady-state parameters, biofilms can become an environmental sink for ENP to accumulate within the extracellular polymeric substances (EPS). Ag-NPs were exposed to model wastewater bacteria *Comamonas testosteroni* in two differently sized bench scale reactors for Ag-NP impact on biomass and removal from suspension. Ongoing work will explore dual and mixed species combinations with additional bacteria *Acinetobacter calcoaceticus* and *Delftia acidovorans* (Andersson et al., 2008).

## Methods

### Experimental design

The three species were first tested for biofilm forming capacity. A biofilm formation assay was conducted in a clear 96 well plate with 2% crystal violet as previously described (Djordjevic et al., 2002; O'Toole, 2011). A control experiment was conducted for 28 days to observe the time for a mature biofilm to form within the CDC biofilm reactor (BioSurface Technologies, Bozeman, MT), and to monitor biological reduction capacity in the absence of Ag-NPs. A non-limiting synthetic wastewater inoculated with *D. acidovorans* was fed and recycled through the CBR as nitrate, phosphate, sulfate, chlorides, COD, and pH were monitored. Shorter experiments with *C. testosteroni* used as a feed into the CBR and the custom flow cell were also performed for 48 hours. For the shorter experiments, the feed was switched to sterile synthetic wastewater to remove planktonic cells from the system. Then, biofilm was exposed to a spike of about  $1 \text{ mg L}^{-1}$  Ag-NPs (CBR) and  $2 \text{ mgL}^{-1}$  (flow cell) for 30 minutes.

### Reactor descriptions and setup

The CBR is a 1 liter glass beaker with a polyethylene lid which holds 8 polyethylene rods, each with three removable polyethylene coupons serving as an attachment site for biofilm growth. The working volume is about 350 mL. The custom flow cell holds three removable polyethylene coupons, and has a working volume of about 2 mL. The synthetic wastewater consisted of nutrient broth ( $300 \text{ mgL}^{-1}$ ),  $\text{KH}_2\text{PO}_4$  ( $44 \text{ mgL}^{-1}$ ),  $\text{NaOH}$  ( $16.7 \text{ mgL}^{-1}$ ),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  ( $132.4 \text{ mgL}^{-1}$ ),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  ( $100 \text{ mgL}^{-1}$ ),  $\text{C}_6\text{H}_{12}\text{O}_6$  ( $140 \text{ mgL}^{-1}$ ),  $\text{KNO}_3$  ( $3 \text{ mg L}^{-1}$ ),  $\text{NaHCO}_3$  ( $175 \text{ mgL}^{-1}$ ),  $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$  ( $12.8 \text{ mgL}^{-1}$ ),  $(\text{NH}_4)_2\text{SO}_4$  ( $118 \text{ mgL}^{-1}$ ), and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  ( $5 \text{ mgL}^{-1}$ ). The CDC biofilm reactor (CBR), flow cell, connectors/tubing, and synthetic wastewater solution were autoclaved at  $121^\circ\text{C}$  for 30 minutes prior to each experiment (Model 522LS Gravity Steam Sterilizer, Getinge, New York). The experimental setup (Figure 2) included the CBR or flow cell connected to a peristaltic pump set at 10 and  $1 \text{ mL min}^{-1}$  flow rate, respectively.

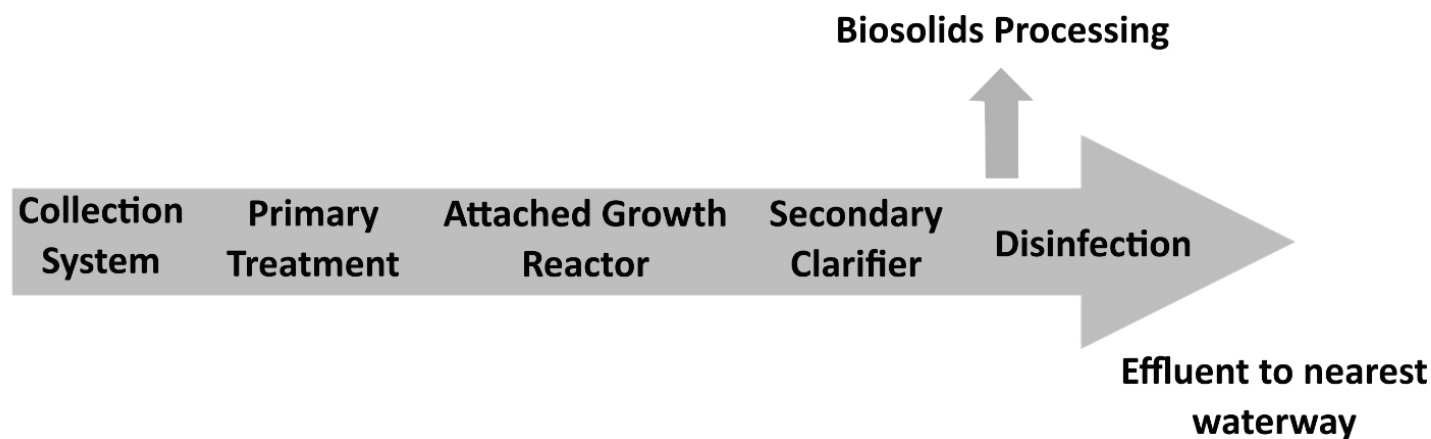


Figure 1. Representative schematic of a typical attached growth wastewater treatment plant.

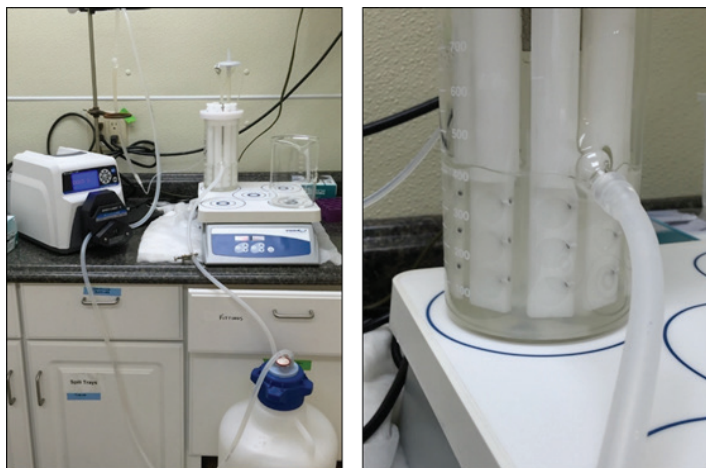


Figure 2. (left) The experimental setup included a peristaltic pump and autoclavable tubing to circulate synthetic wastewater through the CDC biofilm reactor (CBR). (right) A close up shows the detachable polyethylene sampling coupons suspended in the CBR for biofilm testing.

### Biofilm analysis with CBR

Biofilm amount was determined from Hoescht 33342 cell stain with an upright confocal fluorescence microscope (Nikon Eclipse Ni-E upright microscope, Nikon Instruments, Melville, New York). For biofilm stress, a modified dichlorofluorescein (DCF) assay was used as previously described in black-sided clear bottomed 96-well plates (Corning 3603, Corning, MA) and analyzed on a microplate reader (Synergy H1 Multi-Mode Microplate Reader, Biotek Instruments, Inc., VT) (Wang and Joseph, 1999).

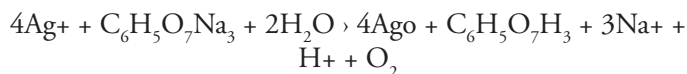
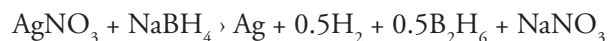
### Biofilm analysis with flow cell

The flow cell system has the advantage of a smaller working volume than the CBR, allowing for quick biofilm formation and simple mass balance measurements. *C. testosteroni* was recycled through the flow cell for 48 hours to establish a mature biofilm. Then, sterile synthetic wastewater was pumped through for 10 minutes to eliminate any planktonic cells. 2 mg L<sup>-1</sup> Ag-NPs were aseptically injected into the cell. After 30 minutes, sterile wastewater was used to flush the flow cell of any unattached Ag-NPs for 10 minutes. All effluent was retained and analyzed for total volume and total silver concentrations. All effluent was collected in sterile centrifuge tubes for mass balance measurements. To remove biofilm from the coupon for ICP-MS, each coupon was aseptically removed from the flow cell and inserted into a sterile tube with 5 mL of DDI water. The tubes were vortexed for 5 minutes. The coupon was removed, and the total volume was brought up to 10 mL and acidified with 2.5% nitric acid for ICP-MS. The concentration of silver ion was measured by centrifugal filtration and ICP-MS.

### Silver synthesis

Silver nanoparticles were formed using sodium borohydride to reduce silver nitrate with sodium citrate as a cap-

ping agent (Mulfinger et al., 2007). All glassware was washed with phosphorus free detergent, rinsed three times with tap water, then rinsed three times with deionized water (Elga Process Water System (18.2 MΩ·cm<sup>-1</sup>) Purelab flex, Veolia, Ireland). The reduction of silver nitrate occurred as follows:



The formation of Ag-NPs was confirmed by scanning the absorbance from 300 – 700 nm with a UV-vis spectrophotometer (Beckman Coulter, CA, USA.). The concentration of Ag-NPs was measured with ICP-MS. Particles size was verified with TEM (Jeol, USA) and DelsaNano (Beckman Coulter, Life Sciences, USA).

### Statistical analysis

All statistics and plots were generated in Sigma-Plot (Systat Software, Inc., version 12.5) where statistic p values less than 0.05 were considered significant.

### Results

Biofilm formation assay. The capability to form biofilm was investigated for the bacteria combinations discussed using a crystal violet microtiter 96-well plate assay. For all single and multiple combinations with these species a strong biofilm was formed. Of the three single assays, *A. calcoaceticus* forms a significantly stronger biofilm than *C. testosteroni* or *Delftia acidovorans* (Figure 3,  $p < 0.05$ ). There was no significant difference

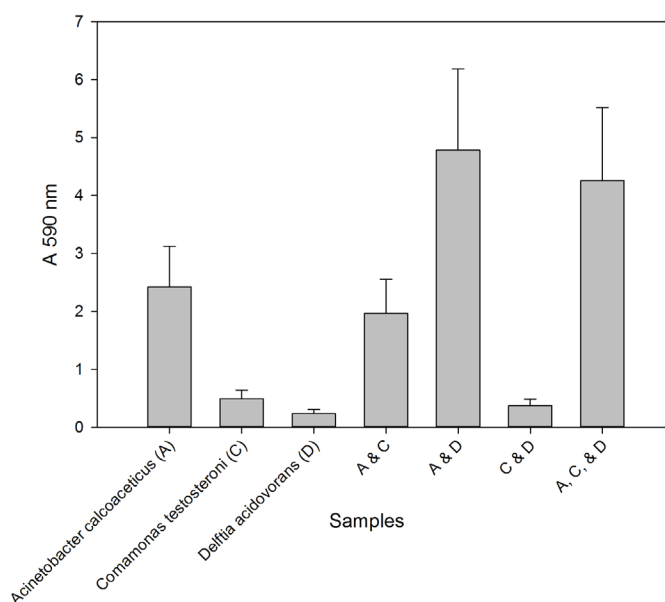


Figure 3. Biofilm formation assay results from crystal violet staining with standard error (n=3) for each species single, duel, and mixed. A greater absorbance reflects increased ability to form biofilm.

between the assay of all three mixed and the assay of *A. calcoaceticus* & *D. acidovorans*. (Stepanovic et al., 2000; O'Toole, 2011).

### Nutrient reduction capacity

The CBR setup as a closed system with recycle was inoculated with *D. acidovorans*; nitrate, phosphate, sulfate, chlorides, COD, and pH were monitored to test for nutrient changes without Ag-NPs present. Minimal or no change was observed for nitrate, phosphate, sulfate, chlorides and pH. COD was reduced to approximately  $18.8 \text{ mgL}^{-1}$  from above detection limit after 10 days. We concluded that the quantity of biofilm formed within this reactor type with single species *D. acidovorans* is not sufficient for nutrient reduction testing.

### Silver nanoparticle formation

The Ag-NPs exhibited the expected UV-vis peak at 395-400 nm for nano-sized silver. The average particle size from photon correlation spectroscopy was 7.9 nm, and confirmed with TEM (Figure 4). ICP-MS measured a stock solution concentration of  $76 \text{ mgL}^{-1}$ , with less than 10% ionic silver present. This stock was stored in the dark and verified as unchanged with UV-vis at each use.

### CBR experiment

In the CBR system, *C. testosteroni* exhibited insignificant change in biomass after Ag-NP exposure ( $p=0.1323$ ).

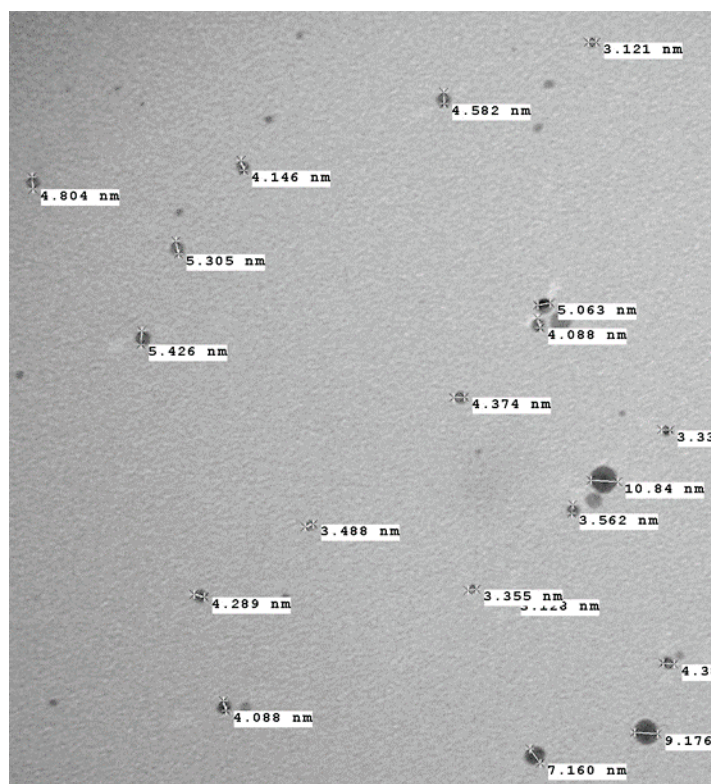


Figure 4. Transmission electron microscope image of silver nanoparticles (Ag-NPs) verifying the formation of nano-sized particles. Embedded within the image are diameters of randomly selected particles.

This is consistent with previous conclusions that wastewater biofilms are tolerant to toxic loadings. However, reactive oxygen species present reflected significant cell stress after the 30-minute treatment (Figure 5,  $p = 0.0132$ ). The CBR experiment addresses the first hypothesis that Ag-NPs can attach without significantly altering biomass.

### Flow cell experiment

The amounts of Ag-NPs per coupon (Table 1) were all less than  $0.1 \text{ ng mm}^{-2}$ . The total silver recovered from biofilms was  $0.172 \text{ ng mm}^{-2}$ . This is a first step toward proving the second hypothesis that biofilms can become a sink for Ag-NPs.

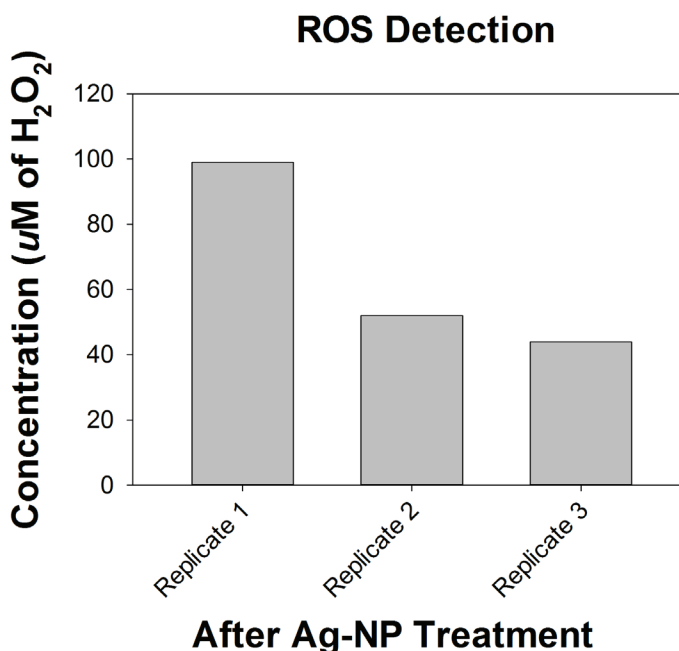
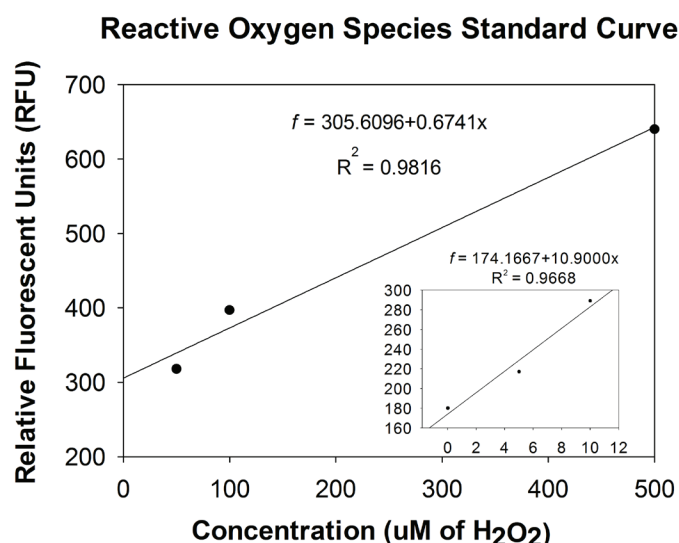


Figure 5. (a) RFU measurements were converted to concentration from the standard curve. (b) Reactive oxygen species measurements after a 30-minute exposure to  $1 \text{ mg L}^{-1}$  Ag-NPs.



	Ag-NPs per coupon (ng/mm <sup>2</sup> )	Ag-NPs per coupon (ng/mm <sup>2</sup> )	Ag-NPs per coupon (ng/mm <sup>2</sup> )	Total Silver in Biofilms (ng)	Percent Recovered	Total Silver Accumulation (ng/mm <sup>2</sup> )
Control (No Biofilm)	-	-	-		104	-
<i>Comamonas testosteroni</i>	0.091	0.053	0.028	21.8	91.7	0.172

Table 1. Silver mass balance: the amount of silver retained on each coupon removed from the flow cell measured by ICP-MS.

## Conclusions, Recommendations and Benefits

Model wastewater biofilm shows potential to resist acute exposure to environmentally relevant quantities of Ag-NPs. Further, this model biofilm can accumulate Ag-NPs into its biofilm structure. This fundamental look at the Ag-NP – biofilm interactions shows minimal potential for Ag-NP accumulation. However, the resistance to detachment in the presence of Ag-NPs shows the capability of even a single wastewater type species to tolerate toxic loadings. We recommend continuing this work with other model species and a more complex biofilm community.

Although ENPs have been commonly referred to as ‘emerging’ contaminants, the presence of ENPs is now a persistent and long term issue that may have already damaged vital microbiological ecosystems. The goal is to explore realistic environmental conditions in wastewater biofilm systems that control the removal and release of potentially toxic ENPs (silver nanoparticles, Ag-NPs), thereby establishing the fundamental groundwork that will enable innovative use of biofilm processes in wastewater treatment for water reuse and recycling in areas of water scarcity. By investigating water supply and quality problems, this research directly addresses the goals of the AWRC. Likewise, by exploring issues that are of immediate concern in arid and semi-arid climates, this research furthers the U.S. Geological Survey’s national water mission to increase knowledge of water quality and quantity. The United States Environmental Protection Agency (EPA) published many examples of current water reuse practice in Region 9 district (serving Arizona, California, Hawaii, Nevada, Pacific Islands and Tribal Nations), and reuse will continue to increase as traditional fresh water sources become increasingly stressed (Fachvereinigung Betriebs- und Regenwassernutzung e, 2005).

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