

3-1-2018

The Effect of MS2 Bacteriophage on the Activity and Performance of Activated Sludge

Sean M. Stuntz

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THE EFFECT OF MS2 BACTERIOPHAGE ON THE ACTIVITY AND
PERFORMANCE OF ACTIVATED SLUDGE

THESIS

Sean M. Stuntz, Captain, USAF

AFIT-ENV-MS-18-M-238

DEPARTMENT OF THE AIR FORCE
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PERFORMANCE OF ACTIVATED SLUDGE

THESIS

Presented to the Faculty

Department of Systems and Engineering Management

Graduate School of Engineering and Management

Air Force Institute of Technology

Air University

Air Education and Training Command

In Partial Fulfillment of the Requirements for the
Degree of Master of Science in Engineering Management

Sean M. Stuntz, BS

Captain, USAF

March 2018

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PERFORMANCE OF ACTIVATED SLUDGE

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Abstract

As a medical waste product, the MS2 bacteriophage has been discharged into wastewater distribution and treatment networks. To categorize its impact to suspended growth treatment methods, this research investigated the effect of the MS2 bacteriophage on activated sludge through a series of respirometry tests. The effects of MS2 bacteriophage at a concentration of 3.2×10^8 PFU/mL were assessed by determining peak O_2 consumption, cumulative O_2 consumption, molar CO_2/O_2 ratios, and shape factors. None of the MS2 samples caused statistically significant effects on the peak or cumulative O_2 consumption. The molar CO_2/O_2 ratios were also not substantially impacted. The skewness and first moment of the area were not significantly impacted by the introduction of the MS2 virus. MS2 bacteriophage did not inhibit chemical oxygen demand (COD) or nitrogen removal. When viewed at 100X amplification and at multiple wavelengths, the MS2 virus appeared to both remain free floating and to adsorb to activated sludge flocculated particles. To the author's knowledge, this is the first study to demonstrate the effect of MS2 bacteriophage on activated sludge activity and the first to use dual fluorescent labeling to examine a mixed consortia of activated sludge microorganisms and MS2. Overall, the results illustrate that the MS2 bacteriophage is unlikely to cause short-term interference with primary biological treatment at an activated sludge treatment plant.

Acknowledgments

I would like to thank the EPA National Homeland Security Research Center, Water Infrastructure Protection Division for financial support of this research effort. Dr. Yun Xing, thank you for your assistance in developing experimental protocols and providing guidance and feedback along the way. Kandace Hunter, thank you for all of your experimental assistance and laughs in lab. Dr. Daniel Felker, thank you for your assistance and logistical support. Dr. Harper, thank you for tolerating all of my “Civil Engineer” questions and for providing me with the tools, knowledge, and mentorship to achieve this academic pursuit. To my loving wife and daughter, for everything you sacrifice and for all of your unceasing support, Thank You! I couldn’t have done it without you!

Sean M. Stuntz

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1. Introduction

1.1 Background

Activated sludge is one of multiple methods used to treat wastewater. In a typical aerobic suspended growth wastewater treatment plant, influent is first fed through a bar screen which removes large debris. Colloidal particles are then allowed to settle in a grit chamber before the effluent reaches a primary clarification tank. Next, wastewater flows into the aeration basin where organic waste products and nutrients are digested by suspended flocculated sludge particles made of a consortium of microorganisms and organic particles. These flocculated sludge particles are then allowed to settle in a secondary clarification tank before the effluent is sometimes disinfected before being discharged to surface waters or underground aquifers.

Activated sludge contains a diverse microbial ecology containing prokaryotic and eukaryotic bacterial cells, microorganisms, intracellular fluids and nanoparticles (Vaccari, Strom, & Alleman, 2006). The diversity of these organisms is significant as a given sample of activated sludge is responsible for degrading a non-specific range of human, animal, and industrial waste products. Organic waste degradation occurs throughout the various stages of the wastewater treatment process. In aerobic regions, dissolved oxygen (DO) concentrations are increased to 2mg/L or higher to facilitate biochemical oxygen demand (BOD) removal via nitrification. In anoxic regions, DO is removed and denitrifying bacteria oxidize NO_3 and NO_2 to produce N_2 gas. Simply put,

the metabolism of specific bacteria is influenced throughout the wastewater treatment process to degrade organic and inorganic waste products (Metcalf & Eddy, 2015).

Although activated sludge waste treatment processes can tolerate changes in environmental conditions and influent characteristics, they are susceptible to degraded performance (Motlagh & Goel, 2014). Anything that disrupts the metabolism of the bacteria or drastically alters environmental conditions conducive to treatment can threaten the underlying biological processes. The inhibitory effects of metals, organic compounds, chemicals, and other microorganisms on treatment processes are well accounted for and documented (Hooper & Terry, 1973; Juliette, Hyman, & Arp, 1993; Kelly, Henriques, & Love, 2004; You, Tsai, & Huang, 2009; Zerdazi, Boutraa, Melizi, Bencheikh Lehocine, & Meniai, 2012). This body of knowledge provides the foundation for discharge limit regulations and treatment facility operations. In addition, past research has helped categorize a waste treatment plant's relative of risk of exposure to these known inhibitors whether due to intentional or inadvertent release. It is the intention of this research effort to contribute to this body of knowledge by determining the degree to which the MS2 bacteriophage inhibits the activity and respiration of activated sludge.

Bacteriophages are viruses that infect bacteria. These viruses can contain either DNA or RNA enclosed in protein capsules called capsids (Kott, Roze, Sperber, & Betzer, 1974). Bacteriophages reproduce by injecting genetic material in host cells and then manipulating cellular functions to support phage replication and eventual release (Grabow, 2001). Replication can occur via lytic or lysogenic mechanisms. During lytic infection, after injecting genetic material, the progeny will open, or lyse, the cell wall and continue to replicate (Joh & Weitz, 2011). Lysogenic infection involves a dormant stage

where virus progeny remain inside the host cell and slowly consume intracellular nutrients before seeking another host for replication (Heath & Wibe, n.d.). While viruses are known to only infect a specific range of bacteria, the microbial diversity of activated sludge and natural variability of biological waste treatment processes drive interest in categorizing risk to conventional treatment processes resulting from exposure to bacteriophages.

1.2 Problem Statement

The U.S. Environmental Protection Agency (EPA) National Homeland Security Research Center (NHSRC) is working with environmental engineering professionals to improve water infrastructure security (U.S. EPA, 2016). One key ongoing initiative concerns high-consequence biological contaminants, including biological weapons, biohazardous or infectious waste, and medical waste. Wastewater treatment plants need to be prepared for scenarios that involve the intentional or inadvertent introduction of biological contaminants into the wastewater collection and treatment system and the U.S. EPA is developing guidance for handling such wastewater. The protocols, policies, or regulations that are needed must be based upon facts related to the effect of the biological contaminant on the treatment system and public and environmental health. This is now the appropriate moment for research that addresses key knowledge gaps. One important issue is the effect of bio contaminants on the activated sludge process. This work will investigate the effect of MS2 bacteriophage on the activity and performance of activated sludge.

1.3 Research Objective

The purpose of this research is to evaluate whether or not a naturally occurring coliphage virus inhibits effective wastewater treatment by activated sludge. Specifically, this study will determine whether or not the MS2 bacteriophage inhibits the respiration, chemical oxygen demand (COD) removal, and nitrification observed as activated sludge degrades organic wastes.

1.4 Scope and Approach

To investigate the effects of the MS2 bacteriophage upon activated sludge activity and performance, quantitative and qualitative methods will be used to analyze data. It is first appropriate, however, to further define the terms activity and performance. Activity of the activated sludge bacteria will be inferred from metrics which describe microbial respiration. This refers to the specific oxygen utilization rate, cumulative O₂ consumption, and shape factors intended to compare respirometry curves between experimental and control groups. These factors will be measured using respirometric equipment with experimental, positive control, and negative control groups. Each group contains three samples monitored over distinct channels in the respirometry equipment. The experimental groups will contain activated sludge pulled from a batch reactor, feed chemicals simulating organic waste, and active MS2 virus particles. The positive control group will contain the activated sludge, feeds, and a known inhibitor of microbial respiration. Finally, the negative control group will contain only the activated sludge and feeds. Each group will be observed for a period of time long enough to allow for degradation of the organic feed chemicals. During the log growth and decay phases of the

microbial life-cycle, respirometric curves of each channel are produced. Statistical comparisons of these respirometric curves will be performed to evaluate significance between experimental and control groups.

Activated sludge bacteria performance will be measured by COD removal, ammonia removal, and NO_3 production. Following each respirometry experiment, samples from the experimental and control groups will be analyzed using spectrometry. Here, comparisons can be drawn between the effectiveness of each group in degrading organic waste. To do so, pre and post COD, ammonia, and NO_3 concentration levels will be measured and graphically compared.

Finally, to serve as a qualitative complement to the aforementioned parametric and graphical approach, microscopy involving dual-fluorescence labeling will be performed. Specifically this will involve using DAPI and FM 4-64 dyes to stain the SSRNA of the MS2 and bacterial cell walls, respectively. The intent is to uncover the extent of infection and qualitatively account for the virus' tendencies within the activated sludge floc and supernatant.

1.5 Summary

Wastewater treatment mediates the interface between human development and the natural environment. The suspended growth activated sludge treatment method is commonly used to treat human, animal, and industrial waste products. Activated sludge treatment is a biological process subject to variability and degraded performance resulting from exposure to biological contaminants. The purpose of this research effort is to evaluate the effect of MS2 on activated sludge and categorize its associated risk to

wastewater infrastructure. This will be performed using a variety of quantitative and qualitative techniques intended to link observations and report meaningful findings.

2. Literature Review

2.1 Chapter Overview

To provide context for the activated sludge treatment method and microbial environment, several topics merit discussion. Within suspended growth treatment processes, several methods are commonly used to influence the metabolism of the microbes responsible for consuming organic waste. These microbes, and subsequent unit processes, are also susceptible to inhibition from a range of constituents. These constituents, and their influence upon the fate of commonly encountered microorganisms, have been well documented (Metcalf & Eddy, 2015; Ricco, Tomei, Ramadori, & Laera, 2004; Crites, 1998; Vaccari, Strom, Alleman, 2006).

2.2 Microbial Mediated Treatment Processes

In the activated sludge process, microorganisms remove organic and inorganic pollutants from wastewater. A conventional activated sludge wastewater treatment plant (WWTP) schematic is shown in Figure 1. Influent wastewater is first treated within a primary clarifier to remove settleable particles. The primary effluent is then fed to an aeration basin where soluble organics and nutrients are removed by a flocculent suspension of microorganisms. The wastewater is then routed to a secondary clarifier for solids separation and biomass recycle (Metcalf & Eddy, 2015).

In conventional processes, activated sludge bacteria grow by using oxygen as the electron acceptor and by using organic carbon within wastewater pollutants as the electron donor. Wastewater treatment is achieved by microbial degradation of these wastewater pollutants via heterotrophic and autotrophic microorganisms. Heterotrophs are responsible for the removal of organic chemicals while autotrophs are responsible for nitrification. Aerobic conditions are required for nitrifying bacteria to convert this ammonia-nitrogen into nitrite and then nitrate as part of the nitrification process (Crites, 1998).

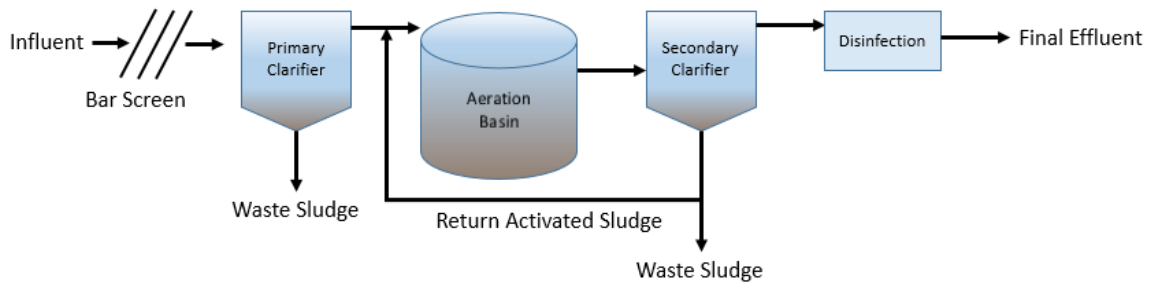
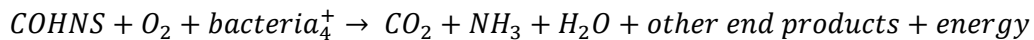
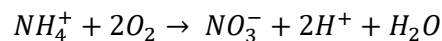
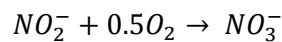


Figure 1 Conventional aerobic treatment schematic (Metcalf & Eddy, 2015)

The stoichiometry for removal of organic chemicals (measured as biochemical oxygen demand) and nitrification are as follows (Metcalf & Eddy, 2015):



The stoichiometry for nitrification is as follows (Crites, 1998):



Autotrophic bacteria capable of oxidizing ammonia include *Nitospira*, *Nitrosomonas*, *Nitrosococcus*, *Nitrosolobus*, and *Nitrosorobrio* (Avrahami & Bohannan, 2007; Metcalf & Eddy, 2015). Other autotrophic bacteria are responsible for oxidizing nitrite and include *Nitrobacter*, *Nitrococcus*, *Nitrospira*, and *Nitroeystis* (Metcalf & Eddy, 2015). While autotrophic bacteria perform nitrification, heterotrophic bacteria remain active within the activated sludge consortia. Heterotrophic microorganisms consume carbon from organic chemicals and consist of protozoa, fungi, and most other bacteria genera (Crites, 1998). Some of these include *Acrhomobacter*, *Acinetobacter*, *Agrobacterium*, *Alcaligenes*, *Bacillus*, *Chromobacterium*, *Moraxella*, *Paracoccus*, *Pseudomonas*, *Spirillum*, and *Vibrio* (Metcalf & Eddy, 2015).

2.3 Inhibition of Activated Sludge

Microbial mediated processes rely upon manipulating various environmental conditions to provide competitive advantages for particular bacteria. These specific bacteria are targeted because each is intended to further purify effluent before it is reintroduced to the natural environment. Because each of these processes are biological, enzyme-driven reactions, they are subject to variability and vulnerable to metabolic inhibition of substrate consumption (Vaccari, Strom, & Alleman, 2006).

2.3-A Metals and Inorganics

Metals and inorganic particles inhibit waste treatment plant operations by disrupting the oxygen transfer process necessary to sustain microbial life. Some metals more readily accept electrons from organic wastes in the influent. Others disrupt the metabolism of activated sludge bacteria or influence the functional community of

bacteria which thereby retards or disrupts plant operations. Regardless of the manner of inhibition, much is known about the tendencies of soluble metals as they interact with treatment processes.

Under laboratory conditions, chromium (VI), copper, nickel, and zinc were observed to reduce the efficiency of aerobic treatment at the threshold concentrations indicated in Table 1 (Barth, Ettinger, Salotto, & McDermott, 1965). Higher final effluent chemical oxygen demand (COD) concentrations were observed as influent metal concentrations increased. However, concentrations higher than these threshold values within the “plateau region” did not significantly reduce aerobic operation efficiency. This suggests that the activated sludge demonstrated an ability to adapt to continuous exposure to soluble metal concentrations (Barth et al., 1965).

Table 1 Threshold Influent Concentrations for Continuous Exposure

Metal	Concentration in Influent Sewage (mg/l)
Chromium (VI)	10
Copper	2
Nickel	1 to 2.5
Zinc	5 to 10

ZnO nanoparticles, although at much higher concentrations, exhibited similar inhibitory characteristics on raw and filtered wastewater. During treatment of raw wastewater, oxygen uptake inhibition as high as 35% was observed when a 2 L sequencing batch reactor was exposed to ZnO nanoparticles at a concentration of 1500 mg/l (Cervantes-Avilés & Cuevas-Rodríguez, 2017). ZnO-NP also inhibited COD removal at concentrations of 450 mg/L within the same study. Manganese II, at 2 mg/L,

was observed to inhibit activated sludge respiration after prolonged exposure of 24 hours (Aragón, Coello, & Quiroga, 2010). You et al. (2009) observed similar inhibitory effects using Cd and Pb.

In addition to degraded COD and oxygen uptake efficiencies, metals have been observed to alter bacterial communities. In one study, exposure to ZnO and Ag nanoparticles increased populations of metal resistant, sludge bulking, and biosorption bacteria (Chen et al., 2014). The resulting overproduction of bulking bacteria can lead to high effluent suspended solids and poor settling (Metcalf & Eddy, 2015).

2.3-B Organics

While most organic wastes are degraded within wastewater treatment applications, several organic compounds have demonstrated the ability inhibit unit processes or resist treatment (Vaccari, Strom, & Alleman, 2006). Commonly encountered industrial waste products such as 3,5-chlorophenol, formaldehyde, 4-nitrophenol, and dichloromethane were observed to disrupt waste degradation and sludge settling (Ricco, Tomei, Ramadori, & Laera, 2004). In another study, long-term continuous exposure to malathion was observed to alter activated sludge bacterial communities, thereby impacting treatment performance (Rauglas et al., 2016). In yet another study, maximum inhibitory concentrations of cyanide, phenol, and 4-nitrophenol were determined after exposure to activated sludge in batch reactors (Inglezakis et al., 2017).

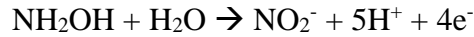
2.3-C N-allylthiourea

Allylthiourea (ATU) is commonly known to inhibit the oxidation of ammonia. The enzyme ammonia monooxygenase, within nitrifying bacteria like *Nitrosomona*,

catalyzes ammonia according to the following chemical reaction (Juliette, Hyman, & Arp, 1993).



Hydroxylamine (NH_2OH) is then produced and oxidized by the enzyme hydroxylamine oxio-reductase according to the following chemical reaction (Juliette et al., 1993).



When introduced to activated sludge, ATU consumes the reactive capacity of the ammonia oxygenase enzyme prior to the complete digestion of organic waste products. This is a result of ATU's high affinity for the copper present within the ammonia monooxygenase enzyme (Hofman & Lees, 1953).

2.4 Pollutants Found in Wastewater

A conventional activated sludge treatment plant is often exposed to a non-specific range of contaminants in addition to organic wastes. The term biological contaminants is applied to numerous chemicals and microorganisms which could potentially degrade unit process effectiveness. Describing the effect of these contaminants on conventional treatment systems also has been the topic of several investigative efforts. These efforts are summarized according to four categories: priority pollutants, bacteria, viruses, and emergent organic compounds.

2.4-A Priority Pollutants

The U.S. EPA has designated 126 wastewater constituents as priority pollutants (US-EPA, 2004). These constituents include inorganic and organic compounds selected for their potential adverse human health impacts due to exposure (Metcalf & Eddy,

2015). Many of these pollutants are considered volatile organic compounds (VOC). VOCs have a boiling point less than or equal to 100°C and/or a vapor pressure greater than 1 mm Hg at 25 °C (Vaccari, Strom, & Alleman, 2006). VOCs present a concern for release into the environment and adverse health risks for treatment plant employees (Metcalf & Eddy, 2015). As a result of the hazardous nature of the chemicals, the EPA has included appropriate regulations in their National Pollution Discharge Elimination System (NPDES) and developed test methods for their detection (U.S. EPA, 2002).

2.4-B Bacteria

Influent wastewater contains a diverse microbial ecology with many pathogenic and non-pathogenic bacteria (Metcalf & Eddy, 2015). Some bacteria have demonstrated a degree of resistance to conventional aerobic treatment. Of particular concern in developed countries is the occurrence of bacteria within the *Salmonella* genus being found in treated sludge or discharge waters (Santiago et al., 2017). Typically, investigators look to determine the degree of infection by enumerating total or fecal coliforms. Coliforms are aerobic rod-like bacteria that ferment lactose within 48 hours at 35°C (Vaccari, Strom, & Alleman, 2006). Testing for coliforms is used to determine the amount of bacteria, usually found in the human intestinal tract, which prefer the same environmental and growth stimulating conditions as more harmful and infectious bacteria (Metcalf & Eddy, 2015). Most strains of *Escherichia Coli* (*E. coli*) fit into this category. Santiago et al. discovered fecal coliforms in concentrations as high as 100 CFU/ml in treated sludge used for food crops. Further, Wen et al. (2009) determined *E.coli* log removal values of only 2.06 ± 0.26 when treated with a lab-scale sludge treatment

process. Effluent readings like these indicate that some form of disinfection or tertiary treatment is necessary prior to discharging into surface or ground waters.

2.4-C Viruses

The removal of viruses from wastewater varies depending on the treatment regime. Zhang and Farahbakhsh (2007) examined the virus removal effectiveness of a conventional activated sludge (CAS) treatment plant which included primary and secondary treatment followed by sand filtration and chlorination. The plant was able to achieve between a 3.2 and 5.5 log reduction in F⁺ specific coliphage (Zhang & Farahbakhsh, 2007). Advanced water treatment (AWT) unit processes have been modeled to approximate virus removal effectiveness of varying treatment trains. Olivieri et al. (1999) seeded several pilot scale AWT unit processes using MS2 and approximated the distributions of virus log removal values (LRV). LRVs for the varying treatment trains were then modeled using a Monte Carlo simulation based on the approximated distributions. The results of this simulation allowed the direct comparison of the effectiveness of the various treatment schemes. Median values of the treatment trains varied between 13.6 and 21.6 logs (Olivieri et al., 1999). Results from this analysis are not intended to communicate expected performance of installed systems; however, the overall effectiveness of AWT unit processes on removal of influent virus concentrations can be inferred. Other studies have investigated virus removal effectiveness using membrane bioreactors (MBR). Ueda and Horan 2000 found the biofilm accumulation on MBRs to be significant in the removal of coliphage. Without the biofilm accumulation, LRVs ranging from 2.3-5.9 were achieved. However, with the biofilm, up to 7 log removal was achieved (Ueda & Horan, 2000).

2.4-D Emergent Organic Compounds

Numerous compounds, for which regulations have not been developed, are being encountered at waste treatment facilities and are subsequently discharged to effluent waters (Metcalf & Eddy, 2015; Montes-Grajales, Fennix-Agudelo, & Miranda-Castro, 2017). Kolpin et al. (2002) investigated 139 effluent streams for the presence of 95 emergent compounds. Chemicals selected for investigation were determined to be potentially hazardous to environmental ecology and human health. These included antibiotics, prescription and non-prescription drugs, endocrine-disrupting chemicals, and combustion by-products. Within 80% of the 139 streams sampled within the Kolpin et al. study, at least one of the 95 contaminants of concern was observed (Kolpin et al., 2002). This suggests that these chemicals are resistant to degradation by waste treatment unit processes. While potable reuse of susceptible receiving waters can pose a health risk to humans, of equal concern are the long-term environmental and ecological effects of exposure upon local flora and fauna.

Effects of emergent compounds upon aquatic wildlife have been well documented (Núñez, Borrull, Pocurull, & Fontanals, 2017; Pal, Gin, Lin, & Reinhard, 2010). The overarching theme within this body of research is that these contaminants are hazardous to aquatic wildlife at very low ($\mu\text{g/L}$) concentrations and are often resistant to conventional activated sludge treatment methods (Carlsson, Johansson, Alvan, Bergman, & Kühler, 2006; Hoeger, Köllner, Dietrich, & Hitzfeld, 2005; Tran & Gin, 2017). Risks due to exposure have ranged from gene expression in fish to inhibited growth of human kidney cells (Pal et al. 2010). As these chemicals continue to be introduced into the

environment, future regulations of the use and discharge of these emergent compounds are anticipated (Metcalf & Eddy, 2015).

2.5 The Use of Respirometry to Detect Microbial Inhibition

The bacteria responsible for the degradation of substrates during nitrification require the presence of oxygen. Respirometry equipment allows for the measurement of respiration of activated sludge (Spanjers, Vanrolleghem, Olsson, & Dold, 1996). This research will incorporate experiments using respirometry equipment to account for the oxygen consumed and carbon dioxide produced to develop insights into the microbial activity and metabolism underlying the nitrification process.

The respirometry experiment has been implemented in diverse studies to examine whether or not specific compounds or biological contaminants impact respiration rates and to model the interaction between these substances and activated sludge. Aragon et al. (2010) investigated the effect of manganese II on activated sludge sampled from two wastewater treatment plants. Respirometry equipment was used to expose the sludge samples to manganese(II) at times ranging from 30 minutes to 24 hours (Aragón et al., 2010). A similar study attempting to determine the toxicity of volatile organic compounds when exposed to activated sludge also used respirometry equipment. From this study, 3,5-dichlorophenol was determined to be more toxic than other compounds investigated (Ricco, Tomei, Ramadori, & Laera, 2004). Further, Rauglas et al. (2016) examined the effect of malathion exposure on the activity and performance of activated sludge. Using data collected during a respirometry experiment, Rauglas et al. (2016)

developed respirometry profiles and molar O₂ to CO₂ ratios to observe impacts to microbial metabolism.

2.6 The Use of MS2 Bacteriophage as a Surrogate

The U.S. EPA is responsible for implementing the Safe Drinking Water Act (SDWA) which governs contaminant levels and treatment methods used for the provision of potable water. However, even with the implementation of the SDWA and World Health Organization regulations, around the world, enteroviruses like the MS2 bacteriophage are still being detected in treated water (Vivier, Ehlers, & Grabow, 2004). This, along with the complexities associated with enumerating enteric viruses, has spurred interest among researchers to investigate and even model the inactivation of enteric viruses using surrogates (Kim, Kim, & Kang, 2017). Surrogate viruses are selected based on the similarity of their reproductive methods, shape, size, preferred environmental conditions, and relative ease of enumeration (Grabow, 2001). Based on this supporting research, the U.S. EPA has recommended using the MS2 bacteriophage to model the fate of human enteric viruses in wastewater treatment processes (U.S. EPA, 2004).

Bacteriophages are viruses that infect bacterial cells (Metcalf & Eddy, 2015). The MS2 virus is a male-specific, single stranded RNA phage with an icosahedral capsid (Grabow, 2001). It belongs to the Leviviridae family of viruses and is commonly known to infect the pilus appendage of male Escherichia Coli cells (Metcalf & Eddy, 2015; Withey, Cartmell, Avery, & Stephenson, 2005). Early studies devoted to evaluating MS2 as a surrogate for human enteric virus found sewage concentrations of MS2 to be higher

than fecal concentrations. This implies that the virus was able to replicate in the aquatic sewage environment similar to human enteric viruses like norovirus, thus indicating a similarity in preferred environmental conditions (IAWPRC, 1991). The similar physical characteristics and environmental preferences, along with the ease of enumerating the virus, has led to its wide acceptance and use as a surrogate for human enteric viruses.

Amarasiri et al. (2017) investigated the use of MS2 as an indicator virus to approximate the removal efficiencies of conventional activated sludge and membrane bioreactor treatment methods. Their intention was to first determine whether or not there exists a high correlation between the LRV of bacteriophages and human enteric viruses then use this to estimate plant effectiveness at removing human enteric viruses. Notably, similar LRVs for bacteriophages and human enteric viruses were not achieved. In fact, they determined that the average LRV for MS2 averaged 1.99 which was lower than all other human enteric viruses examined. Because of this, MS2 was determined to be suitable, and conservative, surrogate for approximating human enteric virus removal at waste reclamation facilities (Amarasiri, Kitajima, Nguyen, Okabe, & Sano, 2017).

In another application using MS2 as a surrogate for norovirus, Tanner et al. investigated the viability of surface-mediated infectivity of bio contaminants following treatment using a novel disinfection technique (Tanner, 2009). The MS2 virus was again selected for its physical similarity to norovirus and ease of enumeration post-treatment. Tanner (2009) achieved disinfection of virus particles and concluded that the treatment method should be as effective when targeting other encapsulated virus particles like HIV (Tanner, 2009). In addition to serving as a surrogate for human enteric or other viruses,

bacteriophages are also used to investigate the virus-host relationship within treatment processes.

Withey et al. (2005) asserted that bacteriophages could play an important role in mediating the bacterial ecology within activated sludge treatment systems (Withey, Cartmell, Avery, & Stephenson, 2005). For instance, when exposed to environmental stressors such as extreme pH levels, heavy metals, or toxic chemicals, certain bacteria induce phage that otherwise would remain dormant (Choi, 2012). More specifically, Choi (2012) investigated, and demonstrated success in, the application of lytic viruses in the control of one species of filamentous bacteria.

2.7 Bacteriophage Concentrations Observed in Wastewater

The MS2 virus, and other bacteriophages, are encountered in varying concentrations at wastewater treatment plants. Within the literature, influent concentrations ranged from 1.7×10^3 to 1.8×10^8 plaque forming units (PFU) ml^{-1} (Ueda & Horan, 2000). Seed concentrations within the Olivieri study, which modeled MS2 removal by various treatment regimes, were between 1×10^{10} and 1×10^{11} PFU ml^{-1} (Olivieri et al., 1999). Further, Ueda and Horan (2000) observed concentrations of bacteriophage ranging from 5.2×10^1 to 1.1×10^8 PFU ml^{-1} within a membrane bioreactor. A high concentration of MS2 was desirable in this study to best observe impacts attributable to infection. A similar concentration of seed virus to the Olivieri et al. (1999) study, 3.2×10^8 PFU ml^{-1} , was selected for use in this experiment.

2.8 Viral Infection of Activated Sludge Bacteria

Viruses are naturally occurring in the activated sludge consortium. While host specificity largely drives the interaction between virus and bacteria species, this relationship has exhibited the potential to impact waste treatment processes. Recently, investigations have focused on how to operationalize viruses known to infect filamentous bacteria (Kotay, Datta, Choi, & Goel, 2011). Researchers achieved improved settling within experimental groups exposed to lytic viruses compatible with the bulk forming bacteria. Further, bacteria responsible for ammonia degradation have also been observed to induce lysogenic bacteriophages in the presence of chemical and environmental stressors (Choi, Kotay, & Goel, 2010). Choi et al. (2010) investigated the performance of ammonia oxidizing bacteria exposed to environmental agents intended to induce phage induction. These environmental stressors included extreme pH conditions, chromium (VI), and potassium cyanide. Researchers observed phage induction in all tested environmental stress conditions and concluded that lysogeny could plausibly degrade treatment efficiency (Choi et al., 2010).

2.9 Summary

While any biological process is subject to variability, a great deal is known about the enzyme-driven processes of activated sludge bacteria. Further, there are numerous constituents that exhibit inhibitory effects of this process through various mechanisms. Investigating the risk of exposure to these volatile, toxic, and hazardous constituents necessitates the use of surrogates. The MS2 virus has been studied previously as a norovirus surrogate and used as an indicator virus because of its physical characteristics

and preferred environmental conditions. Conclusions from this data will serve to contribute to the body of knowledge characterizing the risk to treatment processes resulting from exposure to high consequence biological contaminants.

3. Methodology

3.1 Overview

The intent of this research is to describe the effect of the norovirus surrogate, MS2, on the activity and performance of activated sludge. The effect of the MS2 virus on activated sludge activity was measured by respirometry. The effect of the MS2 virus on activated sludge performance was measured by analyzing final effluent chemical oxygen demand (COD), ammonia, and nitrate via spectrometry. Additionally, microscopy using dual fluorescence labeling was used to describe the interaction between the MS2 virus and activated sludge.

3.2 SBR Operation

Two two-liter sequencing batch reactors (SBR) were used to simulate a suspended growth waste treatment process. The SBRs contain activated sludge sampled from the Fairborn, Ohio wastewater treatment plant (WWTP) and were seeded on 4 January 2016. Every eight hours, the SBRs were injected with 9 mL of feed A, 18 mL feed B1, and 18 mL feed B2 in the concentrations enumerated in Table 2.

Table 2 Sequencing Batch Reactor Feed Concentrations

Name	Concentration (g/L)
Feed A Bicarbonate (0.5L/month)	
Sodium bicarbonate	44.6
Feed B1 Macronutrients (3.0L/month)	
Peptone	6
Sodium Acetate	125
Ammonium Chloride	2.26
Magnesium Chloride	6.86
Calcium Chloride	1.72
Potassium Phosphate	0.6675
Feed B2 Trace Elements (60mL/month)	
Citric Acid	5.46
Hippuric Acid	4
Nitriloacetic Acid	0.72
Sodium EDTA	0.3
Ferric Chloride	3
Boric Acid	0.5
Zinc Sulfate	0.3
Manganese Chloride	0.24
Copper (II) Sulfate	0.14
Potassium Iodide	0.06
Sodium Molybdate	0.06
Cobalt Chloride	0.06
Nickel Chloride	0.06
Sodium Tungstate	0.06

After being fed and aerated, the reactors were allowed to settle for one hour. On average, 796 mL and 800 mL of effluent was then drawn from reactor 1 and 2 respectively. Eight hundred mL of deionized water was then added to each reactor before the feeding, decanting, and effluent discharge cycle repeated. Total suspended solids (TSS) and volatile suspended solids (VSS) tests were performed on the effluent to determine the effectiveness of treatment and overall condition of the reactors. Additionally, TSS/VSS testing was performed on the sludge to categorize the amount of biomass in the reactors.

Sludge VSS readings required 25 ml of sludge drawn from each reactor to provide duplicate 10 ml samples. Two hundred ml of effluent samples were taken from each SBR and applied to 1.5 μm glass microfiber filters. Each sample was then heated to 106°C for 2 hours in a Fisher Scientific Isotemp oven. Following the oven, the samples were immediately weighed using a Mettler AT 261 Delta Range scale (0-205 g). The sludge VSS samples were then placed in a Vettler 3-31D Box Furnace for 20 min at 550°C. Afterwards, pre and post treatment filter weight measurements were compared along with the effluent and sludge accumulations. In addition to the TSS and VSS readings, effluent pH was measured using a Mettler Toledo Seven Multi pH mVORP. Ranges of TSS, VSS, DO, pH, and solids retention time (SRT) values are displayed in Table 3.

Table 3 Ranges for Descriptive Metrics of Sludge and Effluent Samples

Measure	Units	Sludge Ranges		Effluent Ranges	
		Reactor 1	Reactor 2	Reactor 1	Reactor 2
TSS	mg/L	3030	4454	25.42	39.04
VSS	mg/L	1923	1687	35.08	29.84
DO	mg/L	8.268	8.251	-	-
pH	-	6.939-9.124	7.646-8.956	6.876	7.406
SRT	Days	52.22	50.43	52.22	50.43

3.3 Respirometry

Respirometry experiments were conducted using a Columbus Instruments Oxymax Respirometer made in Columbus, OH. Each experiment used 9 of the 20 available channels from which to monitor – three negative control channels, three positive control channels, and three experiment channels. The Oxymax equipment was connected to two pressurized containers with nitrogen gas and mixed gas with the following proportions: 0.5% CO₂, 20.5% O₂, and 79% N. The total atmospheric headspace within each bottle was approximately 350 ml. Prior to respirometry

experiments, this headspace was calibrated to ensure atmospheric ratios were as close as possible to the aforementioned proportions. The test chamber headspace sensitivity, or expected noise, is between 0.01-0.02 $\mu\text{l}/\text{min}$ given the duration of the experiment according to the Columbus Instruments Micro Oxyman V6.03 Software Manual.

Following calibration, 475 ml of sludge was pulled from reactor one and contained within a 500 ml beaker. 50 ml of sludge was then added to each of the nine bottles used during the experiment. Next, the feed solutions were added. Each bottle received 200 μl of feed A, 425 μl of feed B1, and 425 μl of feed B2. The three positive control bottles received 250 μl of allylthiourea (ATU). The three experimental bottles received 1000 μl of 1.6×10^{10} PFU/ml MS2. The MS2 virus was stored in a refrigerator in a largely glucose media. Prior to experimentation, the MS2 was isolated from the media solution via a series of centrifuge filtrations. Once separated from the media, the MS2 were injected into testing sample bottles which contained 50 ml of sludge. The resulting MS2 concentration within each testing sample was 3.2×10^8 PFU/ml.

Each bottle was aerated with a rotating magnetic stir bar for the duration of the experiment, which typically lasted between 17 and 22 hours. O_2 consumption and CO_2 production rates were recorded in $\mu\text{g}/\text{min}$ and plotted versus time. Readings were taken from each bottle via Oxyman Respirometry equipment approximately every hour. The resulting O_2 and CO_2 profiles were compiled to include all nine channels on one graph. The resulting data were analyzed to determine whether or not there were significant differences between the experimental and control groups.

3.4 Analysis

Several methods were used to analyze respirometric data. These methods are employed to detect differences between respiration rates and quantities in order to describe the ability of the microbial population to transfer oxygen during waste oxidation.

3.4-A Shape parameters

The first moment of area (FrM) is commonly used to measure the distribution or centroid of an area relative to an axis. Using Equation 1, a discretized approximation of the integral was used to quantify the shape of oxygen consumption data. The first seven intervals of the O₂ consumption rate were used to describe the exponential growth curves preceding peak oxygen consumption. The “tail” of the data was omitted from this calculation to avoid distorting detectable effects on respiration from the MS2 virus during peak respiration.

$$Y = \int_b^a xf(x) \quad (1)$$

Where: $\int_b^a xf(x)$ = area under the curve and x = distance from the vertical axis.

The skewness of a data set is the measurement of asymmetry of a probability distribution about its mean given by Equation 2. The skewness metric was captured using the first five oxygen consumption rate data points. These points were then averaged and compared between experiment and negative control groups using a Student's t-test.

$$X = \frac{n}{(n-1)(n-2)} \sum \frac{(x_i - \bar{x})^3}{s} \quad (2)$$

Where n = number of elements s = standard deviation x_i = element of interest \bar{x} = mean.

3.4-B Peak oxygen consumption

Peak oxygen consumption was measured by selecting the maximum oxygen consumption rate ($\mu\text{g}/\text{min}$) for each sample regardless of the time interval during which it occurred. This peak rate was converted to a specific oxygen utilization rate (SOUR) after dividing by the reactor MLSS VSS and converting minutes to hours with units of $\text{mg O}_2\text{-g}^{-1}\text{ VSS-hr}^{-1}$. Once the peak SOUR was determined for each channel of the three samples, the mean was compared using a Student's t-test.

3.4-C Cumulative oxygen consumption

Cumulative oxygen consumption was measured for each channel over the duration of the experiment. The fourth time interval from each sample was used for comparison because this was typically the interval in which peak respiration occurred. Experiment and negative control samples' average cumulative oxygen consumption was then compared using a Student's t-test. These values were then plotted with error bars representing the respective standard deviations.

3.4-D Molar CO_2/O_2 ratio

Molar values for CO_2 and O_2 were reported by the Oxymax software. The molar CO_2/O_2 ratio was calculated by dividing the molar CO_2 production by its corresponding molar O_2 consumption rate. The molar ratios for each channel were then plotted against time. The corresponding plot offers insight into the underlying microbial metabolism within each sample.

3.5 Dual Fluorescence Labeling

To qualitatively analyze the interaction between the MS2 virus and activated sludge floc, dual fluorescence labeling microscopy was performed. For this qualitative assessment, MS2 was stained with DAPI dye while sludge samples were stained with FM 4-64. DAPI dye ($C_{16}H_{15}N_5$) stains the AT regions of nucleic acid material and is commonly used in fluorescence microscopy applications (Vaccari, Strom, & Alleman, 2006). FM 4-64 ($C_{30}H_{45}Br_2N_3$) is a lipophilic dye which emits red fluorescence under blue light excitation and can be used to outline cell membranes. The intent of the microscopy was to determine whether or not the MS2 virus infected bacterial cells within the activated sludge floc. The protocols for staining and imaging are outlined below.

In low light conditions, media solution containing 1.6×10^{10} PFU/ml MS2 was first purified of residual *Escherichia coli* (E.coli) cells by filtration using a sterile $0.22 \mu\text{m}$ filter. Media components in the solution were removed by using centrifugal filters with a molecular weight cut-off of 100 K. The sample was then placed into a 4-ml centrifugal filter unit, centrifuged at 4000 rpm for 20 minutes. Media components will pass through the filter membrane, while MS2 were retained on the filter. The filters were then washed with DI water, the washing solution contains purified MS2. Purified MS2 were then diluted with 5 ml of DI water and centrifuged at the same speed and duration. This DI rinse was performed three times. Five ml DI water was then added to the rinsed MS2 and $1 \mu\text{l}$ of $3 \mu\text{M}$ DAPI dye solution was added to the MS2 solution and incubated for 10 minutes. Next, the virus solution was washed twice in a centrifugal filter unit (MWCO: 100K) with 5 ml DI water and twice with 5 ml PBS at 4000 rpm for 15 minutes. Centrifuging the MS2, DI water, and DAPI stain solution at 4,000 rpm for 15 minutes

removes residual DAPI stain by membrane filtration. The filter used in the experiment has a molecular weight cut-off (MWCO) of 100 kilo-Daltons. The 100 kDa designator is assigned to a filter which retains 90% of a solute at or above the MWCO of 100 kDa. This means that 90% of a solute constituent with a molecular weight greater than 100 kDa will be retained by the filter following centrifugation. Because the molecular weights of DAPI dihydrochloride and DAPI dilactate are 350.3 and 457.5 respectively, these constituents, when unbound to MS2 ssRNA, will be removed by the iterations of centrifugation.

These washing steps remove free unbound DAPI from the solution. Finally, 5 ml DI water was added to the DAPI-labeled MS2. The tube containing labeled MS2 was then wrapped in aluminum foil, refrigerated and stored overnight.

Next, a respirometry experiment was performed using the purified and DAPI-labeled MS2 solution. Nine channels were used for the experiment. These respirometry protocols were the exact same as other experimental trials that did not use DAPI labeled MS2. Each channel was administered 50 ml of sludge from reactor 1, 250 μ l of feed A, 425 μ l of feed B1, and 425 μ l of feed B2. The experimental group received 1000 μ l of dyed MS2 solution. The positive control group received 250 μ l ATU. Experimental group bottles were covered with aluminum foil to prevent photo bleaching.

Two 1.5 ml samples were pulled from channels 1-3, then diluted with 1.5 ml of DI water. Afterwards, 10 μ l samples pulled and allowed to dry after being added to a glass slide. Next, 10 μ l of 1:199 FM 4-64/PBS solution were then added to the air-dried sample. Slides were then imaged at three different wavelengths using a 100X oil-

immersion objective: blue excitation with 10,000 ms exposure; ultraviolet excitation with 2,000 ms exposure; and brightfield with 200 ms exposure.

Blue light and UV light images were overlaid to observe whether or not the MS2 virus infected host bacterial cells. If infection were evident, the overlaid image would exhibit both a definable cell wall and a blueish-white fluorescing MS2 virion inside. This phenomena was demonstrated during the development of the dual-fluorescence protocols. In this example, Log phase E. coli was cultured and sampled prior to being dyed with FM 4-64. MS2 virus was stained using both DAPI and syto 9 nucleic acid dyes. The E. coli aliquot was then spiked with the labeled MS2 and imaged immediately. Figure 2 is intended to convey the nature of the overlaid UV and blue filter images. The exposure times for the image below were 4,000ms under blue excitation and 2,000ms under UV excitation.

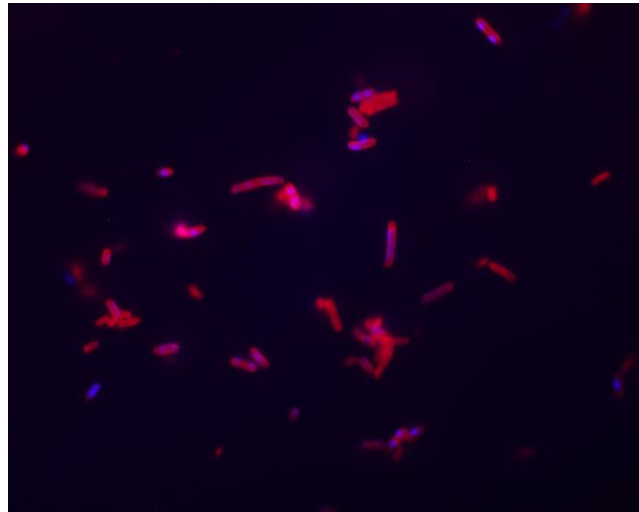


Figure 2 MS2 Infection of host E.coli bacteria

3.6 Summary

In this application, MS2 was introduced into a replicated activated sludge treatment process at a relevant concentration to simulate the advertent or inadvertent

release of norovirus into wastewater infrastructure. Determining the effect of the MS2 bacteriophage on the activity and performance of activated sludge required both quantitative and qualitative analytical techniques. Respirometric data was compared to spectrometry and microscopy in an attempt to provide a cohesive dialogue. The hypothesis for this research effort was that the experimental and negative control groups will not exhibit any significant differences when compared using the quantitative analytical methods presented in this section.

4. Analysis and Results

4.1 Chapter Overview

Five experimental trials and subsequent analysis are presented. One experiment was performed using MS2 suspended in media solution. Four experiments were performed using 3.2×10^8 PFU/ml of MS2. Simple t-tests were used to compare peak respiration rates and shape factors of the three experimental and three negative control samples. Only the first eight sampling intervals of each O₂ respiration curve were used for first moment of the area and skewness analysis to mitigate distorting effects of endogenous respiration rates. The CO₂/O₂ profiles for each experiment are also presented. Summary graphs containing quantitative comparison data are included in Appendix A.

4.2 Microbial Respiration

4.2-A Trial 1 - The effect of MS2 at unknown concentration and media on microbial respiration

During Trial 1, experimental bottles received 1000 µl of an unknown concentration of MS2 and media solution. This concentration was unknown to researchers at the time as the protocols for isolating the MS2 from media were still being developed. The effect of MS2 suspended in media did not exhibit a significant effect on microbial respiration during the first seven sampling intervals. Afterwards, however, the largely glucose media drastically impacted the metabolism of the bacteria. This is evidenced by the oscillating tendency of the experimental O₂ curve after the seventh

sampling interval as evidenced in Figure 3. Once all of the media was consumed by the bacteria, the O₂ consumption rate decreased to endogenous respiration rates consistent with microbial decay. Channel 8 exhibited the highest O₂ respiration rate during the fifth hour of the experiment although Channels 2 and 3 of the experimental groups exhibited the highest endogenous respiration rates. Significant differences were detected using a Student's t-test means comparison at 95% confidence. Comparing the experimental and negative control groups resulted in a p-value of 1.5×10^{-8} . The experimental and positive control groups resulted in a p-value of 1.6×10^{-15} . The comparison between the negative and positive control groups resulted in a p-value of 0.0003. The significant difference observed when comparing all groups indicates that the microbial respiration tendencies were different. This significance is reported using a violin plot in Figure 4. The largely glucose media contributed significantly to the microbial respiration in the experimental samples while the ATU inhibited respiration within the positive control.

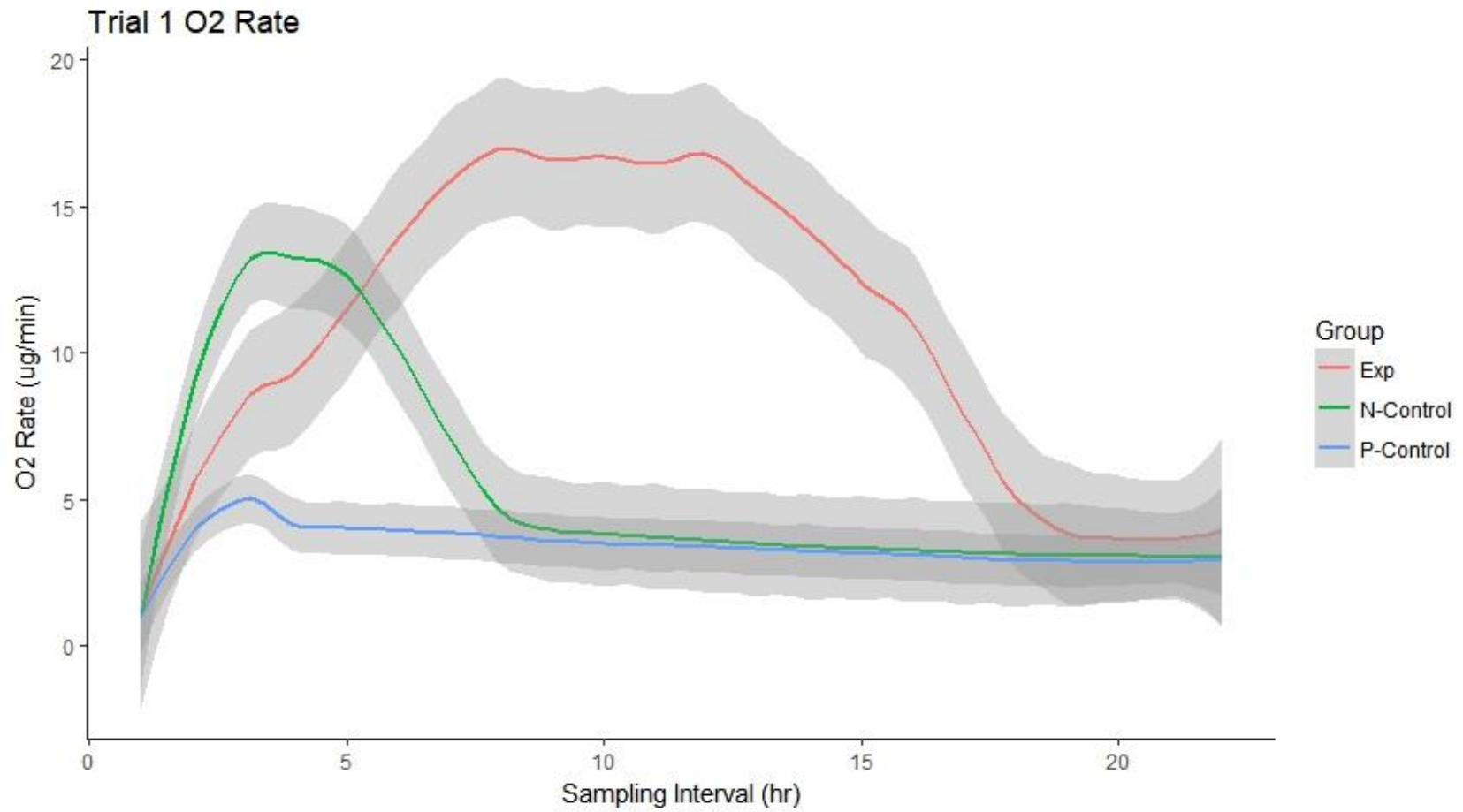


Figure 3 Trial 1 O₂ Consumption with MS2 at unknown concentration with media. Error regions represent 95% confidence interval determined by three replicate data points.

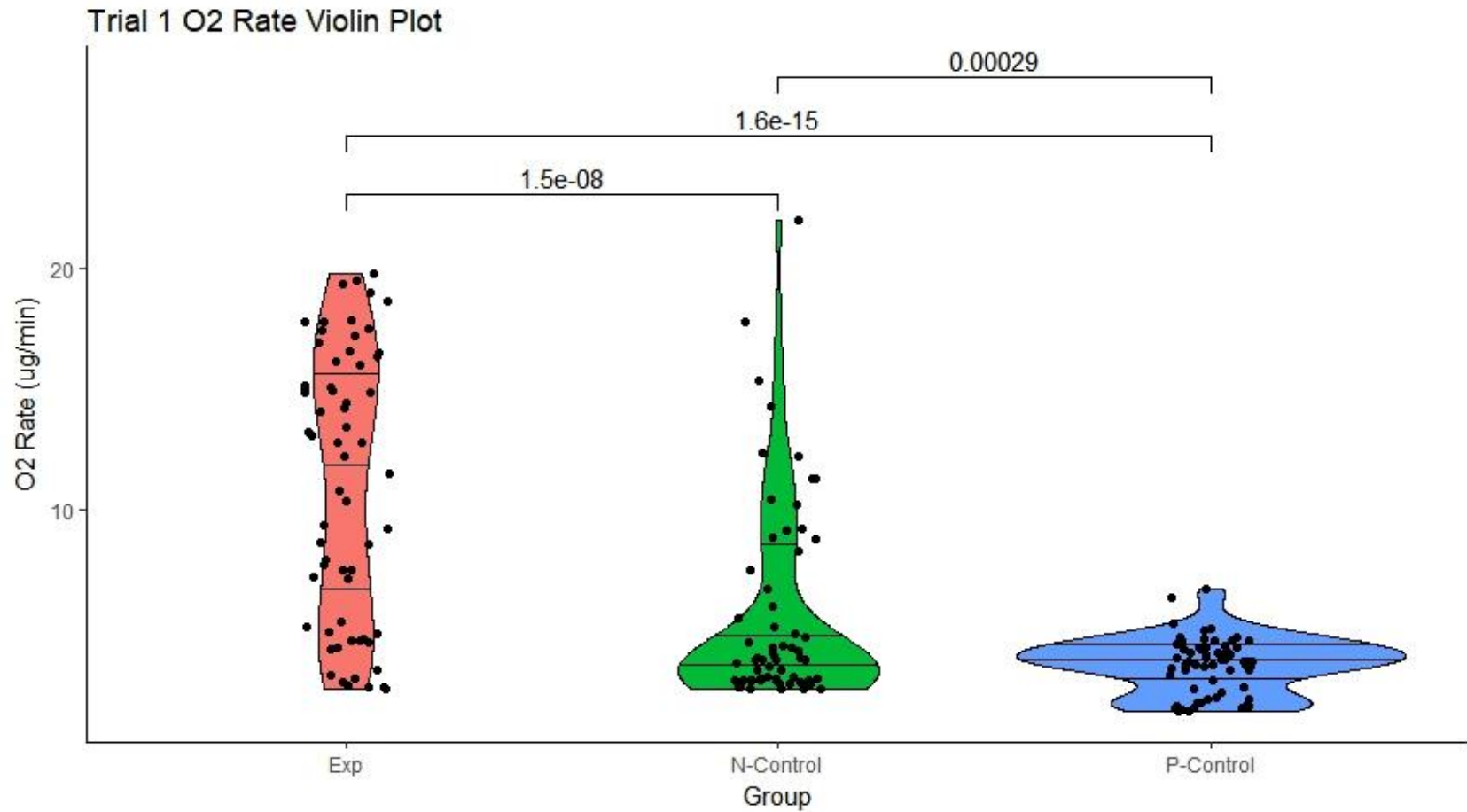


Figure 4 Trial 1 O2 Rate Violin Plot. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

Shape Parameters

The shape parameters of the O₂ consumption profiles were not statistically different from the negative control. The mean FrM of the experiment group was 394.04 $\mu\text{g O}^2/\text{min-hr}^2$ with a variance of 1479.3 ($\mu\text{g O}^2/\text{min-hr}^2$)². The mean FrM of the negative control was 340.21 $\mu\text{g O}^2/\text{min-hr}^2$ with a variance of 6955.8 ($\mu\text{g O}^2/\text{min-hr}^2$)². Comparing the FrM of the experimental and control groups yielding a p-value of 0.64. Figure 5 contains the bar chart as well as the statistical comparison information. The mean skewness of the experimental group was -0.635 with a variance of 0.0084. The mean skewness of the control group was -0.934 with a variance of 0.529. The T-test of the skewness shape factors resulted a p-value of 0.68. Figure 6 contains the skewness comparison bar chart. While a difference in shape factors exists as a result of the media after peak respiration, the initial seven hours of the experiment yielded results that were not statistically different between the experimental and negative control groups. This evidence suggests that the O₂ transfer mechanisms within the negative control and experimental samples were similar during the first hours of the test. Following this time period, however, a clear difference in shape is observed between the two groups.

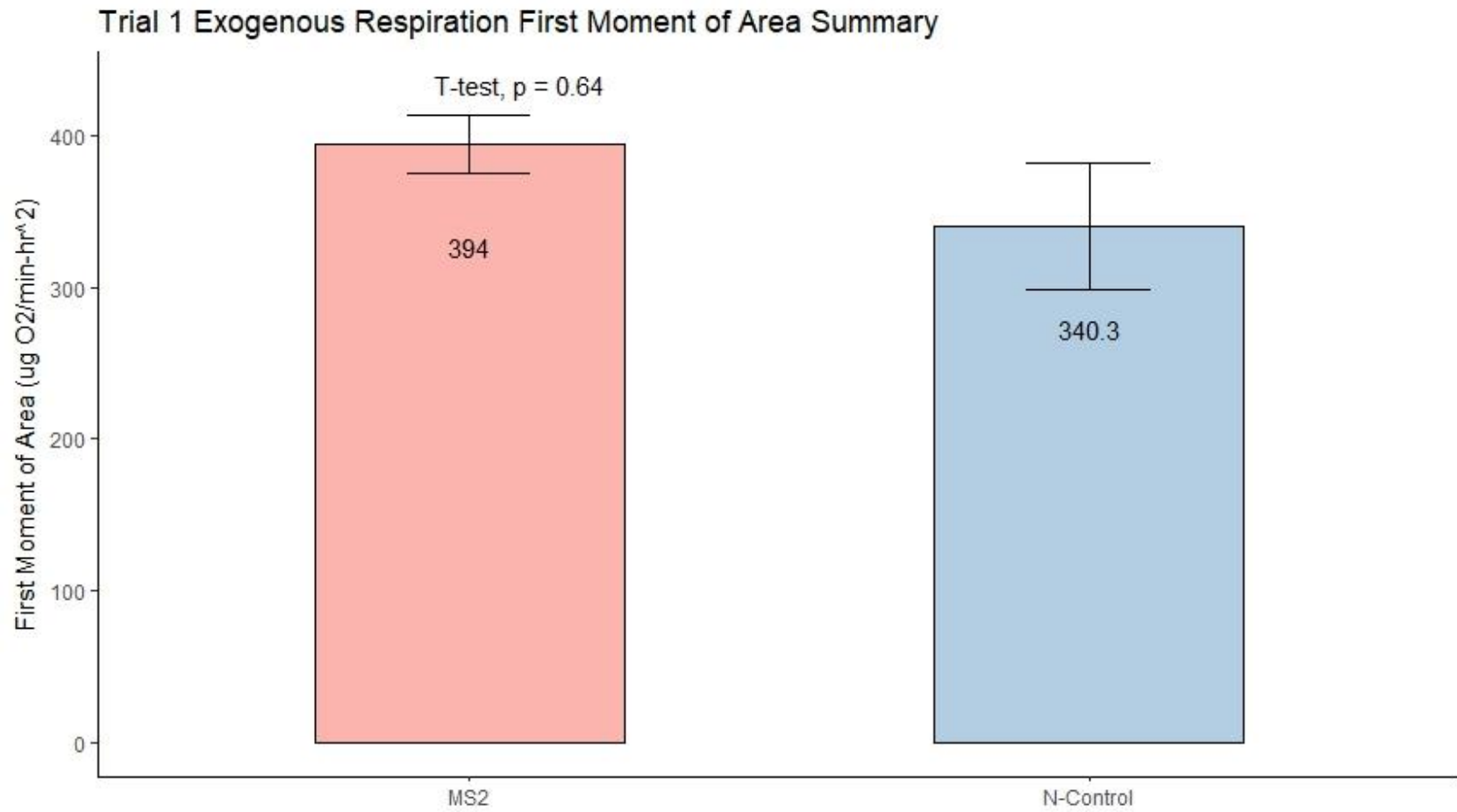


Figure 5 Trial 1 First Moment of Area. Error bars indicate standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

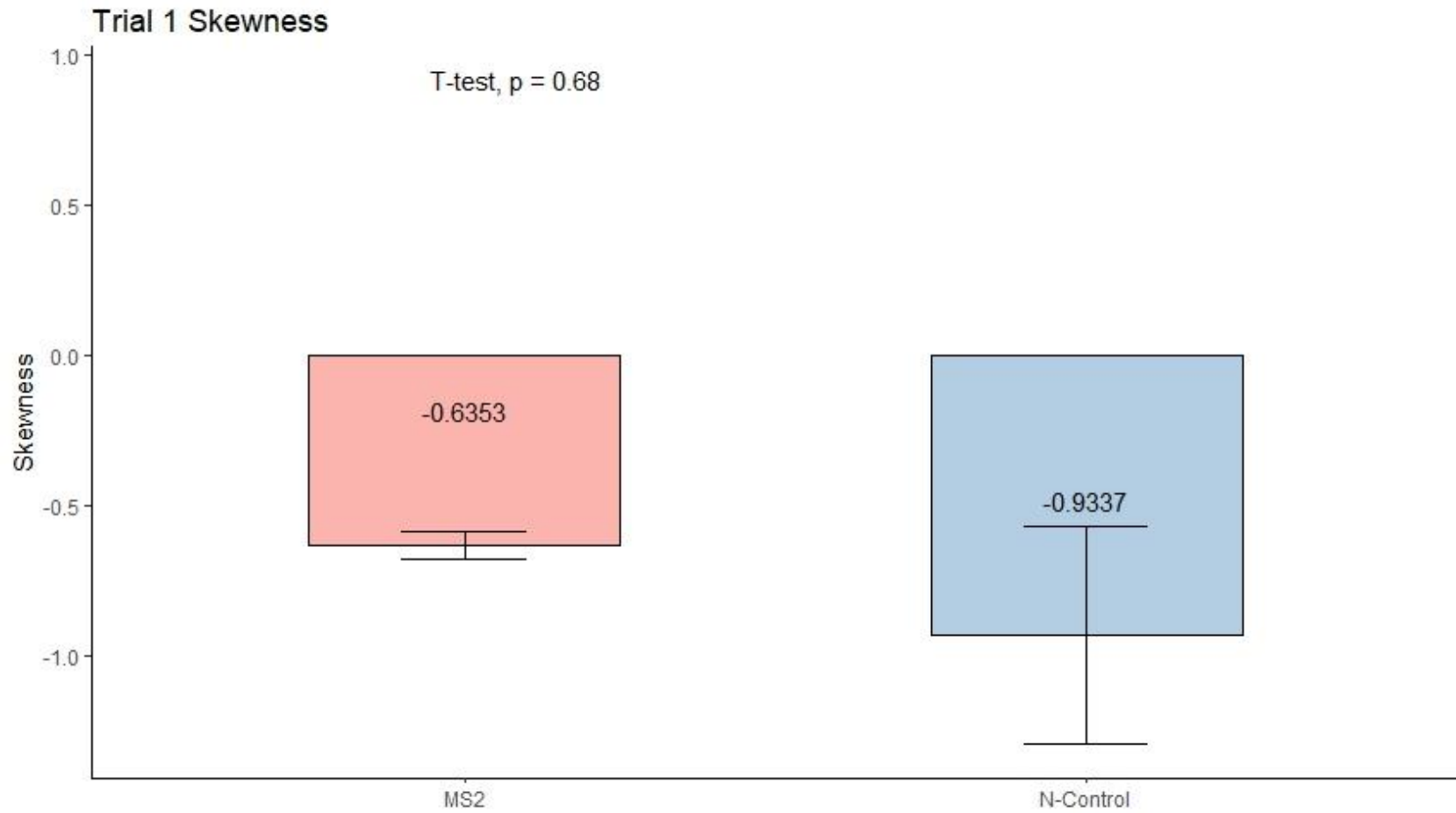


Figure 6 Skewness. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

Peak Oxygen Consumption Rates

Figure 7 presents the Trial 1 Peak O₂ consumption rate comparison data. The maximum mean SOUR of the experiment group was mg 13.1 O₂-g VSS⁻¹-hr⁻¹ with a variance of 0.91 (O₂-g VSS⁻¹-hr⁻¹)². The maximum mean SOUR of the negative controls was 10.4 O₂-g VSS⁻¹-hr⁻¹ with a variance of 23.0 (O₂-g VSS⁻¹-hr⁻¹)². A simple t-test comparing these peak rates of the three experimental samples and three control samples resulted in a p-value of 0.082. There was no statistically significant difference between the O₂ utilization rates for the experiment and negative control samples when comparing peak respiration rates normalized to SOUR.

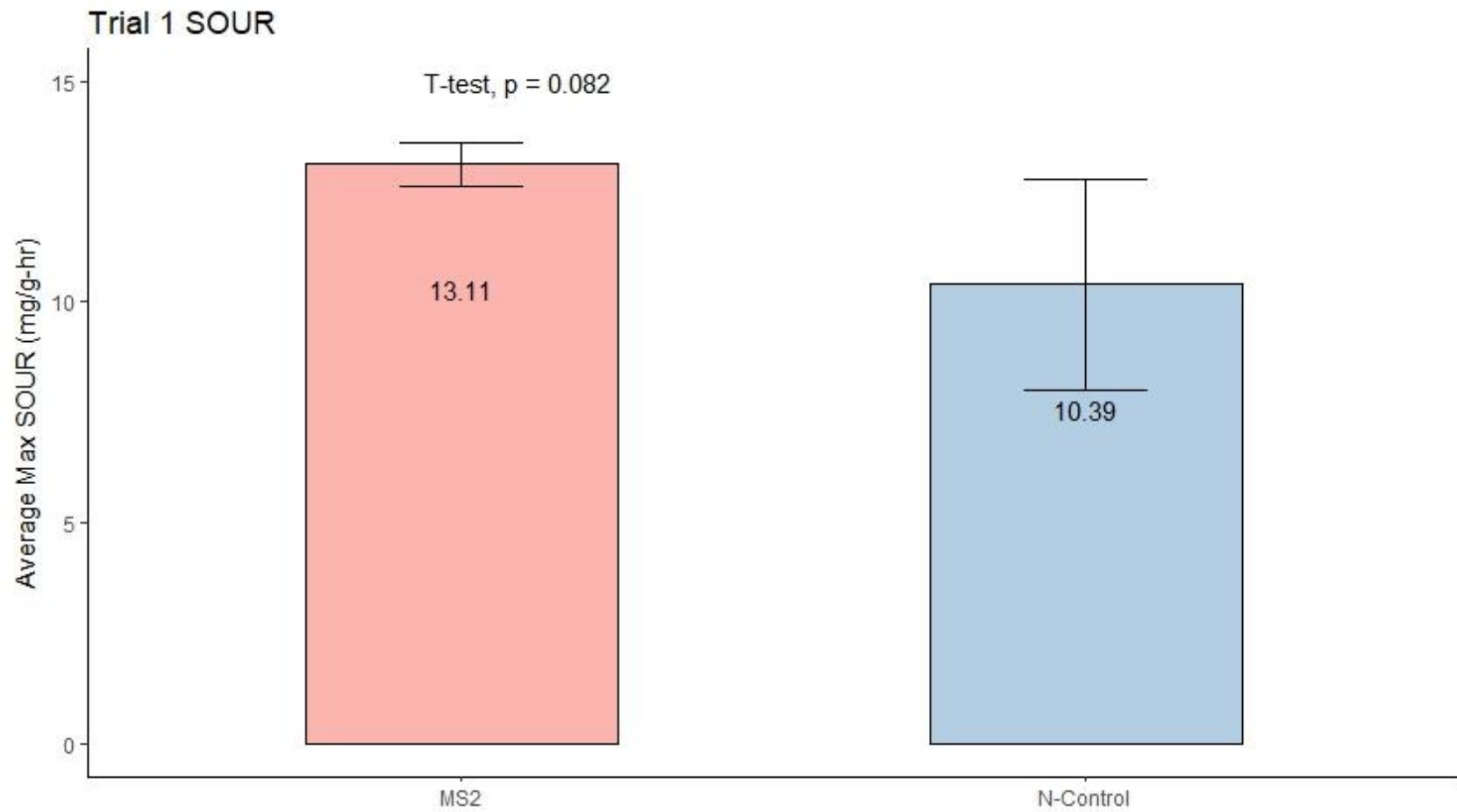


Figure 7 Specific Oxygen Utilization Rate. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

Cumulative Oxygen Consumption

Figure 8 contains the cumulative O₂ consumption comparison between the experimental and negative control groups. The mean cumulative oxygen consumption recorded during the fourth hour of respirometry for the experiment group was 2316.88 µg with a variance of 47308.15 µg². The mean cumulative oxygen consumption of the negative controls was 3216.05 µg with a variance of 1374704.30 µg². A simple t-test comparing the experimental and negative control samples resulted in a p-value of 0.354. There is no statistically significant difference between the cumulative O₂ consumption for experiment and negative control groups when measured through the fourth time interval. Inspecting the reprogram output, one could easily infer that the experimental groups consumed more O₂ than either of the controls due to the cyclic nature of growth and decay observed during the negative control group's endogenous respiration rate.

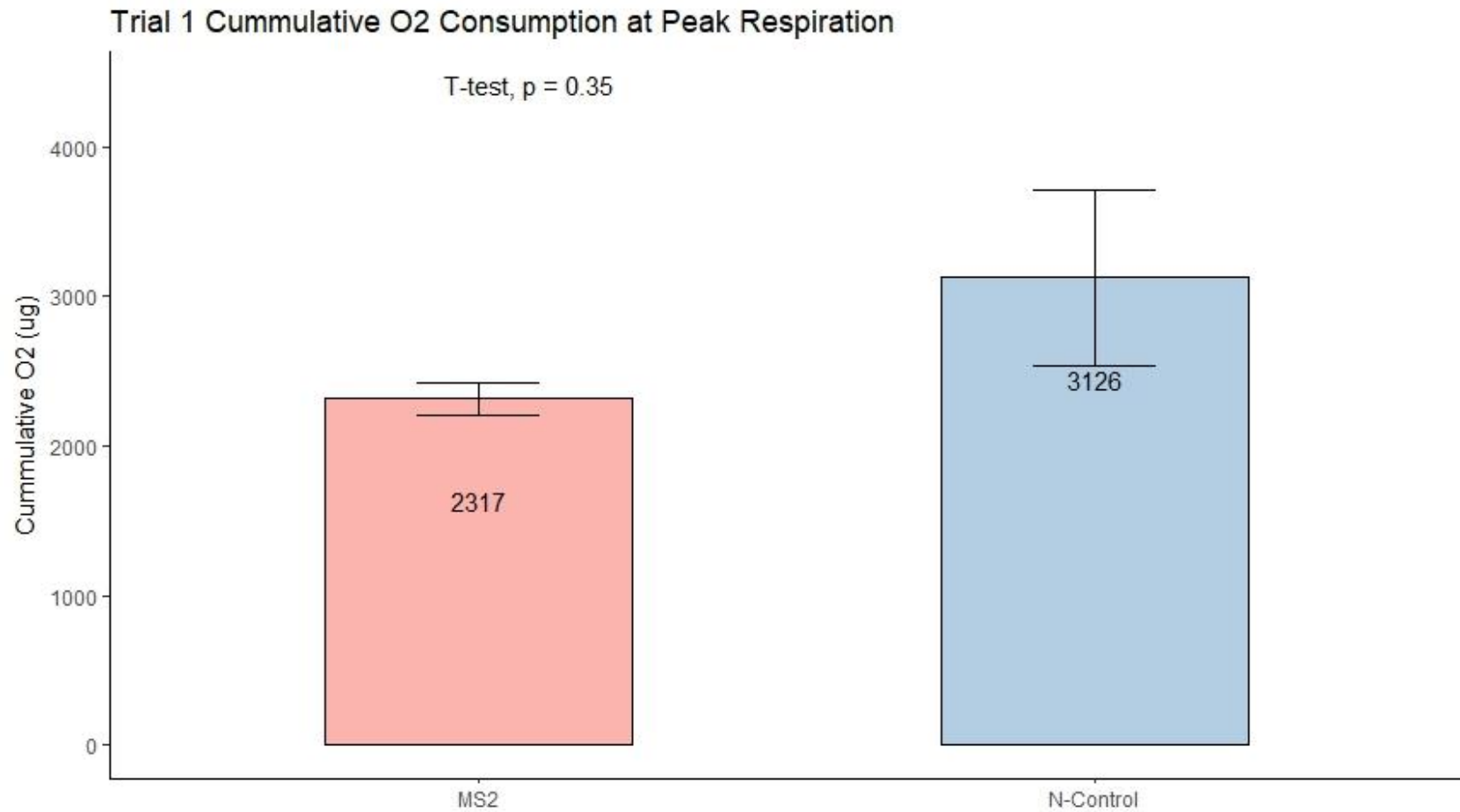


Figure 8 Trial 1 Cumulative O₂ Consumption. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

Molar CO₂/O₂ Ratios

Figures 9 and 10 display the molar ratio data for Trial 1. The molar ratio of CO₂ produced to O₂ consumed at an unknown concentration of MS2 with media did appear different from the negative control group during the first five hours of the experiment. This implies that the substrate media impacted the metabolic activity of the activated sludge. The CO₂/O₂ molar ratio is lower during these initial hours and suggests that while oxidation may be occurring, the substrates within the media solution disrupted the typical enzymatic reactions responsible for respiration. However, throughout the remaining hours of the experiment, the CO₂/O₂ molar ratio did not appear to be different from the negative control group. Typical values for each ranged from 1.75 to 2.17. Figure 10 shows the violin plot comparison using the molar ratio data. A simple mean's comparison using a Student's t-test at 95% confidence yielded p-values of 0.083, 0.88, and 0.0013 when comparing experimental and negative control, experimental and positive control, and negative and positive controls respectively. No significant difference is observed when comparing the molar ratios of the experimental group to either of the control groups. This similarity indicates that the microbial metabolism within the experimental group, in spite of the apparent respiration differences, was still similar to both controls.

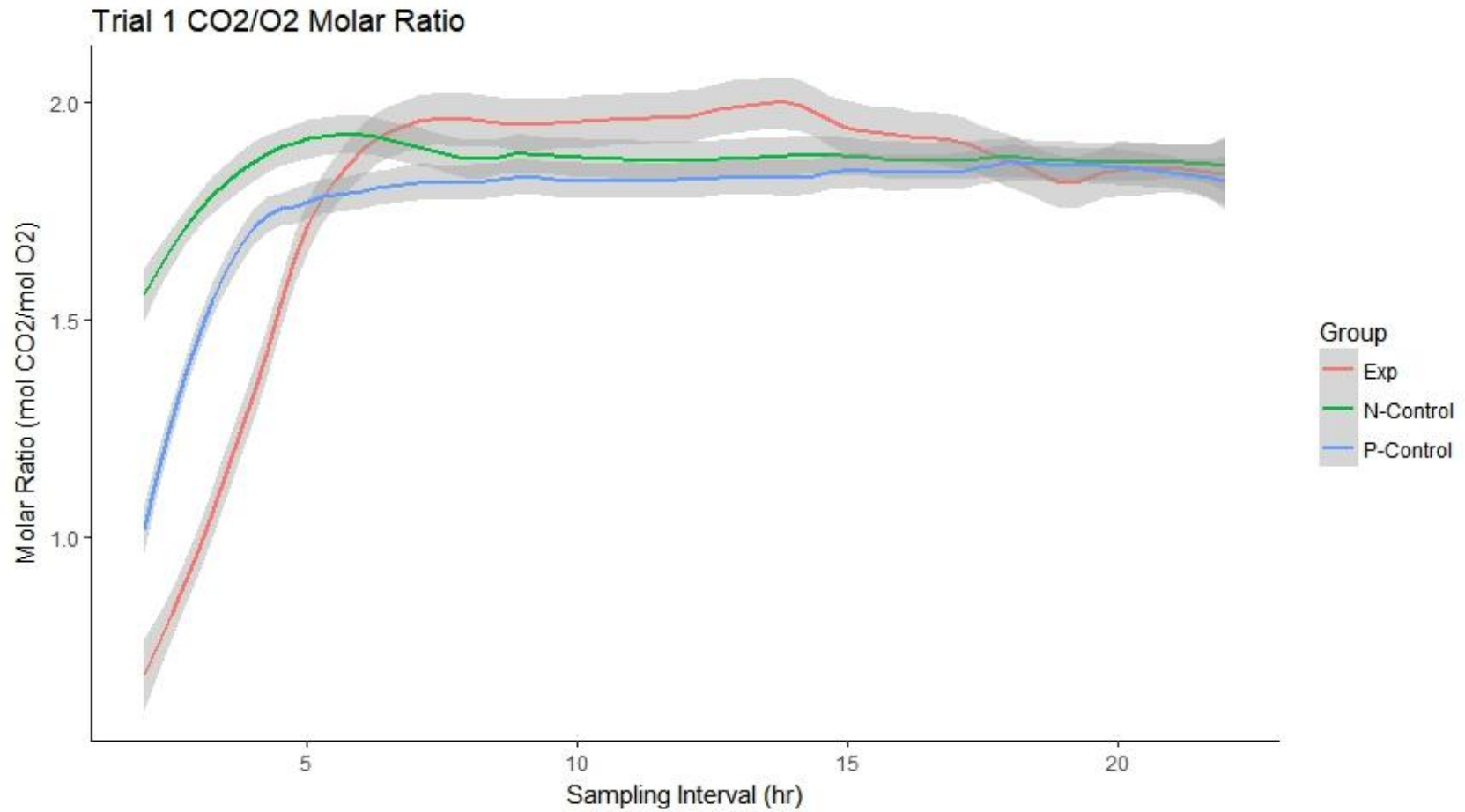


Figure 9 Trial 1 Molar CO₂/O₂ Ratios. Error regions represent 95% confidence interval determined by three replicate data points.

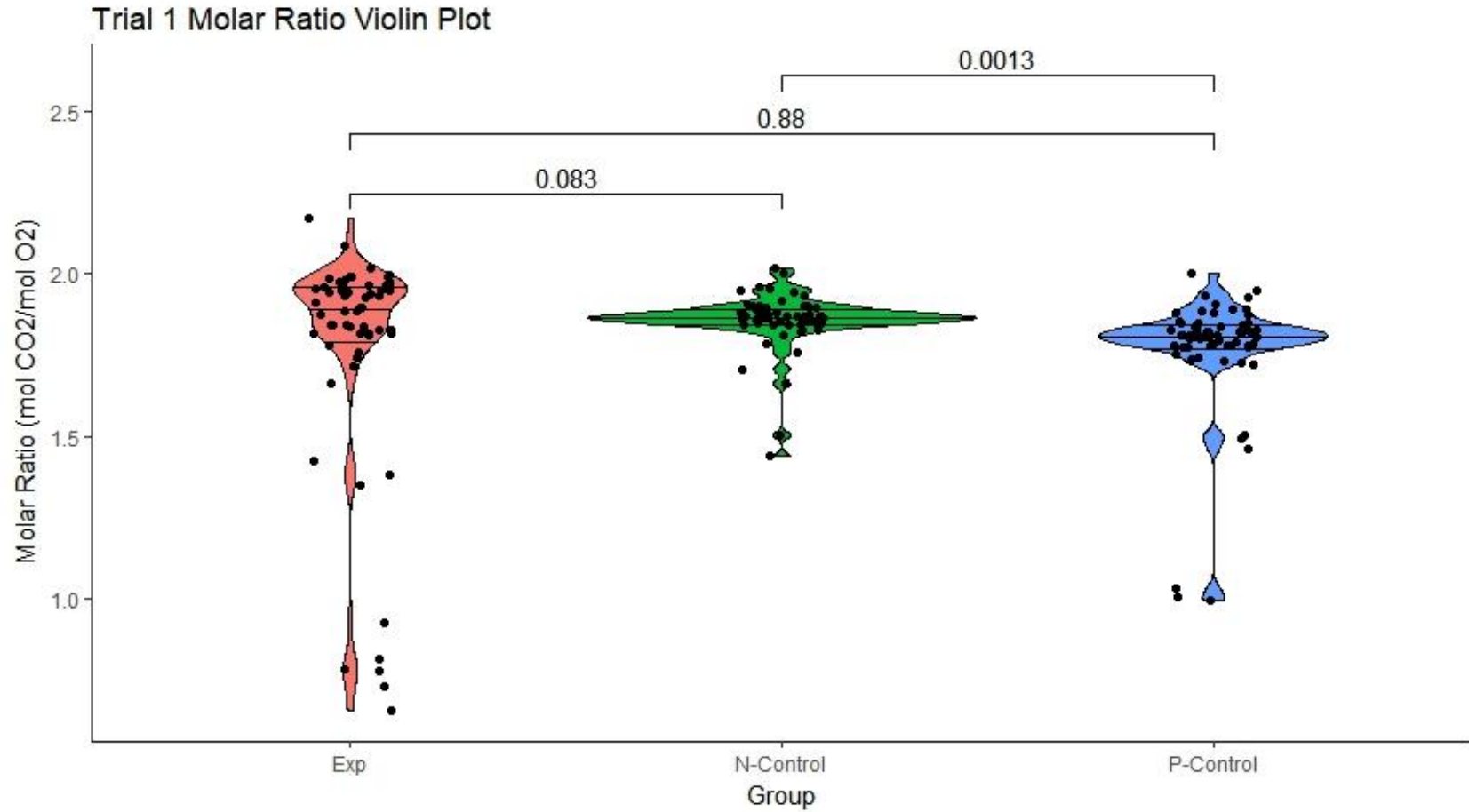


Figure 10 Trial 1 Molar Ratio Violin Plot. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

4.2-B Trial 2 - The effect of 3.2×10^8 PFU/ml MS2 washed on microbial respiration

Figures 11 and 12 present the O₂ consumption rate curves and mean's comparisons respectively. The 3.2×10^8 PFU/ml washed MS2 did not exhibit a significant effect on microbial respiration during exogenous respiration. As the microbes digest the substrate feeds, each curve exhibits a sharp increase in O₂ respiration. The experimental and negative control groups' O₂ curves peaked between hours 4 – 8. The positive control groups containing ATU peaked within the second hour of the test. Following peak respiration, each sample exhibited a decreasing rate of O₂ consumption until endogenous respiration was reached. During endogenous respiration, bacteria are consuming O₂ as they digest other bacteria in the substrate deficient mixture. The trial was terminated after a period of approximately 23 hours. When comparing the O₂ rates using a Student's t-test at 95% confidence, no significant difference is observed between the experimental and negative control groups. A significant difference is observed between the experimental and positive control groups. This suggests that the microbial activity of the experimental group is similar to that of the negative control group and different from the samples containing ATU. Further, this is evidence that microbial respiration was not inhibited in the experimental samples containing the MS2 bacteriophage.

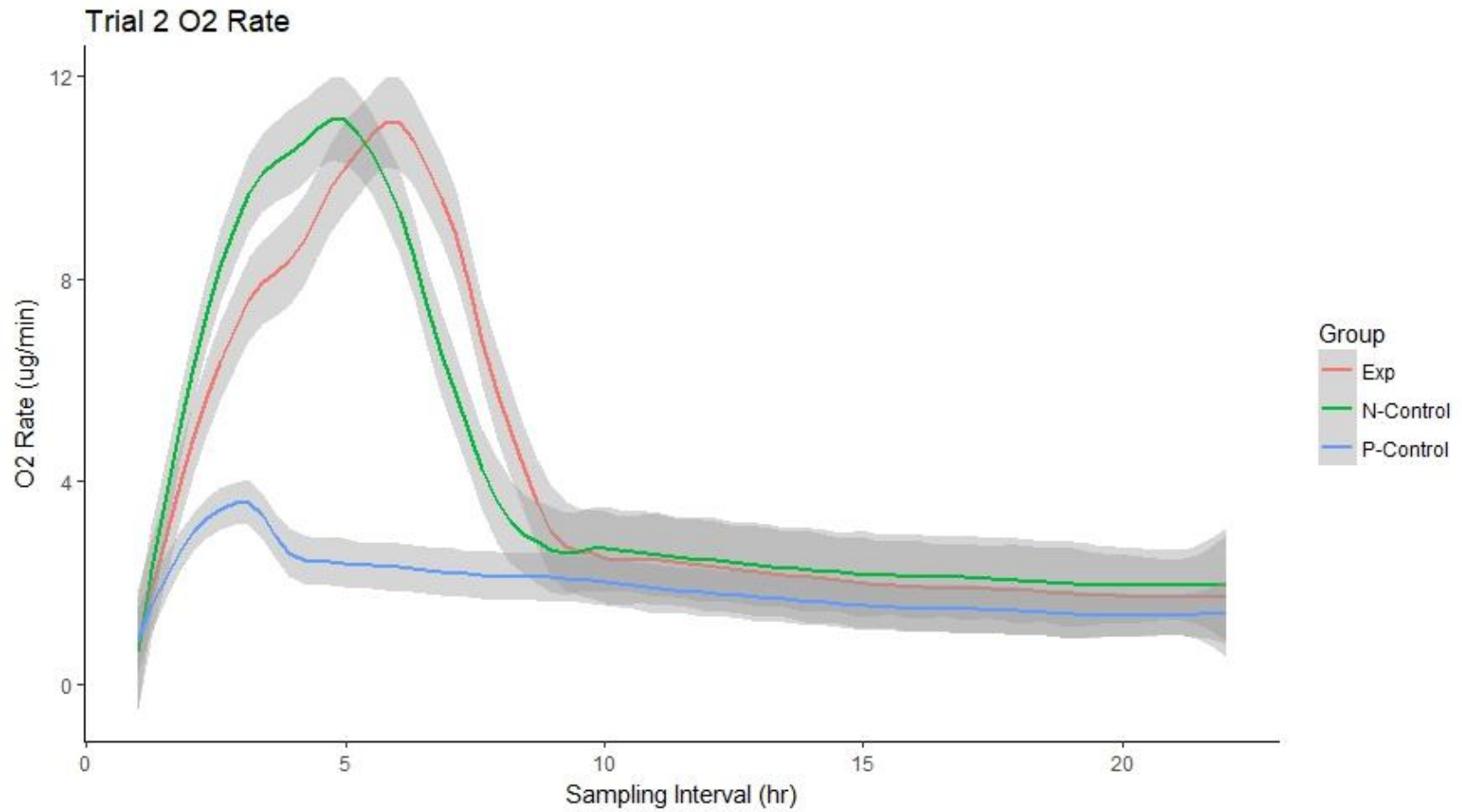


Figure 11 O2 curve with washed MS2 at 3.2×10^8 PFU/ml. Error regions represent 95% confidence interval determined by three replicate data points.

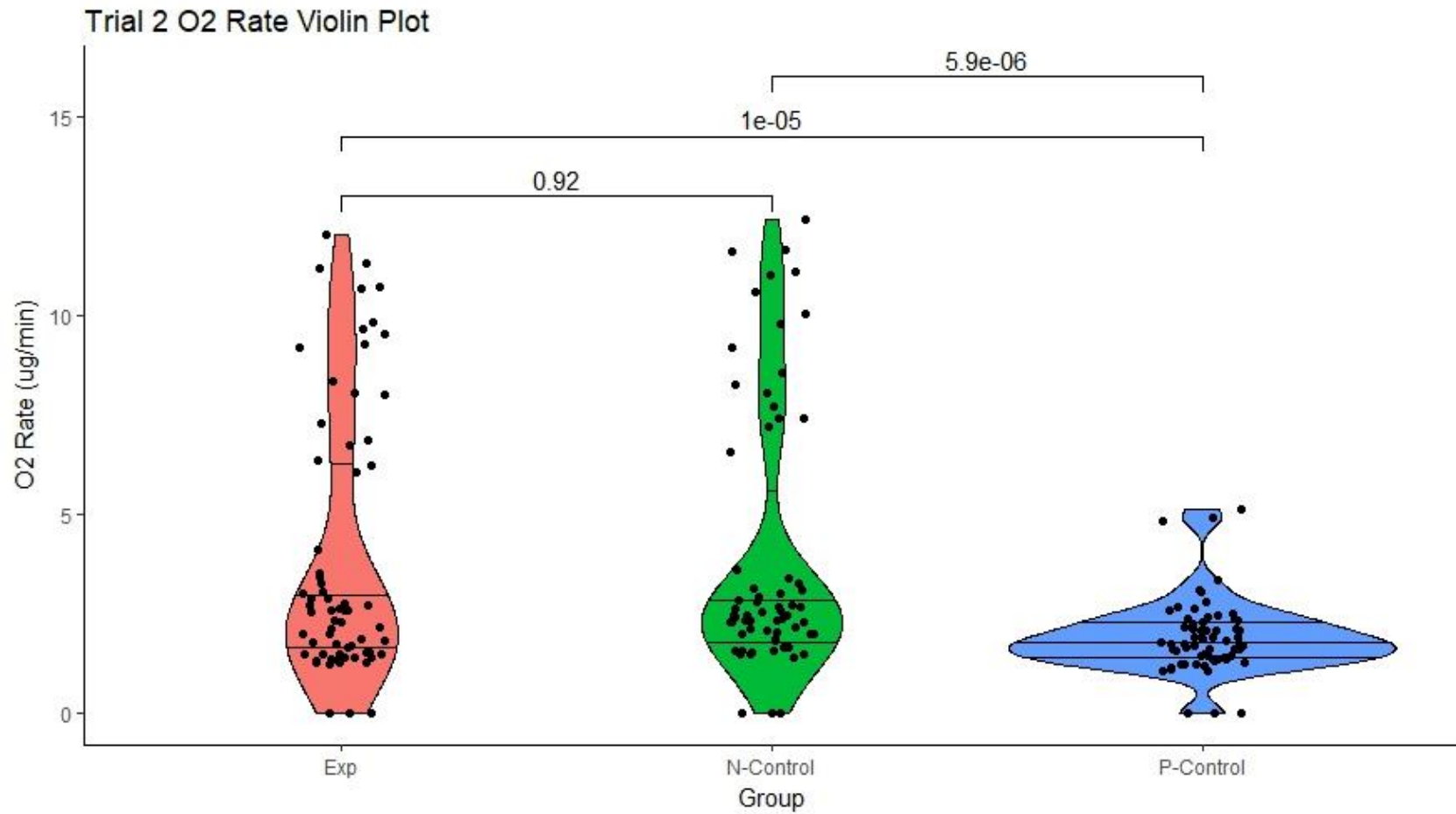


Figure 12 O2 violin plot with washed MS2 at 3.2×10^8 PFU/ml. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

Shape Parameters

The shape parameters of the 3.2×10^8 PFU/ml MS2 O₂ consumption profiles were not statistically different from the negative control. The mean FrM of the experiment group was $269 \mu\text{g O}_2/\text{min-hr}^2$ with a variance of $311.9 (\mu\text{g O}_2/\text{min-hr}^2)^2$. The mean FrM of the negative control was $281.2 \mu\text{g O}_2/\text{min-hr}^2$ with a variance of $700.4 (\mu\text{g O}_2/\text{min-hr}^2)^2$. Comparing the FrM of the experimental and control groups yielding a p-value of 0.545. The FrM analysis is presented in Figure 13. The mean skewness of the experimental group was -1.06 with a variance of 0.235. The mean skewness of the control group was -0.802 with a variance of 0.319. The T-test comparison of the skewness shape factors resulted a p-value of 0.580 and is displayed in figure 14. The initial seven hours of the experiment yielded results that were not statistically different between the experimental and negative control groups suggesting that the underlying O₂ mechanisms were similar. This is further evidence that inhibition is not suspected within samples containing MS2.

Peak Oxygen Consumption Rates

The peak measured SOUR mean of the experiment group was $9.45 \text{ O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$ with a variance of $0.138 (\text{O}_2\text{-g VSS}^{-1}\text{-hr}^{-1})^2$. The peak measured SOUR mean of the negative controls was $9.58 \text{ O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$ with a variance of $0.32 (\text{O}_2\text{-g VSS}^{-1}\text{-hr}^{-1})^2$. Comparing these peak rates of the three experimental samples and three control samples resulted in a p-value of 0.756 as depicted in Figure 15. There is no statistically significant difference between the peak O₂ utilization rates normalized to SOUR for the experiment and negative control samples.

Trial 2 Exogenous Respiration First Moment of Area Summary

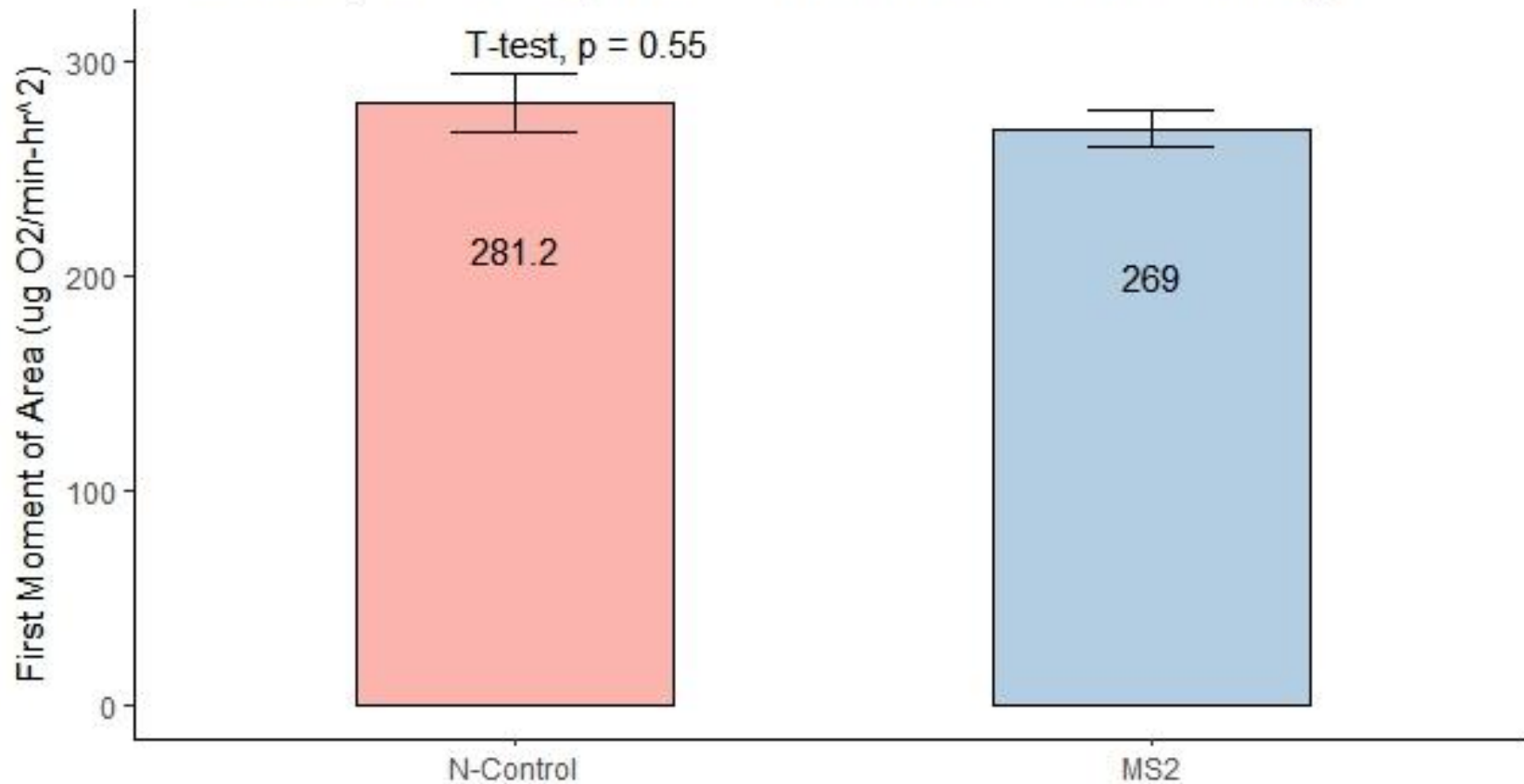


Figure 13 Trial 2 First moment of area. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

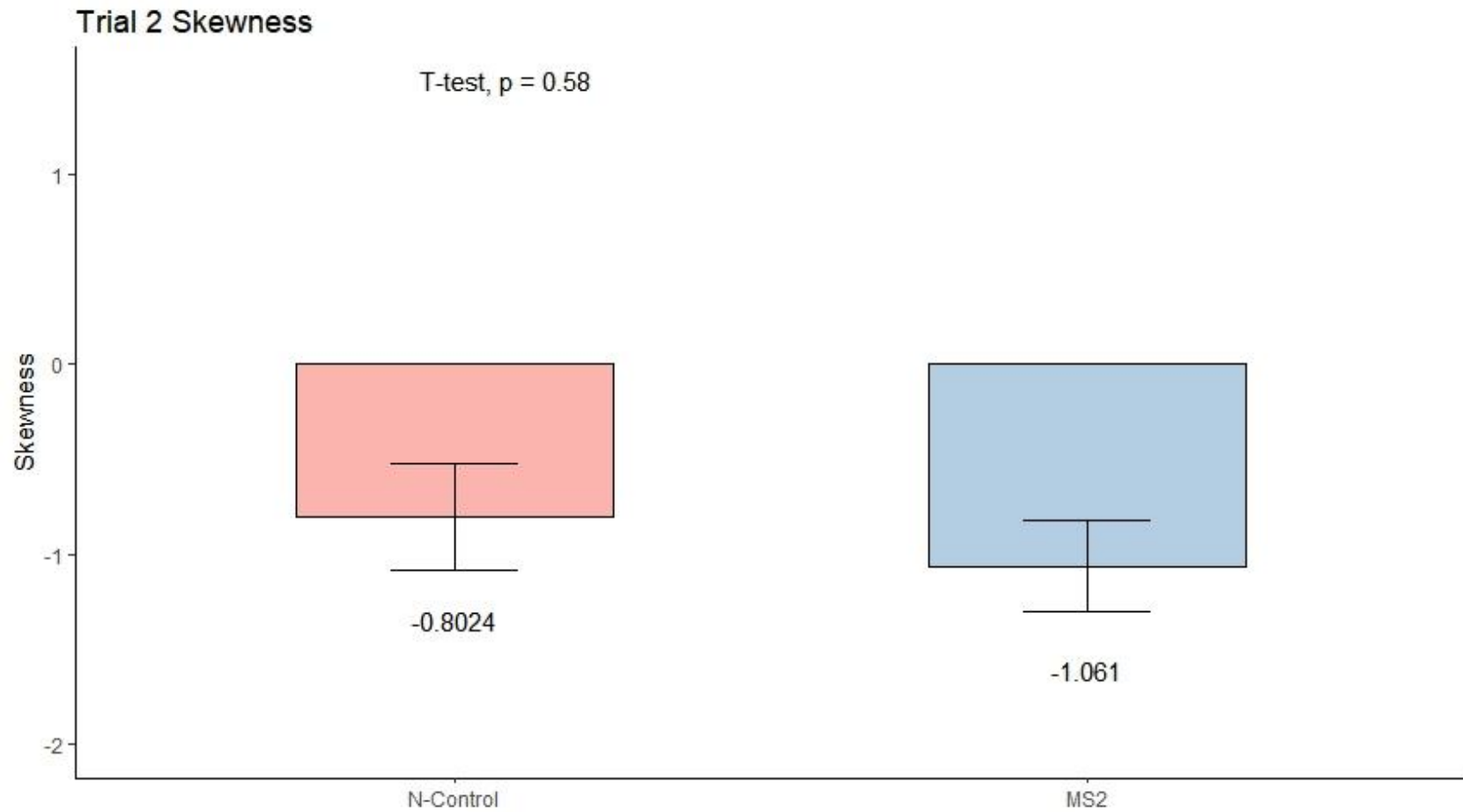


Figure 14 Trial 2 Skewness. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

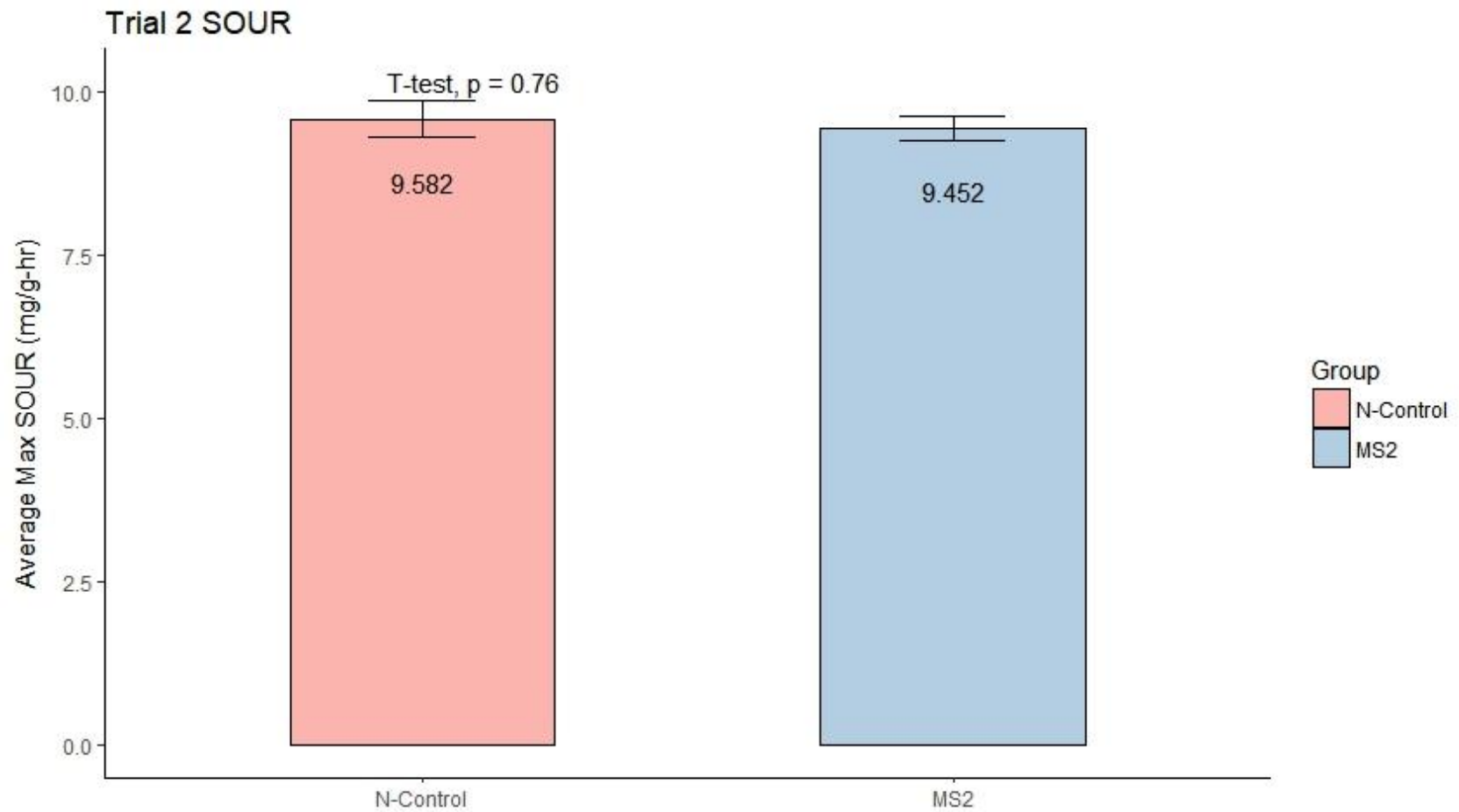


Figure 15 Trial 2 Specific oxygen utilization rate. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

Cumulative Oxygen Consumption Rates

Figure 16 presents the cumulative O₂ comparison for negative and experimental groups in Trial 2. The mean cumulative oxygen consumption recorded during the fourth hour of respirometry for the experiment group was 2001.80 µg with a variance of 10108.60 µg². The mean cumulative oxygen consumption of the negative controls was 2415.25 µg with a variance of 42526.51 µg². A simple t-test was performed comparing the experimental and negative control samples and resulted in a p-value of 0.055. There is no statistically significant difference between the cumulative O₂ consumption for experiment and negative control groups.

Molar CO₂/O₂ Ratios

Figure 17 and 18 display the molar ratio plot and the means comparison violin plot respectively. The molar ratio of CO₂ produced to O₂ consumed at 3.2x10⁸ PFU/ml washed MS2 did not appear significantly different from the negative control group. Both exhibited an increase during the first five hours of the experiment which then stabilized. A simple mean's comparison using a Student's t-test at 95% confidence yielded p-values of 0.61, 0.82, and 0.64 when comparing experimental and negative control, experimental and positive control, and negative and positive controls respectively. No significant difference is observed when comparing the molar ratios of the experimental group to either of the control groups. This similarity indicates that the microbial metabolism within the experimental group, in spite of the apparent respiration differences, was still similar to both controls.

Trial 2 Cumulative O2 Consumption at Peak Respiration

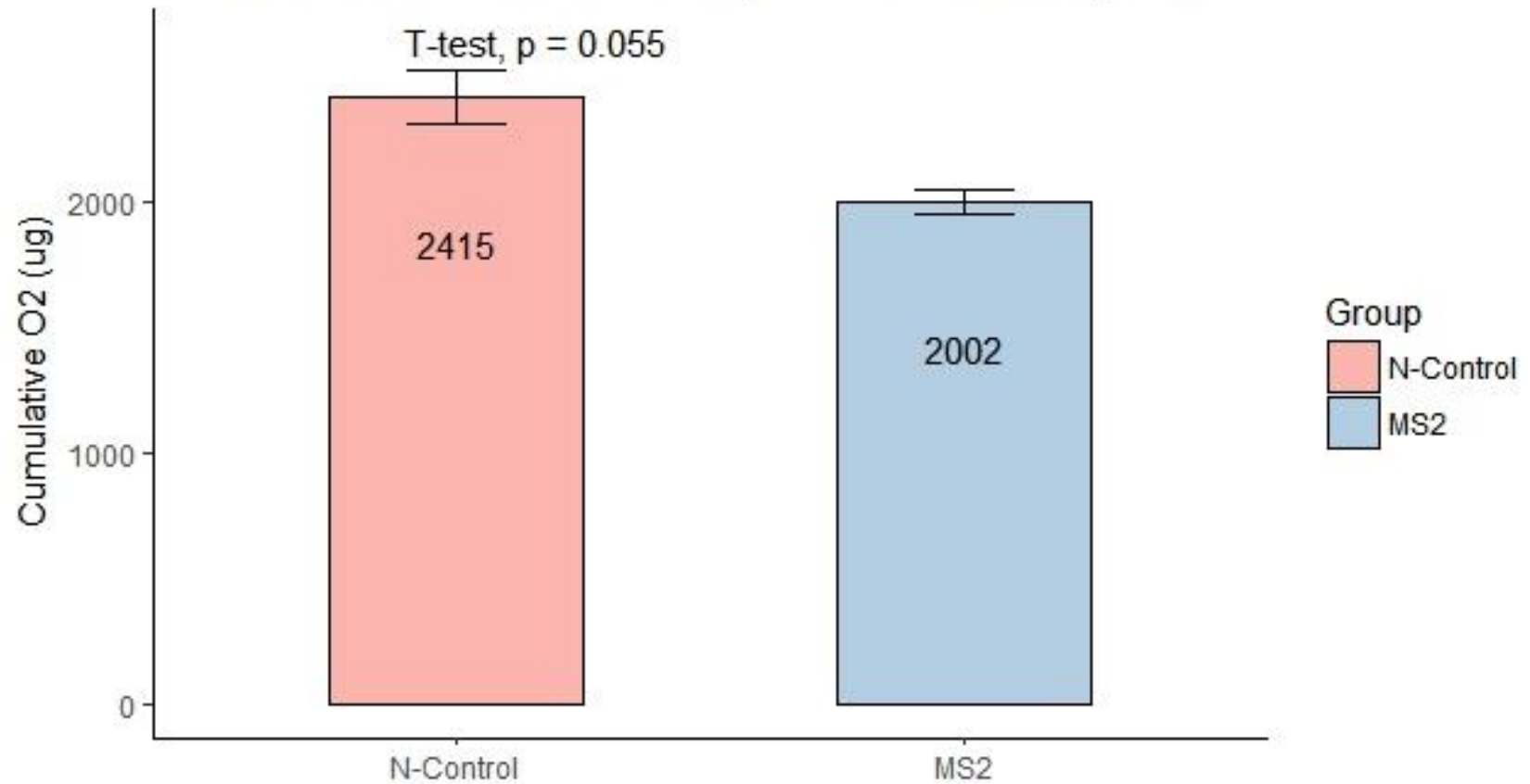


Figure 16 Cumulative O2 Consumption. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

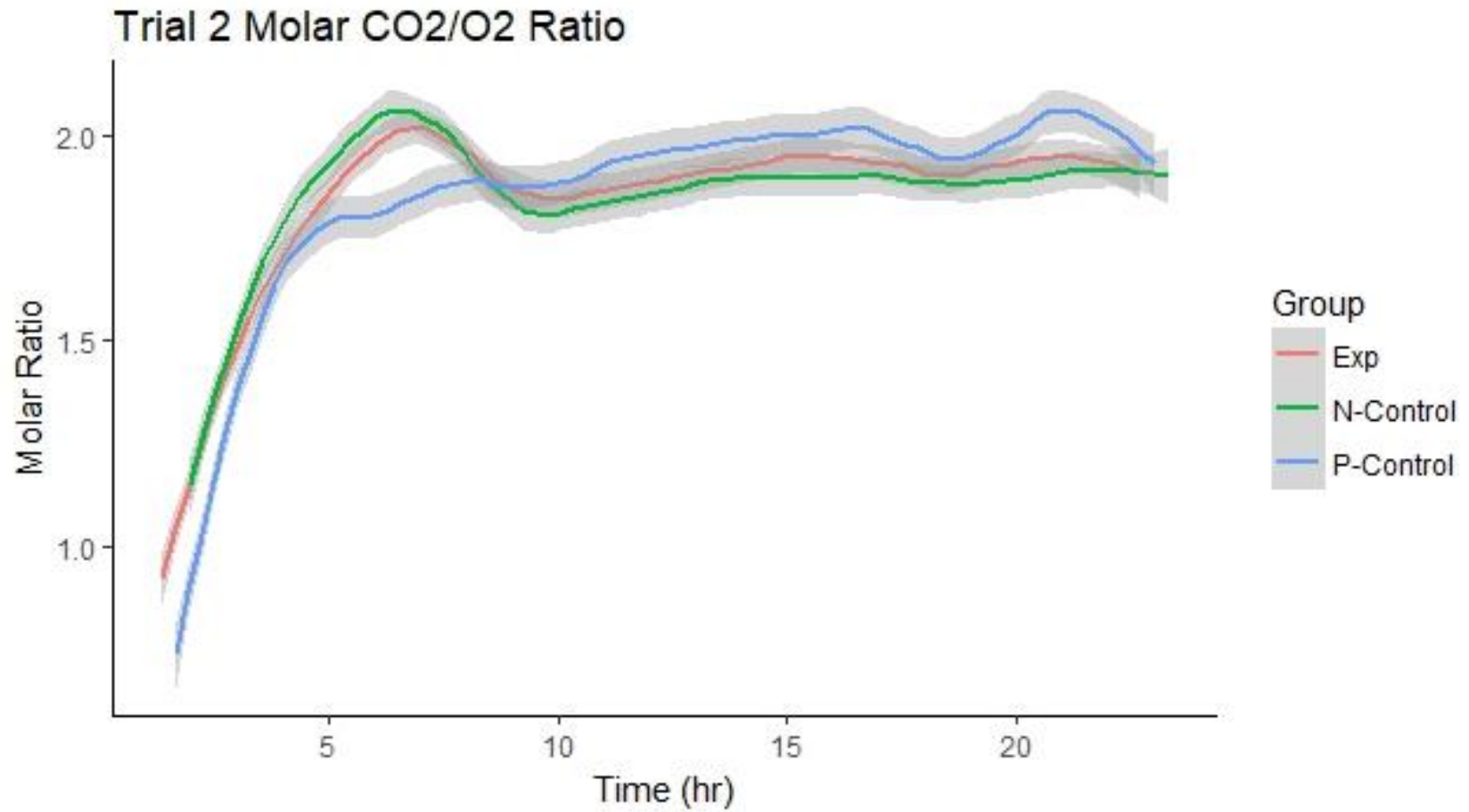


Figure 17 Trial 2 Molar CO₂/O₂ ratios. Error regions represent 95% confidence interval determined by three replicate data points.

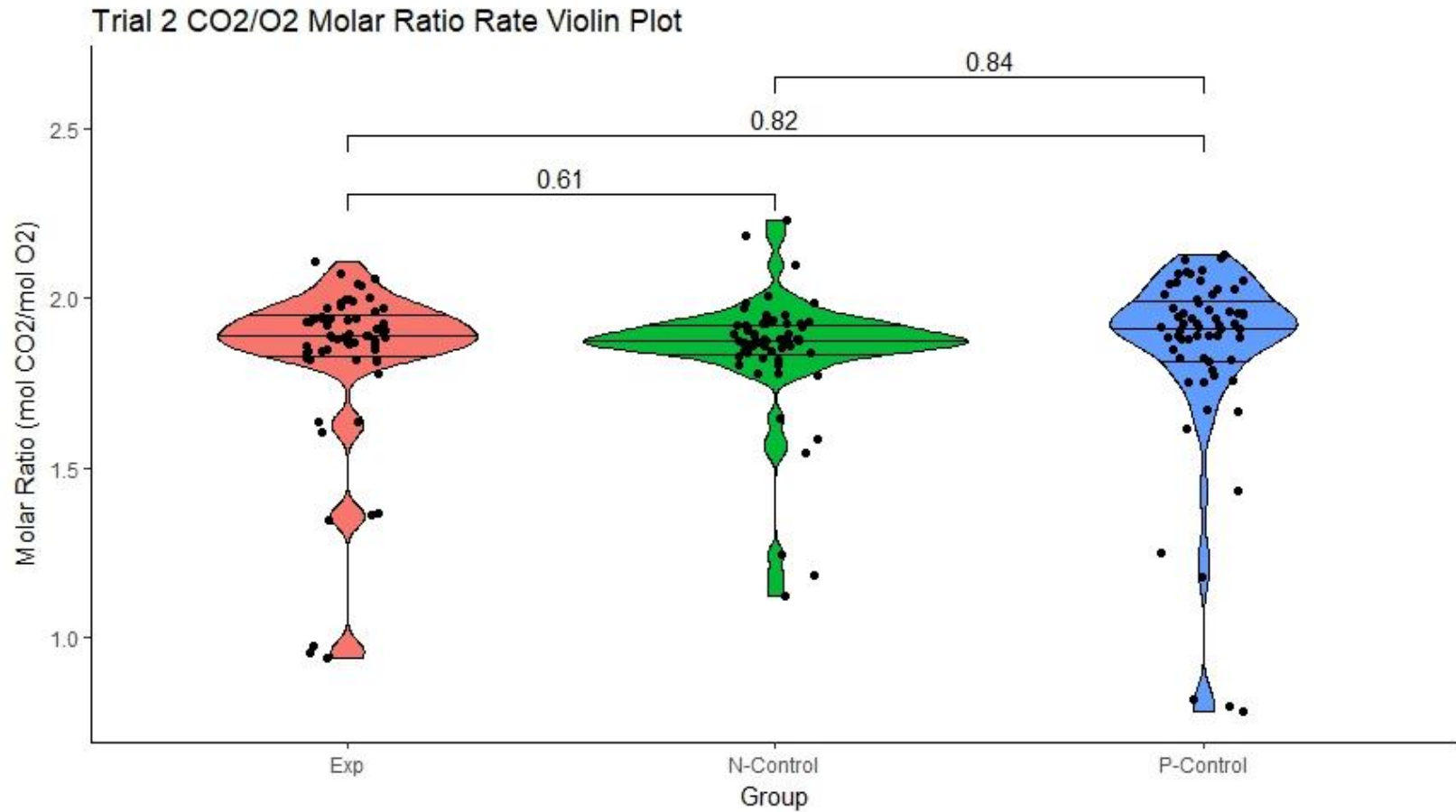


Figure 18 Trial 2 Molar CO₂/O₂ Ratio Violin Plot. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

4.2-C Trial 3 - The effect of 3.2×10^8 PFU/ml MS2 washed on microbial respiration

The 3.2×10^8 PFU/ml washed MS2 did not exhibit a significant effect on microbial respiration during the first eight sampling intervals. With the feeds added to each bottle, each curve exhibits a sharp increase in O_2 respiration rate. The experimental and negative control groups' O_2 curves peaked between hours 3 – 6. The positive control groups containing ATU peaked within the second hour of the test. Following peak respiration, each sample exhibited a decreasing rate of O_2 consumption until endogenous respiration was reached. This endogenous respiration rate varied between each channel and indicates the absence of the feed. During this time, bacteria are consuming O_2 as they digest other bacteria in the substrate deficient mixture. The trial was terminated after a period of approximately 23 hours. The reprogram output is presented in Figure 19. When comparing the O_2 rates using a Student's t-test at 95% confidence, no significant difference is observed between the experimental and negative control groups. A significant difference is observed between the experimental and positive control groups. This suggests that the microbial activity of the experimental group is similar to that of the negative control group and different from the samples containing ATU. Further, this is evidence that microbial respiration was not inhibited in the experimental samples containing the MS2 bacteriophage. This means comparison is presented within Figure 20.

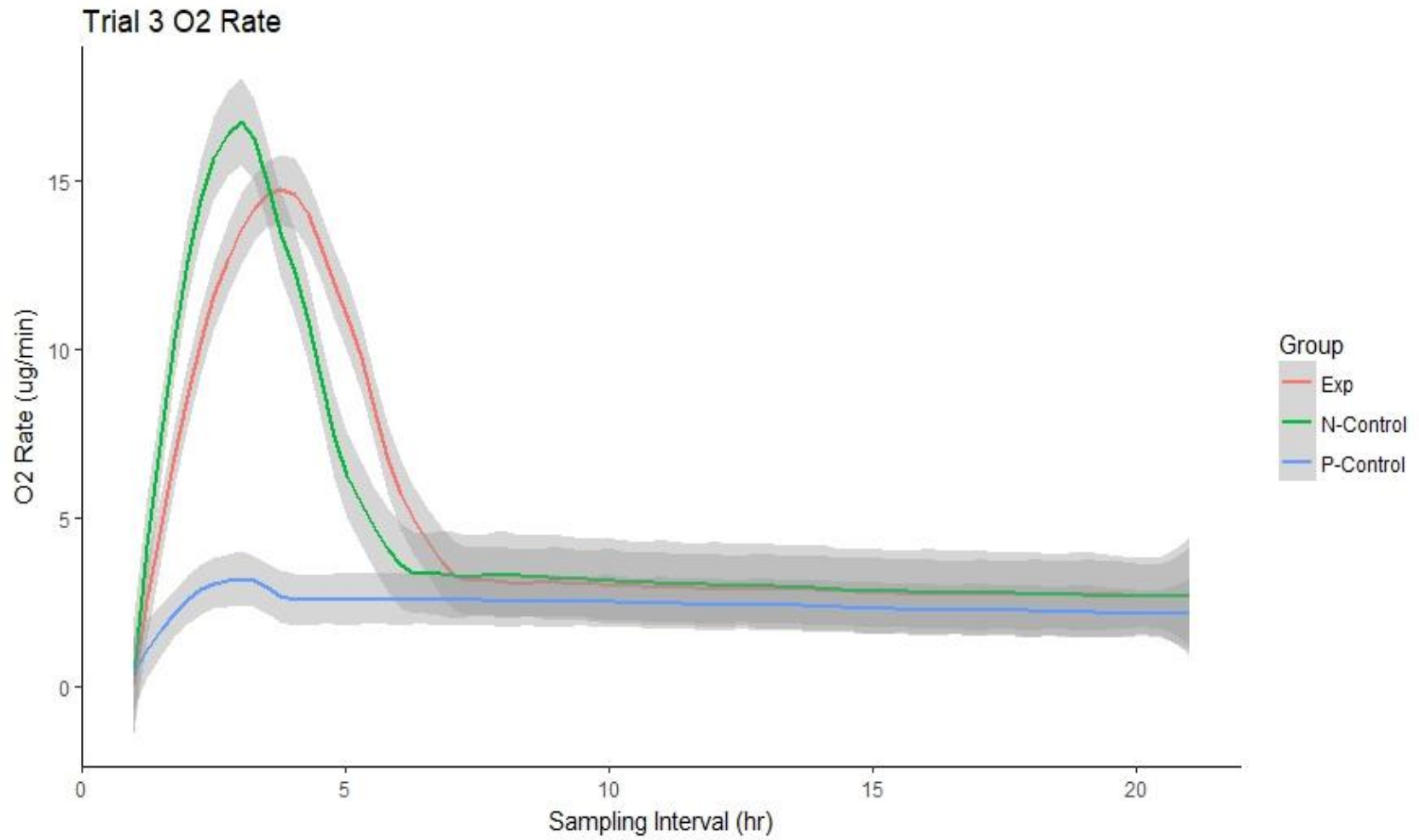


Figure 19 O2 curve with washed MS2 at 3.2×10^8 PFU/ml. Error regions represent 95% confidence interval determined by three replicate data points.

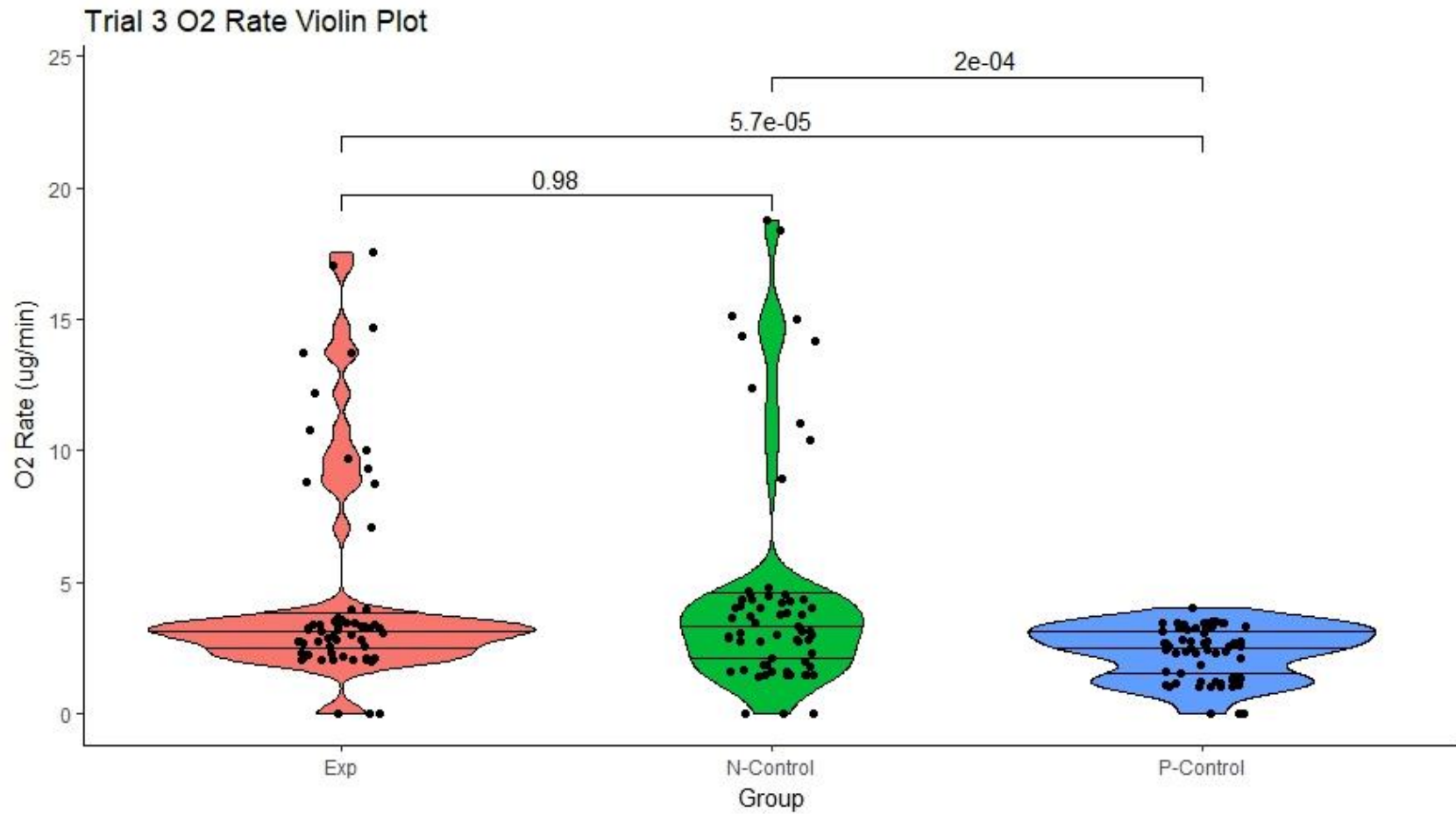


Figure 20 O2 violin plot with washed MS2 at 3.2×10^8 PFU/ml. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

Shape Parameters

Figures 21 and 22 present the shape parameter analysis for the exogenous rate. The shape parameters of the 3.2×10^8 PFU/ml MS2 O₂ consumption profiles were not statistically different from the negative control. The mean FrM of the experiment group was 236.04 $\mu\text{g O}_2/\text{min-hr}^2$ with a variance of 99.6 $(\mu\text{g O}_2/\text{min-hr}^2)^2$. The mean FrM of the negative control was 243.6 $\mu\text{g O}_2/\text{min-hr}^2$ with a variance of 2186.3 $(\mu\text{g O}_2/\text{min-hr}^2)^2$. Comparing the FrM of the experimental and control groups yielding a p-value of 0.855. The mean skewness of the experimental group was 0.233 with a variance of 0.146. The mean skewness of the control group was 0.600 with a variance of 0.117. The T-test comparison of the skewness shape factors resulted a p-value of 0.54. The initial seven hours of the experiment yielded results that were not statistically different between the experimental and negative control groups.

Peak Oxygen Consumption Rates

The peak measured SOUR mean of the experiment group was mg 9.90 O²-g VSS⁻¹-hr⁻¹ with a variance of 1.59 (O²-g VSS⁻¹-hr⁻¹)². The peak measured SOUR mean of the negative controls was 10.7 O²-g VSS⁻¹-hr⁻¹ with a variance of 1.62 (O²-g VSS⁻¹-hr⁻¹)². Comparing these peak rates of the three experimental samples and three control samples resulted in a p-value of 0.487. Figure 23 displays the means comparison between the negative and experimental control groups. There is no statistically significant difference between the O₂ utilization rates for the experiment and negative control samples.

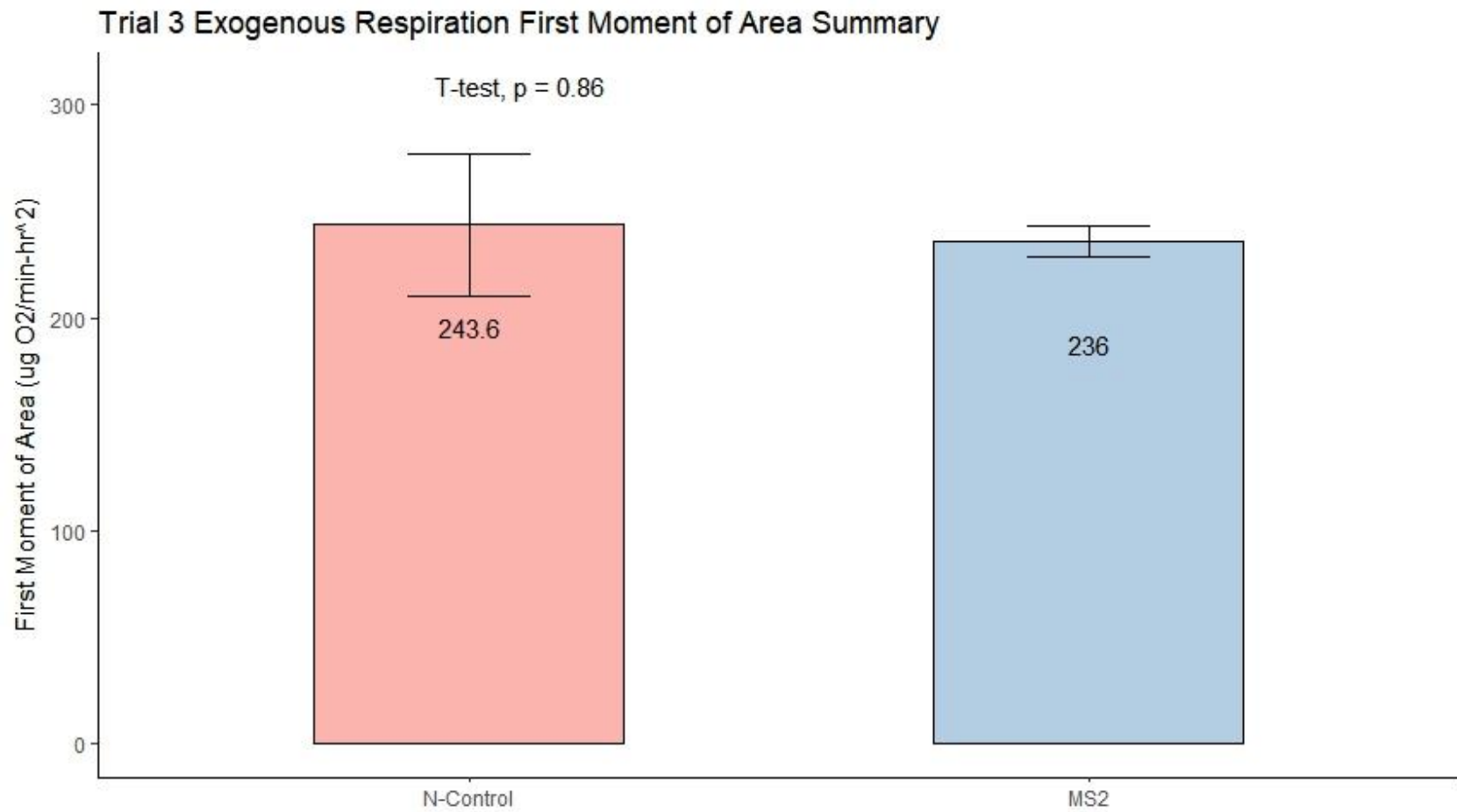


Figure 21 Trial 3 First moment of area. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

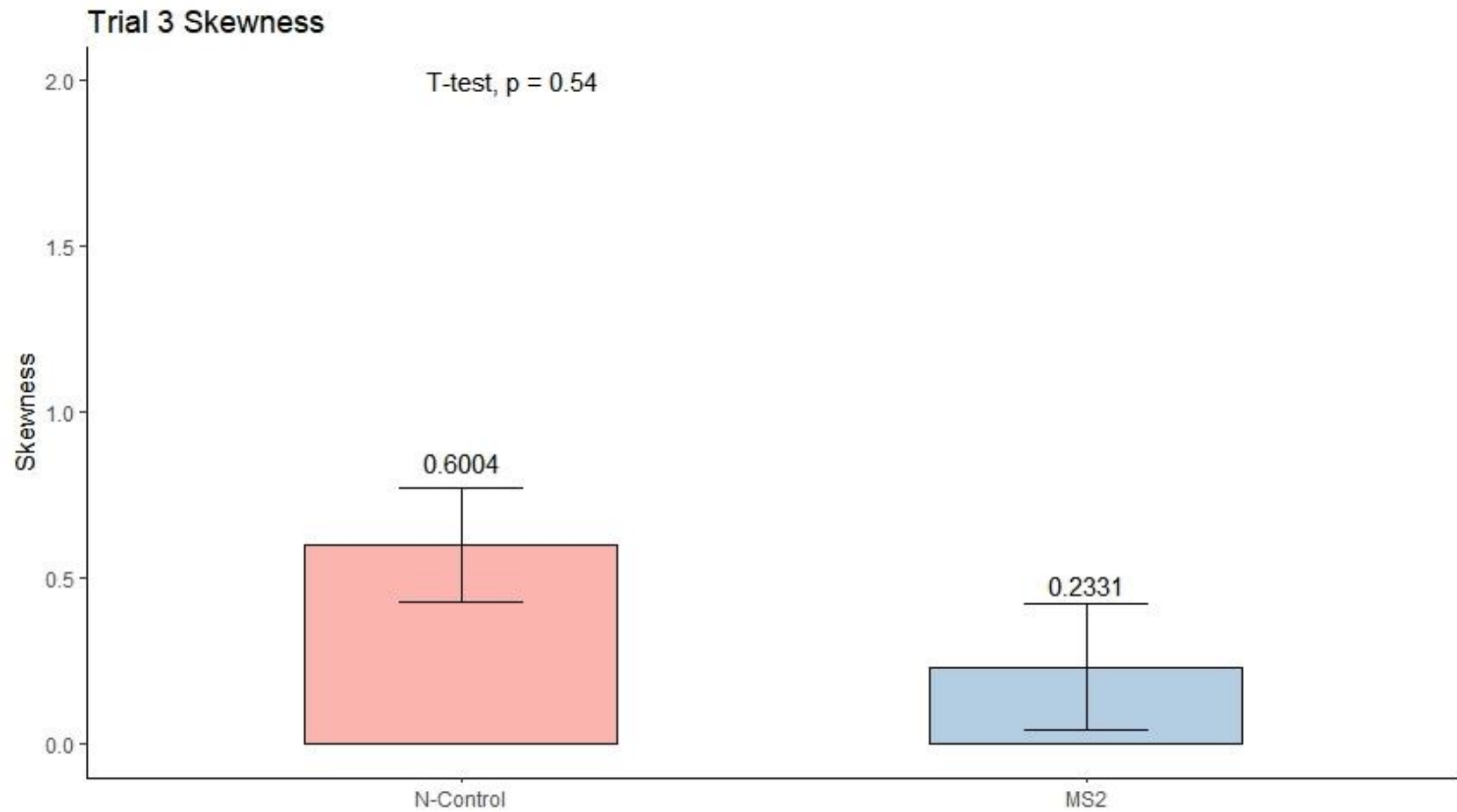


Figure 22 Trial 3 Skewness. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

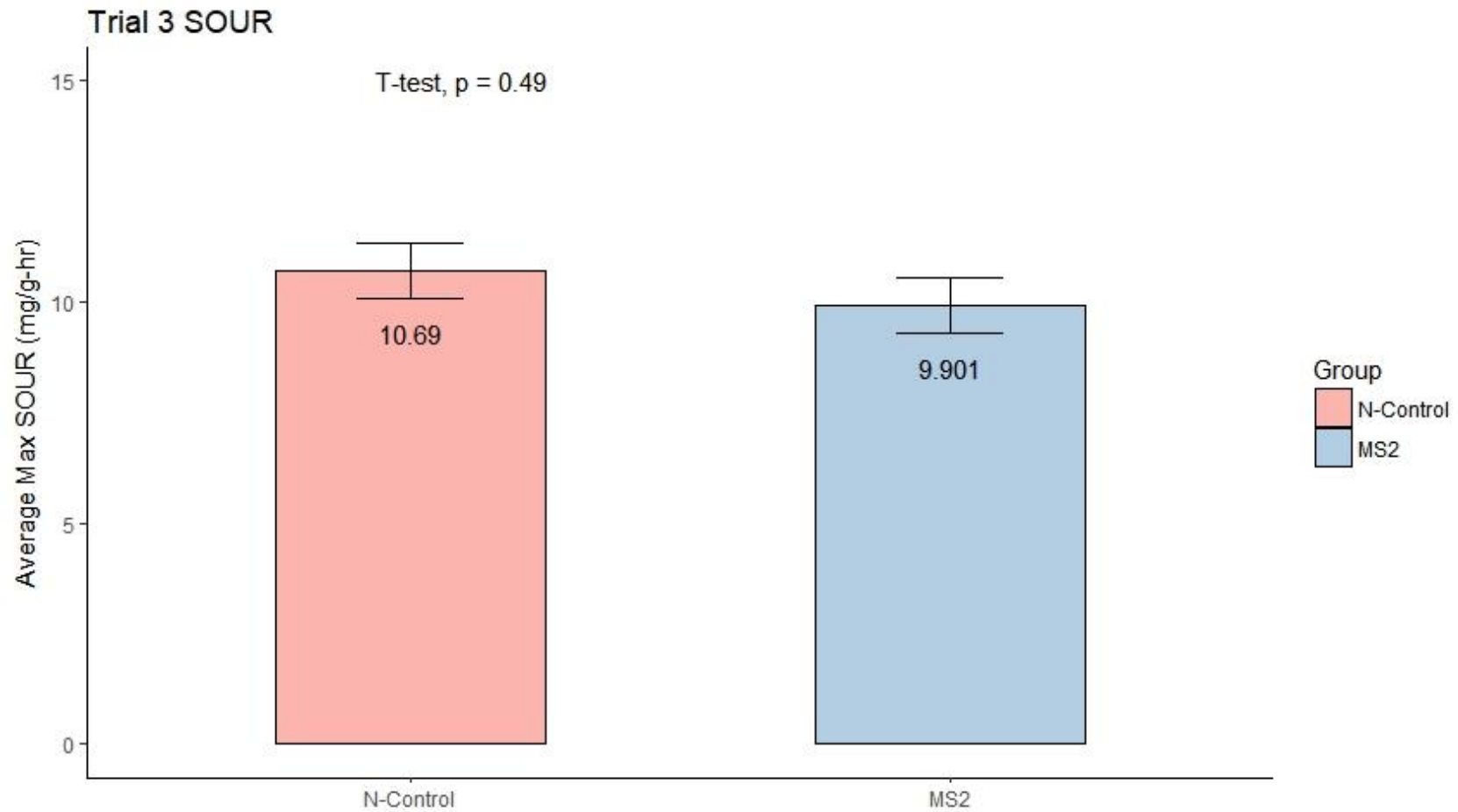


Figure 23 Trial 3 Specific oxygen utilization rate. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

Cumulative Oxygen Consumption Rates

The mean cumulative oxygen consumption recorded during the fourth hour of respirometry for the experiment group was 3101.93 μg with a variance of 89099.28 μg^2 . The mean cumulative oxygen consumption of the negative controls was 3118.22 μg with a variance of 472271.80 μg^2 . A simple t-test was performed comparing the experimental and negative control samples and resulted in a p-value of 0.973. There is no statistically significant difference between the cumulative O_2 consumption for experiment and negative control groups. This data is captured in the bar chart in Figure 24.

Molar CO_2/O_2 Ratios

Figures 25 and 26 present data comparing the molar ratios of the experimental groups. The molar ratio of CO_2 produced to O_2 consumed at 1.6×10^{10} PFU/ml washed MS2 did not appear significantly different from the negative control group. Both exhibited an increase during the first five hours of the experiment and then stabilized. A simple mean's comparison using a Student's t-test at 95% confidence yielded p-values of 0.051, 0.58, and 0.013 when comparing experimental and negative control, experimental and positive control, and negative and positive controls respectively. No significant difference is observed when comparing the molar ratios of the experimental group to either of the control groups. This similarity indicates that the microbial metabolism within the experimental group, in spite of the apparent respiration differences, was still similar to both controls. This implies that the enzymatic reactions responsible for respiration within the activated sludge were not affected by the addition of washed MS2 virus.

Trial 3 Cumulative O2 Consumption at Peak Respiration

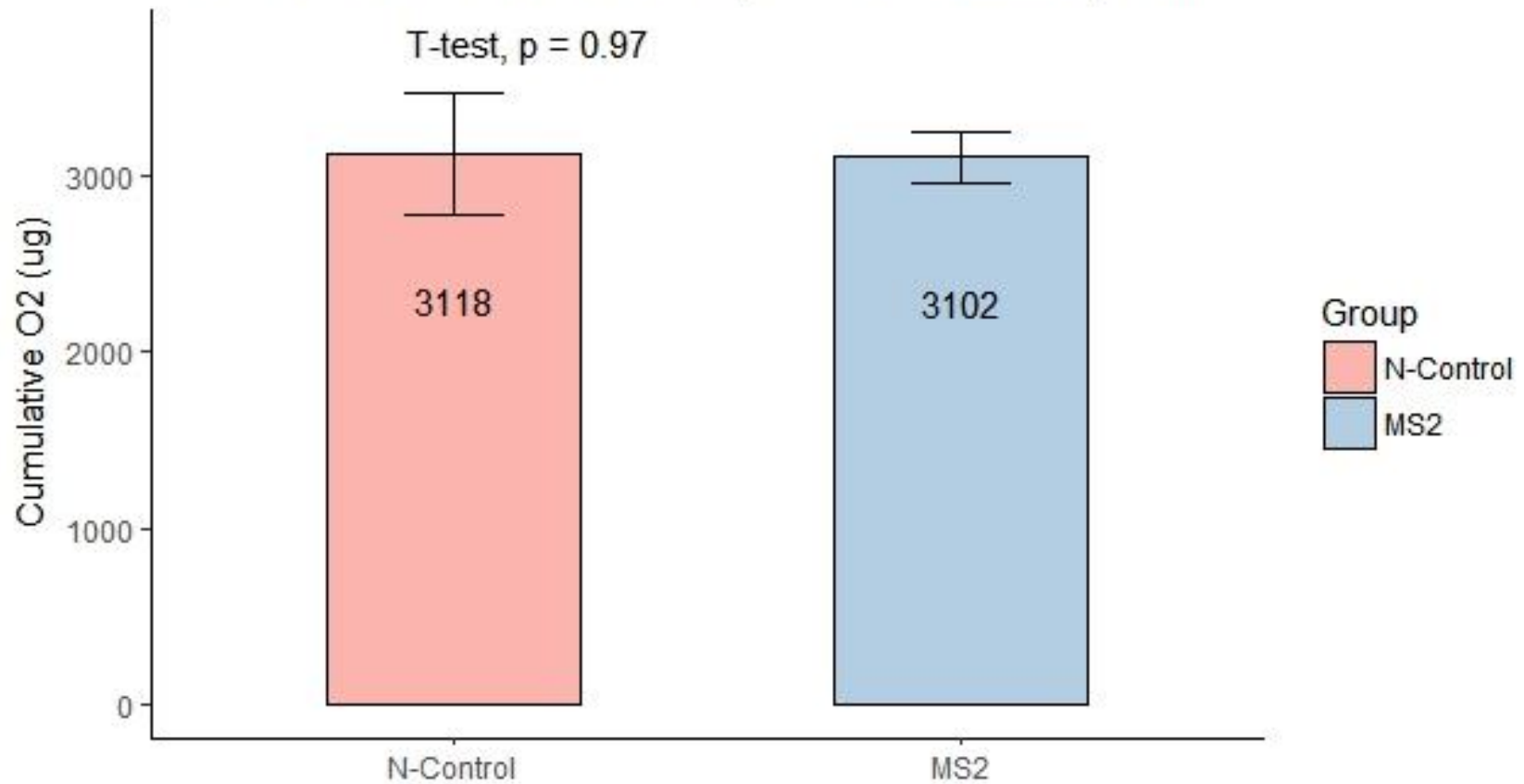


Figure 24 Trial 3 Cumulative O2 Consumption. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

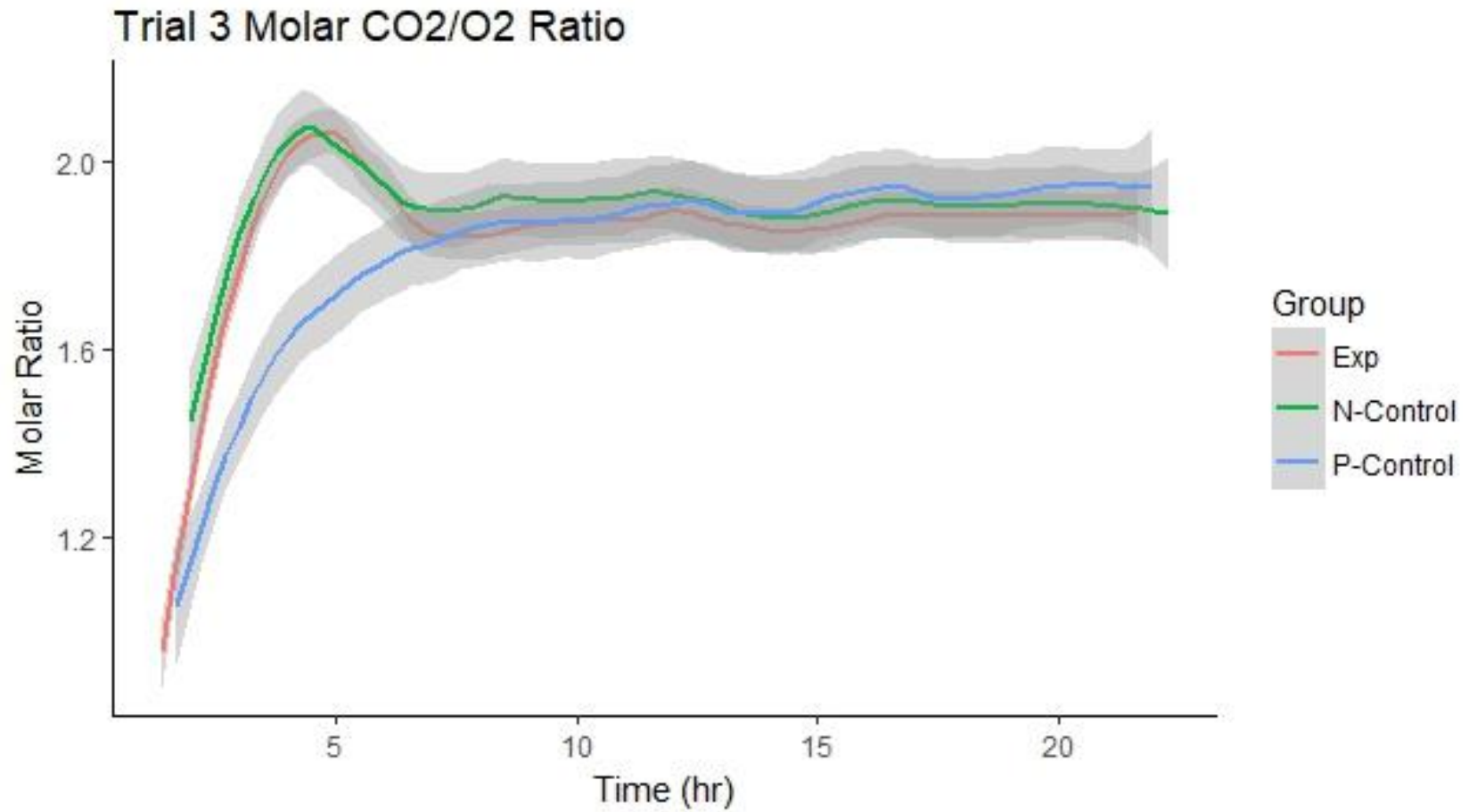


Figure 25 Molar CO₂/O₂ ratio. Error regions represent 95% confidence interval determined by three replicate data points.

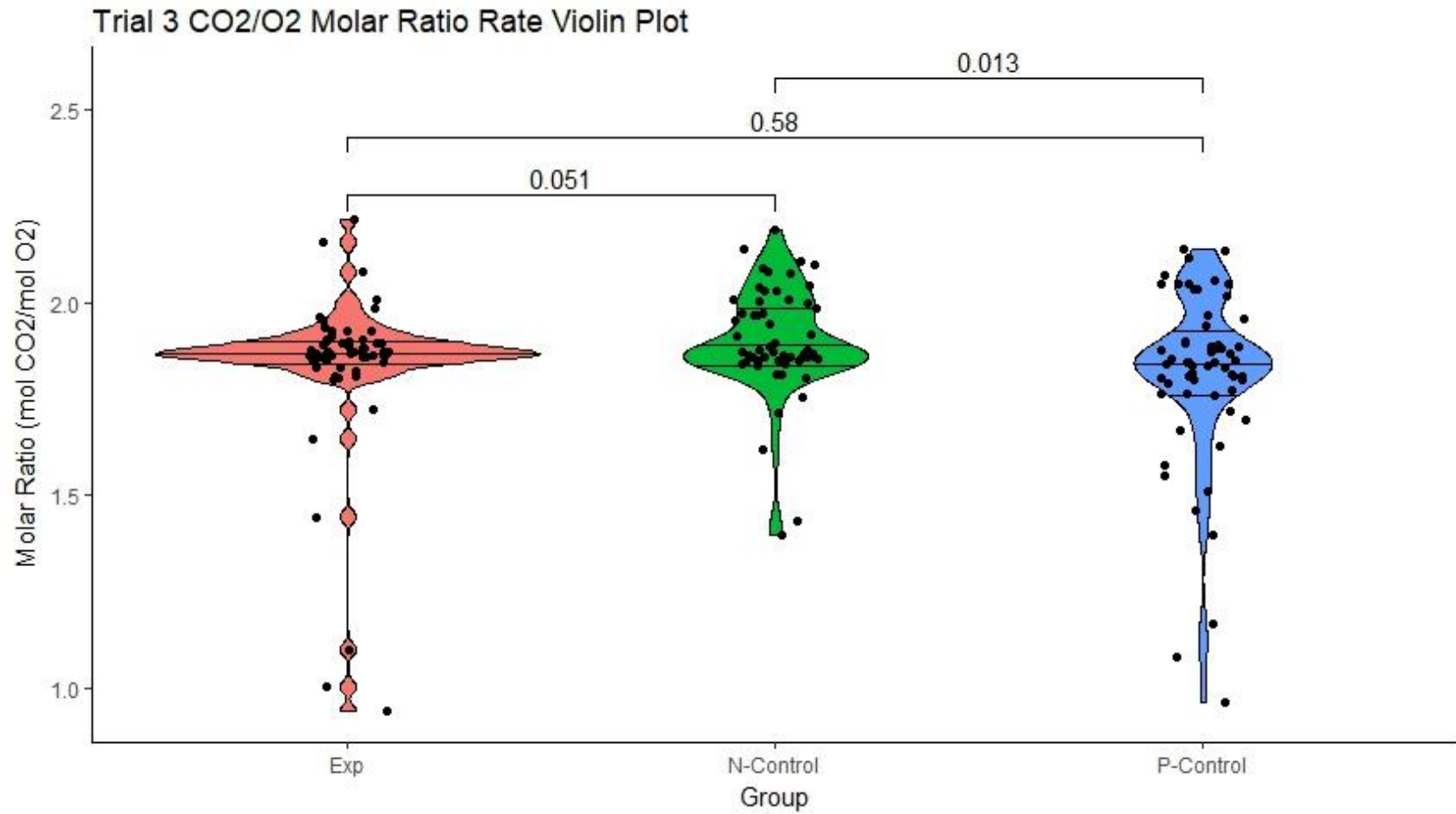


Figure 26 Trial 3 CO₂/O₂ Molar Ratio Violin Plot. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

4.2-D Trial 4 - The effect of 3.2×10^8 PFU/ml MS2 washed on microbial respiration

The 3.2×10^8 PFU/ml washed MS2 did not exhibit a significant effect on microbial respiration during the first eight sampling intervals. With the feeds added to each bottle, each curve exhibits a sharp increase in O₂ respiration rate. The experimental and negative control groups' O₂ curves peaked between hours 3 – 5. The positive control groups containing ATU peaked within the second hour of the test. Following peak respiration, each sample exhibited a decreasing rate of O₂ consumption until endogenous respiration was reached. This endogenous respiration rate varied between each channel and indicates the absence of the feed. During this time, bacteria are consuming O₂ as they digest other bacteria in the substrate deficient mixture. The trial was terminated after a period of approximately 17 hours. The reprogram output for Trial 4 is displayed in Figure 27. When comparing the O₂ rates using a Student's t-test at 95% confidence, no significant difference is observed between the experimental and negative control groups. A significant difference is observed between the experimental and positive control groups. This suggests that the microbial activity of the experimental is similar to that of the negative control group and different from the samples containing ATU. Further, this is evidence that microbial respiration was not inhibited in the experimental samples containing the MS2 bacteriophage. The O₂ rate means comparison is displayed in Figure 28.

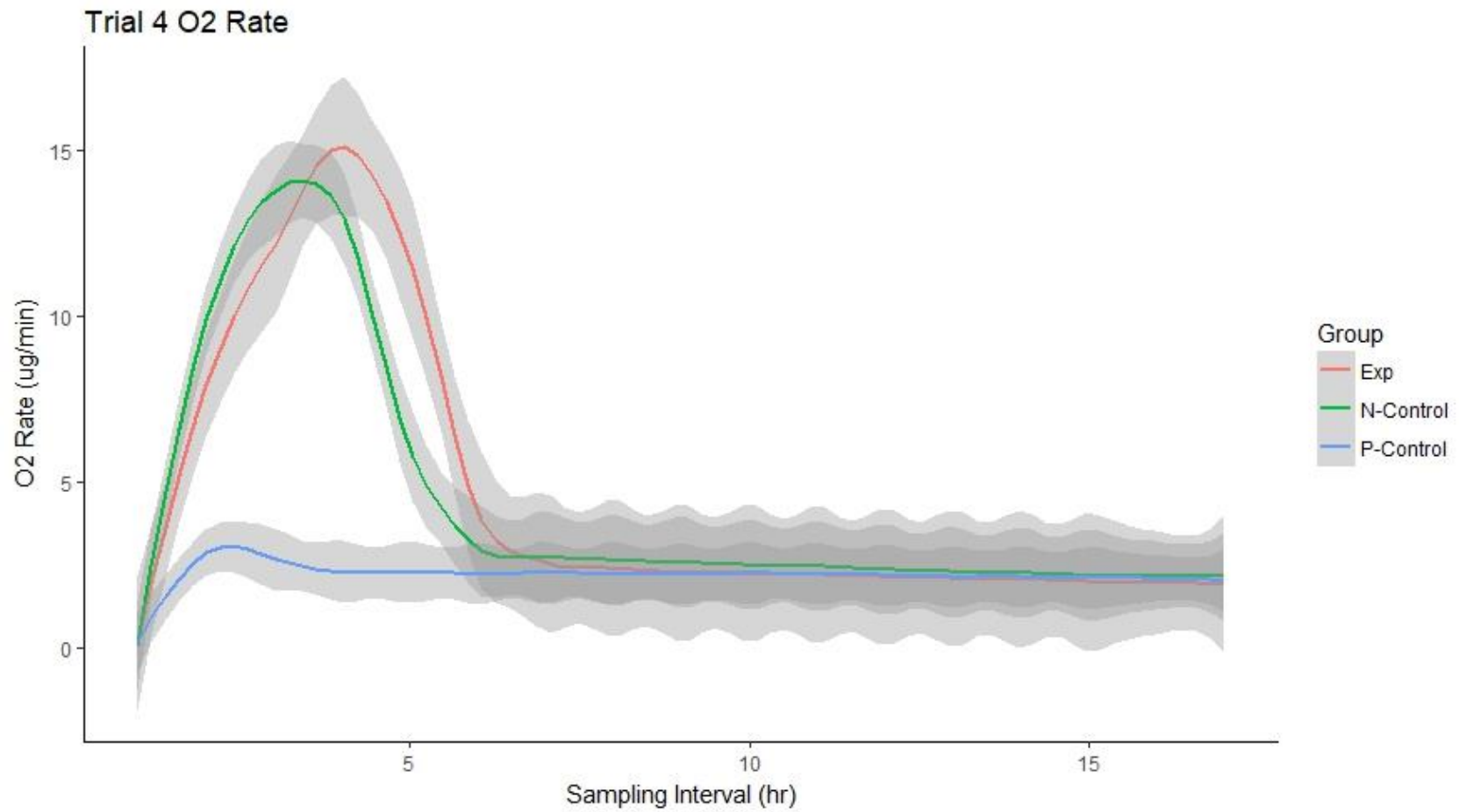


Figure 27 Trial 4 O₂ rate curve with washed MS2 at 1.6×10^{10} PFU/ml. Error regions represent 95% confidence interval determined by three replicate data points.

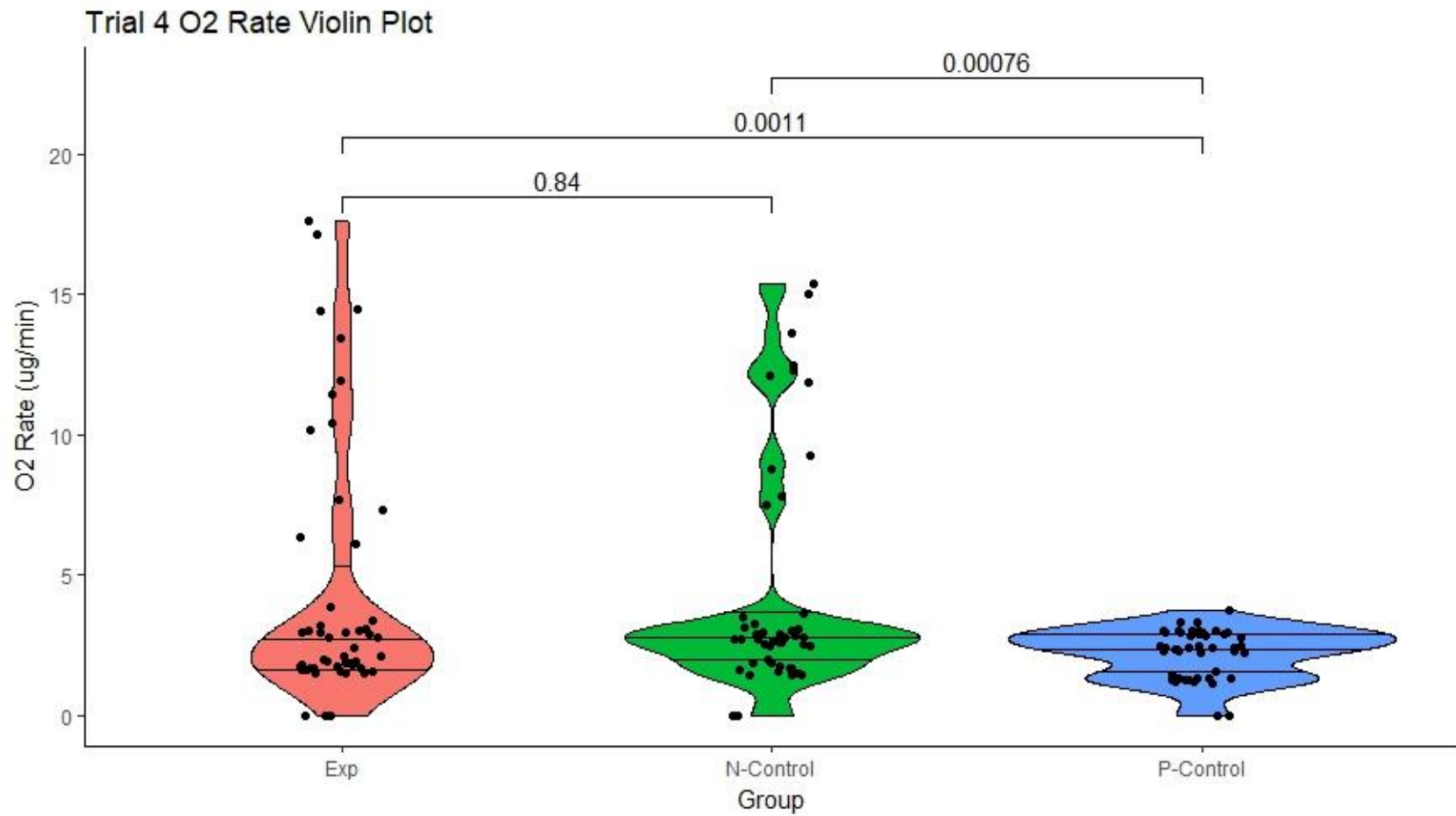


Figure 28 Violin plot with washed MS2 at 3.2×10^8 PFU/ml. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

Shape Parameters

Figures 29 and 30 display the shape parameter comparisons for Trial 4. The shape parameters of the 3.2×10^8 PFU/ml MS2 O₂ consumption profiles were not statistically different from the negative control. The mean FrM of the experiment group was 214.82 $\mu\text{g O}_2/\text{min-hr}^2$ with a variance of 2128 ($\mu\text{g O}_2/\text{min-hr}^2$)². The mean FrM of the negative control was 214.27 $\mu\text{g O}_2/\text{min-hr}^2$ with a variance of 1320.8 $\mu\text{g O}_2/\text{min-hr}^2$. Comparing the FrM of the experimental and control groups yielding a p-value of 0.988. The mean skewness of the experimental group was 0.311 with a variance of 0.133. The mean skewness of the control group was 0.489 with a variance of 0.042. The T-test comparison of the skewness shape factors resulted a p-value of 0.51. The initial seven hours of the experiment yielded results that were not statistically different between the experimental and negative control groups.

Peak Oxygen Consumption Rates

The peak measured SOUR mean of the experiment group was $11.9 \text{ O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$ with a variance of 2.93 ($\text{O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$)². The peak measured SOUR mean of the negative controls was $11.3 \text{ O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$ with a variance of 1.59 ($\text{O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$)². Comparing these peak rates of the three experimental samples and three control samples resulted in a p-value of 0.608. The SOUR comparison for Trial 4 is displayed in Figure 31. There is no statistically significant difference between the O₂ utilization rates for the experiment and negative control samples.

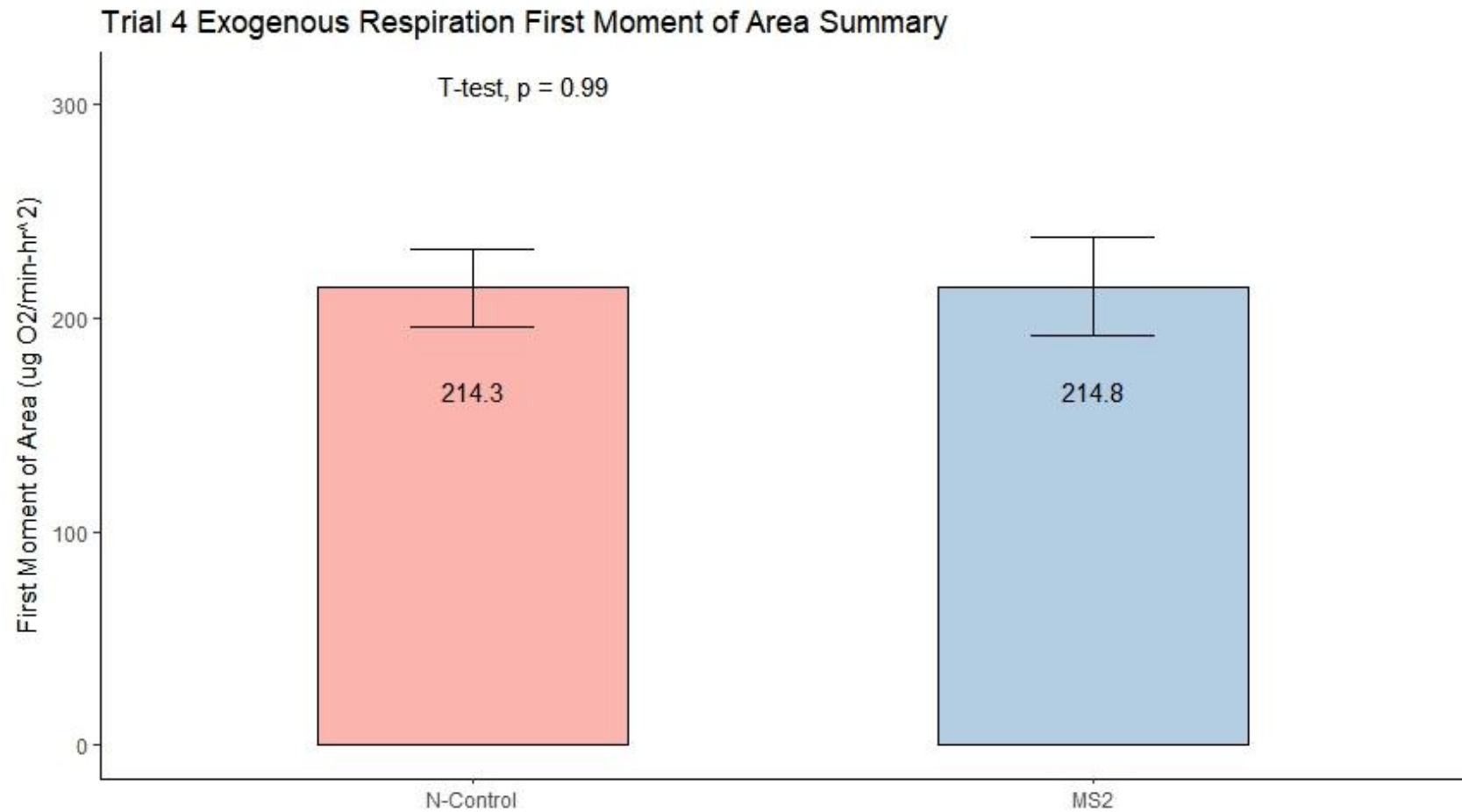


Figure 29 Trial 4 First moment of area. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

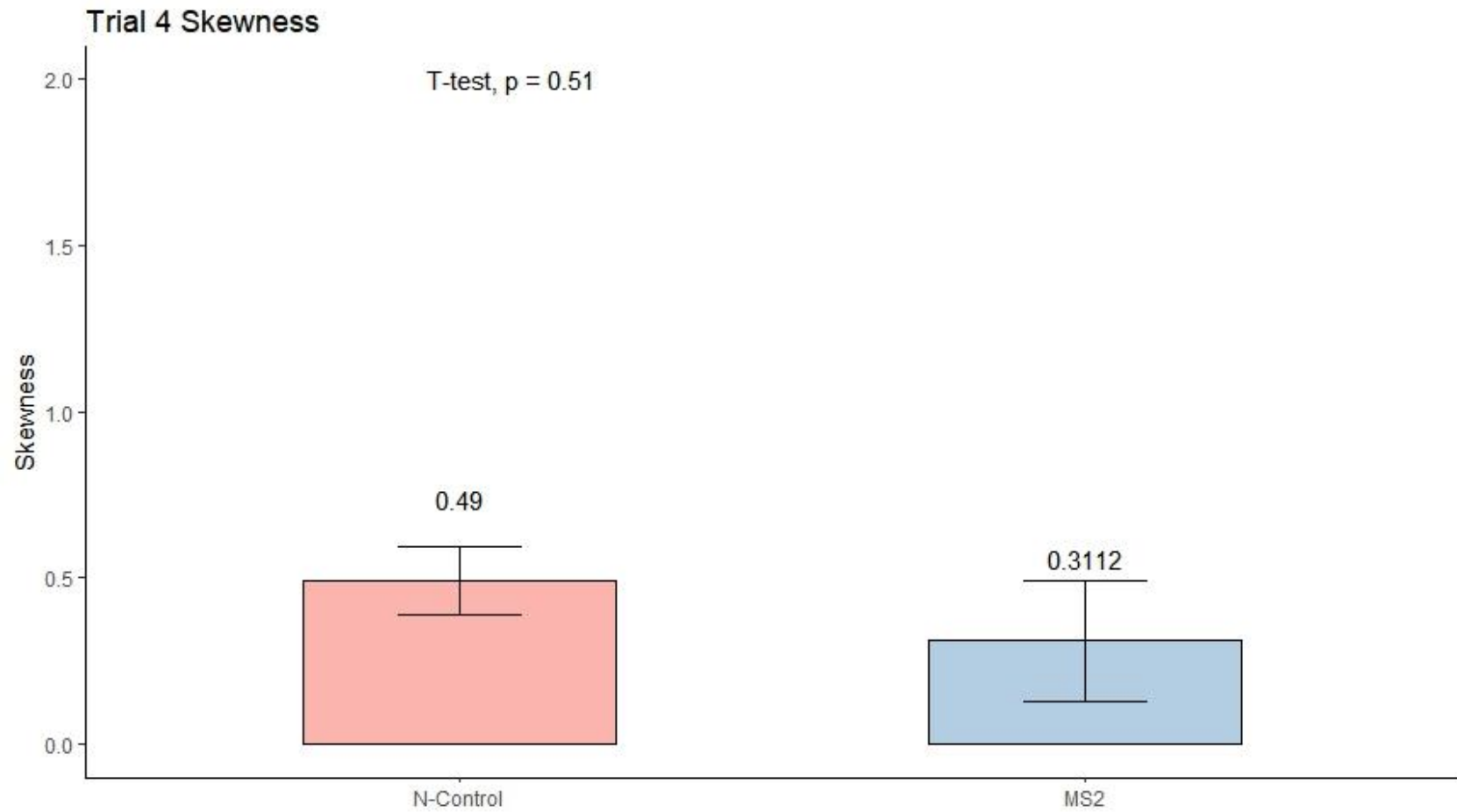


Figure 30 Trial 4 Skewness. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

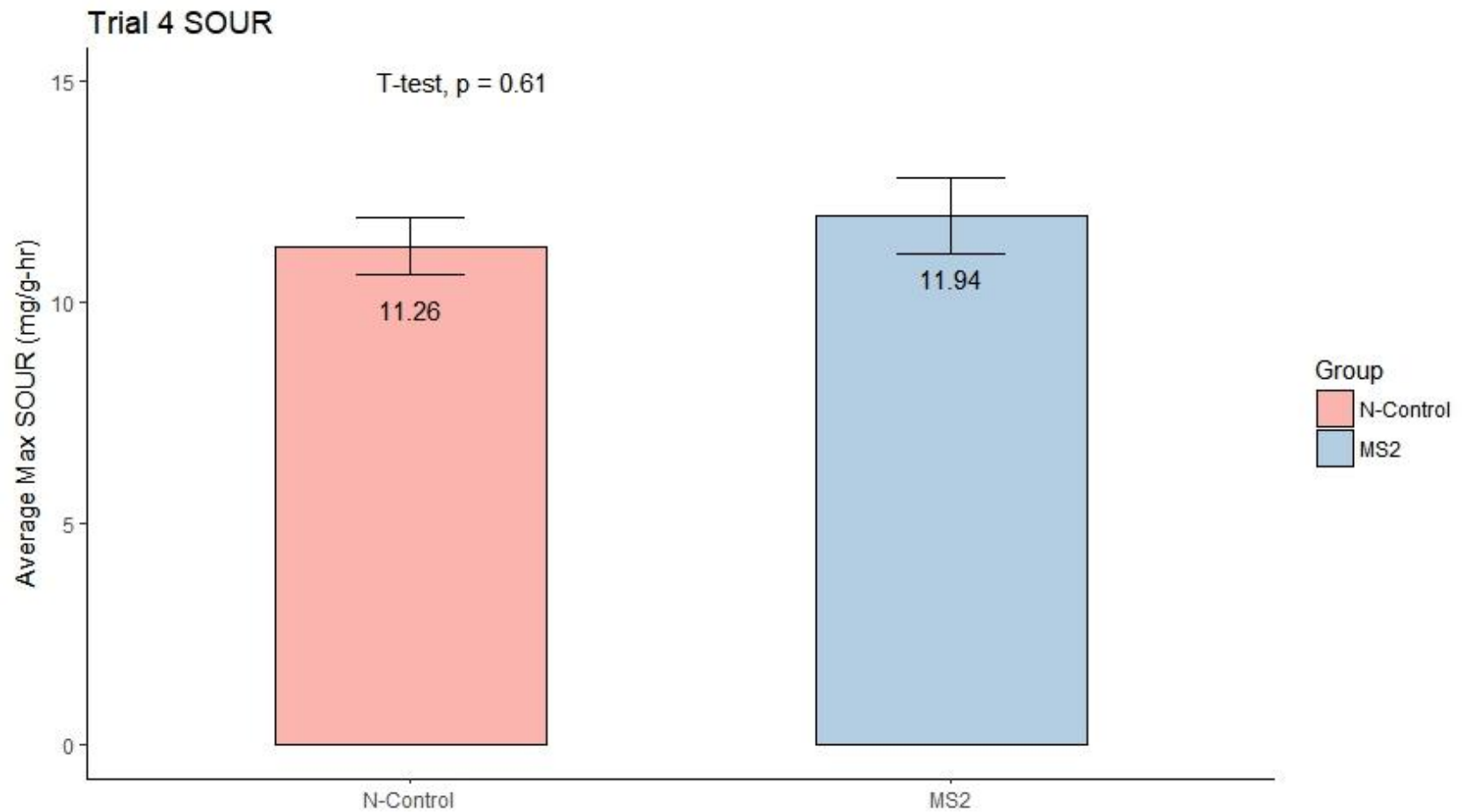


Figure 31 Trial 4 Specific oxygen utilization rate. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

Cumulative Oxygen Consumption Rates

The mean cumulative oxygen consumption recorded during the fourth hour of respirometry for the experiment group was 3023.59 μg with a variance of 446812.40 μg^2 . The mean cumulative oxygen consumption of the negative controls was 2743.18 μg with a variance of 138336.7 μg^2 . A simple t-test was performed comparing the experimental and negative control samples and resulted in a p-value of 0.569. Figure 32 presents the cumulative O₂ comparison. There is no statistically significant difference between the cumulative O₂ consumption for experiment and negative control groups.

Molar CO₂/O₂ Ratios

Figure 33 and 34 present data for the Trial 4 molar ratio comparison. The molar ratio of CO₂ produced to O₂ consumed at 1.6×10^{10} PFU/ml washed MS2 did not appear significantly different from the negative control group. Both exhibited an increase during the first five hours of the experiment and then stabilized. This implies that the enzymatic reactions responsible for respiration within the activated sludge were not affected by the addition of washed MS2 virus. A simple mean's comparison using a Student's t-test at 95% confidence yielded p-values of 0.52, 0.048, and 0.0025 when comparing experimental and negative control, experimental and positive control, and negative and positive controls respectively. No significant difference is observed when comparing the molar ratios of the experimental group to the negative control group. This similarity indicates that the microbial metabolism within the experimental group was still similar to the negative control. This implies that the enzymatic reactions responsible for respiration within the activated sludge were not affected by the addition of washed MS2 virus.

Trial 4 Cumulative O₂ Consumption at Peak Respiration

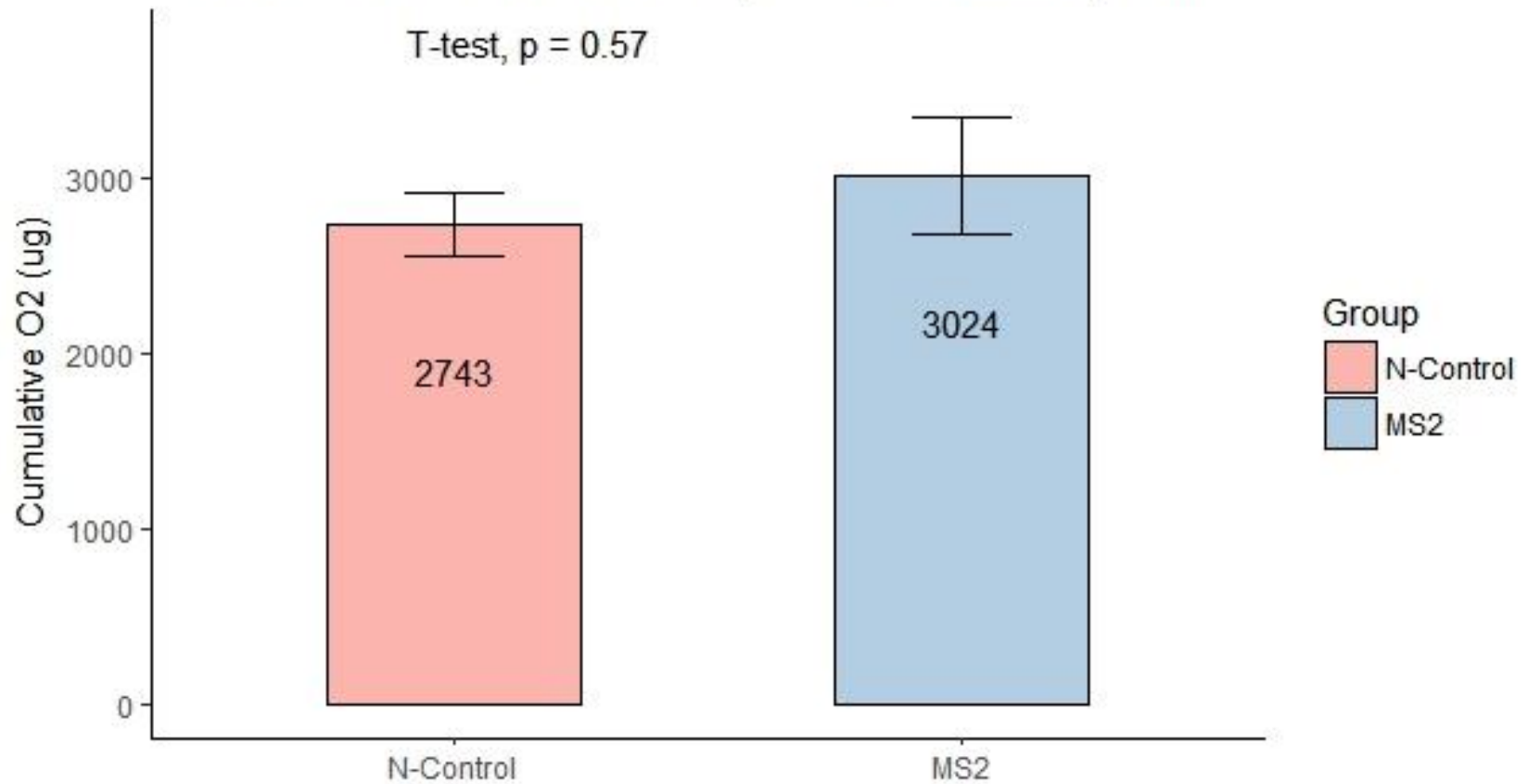


Figure 32 Trial 4 Cumulative O₂ consumption. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

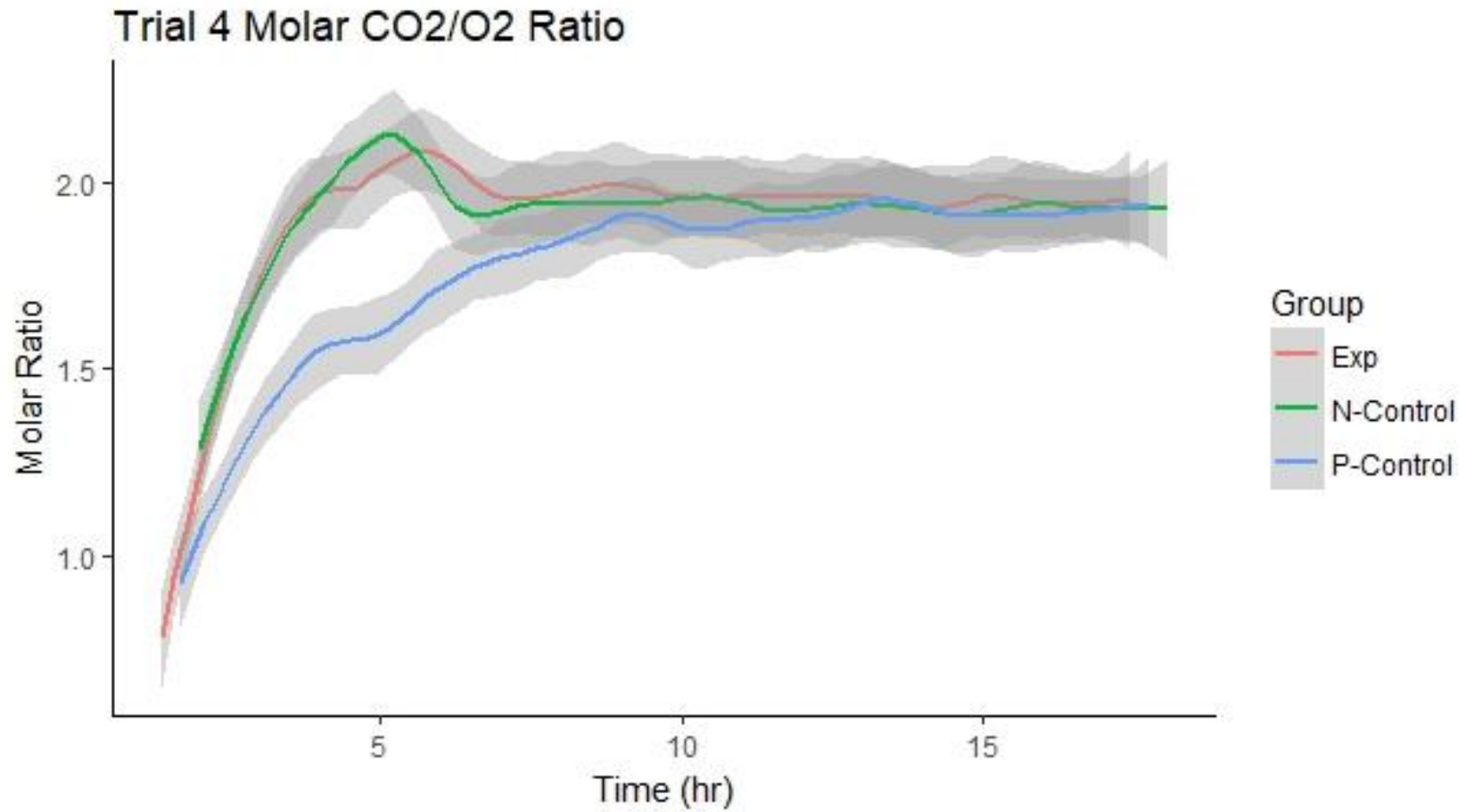


Figure 33 Molar CO₂/O₂ ratio. Error regions represent 95% confidence interval determined by three replicate data points.

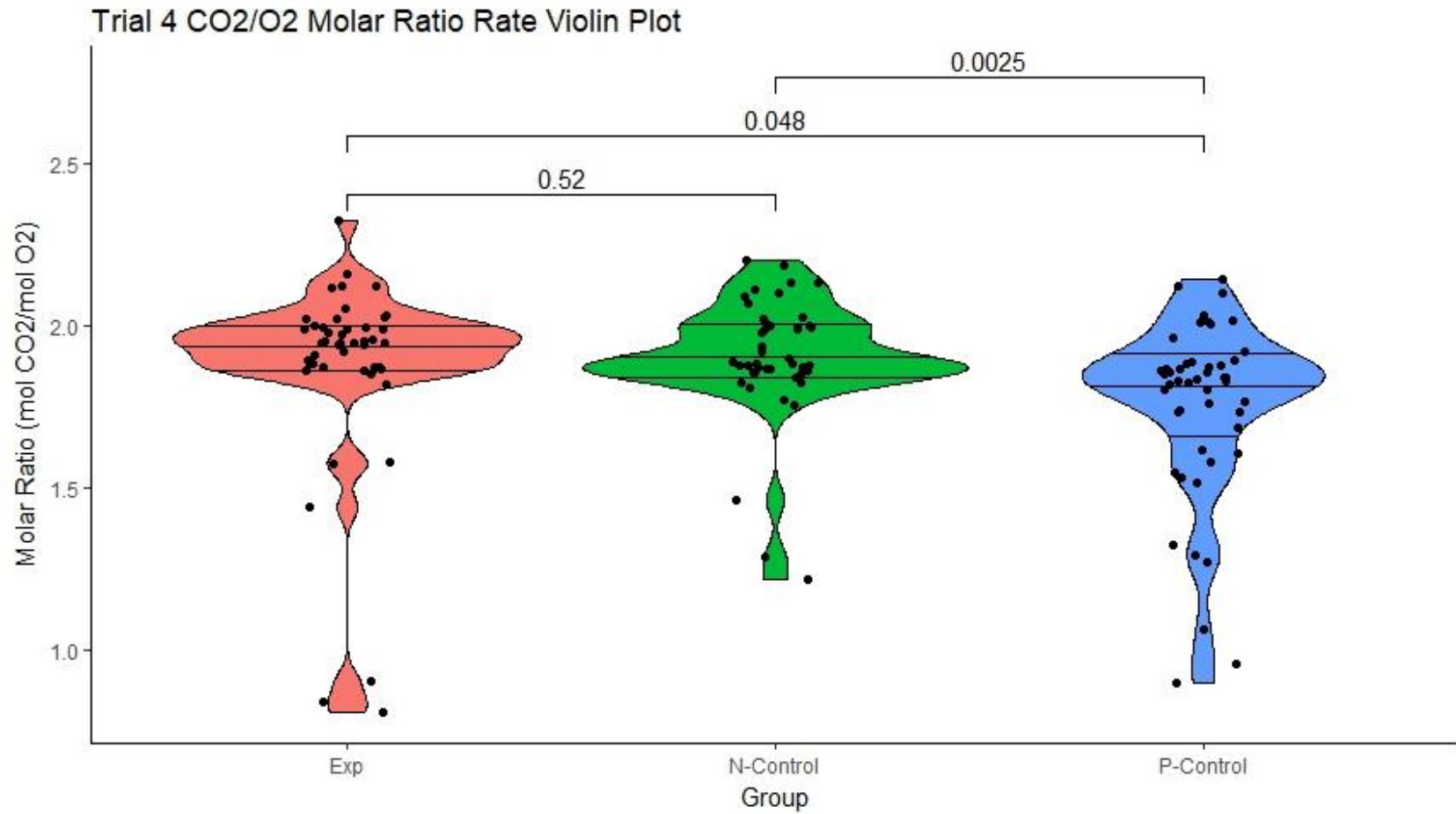


Figure 34 Trial 4 CO₂/O₂ Molar Ratio Violin Plot. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

4.2-E Trial 5 - The effect of 3.2×10^8 PFU/ml MS2 washed on microbial respiration

The 3.2×10^8 PFU/ml washed MS2 did not exhibit a significant effect on microbial respiration during the first eight sampling intervals. With the feeds added to each bottle, each curve exhibits a sharp increase in O₂ respiration rate. The experimental and negative control groups' O₂ curves peaked between hours 3 – 6. The positive control groups containing ATU peaked within the second hour of the test. Following peak respiration, each sample exhibited a decreasing rate of O₂ consumption until endogenous respiration was reached. This endogenous respiration rate varied between each channel and indicates the absence of the feed. During this time, bacteria are consuming O₂ as they digest other bacteria in the substrate deficient mixture. The trial was terminated after a period of approximately 17 hours. Figure 35 presents the reprogram output for Trial 5. When comparing the O₂ rates using a Student's t-test at 95% confidence, no significant difference is observed between the experimental and negative control groups. A significant difference is observed between the experimental and positive control groups. This suggests that the microbial activity of the experimental is similar to that of the negative control group and different from the samples containing ATU. Further, this is evidence that microbial respiration was not inhibited in the experimental samples containing the MS2 bacteriophage. The O₂ means comparison violin plot is included in Figure 36.

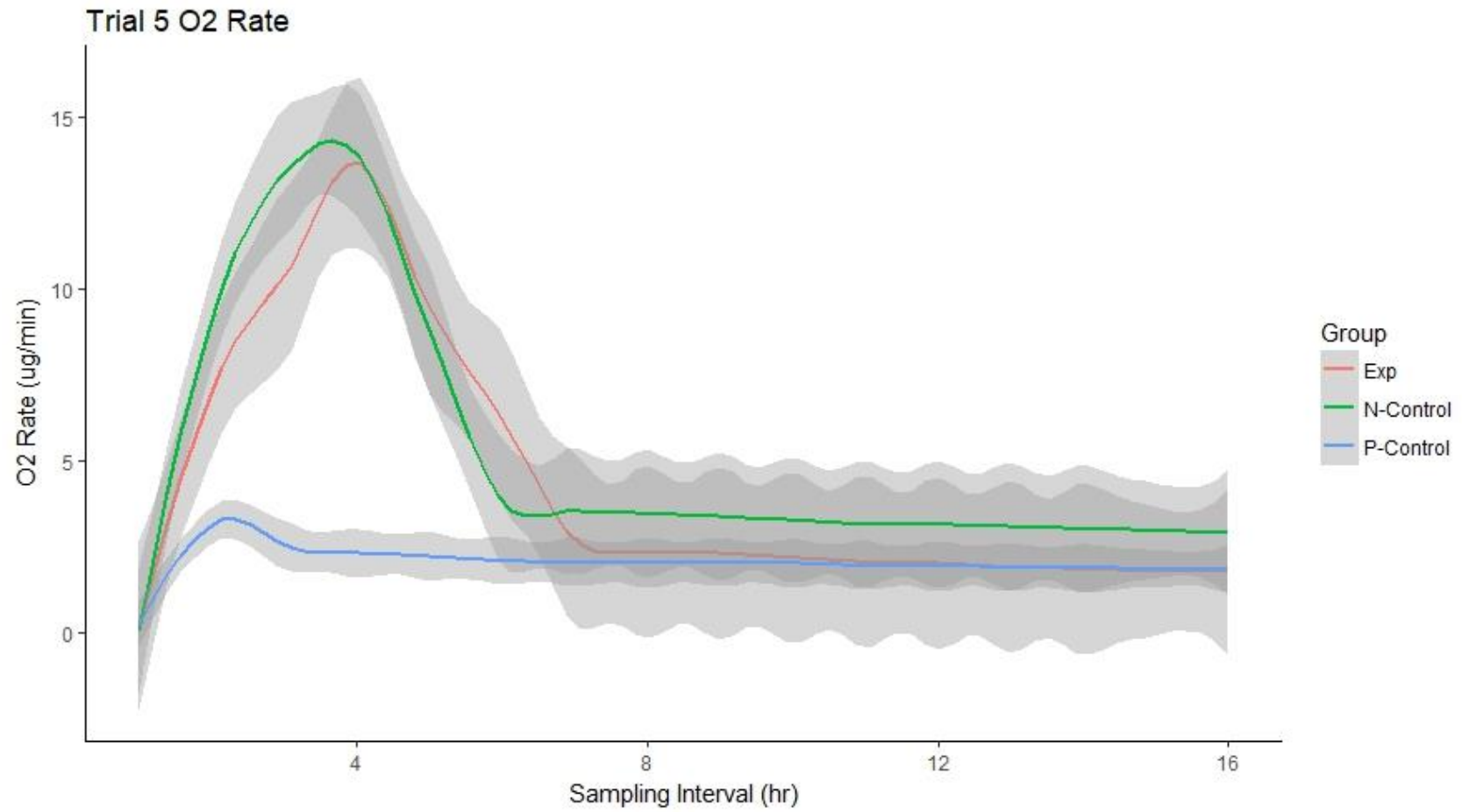


Figure 35 O2 curve with washed MS2 at 3.2×10^8 PFU/ml. Error regions represent 95% confidence interval determined by three replicate data points.

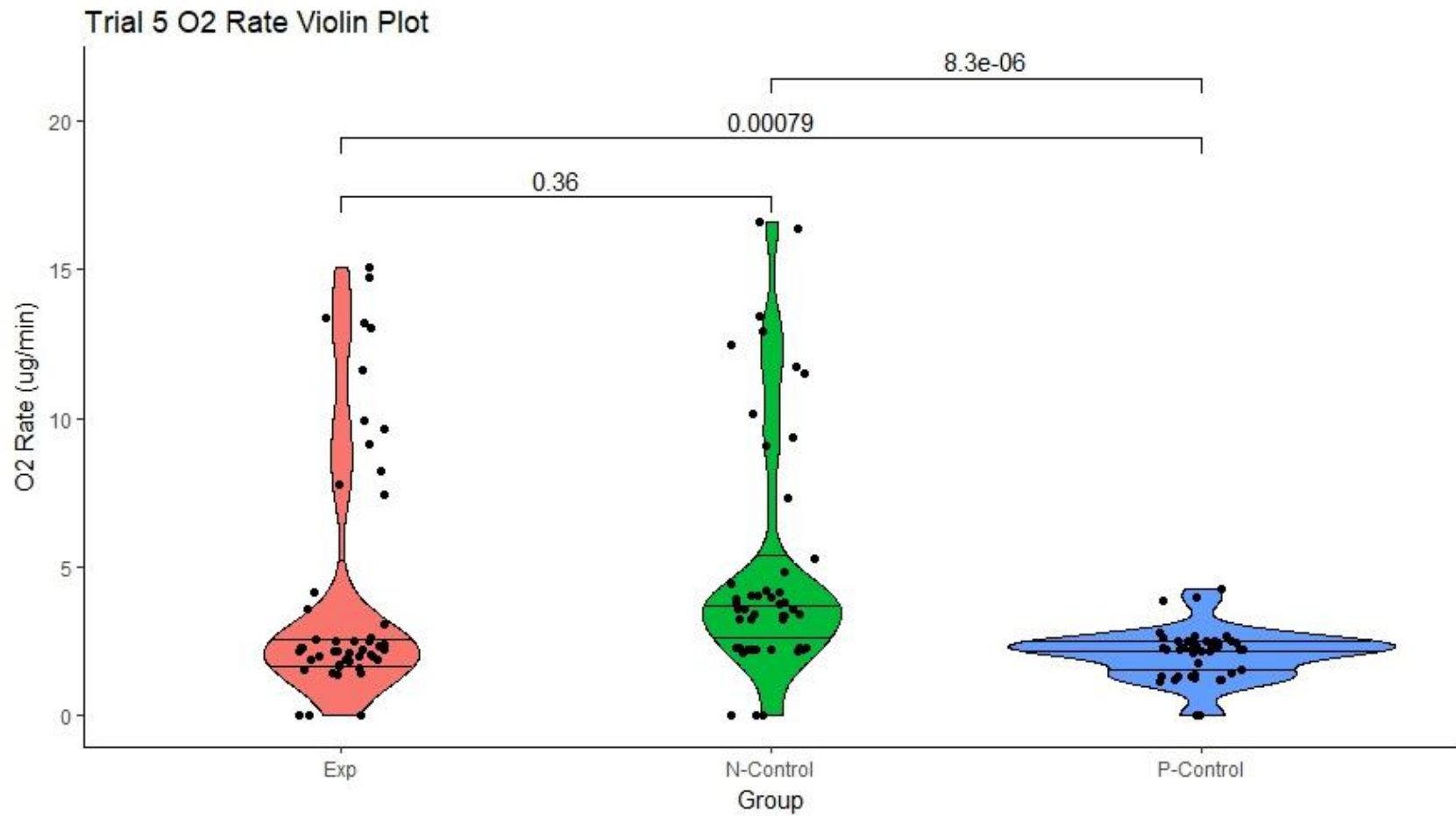


Figure 36 O2 violin plot with washed MS2 at 3.2×10^8 PFU/ml. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

Shape Parameters

The shape parameter comparisons are presented in Figures 37 and 38. The shape parameters of the 3.2×10^8 PFU/ml MS2 O₂ consumption profiles were not statistically different from the negative control group. The mean FrM of the experiment group was $204.54 \mu\text{g O}_2/\text{min-hr}^2$ with a variance of $4264.76 (\mu\text{g O}_2/\text{min-hr}^2)^2$. The mean FrM of the negative control was $243.52 \mu\text{g O}_2/\text{min-hr}^2$ with a variance of $3781.15 (\mu\text{g O}_2/\text{min-hr}^2)^2$. Comparing the FrM of the experimental and control groups yielding a p-value of 0.493. The mean skewness of the experimental group was 0.319 with a variance of 0.295. The mean skewness of the control group was 0.370 with a variance of 0.079. The T-test comparison of the skewness shape factors resulted a p-value of 0.68.

Peak Oxygen Consumption Rates

The average maximum SOUR of the experiment group was $10.8 \text{ O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$ with a variance of $0.46 (\text{O}_2\text{-g VSS}^{-1}\text{-hr}^{-1})^2$. The average maximum SOUR of the negative controls was $11.5 \text{ O}_2\text{-g VSS}^{-1}\text{-hr}^{-1}$ with a variance of $2.37 (\text{O}_2\text{-g VSS}^{-1}\text{-hr}^{-1})^2$. Comparing these peak rates of the three experimental samples and three control samples resulted in a p-value of 0.53. As a result, there is no statistically significant difference between the O₂ utilization rates for the experiment and negative control samples. This comparison is presented in Figure 39.

Trial 5 Exogenous Respiration First Moment of Area Summary

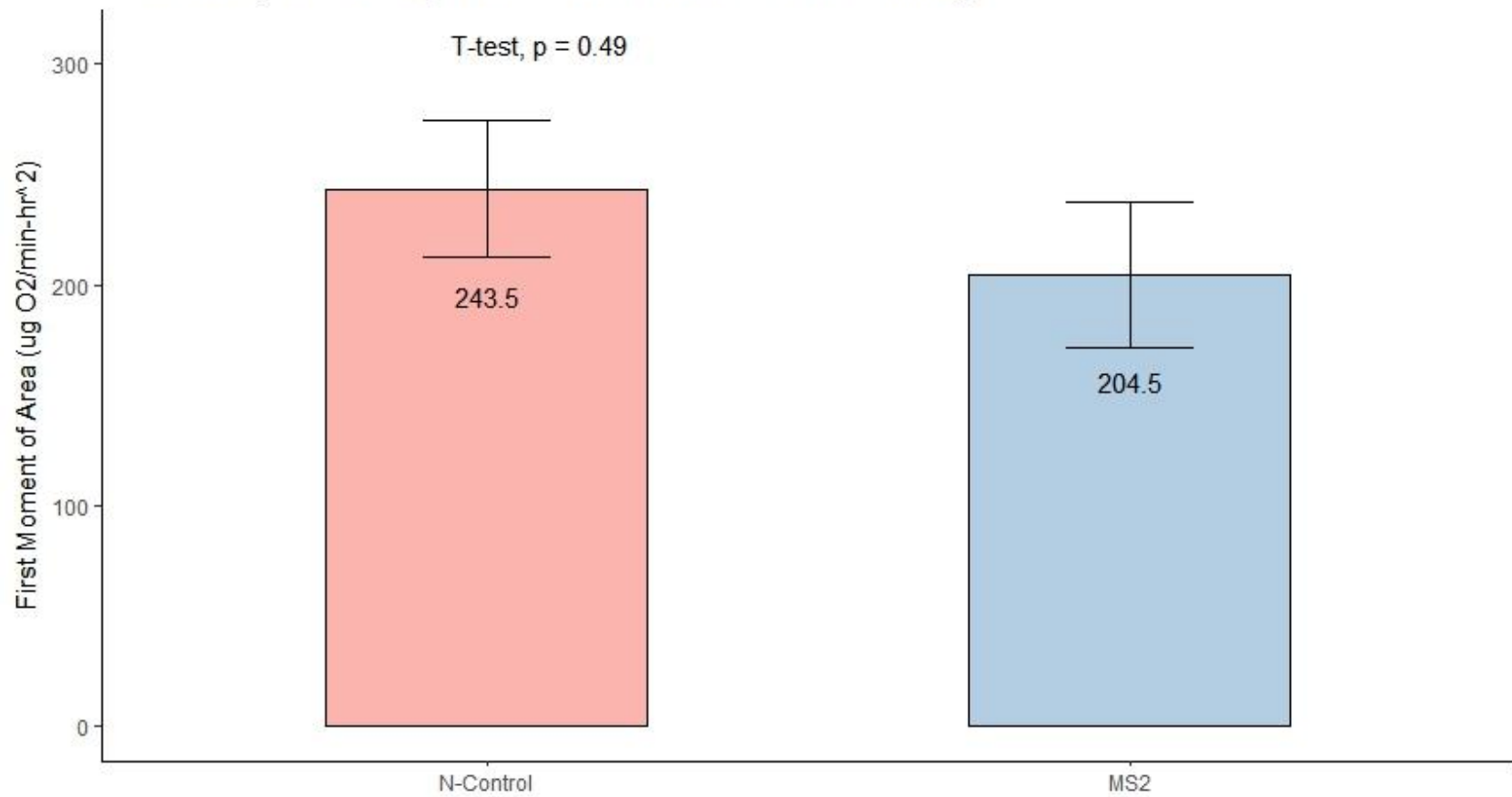


Figure 37 Trial 5 First moment of area. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

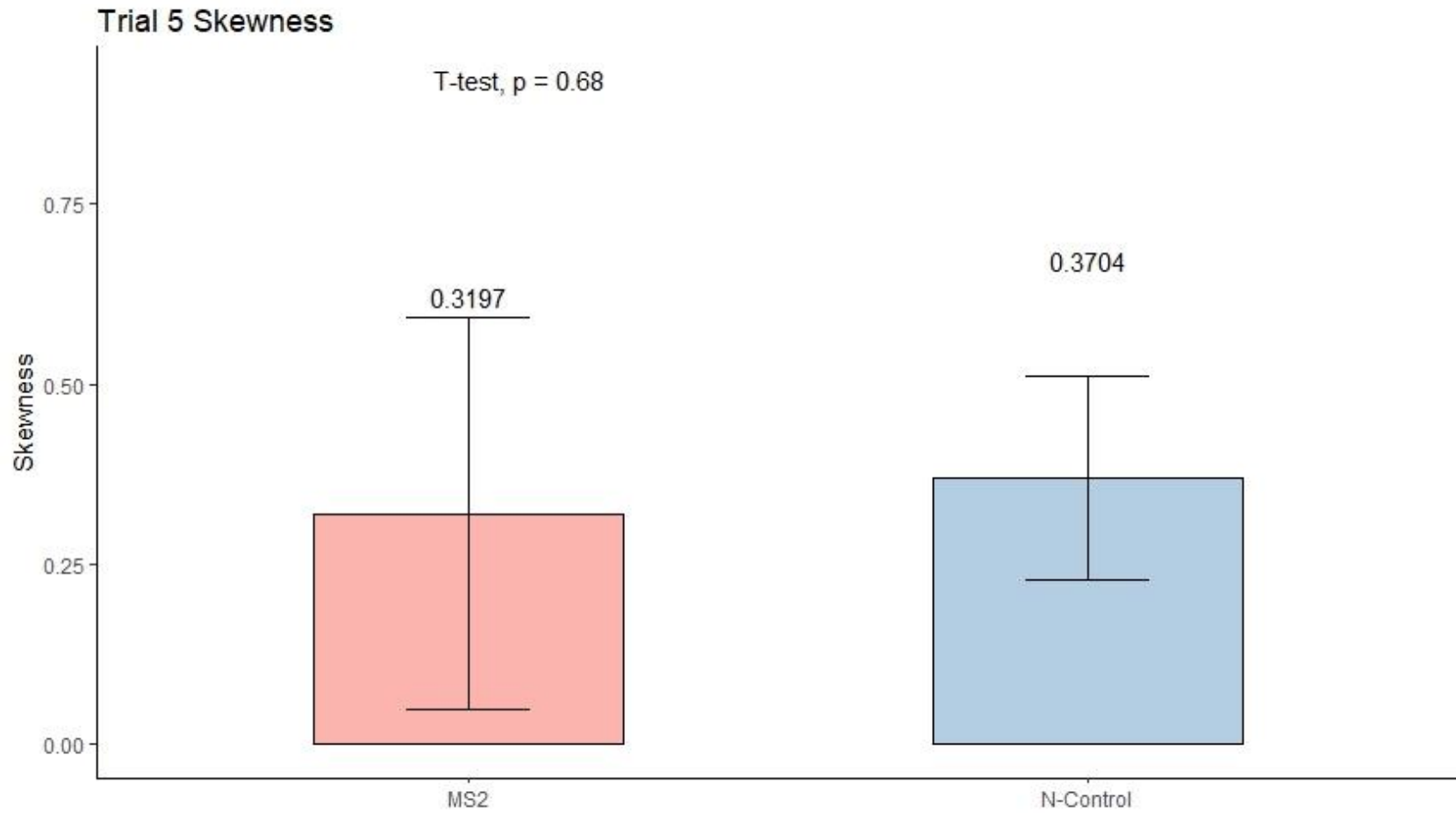


Figure 38 Trial 5 Skewness. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

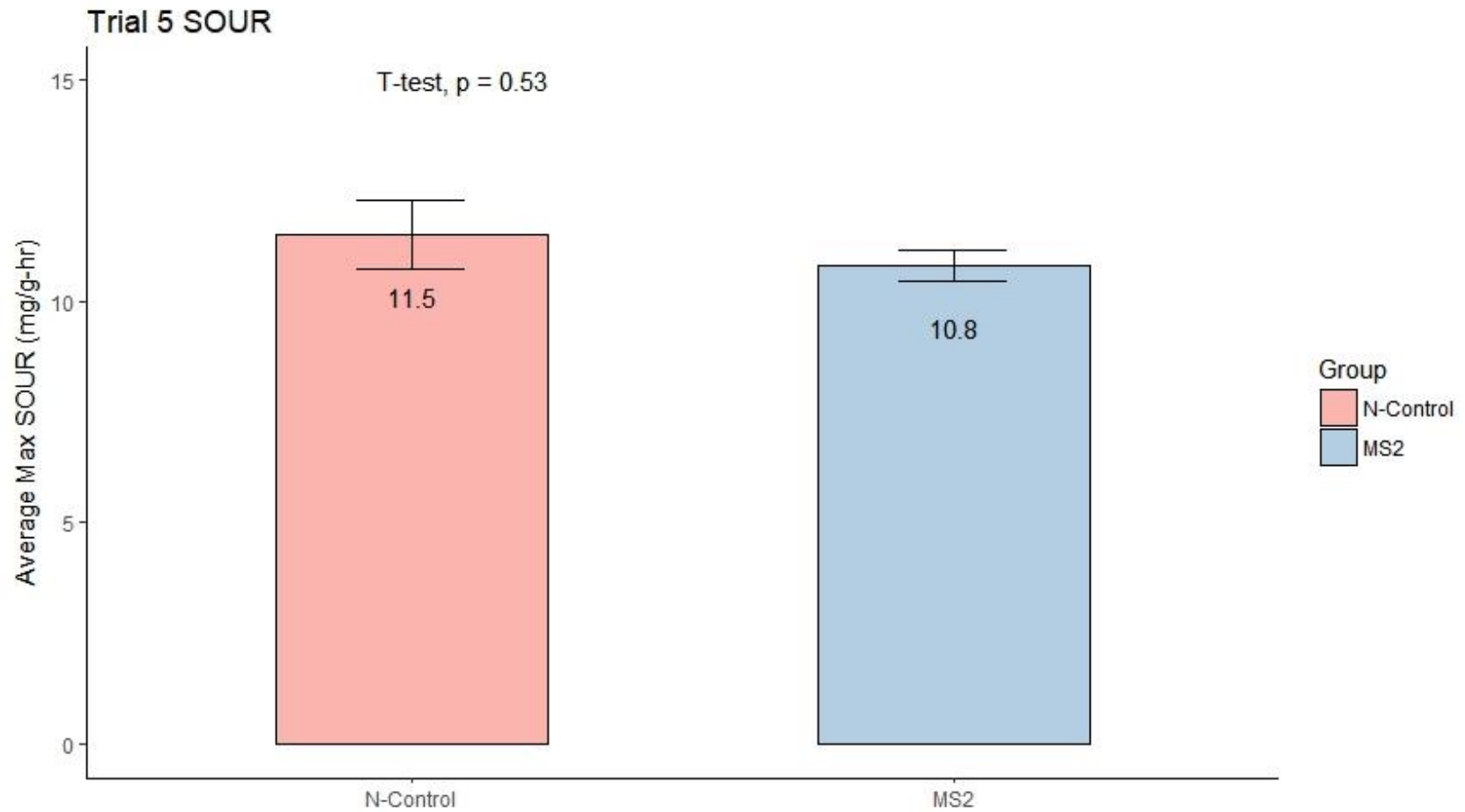


Figure 39 Trial 5 Specific oxygen utilization rate. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

Cumulative Oxygen Consumption Rates

The cumulative O₂ consumption rate comparison is presented in Figure 40. The mean cumulative oxygen consumption recorded during the fourth hour of respirometry for the experiment group was 2631.71 µg with a variance of 60019.91 µg². The mean cumulative oxygen consumption of the negative controls was 2888.59 µg with a variance of 292215.40 µg². A simple t-test comparing the experimental and negative control samples and resulted in a p-value of 0.512. There is no statistically significant difference between the cumulative O₂ consumption for experiment and negative control groups.

Molar CO₂/O₂ Ratios

Figures 41 and 42 present the molar ratio comparison data for Trial 5. The molar ratio of CO₂ produced to O₂ consumed at 3.2x10⁸ PFU/ml washed MS2 did not appear significantly different from the negative control group. Both exhibited an increase during the first five hours of the experiment and then stabilized. This implies that the enzymatic reactions responsible for respiration within the activated sludge were not affected by the addition of washed MS2 virus. A simple mean's comparison using a Student's t-test at 95% confidence yielded p-values of 0.63, 0.038, and 0.04 when comparing experimental and negative control, experimental and positive control, and negative and positive controls respectively. No significant difference is observed when comparing the molar ratios of the experimental group to the negative control group. This similarity indicates that the microbial metabolism within the experimental group was still similar to the negative control. This implies that the enzymatic reactions responsible for respiration within the activated sludge were not affected by the addition of washed MS2 virus.

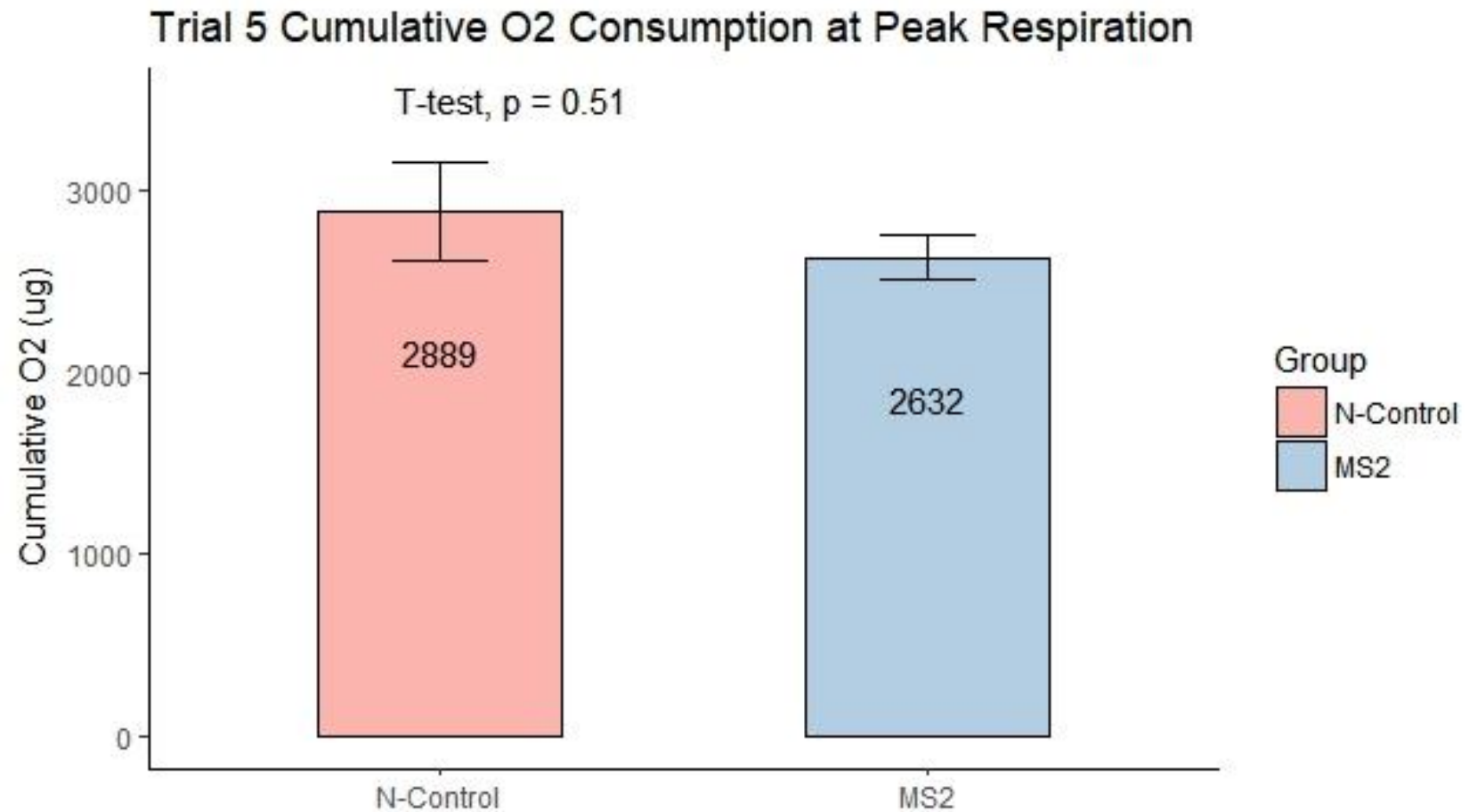


Figure 40 Trial 5 Cumulative O₂ consumption. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

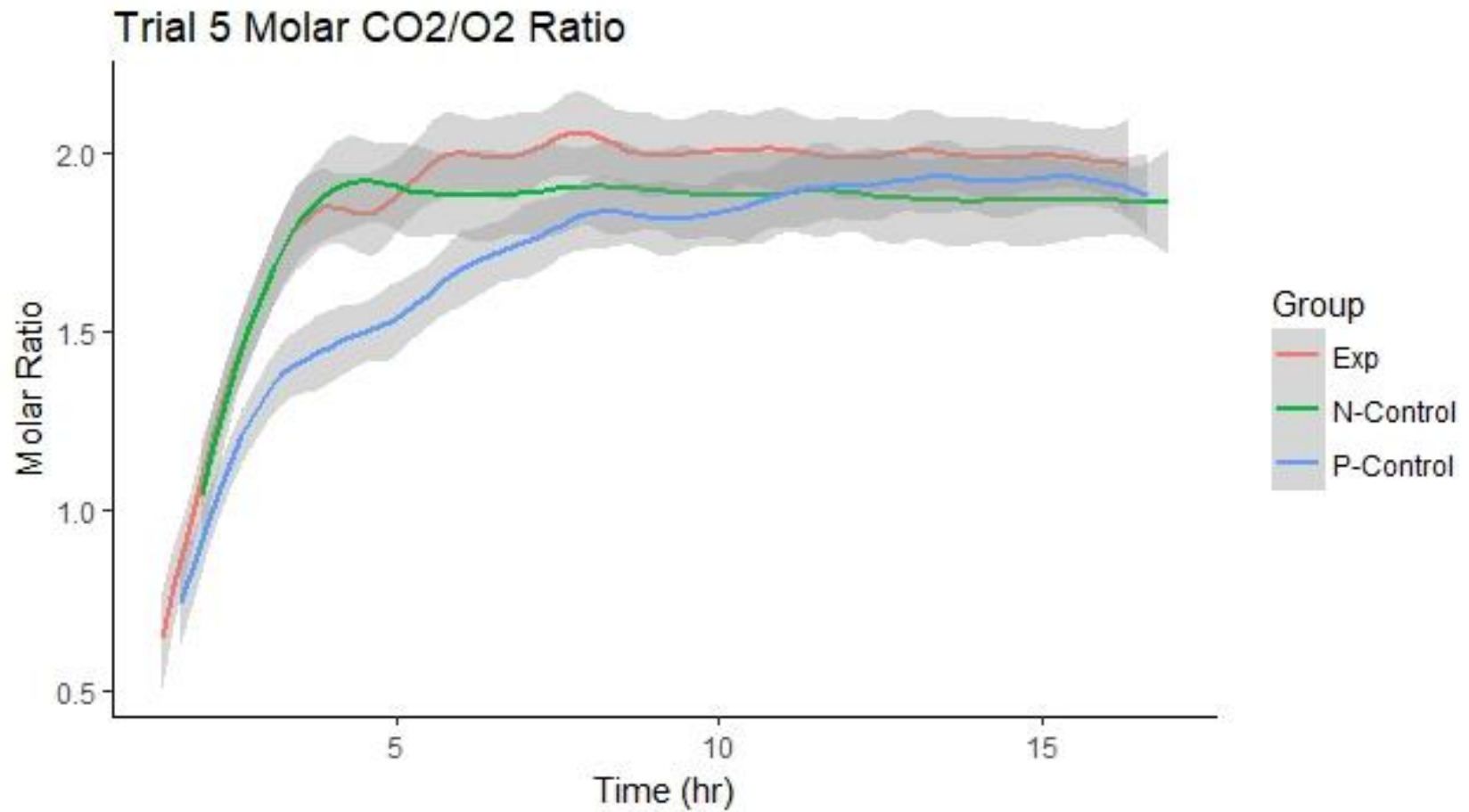


Figure 41 Molar CO₂/O₂ ratio. Error regions represent 95% confidence interval determined by three replicate data points.

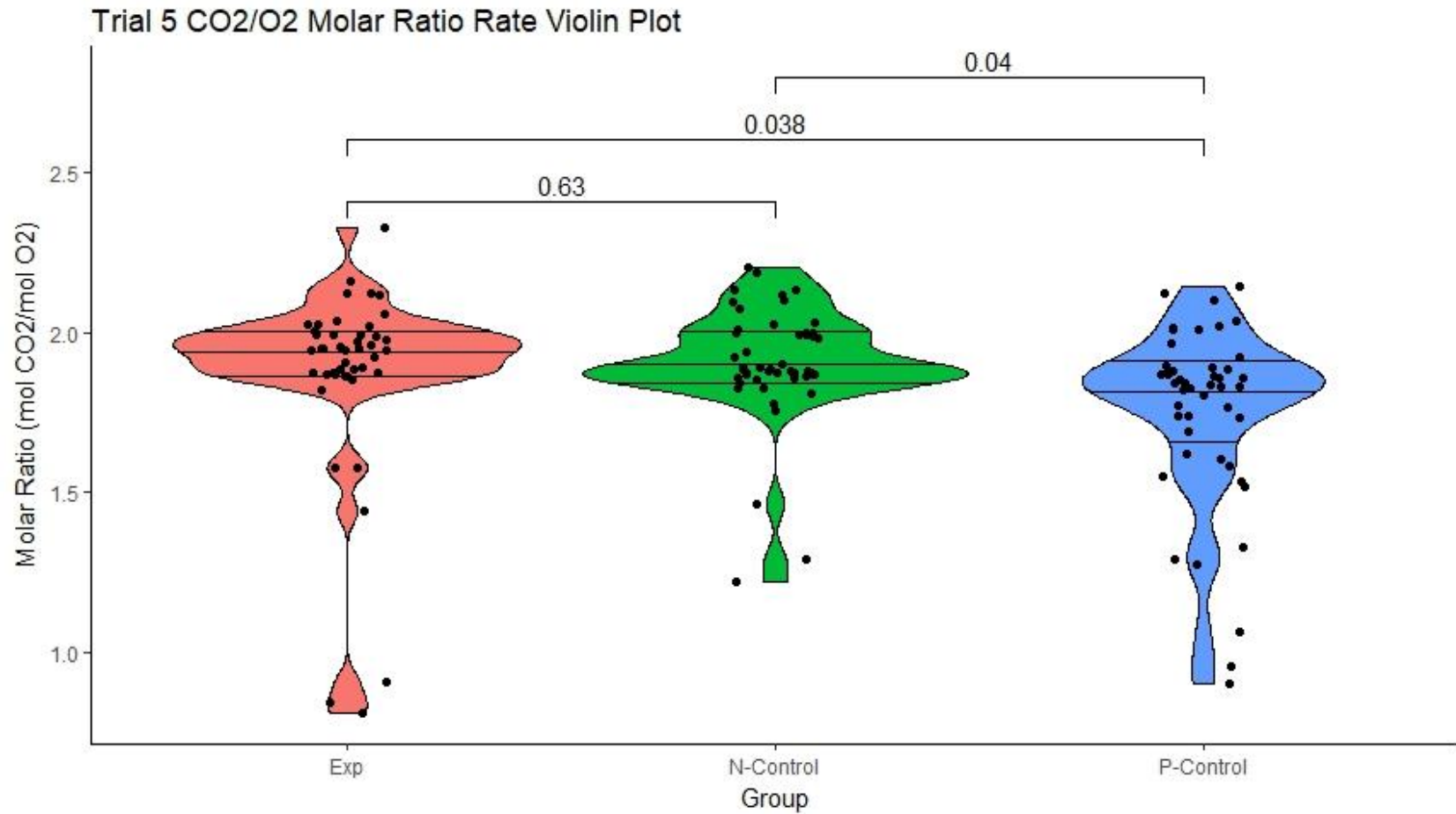


Figure 42 Trial 5 CO₂/O₂ Molar Ratio Violin Plot. Horizontal lines indicate the 0.75, 0.5, and 0.25 quantiles. P-values associated with Student's t-test conducted at 95% confidence are presented.

4.3 Spectrometry Analysis

4.3-A COD Concentration

Figure 43 presents the post-treatment COD removal analysis. The MS2 virus did not inhibit COD consumption at a concentration of 3.2×10^8 PFU/ml. A two-tailed, Student's t-test assuming equal variance at 95% confidence yielded a p-value of 0.932 when comparing the post-treatment COD concentration of MS2 and negative control groups. This similarity implies that MS2 did not disrupt the ability of activated sludge samples to degrade COD.

4.3-B Nitrate Concentration

Figure 34 presents the post-treatment nitrate nitrogen concentration analysis. MS2 did not inhibit nitrification at a concentration of 3.2×10^8 PFU/ml. A two-tailed, Student's s-test assuming equal variance at 95% confidence yielded a p-value of 0.635 when comparing the post-treatment nitrate concentration of MS2 and negative control groups. This suggests that nitrification occurred in experimental samples to an extent similar to the negative control group.

4.3-C Ammonia Concentration

Figure 35 presents the post-treatment ammonia nitrogen concentration analysis. The MS2 virus did not inhibit ammonia consumption at a concentration of 3.2×10^8 PFU/ml. A two-tailed, Student's t-test assuming equal variance at 95% confidence yielded a p-value of 0.859 when comparing the post-treatment ammonia concentration of MS2 and negative control groups. This suggests that MS2 did not inhibit the ability of activated sludge within the experimental groups to degrade ammonia. As expected, ATU

demonstrated inhibition of ammonia removal. In fact, as a result of endogenous respiration, ammonia levels increased to an average of 83.5 mg/L between the positive control samples.

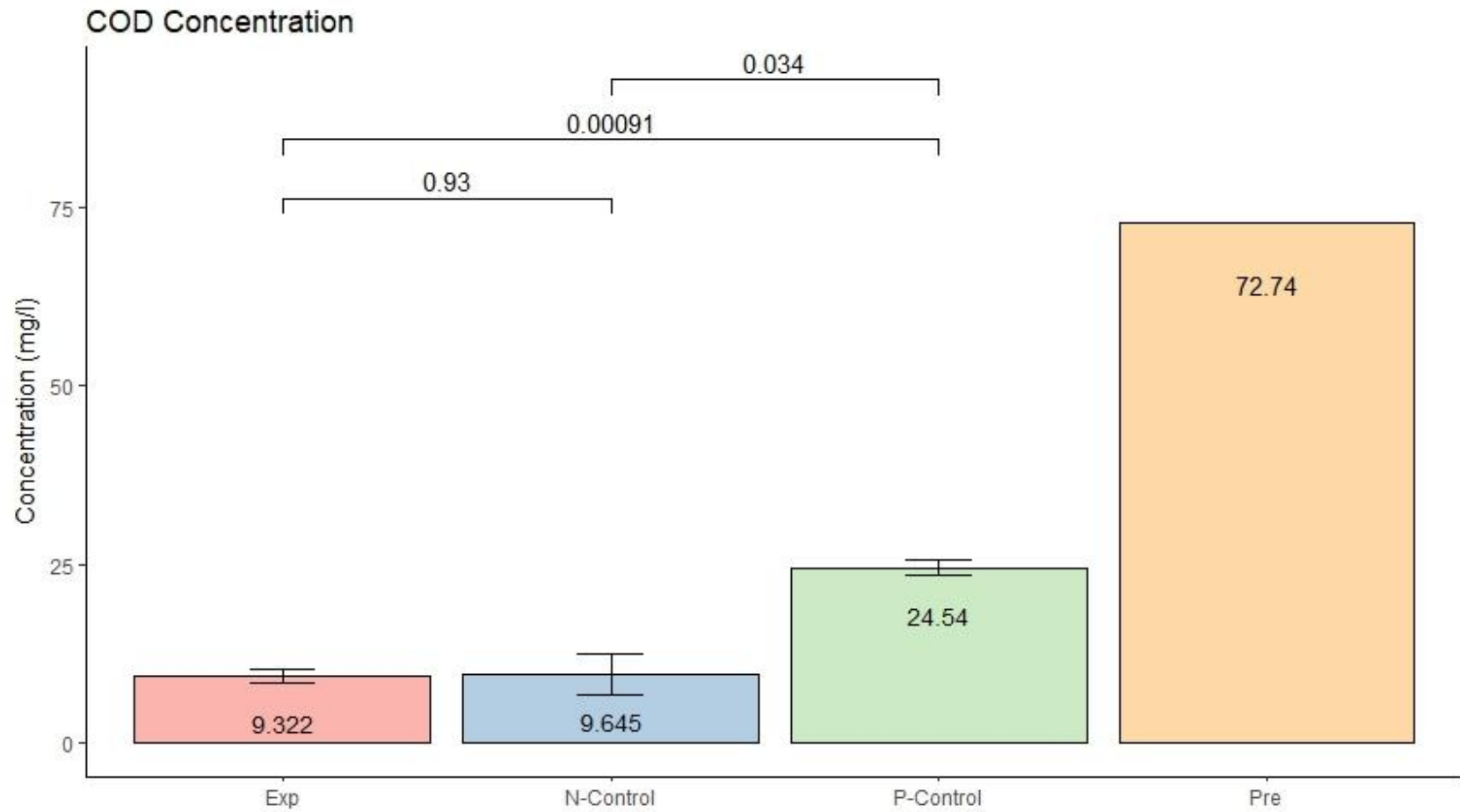


Figure 43 COD Concentration. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

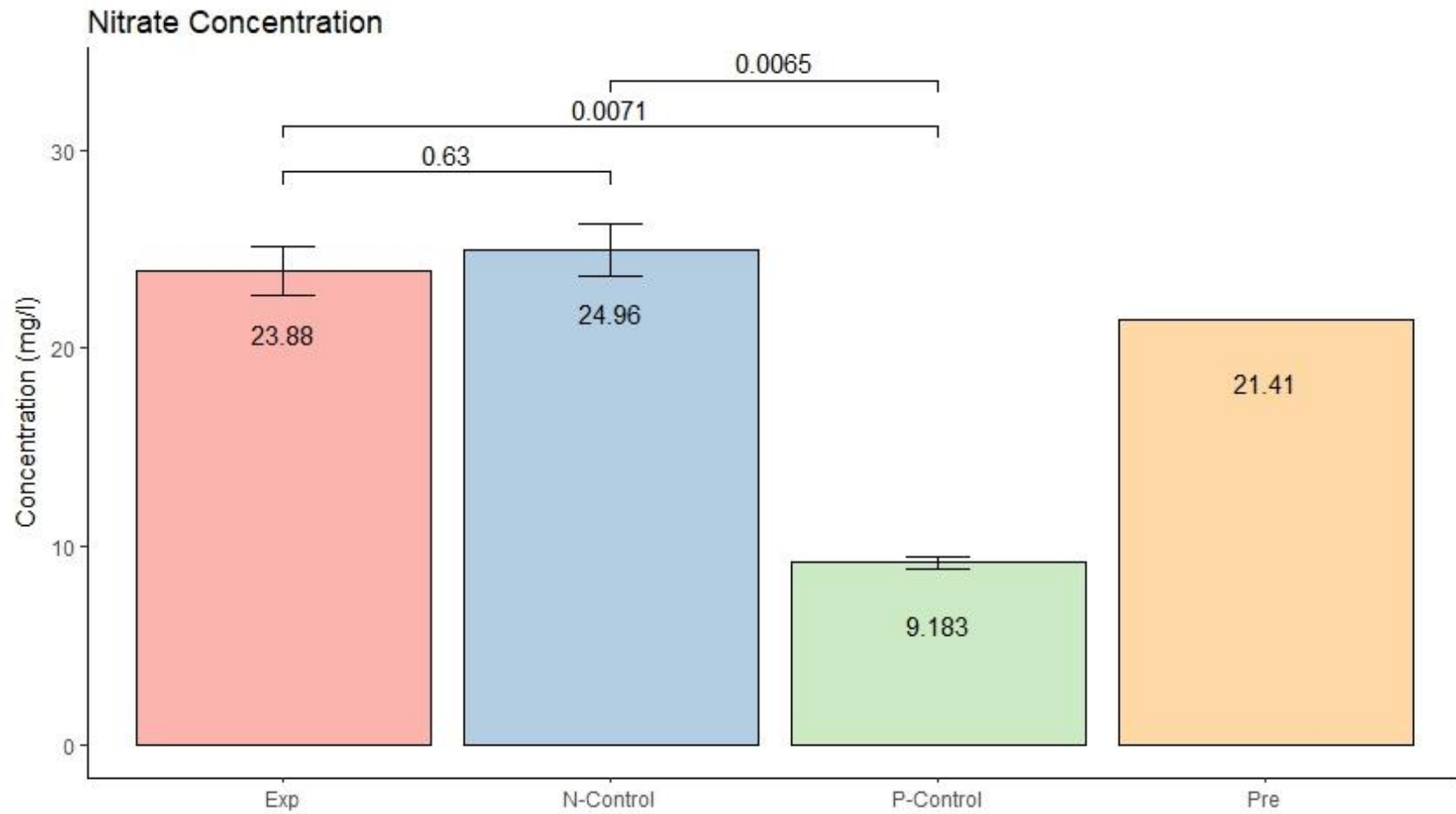


Figure 44 Nitrate-Nitrogen Concentration. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

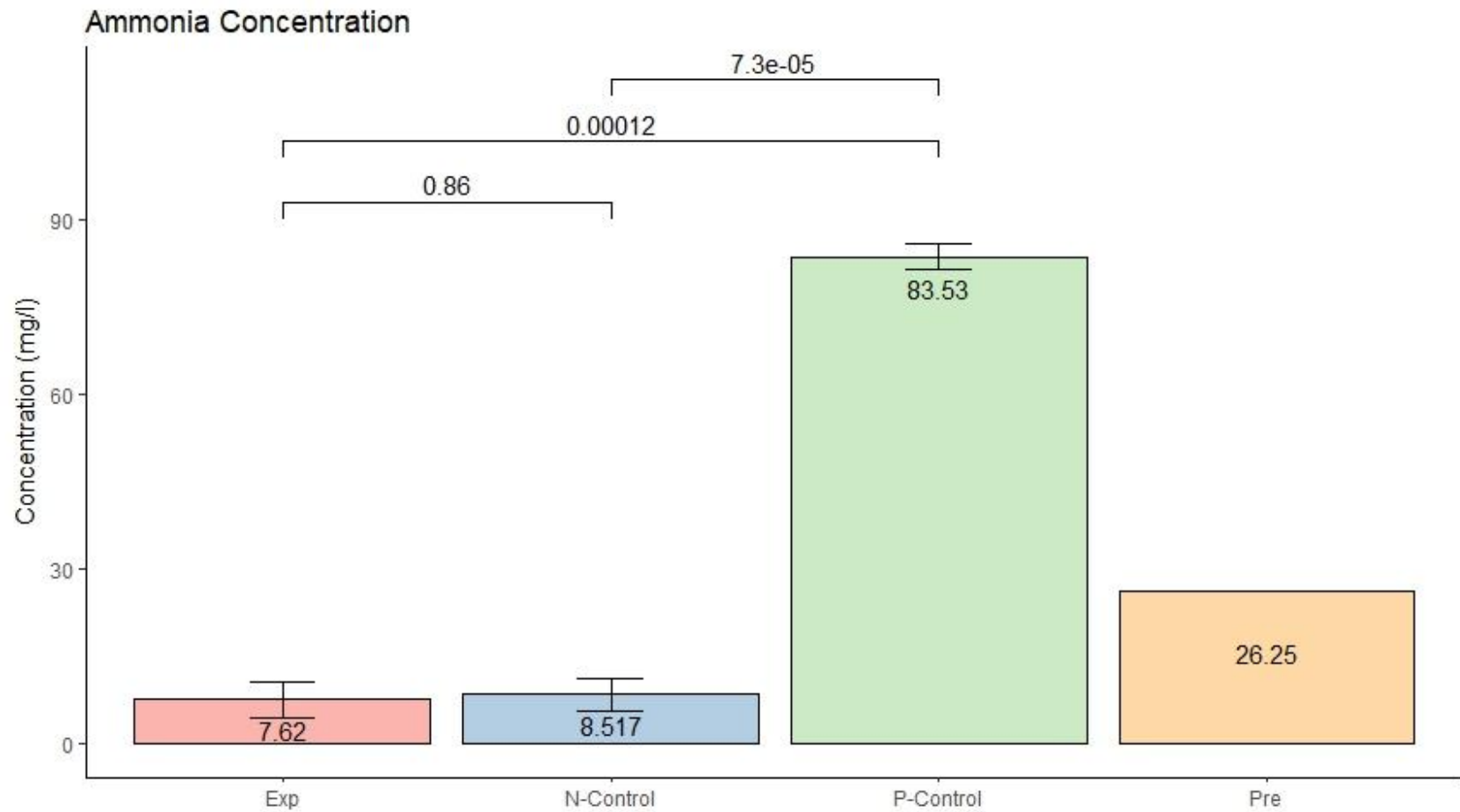


Figure 45 Ammonia Concentration. Error bars represent standard deviation. P-values associated with Student's t-test conducted at 95% confidence are presented.

4.4 Dual Fluorescence Labeling

Because of the diversity within a given flocculated sludge particle, the dual fluorescence labeling protocols results must be qualified prior to making any determinations regarding infection. Several trends were consistently observed which offer context to be considered when viewing the imagery. Specifically, the occurrence of auto-fluorescence and free floating lipids was common and are evidenced by Figure 46 and Figure 47, respectively. Auto-fluorescence can be described as the tendency of a particle to reflect a wide range of wavelengths of the electromagnetic spectrum without the addition of dyes. This means that the same particles can be viewed under blue light and UV light exposure. In the blue light imagery at left in Figure 46, the sludge floc fluoresces as a green mass. Red areas are also observed either indicating cellular structures or free floating fatty acids. The image at right in Figure 46 is the same sludge floc particle viewed under UV light exposure. Notably, distinguishable features of the two masses are visible under both wavelengths and are thus auto-fluorescing.



Figure 46: Auto fluorescence of activated sludge floc. Left panel: blue light excitation. Right panel: UV light excitation.

While this occurred frequently, it did not limit the observer's ability to identify MS2 virus particles or fluorescing lipids. However, as evidenced by Figure 47, free floating lipids were commonly observed as cloudy red areas both within flocculated particles and within the supernatant.

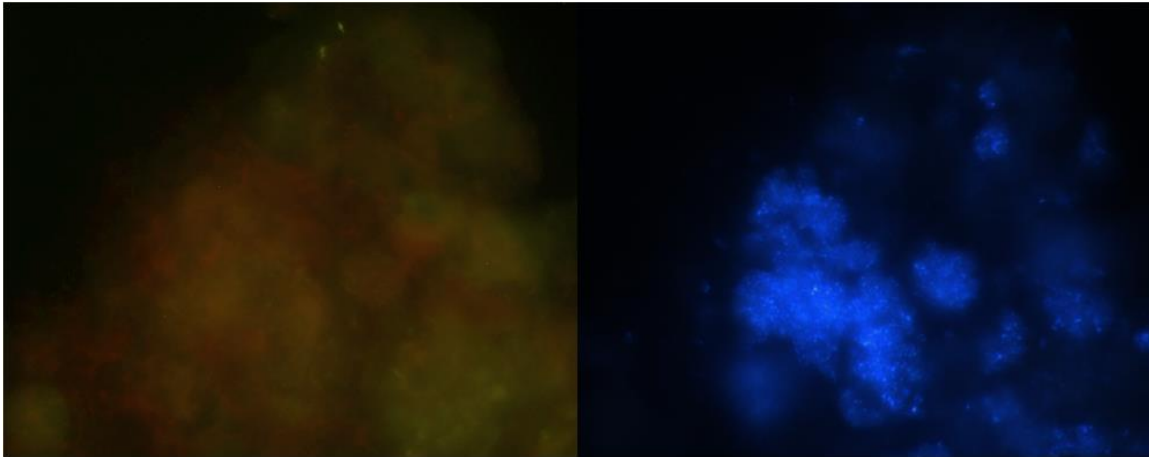


Figure 47: Free floating lipids stained with FM4-64 dye viewed under blue light at 10,000 ms exposure. Left panel: blue light excitation. Right panel: UV light excitation.

This phenomena made it more difficult to identify cellular structures with a high degree of certainty. This is because the FM 4-64 lipophilic dye is not specific to the lipids of cellular membranes. However, in many images, defined, circular, red fluorescence is observed as in Figure 48. It is assumed that this occurrence is less likely to be the result of free floating lipids and more likely indicative of cellular structures.

In Figure 48, the image at left is viewed under blue light. Well defined, red fluorescing, circular objects are observed. It is believed that these are more likely to be cellular structures because of their defined shape. In the right panel, two fluorescing MS2 particles are clearly observed at the center of the image. This example of MS2 fluorescence was ubiquitously observed on both large and small flocculated particles.

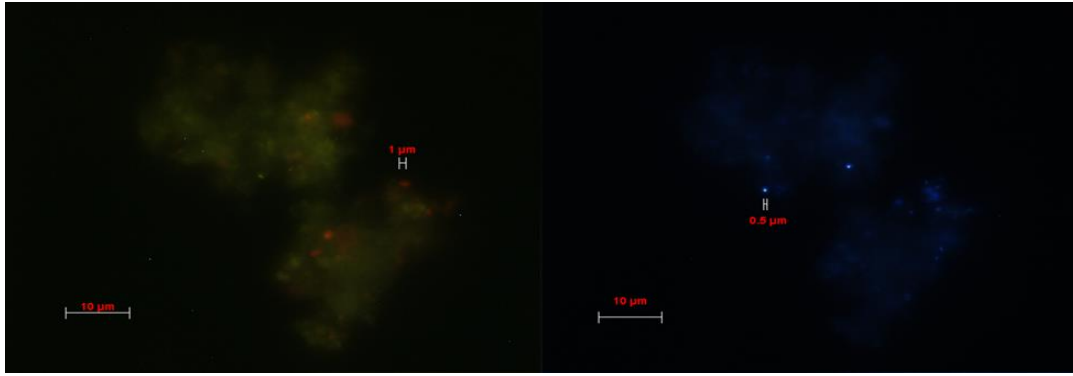


Figure 48: Occurrence of apparent cellular structures as small red circles viewed under blue light. Left panel: blue light excitation. Right panel: UV light excitation.

Because they were stained with DAPI dye, the MS2 virus particles fluoresced as expected when exposed to UV wavelengths. While usually viewed against the backdrop of an auto-fluorescing floc particle, the MS2 appears throughout as white or bright blue dots. Commonly, the microscope's focus on the foreground of the floc particle would leave some MS2 particles blurry with others in relatively good focus. The brightly fluorescing areas of the image in the right panel are closely adjacent to other MS2 particles along the perimeter of the floc which are not fluorescing as brightly.

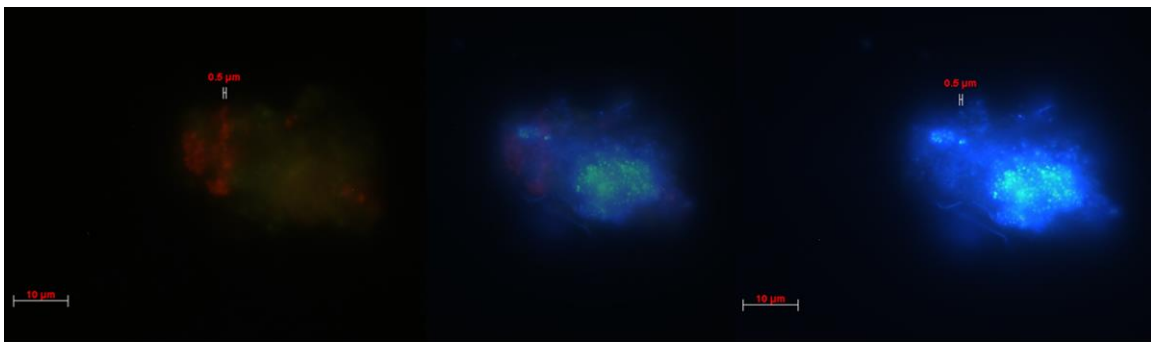


Figure 49: MS2 virus adherence to small sludge floc particle. Left panel: blue light excitation. Center panel: Image overlay of blue light and UV light. Right panel: UV light excitation.

MS2 particles were observed on both large, aggregated floc particles, and small, separated floc particles. The blue excitation image at left in Figure 49 exhibits red fluorescence. Comparing this relatively defined fluorescence to the cloudy appearance of Figure 47, the presence of cellular structures is supported. While the presence of cellular structures is supported and the occurrence of the MS2 virus is observed, infection is not evident as the two do not appear in the same location. Were infection evident, the white fluorescing particles would appear within the defined cellular membranes. The image overlay in the center panel of Figure 49 supports this claim.

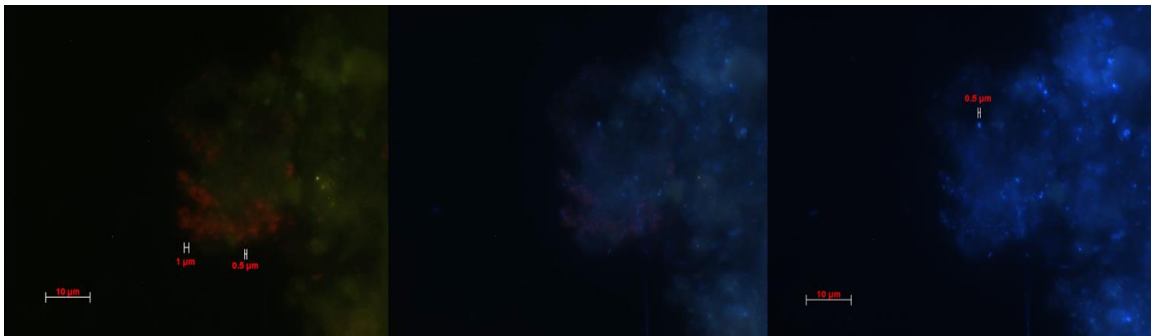


Figure 50: MS2 adherence to large sludge floc particle. Left panel: blue light excitation. Center panel: Image overlay of blue light and UV light. Right panel: UV light excitation.

MS2 virus particles were also observed on larger floc as seen in Figure 50 and Figure 51. Again, the brightly fluorescing dots in the UV image in the right panel are the MS2 virus particles. In the blue light image at left, green auto-fluorescence is observed along with relatively defined areas of red fluorescence. This supports the claim that cellular structures are present. In this instance, no apparent occurrence of MS2 virus particles is observed within the red fluorescing cellular structures. Although, MS2 particles are observed near the red fluorescing areas. While this occurrence suggests that infection is possible, MS2 virus particles are not observed in each structure. This is

important because, given the occurrence of free floating lipids and the biodiversity of sludge particles, infection would have to be consistently observed before a claim could be supported. It appears that the relative co-location of both red circular structures and MS2 virus particles is coincidental, not an indication of active infection.

Figure 51 exhibits trends similar to those of Figure 50. The brightly fluorescing MS2 particles are easily viewed in the UV image at right. At left, circular clusters of red fluorescence are observed but these occurrences are also ill-defined and cloudy. Certainly, MS2 virus particles are not occurring within every red, circular structure. However, there are occasions where the MS2 particles appear very near, on top of, or adjacent to these red fluorescing areas. Considering the limitations previously discussed, it seems pertinent to suggest that infection may be possible but is unlikely, given the specificity of the MS2 virus for *E. coli* strains. Additional imagery is included in the Appendix.

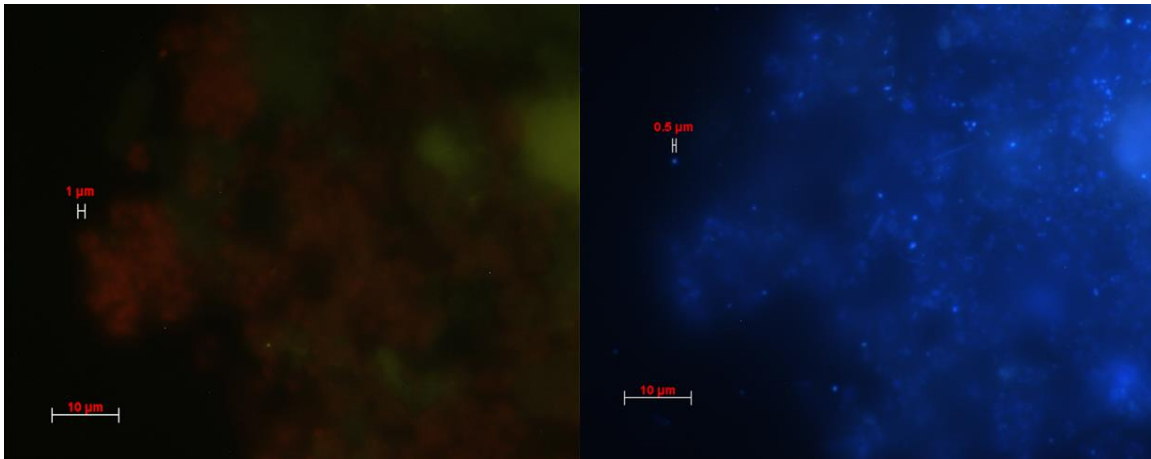


Figure 51: MS2 adherence to large floc particle. Left panel: blue light excitation. Center panel: Image overlay of blue light and UV light. Right panel: UV light excitation.

The next phenomenon commonly observed was free floating MS2 virus particles. Figure 52 and Figure 53 both exhibit pictures taken that account for these observations. In Figure 52, the bright filter image in the right panel exhibits a sludge floc in the bottom half of the image and supernatant in the upper half of the image. Within the supernatant, small circular objects were regularly observed to be free floating. At right is the overlaid image containing both the UV and blue light images for a given sludge floc particle. In this image, the small circular objects are not detected as a result of the microscope's foreground focus.

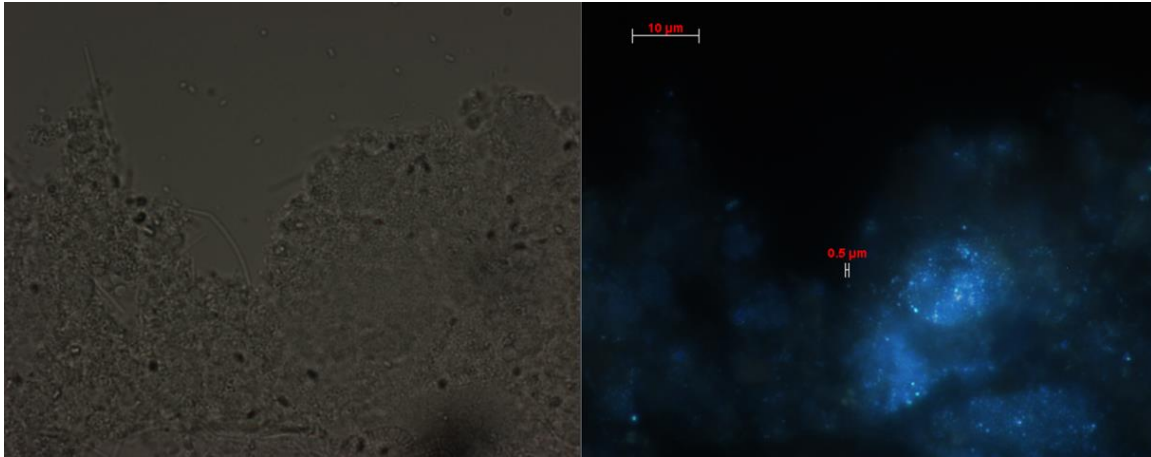


Figure 52: Free floating MS2 virus viewed under bright filter. Not visible under UV light due to focus. Left panel: bright filter excitation. Right panel: UV light excitation.

Figure 53 exhibits similar circles in the bright filter image at left. Although this time, the UV image exhibits the brightly fluorescing MS2 particles floating in the same area of supernatant. This suggests that while MS2 will adhere to activated sludge floc particles, there is a potential for a residual concentration after secondary treatment which will need to be dealt with prior to discharge.

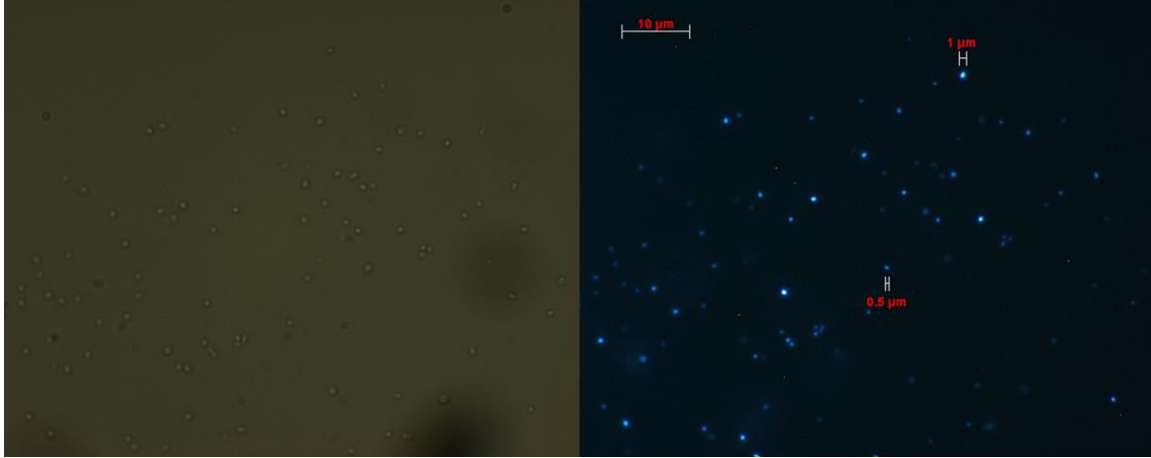


Figure 53: Free floating MS2 viewed under bright filter and UV light. Left panel: bright filter excitation. Right panel: UV light excitation.

Infection of host bacterial cells was not supported by the imagery but also cannot be completely ruled out because of the difficulty interpreting the observed red fluorescence. MS2 was successfully observed to adhere to flocculated particles but also occurred in the supernatant. The occurrence of MS2 and red fluorescing cell wall structures in the same location was only infrequently observed. While some relative MS2 and host cell collocation occurs, a pervasive occurrence of MS2 within a definable cell wall is not apparent. Although the conclusions here are limited, the well-known virus-host specificity of MS2 supports these observations. The tendency of coliphages to adsorb diverse particles is supported by relevant literature describing coliphage removal in conventional activated sludge treatment plants (Preston & Farrah, 1988; Zhang & Farahbakhsh, 2007).

4.5 Summary

The MS2 virus does not negatively affect the O₂ consumption rate, cumulative O₂ consumption, molar O₂/CO₂ ratios, or nitrification processes in activated sludge.

Fluorescing MS2 virus particles as well as stained lipids were successfully observed using dual fluorescence labeling and appeared to adhere to activated sludge floc particles. Although, there is not overwhelming visual evidence of MS2 infection of bacteria within the flocculated sludge particles.

5. Conclusions and Recommendations

This research effort examined MS2 as a biological contaminant to determine its effect on the microbial respiration and waste oxidation capabilities of activated sludge. Respirometry, spectrometry, and dual fluorescence labeling microscopy were employed to gather data. Several methods of quantitative analysis were performed in order to compare experimental groups in order to detect differences in the respiration and microbial metabolism of the microorganisms responsible for waste oxidation. Throughout all of these efforts, the experimental and negative control groups did not exhibit any significant differences. The microscopy results were not indicative of infection although the conclusions able to be drawn from the imager must be qualified for reasons previously discussed.

5.1 Conclusions of Research

The MS2 virus does not negatively affect the short term activity or performance of activated sludge. If infection of activated sludge bacteria were suspected, several observations would be apparent. First, the experimental group O₂ respirometric profiles would exhibit significant differences when compared to negative control groups. This expected difference is reasonable because, as a result of the viral infection, there would be fewer autotrophic bacteria available to take part in the nitrification. Experimental groups would also exhibit trends similar to the positive control groups in the spectrometry analysis. As a known inhibitor, ATU disrupts the oxidation of ammonia-N by interfering with the ammonia monooxygenase enzyme in nitrifying bacteria. While this method of disrupting nitrification is very different than the method employed by lytic

or lysogenic viruses, it was used here as a baseline for comparison. None of the experimental groups behaved in a manner similar to those injected with the known inhibitor. Furthermore, there were no significant differences between the experimental and negative control groups when evaluated using the diverse quantitative methods employed. Therefore, the MS2 bacteriophage does not represent a high consequence threat to wastewater treatment processes or infrastructure. This appears to be the first study to demonstrate the effect of MS2 bacteriophage on activated sludge activity and the first to use utilize dual fluorescent labeling to examine a mixed consortia of activated sludge microorganisms and the MS2 bacteriophage.

5.2 Significance of Research

In this application, MS2 was examined as a biological contaminant. Because of its use in medical applications, MS2 is increasingly discharged into wastewater distribution networks. The preponderance of evidence provided by parametric methods and microscopy suggest that the effect of MS2 on the respiration and performance of activated sludge is negligible, if not non-existent. It demonstrated similarity to negative control groups in every experimental replicate when analyzed using diverse methods. The MS2 bacteriophage is therefore not a threat to wastewater treatment operations in the short term.

5.3 Recommendations for Future Research

Determining whether or not activated sludge treatment inactivates the MS2 virus would meaningfully contribute to understanding the risks of using recovered biosolids from a waste treatment facility. A study could investigate this using a single agar layer

virus enumeration procedure based upon EPA Method 1602. The MS2 virus will be introduced to the activated sludge slurry and respirometry experiment performed. MS2 will then need to be isolated from the sludge, combined with log-phase E. Coli host cells and molten tryptic soy agar, and then incubated overnight (EPA, 2001). The number of plaque forming units will then be counted and compared to control samples.

Mathematical virus-host modeling has been implemented in several instances and serves to provide a foundation for understanding and communicating microbial interactions. Merikanto et al. (2007) investigated natural predatory kinetics and decay using a deterministic continuous time model with several rate parameters describing transmission, growth and mortality rates. Beke et al. (2016) combined two models describing phage-host interactions using a similar mathematical approach. Future research into the use of surrogate viruses could employ these or similar mathematical models in an attempt to describe degradation, inactivation, or exposure risks. Other methods involve the use of Monte Carlo simulation to model treatment effectiveness of various unit processes (Olivieri et al., 1999). Monte Carlo simulations could be particularly useful to virus enumeration studies because of the complexity associated with replicating the experiment. To incorporate this method, one could carry out the virus enumeration protocols previously mentioned, gather data related to MS2 inactivation resulting from treatment, assign a distribution to this data, and model the inactivation of MS2. In addition, other investigations using virus surrogates could also incorporate the addition of environmental stress factors to observe potential impacts to activated sludge activity and performance. Choi et al (2010) observed phage induction in the presence of toxic chemicals, extreme pH levels, and certain metals. Similarly introducing these

conditions during a respirometry experiment could produce interesting and meaningful results thereby allowing insight into the susceptibility of underlying microbial interactions.

Appendix A

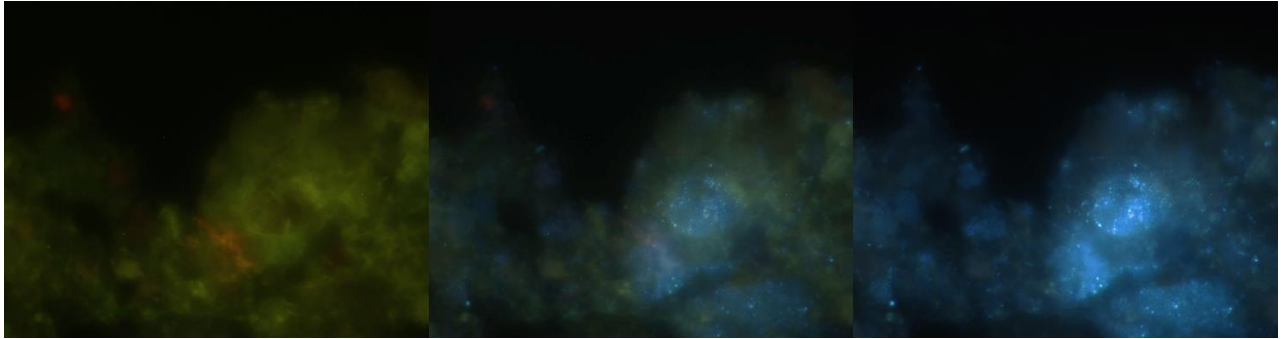


Figure 54 Large Flocculated Particle with MS2 and Red Fluorescing Areas

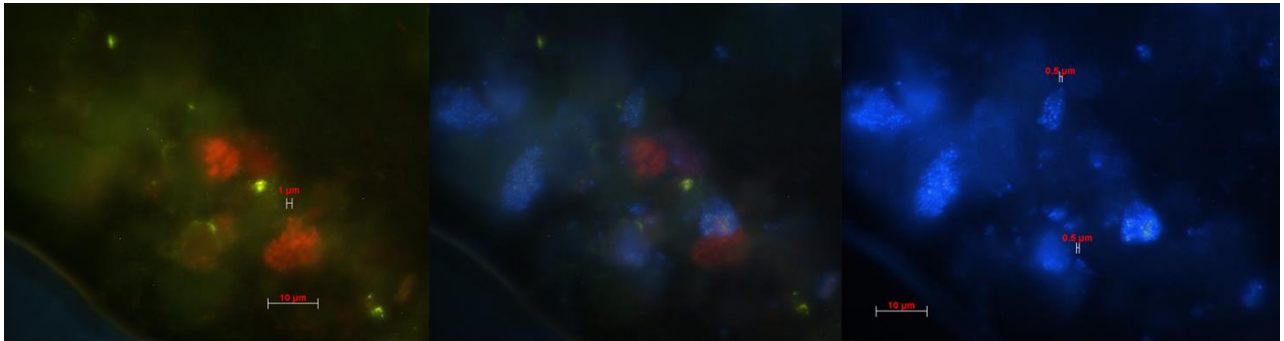


Figure 55 Fluorescing MS2 and Apparent Cellular Structures



Figure 56 Large Flocculated Sludge Particle with Red Fluorescence and MS2



Figure 58 Bright Filter and UV Images Showing Aggregated MS2 Particles

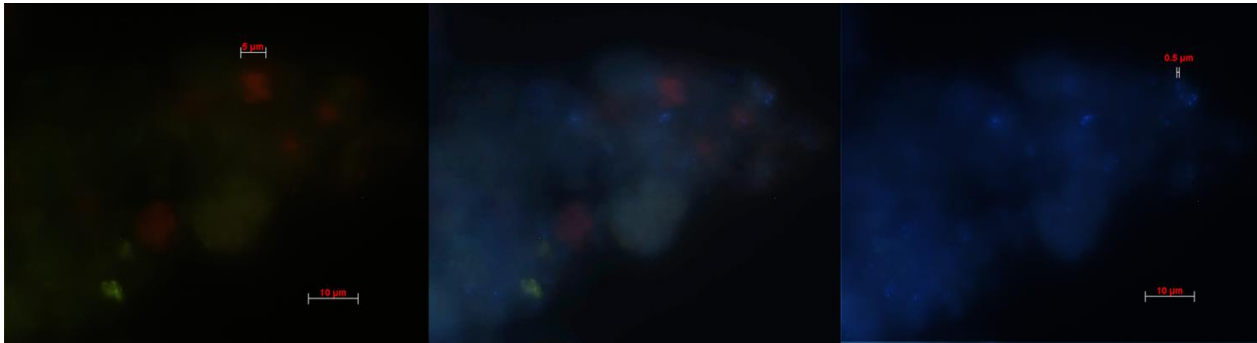


Figure 57 Large Flocculated Particle with Red Fluorescence and MS2

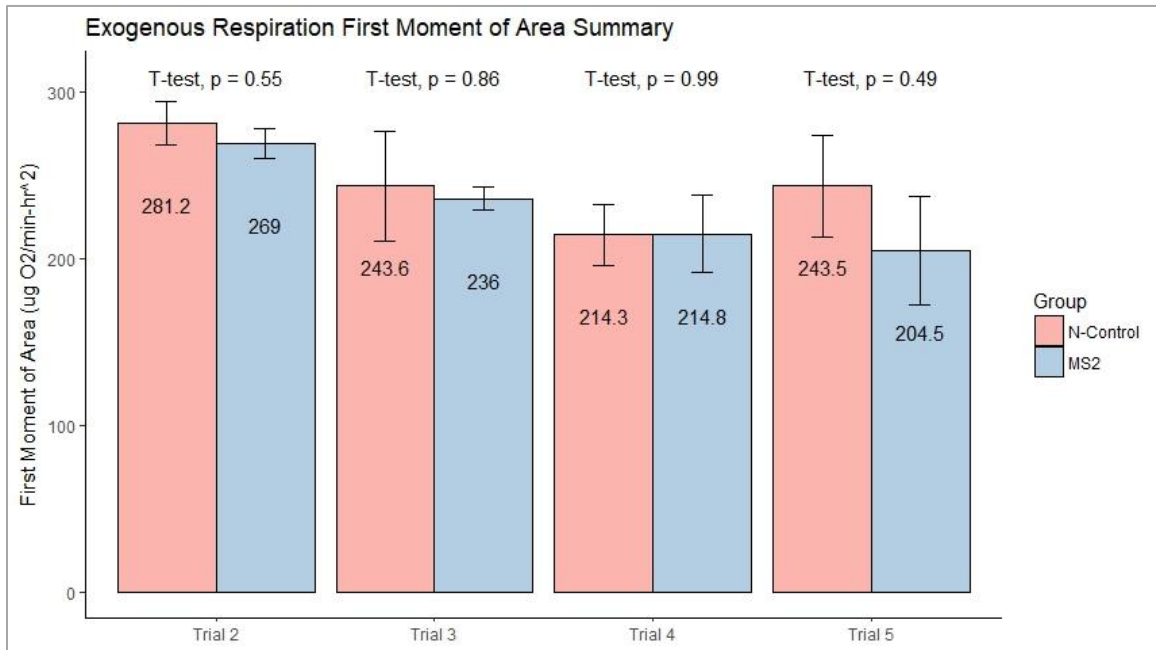


Figure 59 First Moment of Area Summary

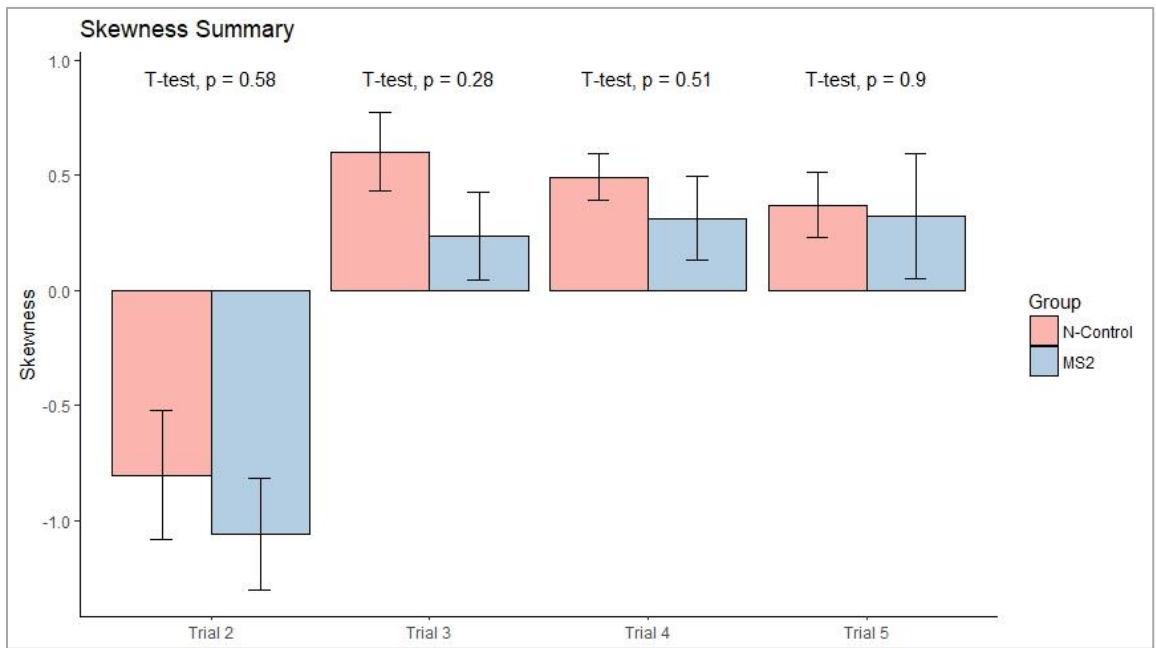


Figure 60 Skewness Summary

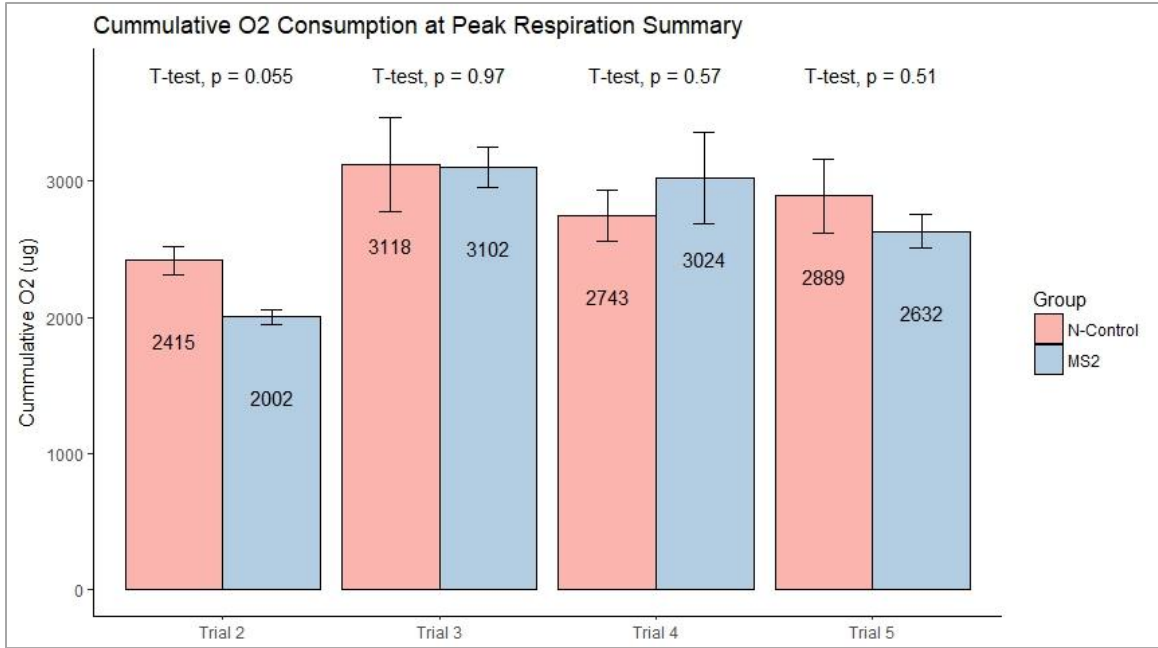


Figure 61 Cumulative O2 Consumption Summary

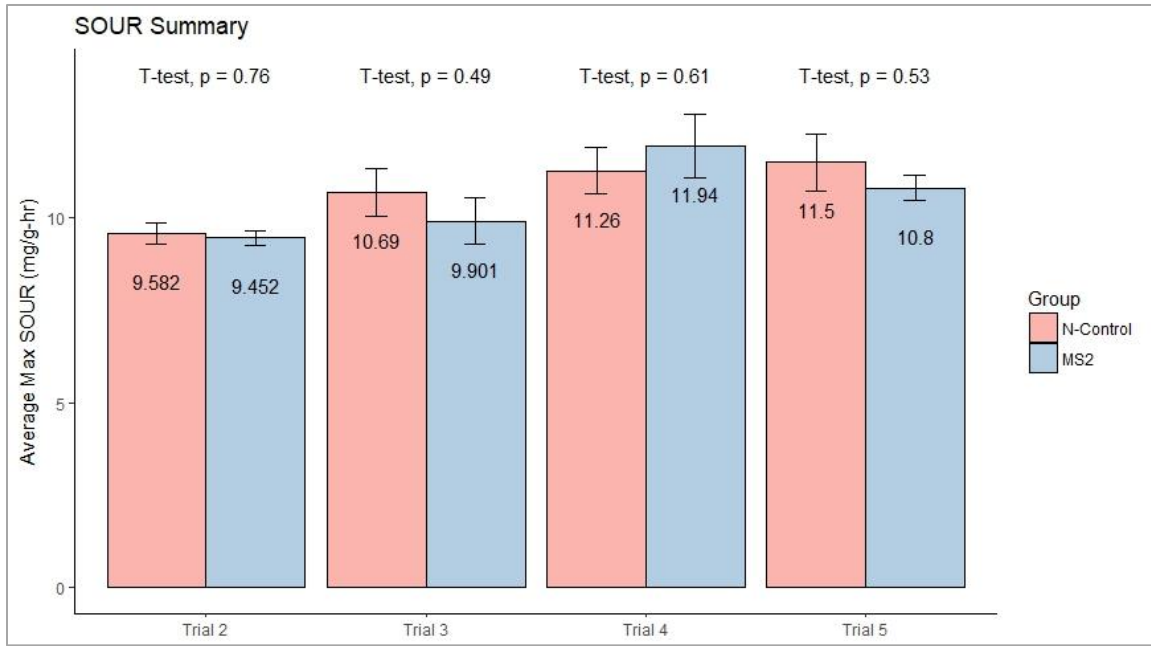


Figure 62 SOUR Summary

MS2 DAPI staining flow chart

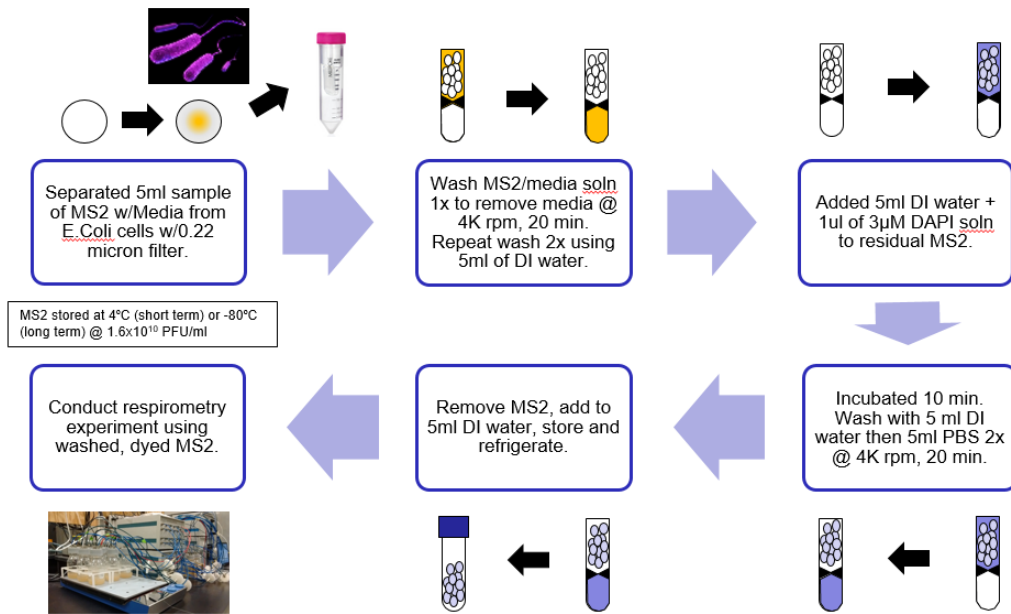


Figure 63 MS2 DAPI staining flow chart.

Sample dilution & FM 4-64 lipid staining flow chart

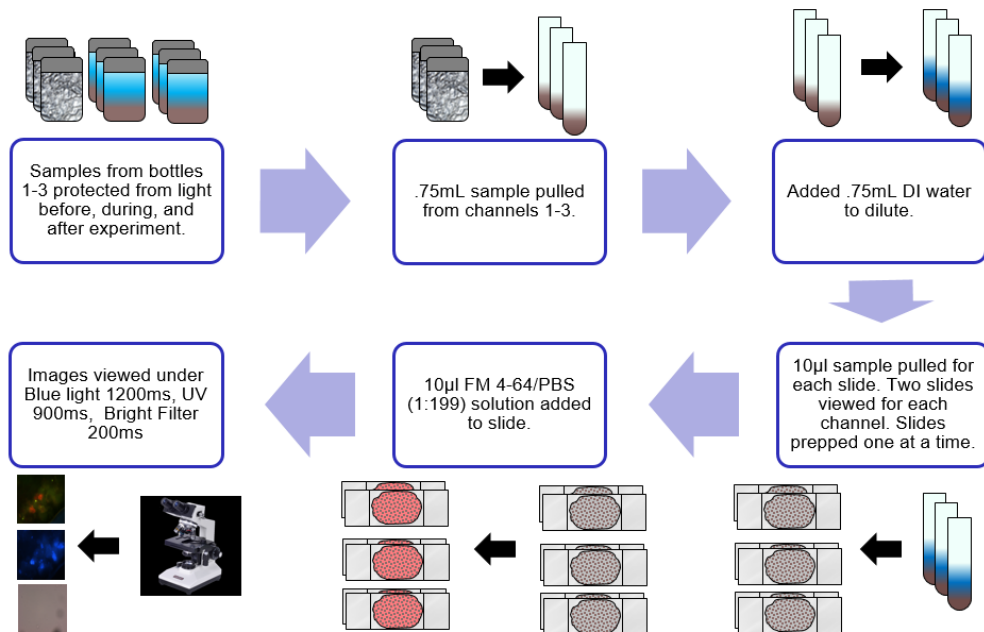


Figure 64 Sample dilution and lipid staining flow chart.

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1. REPORT DATE (DD-MM-YYYY) 23 Mar 2018		2. REPORT TYPE Master's Thesis		3. DATES COVERED (From - To) 8 August 2016 – 23 March 2018	
4. TITLE AND SUBTITLE The Effect of MS2 Bacteriophage on the Activity and Performance of Activated Sludge			5a. CONTRACT NUMBER		
			5b. GRANT NUMBER		
			5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Stuntz, Sean M., Captain, USAF			5d. PROJECT NUMBER		
			5e. TASK NUMBER		
			5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAMES(S) AND ADDRESS(S) Air Force Institute of Technology Graduate School of Engineering and Management (AFIT/ENV) 2950 Hobson Way, Building 640 WPAFB OH 45433-8865			8. PERFORMING ORGANIZATION REPORT NUMBER AFIT-ENV-MS-18-M-238		
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) Environmental Protection Agency National Homeland Security Research Center 26 West Martin Luther King Drive Cincinnati, OH 45268 513-569-7321, magnuson.matthew@epa.gov			10. SPONSOR/MONITOR'S ACRONYM(S) U.S. EPA/NHRSC		
			11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION/AVAILABILITY STATEMENT Distribution Statement A. Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES This work is declared a work of the U.S. Government and is not subject to copyright protection in the United States.					
14. ABSTRACT The effects of MS2 bacteriophage at a concentration of 3.2x10 ⁸ PFU/mL were assessed by determining peak O ₂ consumption, cumulative O ₂ consumption, molar CO ₂ /O ₂ ratios, and shape factors. None of the MS2 samples caused statistically significant effects on the peak or cumulative O ₂ consumption. The molar CO ₂ /O ₂ ratios were also not substantially impacted. The skewness and first moment of the area were not significantly impacted by the introduction of the MS2 virus. MS2 bacteriophage did not inhibit chemical oxygen demand (COD) or nitrogen removal. When viewed at 100X amplification and at multiple wavelengths, the MS2 virus appeared to both remain free floating and to adsorb to activated sludge flocculated particles. To the author's knowledge, this is the first study to demonstrate the effect of MS2 bacteriophage on activated sludge activity and the first to use dual fluorescent labeling to examine a mixed consortia of activated sludge microorganisms and MS2. Overall, the results illustrate that the MS2 bacteriophage is unlikely to cause short-term interference with primary biological treatment at an activated sludge treatment plant.					
15. SUBJECT TERMS MS2, Activated Sludge, Respirometry, Dual Fluorescence Labeling Microscopy					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER (Include area code)
U	U	U	UU	118	Dr. Willie F. Harper Jr., AFIT/ENV (937) 255-3636 x4528 willie.harper@afit.edu

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