THE EFFECT OF BACILLUS GLOBIGII SPORES ON THE ACTIVITY AND PERFORMANCE OF ACTIVATED SLUDGE

THESIS

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Abstract

This research investigated the effect of a biological warfare agent surrogate (*Bacillus globigii*) on activated sludge through a series of respirometry tests. The effects of *B. globigii* concentrations ranging from $2x10^1$ CFU/ml to $2x10^7$ CFU/ml were assessed by determining peak O_2 consumption, cumulative O_2 consumption, molar O_2/CO_2 ratios, and shape factors. None of the concentrations tested caused statistically significant effects on the peak (p-values, 0.11 - 0.32) or cumulative O_2 (p-values, 0.21 - 0.62) consumption. The molar O_2/CO_2 ratios were also not substantially impacted. The shape factors were significantly impacted by the introduction of spores (p-values, 8.53 E-06 – 0.95) but these factors proved to be a poor indicator of biological inhibition when considered in light of the other factors analyzed. *B. globigii* did not inhibit COD or N removal.

However, when unwashed spores of the same concentrations in their 40% ethanol storage solution were tested, the results at $2x10^7$ CFU/ml were significantly inhibited. Initial peak O_2 consumption was 34% to 44% less than the control. Final effluent COD increased to 136.3 mg/l, while NO_3-N, on the other hand, was reduced to negligible levels unlike other trials. Biological treatment was inhibited by ethanol, not *B. globigii*.

Spore germination was observed at 0.88% to 1.7%, and spores preferentially adhered to the floc (81%) as opposed to remaining in the bulk liquid (19%).

Overall, these results illustrate that *B. globigii* is unlikely to cause short-term interference with biological treatment at an activated sludge plant, but organic co-

contaminants may cause inhibition, and spore germination may cause the wastewater treatment plant to become a longer-term source of biocontamination.

Ad Majorem Dei Gloriam

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Matthew D. Smith

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THE EFFECT OF BACILLUS GLOBIGII SPORES ON THE ACTIVITY AND PERFORMANCE OF ACTIVATED SLUDGE

I. Introduction

Background

Activated sludge is a biological process used to treat contaminated wastewater. A typical process schematic used at a wastewater treatment plant (WWTP) is shown in Figure 1. Influent wastewater is fed to a primary clarifier for removal of large particles. The primary effluent is then routed into a bioreactor, which contains a flocculent suspension of microorganisms that are cultivated and nourished with the pollutants found in wastewater. This bioreactor is the central feature of the activated sludge process and is controlled to provide proper growth conditions. Bacteria reproduce and simultaneously remove a wide range of soluble pollutants, including organic chemicals and inorganic constituents such as ammonia and phosphorus. The bioreactor is typically followed by a secondary clarifier for biomass recycle and for solids separation. The secondary effluent typically receives further treatment (e.g. filtration, disinfection) before being discharged to a receiving water. Activated sludge is an essential component of modern water infrastructure and is now the most routine way to treat municipal wastewater (Metcalf & Eddy, 2002).

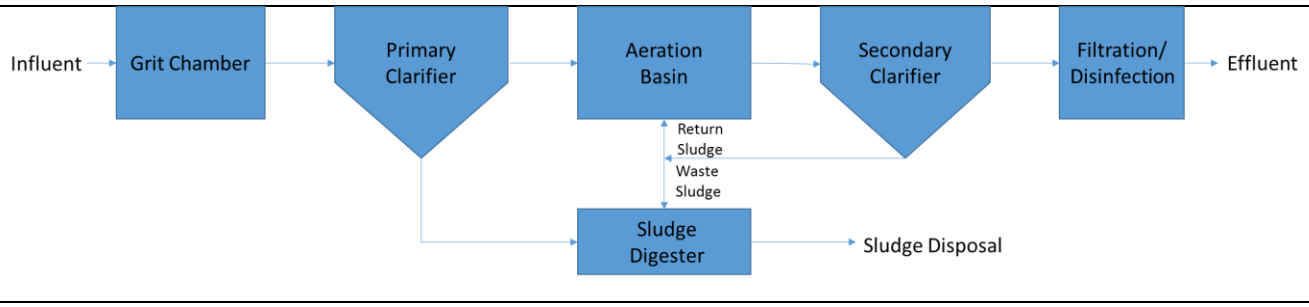

Figure 1: Typical WWTP process diagram.

Problem Statement

The U.S. Environmental Protection Agency (EPA) National Homeland Security Research Center (NHSRC) is working with the water quality community to improve water infrastructure security (WER7F7W15, 2016). One key ongoing initiative concerns high-consequence biocontaminants, including biological weapons, biohazardous or infectious waste, and medical waste. WWTPs need to be prepared for scenarios that involve the intentional or inadvertent introduction of biocontaminants into the wastewater collection and treatment system and the EPA is working toward developing the guidance for the handling of such wastewater. The protocols, policies, or regulations that are needed must be based upon scientifically-based facts related to the effect of the bio-contaminant on the treatment system and public and environmental health. The Department of Defense (DoD) is also keenly interested in proper treatment of high-consequence biocontaminants in wastewater. The DoD's gross decontamination response to a biological warfare attack would produce copious amounts of contaminated wash-down water (D.O.D., 2006). This is now the appropriate moment for research that addresses key knowledge gaps. One important issue is the effect of biocontaminants on the activated sludge process.

Research Objective

The purpose of this research is to evaluate whether or not a proxy to a biological warfare agent will inhibit effective wastewater treatment by activated sludge in a WWTP. Specifically, this study will determine whether or not *Bacillus globigii* spores inhibit the respiration, COD removal, and nitrification observed in activated sludge.

Scope and Approach

This research effort used *Bacillus globigii* as a surrogate for weapons-grade *Bacillus anthracis*. *B. globigii* is a common heterotrophic organotroph found outdoors in dirt, hay, and other natural environments. It has similar spore-forming capabilities as *B. anthracis* but has a much lower toxicity to human health (Miller, 2004). When presented with unfavorable conditions, *B. globigii* cells will go dormant and form a hard protective layer making it resistant to drying, heat, cold, etc. The spores will rejuvenate and resume metabolic activity when favorable conditions allow. *B. globigii* were previously investigated as a tracer for WWTPs and were found to adhere to floc in activated sludge and remain in the plant for extended lengths of time of up to one year (Horan, et al., 1991). However, respirometry experiments were not conducted and the effect of the spores not recorded.

Activated sludge for this project was cultivated from the operation of a bench-scale activated sludge sequencing batch reactor (SBR). The reactor was seeded with activated sludge from the Fairborn, OH WWTP collected in January 2016. The SBC was operated on an eight-hour cycle. Each cycle included settling, effluent decanting, make-up water inflow, feeding, and aeration. The reactor was monitored daily with volatile

suspended solids (VSS) and total suspended solids (TSS) measurements taken three times per week.

Respirometry was conducted using a Columbus Instruments Respirometer. The apparatus consisted of multiple 250 ml glass bottles, an O_2 sensor, a CO_2 sensor, and various sampling pumps, dryers, and filters. The instrument measured O_2 consumption and CO_2 production over a set time interval. Nine bottles (channels) were used for each experiment. Fifty ml of mixed liquor suspended solids (MLSS), a magnetic stir bar, and feed solutions were added to each channel. Channels 1-3 received a set concentration of spores to establish the experiment. Channels 4-6 received 250 µl of allylthiourea (ATU) in order to positively demonstrate known inhibition – positive control. Channels 7-9 served as the negative control – no inhibition expected. Each tested lasted approximately 16 hours with O_2 consumption and CO_2 production rates measured at one hour intervals. Chemical oxygen demand (COD), NH_3-N, and NO_3-N measurements were taken before and after each experiment to determine the effectiveness of the activated sludge.

Summary

Wastewater treatment plants take advantage of the symbiotic relationship between man and bacteria to effectively clarify and decontaminate sewage. However, activated sludge is vulnerable to bio-contaminated waters from accidental release or manmade weaponized bacteria. *B. globigii* was used as a surrogate for *B. anthracis* in an attempt to model the impact of bio-contaminated waters on activated sludge.

II. Literature Review

Chapter Overview

This chapter will cover several main topics. First, it will review the compounds that are known to cause inhibition. Second, it will review the known fate of biocontaminants in the wastewater treatment process. Third, this chapter will discuss respirometry and the factors that influence oxygen consumption profiles. Finally, it will investigate likely spore concentrations in decontamination wash-down water.

Inhibition of Activated Sludge

Metals and Other Inorganics

Heavy metals such as lead, mercury, and chromium are detrimental to activated sludge (Fristoe & Nelson, 1983). Other metals are essential to life in trace amounts but become toxic in large concentrations. For example, Manganese (II) was found to increase cellular respiration at concentrations less than 1 ppm but decrease respiration at 2 ppm (Aragón, et al., 2010). Their research also documented that activated sludge harvested from a WWTP regularly receiving industrial wastewater was preconditioned to the presence of Mn (II). The activated sludge from a second WWTP, not acclimated with industrial pollutants, did not display the increased respiration at low Mn (II) concentrations, nor did it display as great of inhibition at higher concentrations (Aragón et al., 2010). A decrease in affect after acclimation to a normally toxic substance is common to the robust, diverse microbial community of activated sludge. For example, Chromium (VI) displayed a similar, albeit lessor, affect after an acclimation time period (Stasinakis, et al., 2002).

Cadmium, copper, and zinc are other heavy metals of concern often found in industrial wastewater. Cadmium has been shown to inhibit nitrification by targeting ammonia oxidizing bacteria (AOB) unlike other compounds that inhibit both AOB and nitrite oxidizing bacteria (NOB) (Kelly, et al., 2004). Copper and zinc have long been understood to inhibit activated sludge (Barth, et al., 1965). More recently, the impact of copper and zinc was determined to be greater on nitrifying bacteria than on heterotrophs (Principi, et al., 2006).

Nanoparticles can inhibit wastewater treatments. Sibag et al. (2015) suggest that silica nanoparticles impact biological activity by imbedding in the microbial cell membrane. The smaller nanoparticles seemed to impart the greater effect. Silver and titanium oxide nanoparticles exhibited inhibitory tendencies towards denitrification, which decreased the population of denitrifying bacteria (Chen et al., 2014). However, the same experiment showed the nanoparticles to aid the growth of sludge bulking, heavy metal resistant, and biosorption bacteria. The effect of nanoparticles on activated sludge is an emerging field and has not yielded completely conclusive findings.

Organics

When exposed to a new organic substrate, activated sludge typically requires an acclimation phase. During this time (typically referred to as lag phase), the bacteria begin to produce the enzymes necessary to metabolize the unfamiliar compound (Vaccari, et al., 2006). Many inhibitory biodegradable substrates impart an extended lag growth phase as the activated sludge adjusts (Rozich & Gaudy, 1992). Twenty-four different chemicals typically found in industrial waste from petrochemical processing adhered to this pattern (Cai, et al., 2010). All 24 displayed greater acute toxicity to

activated sludge at 30 mins after exposure rather than after 24 hours measured via respiration and dissolved oxygen concentration. However, other organics show little impact during short-term exposure but do display a negative effect during long-term tests. Malathion, an organophosphate often used as a surrogate for chemical weapons, was shown to have little effect on activated sludge during short term tests but displayed chemical oxygen demand (COD) removal and ammonia removal inhibition during longer duration tests (Rauglas, et al., 2016).

Other research observed immediate inhibition of nitrifying activated sludge followed by eventual recovery. In testing a representative selection of common industrial wastewater components, Kelly et al. (2004) documented AOB and NOB inhibition by shock treatments of 1-chloro-2,4-dinitrobenzene, pH 11, and cyanide. 1-octanol, on the other hand, only inhibited AOB while leaving NOB unaffected. All of the activated sludge communities showed trends of recovery, albeit at varying rates (Kelly et al., 2004).

N-allylthiourea

N-allylthiourea (ATU) is an ammonia oxidation inhibitor often used to isolate the effect of ammonia oxidizing microbes in activated sludge (Men, et al., 2017). ATU is thought to bind with the copper in the active site of the ammonia monooxygenase operon thereby inhibiting nitrification (Bedard & Knowles, 1989). It has been regularly used to demonstrate positive inhibition during respirometry for a variety of different research tracks involving activated sludge (Hofman & Lees, 1953; Ning, 2000; Wu, et al., 2016; Rauglas et al., 2016).

Summary

As a consortium of bacteria, there are many different factors that can inhibit the performance of activated sludge. Heavy metals, nanoparticles, and various organics all can inhibit certain aspects of wastewater treatment. Fortunately the great diversity of bacteria present in activated sludge provides a robust defense against complete failure. The substance that inhibits one type of bacteria often leaves other bacteria unaffected. As long as toxic shock is avoided, the microbial community will compensate over time for the inhibition.

Fate of Biocontaminants in Wastewater Treatment

The threefold focus of wastewater treatment over the past hundred years has been the removal of pathogens, unsightly solids, and nutrients from receiving waters. Federal effluent regulations typically focus on the three factors of biological oxygen demand, suspended solids, and nitrogen levels (Metcalf & Eddy, 2002). However, the fate of pathogens (bacterial, viral, and multicellular) along with other biocontaminants such as pharmaceuticals has recently become of great interest. Direct water reuse for potable treatment necessitates adequate biocontaminant removal. Although the focus of most research studies revolve around the effect of the WWTP on the biocontaminant, it can still provide insight as to how the biocontaminant can impact the bacteria of activated sludge.

Biocontaminants

Biocontaminants in wastewater treatment is a broad term for any undesirable organism or compound that interferes with desirable biological function. It includes

bacteria, viruses, protozoa, fungi, and helminths. It also includes chemical toxins and pharmaceuticals as well. Human pathogens are passed into the WWTP through the waste of infected persons. Human waste is also the typical point of entry for many pharmaceuticals including antibiotics and synthetic estrogens from birth-control pills (Weber, et al., 2005). Hospital wastewater can be a source of high consequence biocontaminants such as severely toxic pathogens and antibiotic resistant bacteria, however, the volume of hospital effluent is significantly less than traditional municipal wastewater (Reinthaler et al., 2003). Other biocontaminants enter the waste stream from industrial wastes.

A typical WWTP has four treatment stages: preliminary, primary, secondary, and tertiary. Preliminary and primary treatment rely on the physical separation and settling of dense pieces of matter. Secondary treatment is biological degradation of organics by bacteria as activated sludge in an aeration basin or bacteria attached to a biofilm on a trickling filter. Tertiary treatment is a final polishing of the treated water with either filtration or disinfection or a combination of the two. Various sorts of matter can be used for filtration including sand, activated carbon, and semipermeable membranes (Metcalf & Eddy, 2002). Any of the standard disinfection methods can be utilized: chlorination, ozone, UV, etc. Geldreich (1978) summarized the removal of indicator and pathogenic organisms for typical sewage treatment processes in Table 1.

Table 1: Removal of indicator and pathogenic organisms for typical sewage treatment processes.

Type of sewage treatment	Removal range for various organisms, %
Septic tanks	25-75
Primary	5-40
Activated sludge	25-99
Trickling filters	18-99
Anaerobic digestion	25-92
Waste stabilization ponds	60-99
Tertiary	93-99.99

Recreated from (Droste, 1997)

Bacteria

The most studied bacteria in wastewater treatment is the standard indicator bacteria *Escherichia coli*. Well established analysis methods for *E. coli* make it the predominant indicator of fecal contamination. While not necessarily a pathogen itself, *E. coli* share common traits of other pathogenic bacteria including survivability rates outside their host. *E. coli* is largely removed by biological treatment of wastewater. Marin et al. (2015) observed a 1.41 log reduction of coliforms for a trickling filter in a WWTP that achieved an overall 2.34 log reduction in *E. coli*. Similar log reductions from biological treatment using activated sludge of 2.06 and 2 were reported by Wen et al. (2009) and Reinthaler et al. (2003) respectively. These reductions were all based on influent to effluent comparisons. However, Marin et al. confirmed the general assumptive consensus that the removal is largely a physical phenomenon by observing an increase in *E. coli* in the sludge collected from the trickling filters (Marín et al., 2015). Sludge from an aeration basin system showed similar high *E. coli* concentrations (Reinthaler et al., 2003). It is not until thermophilic sludge digestion and dewatering that *E. coli* is substantially destroyed (Marín et al., 2015). Primary and secondary treatment greatly

reduce the number of bacteria present, but there is still a great enough concentration in the effluent for concern. It is not until tertiary treatment that levels of bacteria are lowered to meet potable water source standards. Although they document the physical presence of *E. coli* quite well, the listed articles did not address what affect, if any, the *E. coli* had on the activated sludge.

Viruses

Viruses are another potentially harmful constituent of wastewater that is ideally removed during treatment. 1.85 log (MS-2 bacteriophage) to 5.5 log (coliphage) reductions during primary and secondary treatment are well documented with the greatest removal in the secondary clarifier (Marín et al., 2015; Zhang & Farahbakhsh, 2007). Similar to bacteria, virus concentrations are reduced when processed through primary and secondary treatment but remain in significant quantities that often times require tertiary treatment to meet treatment objectives.

While researching basin mixing modeling, Hornan et al (1991), investigated the use of *Serratia* phage as a potential tracer. As a bacteriophage *Serratia* has a specific host bacteria which is not commonly present in activated sludge. *Serratia* demonstrated a great adherence to activated sludge floc (95%) but had an exceptionally high die off rate of 90%. The research confirmed the tendency for virus removal by activated sludge.

Pharmaceuticals

Pharmaceuticals are not true pathogens, but they can have a grave effect on the environment. Antibiotics released in human excrement or from flushing unused medications down the toilet contribute to the growing concern of antibiotic resistant bacteria (Glassmeyer, et al., 2008). Others pharmaceuticals mimic hormones or steroids

which greatly disrupts the endocrine system. Endocrine disrupting compounds (EDC), whether natural or synthetic, can have a significant impact at low concentrations on organ and anatomical development (Colborn, et al., 1994). EDCs are detectable even in the most remote parts of the earth and can have an ill effect on wildlife and humans. Interest in EDCs will continue to grow as a contaminant of concern as the world's water supply deteriorates and water reuse becomes more necessary (Noguera-Oviedo & Aga, 2016).

In testing five anticancer drugs, Mahnik et al. (2007) witnessed a 90% removal rate by activated sludge. The researchers attributed this to some biodegradation but to adsorption as well. Joss et al. (2006) researched 35 different pharmaceuticals and found that only four were degraded by more than 90% while 17 were degraded by less than 50%. Although not true pathogens, pharmaceuticals follow the same pattern of removal during secondary treatment of both biodegradation and adsorption, but with significant amounts remaining.

Endospores

The most pertinent literature on the fate of biocontaminants in wastewater treatment to this research investigating the effect of *B. globigii* on activated sludge was the work by Dr. Horan (1991). The same project that investigated the use of *Serratia* phage as a tracer in WWTPs examined *B. globigii* spores for the same purpose. The spores exhibited the same tendency to adhere to the floc (92%) as *Serratia* phage (consequently lowering the prospects of both as desirable tracers). However, unlike *Serratia* phage, which exhibited substantial die off, *B. globigii* spores showed no loss of viability. The spores exhibited survival of up to one year in the plant after the experiment

was conducted. Horan et al. did not report any effects of the spores on the efficacy of the activated sludge or conduct respirometry experiments (Horan et al., 1991).

Summary

Bacteria, viruses, and pharmaceuticals are substantially removed (but not completely or even adequately in some circumstances) by conventional wastewater treatment. Although some biodegradation has been reported, the primary removal pathway seems to be adherence to the floc. However, what is not thoroughly documented is the impact a biocontaminant has on the bacteria once it has adhered to the floc in activated sludge.

Respirometry

This research investigated the effect of a biological warfare agent surrogate (*B. globigii*) on activated sludge through a series of respirometry tests. Respirometry is a well-established method for evaluating microbial activity. This technique uses electrical probes to monitor the disappearance of oxygen and appearance of carbon dioxide to determine respiration rates. The oxygen consumption profiles are plotted verse time to create figure known as a respirogram. The observed profiles are driven by microbial activity, however, their shape can be impacted by hydrodynamic factors (e.g. mixing) that control transport of oxygen and carbon dioxide (Vanrollegham, 2002). Respirometry has been used extensively to evaluate inhibition of activated sludge (Aragón et al., 2010; Rauglas et al., 2016). It has also been used to study process control in a WWTP such as food to biomass ratio and hydraulic retention time (Ning, 2000; Wu et al., 2016). Finally,

respirometry is critical to the develop growth models for modern WWTP design (Rozich & Gaudy, 1992; Strotmann, et al., 1999).

Spore Concentration

Fortunately, a largescale biological warfare attack has not recently occurred. Unfortunately, this presents a knowledge gap as to the concentration of spores in the wash-down water from a large decontamination effort. The vast body of research on biological weapons focuses on the detection or immediate decontamination thereof. Far less information is available on the proper treatment and disposal of the contaminated wash-down water itself. Spore concentration depends on the widely variable factors of both the amount of agent dispersed and quantity of water used during decontamination. Further dilution from mixing with regular sewage would result in very low influent concentrations when the wash-down water enters a WWTP (Macintyre et al., 2000). Although real-world historical data is lacking, various studies on decontamination procedures begin with a starting concentration in the $10^7 – 10^8$ CFU/ml range (Raber & McGuire, 2002; Buhr, 2006; Amitai et al., 2010). To test detection methods of biological warfare agents in landfill leachate, Saikaly et al. (2007) used a series of concentrations ranging from 10^0 to 10^7 CFU/ml. In order to account for dilution from decontamination efforts and combination with wastewater influent, this study used concentrations of *B. globigii* spores from $2x10^1$ to $2x10^7$ CFU/ml.

Conclusion

Secondary treatment at a WWTP may be primarily designed to remove organic molecules from wastewater, but it is also very effective at removing biocontaminants.

Numerous studies have shown the bacteria in activated sludge to be greatly susceptible to inhibition by both inorganic and organic compounds. Other studies have shown significant removal of biocontaminants during the activated sludge process. However, minimal research has focused on the effect of the biocontaminants on the activity of the activated sludge. Specifically, this is the first study to investigate the effect of *B. globigii* spores on activated sludge.

III. Methodology

Overview

Of the many biocontaminants that could infiltrate a wastewater treatment plant (WWTP), the DoD is greatly concerned about militarized pathogens such as anthrax. In lieu of the highly dangerous *Bacillus anthracis* bacterium, this research used *Bacillus globigii* to simulate *B. anthracis* introduction to a WWTP. *B. globigii* forms durable endospores able to tolerate inhospitable conditions similar to *B. anthracis*; however, *B. globigii* is much less virulent (Miller, 2004). *B. globigii* spores in varying concentrations were added to activated sludge cultivated in the laboratory. The effect of the spores was measured by respirometry and final effluent COD, ammonia, and nitrate analysis.

SBR Operation

The activated sludge for the experiment was cultivated in two identical 2.0 L bench-scale sequencing batch reactors (SBR) constructed and operated as previously described by Janeczko et al. (2014) except as noted, Figure 2. All sludge for respirometry experiments was harvested from reactor 1, but both reactors were operated identically for redundancy. The reactors were seeded with activated sludge collected from the Fairborn, OH, WWTP. Eight liters were collected on 4 January 2016; after resuspension and homogenization in the laboratory two liters were added to each reactor.

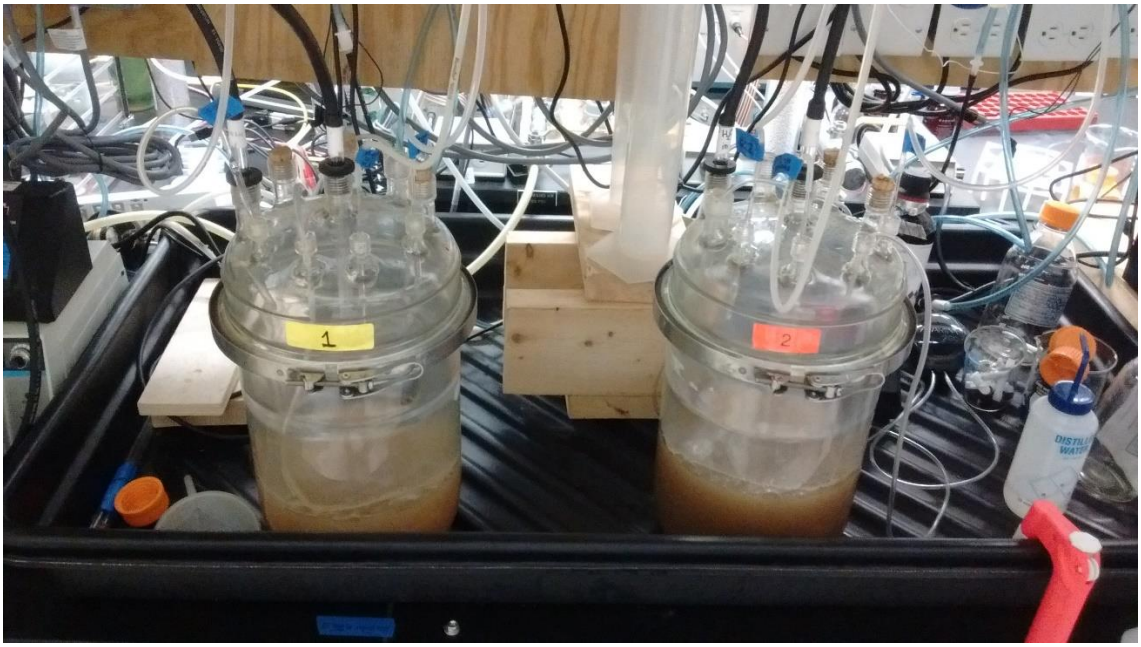

Figure 2: AFIT laboratory sequencing batch reactors.

The SBRs were operated on an eight-hour cycle. Each cycle began with a one-hour settling period. After settling, approximately 800 ml of effluent was drawn off. Following the effluent draw off, the reactor began a seven-hour aeration period. During the aeration period, approximately 800 ml of makeup deionized (DI) water was pumped into the reactor and feed solutions were administered to the reactor as well.

The synthetic wastewater consisted of two feed types: A – sodium bicarbonate, B – macro and micro nutrients, adapted from Rauglas et al. (2016). Feed B was subsequently divided into two subsets B1 and B2 in order to reduce spoiling. Feed A consisted 44.6 g of sodium bicarbonate dissolved in 1 L of DI water. It was added at a rate of nine ml per cycle. Feed B1 consisted of 12.0 g casamino acid and 2.5 g sodium acetate dissolved in one liter of DI water. B1 was fed to the reactors at a rate of 18 ml per cycle. Feed B2 consisted of 4.52 g ammonium chloride, 13.72 g magnesium chloride,

3.44 g calcium chloride, 1.4 g potassium dihydrogen phosphate, and 40 ml trace element solution (Appendix A: Feed Solutions) dissolved in 1 liter of DI water. 18 ml of Feed B2 was added per SBR per cycle. Feeds A and B2 were changed out monthly. Feed B1 would become cloudy after three days of use (presumably from undesired bacterial growth) and thus was changed out three times per week. B1 feed lines were cleaned weekly while feed lines for A and B2 were cleaned monthly. All feed lines were cleaned with a 1% bleach solution followed by flushing with 8-10 liters of DI water.

Daily monitoring of the SBRs included TSS and VSS readings three times per week, excess water decanted or additional makeup water added as needed, and rinsing of the interior walls of the reactor to remove biofilm buildup. TSS and VSS were measured according to the standard methods for the examination of water and wastewater methods 2540D and 2540E (APHA et al., 2016). Dissolved oxygen averaged 8.3 mg/l and 8.4 mg/l for reactors one and two respectively. Solid retention times averaged 55 days for Reactor 1 and 38 days for Reactor 2.

The SBR system used Cole Parmer Masterflex easy load II Model 77200-50 pumps. Automatic timing control was provided by ChemTrol Model XT4s. Each reactor had a Marina 200 air pump and two Topfin aerator stones.

Respirometry

Respirometry experiments were conducted using a Columbus Instruments Respirometer (see Figure 3). The apparatus consisted of two, ten-channel interface units, an O_2 sensor, a CO_2 sensor, and various sampling pumps and filters. The respirometer measured O_2 consumption and CO_2 production in µg/min over the approximate 14-20

hour duration of the experiment. The respirometer was calibrated with a laboratory grade pure N_2 gas and a laboratory grade calibration standard (Matheson Tri Gas, CO_2 0.500%, O_2 20.50%, N_2 79.00%) gas before each experiment.

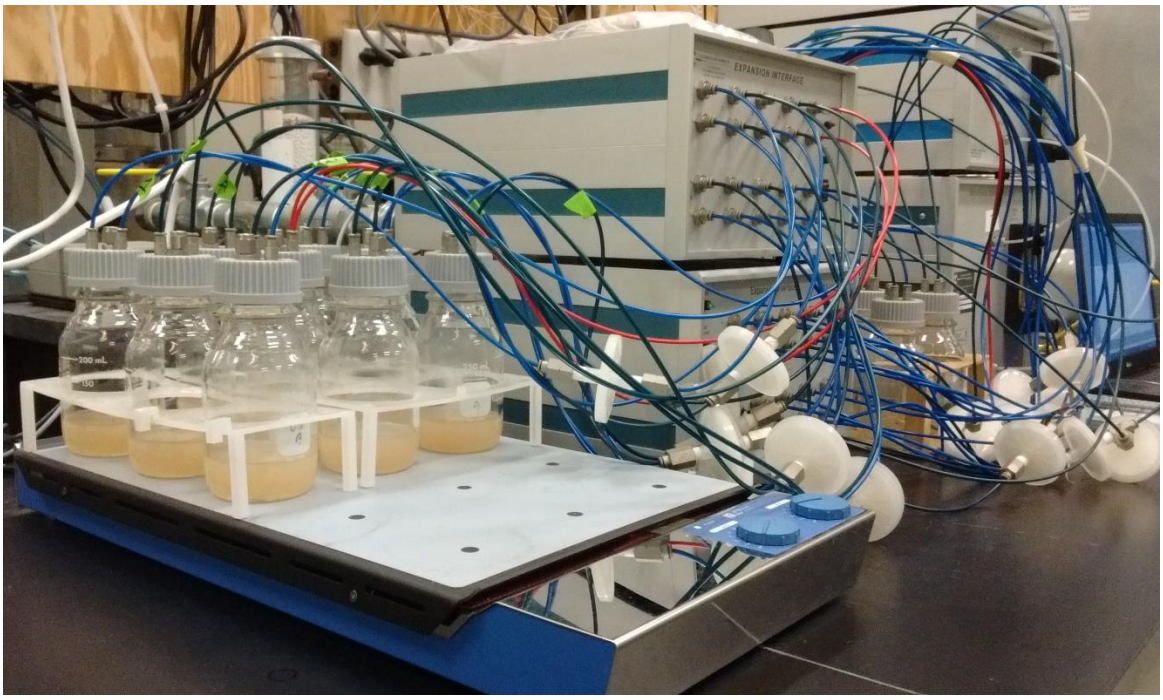

Figure 3: Respirometry experiment. 9 channels containing activated sludge, feed solutions, magnetic stir bar, and *B. globigii* spores or ATU as appropriate. Placed on stir plate to provide constant mixing. Connected to CI Respirometer to measure O_2 consumption and CO_2 production.

Nine channels on the respirometer were used during each experiment. Each channel was connected to a 250 ml glass Pyrex bottle. 50 ml of activated sludge, a magnetic stir bar, 200 µl Feed A, 425 µl Feed B1, and 425 µl of Feed B2 were added to each bottle. Channels 1-3 were the experiment and received a set dose of spores. Spore concentrations were added to achieve $2x10^7$, $2x10^5$, $2x10^3$, and $2x10^1$ CFUs/ml for the multiple experiments. Channels 4-6 functioned as a positive (i.e. inhibition expected) control set and received 250 µl of 0.01M ATU to establish and ATU concentration of 5 µM. Channels 7-9 served as the negative (i.e. no inhibition) control and only received

the feed solutions. The bottles were placed on a magnetic stir plate, which stirred the sludge mixture at approximately 250 rpm.

Post respirometry, the activated sludge from each bottle was transferred to a 50 ml conical tube. The conical tubes were centrifuged at 4000 rpm for 15 minutes and then stored at 4º C for 1-4 hours until COD, NH_3-N, and NO_3-N tests could be performed. COD was measured using the Hach™ Method 8000 kit (USEPA approved, Standard Method 5220 D). NH_3-N was measured using Hach™ Method 10031, and NO_3-N was measured using Hach™ Method 10020. The Hach™ kit test tubes were read using an Agilent Cary-60 UV-Vis Spectrometer. All Hach™ measurements were conducted in triplicate except as noted. See Appendix B: UV-Vis Spectrometer Calibration Curves for calibration curves. Initial conditions for pre-respirometry comparison were measured using collected effluent from Reactor 1 with appropriate feed solutions added.

Analysis

The effect of *B. globigii* spores on microbial activity was analyzed quantitatively by comparing the shape parameters, peak oxygen consumption, and cumulative oxygen consumption of the experiment and control using a Student's t-test, $\alpha = 0.05$. Qualitative analysis consisted of visual comparison of the experiment and control oxygen consumption profiles and molar O_2/CO_2 ratios. To evaluate the effectiveness of the activated sludge, Student's t-tests, $\alpha = 0.05$, compared final COD, NH_3-N, and NO_3-N amounts of the experiment and control groups.

Shape parameters

The first moment of area (FrM) is often used to determine the center of mass or centroid of a shape. In this case it was used to quantify the shape of the O_2 consumption profile for comparisons sake. The FrM was calculated about the y-axis using a mid-point approximation for the integral in $\boldsymbol{Q_y = \int_a^b xf(x)}$ Equation 1, see Figure 4. A discretized approximation of the integral was chosen in order to solely relay on the collected data points. Although software is available to "connect the dots" between discreet data points and create a smooth curve, it presents the possible introduction of fictitious data. The FrM was calculated using only the first three sampling intervals in order to capture most of the "peak" of the curve while omitting as much of the "tail" as possible. The "tail" represented the endogenous respiration of the sample which varied amongst channels. Due to its distance from the y-axis, a sample with a high endogenous respiration rate would have a greater effect on the FrM muddling any differences in microbial activity from the *B. globigii* spores.

$\boldsymbol{Q_y = \int_a^b xf(x)}$ **Equation 1: First Moment of Area**

$$Where: \int_a^b f(x) = area\ under\ the\ curve$$

$$x = distance\ from\ veritical\ axis$$

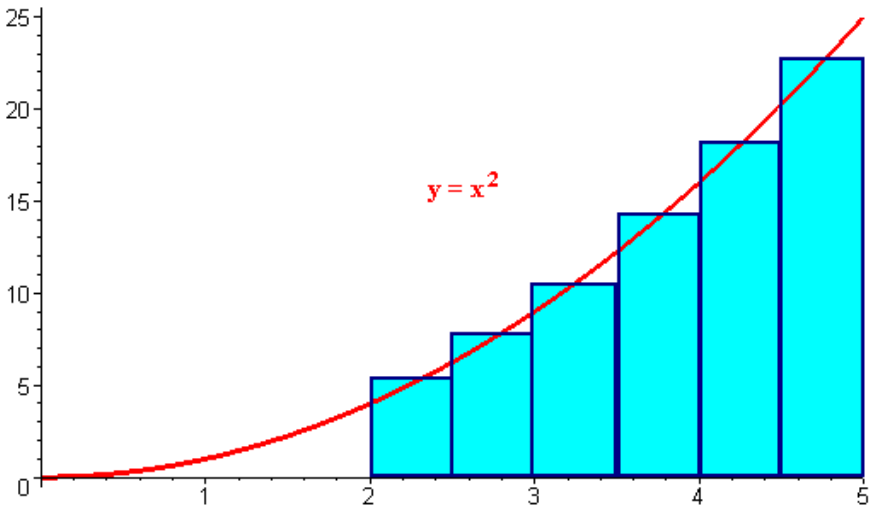

Figure 4: Simple illustration of midpoint approximation example.

Skewness is a measurement of the asymmetry of a probability distribution around its mean, $\boldsymbol{g_1 = \frac{n}{(n-1)(n-2)} \sum \left(\frac{x_i - \bar{x}}{s}\right)^3}$ Equation 2. In this case, it was used to quantify the shape of the O_2 consumption profile during the first five sampling intervals of each trial. Although perhaps not a true measurement of skewness in the strict sense of the term, it served as another quantifiable factor for the comparison of the experiment and control O_2 consumption profiles using a Student's t-test.

$$\boldsymbol{g_1 = \frac{n}{(n-1)(n-2)} \sum \left(\frac{x_i - \bar{x}}{s}\right)^3}$$ **Equation 2: Skewness**

$$Where: n = number\ of\ elements$$

$$s = standard\ deviation$$

$$x_i = element\ of\ interest$$

$$\bar{x} = mean$$

Peak oxygen consumption

Peak oxygen consumption was measured by selecting the greatest measured oxygen uptake rate (OUR) from the oxygen consumption profile curve regardless of the sampling interval in which it occurred. The peak OUR was converted to a specific oxygen uptake rate (SOUR) via normalizing by the reactor MLSS VSS. Units of SOUR are mg O_2-g^{-1} VSS-hr^{-1}. The mean peak SOUR of the experiment and the control where then compared using a Student's t-test.

Cumulative oxygen consumption

The cumulative oxygen consumption after the fourth sampling interval was selected to once again capture the "peak" without too much distortion from variation in endogenous respiration "tails." The mean cumulative oxygen consumed between the experiment and the control was then compared using a Student's t-test. Due to the large values, plotting error bars as variance tended to obscure the meaningfulness of the graph so standard deviation was used instead.

Molar O_2/CO_2 ratio

The molar O_2/CO_2 ratio was calculated by dividing the CO_2 production rate by its corresponding O_2 consumption rate. The molar ratios were then plotted verses the corresponding time. In order to provide reference for the activated sludge molar O_2/CO_2 ratios, the theoretical stoichiometric molar O_2/CO_2 ratios of acetate (2.48) and peptone (0.98) were plotted as well. Additionally, a 13% acetate and 87% peptone mixture (molar O_2/CO_2 ratio 1.76) was plotted to represent the theoretical O_2/CO_2 ratio of the feed solutions.

Summary

In order to investigate the impact on a WWTP receiving contaminated wash-down waters from a biological warfare attack, *B. globigii* spores were used as a surrogate for *B. anthracis*. Different concentrations of spores were added to activated sludge and then monitored for respiration rates. Respirometry measured overall microbial activity; effectiveness of the activated sludge was measured by COD, NH_3-N, and NO_3-N removal. The data was analyzed using Student t-tests to assess differences between the experiment and the control.

IV. Results

Chapter Overview

Spore concentrations ranging from $2x10^1$ to $2x10^7$ CFU/ml were tested both in the 40% ethanol storage solution and after washing with DI water. None of the concentrations tested caused statistically significant effects on the peak O_2 consumption rate (p-values, 0.11 - 0.32) or the cumulative O_2 consumption (p-values, 0.21 - 0.62). The molar O_2/CO_2 ratios were similarly unaffected. The shape factors were significantly impacted by the introduction of spores (p-values, 8.53 E-06 – 0.95) but these factors did not reflect biological inhibition. *B. globigii* did not inhibit COD or N removal. Unwashed spores inhibited initial O_2 consumption rates when $2x10^7$ CFU/ml were injected into the activated sludge. Spore germination was observed at 0.88% to 1.7%, and spores preferentially adhered to wastewater floc (81%) as opposed to remaining in the bulk liquid (19%).

Typical SBR Operations

Sequencing batch reactor operation began 12 Jan 2016. The activated sludge was allowed to acclimate to the SBR for approximately three months. Experiments were conducted from 14 April 2016 to 12 October 2016. During the experiment duration, total suspended solids (TSS) of the mixed liquor suspended solids (MLSS) averaged 2870 ± 374 mg/l, of which 87.3% were volatile suspended solids (VSS). SBR effluent TSS averaged 25.4 ± 9.6 mg/l, a 99.1% solids removal rate. During the eight hour cycle time, the SBR facilitated average COD reduction from 184.2 mg/l to 34.0 mg/l, an 81.5% reduction. Nitrification reduced ammonia levels from 23.2 mg/l as N to below the LOD.

Nitrate, on the other hand, increased from 0.18 mg/l as N to 33.8 mg/l as N. Denitrification was not anticipated due to the high levels of dissolved oxygen (8.3 mg/l).

Microbial Respiration

The effect of $2x10^1$ CFU/ml B. globigii on microbial respiration

The respirogram of $2x10^1$ CFU/ml *B. globigii* in activated sludge exhibited typical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution a sharp increase in respiration occurred in response to the injection of substrate as shown in Figure 5. Following the substrate uptake, the O_2 consumption curves peaked between 2-4 hours and then sharply declined to a gradually decreasing endogenous respiration rate, creating the familiar "peak and tail" respirogram profile (Dircks, et al., 1999). Two members of the experiment group, channels 1 and 3 had rather wide, flat curves with three measurements near the peak value whereas all other channels only had two measured points near their respective peak respiration rates. Of the control group, channels 8 and 9 stand out from channel 7 as having a higher peak in their curves. These two channels also clearly have a higher endogenous respiration rate observed in the tail sections of the profile curves.

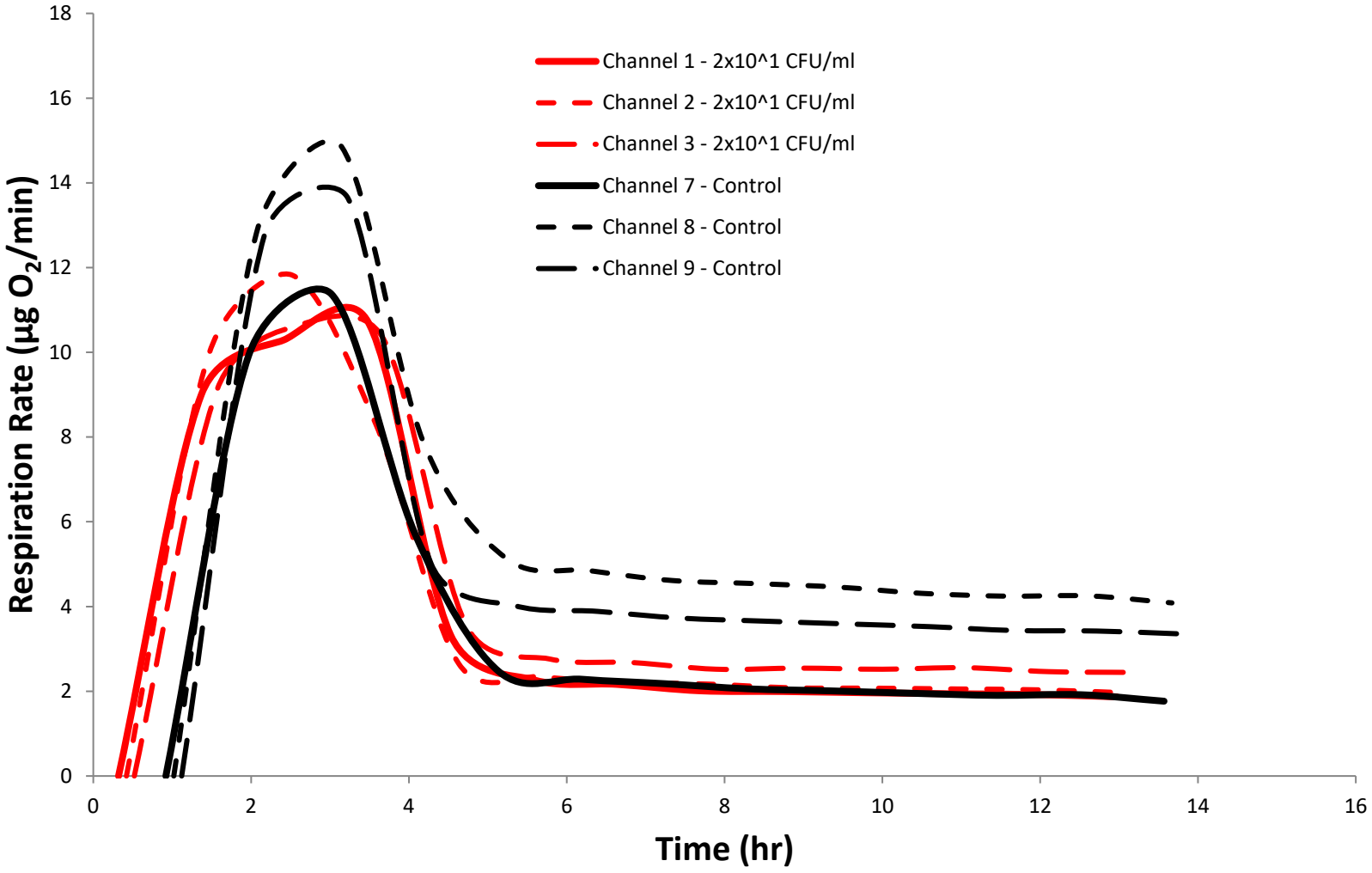

Figure 5: O_2 consumption profile of $2x10^1$ CFU/ml *B. globigii* spores in activated sludge.

Shape parameters

The shape parameters of the $2x10^1$ CFU/ml *B. globigii* O_2 consumption profiles were statistically different from the control profile curves. The mean FrM of the experiment group was 59.4 µg O_2/min-hr^2 with a variance of 8.7 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 81.8 µg O_2/min-hr^2 with a variance of 164.1 (µg O_2/min-hr^2)^2. Although the large difference between the channels in the control group resulted in a large variance, the p-value from comparing the FrM was 0.04, Figure 6. The mean skewness of the experiment group was -0.71 with a variance of 0.038. The mean skewness of the control group was -0.076 with a variance of 0.052. Comparing the skewness of the curves also resulted in a statistically significant difference with a p-value of 0.02, Figure 7. These results show that there were statistically significant differences in the shape parameters between the test and control respirograms, indicating factors that influenced the transport of oxygen were likely present.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 13.3 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 0.62 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 15.9 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 4.57 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of $2x10^1$ CFU/ml *B. globigii* and activated sludge was not statistically significantly different from the control. The p-value from comparing the maximum SOURs was 0.11, Figure 8. The lack of a statistically significant difference between the experiment and control groups at $2x10^1$ CFU *B. globigii*/ml activated sludge indicate that the bacteria responded to the presence of the substrate the same with or without the spores.

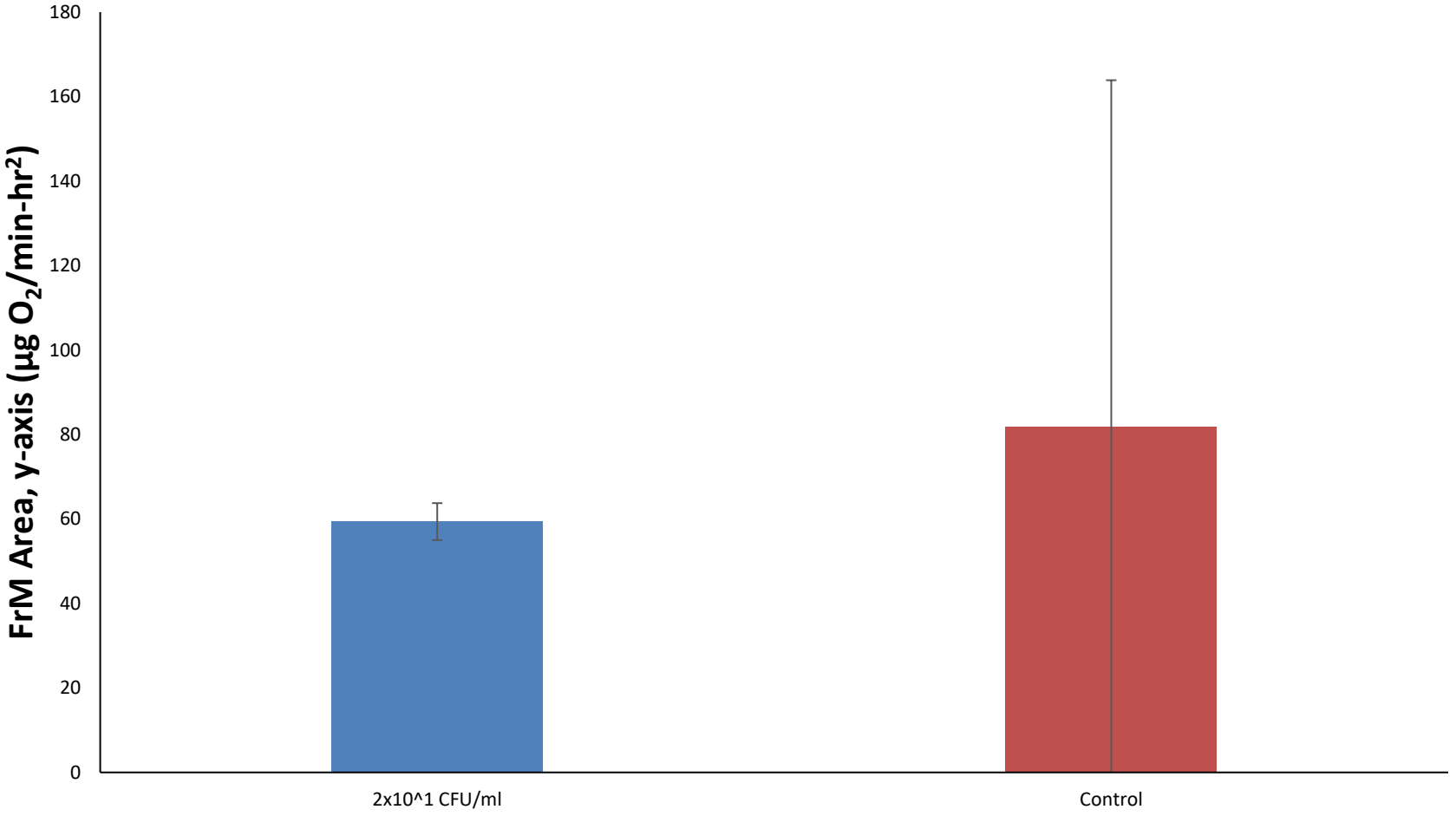

Figure 6: Comparison of 2x10[1] CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

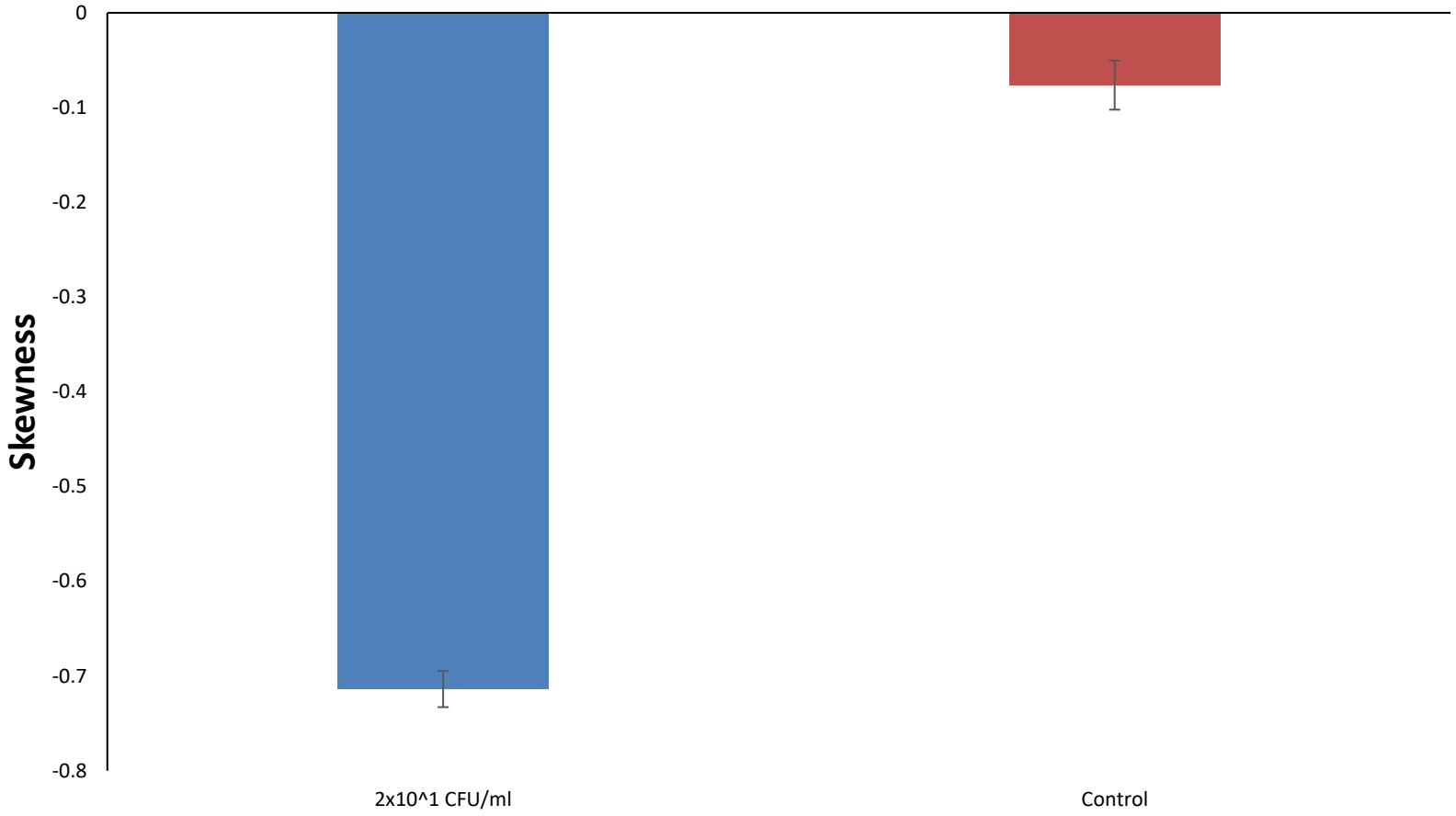

Figure 7: Comparison of $2x10^1$ CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

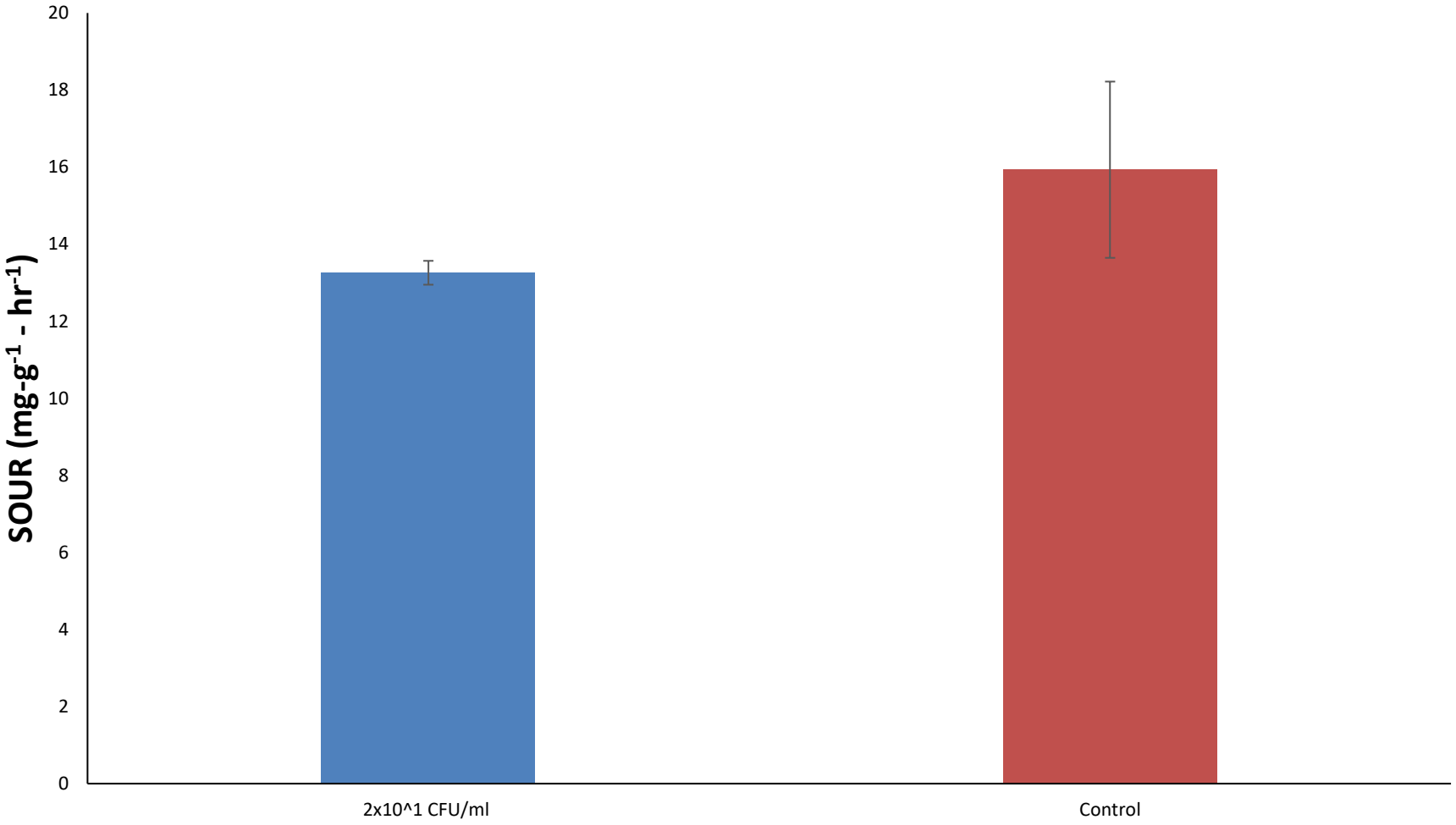

Figure 8: Peak SOUR of 2x10[1] CFU *B. globigii*/ml activated sludge. Error bars represent sample variance.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2101 µg with a standard deviation of 27 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2227 µg with a standard deviation of 356 µg. The cumulative O_2 consumption of $2x10^1$ CFU/ml *B. globigii* and activated sludge was not statistically significantly different from the control, Figure 9. The p-value from a student t-test comparison was 0.58. The lack of a statistically significant difference in the cumulative O_2 consumed at $2x10^1$ CFU *B. globigii*/ml activated sludge indicates that the bacteria were not inhibited by the presence of the spores.

Molar O_2/CO_2 ratios

The molar ratio of O_2 consumed-to-CO_2 produced at $2x10^1$ CFU *B. globigii*/ml activated sludge did not appear to be substantially different from the control. Both the control and the experiment groups exhibited a rise in the molar O_2/CO_2 ratio for approximately the first three hours of the test but then minimal undulation for the remaining duration. Typical values ranged from 1.89 to 2.02 mole O_2/mole CO_2, Figure 10. The unaffected molar O_2/CO_2 ratio indicates that the underlying metabolic pathway of the activated sludge was not affected by the *B. globigii* spores. The results also show that the measured stoichiometric O_2 consumption-to-CO_2 production ratio was similar to the value (approx. 1.8 mole O_2/mole CO_2) that would be bioenergetically expected when the substrate is a mixture of peptone and acetate (Rauglas et al., 2016). The theoretically-expected stoichiometric ratios for acetate and peptone are 2.48 mole O_2/mole CO_2 and 0.98 mole O_2/mole CO_2 respectively.

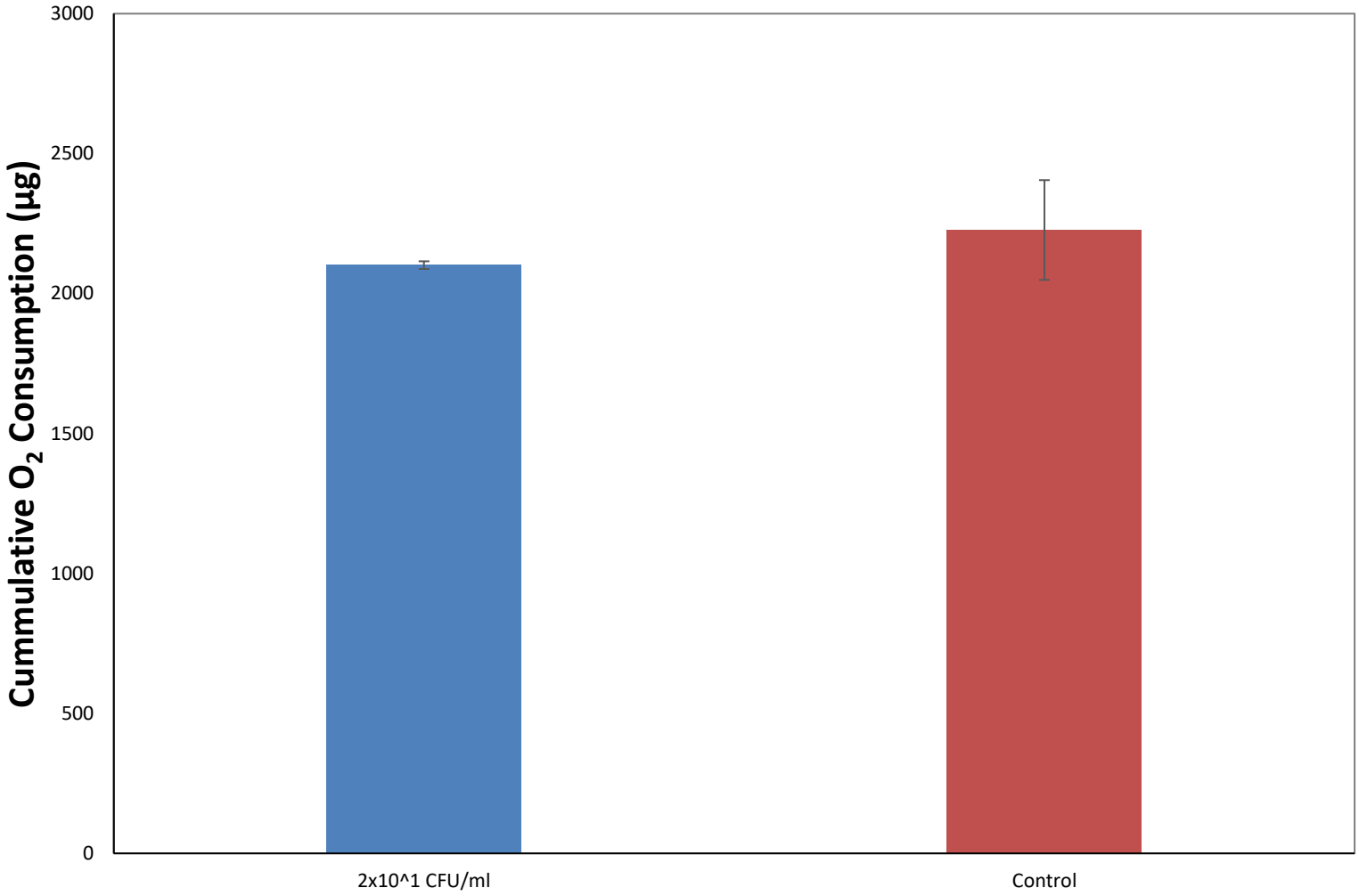

Figure 9: Cumulative O_2, $2x10^1$ CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

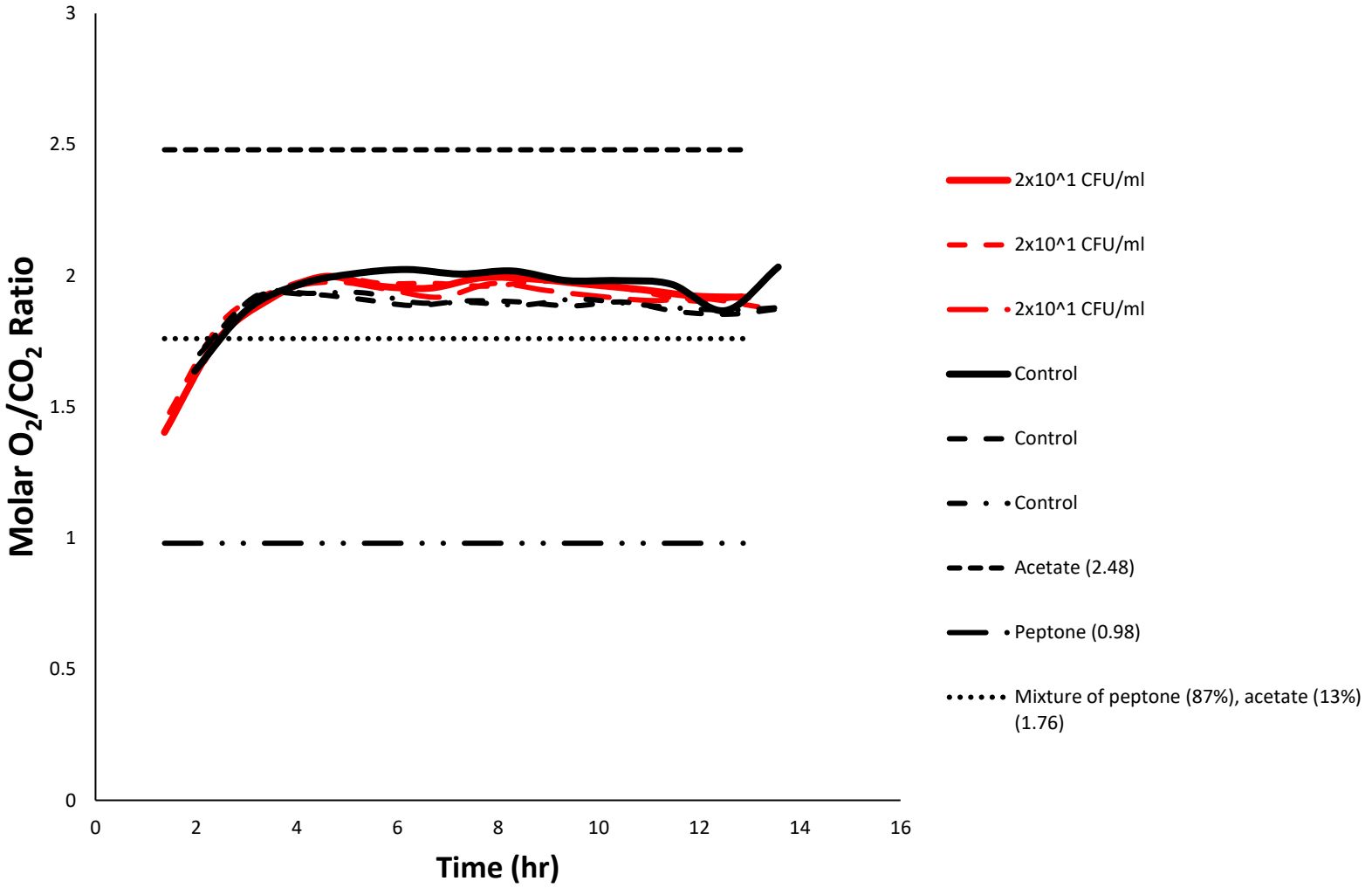

Figure 10: Molar O_2 to CO_2 Ratio, $2x10^1$ CFU *B. globigii*/ml activated sludge.

The effect of 2x10^3 CFU/ml B. globigii on microbial respiration

The respirogram of 2x10^3 CFU/ml *B. globigii* in activated sludge exhibited typical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution a sharp increase in respiration occurred in response to the injection of substrate as shown in Figure 11. Following the substrate uptake, the O_2 consumption curves peaked between 2-4 hours and then sharply declined to a gradually decreasing endogenous respiration rate, creating the familiar "peak and tail" respirogram profile. Channels 4 and 6 had the highest peak respiration rates and also the greatest endogenous respiration tail indicating greater overall microbial activity. Channel 8 of the control group similarly had a higher peak respiration rate and a greater endogenous respiration tail than the other two channels in the control group. Channels 5 and 9 both have an unusual rise in their tails. This is most likely the result of a delayed release of secondary metabolites in those specific samples.

Shape parameters

The shape parameters of the 2x10^3 CFU/ml *B. globigii* O_2 consumption profiles were not statistically different from the control profile curves. The mean FrM of the experiment group was 83.7 µg O_2/min-hr^2 with a variance of 108.0 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 78.2 µg O_2/min-hr^2 with a variance of 109.8 (µg O_2/min-hr^2)^2. The p-value from comparing the FrM was 0.55, Figure 12. The mean skewness of the experiment group was -0.10 with a variance of 0.17. The mean skewness of the control group was 0.33 with a variance of 0.11. Comparing the skewness of the curves also did not result in a statistically significant difference with a

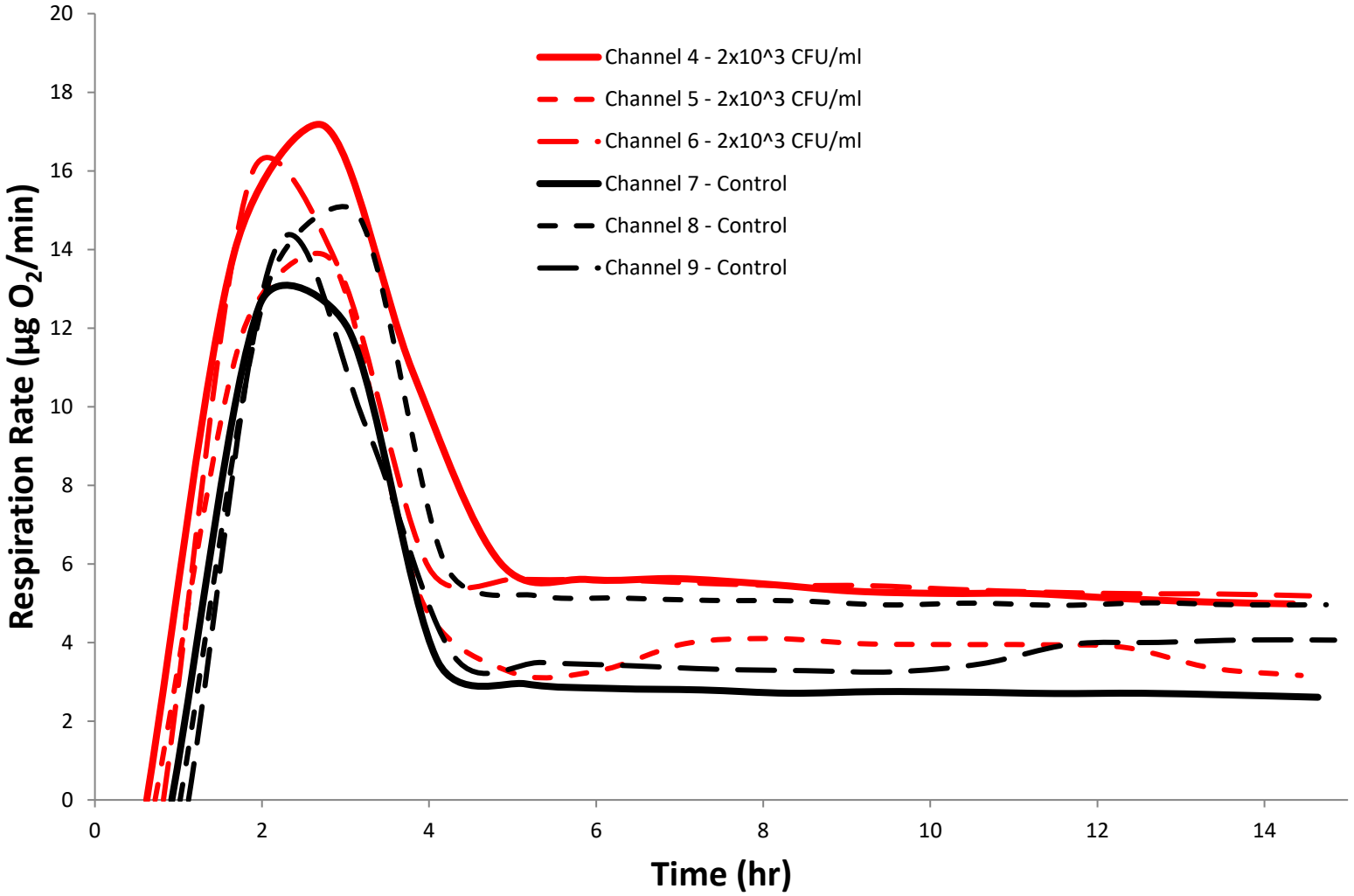

Figure 11: O_2 consumption profile of $2x10^3$ CFU/ml *B. globigii* spores in activated sludge.

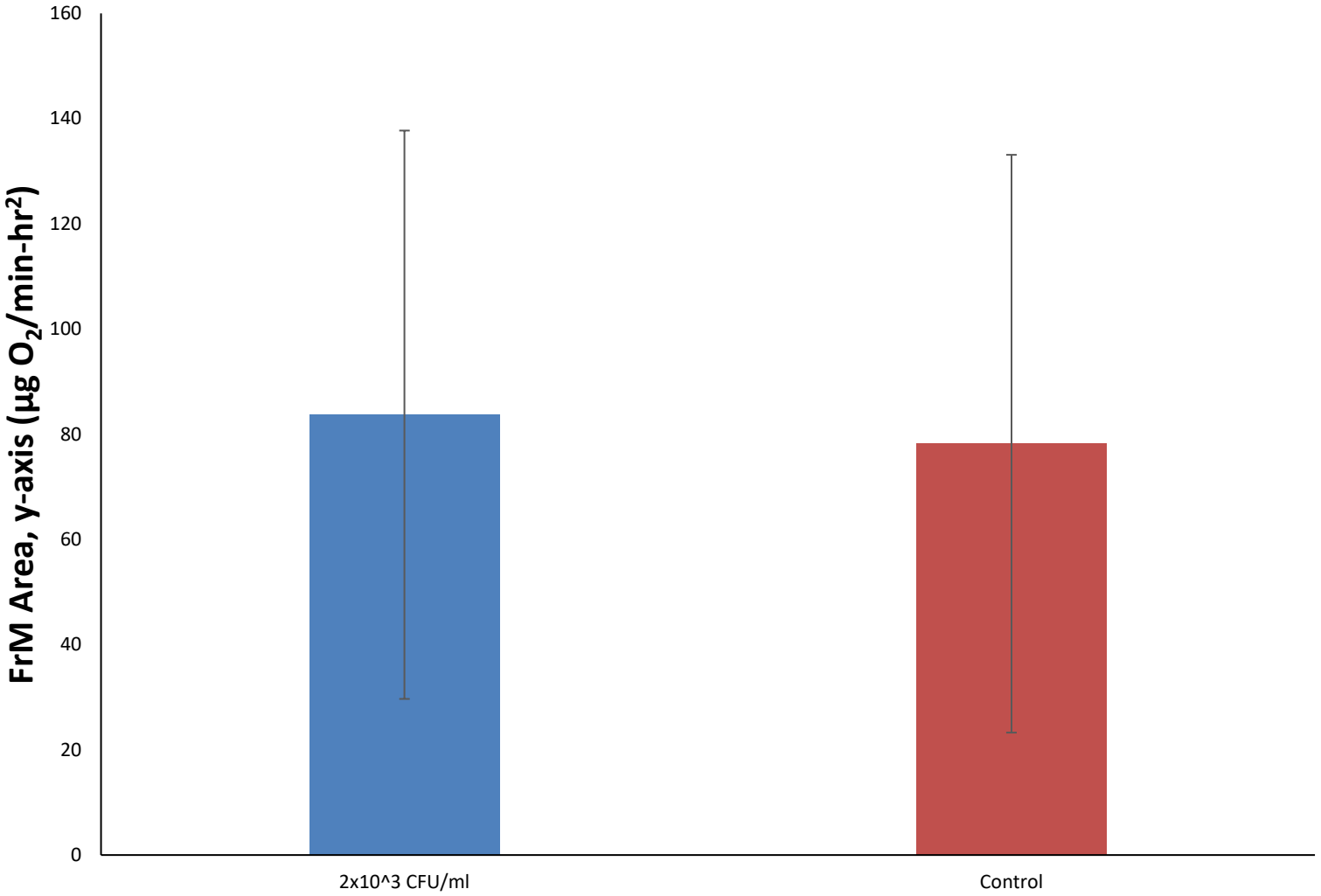

Figure 12: Comparison of $2x10^3$ CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

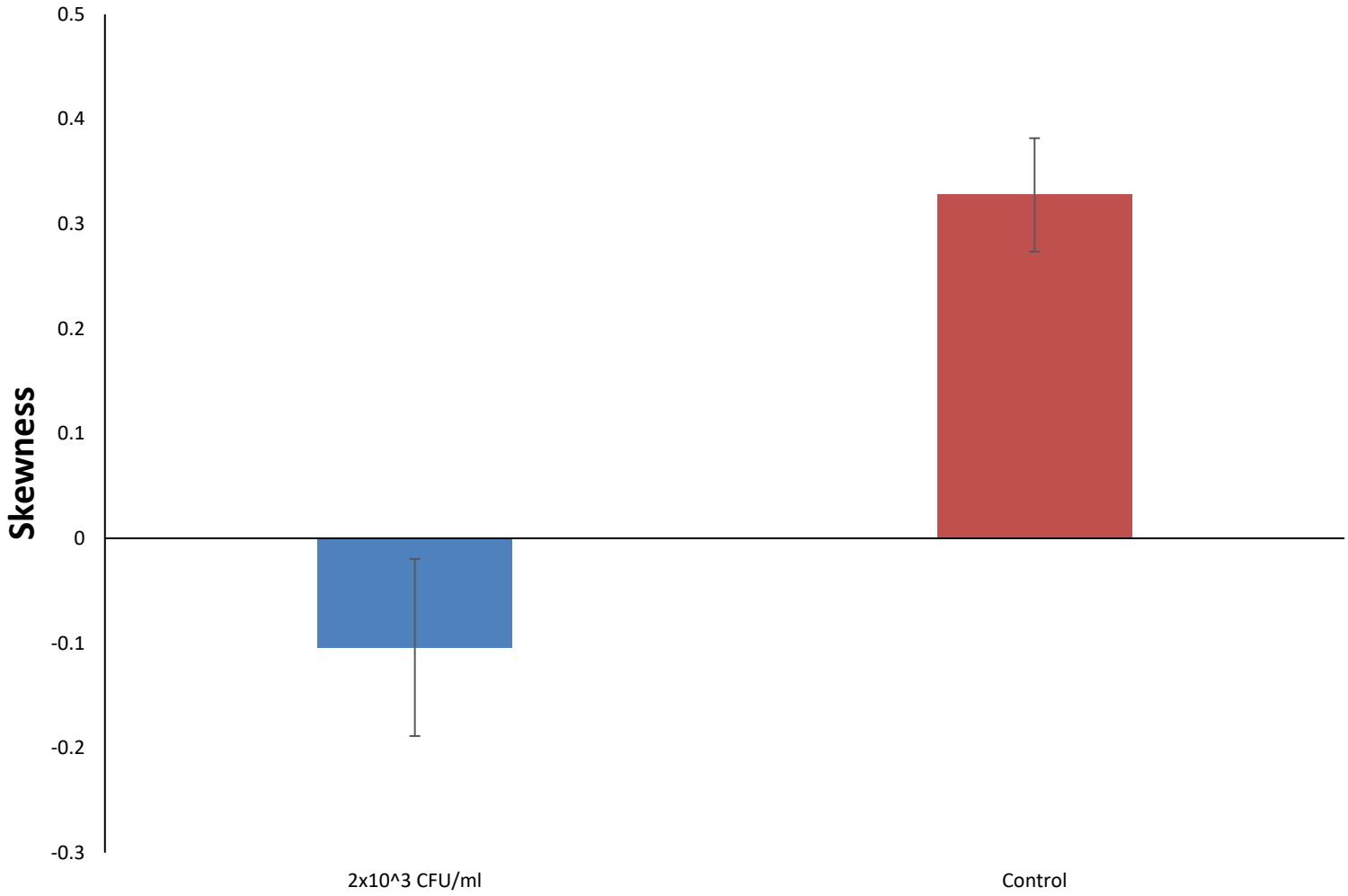

Figure 13: Comparison of 2x10^3 CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

p-value of 0.23, Figure 13. Neither of these shape parameters point to statistically significant differences between the experiment and the control groups.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 16.5 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 3.4 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 13.8 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 1.4 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of $2x10^3$ CFU/ml *B. globigii* and activated sludge were not statistically significantly different from the control. The p-value from comparing the maximum SOURs was 0.10, see Figure 14 . The lack of a statistically significant difference between the experiment and control groups at $2x10^3$ CFU *B. globigii*/ml activated sludge indicate that the bacteria responded to the presence of the substrate the same with or without the spores.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2607 µg with a standard deviation of 448 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2139 µg with a standard deviation of 306 µg. The cumulative O_2 consumption of $2x10^3$ CFU/ml *B. globigii* and activated sludge was not statistically significantly different from the control, see Figure 15. The p-value from a student t-test comparison was 0.21. The lack of a statistically significant difference in the cumulative O_2 consumed at $2x10^3$ CFU *B. globigii*/ml activated sludge indicates that the bacteria was equally effective at incorporating the substrate with or without the presence of the spores.

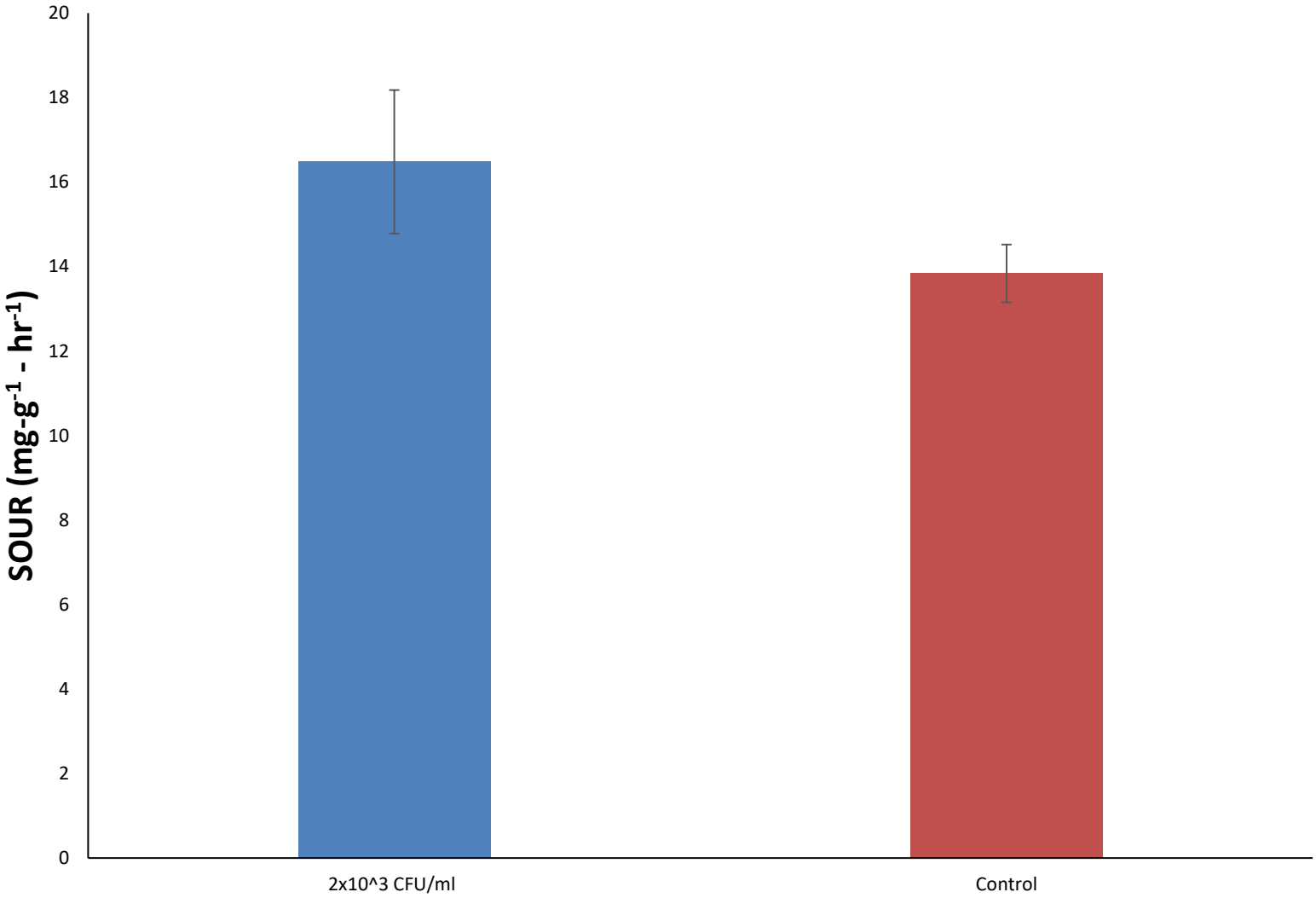

Figure 14: Peak SOUR of $2x10^3$ CFU *B. globigii*/ml activated sludge. Error bars represent sample variance

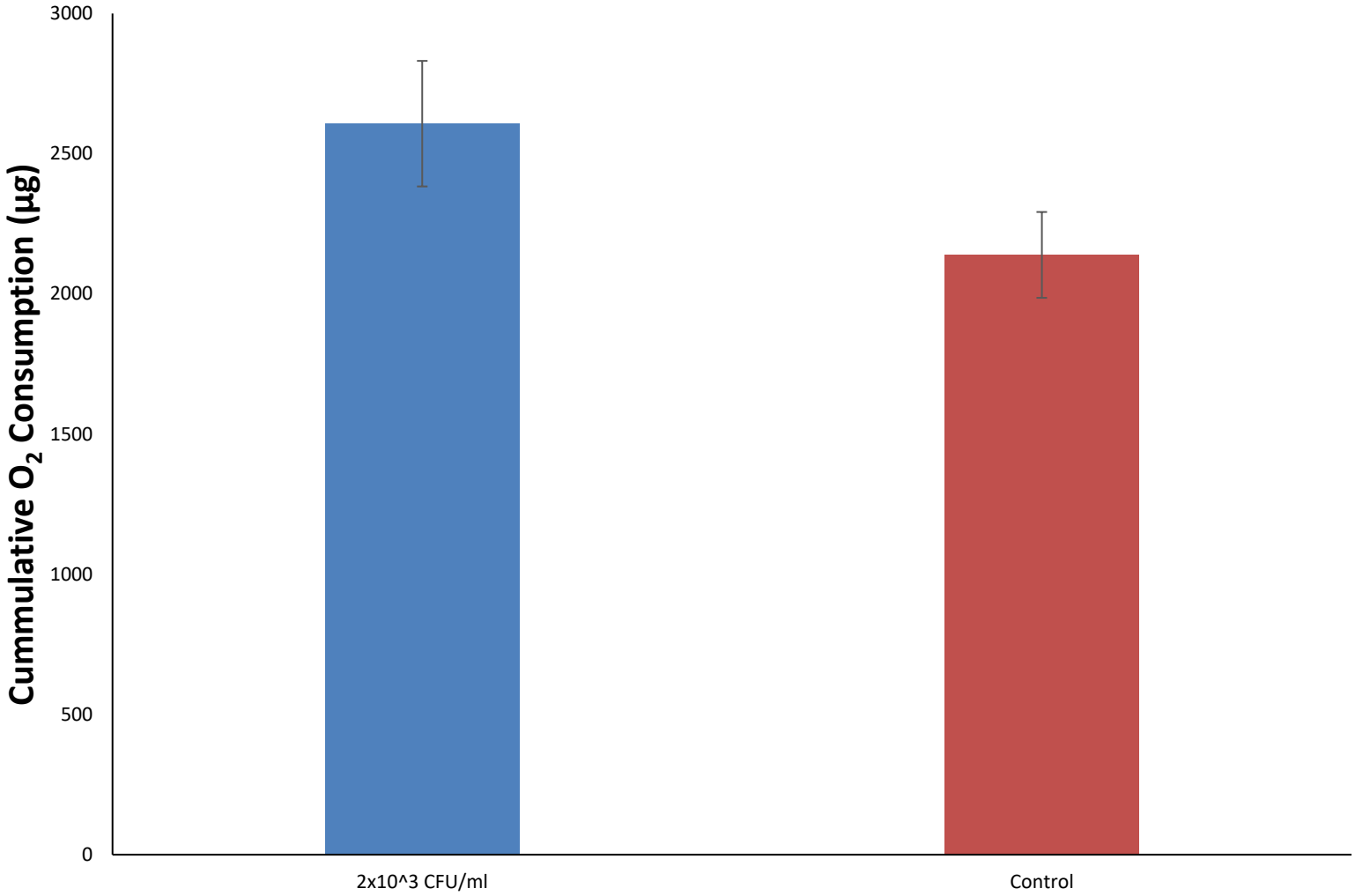

Figure 15: Cumulative O_2, 2x10^3 CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

Molar O_2/CO_2 ratios

The molar ratio of O_2 consumed-to-CO_2 produced at $2x10^3$ CFU *B. globigii*/ml activated sludge did not appear to be significantly different from the control. Both the control and the experiment groups exhibited a rise in the molar O_2/CO_2 ratio for approximately the first three hours of the test but then minimal undulation for the remaining duration. The exception is channel 4 which has a rise and dip from the 4-5 hour point which corresponds to a slight inflection point on the O_2 consumption profile for that channel. This is most likely due to the refreshing the headspace on the sample container. Typical values ranged from 1.88 to 1.94, see Figure 16. The unaffected molar O_2/CO_2 ratio indicates that the underlying metabolic pathway of the activated sludge was not affected by the *B. globigii* spores.

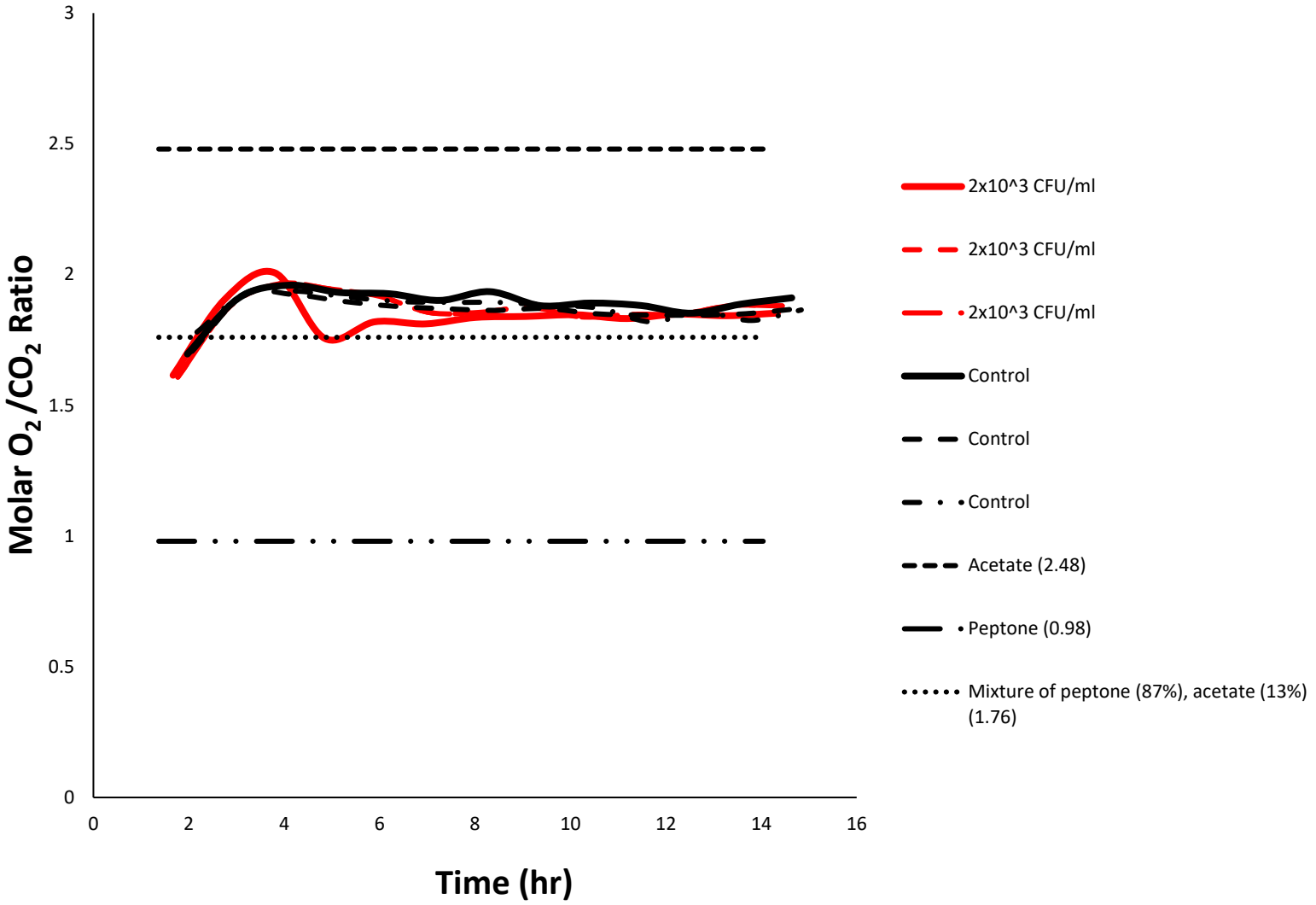

Figure 16: Molar O_2 to CO_2 Ratio, $2x10^3$ CFU *B. globigii*/ml activated sludge.

The effect of 2x10^5 CFU/ml B. globigii on microbial respiration

The respirogram of 2x10^5 CFU/ml *B. globigii* in activated sludge exhibited typical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution a sharp increase in respiration occurred in response to the injection of substrate as shown in Figure 17. The O_2 consumption curves peaked between 2-4 hours and then sharply declined to a gradually decreasing endogenous respiration rate, creating the familiar "peak and tail" respirogram profile. Channel 2 had the highest peak respiration rate and also the greatest endogenous respiration tail indicating the greatest overall microbial activity. Channel 8 of the control group did not have as high of a peak respiration rate but had a similar greater endogenous respiration tail than the other channels. Channel 9 had an unusual rise in its tail. This is most likely the result of a delayed release of secondary metabolites in that specific sample.

Shape Parameters

The shape parameters of the 2x10^5 CFU/ml *B. globigii* O_2 consumption profiles were not statistically different from the control profile curves. The mean FrM of the experiment group was 73.5 µg O_2/min-hr^2 with a variance of 88.8 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 78.2 µg O_2/min-hr^2 with a variance of 109.8 (µg O_2/min-hr^2)^2. The p-value from comparing the FrM was 0.59, see Figure 18: Comparison of 2x10^5 CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. The mean skewness of the experiment group was -0.0034 with a variance of 0.097. The mean skewness of the control group was 0.33 with a variance of 0.11. Comparing the skewness of the curves also did not result in a statistically significant difference with a p-

value of 0.27, see Figure 19. Neither of these shape parameters point to statistically significant differences between the experiment and the control groups.

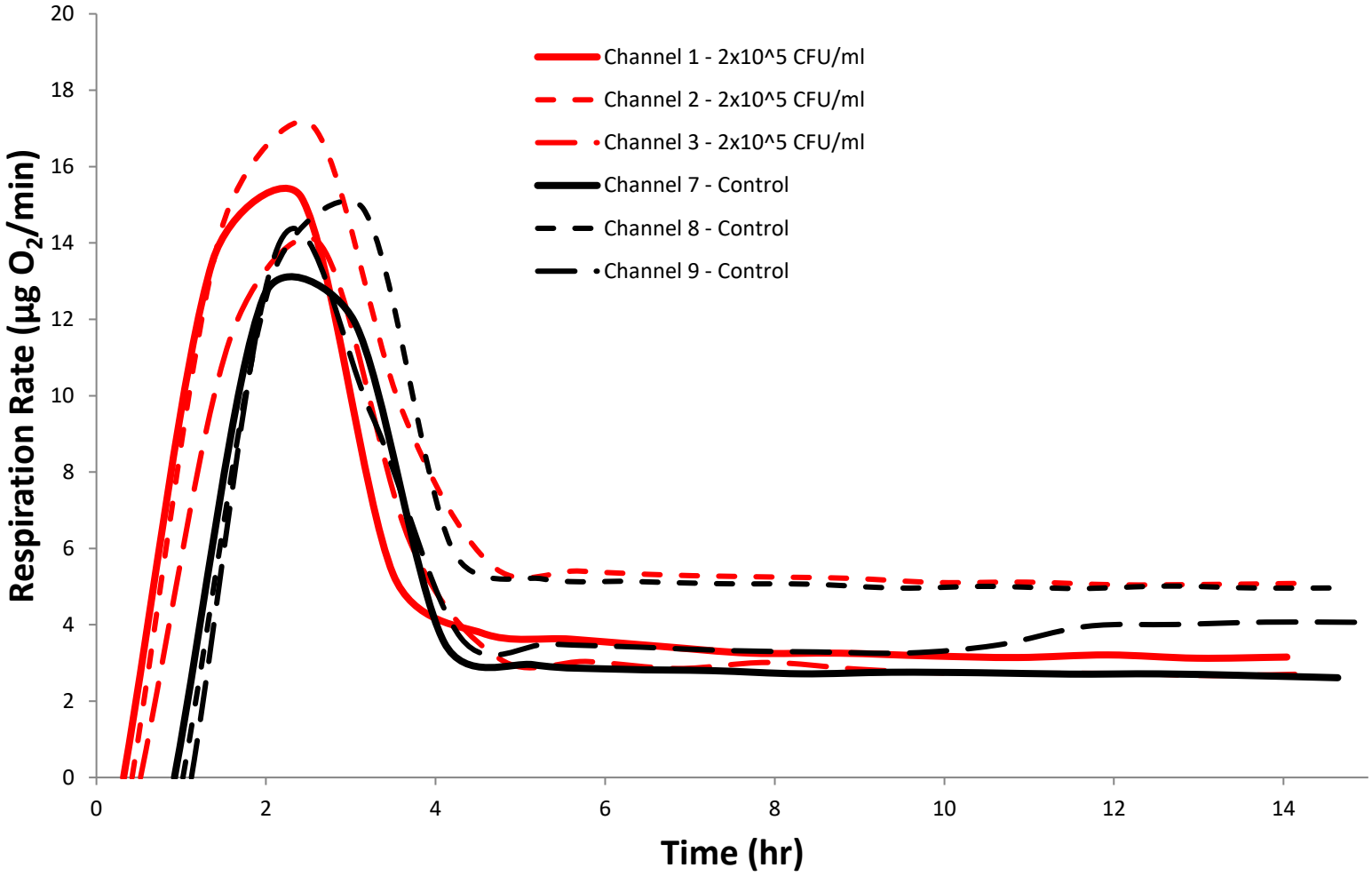

Figure 17: O_2 consumption profile of $2x10^5$ CFU/ml *B. globigii* spores in activated sludge.

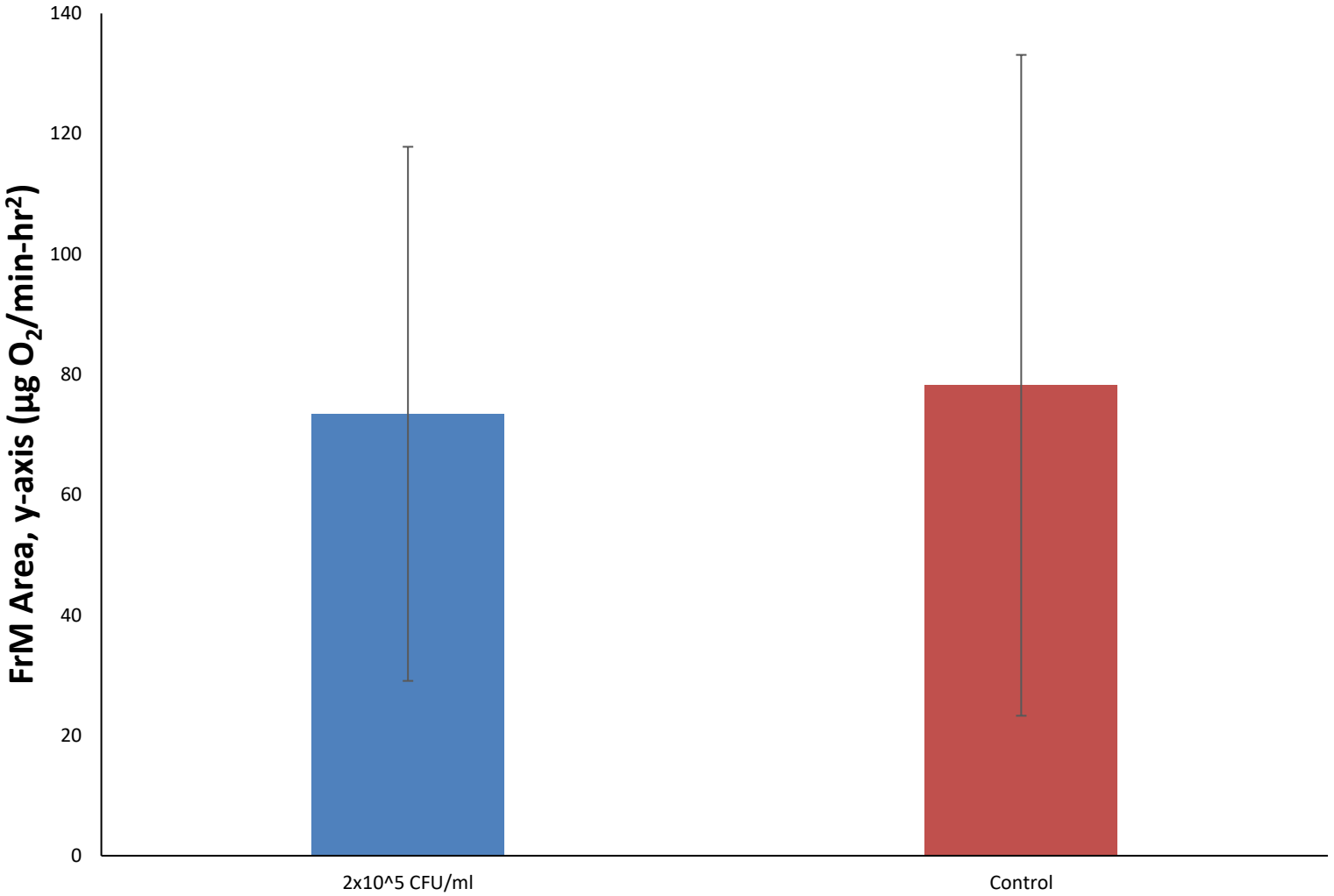

Figure 18: Comparison of 2x10^5 CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

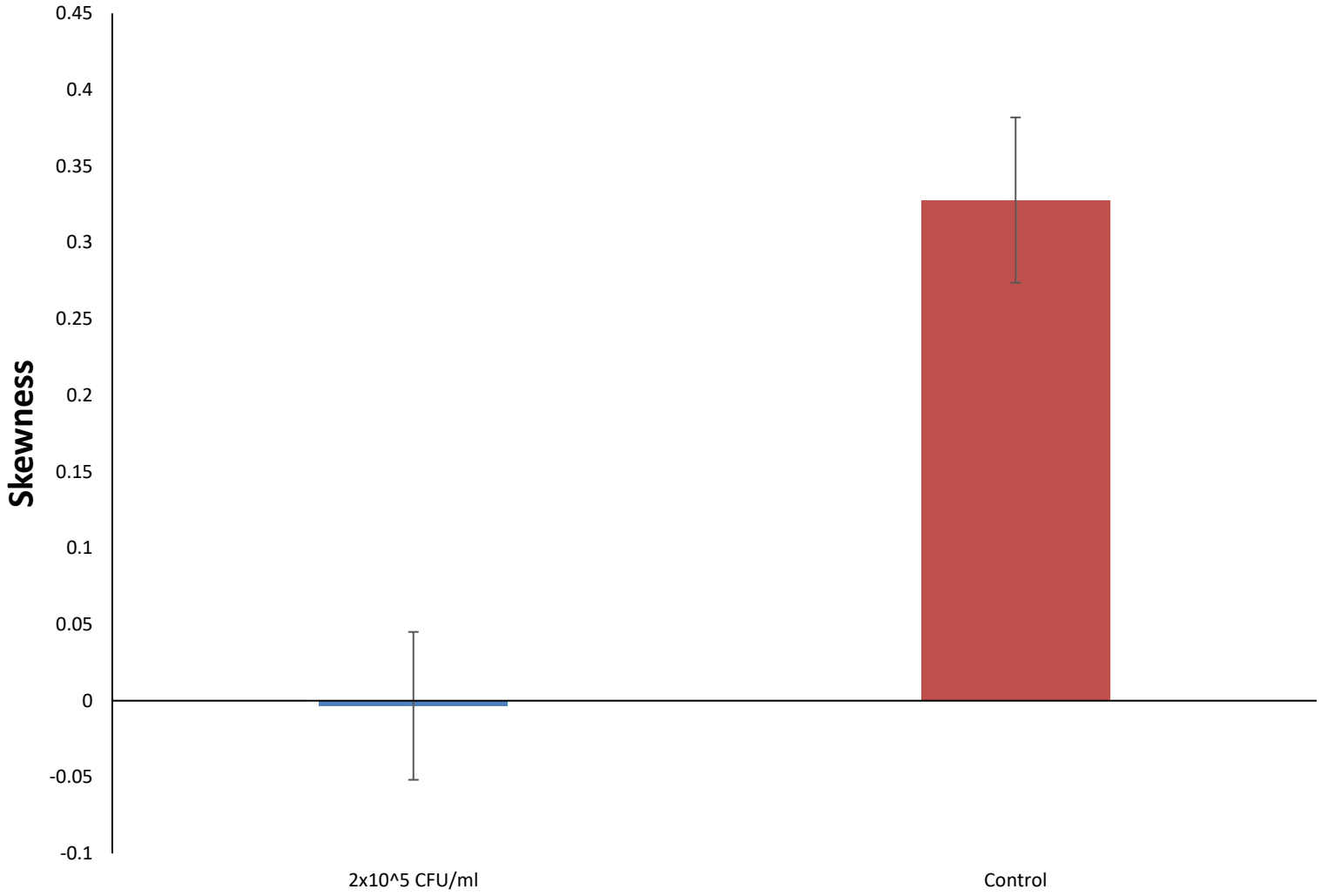

Figure 19: Comparison of $2x10^5$ CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 16.3 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 2.7 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 13.8 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 1.37 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of the activated sludge in the presence of $2x10^5$ CFU/ml *B. globigii* were not statistically significantly different from the control. The p-value from comparing the maximum SOURs was 0.10, see Figure 20. The lack of a statistically significant difference between the experiment and control groups at $2x10^5$ CFU *B. globigii*/ml activated sludge indicate that the bacteria responded to the presence of the substrate the same with or without the spores.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2524 µg with a standard deviation of 369 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2139 µg with a standard deviation of 306 µg. The cumulative O_2 consumption of the activated sludge in the presence of $2x10^5$ CFU/ml *B. globigii* was not statistically significantly different from the control, see Figure 21. The p-value from a student t-test comparison was 0.25. The lack of a statistically significant difference in the cumulative O_2 consumed indicates that the bacteria was equally effective at incorporating the substrate with or without the presence of the spores.

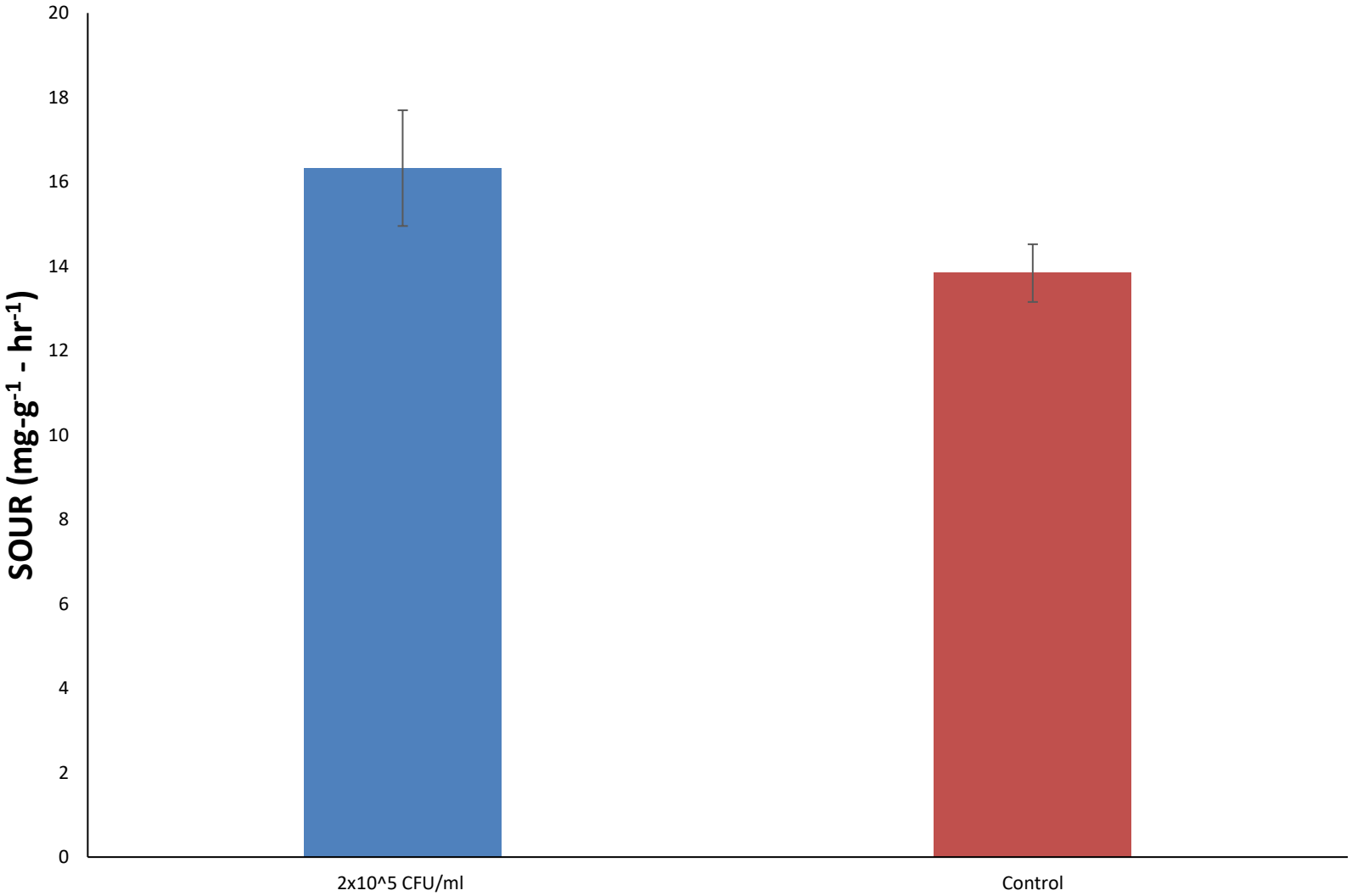

Figure 20: Peak SOUR of 2x10^5 CFU *B. globigii*/ml activated sludge. Error bars represent sample variance.

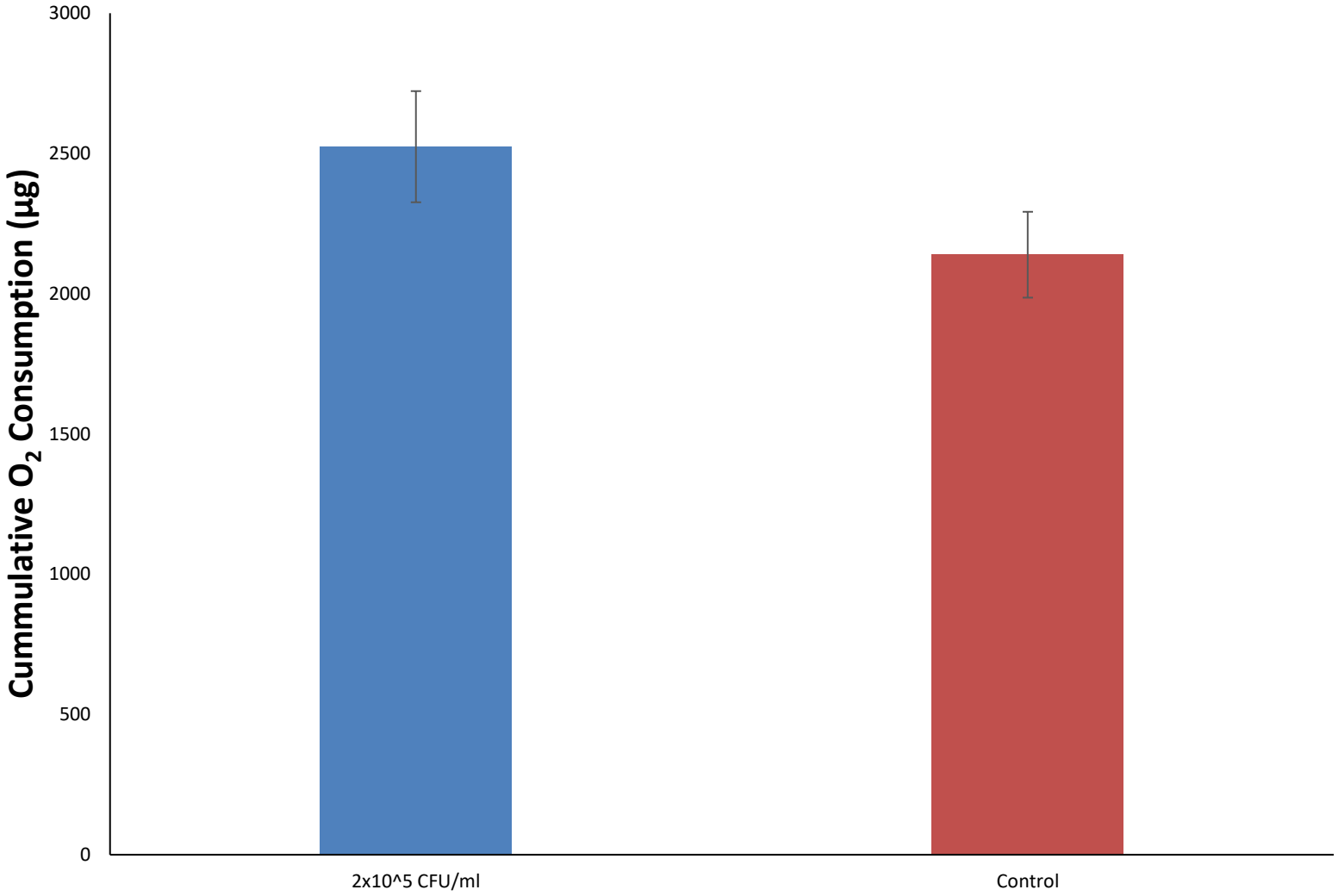

Figure 21: Cumulative O_2, 2x10^5 CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

Molar O_2/CO_2 ratios

The molar ratio of O_2 consumed-to-CO_2 produced at $2x10^5$ CFU *B. globigii*/ml activated sludge was similar to that of the control. Both the control and the experiment groups exhibited a rise in the molar O_2/CO_2 ratio for approximately the first three hours of the test but then minimal undulation for the remaining duration. The exception is channel 2 which has a rise and dip from the 4-5 hour point which corresponds to a slight inflection point on the O_2 consumption profile for that channel. This is most likely due to the refreshing the headspace on the sample container. Typical values ranged from 1.89 to 1.98, Figure 22. The unaffected molar O_2/CO_2 ratio indicates that the underlying metabolic pathway of the activated sludge was not affected by the *B. globigii* spores.

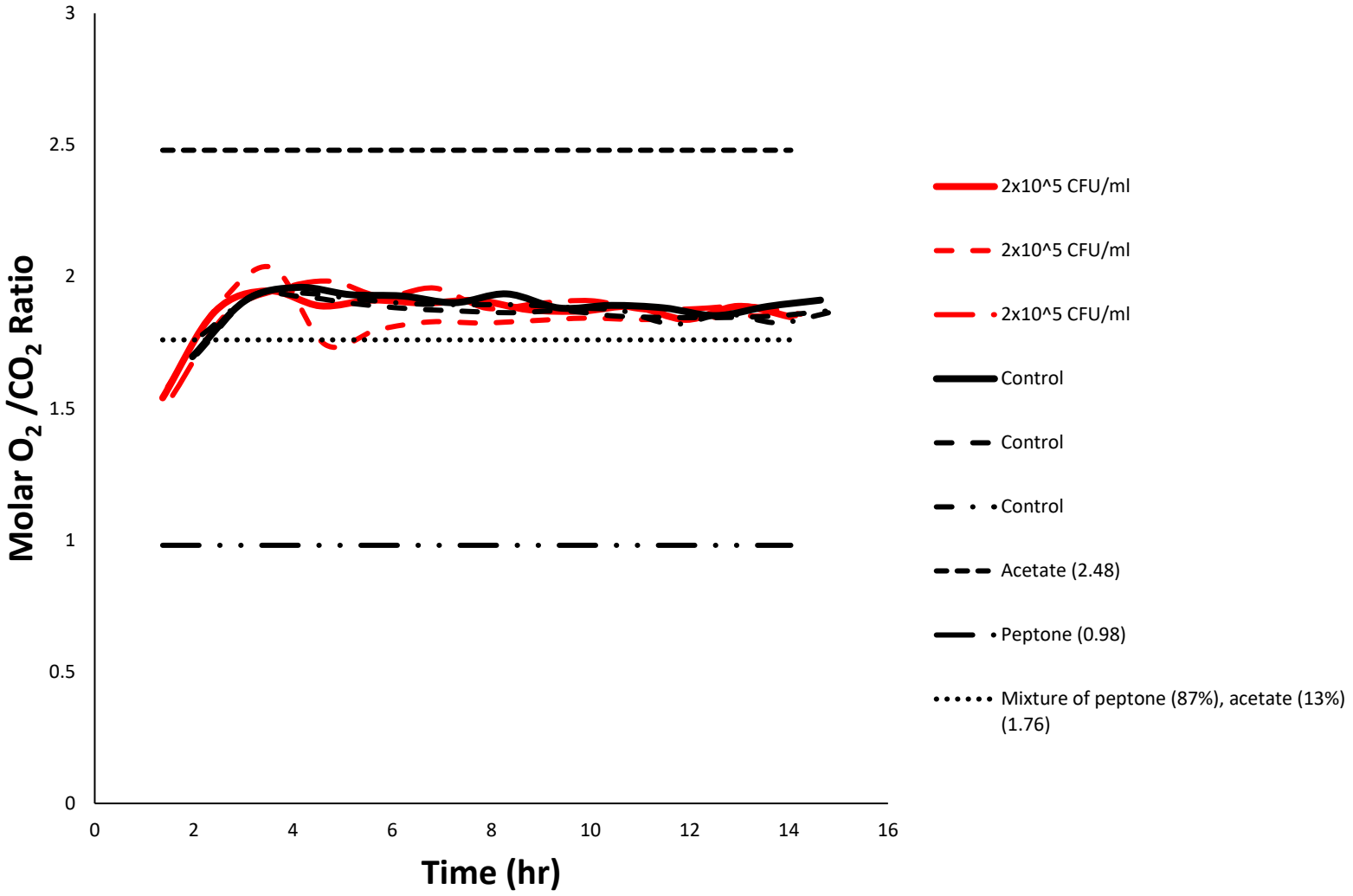

Figure 22: Molar O_2 to CO_2 Ratio, 2x10^5 CFU *B. globigii*/ml activated sludge.

The effect of 2x10^7 CFU/ml B. globigii on microbial respiration

The respirogram of activated sludge in the presence of 2x10^7 CFU/ml *B. globigii* exhibited typical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution a sharp increase in respiration occurred, in response to the presence of the substrate as shown in Figure 23. Following the substrate uptake, the O_2 consumption curves peaked between 2-4 hours and then sharply declined to a gradually decreasing endogenous respiration rate, creating the familiar "peak and tail" respirogram profile. Channel 9 had the highest peak respiration rate and also the greatest endogenous respiration tail indicating the greatest overall microbial activity. The remaining channels had similar peaks but their tails were divided into two groups. Channels 4, 5, and 8 oscillated slightly under 4 µg/min whereas channels 6 and 7 oscillated around 2 µg/min.

Shape parameters

Only one of the shape parameters of the 2x10^7 CFU/ml *B. globigii* O_2 consumption profiles was statistically different from the control profile curves. The mean FrM of the experiment group was 70.3 µg O_2/min-hr^2 with a variance of 13.6 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 72.3 µg O_2/min-hr^2 with a variance of 67.5 (µg O_2/min-hr^2)^2. The p-value from comparing the FrM was 0.72, see Figure 24. The mean skewness of the experiment group was 0.46 with a variance of 0.02. The mean skewness of the control group was 0.91 with a variance of 0.013. The low variance in the skewness of the curves did result in a statistically significant difference when compared resulting in a p-value of 0.016, see Figure 25. These results show that there were statistically significant differences in the shape parameters between the test and control respirograms, indicating factors that influenced the transport of oxygen were likely present

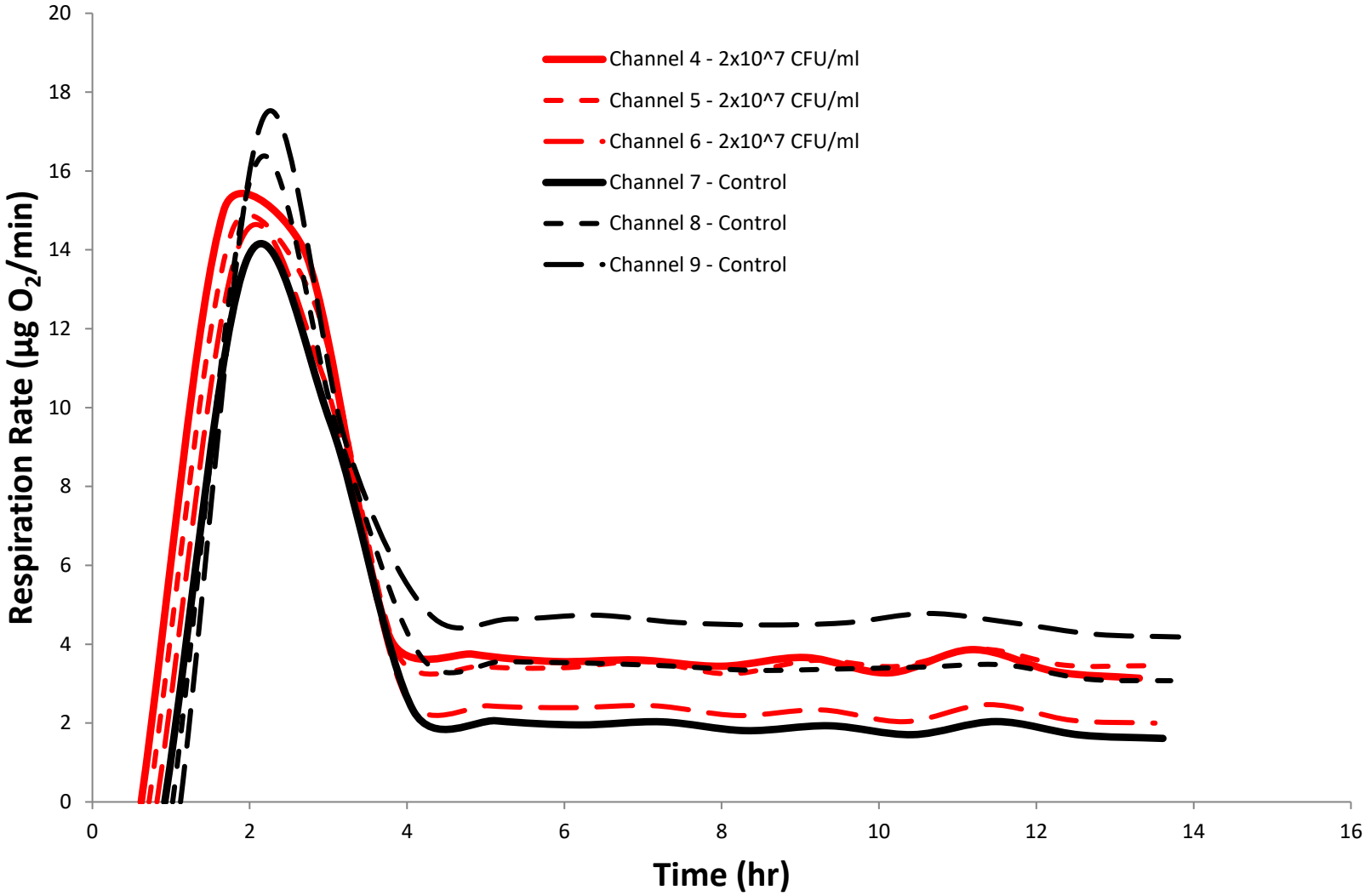

Figure 23: O_2 consumption profile of $2x10^7$ CFU/ml *B. globigii* spores in activated sludge.

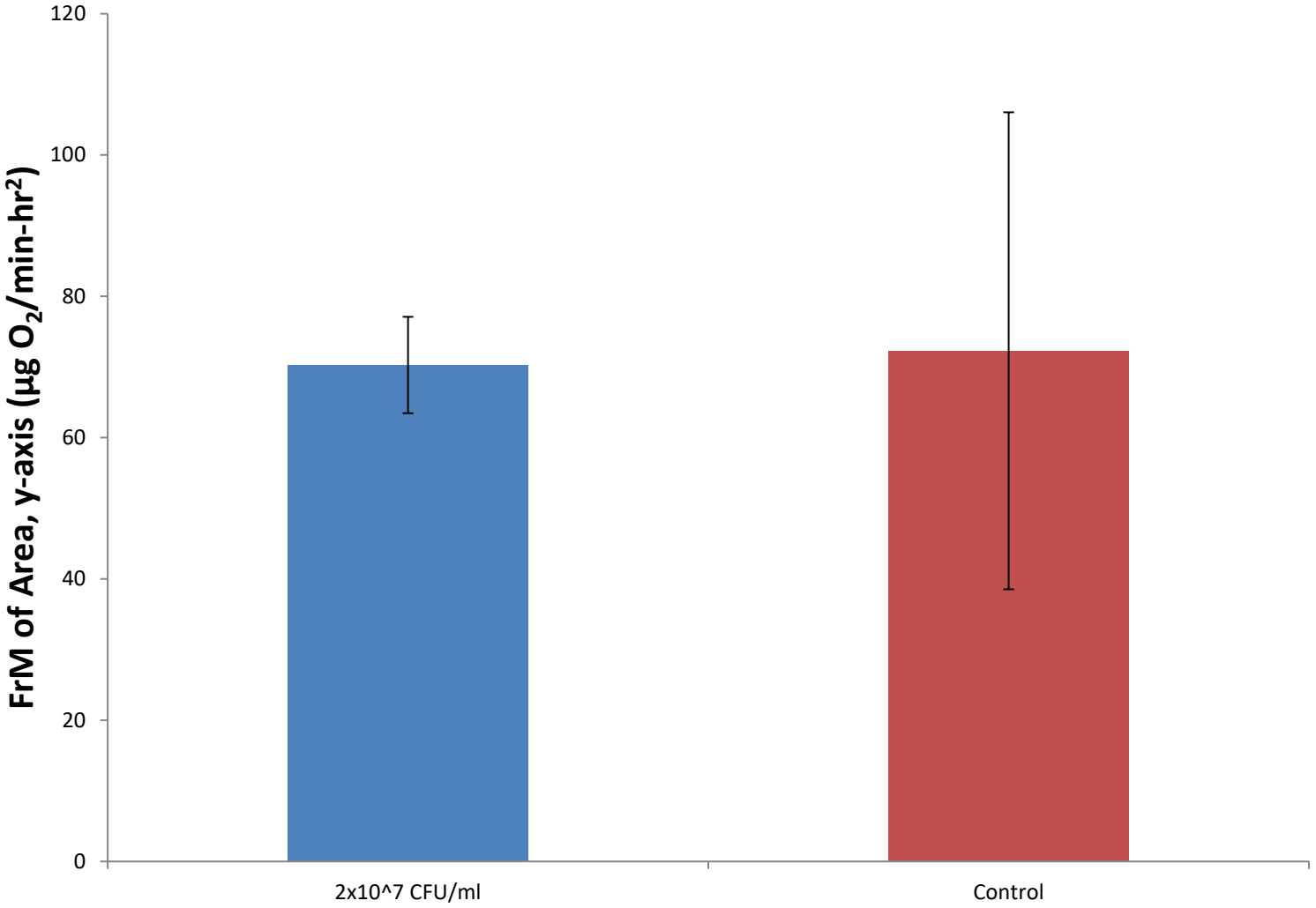

Figure 24: Comparison of $2x10^7$ CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

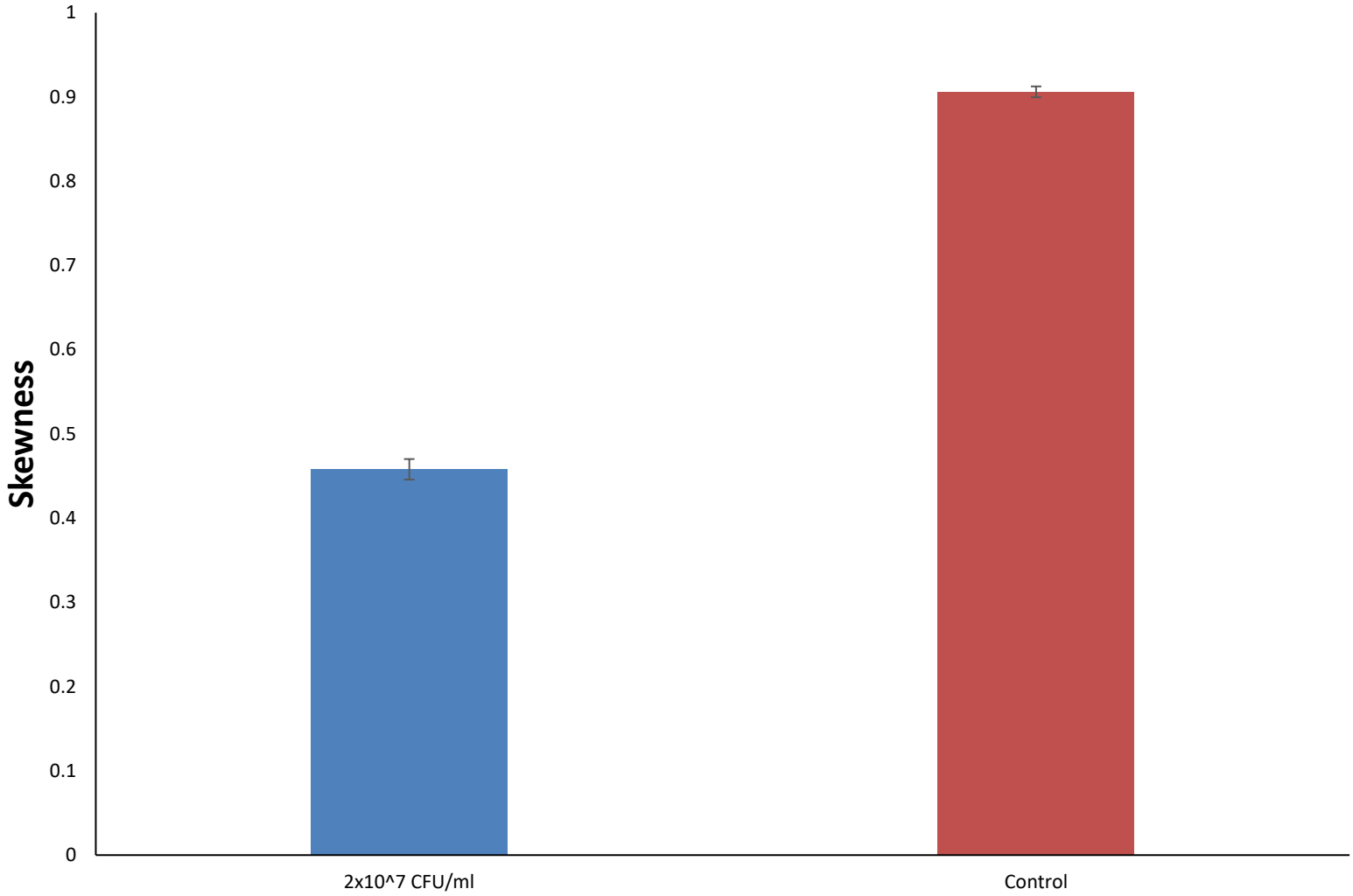

Figure 25: Comparison of $2x10^7$ CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 17.1 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 0.26 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 18.5 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 4.52 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of activated sludge in the presence of $2x10^7$ CFU/ml *B. globigii* were not statistically significantly different from the control. The p-value from comparing the maximum SOURs was 0.32, see Figure 26. The lack of a statistically significant difference between the experiment and control groups at $2x10^7$ CFU *B. globigii*/ml activated sludge indicate that the bacteria responded to the presence of the substrate the same with or without the spores.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2132 µg with a standard deviation of 215 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2030 µg with a standard deviation of 257 µg. The cumulative O_2 consumption activated sludge in the presence of $2x10^7$ CFU/ml *B. globigii* was not statistically significantly different from the control, see Figure 27. The p-value from a Student's t-test comparison was 0.63. The lack of a statistically significant difference in the cumulative O_2 consumed at $2x10^7$ CFU *B. globigii*/ml indicates that the bacteria was equally effective at respiration with or without the presence of the spores.

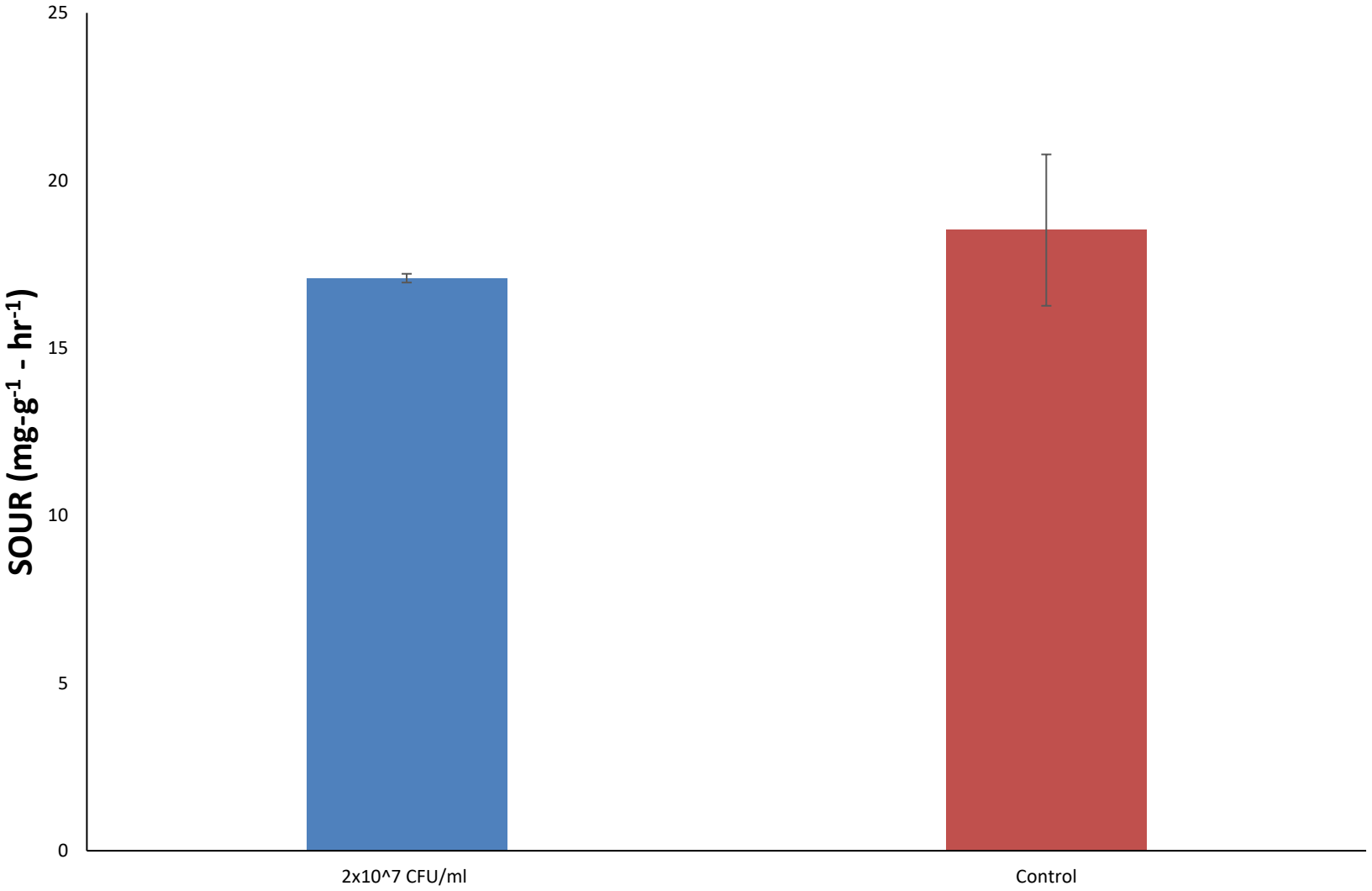

Figure 26: Peak SOUR of 2x10^7 CFU *B. globigii*/ml activated sludge. Error bars represent sample variance.

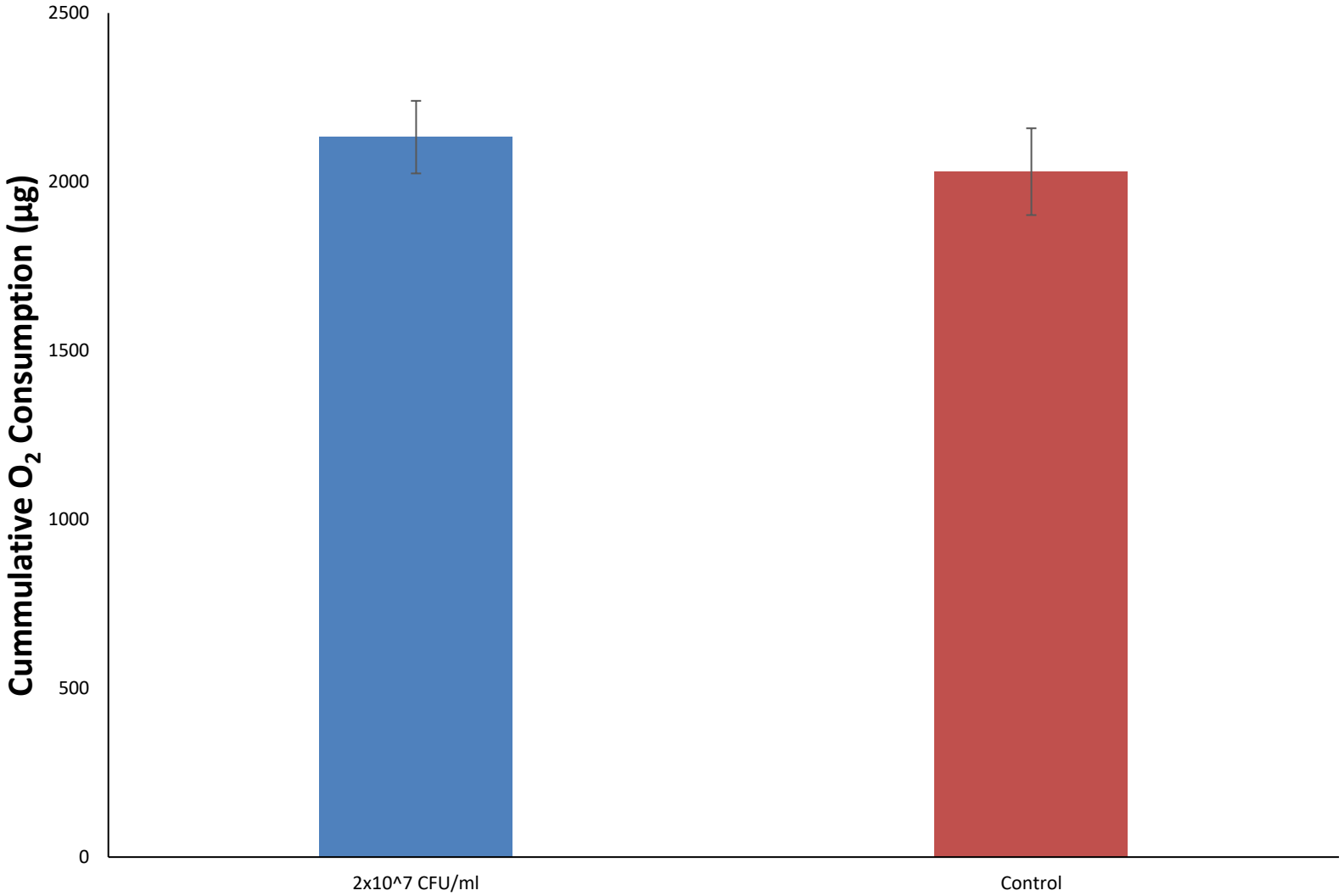

Figure 27: Cumulative O_2, 2x10^7 CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

Molar O_2/CO_2 ratios

The molar ratio of O_2 consumed-to-CO_2 produced of activated sludge in the presence of $2x10^7$ CFU *B. globigii*/ml did not appear to be significantly different from the control. Both the control and the experiment groups exhibited a rise in the molar O_2/CO_2 ratio for approximately the first three hours of the test. Unlike other runs which had minor undulations, this test exhibited a number of spikes in the molar O_2/CO_2 ratio. Channel 7 of the control group had the most extreme spike, but the three experiment channels followed suite, only to a lesser degree. This is most likely due to bleed over channels 1-3 (used for another experiment, discussed elsewhere) when the respirometer refreshed the headspace on the sample container. Values ranged from 1.76 to 2.25, see Figure 28. Because the molar O_2/CO_2 ratio of both the control and the experiment followed the same patterns even with the unexpected peaks, this indicates that the underlying metabolic pathway of the activated sludge was not affected by the *B. globigii* spores.

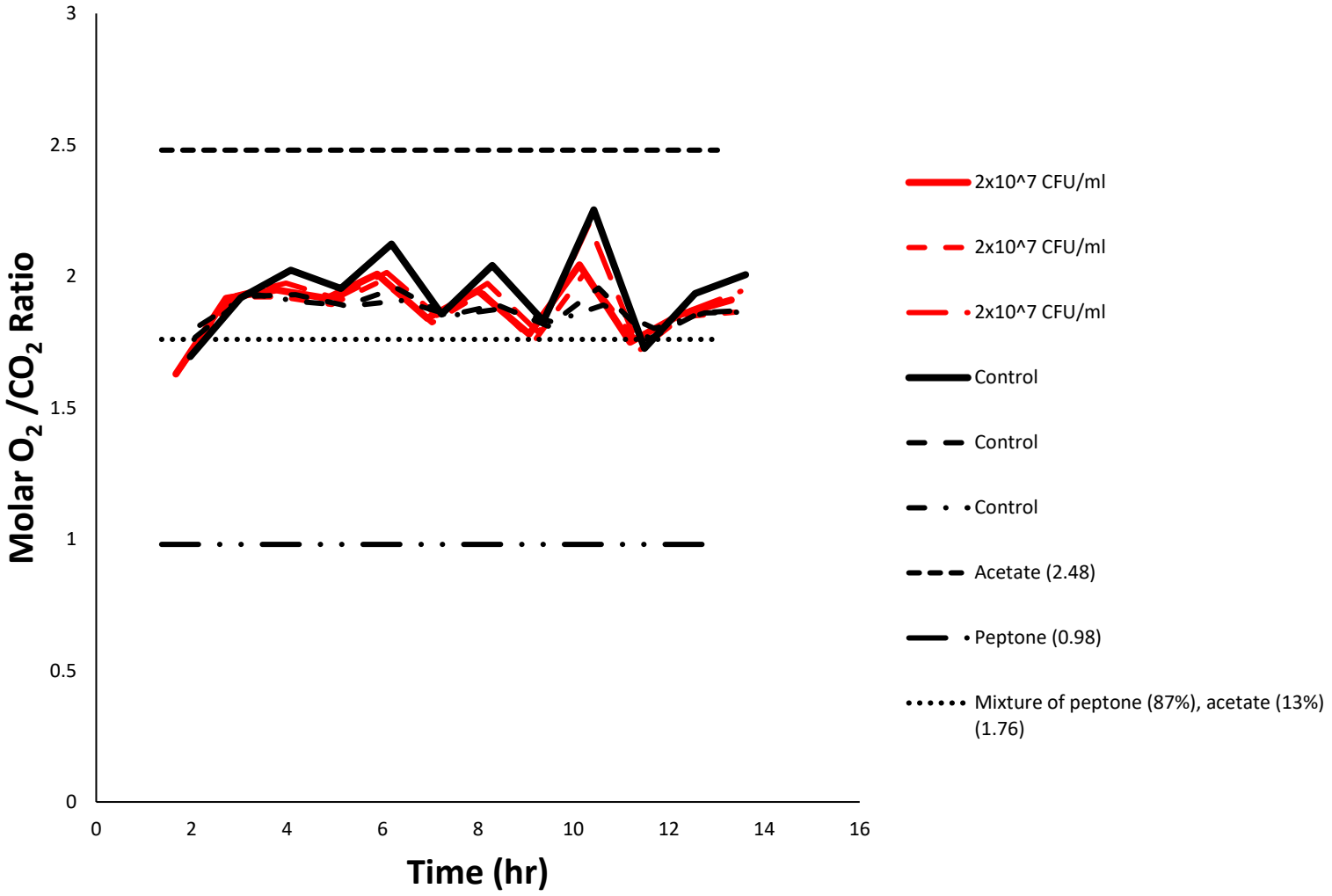

Figure 28: Molar O_2 to CO_2 Ratio, 2x10^7 CFU *B. globigii*/ml activated sludge.

The effect of unwashed 2x10^1 CFU/ml B. globigii on microbial respiration

The three trials of unwashed 2x10^1 CFU/ml *B. globigii* in activated sludge all resulted in similar outcomes. Only the 7 Sep 2016 trial will be discussed in detail. See Appendix C for respirograms of remaining trials (Figure 75 to Figure 80). The respirogram of unwashed 2x10^1 CFU/ml *B. globigii* in activated sludge exhibited typical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution a sharp increase in respiration occurred in response to the presence of the substrate as shown in Figure 29. Following the substrate uptake, the O_2 consumption curves peaked between 2-4 hours and then sharply declined to a gradually decreasing endogenous respiration rate, creating the familiar "peak and tail" respirogram profile. In this trial, channel 8, a member of the control group, stood out as having the highest peak and also the highest tail. Channel 3 of the experiment group had a similar peak to the other channels, but stood out by having a higher endogenous respiration tail, albeit not as high as channel 8. Note that channels 4-6 contained ATU to establish the positive control and will not be discussed.

Shape parameters

Only one of the shape parameters of the activated sludge in the presence of unwashed 2x10^1 CFU/ml *B. globigii* was statistically different from the control profile curves. The mean FrM of the experiment group was 71.6 µg O_2/min-hr^2 with a variance of 52.0 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 73.0 µg O_2/min-hr^2 with a variance of 313.3 (µg O_2/min-hr^2)^2. The p-value from comparing the FrM was 0.91, see Figure 30. The mean skewness of the experiment group was -0.043 with a variance of 0.0072. The mean skewness of the control group was 1.11 with a variance of

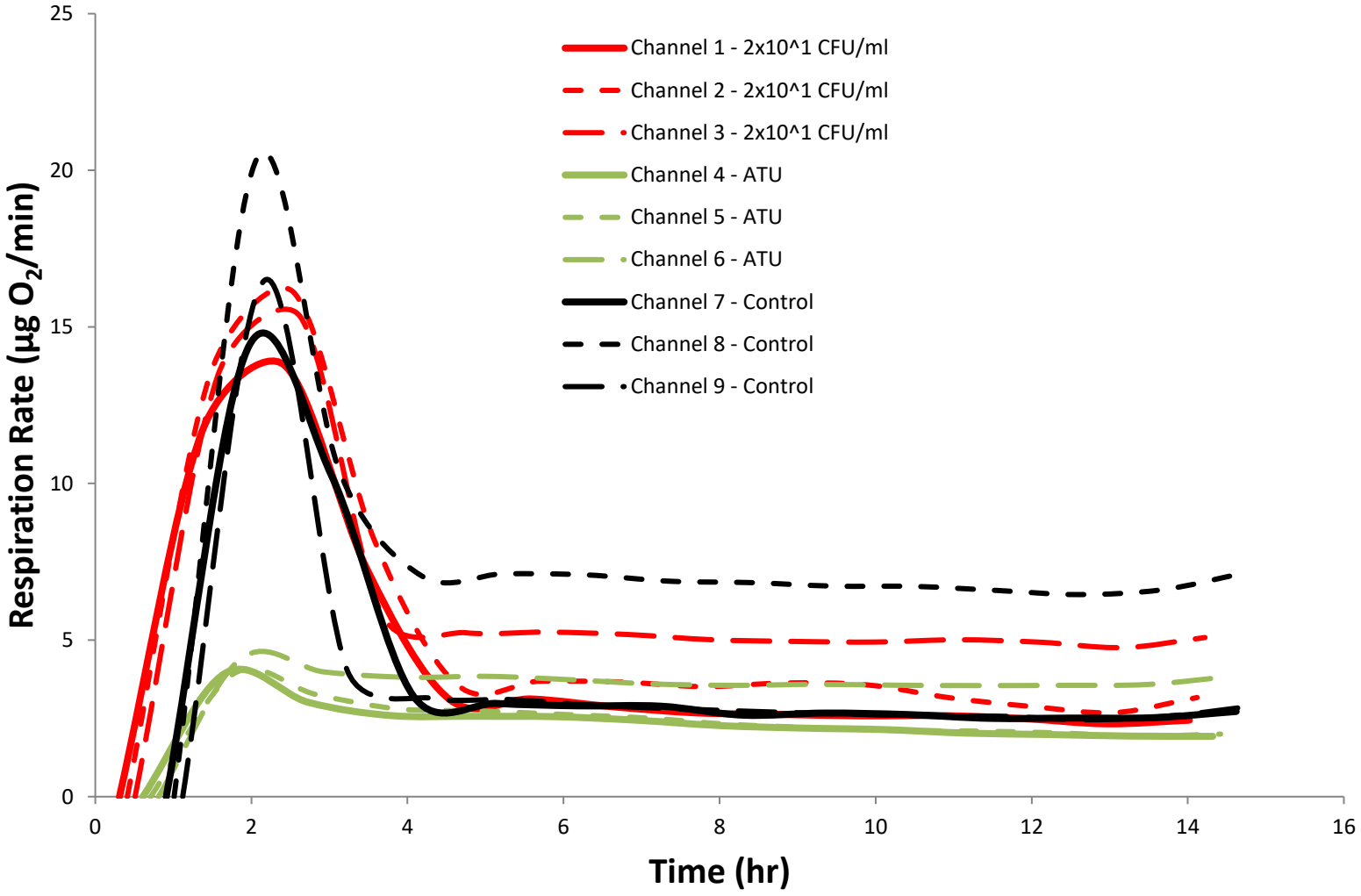

Figure 29: O_2 consumption profile of unwashed $2x10^1$ CFU/ml *B. globigii* spores in activated sludge.

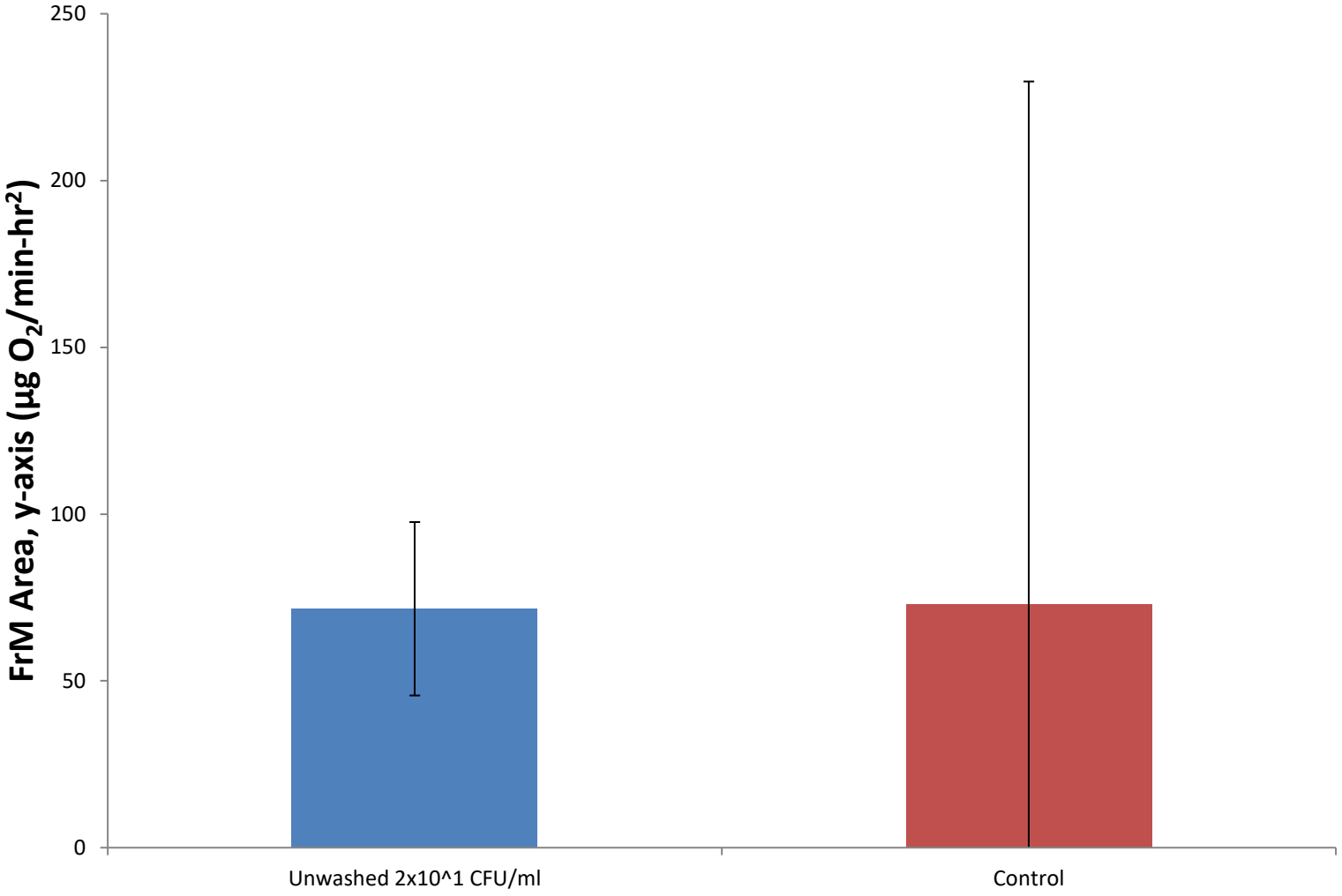

Figure 30: Comparison of unwashed 2x10[1] CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

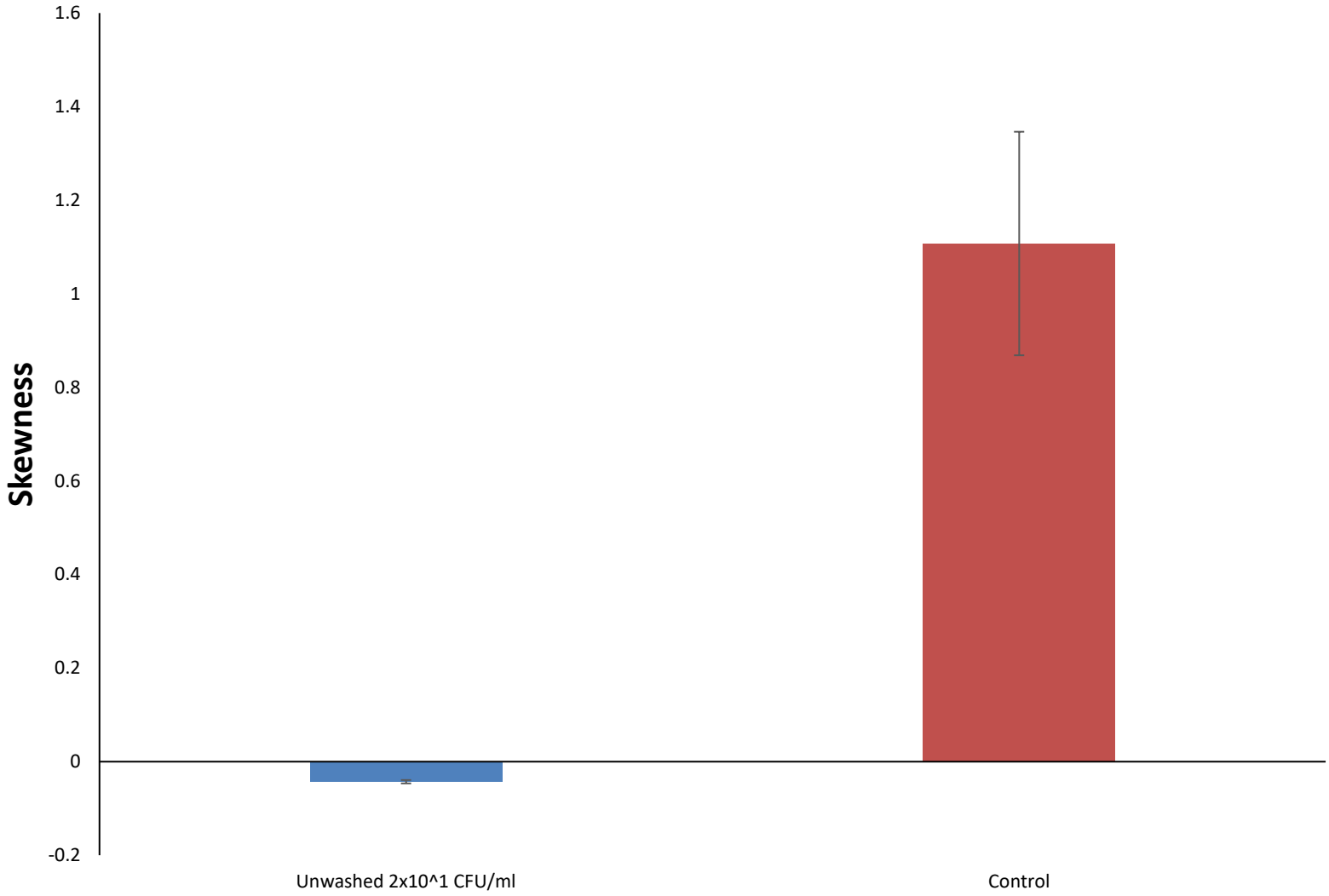

Figure 31: Comparison of unwashed 2x10[1] CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

0.48. The low variance in the skewness of the curves did result in a statistically significant difference when compared resulting in a p-value of 0.046, see Figure 31. These results show that there were statistically significant differences in the shape parameters between the test and control respirograms, indicating factors that influenced the transport of oxygen were likely present.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 14.8 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 1.34 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 16.8 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 8.85 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of activated sludge in the presence of unwashed $2x10^1$ CFU/ml *B. globigii* were not statistically significantly different from the control. The p-value from comparing the maximum SOURs was 0.34, see Figure 32. The lack of a statistically significant difference between the experiment and control groups indicate that the bacteria responded to the presence of the substrate the same with or without the spores.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2472 µg with a standard deviation of 183 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2164 µg with a standard deviation of 605 µg. The cumulative O_2 consumption of activated sludge in the presence of unwashed $2x10^1$ CFU/ml *B. globigii* was not statistically significantly different from the control, see Figure 33. The p-value from a student t-test comparison was 0.45. The lack of a statistically significant difference in the cumulative O_2 consumed indicates that

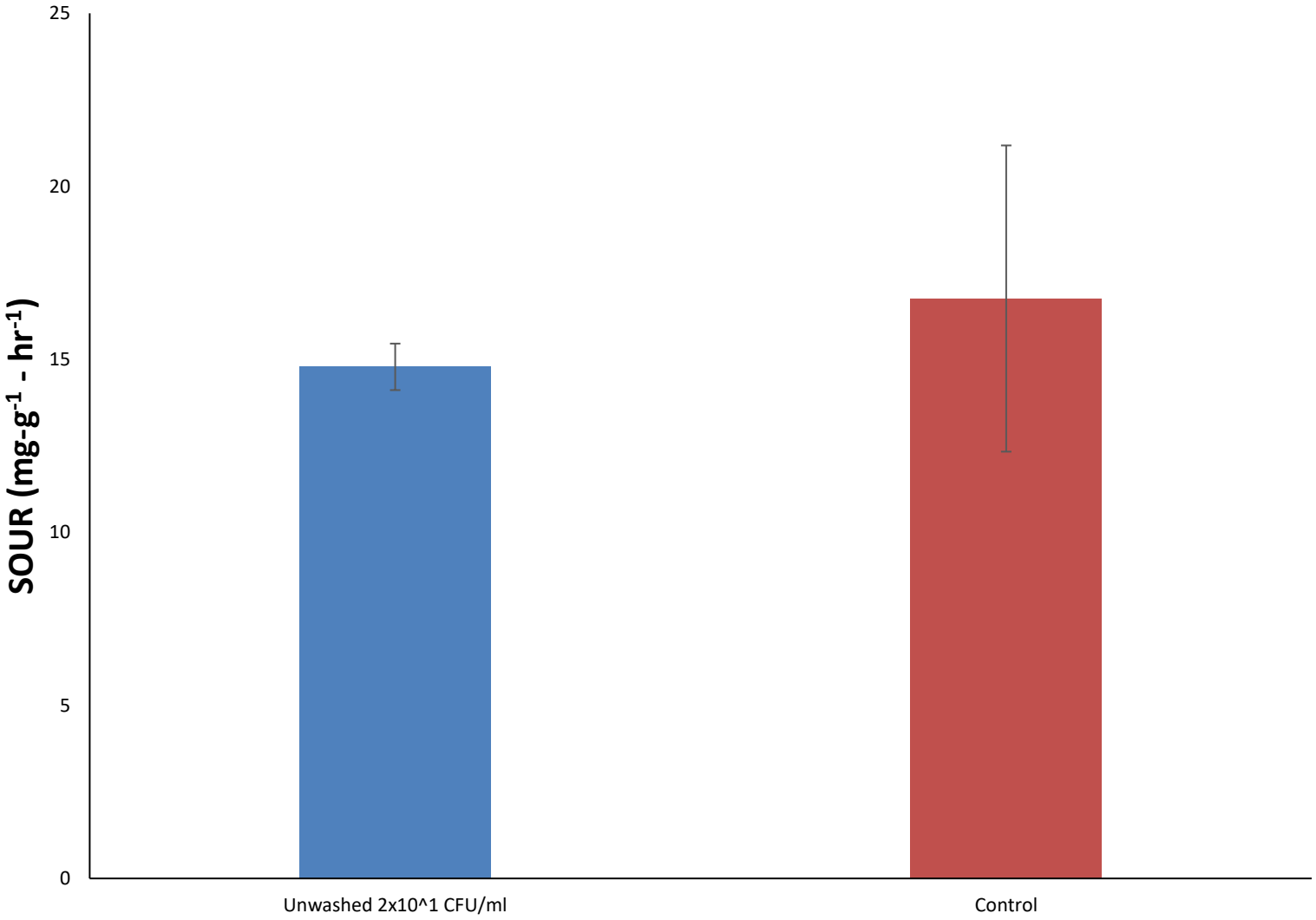

Figure 32: Peak SOUR of unwashed 2x10[1] CFU *B. globigii*/ml activated sludge. Error bars represent sample variance.

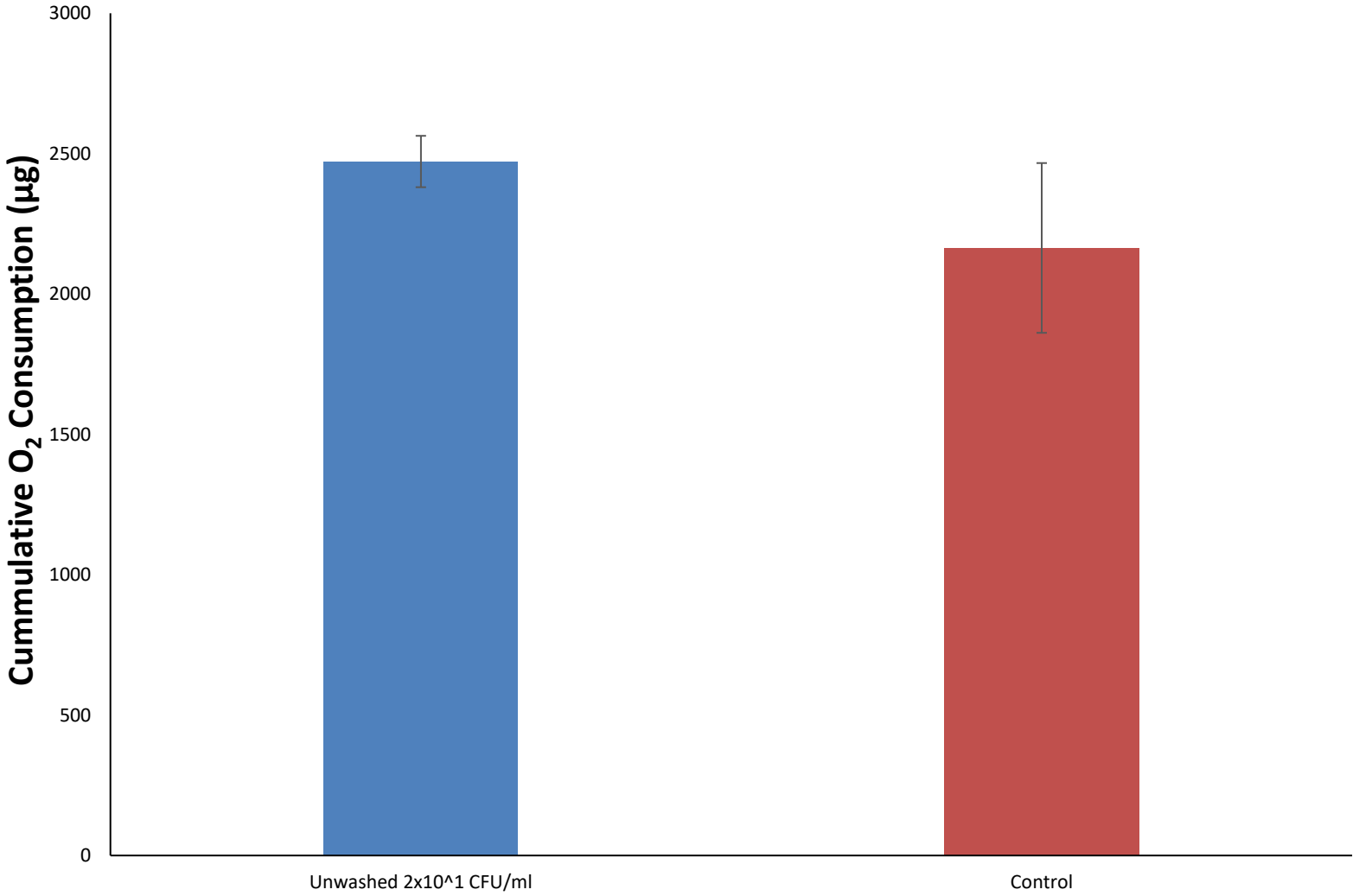

Figure 33: Cumulative O_2, unwashed 2x10^1 CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

the bacteria was equally effective at respiration with or without the presence of the spores.

Molar O_2/CO_2 ratios

The molar ratios of O_2 consumed-to-CO_2 produced by activated sludge in the presence of unwashed $2x10^1$ CFU *B. globigii*/ml were similar to the control. Both the control and the experiment groups exhibited a rise in the molar O_2/CO_2 ratio for approximately the first three hours of the test but then minimal undulation for the remaining duration. The exception is channel 2 which has a rise and dip from the 4-5 hour point which corresponds to a slight inflection point on the O_2 consumption profile for that channel. This is most likely due to the refreshing the headspace on the sample container. Typical values ranged from 1.75 to 1.98, Figure 34. The unaffected molar O_2/CO_2 ratio indicates that the underlying metabolic pathway of the activated sludge was not affected by the *B. globigii* spores.

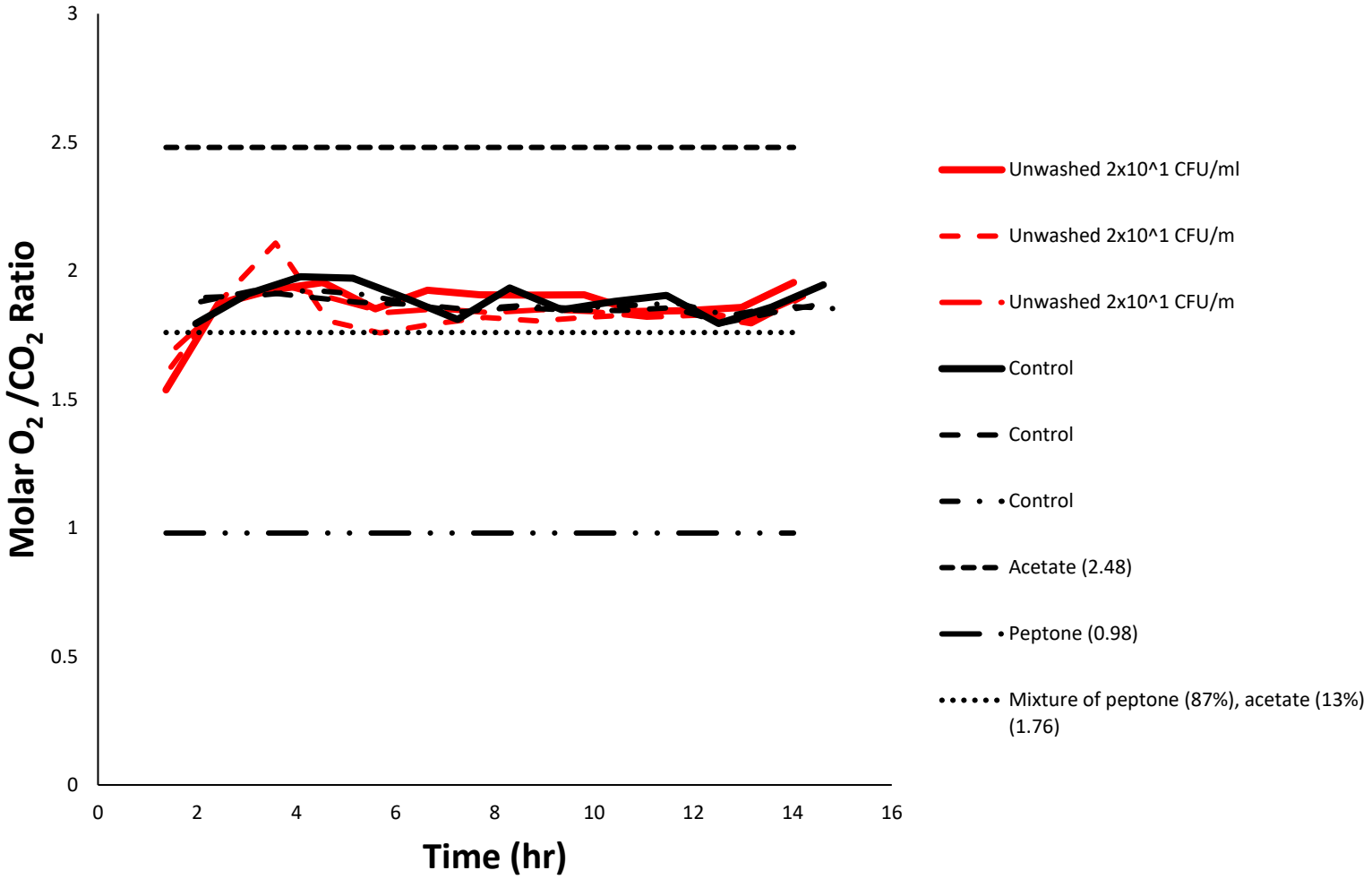

Figure 34: Molar O_2 to CO_2 Ratio, unwashed 2x10^1 CFU *B. globigii*/ml activated sludge.

The effect of unwashed 2x10^3 CFU/ml B. globigii on microbial respiration

The two trials of unwashed 2x10^3 CFU/ml *B. globigii* in activated sludge produced similar outcomes. Only the 31 Aug 2016 trial will be discussed in detail. See Appendix C for results of remaining trial (Figure 81 to Figure 86). The respirogram of activated sludge in the presence of unwashed 2x10^3 CFU/ml *B. globigii* exhibited typical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution a sharp increase in respiration occurred in response to the presence of the substrate as shown in Figure 35. Following the substrate uptake, the O_2 consumption curves peaked between 2-4 hours and then sharply declined to a gradually decreasing endogenous respiration rate, creating the familiar "peak and tail" respirogram profile. In this trial, channel 3 of the experiment group and channel 9 of the control group had similar profiles in that they both were lower and broader then the rest of their respective groups. Note that channels 4-6 contained ATU to establish the positive control and will not be discussed.

Shape parameters

The shape parameters observed in the presence of unwashed 2x10^3 CFU/ml *B. globigii* were not statistically different from the control profile curves. The mean FrM of the experiment group was 72.9 µg O_2/min-hr^2 with a variance of 109.4 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 73.8 µg O_2/min-hr^2 with a variance of 270.1 (µg O_2/min-hr^2)^2. The p-value from comparing the FrM was 0.946, see Figure 36. The mean skewness of the experiment group was 0.081 with a variance of 0.62. The mean skewness of the control group was 1.22 with a variance of 0.83. Comparing the skewness of the curves also did not result in a statistically significant difference with a p-value of

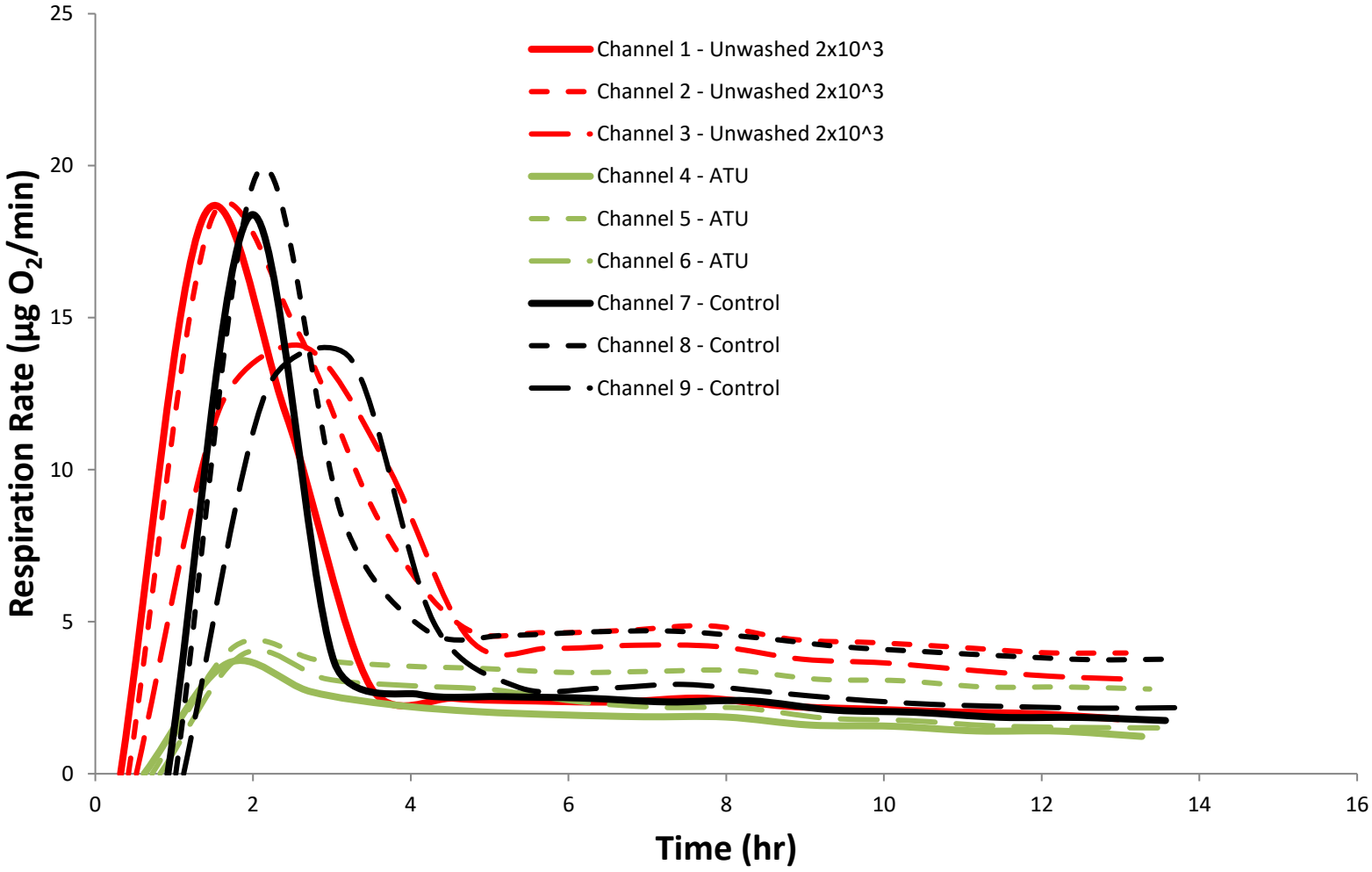

Figure 35: O_2 consumption profile of unwashed $2x10^3$ CFU/ml *B. globigii* spores in activated sludge.

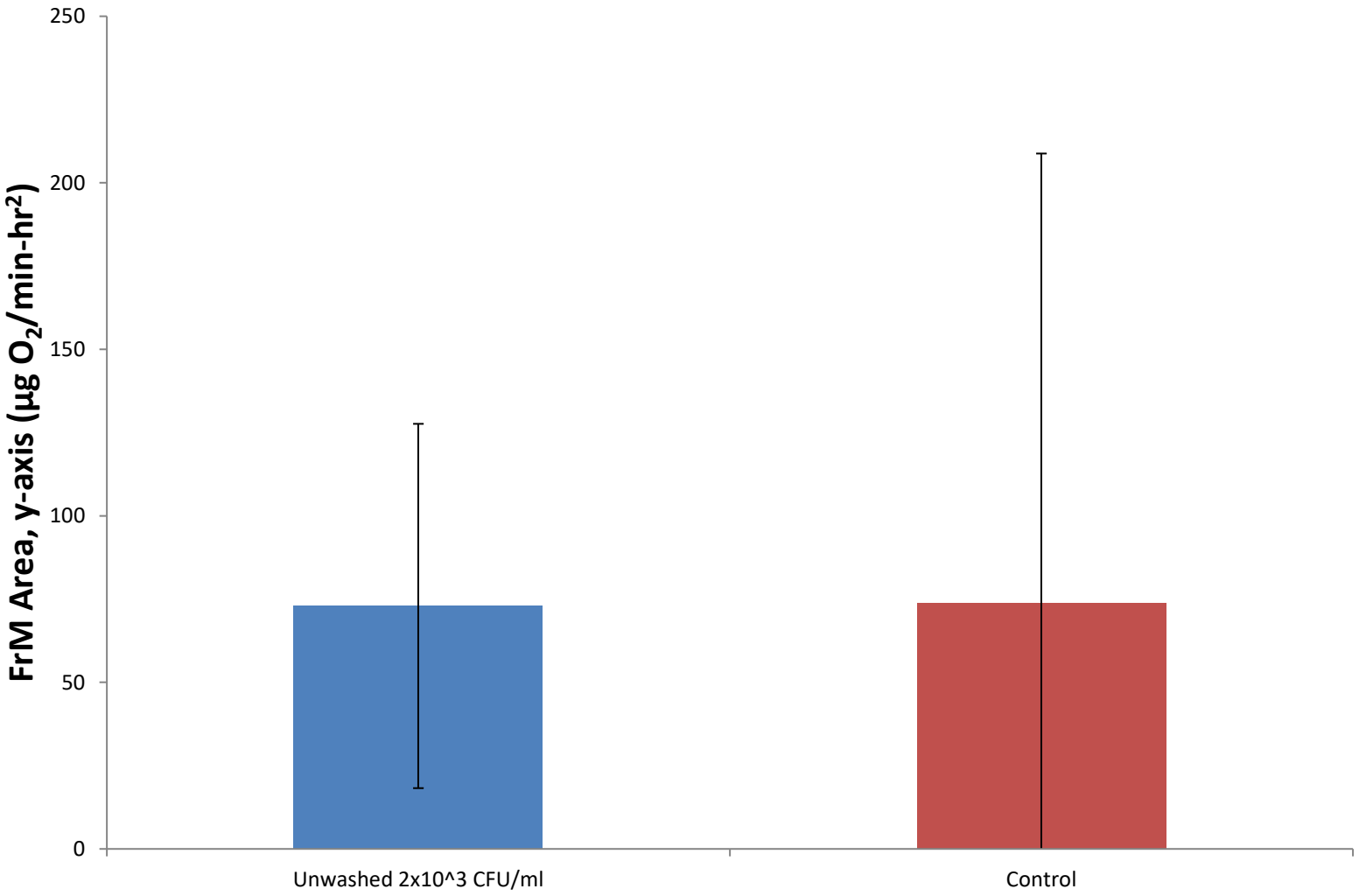

Figure 36: Comparison of unwashed 2x10^3 CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

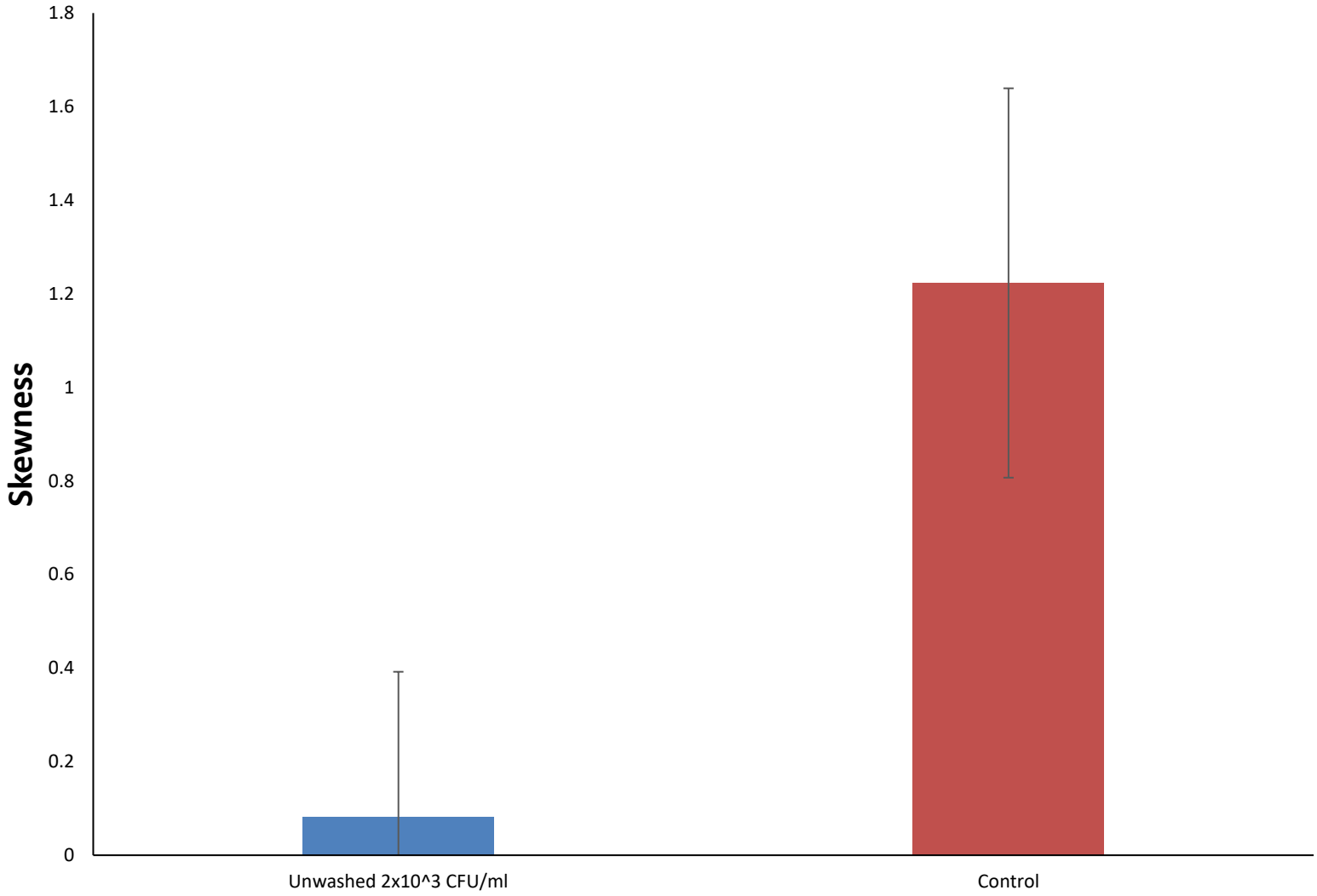

Figure 37: Comparison of unwashed 2x10^3 CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

0.176, see Figure 37. Neither of these shape parameters point to statistically significant differences between the experiment and the control groups.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 19.6 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 7.9 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 20.1 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 14.2 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of activated sludge in the presence of unwashed $2x10^3$ CFU/ml *B. globigii* were not statistically significantly different from the control. The p-value from comparing the maximum SOURs was 0.87, see Figure 38. The lack of a statistically significant difference between the experiment and control groups indicate that the bacteria responded to the presence of the substrate the same with or without the spores.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2584 µg with a standard deviation of 343 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2087 µg with a standard deviation of 333 µg. The cumulative O_2 consumption of activated sludge in the presence of unwashed $2x10^3$ CFU/ml *B. globigii* was not statistically significantly different from the control, see Figure 39. The p-value from a student t-test comparison was 0.15. The lack of a statistically significant difference in the cumulative O_2 consumed indicates that the bacteria was equally effective at respiration with or without the presence of the spores.

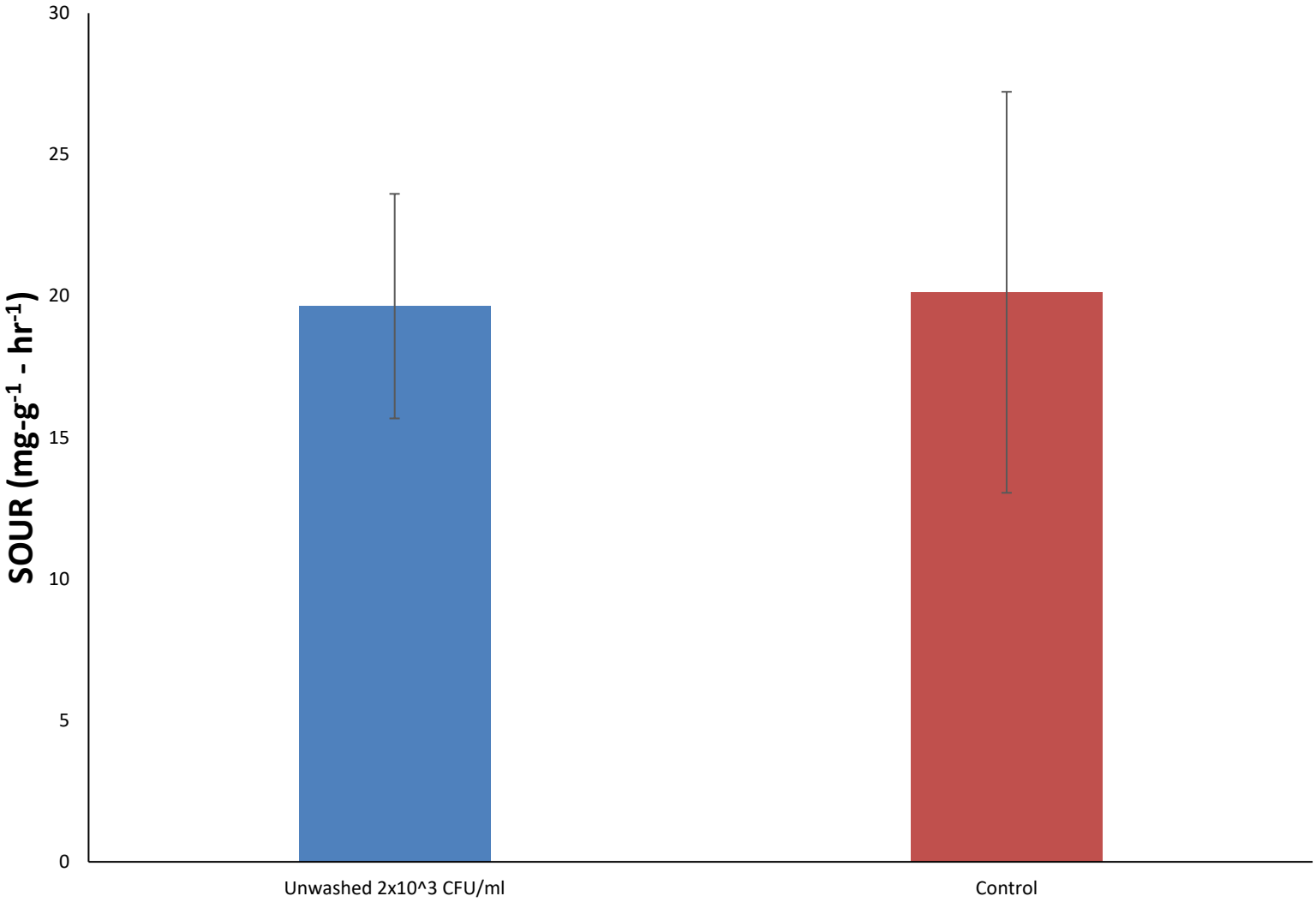

Figure 38: Peak SOUR of unwashed 2x10^3 CFU *B. globigii*/ml activated sludge. Error bars represent sample variance.

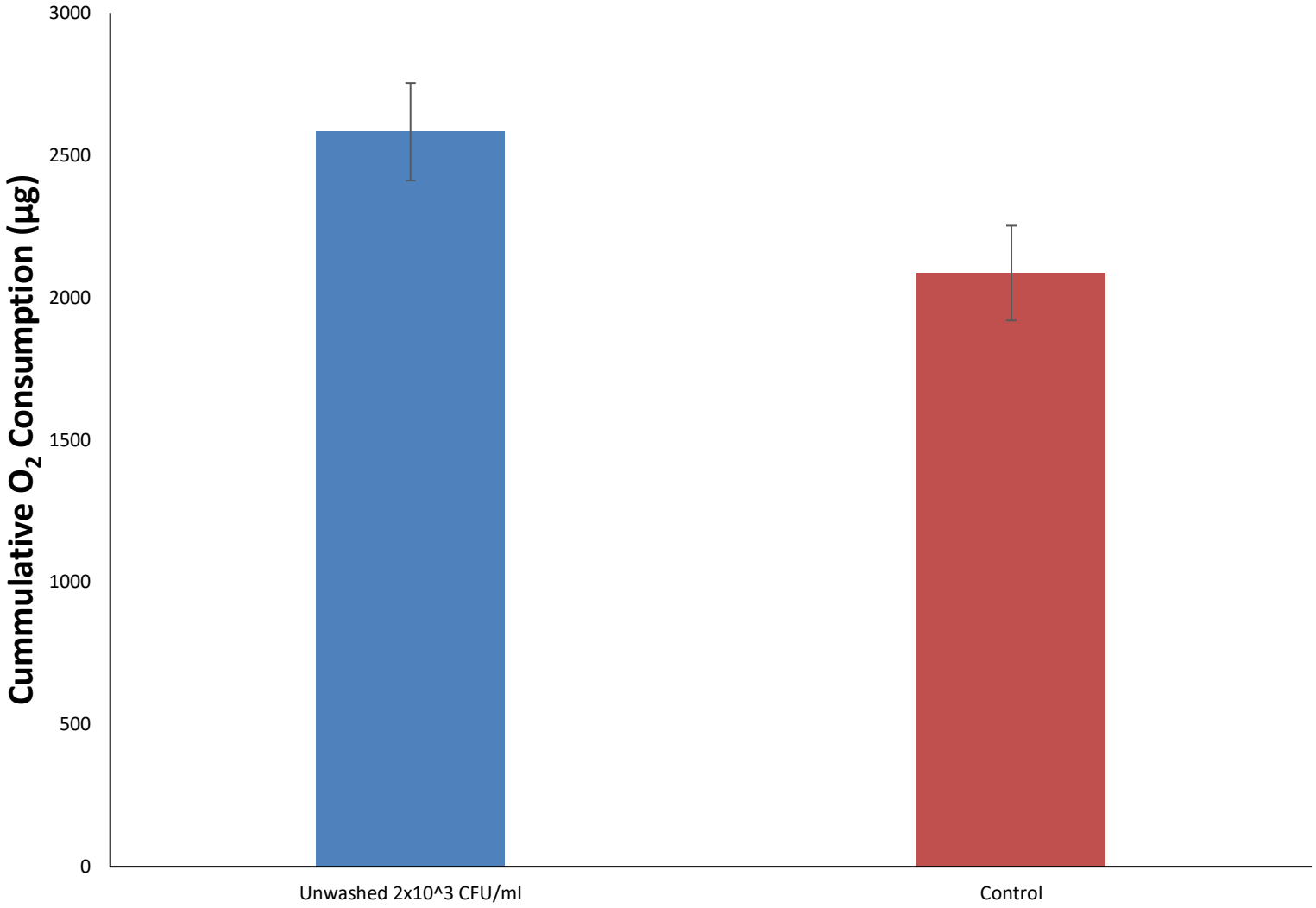

Figure 39: Cumulative O_2, unwashed $2x10^3$ CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

Molar O_2/CO_2 ratios

The molar ratio of O_2 consumed-to-CO_2 produced activated sludge in the presence of unwashed $2x10^3$ CFU *B. globigii*/ml were similar to that of the control. Both the control and the experiment groups exhibited a rise in the molar O_2/CO_2 ratio for approximately the first three hours of the test but then minimal undulation for the remaining duration. Typical values ranged from 1.75 to 2.04, Figure 40. The unaffected molar O_2/CO_2 ratio indicates that the underlying metabolic pathway of the activated sludge was not affected by the *B. globigii* spores.

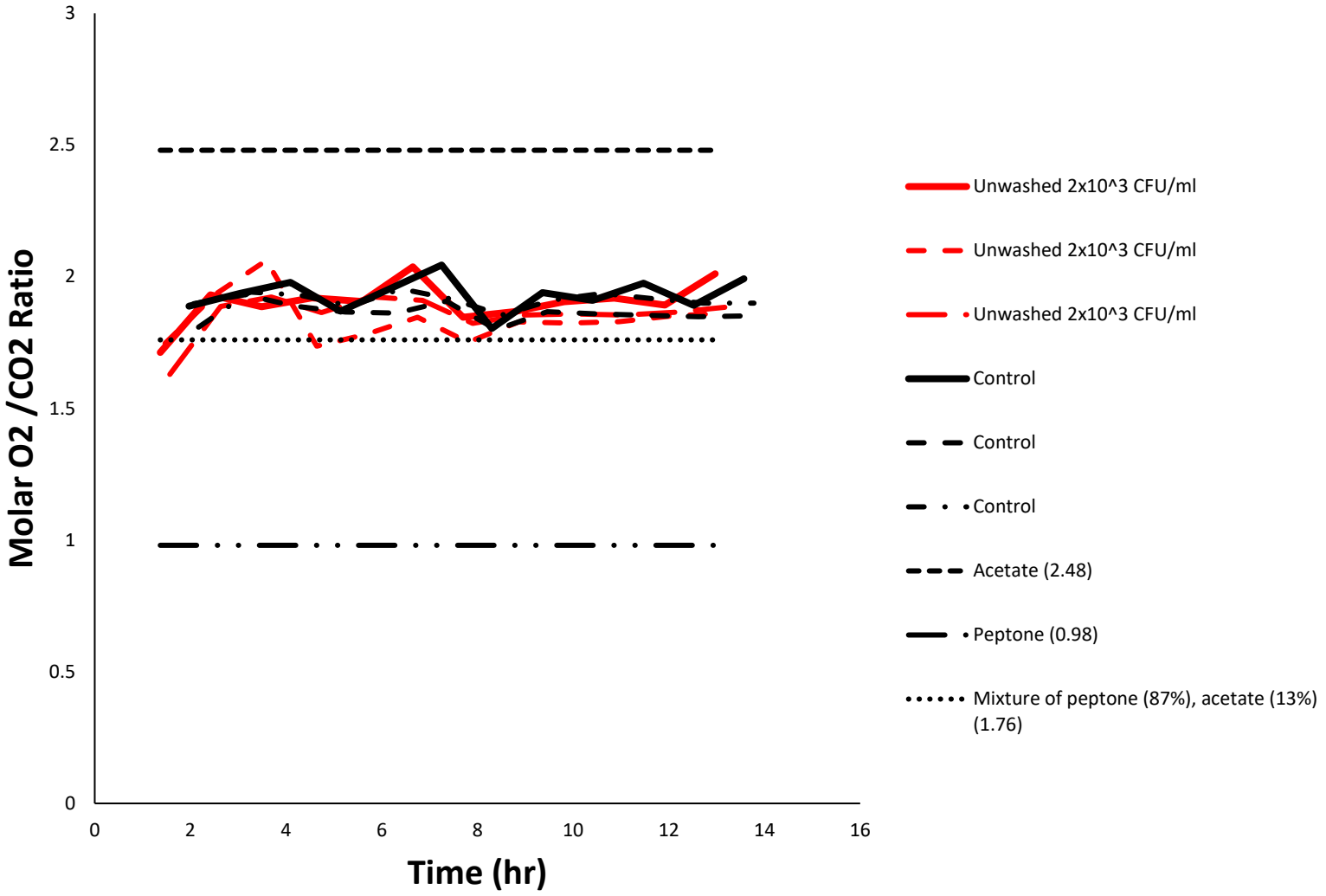

Figure 40: Molar O_2 to CO_2 Ratio, unwashed $2x10^3$ CFU *B. globigii*/ml activated sludge.

The effect of unwashed 2x10^5 CFU/ml B. globigii on microbial respiration

The three trials of unwashed 2x10^5 CFU/ml *B. globigii* in activated sludge all resulted in similar outcomes. Only the 24 Aug 2016 trial will be discussed in detail. See Appendix C for respirograms of remaining trials (Figure 87 to Figure 92). The respirogram of activated sludge in the presence of unwashed 2x10^5 CFU/ml *B. globigii* exhibited typical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution a sharp increase in respiration occurred in response to the presence of the substrate as shown in Figure 41. Following the substrate uptake, the O_2 consumption curves peaked between 1-4 hours and then sharply declined to a gradually decreasing endogenous respiration rate, creating the familiar "peak and tail" respirogram profile. In this trial channel 3 of the experiment group had a lower, wider curve than any other channel. The wideness can be attributed to the respirometer refreshing the sample headspace during the third sampling interval at 3.7 hrs. Note that channels 4-6 contained ATU to establish the positive control and will not be discussed.

Shape parameters

The shape parameters of the activated sludge in the presence of unwashed 2x10^5 CFU/ml *B. globigii* were not statistically different from the control profile curves. The mean FrM of the experiment group was 79.2 µg O_2/min-hr^2 with a variance of 239 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 84.0 µg O_2/min-hr^2 with a variance of 372 (µg O_2/min-hr^2)^2. The p-value from comparing the FrM was 0.76, see Figure 42. The mean skewness of the experiment group was -0.16 with a variance of 2.29. The mean skewness of the control group was 0.83 with a variance of 0.68.

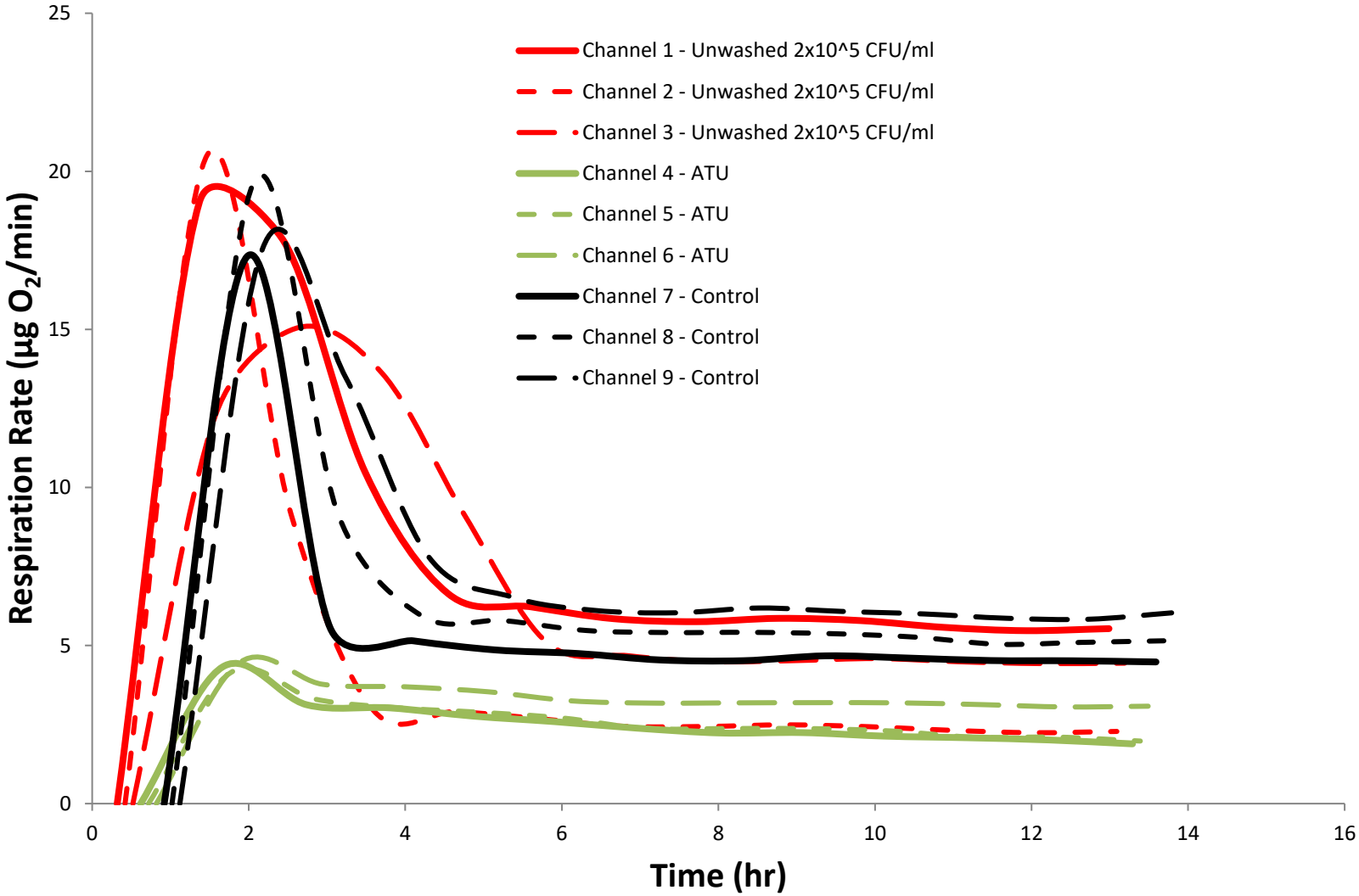

Figure 41: O_2 consumption profile of unwashed $2x10^5$ CFU/ml *B. globigii* spores in activated sludge.

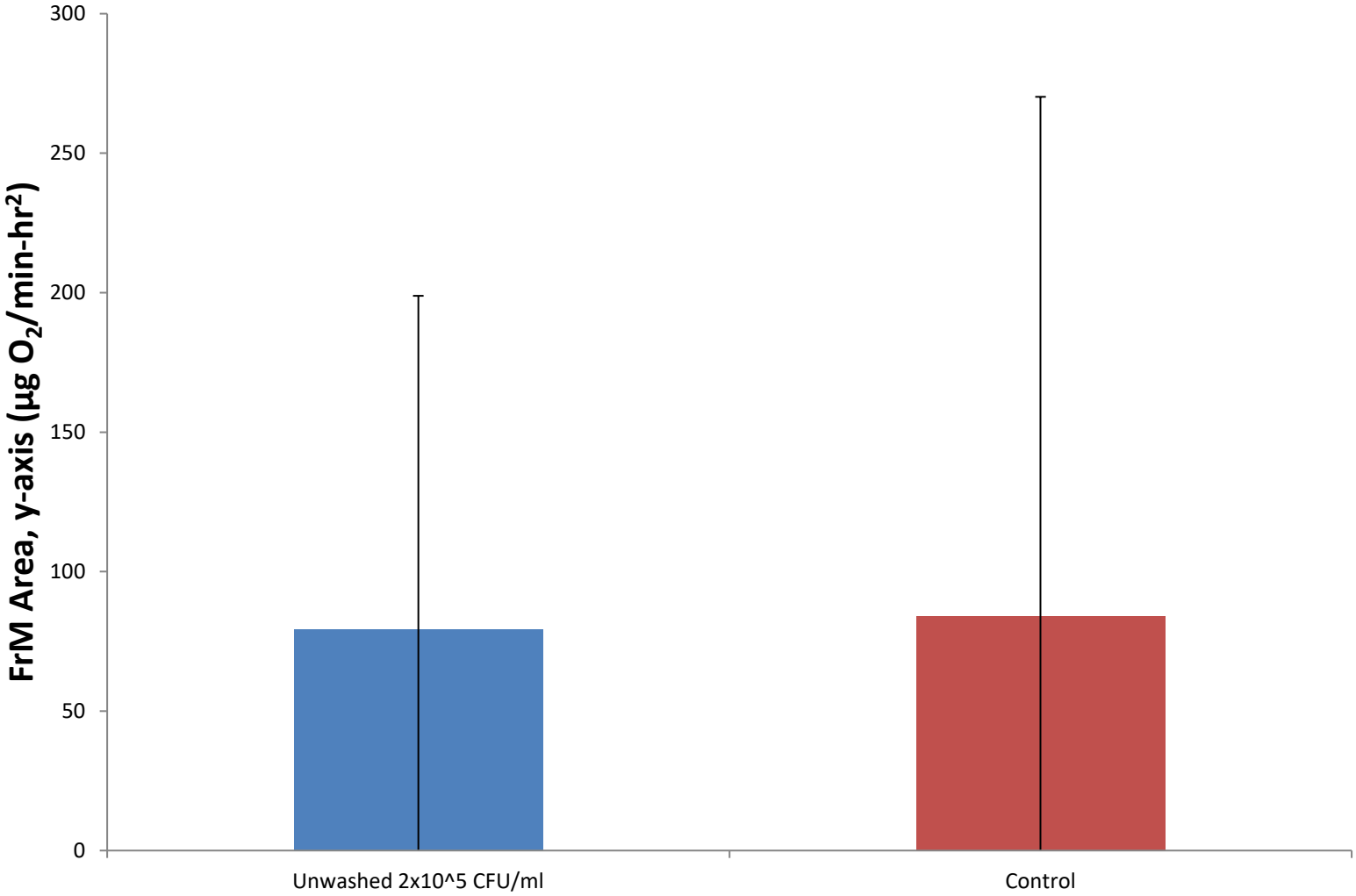

Figure 42: Comparison of unwashed 2x10^5 CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

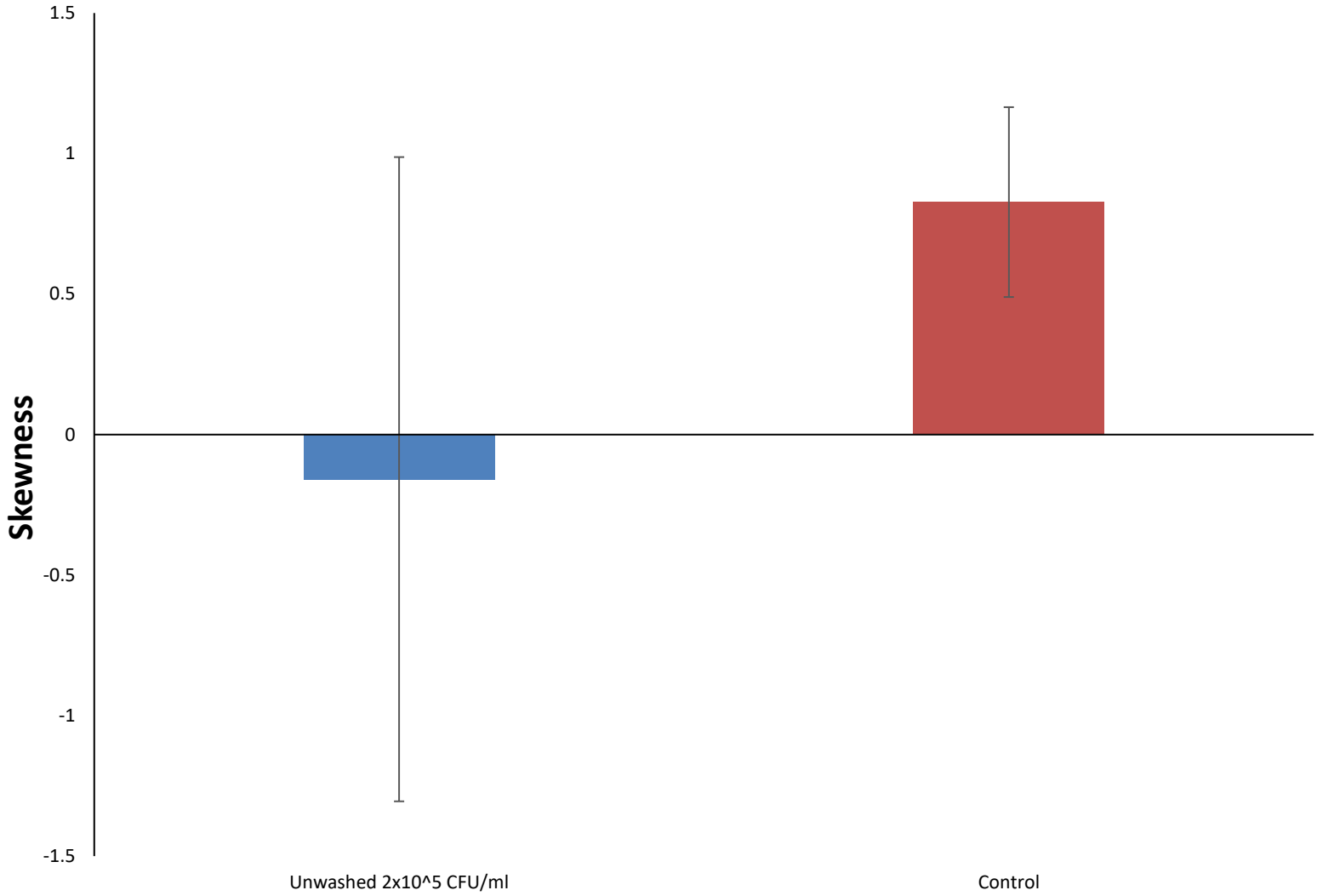

Figure 43: Comparison of unwashed 2x10^5 CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

Comparing the skewness of the curves also did not result in a statistically significant difference with a p-value of 0.38, see Figure 43. Neither of these shape parameters point to statistically significant differences between the experiment and the control groups.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 16.4 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 6.44 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 16.4 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 1.42 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of activated sludge in the presence of unwashed $2x10^5$ CFU/ml *B. globigii* were not statistically significantly different from the control. The p-value from comparing the maximum SOURs was 1.0, see Figure 44. The lack of a statistically significant difference between the experiment and control indicate that the bacteria responded to the presence of the substrate the same with or without the spores.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2954 µg with a standard deviation of 618 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2515 µg with a standard deviation of 403 µg. The cumulative O_2 consumption of activated sludge in the presence of unwashed $2x10^5$ CFU/ml *B. globigii* was not statistically significantly different from the control, see Figure 45. The p-value from a student t-test comparison was 0.36. The lack of a statistically significant difference in the cumulative O_2 consumed of unwashed $2x10^5$ CFU *B. globigii*/ml activated sludge indicates that the bacteria was equally effective at respiration with or without the presence of the spores.

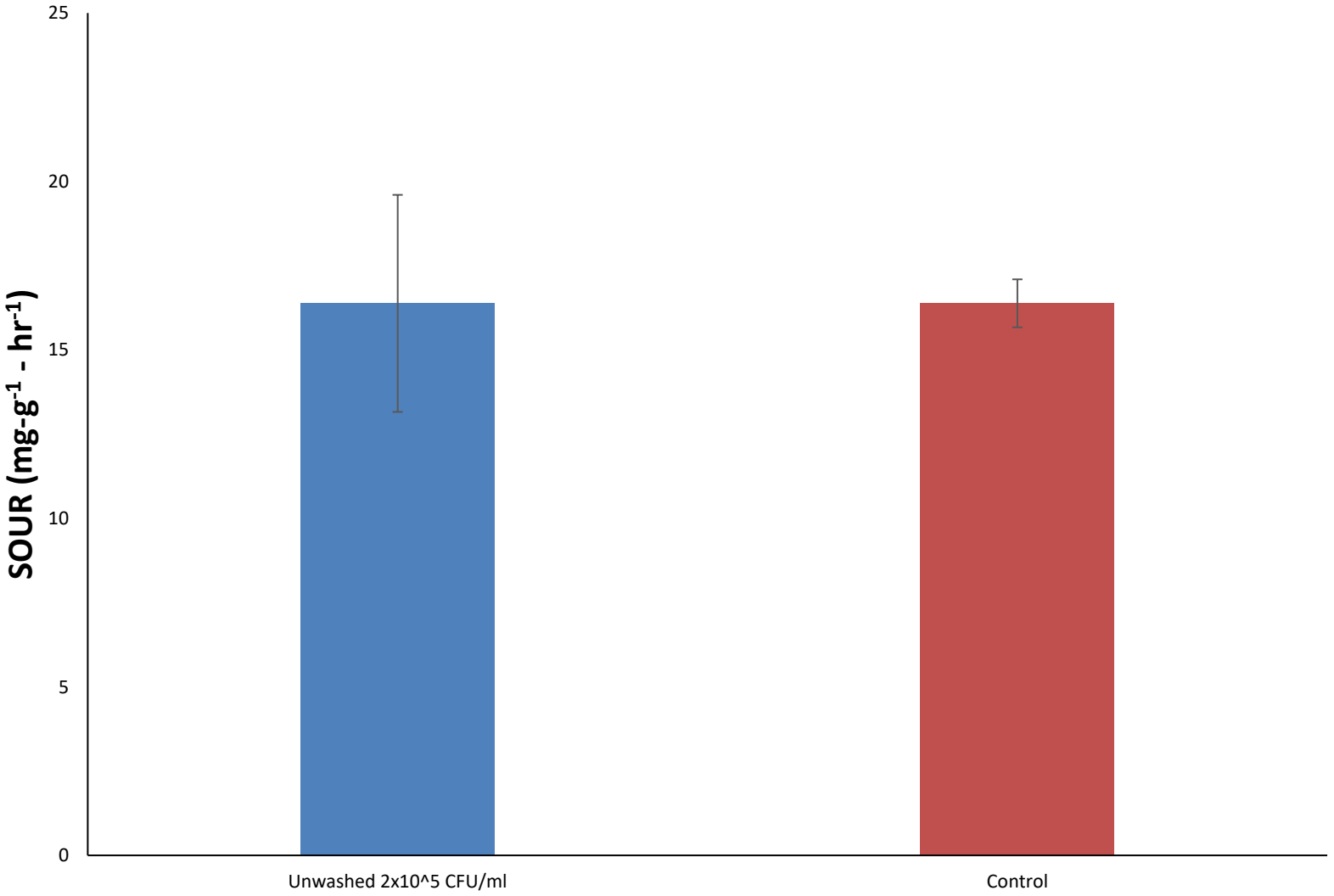

Figure 44: Peak SOUR of unwashed 2x10^5 CFU *B. globigii*/ml activated sludge. Error bars represent sample variance.

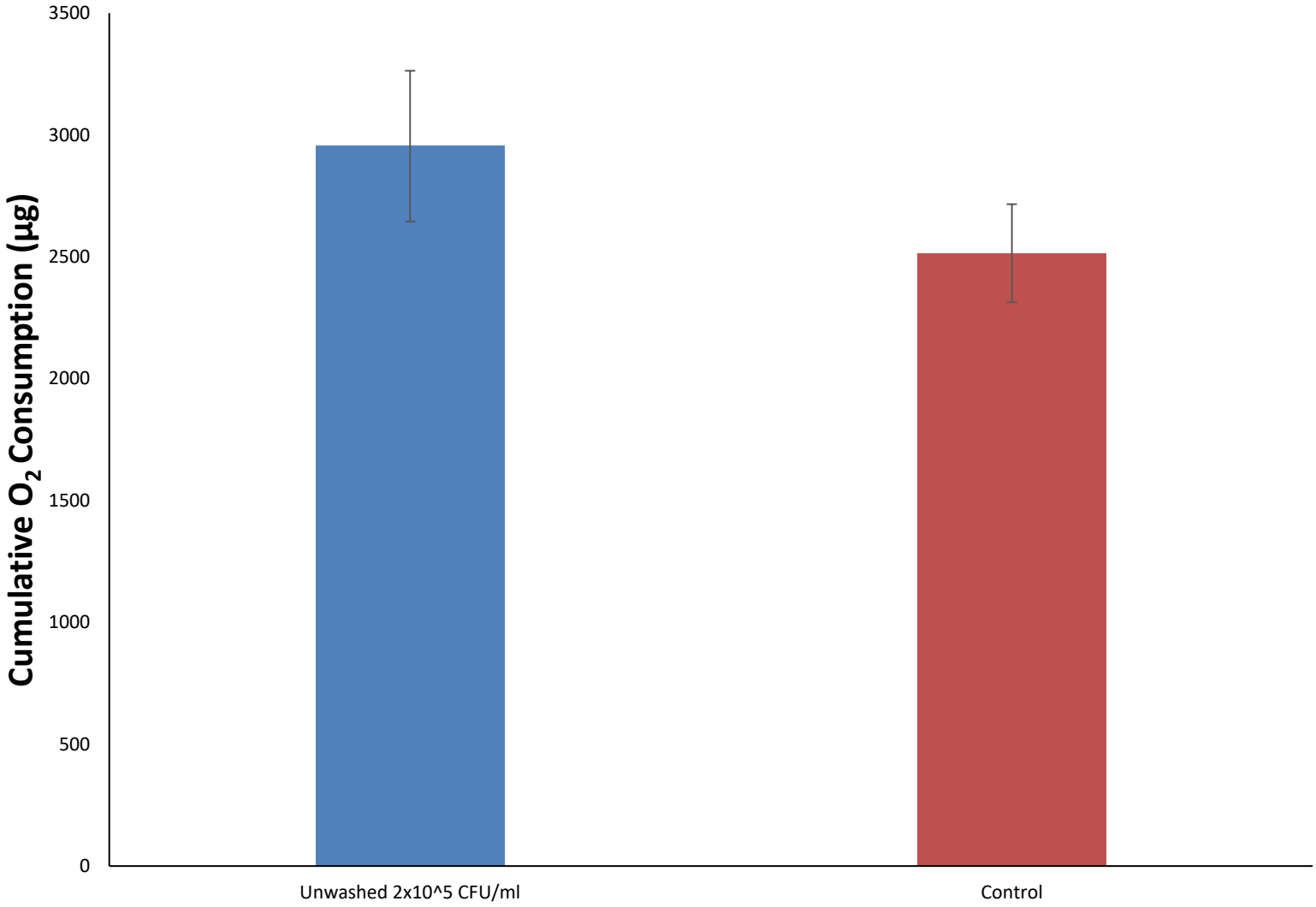

Figure 45: Cumulative O_2, unwashed 2x10^5 CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

Molar O_2/CO_2 ratios

The molar ratio of O_2 consumed-to-CO_2 produced activated sludge in the presence of unwashed $2x10^5$ CFU *B. globigii*/ml was similar to that of the control. Both the control and the experiment groups exhibited a rise in the molar O_2/CO_2 ratio for approximately the first three hours of the test but then minimal undulation for the remaining duration except for channels 1 and 3. Both of these channels had spikes at the second and third sampling intervals respectively corresponding to refreshes of the sample headspace. Typical values ranged from 1.76 to 1.86, Figure 46. The unaffected molar O_2/CO_2 ratio indicates that the underlying metabolic pathway of the activated sludge was not affected by the *B. globigii* spores.

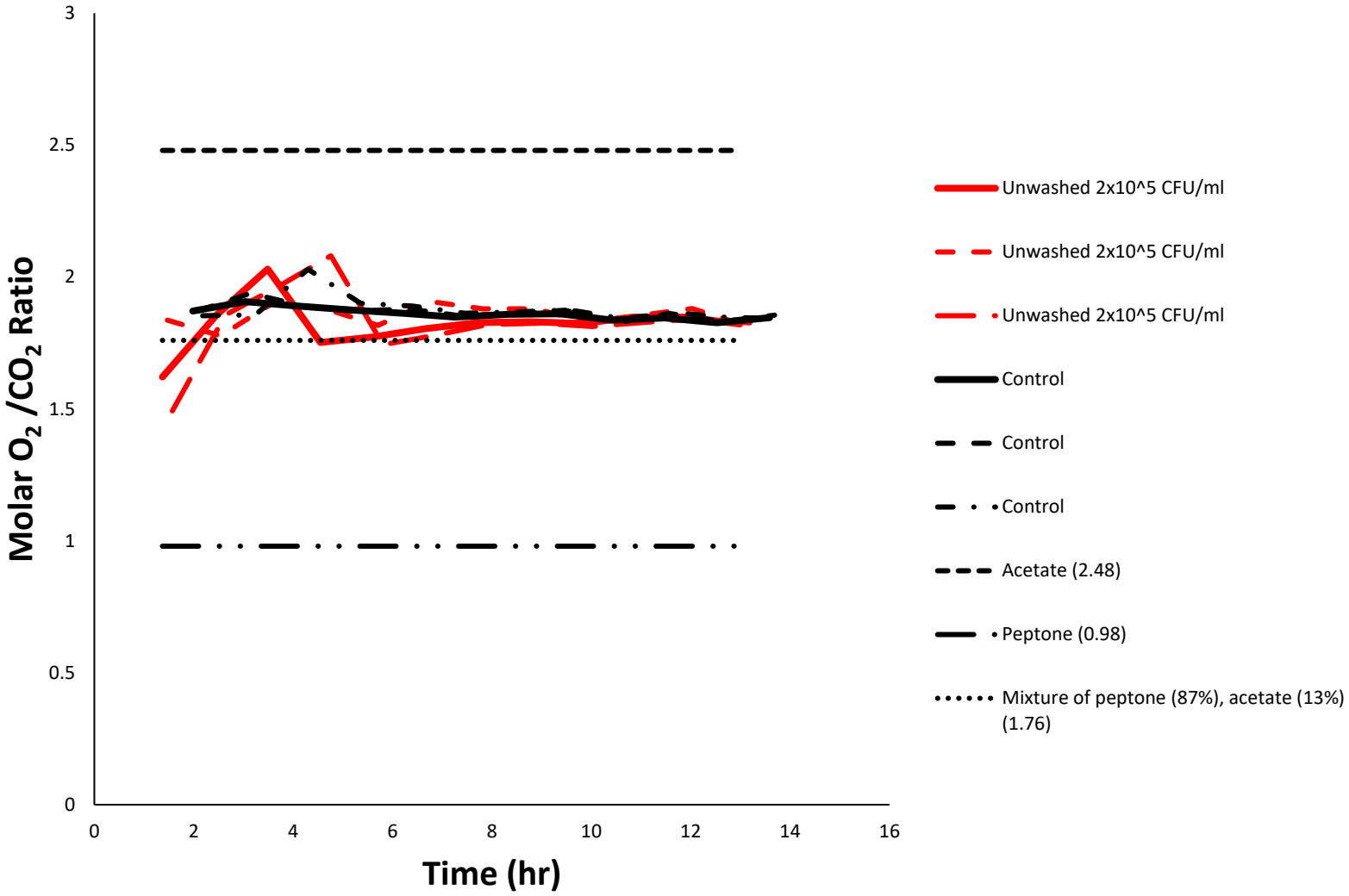

Figure 46: Molar O_2 to CO_2 Ratio, unwashed $2x10^5$ CFU *B. globigii*/ml activated sludge.

The effect of unwashed 2x10^7 CFU/ml B. globigii on microbial respiration

The four trials of observing activated sludge in the presence of unwashed 2x10^7 CFU/ml *B. globigii* resulted in similar outcomes. Only the 14 Sep 2016 trial will be discussed in detail. See Appendix C for respirograms of remaining trials (Figure 93 to Figure 98). Contrary to other experiments, the respirogram with unwashed 2x10^7 CFU/ml *B. globigii* exhibited atypical O_2 consumption profile tendencies. Upon inoculation of the sample with the feed solution an increase in respiration occurred in response to the presence of the substrate as shown in Figure 47. However, the initial O_2 uptake rate was not as high as the control or any other spore concentrations (washed or unwashed) tested. Following the initial peak, the O_2 consumption began to decline for one sampling interval but then rose again dramatically. The O_2 consumption then remained elevated for the remainder of the test. This unexpected phenomenon was presumably caused by the ethanol storage solution. Although the storage solution was only 40% ethanol, it contributed 3952 mg/l of additional COD.

Shape parameters

The shape parameters of the unwashed 2x10^7 CFU/ml *B. globigii* O_2 consumption profiles were both statistically different from the control profile curves. The mean FrM of the experiment group was 44.7 µg O_2/min-hr^2 with a variance of 48.7 (µg O_2/min-hr^2)^2. The mean FrM of the control group was 72.3 µg O_2/min-hr^2 with a variance of 67.5 (µg O_2/min-hr^2)^2. The p-value from comparing the FrM was 0.011, see Figure 48. The mean skewness of the experiment group was -1.67 with a variance of 0.011. The mean skewness of the control group was 0.91 with a variance of 0.013. Comparing the

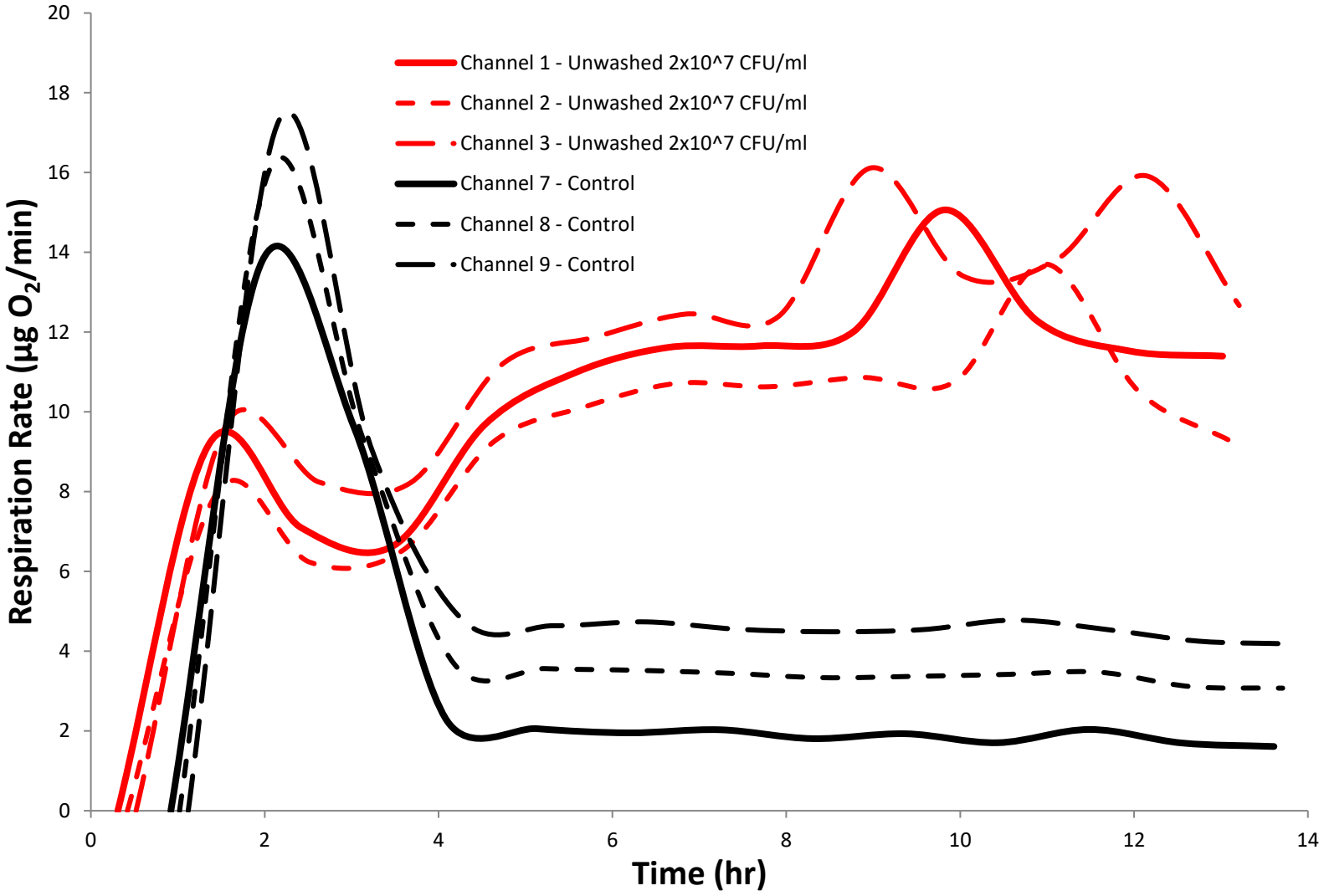

Figure 47: O_2 consumption profile of unwashed $2x10^7$ CFU/ml *B. globigii* spores in activated sludge.

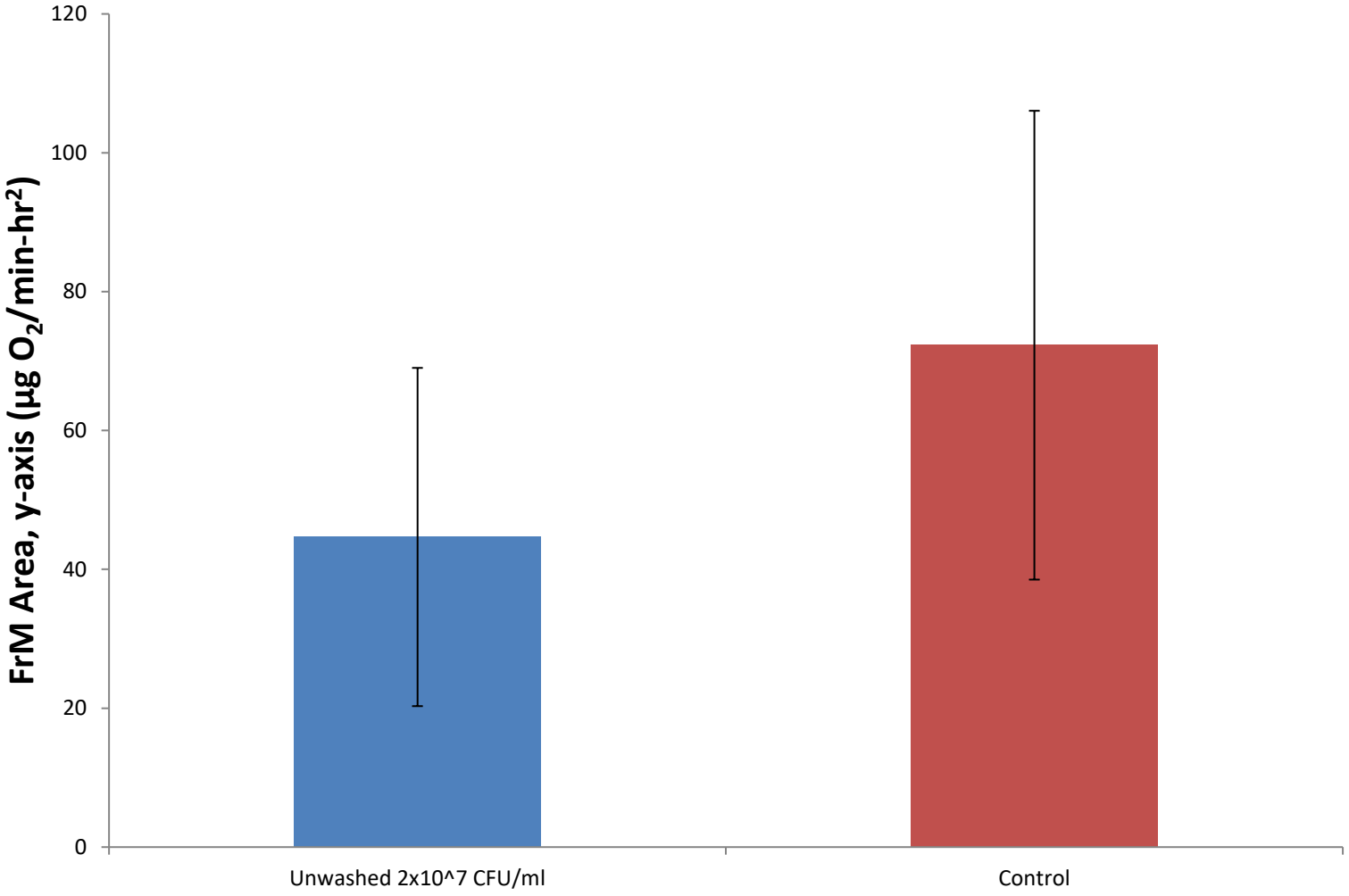

Figure 48: Comparison of unwashed 2x10^7 CFU/ml *B. globigii* and activated sludge First Moment of Area about the y-axis. Error bars represent sample variance.

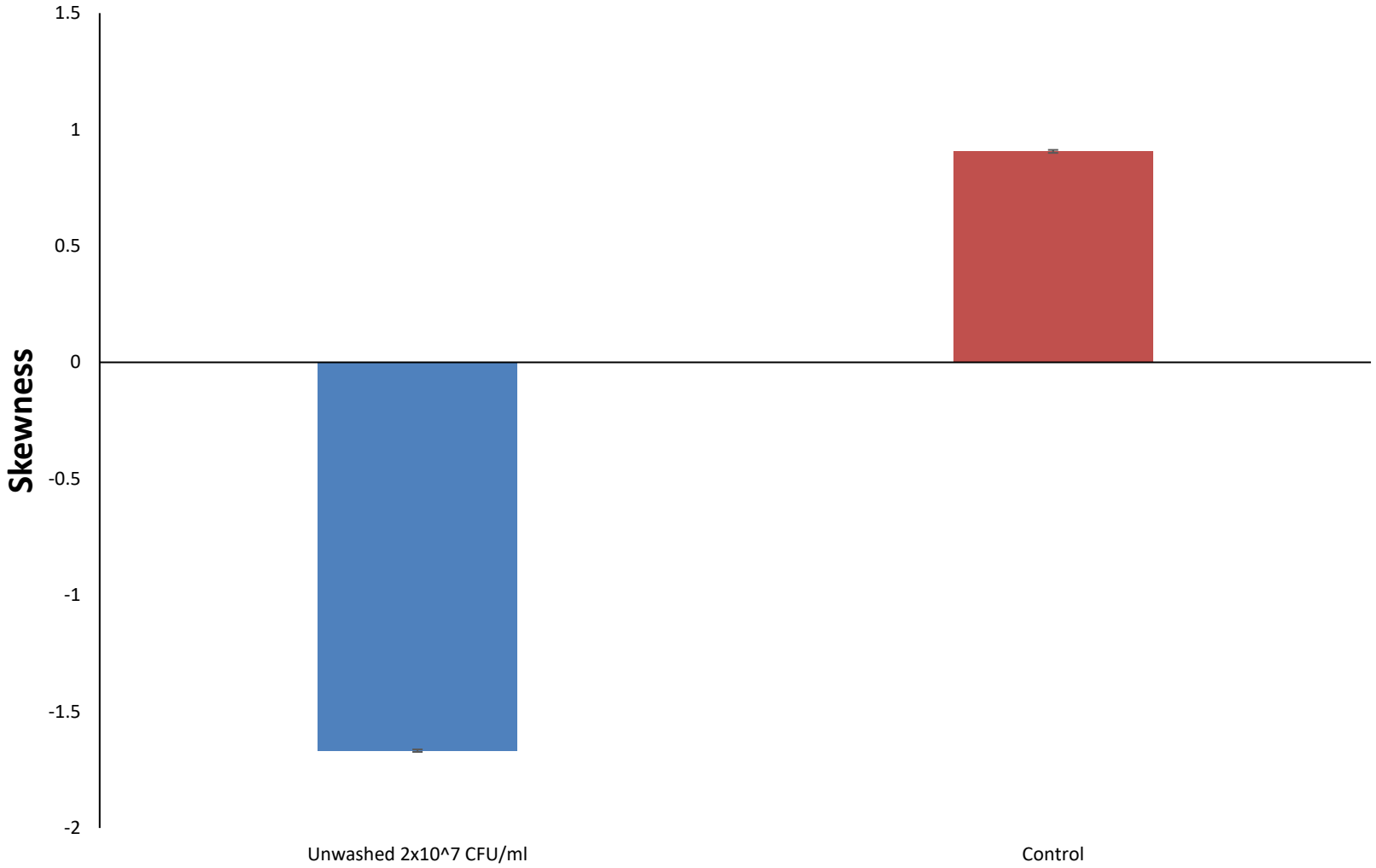

Figure 49: Comparison of unwashed 2x10^7 CFU/ml *B. globigii* and activated sludge Skewness. Error bars represent sample variance.

skewness of the curves resulted in a statistically significant difference with a p-value of 8.53 E-06, see Figure 49. These results show that there were statistically significant differences in the shape parameters between the test and control respirograms, indicating factors that influenced the transport of oxygen were likely present.

Peak oxygen consumption rates

The peak measured SOUR mean of the experiment group was 10.6 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 1.04 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak measured SOUR mean of the control group was 18.5 mg O_2-g VSS^{-1}-hr^{-1} with a variance of 4.52 (mg O_2-g VSS^{-1}-hr^{-1})^2. The peak oxygen consumption rates of activated sludge in the presence of unwashed $2x10^7$ CFU/ml *B. globigii* were statistically significantly different from the control and the associated p-value was 0.0043, see Figure 50. The statistically significant difference between the experiment and control groups suggest that the bacteria were inhibited in the presence of the unwashed spores. This inhibition was most likely due to ethanol.

Cumulative oxygen consumption

The mean cumulative O_2 consumption for the experiment group after the fourth interval was 2110 µg with a standard deviation of 236 µg. The mean cumulative O_2 consumption for the control group after the fourth interval was 2030 µg with a standard deviation of 257 µg. The cumulative O_2 consumption of the activated sludge in the presence of unwashed $2x10^7$ CFU/ml *B. globigii* was not statistically significantly different from the control, see Figure 51. The p-value from a student t-test comparison was 0.71. However, if this analysis was performed after the third or fifth sampling interval, the difference is statistically significant. The fourth interval, intentionally

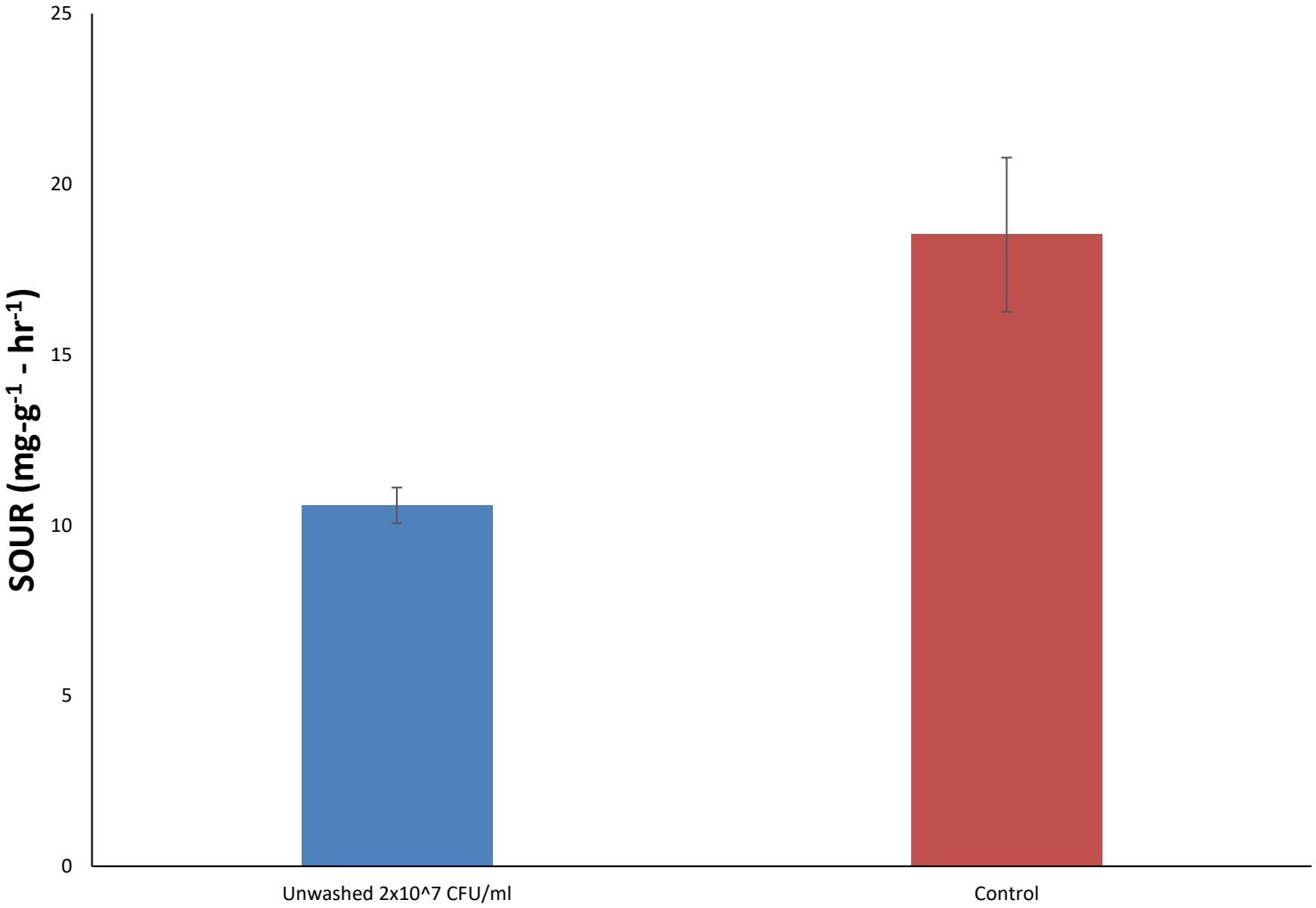

Figure 50: Peak SOUR of unwashed 2x10^7 CFU *B. globigii*/ml activated sludge. Error bars represent sample variance.

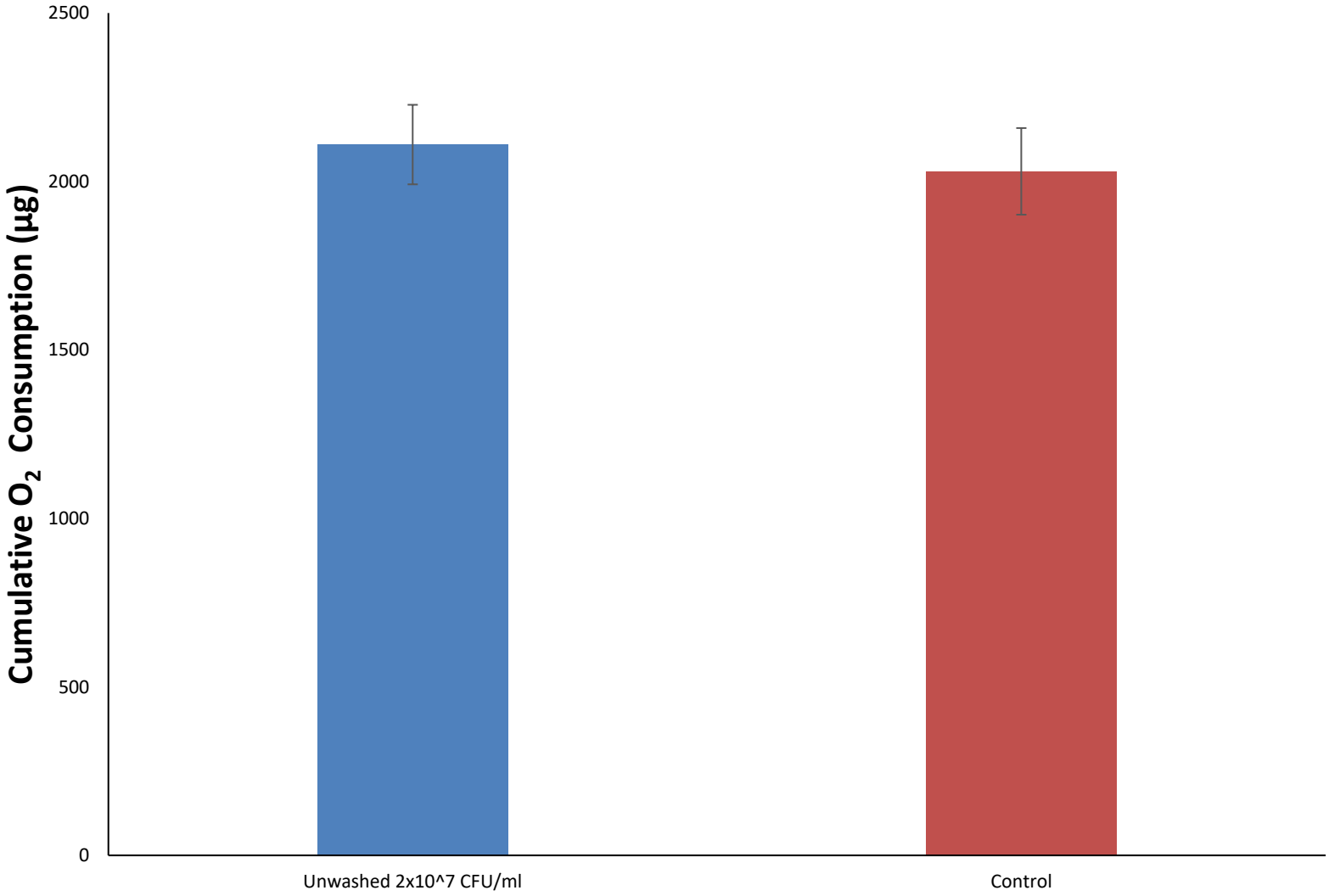

Figure 51: Cumulative O_2, unwashed $2x10^7$ CFU *B. globigii*/ml activated sludge. Error bars represent sample standard deviation.

selected as explained in the Methods section, is where the cumulative oxygen consumption curves intersect. The statistically significant difference in the cumulative O_2 consumed indicates that the respiration was significantly impacted by the presence of the unwashed spores.

Molar O_2/CO_2 ratios

The molar ratio of O_2 consumed-to-CO_2 produced by activated sludge in the presence of unwashed $2x10^7$ CFU *B. globigii*/ml sludge was not similar to the control. The molar O_2/CO_2 ratio of the experiment group began much lower than the control at 0.70 and then declined to as low as 0.27 during the first three hours of the test, Figure 52. At approximately the four hour point it rose to slightly above 1.50 and was relatively stable with minor oscillation near the end of the test. The rise in the molar O_2/CO_2 ratio corresponds to the unexpected second rise in the O_2 consumption profile curve, see Figure 47. Unlike all tests with washed or unwashed spores, at no point did the experimental molar O_2/CO_2 ratio match the control molar O_2/CO_2 ratio.

The effect of the unwashed spores at $2x10^7$ CFU *B. globigii*/ml on the molar O_2/CO_2 ratio indicates a discernable impact on the underlying metabolic pathways occurring the activated sludge. Ethanol is known to drive denitrification (Adouani, et al., 2010), where nitrate, not oxygen, is used as the electron acceptor. When denitrification occurs, CO_2 is produced without the associated consumption of oxygen. This explains why the molar O_2/CO_2 ratio is smaller when the activated sludge is exposed to $2x10^7$ CFU *B. globigii*/ml.

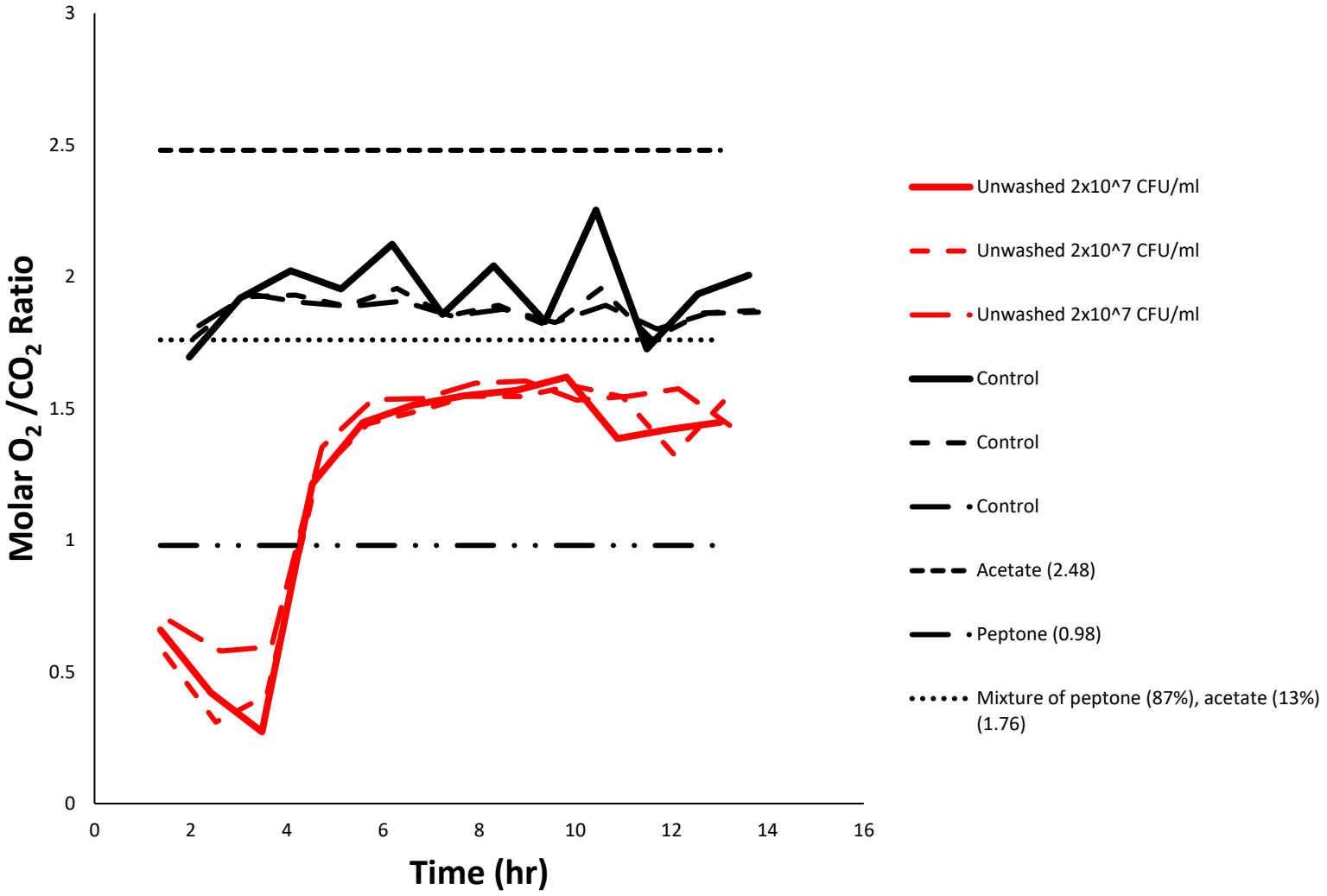

Figure 52: Molar O_2 to CO_2 Ratio, unwashed 2x10^7 CFU *B. globigii*/ml activated sludge.

Ethanol storage solution

In order to determine the impact of the ethanol storage solution, a separate respirometry experiment was conducted using a comparable amount of pure ethanol (2.4 µl/ml) in addition to the standard feed solutions. The results replicated the $2x10^7$ CFU *B. globigii*/ml activated sludge oxygen consumption profiles confirming the influence of ethanol on the activity of activated sludge, see Figure 53.

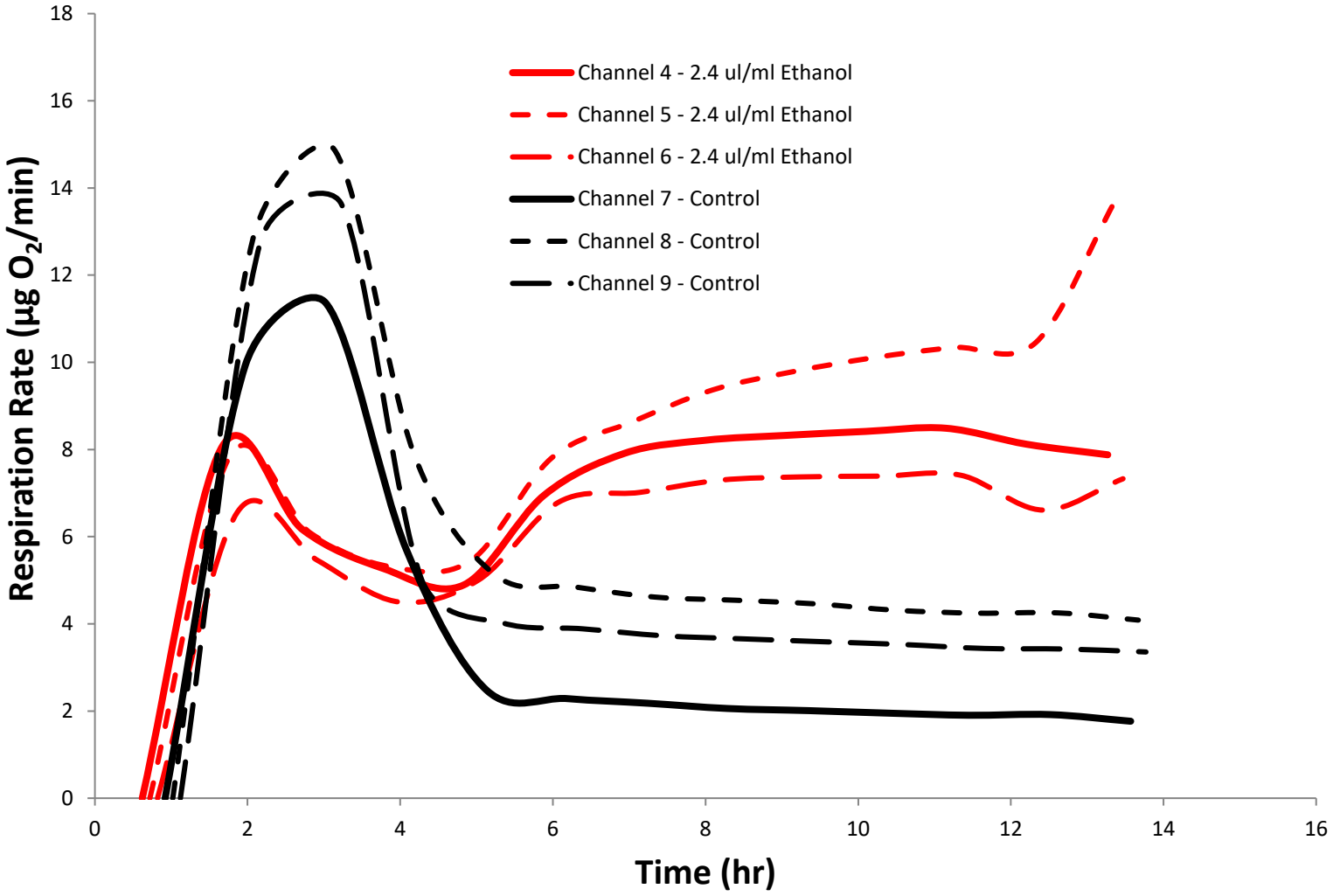

Figure 53: O_2 consumption profile of 2.4 µl/ml ethanol in activated sludge.

COD and nitrogen removal

COD removal

B. globigii spores did not interfere with COD removal, (Figure 54). COD was effectively removed at all spore concentrations tested. However, one anomaly was observed: at $2x10^3$ CFU/ml the final COD in the experiment group (24.8 mg/l) was greater than the final COD of the control group (12.3 mg/l, p-value = 0.044). Although statistically different at $2x10^3$ CFU/ml, both the experiment and control groups performed effectively by removing the majority of the COD present.

Ammonia-N removal

B. globigii spores did not interfere with NH_3-N removal, (Figure 55). Essentially all of the NH_3-N was consumed during the course of the experiment; negative numbers represent readings below the LOD. As expected nitrification occurred in the aerobic environment. One anomaly was observed: at $2x10^7$ CFU/ml, the initial amount of NH_3-N was much lower than the initial condition of all other experiments. The only source of ammonia is the liquor from the SBR so the low initial ammonia concentration presumably was due to a NH_3-N fluctuation in the SBR.

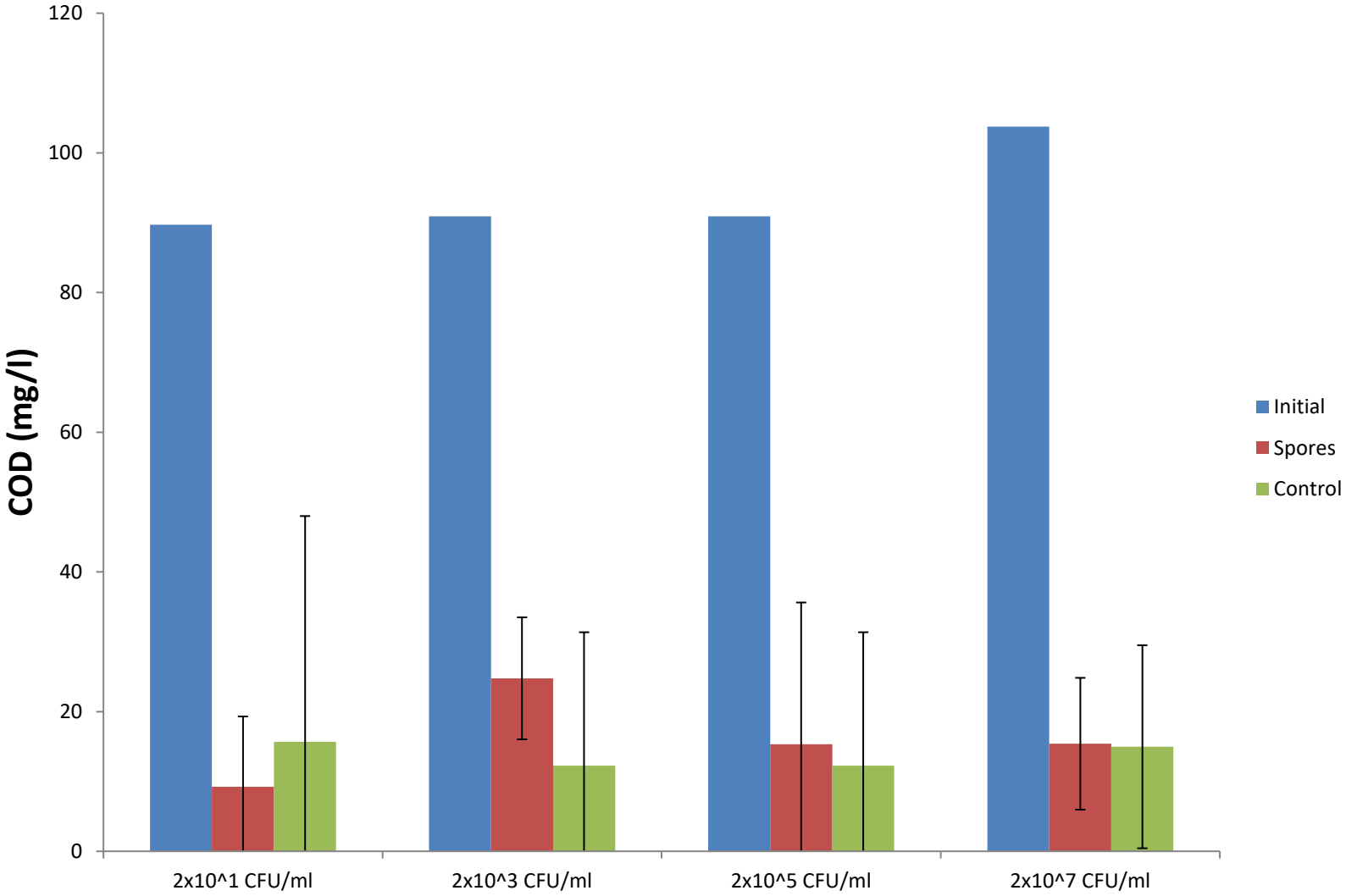

Figure 54: Initial and final COD for $2x10^1$ – $2x10^7$ CFU/ml *B. globigii* in activated sludge. Error bars represent sample variance.

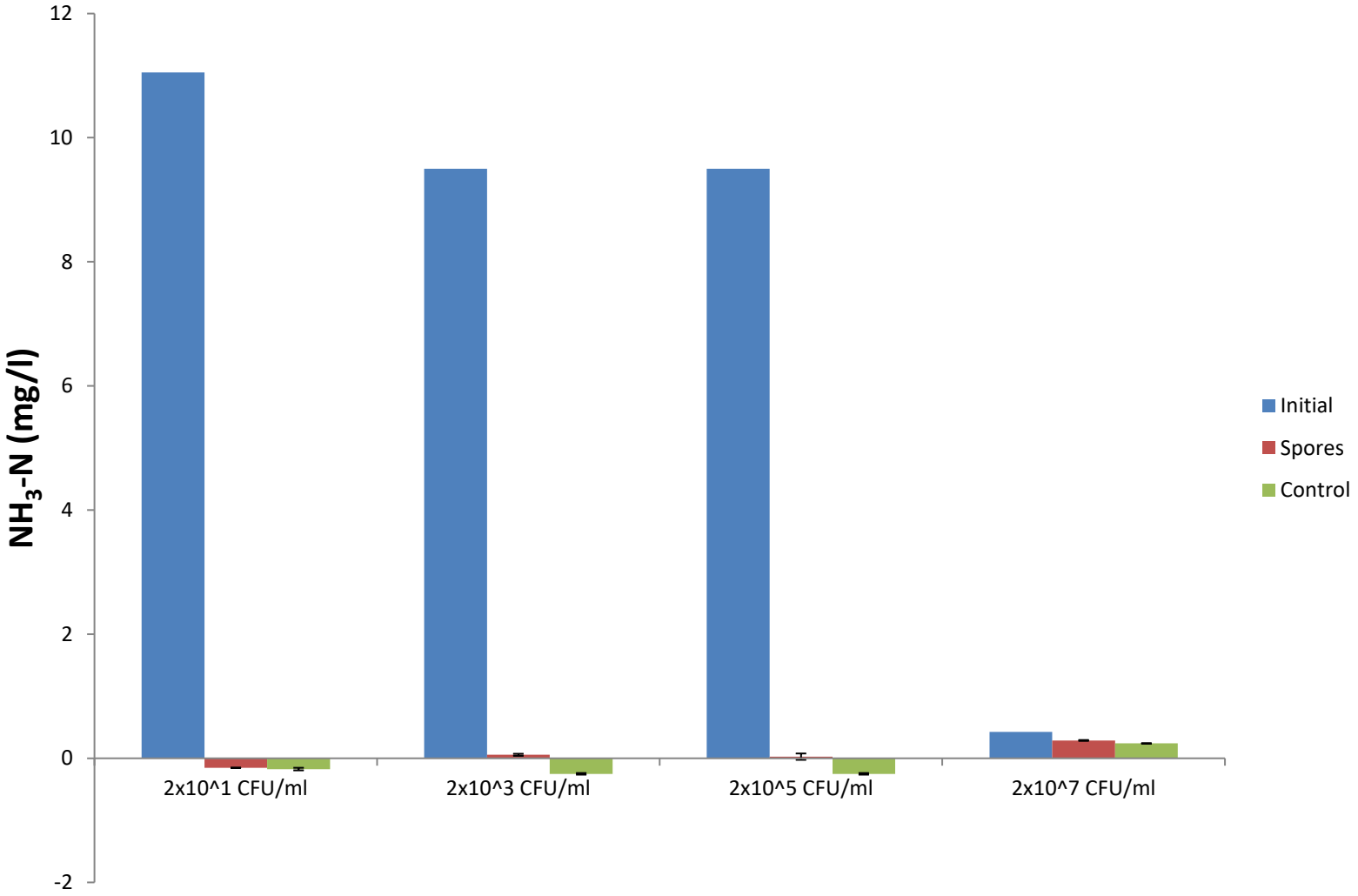

Figure 55: Initial and final NH_3-N for $2x10^1$ – $2x10^7$ CFU/ml *B. globigii* in activated sludge. Error bars represent sample variance.

Nitrate-N removal

B. globigii spores did not interfere with NO3-N production (Figure 56).There were no statistically significant differences between the test and control groups for any of the spore concentrations tested. As expected due to the lack of anoxic conditions, NO_3 only decreased slightly throughout the course of the experiment.

COD and nitrogen removal of unwashed spores

COD removal

Unwashed *B. globigii* spores did not interfere with COD when the concentration was $2x10^5$ CFU/ml or less (Figure 57). There was no statistically significant difference in the final COD concentration of test and control samples. However, when the concentration of unwashed spores was $2x10^7$ CFU/ml, there was a statistically significant difference. The highest concentration of unwashed spores tested exhibited an unexpected rise in final COD (136 mg/l). Addition of 2.4 µl/ml of ethanol exhibited the same COD trend but the magnitude of the COD increase was even greater (>600 mg/l) causing final COD concertation in excess of the MDL even after a 4X dilution. Even though the 2.4 µl/ml of ethanol was proportionate to the ethanol in the spore storage solution, it had a much more dramatic effect on the final COD. The difference is best attributed to the use of fresh laboratory grade ethanol for the 2.4 µl/ml of ethanol respirometry test. This is in contrast to the ethanol in the spore storage solution which was in contact with the spores for six months and could have degraded somewhat overtime. Also, the same stock of *B. globigii* spores was used for every experiment. The opening and closing of the vial for

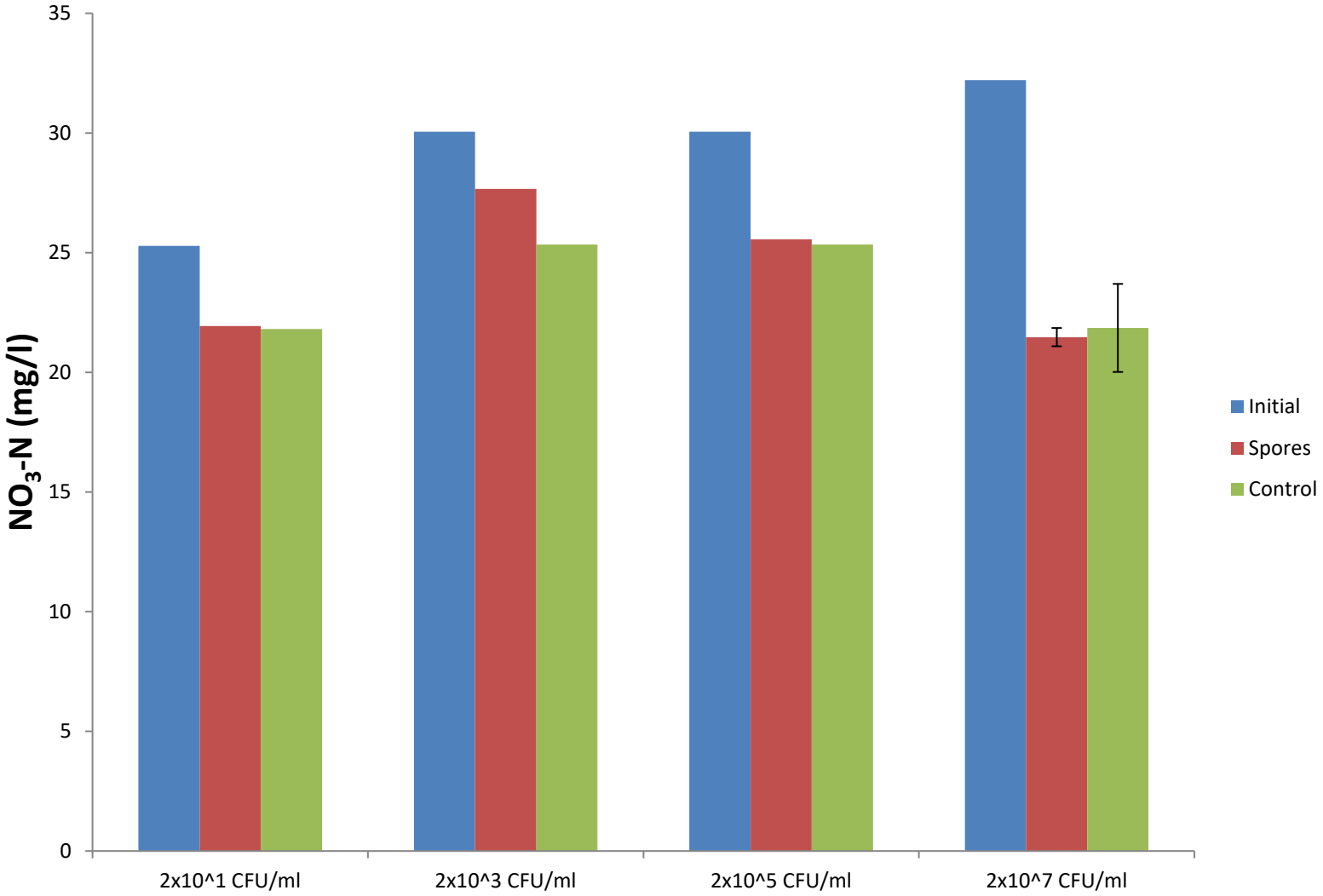

Figure 56: Initial and final NO_3-N for $2x10^1$ – $2x10^7$ CFU/ml *B. globigii* in activated sludge. Error bars represent sample variance.

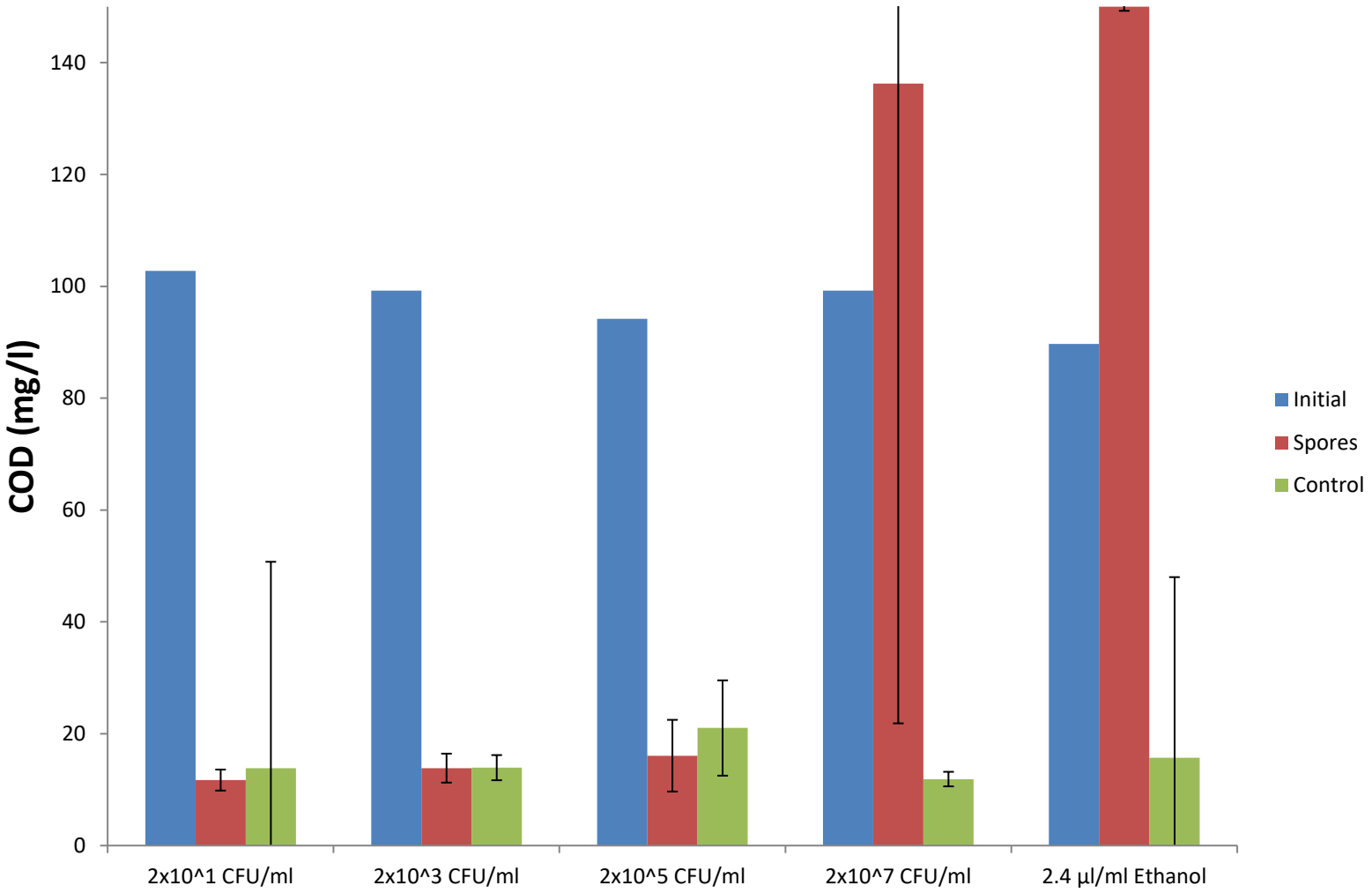

Figure 57: Initial and final COD for unwashed $2x10^1$ – $2x10^7$ CFU/ml *B. globigii* in activated sludge. Error bars represent sample variance. 2.4 µl/ml ethanol exceeded 150 mg/l MDL after 4X dilution.

each experiment could have allowed volatized ethanol to dissipate reducing its concentration overtime.

Ammonia-N removal

Unwashed *B. globigii* spores did not interfere with NH_3-N removal (Figure 58). NH_3-N was effectively removed via nitrification as expected at all spore concentrations tested. 2.4 µl/ml ethanol exhibited the same trend of nitrification removing essentially all of the ammonia as indicated by negative values representing concentrations below the LOD.

Nitrate-N removal

Unwashed *B. globigii* spores did not interfere with NO3-N removal in the presence of $2x10^5$ CFU/ml *B. globigii* or less (Figure 59). NO_3 only decreased slightly throughout the course of the experiment. However, when unwashed spores at $2x10^7$ CFU/ml were introduced the NO_3 was removed to levels below the LOD. The same phenomenon occurred testing 2.4 µl/ml ethanol. This is most likely a result from the large amount of COD contributed by the ethanol, which in turn, supported significant denitrification. Note: initial NO_3 was not measured for the trial of $2x10^5$ CFU/ml due to lack of material. However, it is assumed to be between 27 and 15 mg/l as NO_3-N.

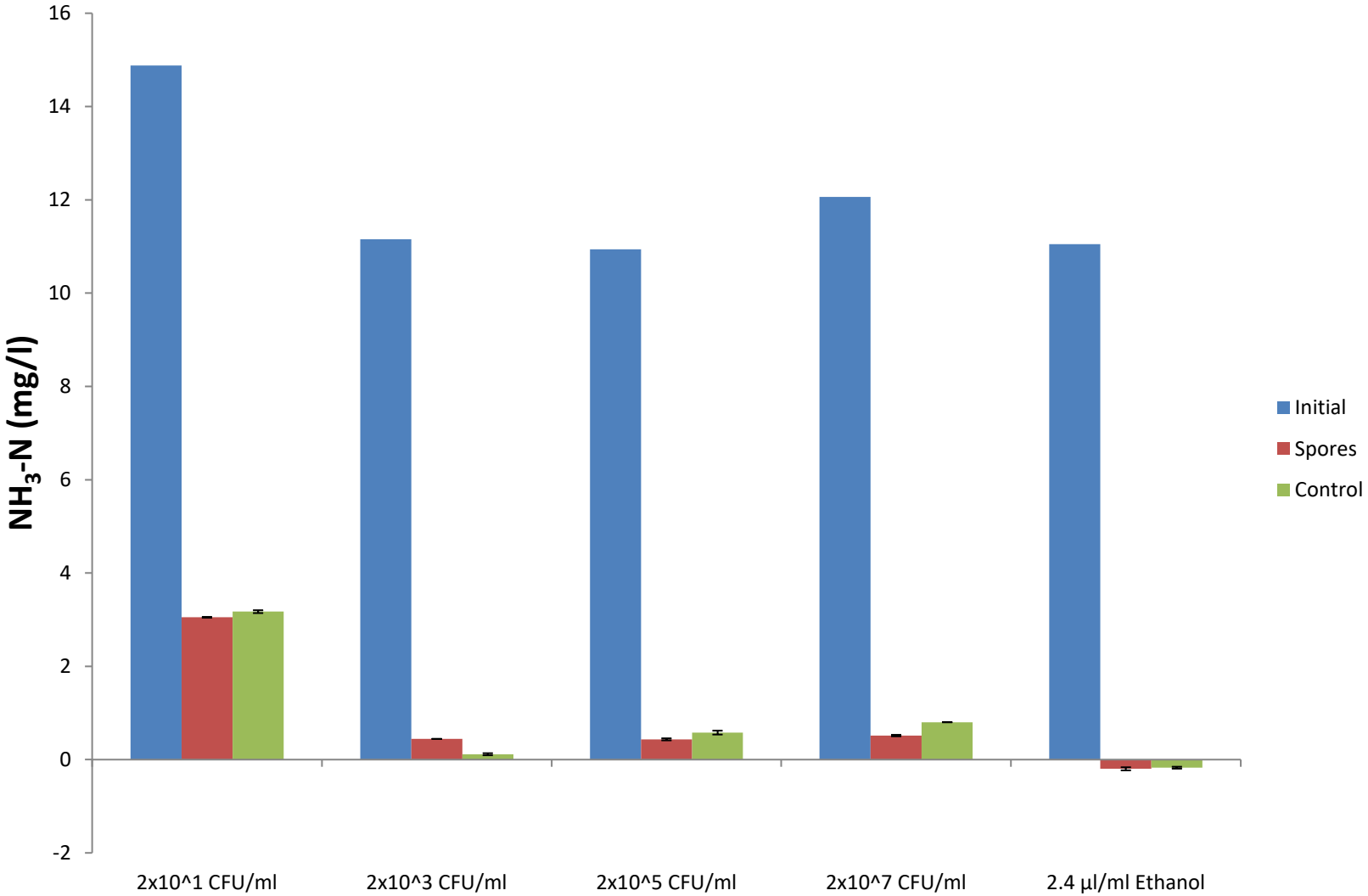

Figure 58: Initial and final NH_3-N for unwashed $2x10^1$ – $2x10^7$ CFU/ml *B. globigii* in activated sludge. Error bars represent sample variance.

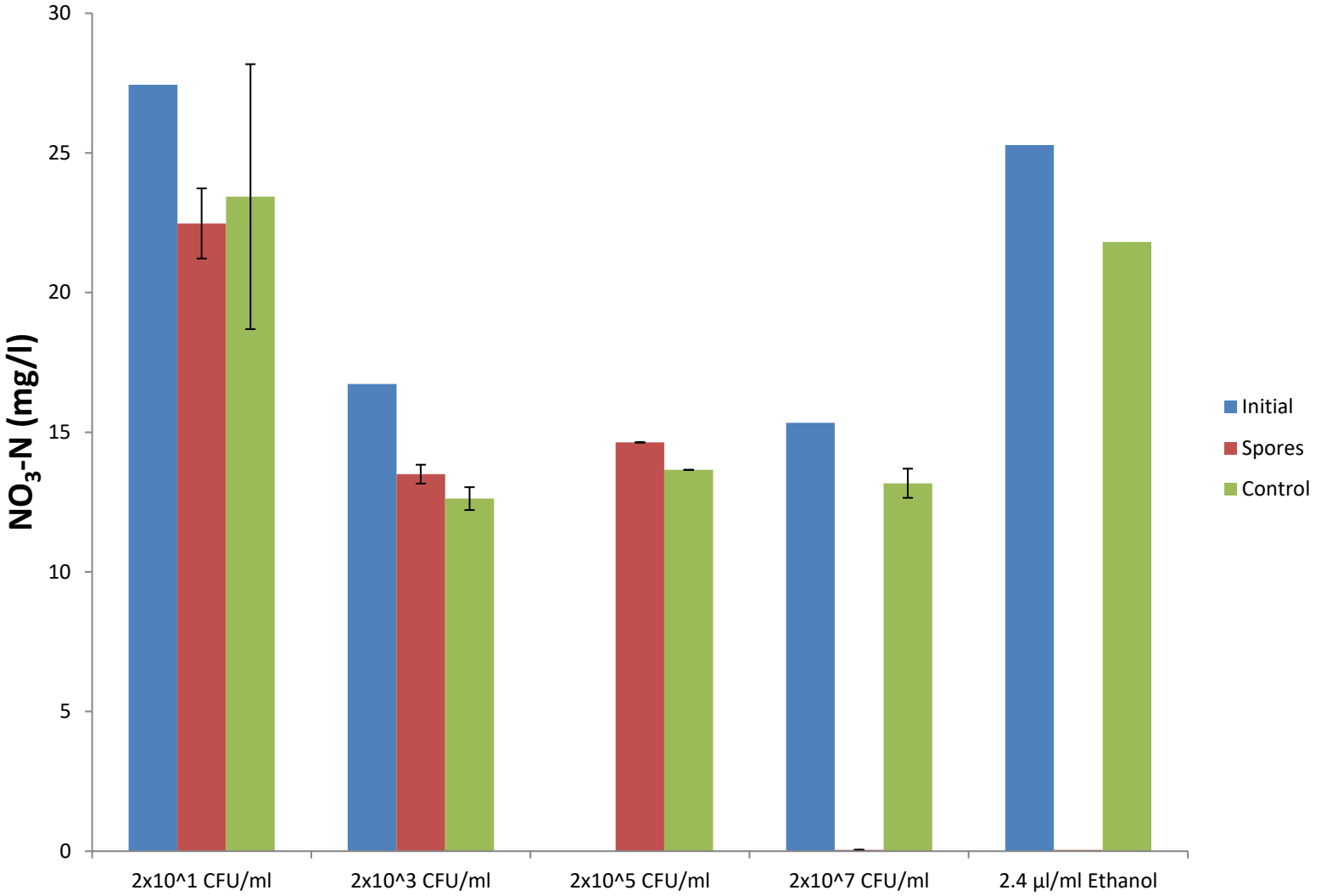

Figure 59: Initial and final NO_3-N for unwashed $2x10^1$ – $2x10^7$ CFU/ml *B. globigii* in activated sludge. Error bars represent sample variance.

Spore Dynamics

Spore germination and growth did not substantially impact the respiration or effectiveness of activated sludge in this experiment. A parallel study in the AFIT laboratory determined that although they remained viable, only 0.88 – 1.7% germinated during respiration (Xing et al.). Germination percentages in spore storage solution were 0.35%. These germination percentages were not substantial enough to impact O_2 or COD, NH_3-N, or NO_3-N measurements. The majority of the spores, 81%, were found to be adsorbed to the floc (Xing et al.). Albeit slightly lower, this coincides with published findings of 92% (Horan et al., 1991).

Functional redundancy

This research treated activated sludge as a community. The community as a whole was not effected by *B. globigii* spores at the concentrations tested. As such, this research cannot speak to whether or not any individual species of bacteria was inhibited. The consortia of bacteria in activated sludge has remarkable functional redundancy. If one species of bacteria was effected, another unaffected species could have benefitted from the decrease in competition for resources and increased its growth instead. The diversity of activated sludge bacteria greatly aids its ability to respond to varying conditions and to recover from a potentially inhibiting biocontaminant.

Shape Factors

The shape factors of FrM and skewness did not appear to have a strong relationship with spore concentrations. Shape factor differences were inconsistent and

did not vary directly with the tested spore concentrations. Even within the same experiment, shape factors varied within the respective experiment and control groups. For example, at $2x10^3$ CFU/ml (Figure 11) the visual difference in the shapes of the O_2 consumption profiles are clearly observable even before calculating the associated variances. Hydrodynamic factors such as mixing are a likely cause of the variability (Vanrollegham, 2002). Other factors that could impact the transfer of oxygen are the size of the sludge floc or any sort of film on the surface of the liquid retarding gas transfer equilibrium. Shape factors are important parameters in determining if a suspected inhibition is due to direct impedance on the activated sludge bacteria or if it is due to another factor limiting molecular O_2 transfer.

From an operational standpoint skewness could prove to be a significant factor. Skewness is an indicator of how quickly the bacteria respond to the substrate. An inhibitor could have no effect on the overall peak respiration rate of the activated sludge, but instead it could delay how quickly it occurred. Depending on the operating parameters of the WWTP this effect may need to be balanced by adjusting the hydraulic retention time of the flow.

Biological Variability

The motif of biological variability was addressed in multiple ways throughout this research. Primarily, all experiments and controls were conducted in triplicate. Secondly, the analysis methods attempted to focus on the immediate response of activated sludge to the substrate (the "peak") while minimizing the impact from the sample's endogenous respiration (the "tail"). Referring to the $2x10^3$ CFU/ml respirogram again (Figure 11),

amongst just the control samples there is an approximate 2.3 µg O_2/min difference in final respiration rates. The observed biological variability has many potential sources. Experimental protocol is one obvious avenue: differing sludge floc size distribution despite homogenization or differing thermal energy dissipation due to location on magnetic stir plate. Biologically, testing in triplicate is not necessarily a large enough sample to insulate the experiment from random events such as DNA replication errors during microbial growth. Another simple explanation is due to a bacterium's physical proximity to the feed solutions. As the substrates were added they were not instantaneously evenly dispersed. Thus the bacteria in contact with the greater substrate concentrations had a longer duration to take up more. If bacteria which produce polyhydroxyalkanoates (PHAs) happened to uptake a greater proportion of the substrate in that sample the endogenous respiration would be greater due to the resultant uptake of the PHAs.

Summary

B. globigii spores did not interfere with the respiration or performance of activated sludge at concentrations ranging between $2x10^1$ CFU/ml to $2x10^7$ CFU/ml. These spores did not significantly impact the peak O_2 consumption rate, the cumulative O_2 consumption, the molar O_2/CO_2 ratios, or the shape of the respirograms as determined by the FrM and the skewness. The spores also did not impact COD removal or nitrification. Ethanol, a possible co-contaminant, inhibit the initial O_2 consumption rates, and it may drive denitrification when introduced at 2.4 µl/ml. Most of the spores adhered to the floc, and a relatively small fraction (approx. 1%) of the spores germinated during the

experimental period. Overall, these results illustrate that *B. globigii* does not cause short-term interference with biological treatment at an activated sludge plant, but organic co-contaminants may cause inhibition, and spore germination may cause the wastewater treatment plant to become a longer-term source of biocontamination.

V. Conclusions

This study provided insight into the effect of *B. globigii* spores on the activity and performance of activated sludge and it demonstrates that spore concentrations of $2x10^1$ CFU/ml – $2x10^7$ CFU/ml do not interfere with the short term behavior of activated sludge organisms. The absence of spore-related inhibition was determined by a detailed analysis of the respirograms, which included determining the peak O_2 consumption rate, the cumulative O_2 consumption, the molar O_2/CO_2 ratio, and the FrM and skewness of the respirogram. The negative and positive controls verified the fidelity of the experimental technique. Unwashed *B. globigii* spores inhibited the initial O_2 consumption rate by 34-44% at $2x10^7$ CFU/ml. This was due to the significant amount of ethanol, which also caused nitrate to be removed via denitrification. The *B. globigii* spores did not cause a statistically significant impact on COD removal or nitrification. A small fraction (approx. 1%) of *B. globigii* spores germinated during respirometry tests, and 81% of the spores adhered to the floc particles. This study shows that *B. globigii* spores will not interrupt biological wastewater treatment but they will remain viable and a fraction will germinate.

VI. Recommendation for Future Research

The following are suggestions for future research:

1. Experiments show be repeated using fresh sludge harvested from an actual WWTP for each respirometry test because the microbial community in full scale treatment systems is more diverse.

2. The tests should be repeated with a quicker sampling respirometer in order to reduce the sampling interval and increase the fidelity of the OUR measurements

3. The tests should be repeated with more intermittent COD measurements in order to better define the COD removal profile.

4. The tests should be repeated with MS2phage, as these biocontaminants may infect key microbial groups and cause disruption to the treatment process.

Appendix A: Feed Solutions

Feed A: Sodium bicarbonate

1 L DI water		
$NaHCO_3$	Sodium Bicarbonate	44.6 g

Feed B1: Macronutrients

1 L DI water		
Casamino acid		12.0 g
$C_2H_3NaO_2$	Sodium acetate	2.5 g

Feed B2: Micronutrients

1 L DI water		
NH_4Cl	Ammonium Chloride	4.52 g
$MgCl_2$	Magnesium Chloride	13.72 g
$CaCl_2$	Calcium Chloride	3.44 g
KH_2PO_4	Potassium Dihydrogen Phosphate	1.335 g
Trace Element Solution		40 ml

Trace Element Solution

1 L DI water		
C6H8O7	Citric acid	2.73 g
C9H9NO3	Hippuric Acid	2.00 g
C6H6NNa3O6 • H2O	NTA trisodium salt	0.36 g
C10H12N2O8Na4 • 2H2O	EDTA tetrasodium salt	0.15 g
H3BO3	Boric Acid	0.25 g
KI	Potassium Chloride	0.03 g
FeCl3 • 6H2O	Ferric Chloride Hexahydrate	1.5 g
ZnSO4 • 7H2O	Zinc Sulfate Heptahydrate	0.15 g
MnCl2 • 4H2O	Manganese Chloride Tetrahydrate	0.12 g
CuSO4 • 5H2O	Copper (II) Sulfate Pentahydrate	0.07 g
Na2MoO4 • 2H2O	Sodium molybdate (VI) dihyrate	0.03 g
CoCl2 • 6H2O	Cobalt (II) chloride hexahydrate	0.03 g
NiCl2 • 6H2O	Nickelous Chloride Hexahydrate	0.03 g
Na2WO4 • 2H2O	Sodium tungstate dehydrate	0.03 g

Appendix B: UV-Vis Spectrometer Calibration Curves

Chemical Oxygen Demand

Three tests at each concentration were averaged to produce the COD calibration curve. Stock solution: Hach™ Cat. 2253929 Chemical Oxygen Demand Standard, 1000 mg/l. Hach™ Method 8000 kit (USEPA approved, Standard Method 5220 D). Agilent Cary-60 UV-Vis Spectrometer: 420 nm.

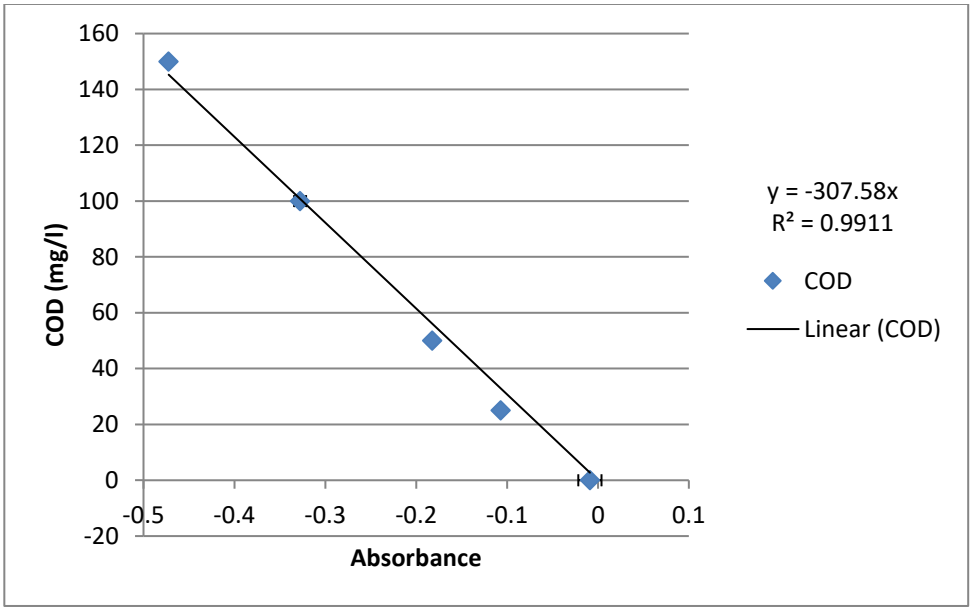

Figure 60: COD calibration curve.

Ammonia

Three tests at each concentration were averaged to produce the NH_3-N calibration curve. Stock solution: Hach™ Cat. 23541-53 Ammonia Standard Solution, 1000 mg/l NH_3-N. Hach™ Method 10031. Agilent Cary-60 UV-Vis Spectrometer: 655 nm.

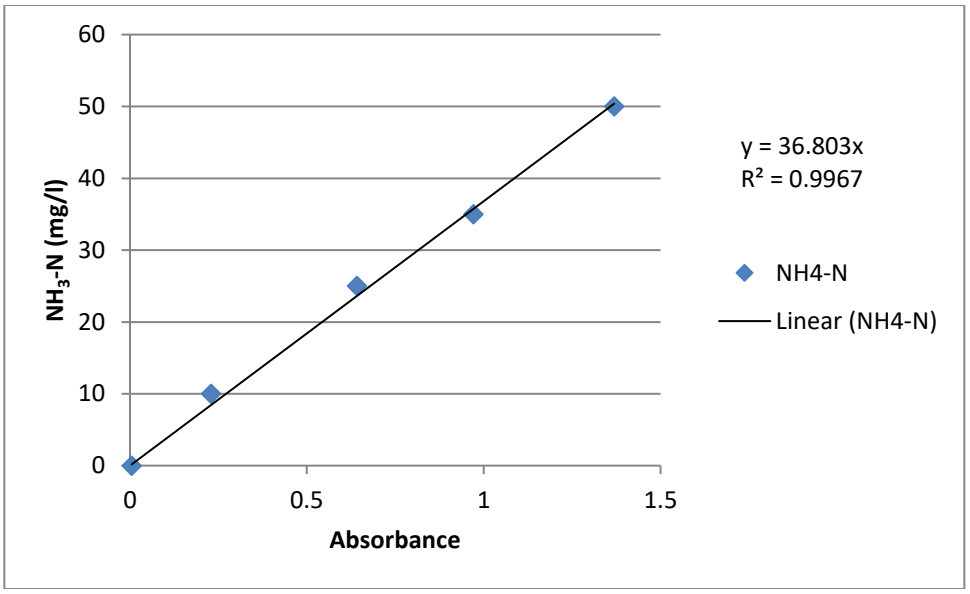

Figure 61: NH_3-N calibration curve.

Nitrate

Three tests at each concentration were averaged to produce the NO_3-N calibration curve. Stock solution: Hach™ 2833149 Wastewater Influent Inorganics, 10 mg/l NO_3-N. Hach™ Method 10020. Agilent Cary-60 UV-Vis Spectrometer: 410 nm.

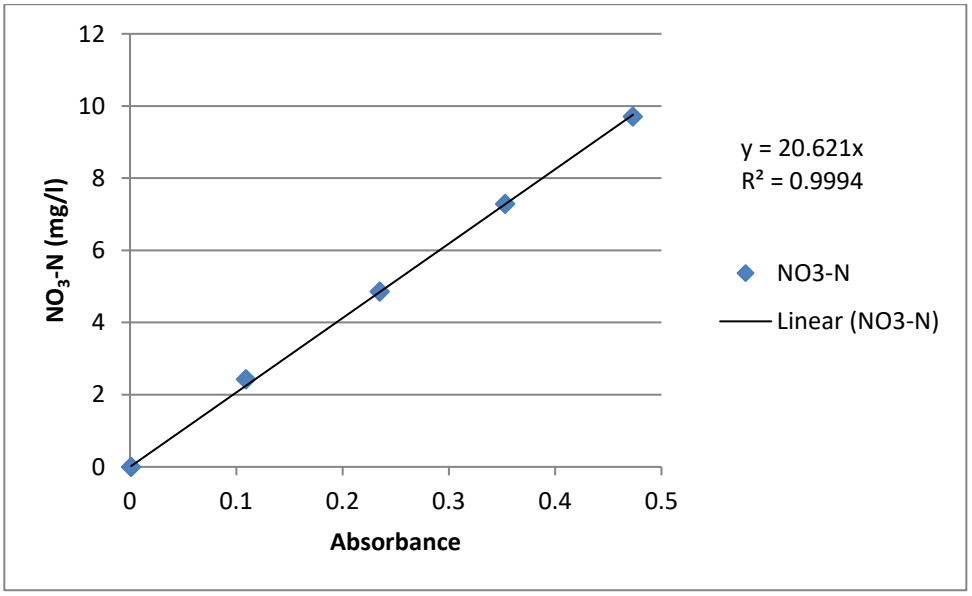

Figure 62: NO_3-N calibration curve.

Appendix C: Respirometry, COD, NH_3-N, and NO_3-N Data

$2x10^1$ CFU/ml and 2.4 µl/ml ethanol: 12 Oct 2016

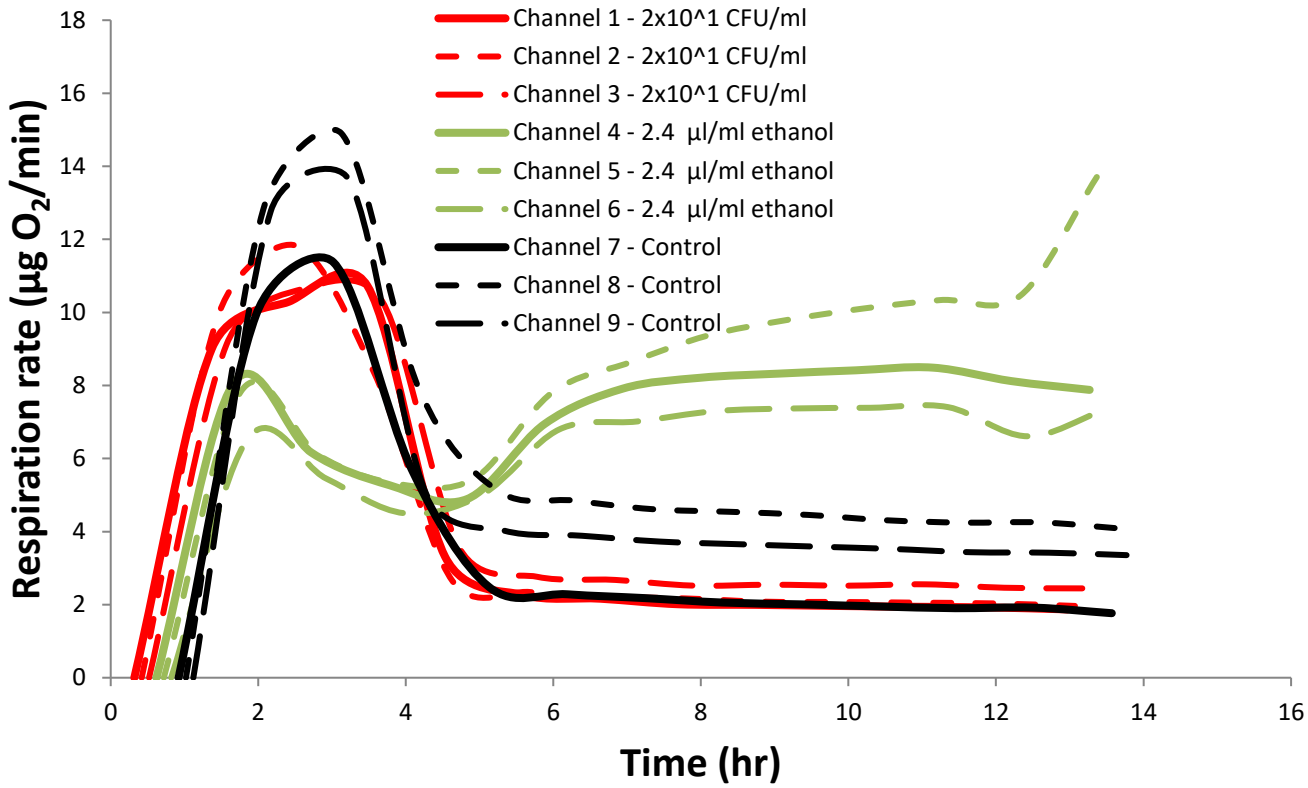

Figure 63: O_2 consumption profile of $2x10^1$ CFU/ml *B. globigii* spores and 2.4 µl/ml ethanol in activated sludge

COD

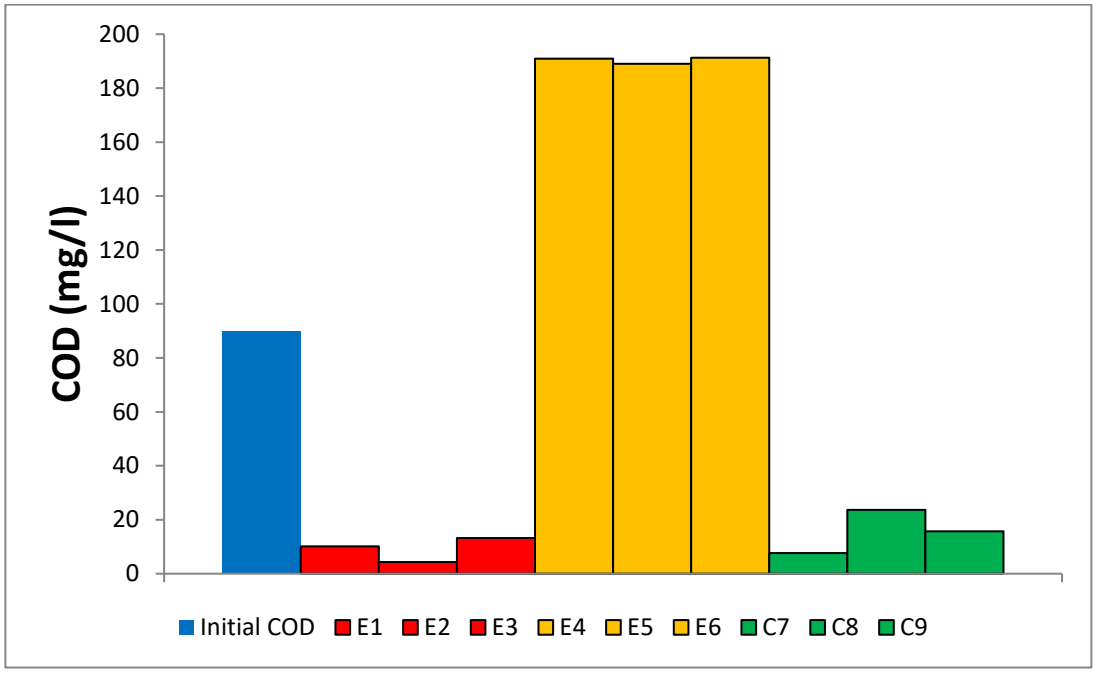

Figure 64: Initial and final COD for 2x10[1] CFU/ml *B. globigii* and 2.4 µl/ml ethanol in activated sludge. 2.4 µl/ml ethanol (E4-E6) exceeded 4x MDL.

NH_3-N

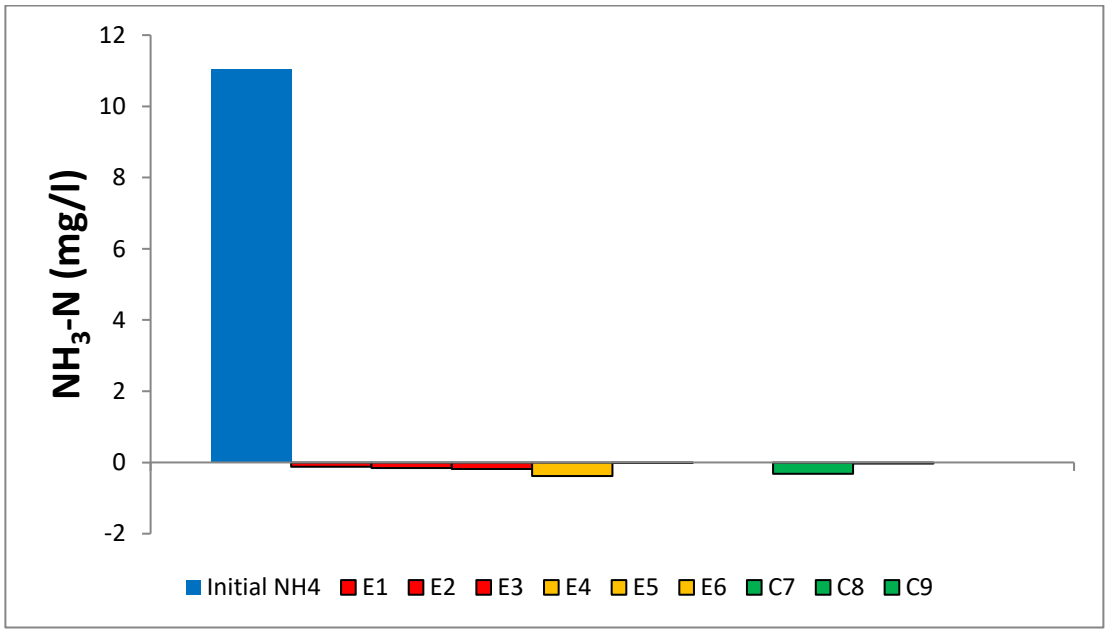

Figure 65: Initial and final NH_3-N for 2x10[1] CFU/ml *B. globigii* and 2.4 µl/ml ethanol in activated sludge. Negative values are below LOD. Channels E6 and C9 were not tested.

NO_3-N

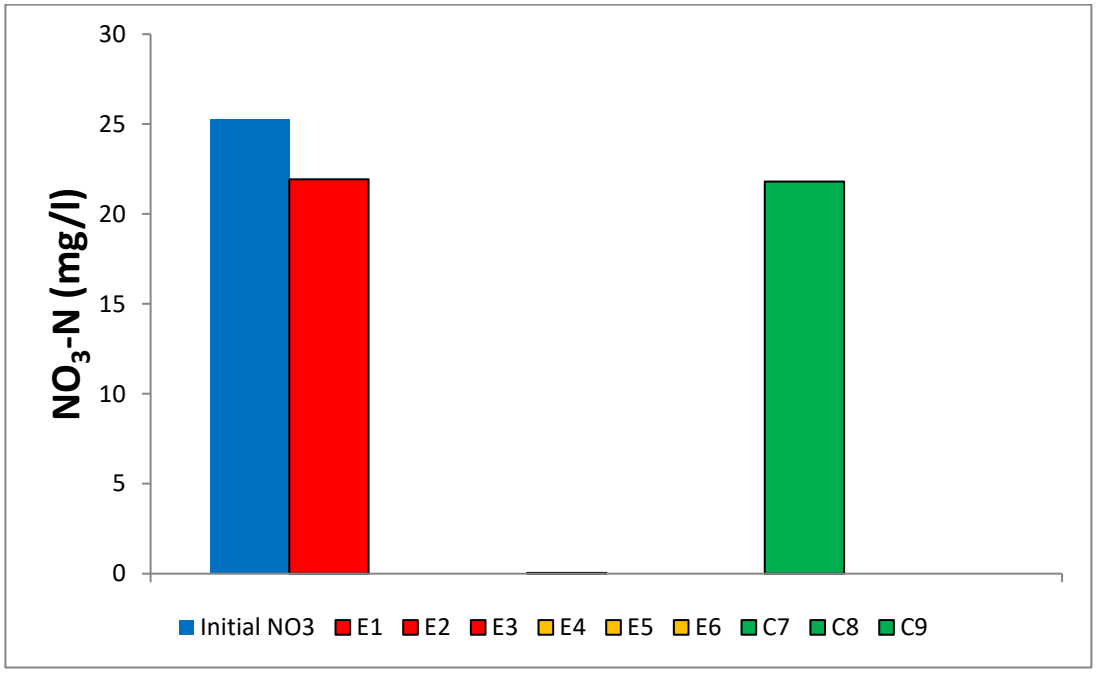

Figure 66: Initial and final NO_3-N for $2x10^1$ CFU/ml *B. globigii* and 2.4 µl/ml ethanol in activated sludge. Only channels E1, E4, and C7 were tested.

2x10^3 CFU/ml and 2x10^5 CFU/ml: 5 Oct 2016

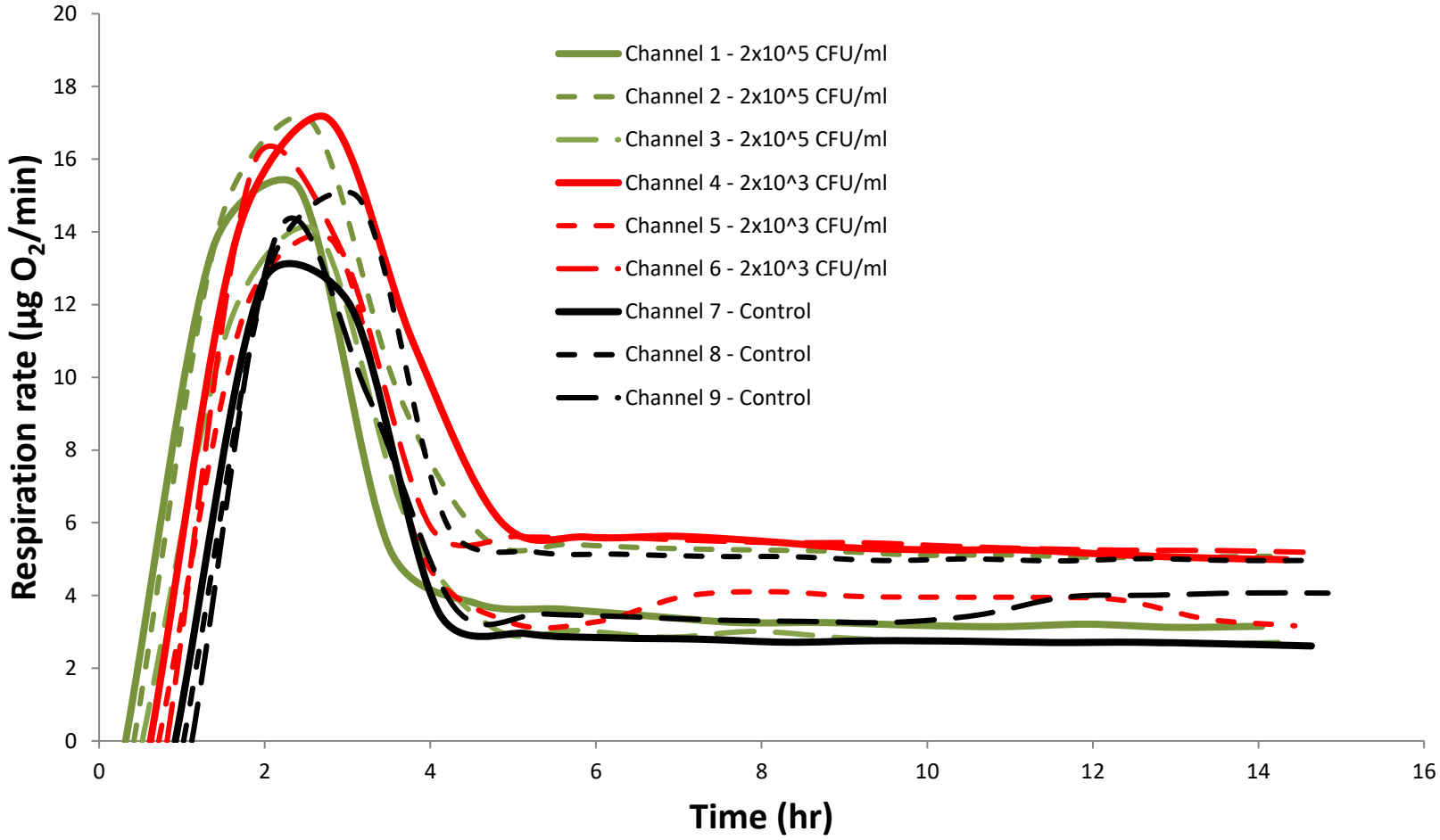

Figure 67: O_2 consumption profile of 2x10^3 CFU/ml and 2x10^5 CFU/ml *B. globigii* spores in activated sludge.

COD

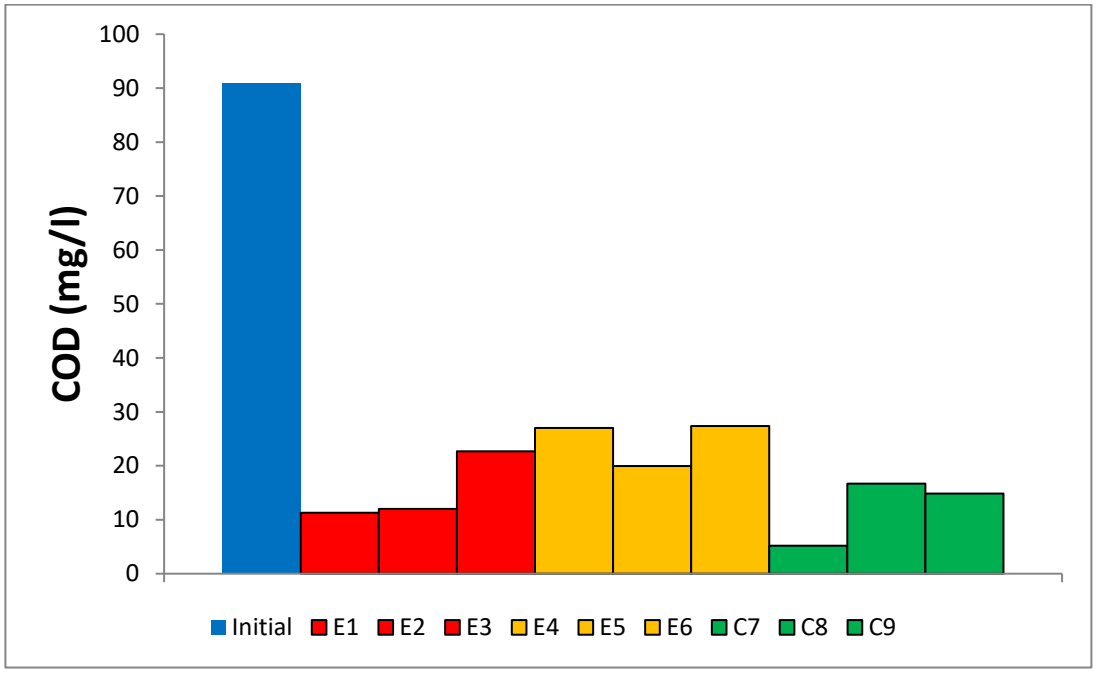

Figure 68: Initial and final COD for $2x10^3$ CFU/ml and $2x10^5$ CFU/ml *B. globigii* in activated sludge.

NH_3-N

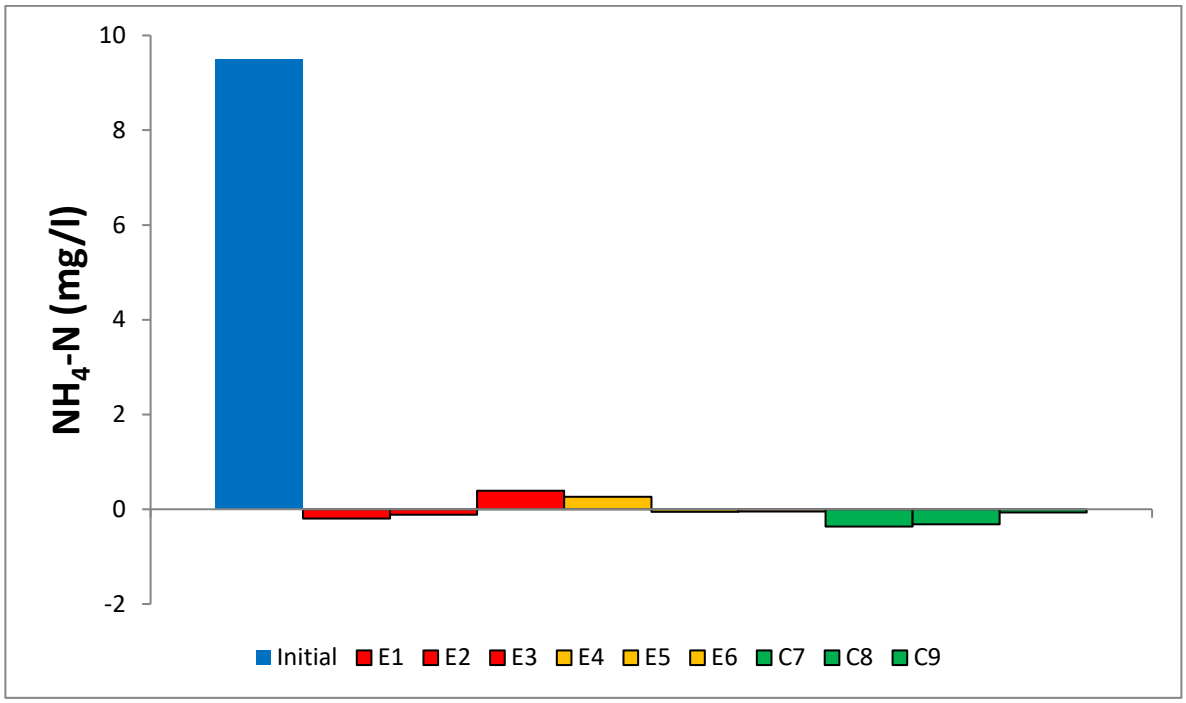

Figure 69: Initial and final NH_3-N for $2x10^3$ CFU/ml and $2x10^5$ CFU/ml *B. globigii* in activated sludge. Negative values are below LOD.

NO_3-N

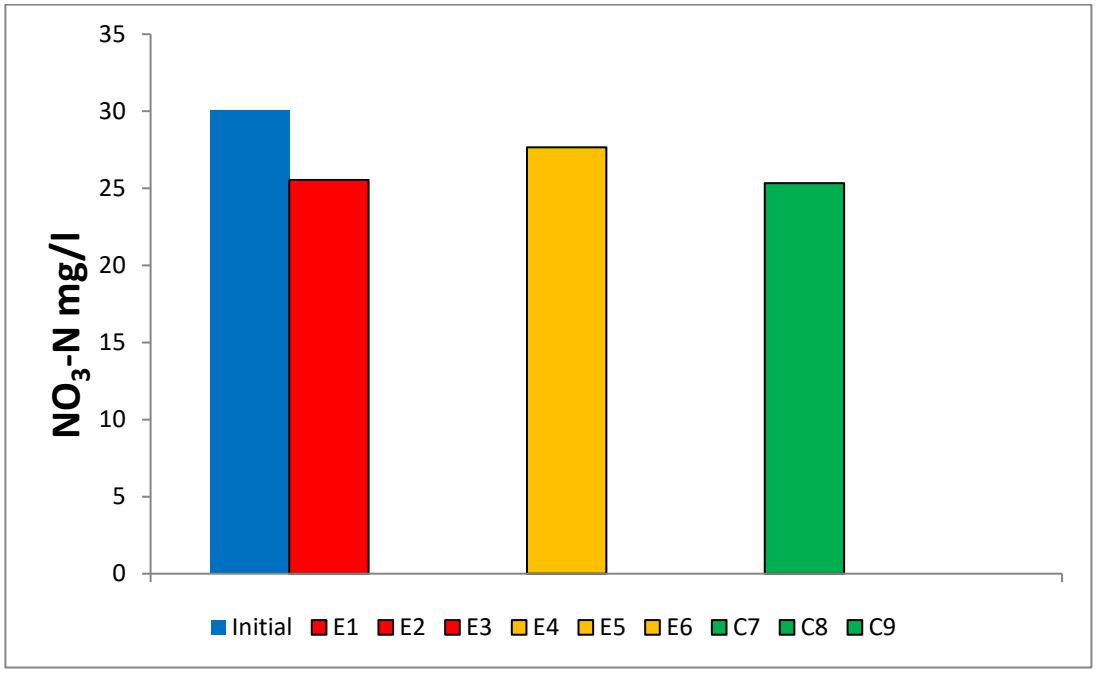

Figure 70: Initial and final NO_3-N for $2x10^3$ CFU/ml and $2x10^5$ CFU/ml *B. globigii* in activated sludge. Only channels E1, E4, and C7 were tested.

2x10^7 CFU/ml and unwashed 2x10^7 CFU/ml: 14 Sep 2016

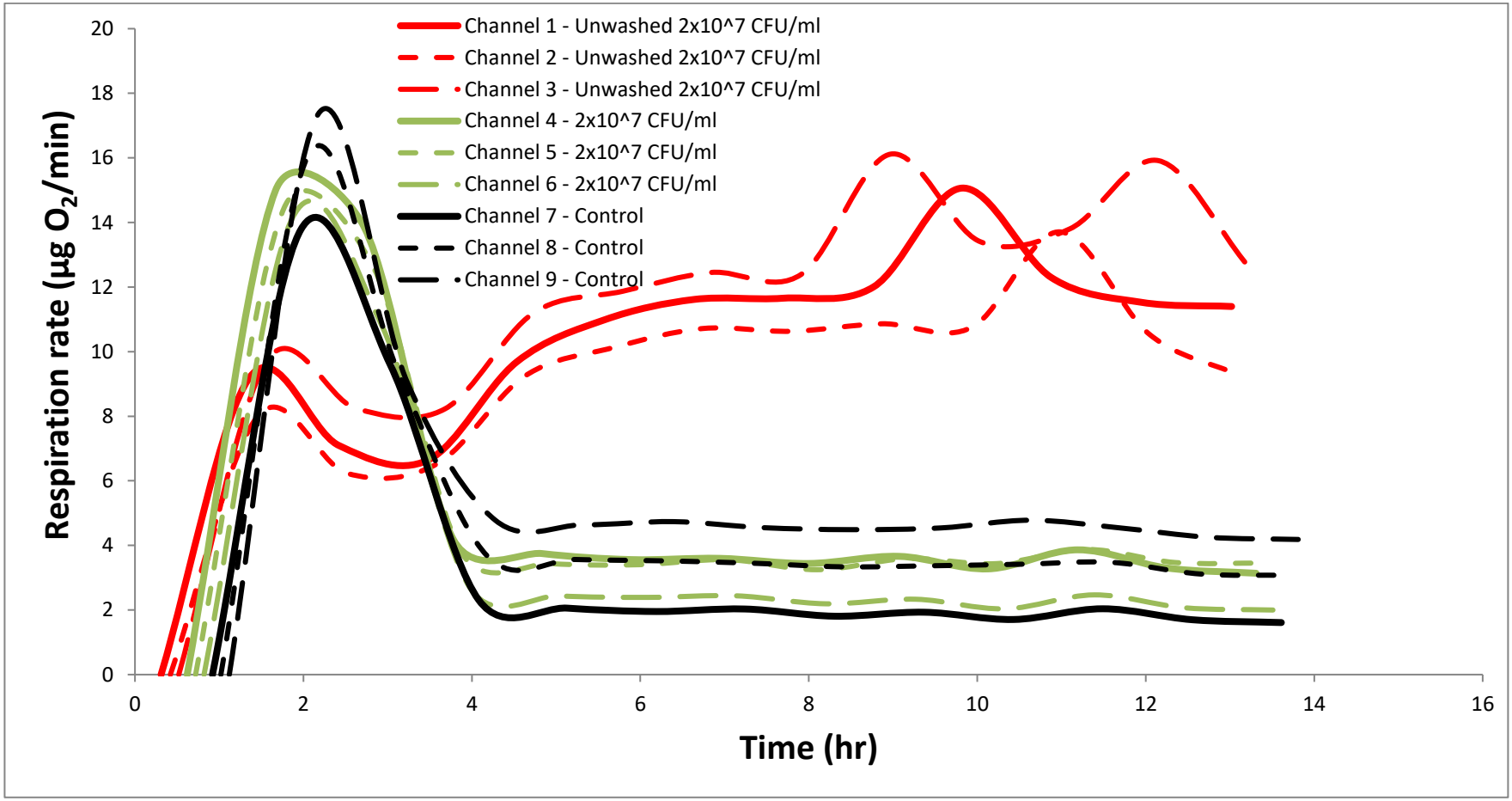

Figure 71: O_2 consumption profile of 2x10^7 CFU/ml and unwashed 2x10^7 CFU/ml *B. globigii* spores in activated sludge.

COD

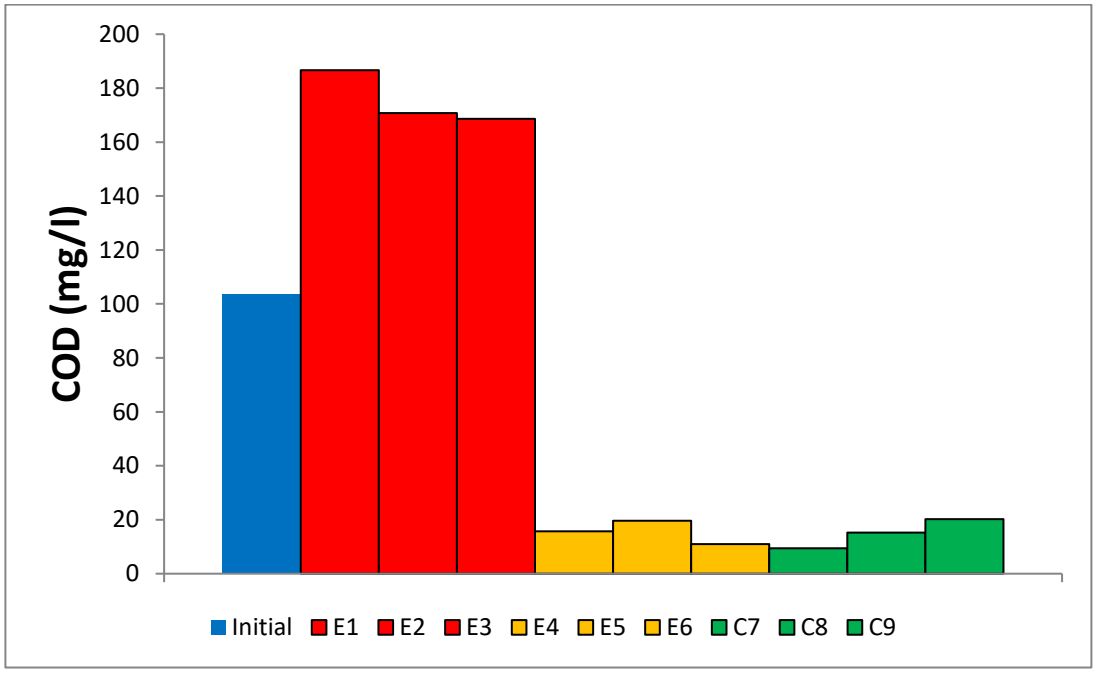

Figure 72: Initial and final COD for $2x10^7$ CFU/ml and unwashed $2x10^7$ CFU/ml *B. globigii* in activated sludge.

NH_3-N

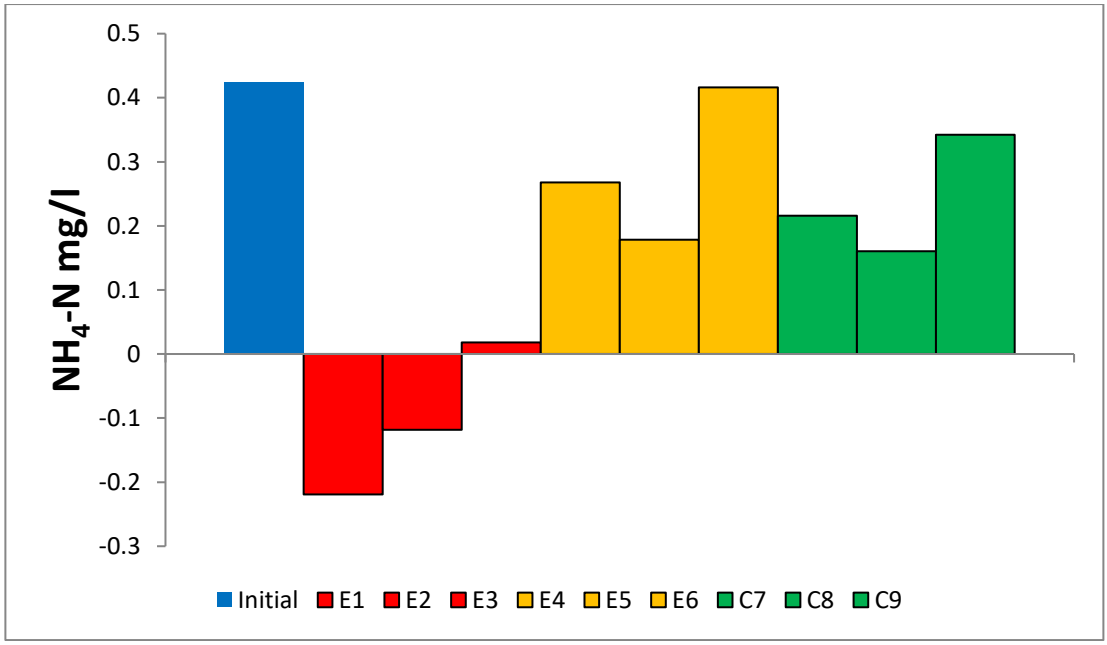

Figure 73: Initial and final NH_3-N for $2x10^7$ CFU/ml and unwashed $2x10^7$ CFU/ml *B. globigii* in activated sludge. Negative values are below LOD.

NO_3-N

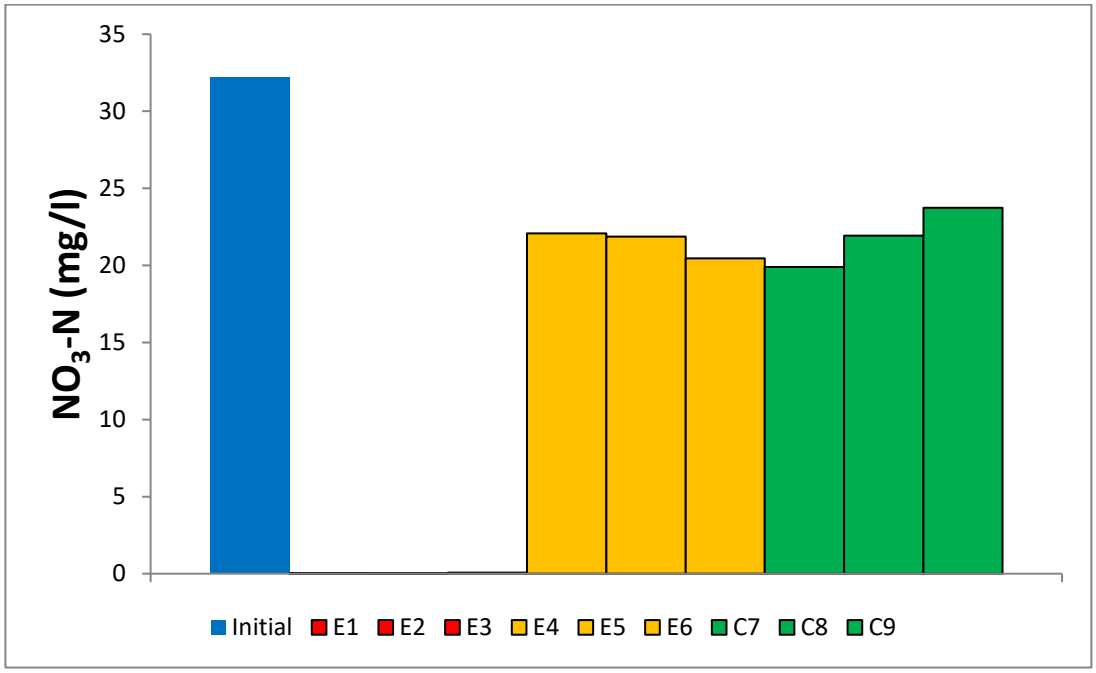

Figure 74: Initial and final NO_3-N for $2x10^7$ CFU/ml and unwashed $2x10^7$ CFU/ml *B. globigii* in activated sludge.

Unwashed 2x10^1 CFU/ml

7 Sept 2016

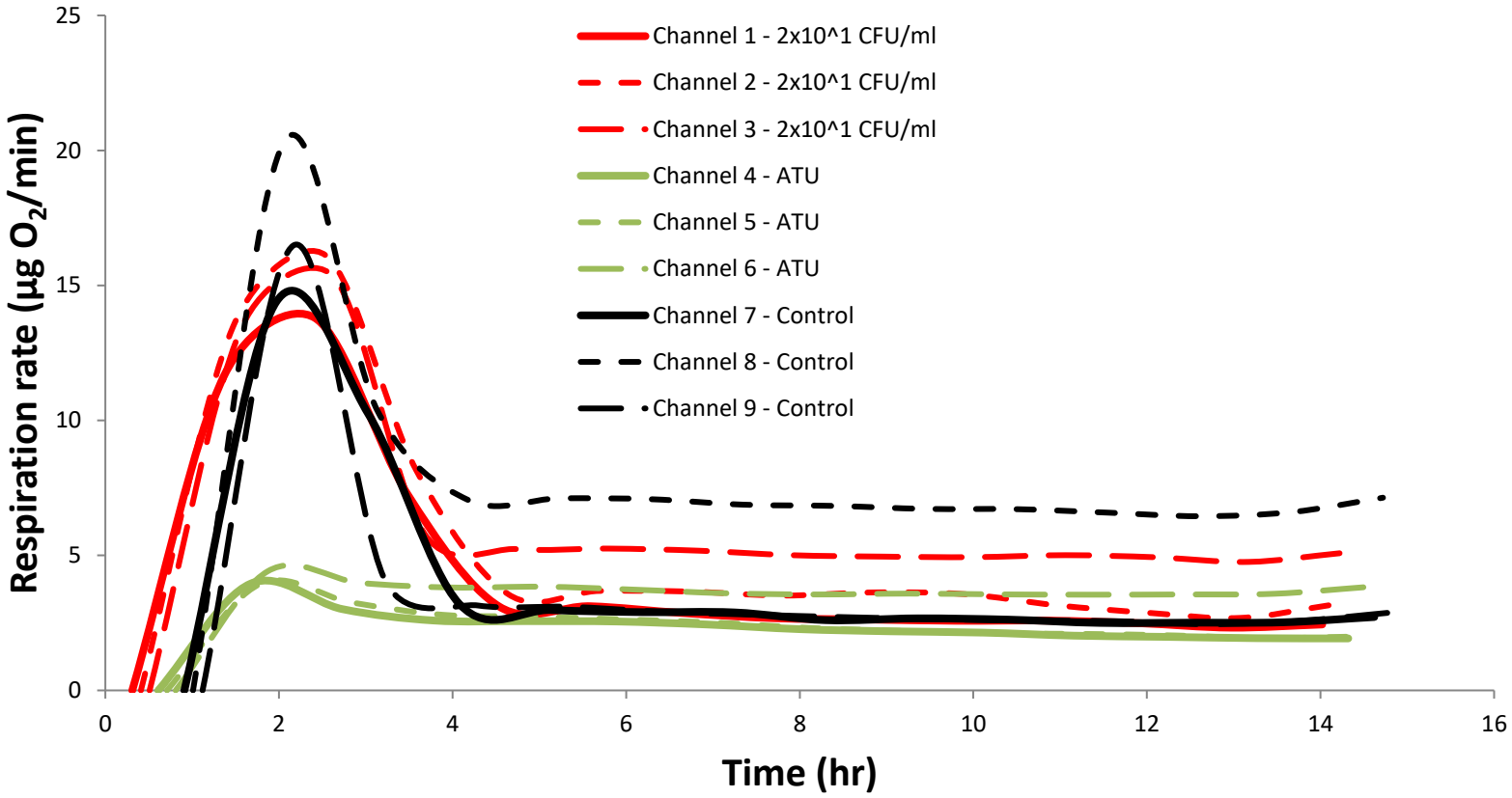

Figure 75: O_2 consumption profile of unwashed 2x10^1 CFU/ml *B. globigii* spores in activated sludge.

COD

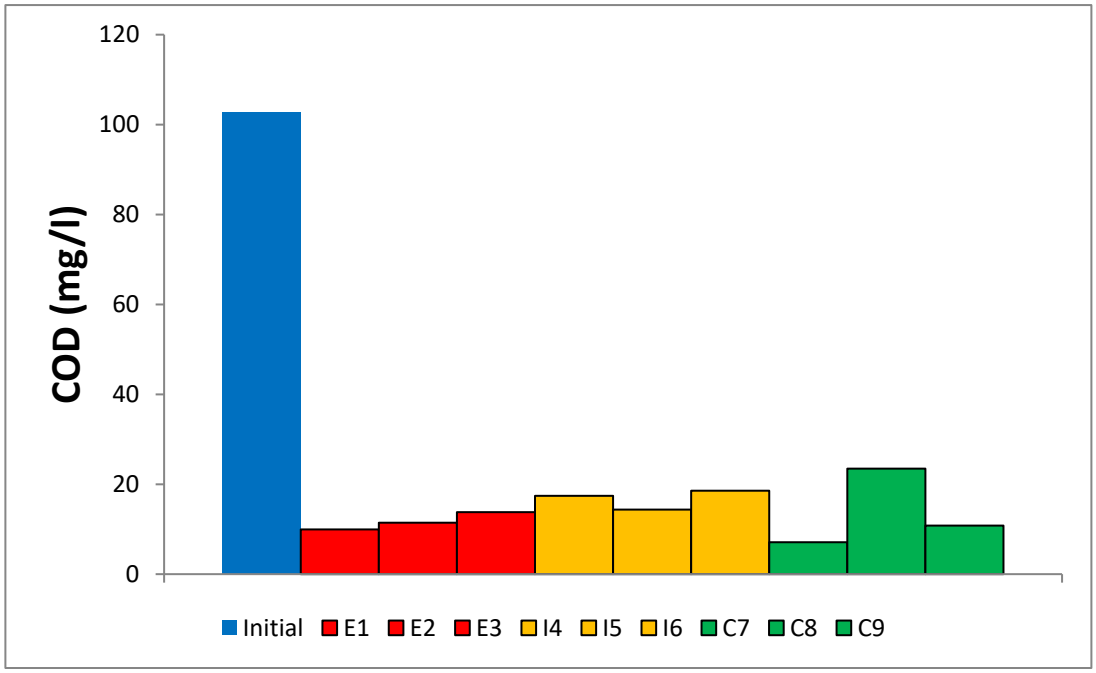

Figure 76: Initial and final COD for unwashed 2x10[1] CFU/ml *B. globigii* in activated sludge.

NH_3-N

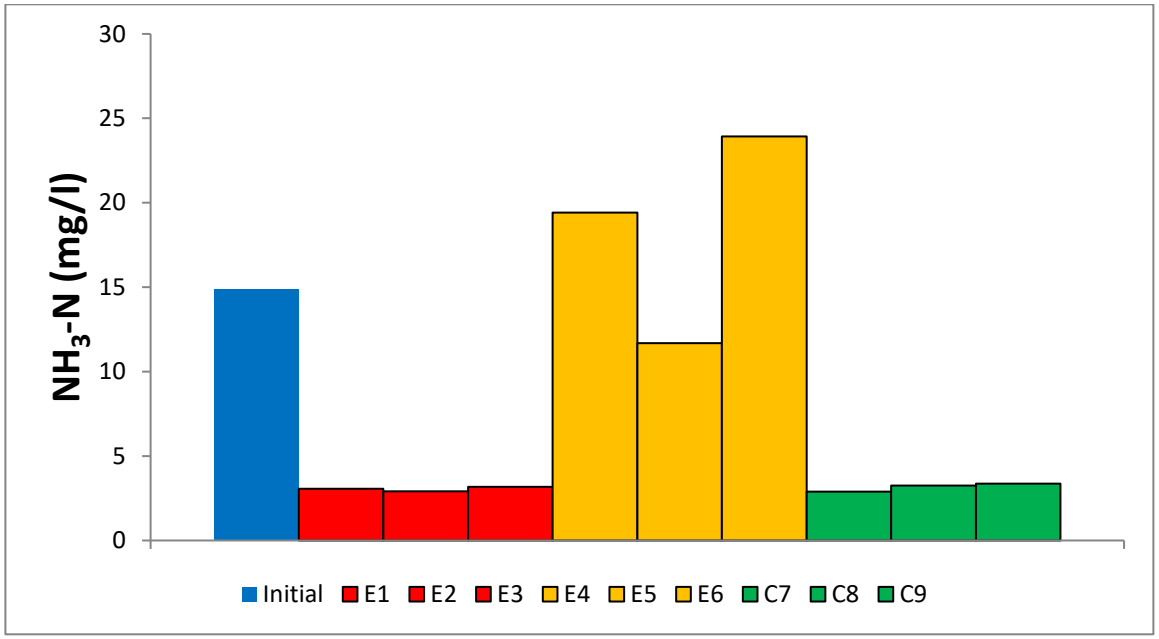

Figure 77: Initial and final NH_3-N for unwashed 2x10[1] CFU/ml *B. globigii* in activated sludge.

NO_3-N

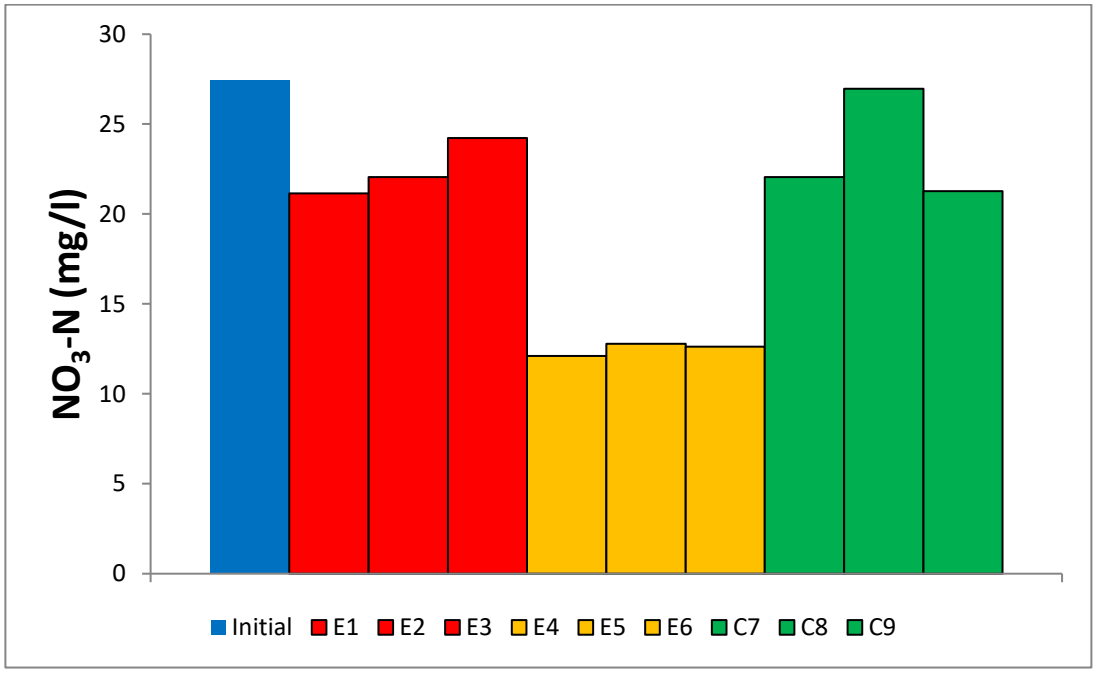

Figure 78: Initial and final NO_3-N for unwashed $2x10^1$ CFU/ml *B. globigii* in activated sludge.

1 Jun 2016

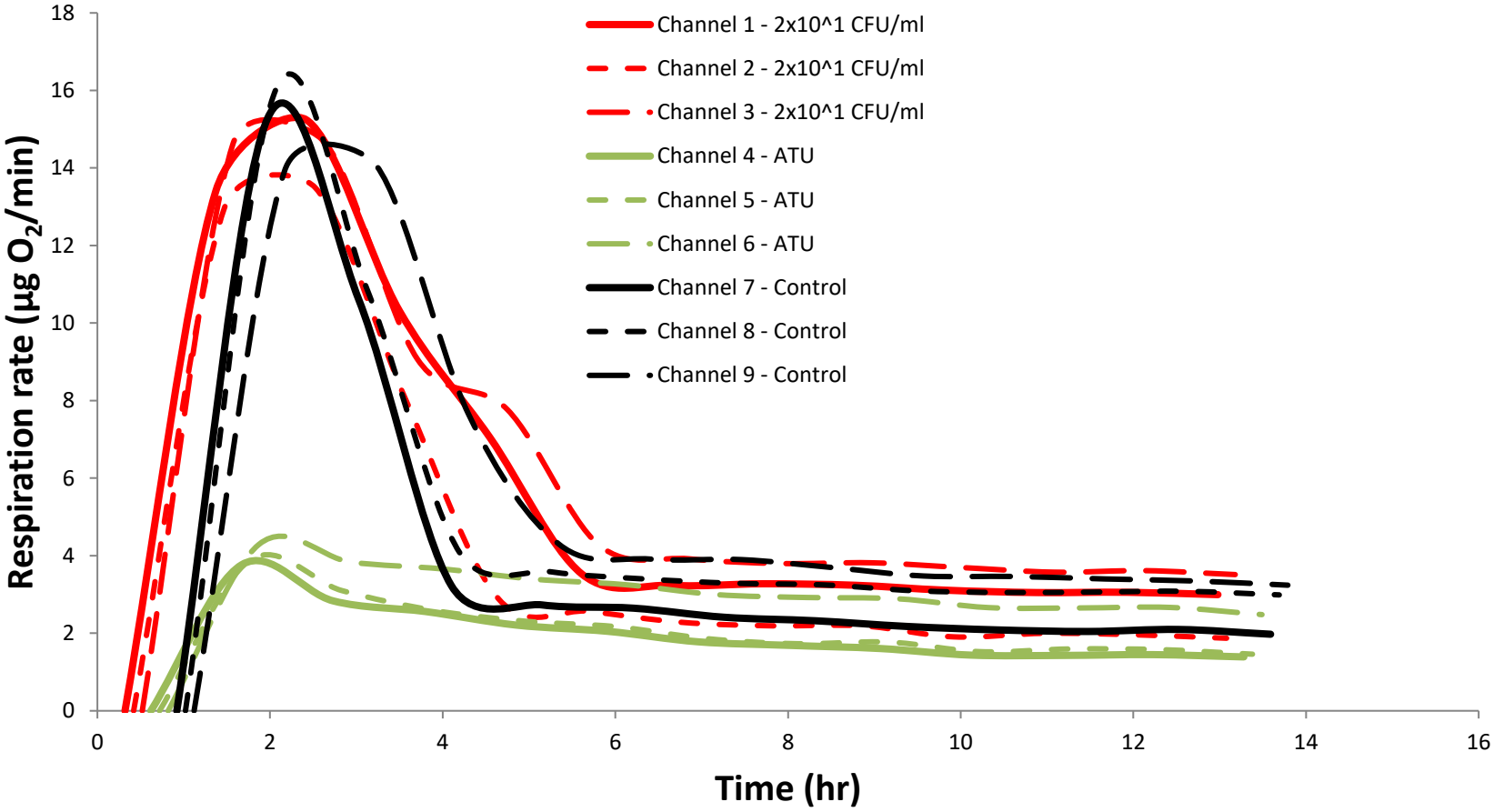

Figure 79: O_2 consumption profile of unwashed $2x10^1$ CFU/ml *B. globigii* spores in activated sludge.

5 May 2016

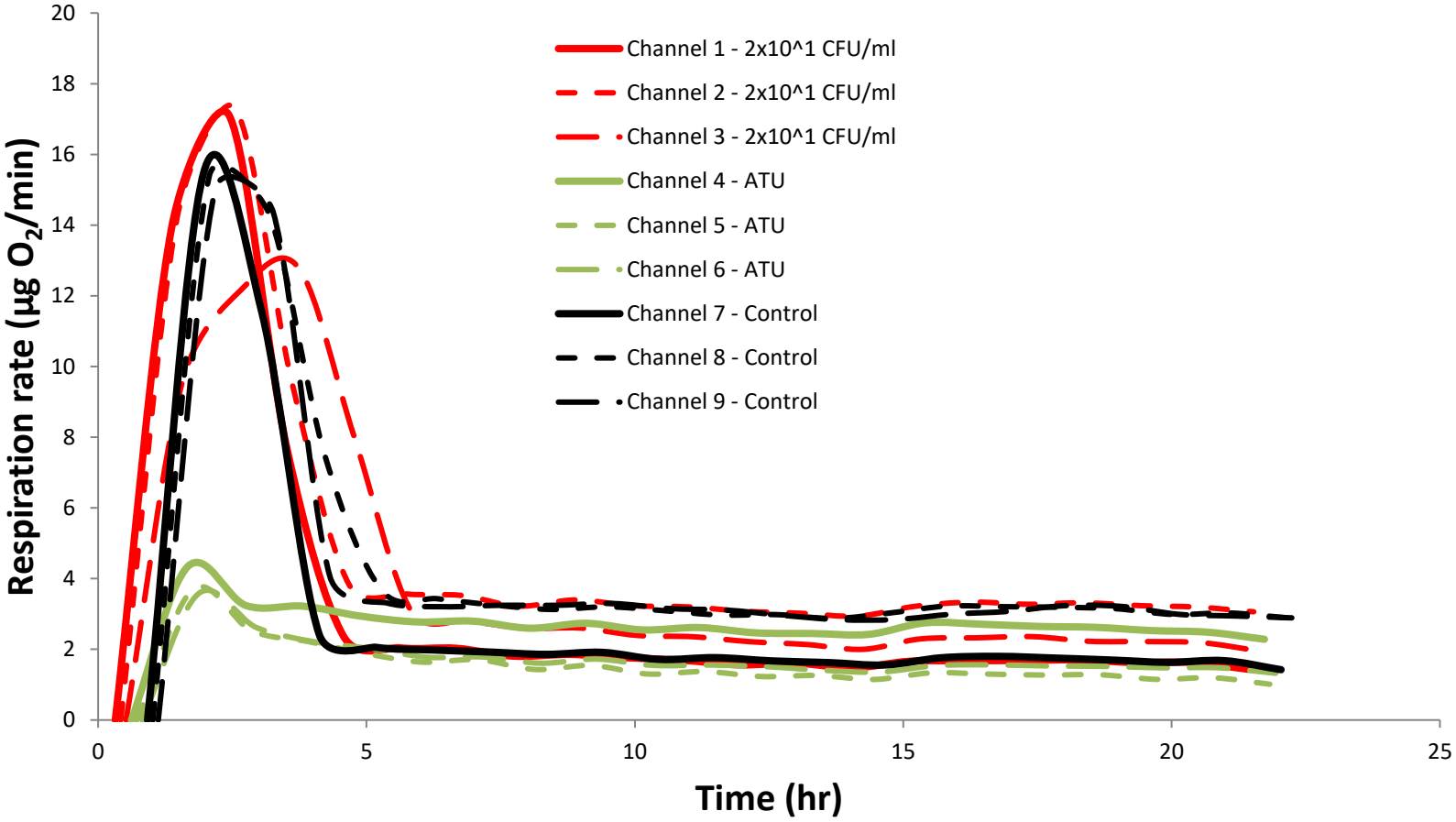

Figure 80: O_2 consumption profile of unwashed $2x10^1$ CFU/ml *B. globigii* spores in activated sludge.

Unwashed 2x10^3 CFU/ml

31 Aug 2016

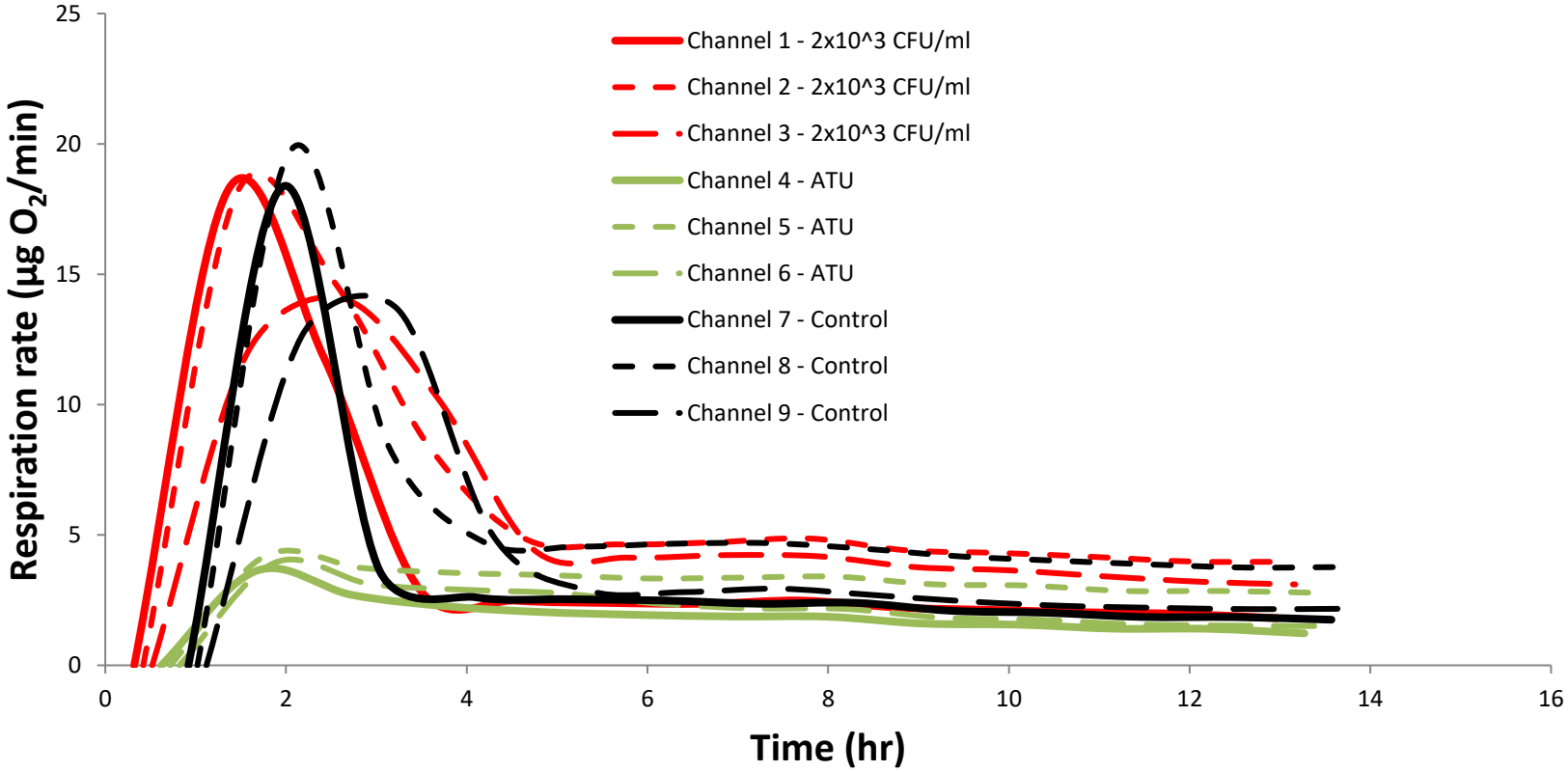

Figure 81: O_2 consumption profile of unwashed 2x10^3 CFU/ml *B. globigii* spores in activated sludge.

COD

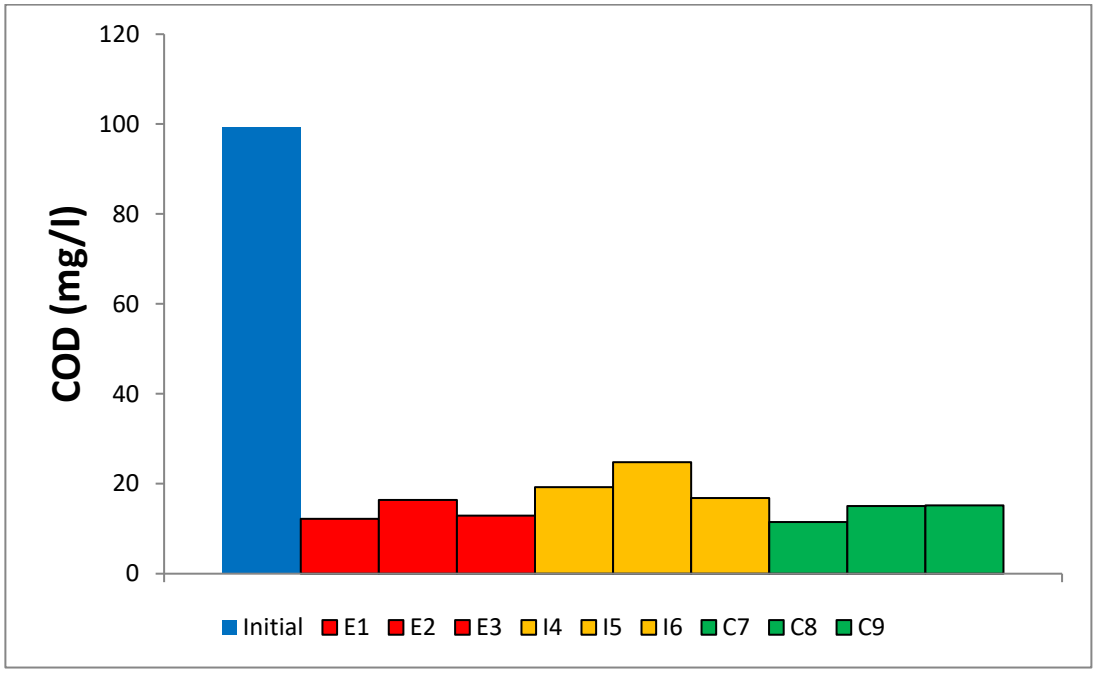

Figure 82: Initial and final COD for unwashed 2x10^3 CFU/ml *B. globigii* in activated sludge.

NH_3-N

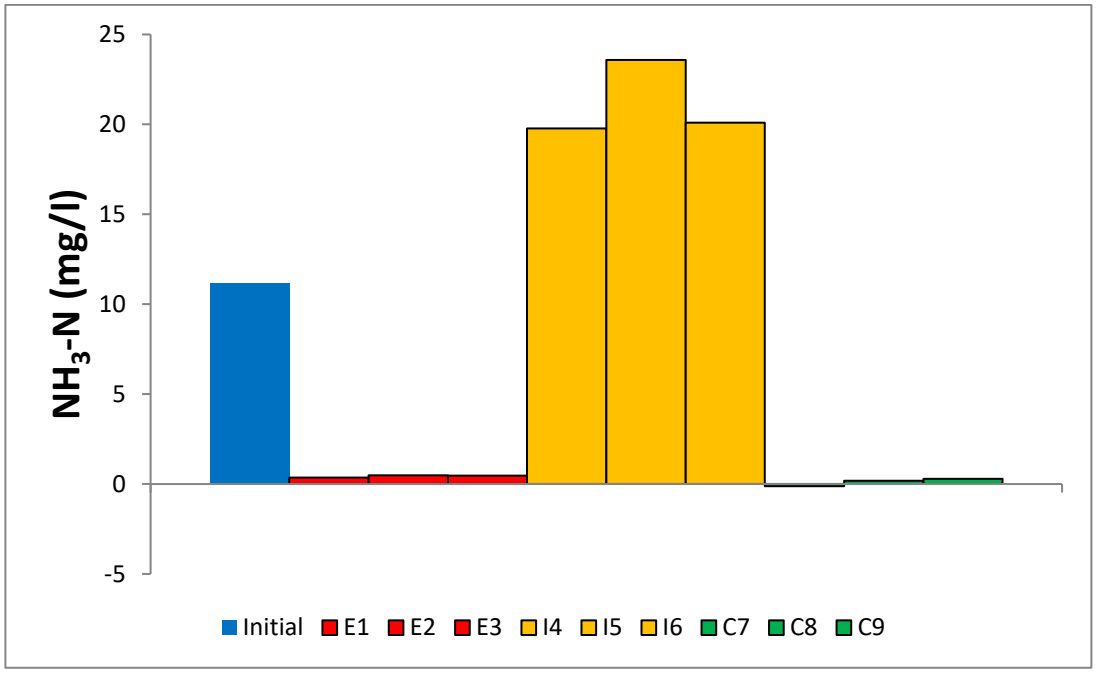

Figure 83: Initial and final NH_3-N for unwashed 2x10^3 CFU/ml *B. globigii* in activated sludge.

NO3-N

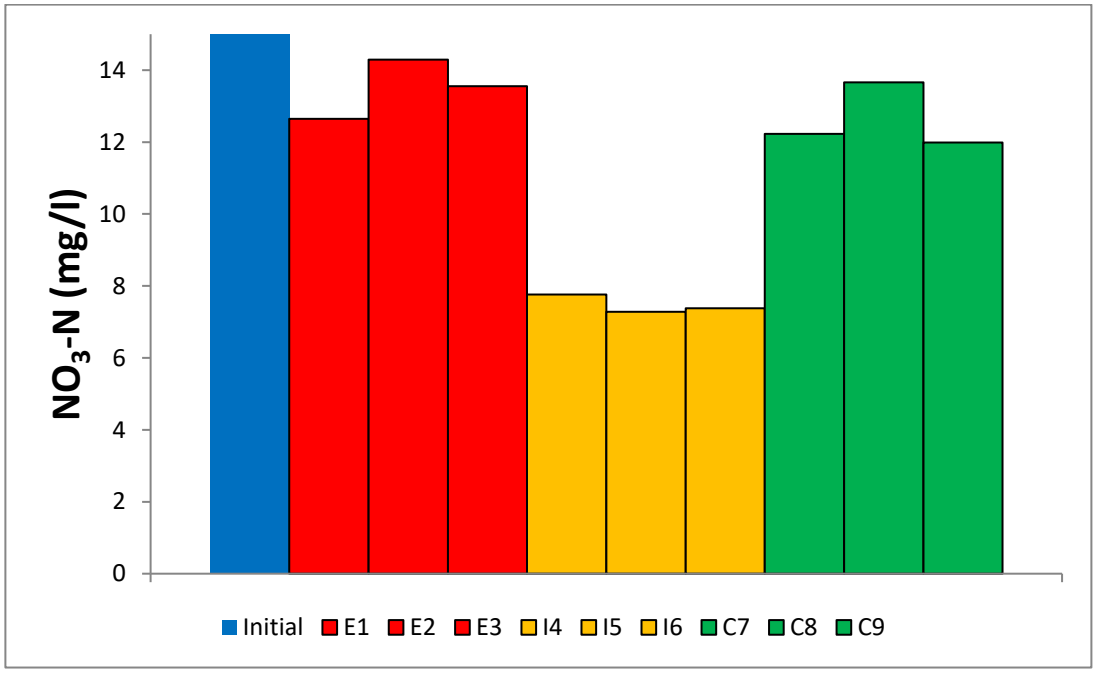

Figure 84: Initial and final NO_3-N for unwashed $2x10^3$ CFU/ml *B. globigii* in activated sludge.

25 May 2016

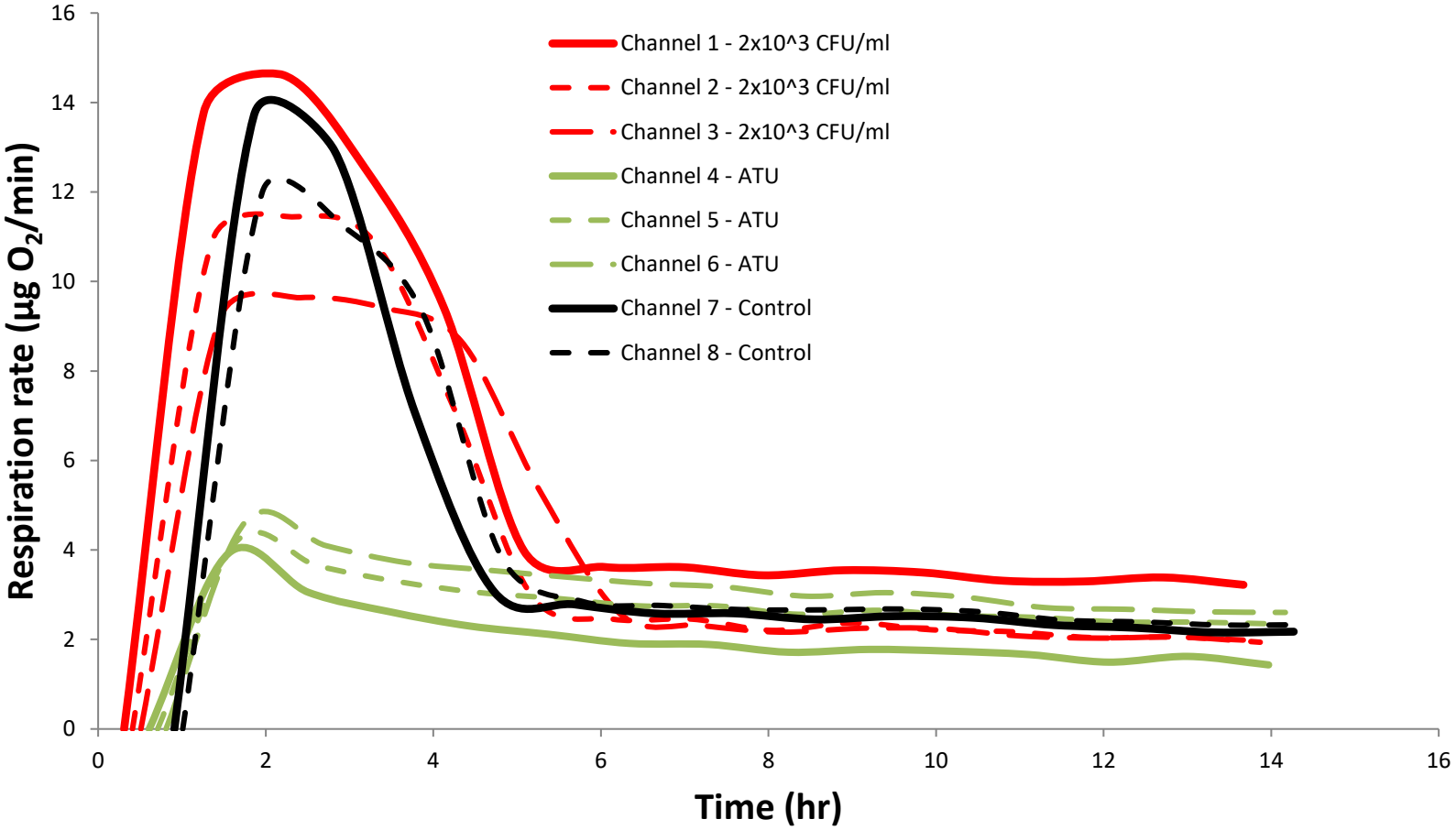

Figure 85: O_2 consumption profile of unwashed $2x10^3$ CFU/ml *B. globigii* spores in activated sludge.

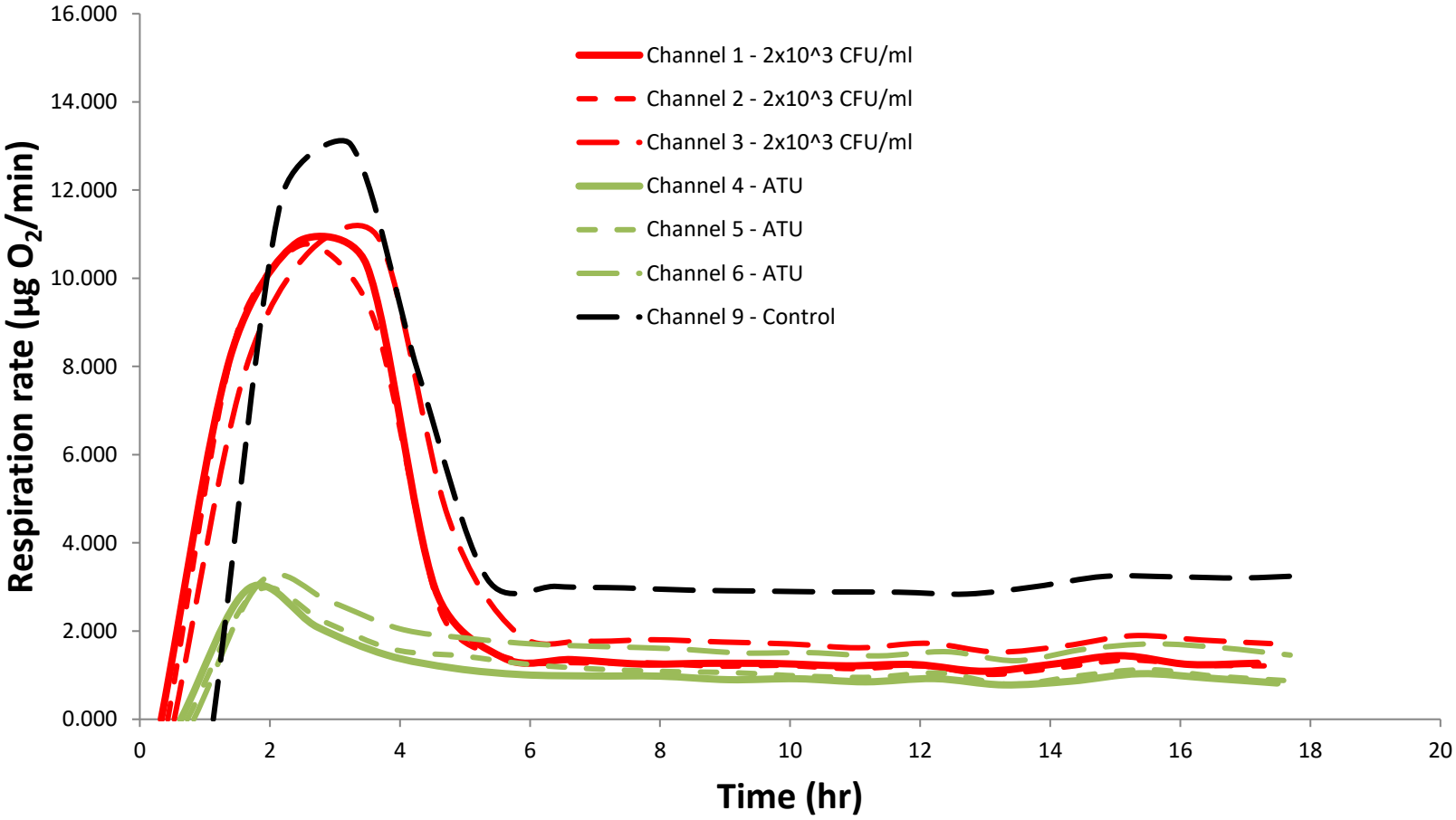

Figure 86: O_2 consumption profile of unwashed $2x10^3$ CFU/ml *B. globigii* spores in activated sludge.

Unwashed 2x10^5 CFU/ml

24 Aug 2016

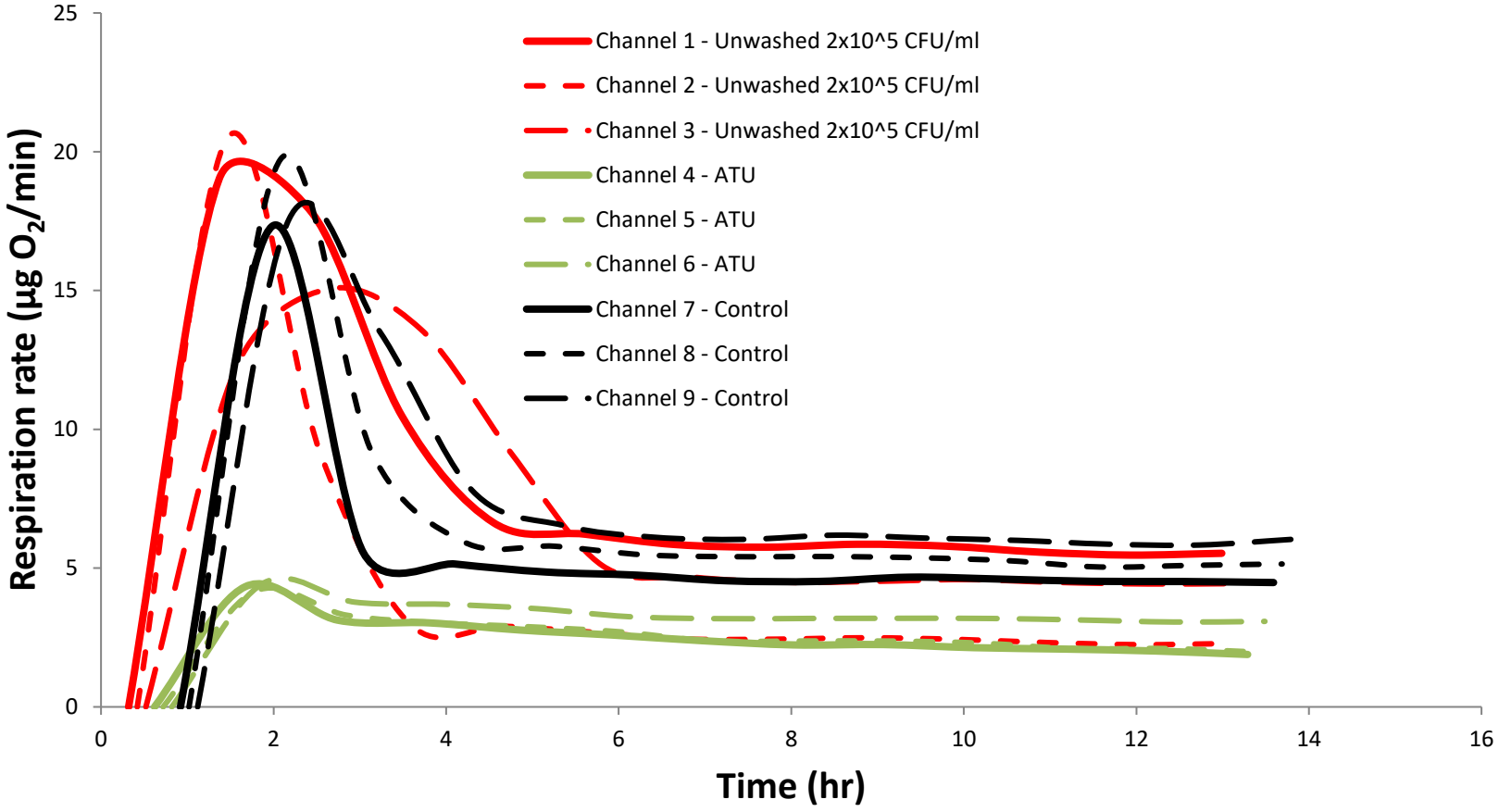

Figure 87: O_2 consumption profile of unwashed 2x10^5 CFU/ml *B. globigii* spores in activated sludge.

COD

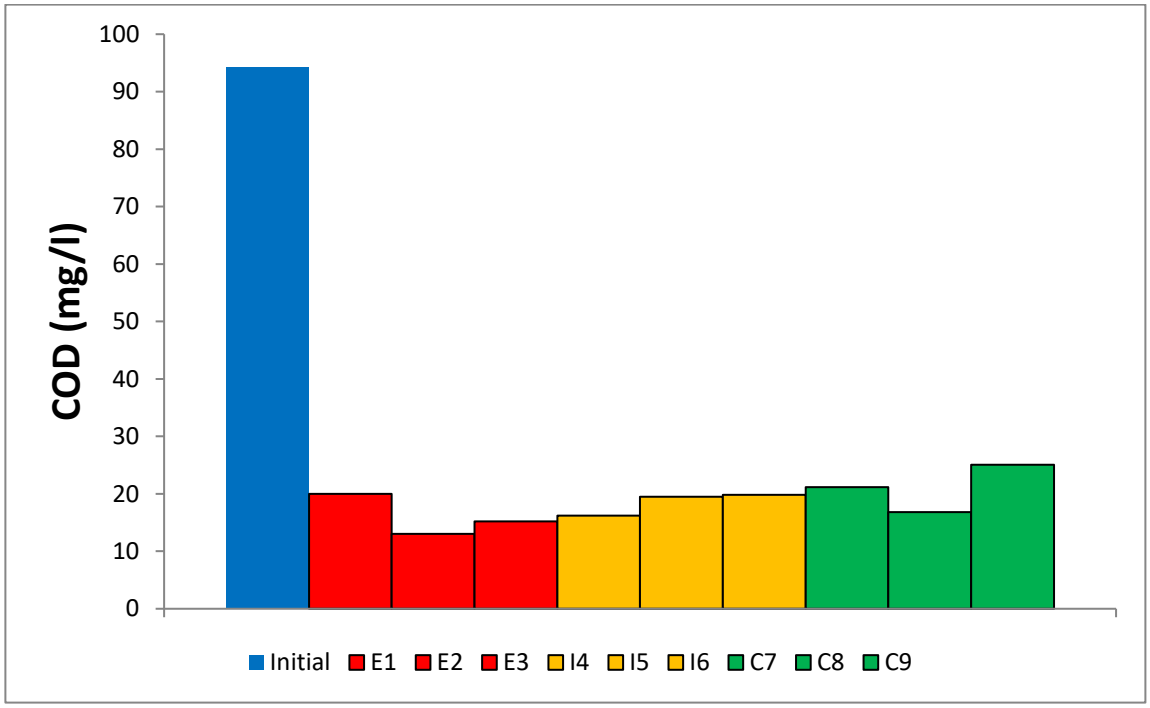

Figure 88: Initial and final COD for unwashed 2x10^5 CFU/ml *B. globigii* in activated sludge.

NH_3-N

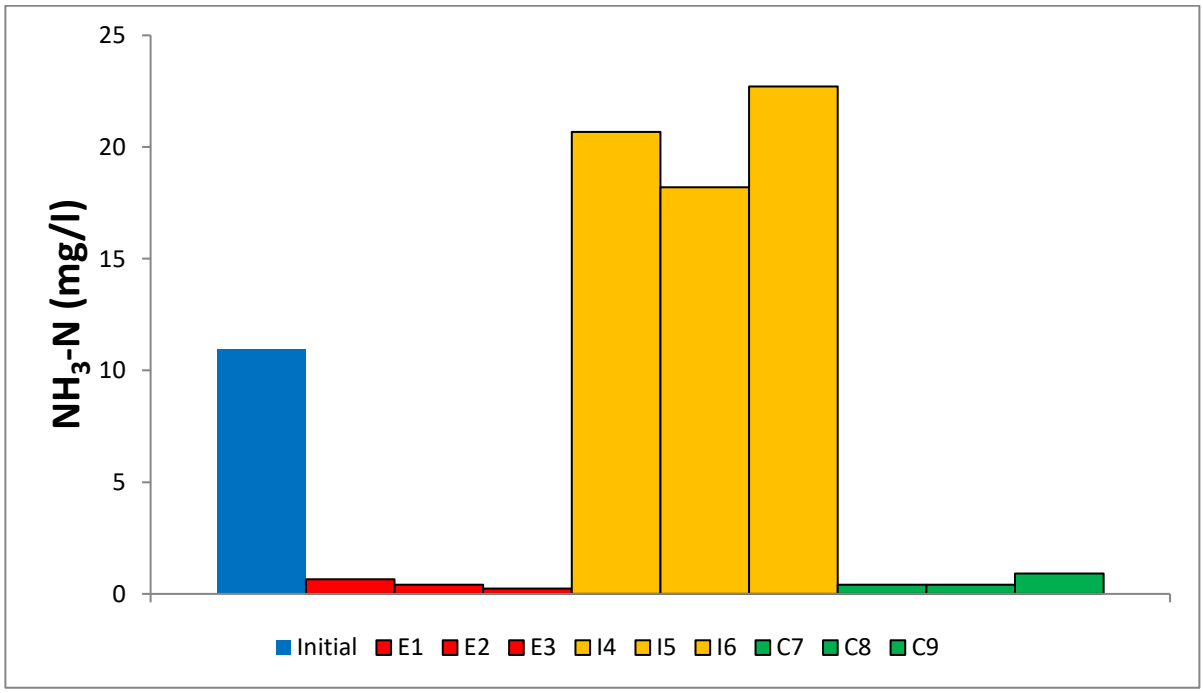

Figure 89: Initial and final NH_3-N for unwashed 2x10^5 CFU/ml *B. globigii* in activated sludge.

NO_3-N

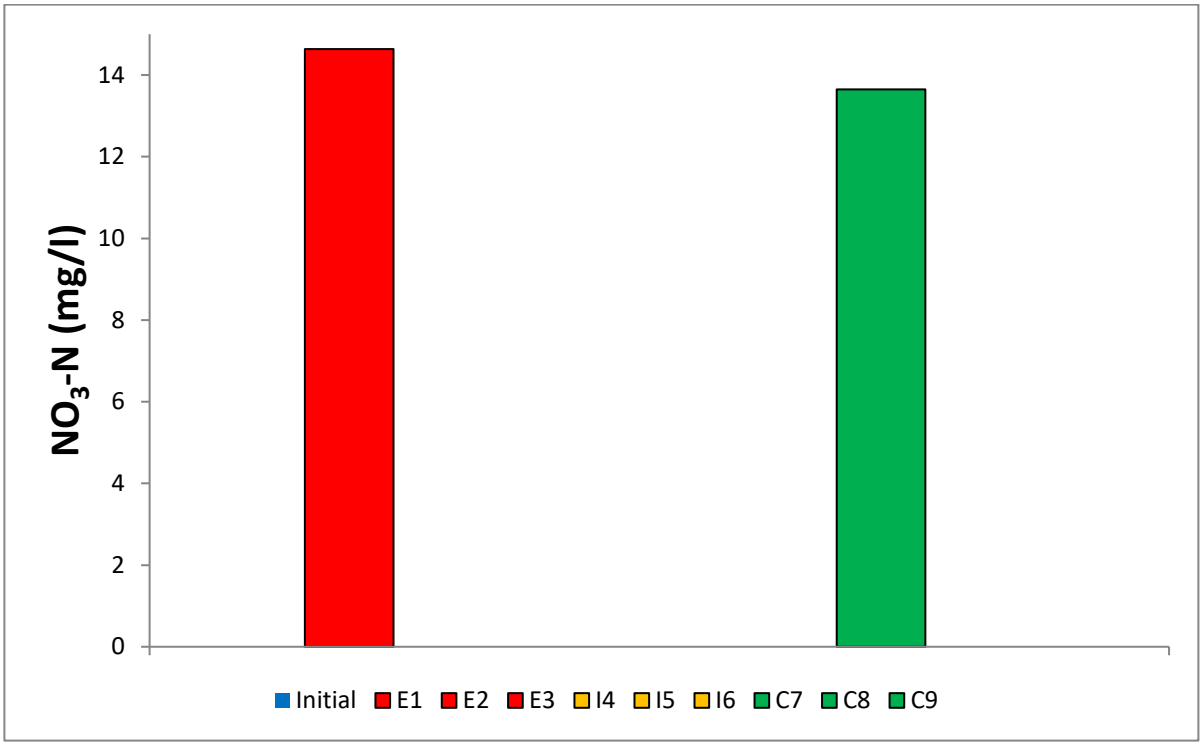

Figure 90: Initial and final NO_3-N for unwashed $2x10^5$ CFU/ml *B. globigii* in activated sludge.

19 May 2016

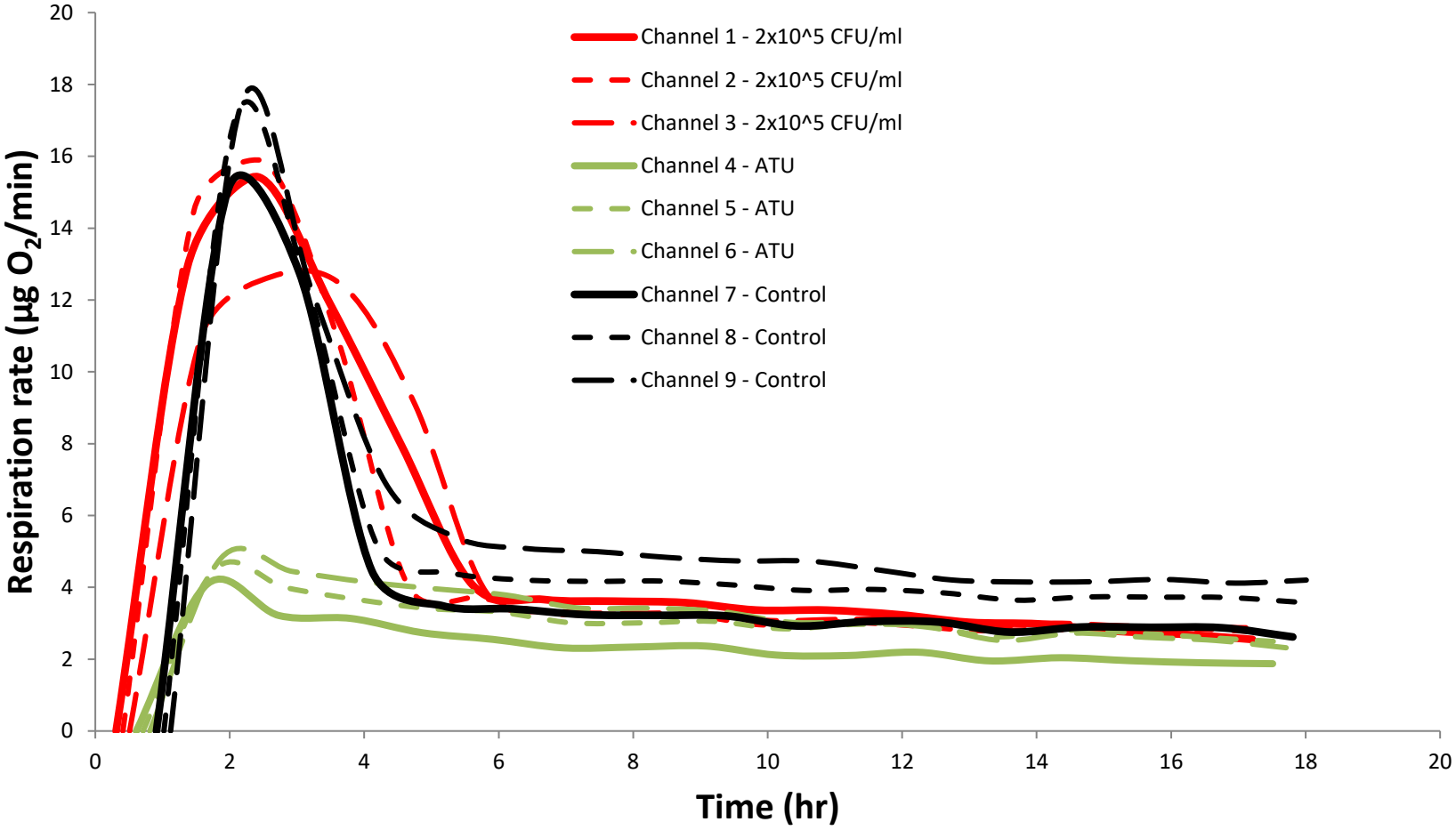

Figure 91: O_2 consumption profile of unwashed $2x10^5$ CFU/ml *B. globigii* spores in activated sludge.

21 Apr 2016

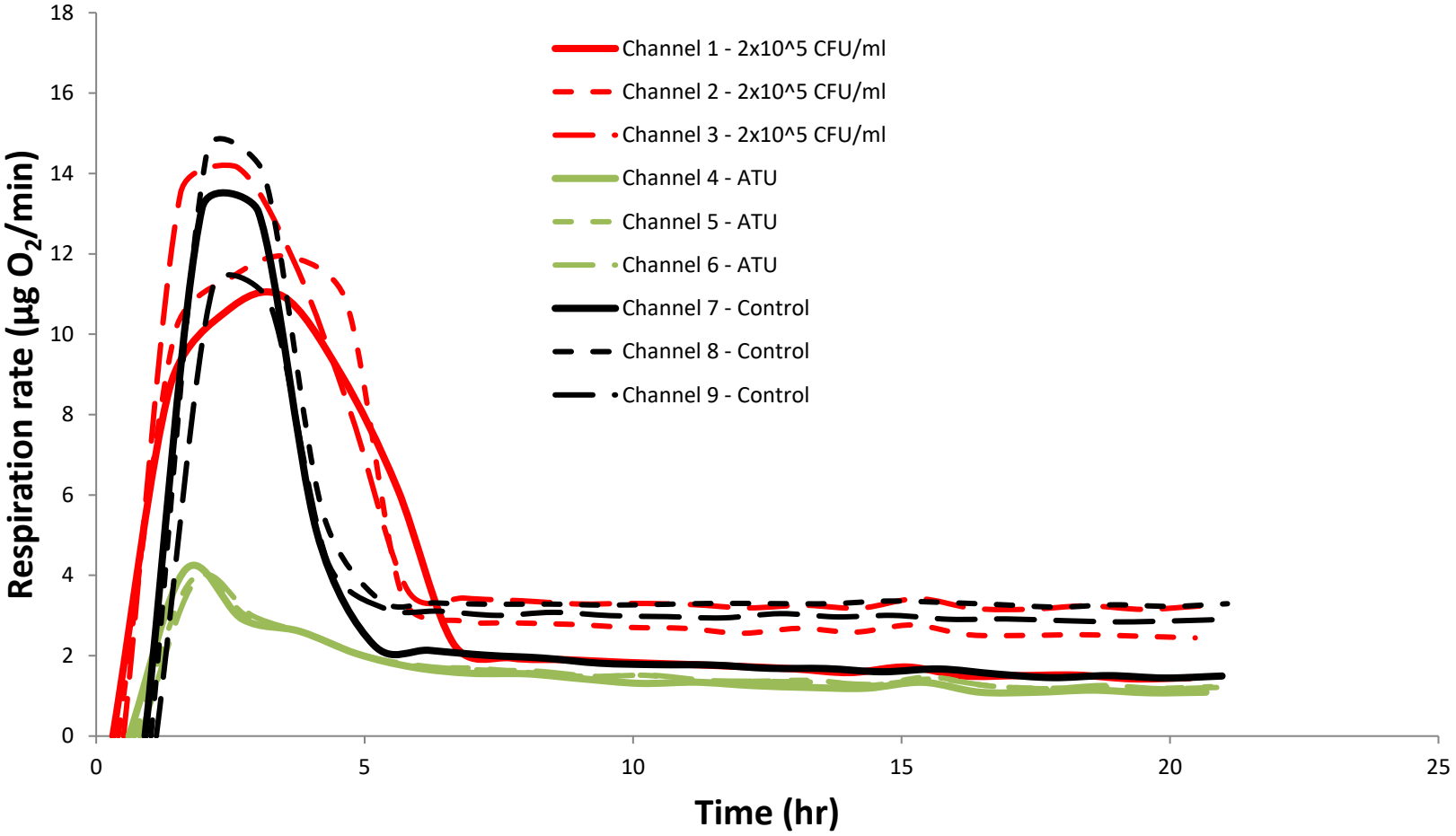

Figure 92: O_2 consumption profile of unwashed 2x10^5 CFU/ml *B. globigii* spores in activated sludge.

2x10^7 CFU/ml with ethanol

17 Aug 2016

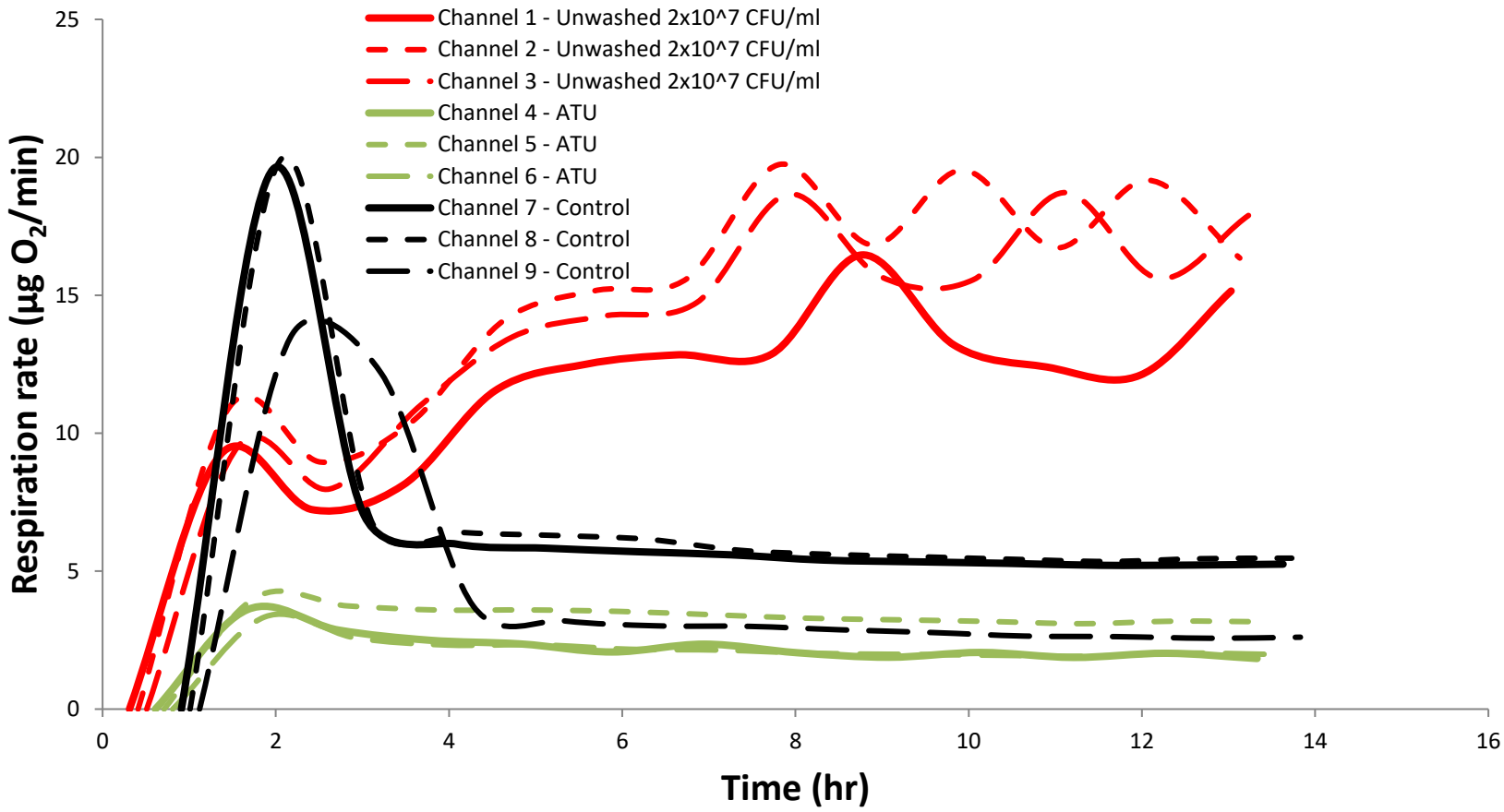

Figure 93: O_2 consumption profile of unwashed 2x10^7 CFU/ml *B. globigii* spores in activated sludge.

COD

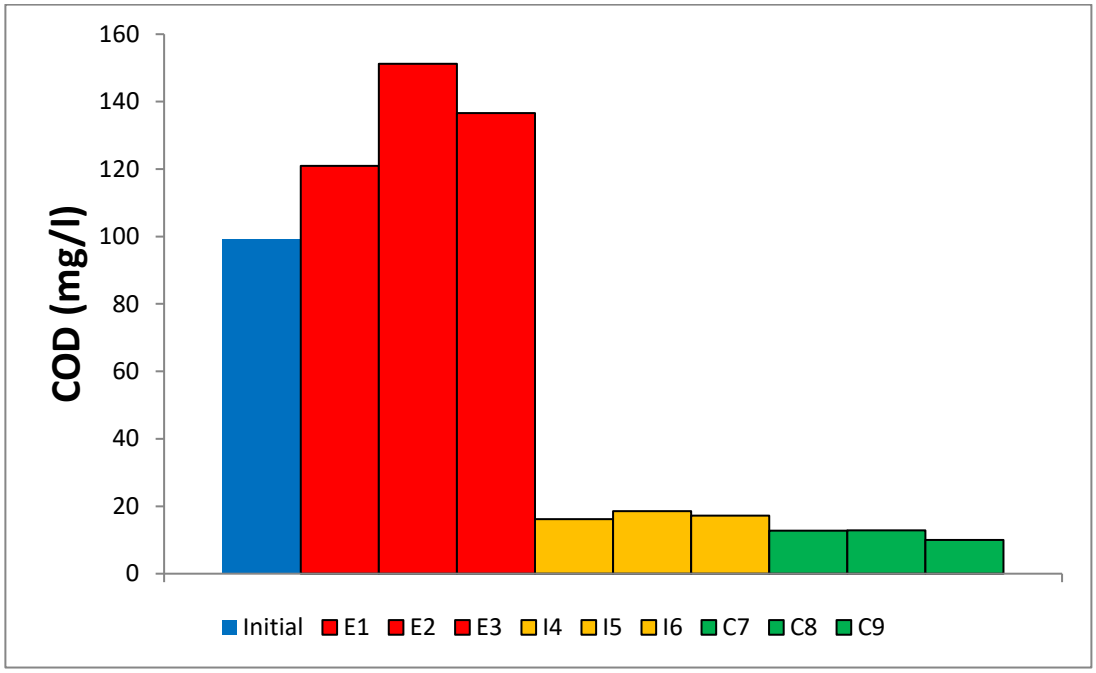

Figure 94: Initial and final COD for unwashed 2x10^7 CFU/ml *B. globigii* in activated sludge.

NH_3-N

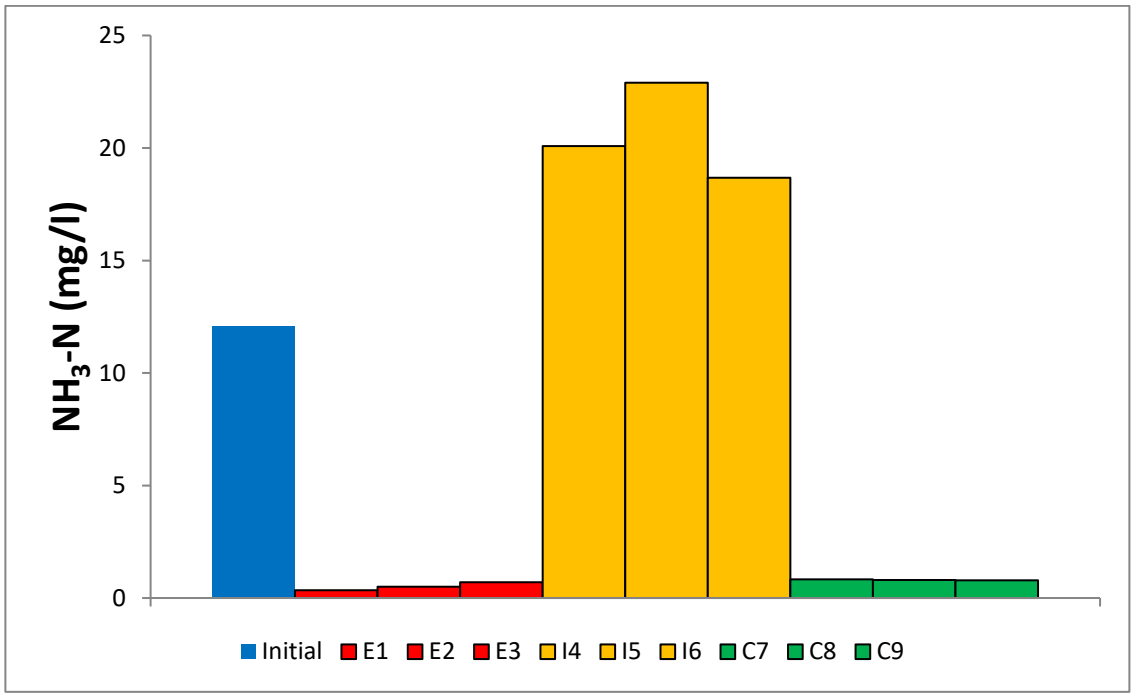

Figure 95: Initial and final NH_3-N for unwashed 2x10^7 CFU/ml *B. globigii* in activated sludge.

NO_3-N

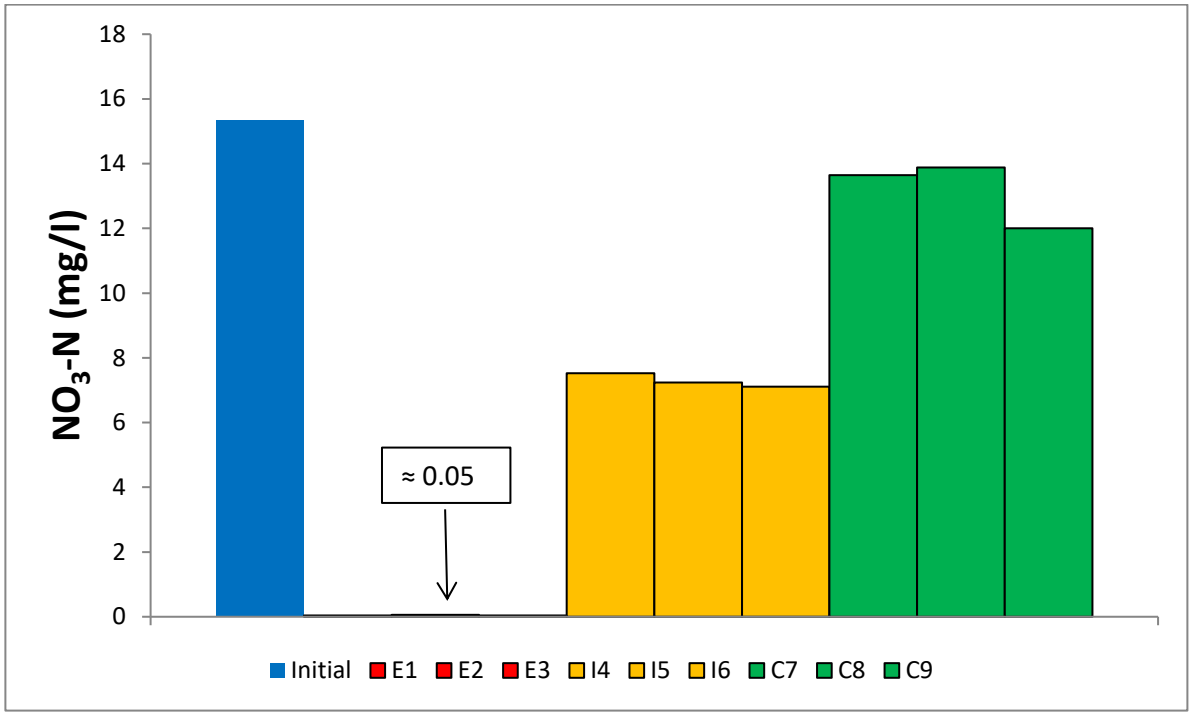

Figure 96: Initial and final NO_3-N for unwashed $2x10^7$ CFU/ml *B. globigii* in activated sludge.

12 May 2016

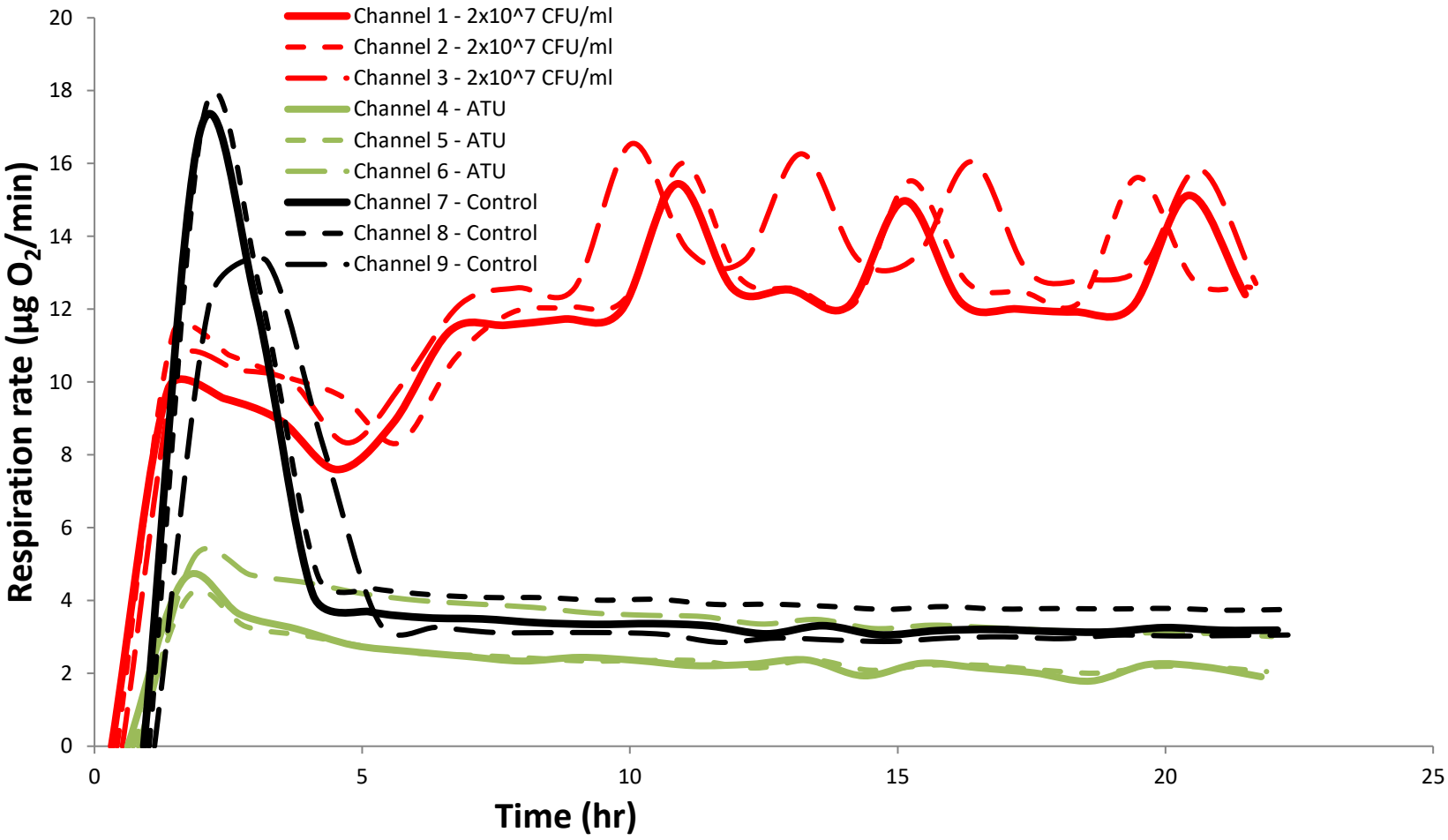

Figure 97: O_2 consumption profile of unwashed $2x10^7$ CFU/ml *B. globigii* spores in activated sludge.

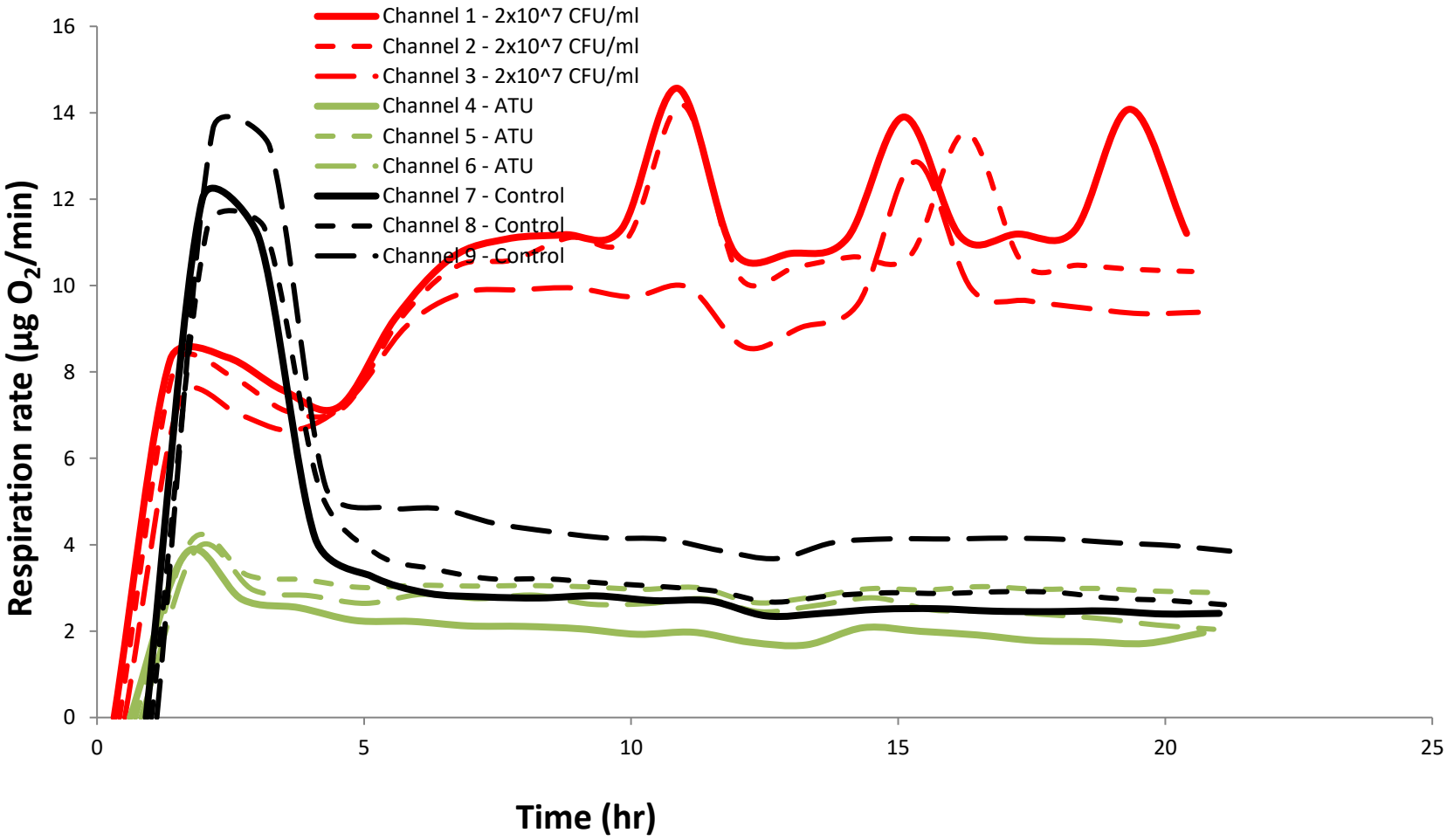

Figure 98: O_2 consumption profile of unwashed $2x10^7$ CFU/ml *B. globigii* spores in activated sludge.

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14. ABSTRACT

This research investigated the effect of a biological warfare surrogate (Bacillus globigii) on activated sludge through respirometry at concentrations from 2x101 CFU/ml to 2x107 CFU/ml. No concentrations caused statistically significant effects on peak (p-values, 0.11 - 0.32) or cumulative O2 (p-values, 0.21 - 0.62) consumption. Molar O2/CO2 ratios were also not impacted. Shape factors were impacted by the introduction of spores (p-values, 8.53 E-06 – 0.95) but these factors proved a poor indicator of biological inhibition when considered in light of other factors. B. globigii did not inhibit COD or N removal. When spores of the same concentrations in their 40% ethanol storage solution were tested, the results at 2x107 CFU/ml were significantly inhibited. Initial peak O2 consumption was 34% to 44% less. Final effluent COD increased to 136.3 mg/l, while NO3-N was reduced. Biological treatment was inhibited by ethanol, not B. globigii. Spore germination was observed at 0.88% to 1.7%. Spores preferentially adhered to the floc (81%) verse bulk liquid (19%). These results illustrate that B. globigii is unlikely to cause short-term interference with biological treatment at an activated sludge plant, but organic co-contaminants may cause inhibition. Spore germination may

15. SUBJECT TERMS

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