THE EFFECTS OF BACTERIAL ROLES ON THE FORMATION OF DISINFECTION BY-PRODUCTS UNDER METAL INDUCTION AND BIOFILM RESPONSE TO ANTIBIOTICS

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Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
MAY, 2019

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ACKNOWLEDGEMENTS

Winston S. Churchill once said, “The greatest lesson in life is to know that even fools are right sometimes.” I was taught to try getting everything right all the time ever since my young ages. For a long time in my past school life, I feel myself like a fool whenever I failed in the exam, made mistakes in experiment, and answered questions wrong. I have passions on research but I was scared of failures. But I am right on choosing my advisor, Dr. Mark J. Krzmarzick, who supports me without condition, bears with all my mistakes, and encourages me all the time. With him, I am able to try my crazy ideas and face every failure, because I know he is always there ready to give me a hug. And even this dissertation is one of my brainstorm that began with “zero” in our lab. Dr. Krzmarzick has never ignored students’ idea, he helped me do literature reviews, logical reasoning, and proposals. There is no way for me to finish this study without all of these. For me, he is my mentor, my friend, and my idol.

I also appreciate the lessons and helps from Dr. Gregory Wilber and Dr. David Lampert. They are always nice and patient to reply my stupid questions. Dr. Wilber taught me a lot of experiment skills and comforted me whenever I lost confidence on myself; Dr. Lampert has many excellent research ideas and very skillful modelling knowledge, his class is always hard but pretty valuable. All of the moments getting along with them are wonderful memories.

Especially, I want to appreciate Dr. Erika Lutter very much, she taught me in protein experiments that I hoped to do since my first year. It was so exciting when I finally run out my SDS-PAGE gel, which was made of compounds that I borrowed from her lab. There is not possibility to make it without her unreserved supports. Her advanced skills and warm smiles are very fantastic impression forever in my mind.

And I will never forget all the days doing study with my colleagues and friends, Brice Fiddler, Tania Lozville, Melissa Evans, Jeanne Carroll, Xuewen Wang, Chunmei Xiong, Matt Brooks, David Leavitt and David Taylor. They brought me go outside of the academy into the field and enjoy the nature; they encourage me to take challenge on discovering the new world. Their companions help me get through those hard days and make me a better man.

Last but not the least, I thank my love, Shun Li, who believes in me without condition and who gives me strength to purchase my dream. Our relationship is the brightest light in my sky whenever I feel sad and suspect myself. There is not any appropriate word to describe how much I cherish it!

Studying in OSU with all of these is such a magic journey, I sincerely appreciate.

Name: Xiang Fu

Date of Degree: MAY, 2019

Title of Study: THE EFFECTS OF BACTERIAL ROLES ON THE FORMATION OF DISINFECTION BY-PRODUCTS UNDER METAL INDUCTION AND BIOFILM RESPONSE TO ANTIBIOTICS

Major Field: CIVIL ENGINEERING

Abstract: This dissertation had studied the differences between chemical and biological reaction of DBP production and provide a mechanistic understanding for the sources of precursors under which DBP formation varies. This study had covered: (1) The formation of DBPs during chlorination due to biological effects studied; (2) the protein expression so as to study the potential mechanisms and further to develop possible methods to predict DBP formation; and (3) bacterial response to antibiotics in biofilm. The relationship of two prevalent bacteria and four common metal elements were studied. And the separation of intracellular organic matter, extracellular organic matters and pure culture organic matters clearly showed the differences in DBP distribution among them. The results imply that (1) the correlation between cells and metals possesses potential mechanism to influence DBP formation; (2) metal-related proteins such as metal-binding proteins and metal activated transporters on membranes could be potential DBP precursors; (3) Bacterial community could be influenced by environmental pressures such as antibiotics. This study proved that bacterial organic matters could be very possible DBP precursors with comparison between *in vivo* bacterial cells and extracted organic matters; and the addition of metals assisted my research to exploit potential cellular mechanism inside cells. And antibiotic resistant biofilm validated some aspects of biological response that possibly relate to DBP formation. This study of the formation of DBPs could be benefited not only for better water treatment, but also for studies of bio-resistance, bio-metabolism and artificial biochemical compounds.

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CHAPTER I

INTRODUCTION

Disinfection is one of the most important process in water treatment plants that guarantees the safety of drinking water. However, alongside with killing pathogens, disinfectants may react with chemical and biological compounds in the water to yield by-products called "Disinfection by-products" (DBPs). DBPs could cause illness; by meta-analyses and pooled analyses of epidemiology studies, scientists have demonstrated consistent associations between DBPs and bladder cancer (Villanueva et al., 2006); and DBPs effects for babies being born small for gestational age (Grellier et al., 2010). Water treatment plants in the U.S. use chlorine and chloramine as disinfectants for decades because they can apply long-lasting residuals in water that continuously protect the drinking water from infecting by pathogens during distribution. UV light and ozone are two other popular disinfectants that result in less DBP yielding, however, there are still some problems entangling in DBPs' studies that not only the formation of DBPs is unclear but also the application of new disinfectants that aimed to decrease the production of DBPs unexpectedly produce new DBPs. Under such situations, the understanding of the mechanism of DBPs formation is mandatory for DBP formation prevention or DBP treatment. Natural organic matter (NOM) is seemed as the major source of DBPs (Siddiqui et al., 2000; Kim et al., 2005; Fang et al., 2010), the reactions between disinfectants (such as chlorine, chloramine and ozone) and NOM had been reported (Li et al., 2017). However, even though a lot of study has paid attention to NOM to figure out the mechanical characteristics and the potential pathways of DBP yielding

(Chang et al., 2001; Xie, 2003; Hua and Reckhow, 2007), the findings are limited because of many unexplained phenomenon. Critically, NOM is a large group of organic complexes, including thousands of carbohydrate compounds such as humic substances, carboxylic acids, amino acids and proteins (Bond et al., 2011; Fu et al., 2017). Also, NOM is structurally diverse, including not only natural chemical compounds, but also biological organic matter from animals, plants and microorganisms. There is a question of what kind of NOM are the most potential precursors of DBPs? It has not yet been well explained until now. Beside the studies of chemical compounds, the DBP precursor aspects of biological matter has also recently been reported, specifically biomolecules in biofilm and bacteria cells (Sharma et al., 2017; Wang et al., 2013; Hong et al., 2008; Navalon et al., 2008; Wang and Zhang, 2010; Wei et al., 2011). Reckhow and Kim (2008) found that two bacterial-organic compounds named l-aspartic acid and l-asparagine could be reactive DBP precursors, and the study of Bond (2011) also revealed that glucose and mannose are potential precursors of DBPs. With these new findings, some researchers debated that bacteria may contain or absorb extracellular matters to react with disinfectants. Williams et al. (2019) indicated that dissolve organic matter (DOM) composition may play an important role in DBP formation, for instance, Human urea, which is a prevalent DOM, can increase the DBPs in swimming pools (Kim et al., 2002; Blatchley and Cheng, 2010; Laat et al., 2011); the DBP production from algal extracellular organic matter (EOM) had revealed a straight relationship (Plummer and Edzwald 1998; Li et al., 2012; Xing et al., 2018); and Wang et al. (2013) indicated that extracellular polymers substances (EPS) from cells may be certain precursors of DBPs. Thus, bacteria or bacterial organic matter may be potential suppliers of DBP precursors which could significantly make a change on DBP formation. In combination of these points, cellular matter of bacteria could be reasonable sources of DBP formation.

Interaction between bacterial matter and metals during disinfection processes could be a new frontier for DBP studies. Variable physical-chemical factors influence bacterial physiology, especially metals. Metal ions play essential roles in nearly all of bacterial metabolism. It is interesting that the

results of investigating metal ions, such as Cu^{2+}, Ca and Fe, into disinfection process had revealed apparent changes of DBPs production under the influences of metal ions (Navalon et al., 2009; Liu et al., 2011; Zhao et al., 2016). Based on the reports of metal ion studies, metal-related proteins are considered to be potential precursors because bacteria matters such as hydrophobic ligands, amino acids and proteins require the binding of metals to activate their functions (Makoto and Katsuyuki, 1996; Biros et al., 2007). However, a lot of research only took this phenomenon as chemical transformation, ignoring the fact that those reactions were mainly performing on the surface of bacteria instead of the liquid (Wang et al., 2012). It can infer that regulatiors of those reactions are just inside the bacterial cell.

Sharma (2017) found that not only water parameters (pH, cationic and anionic constituents, and types and concentration of NOM) could influence the production of DBPs, but also metal ions with moieties of NOM may play important roles. Metals in raw water are certainly consumed by microorganisms because the many gene regulators in bacteria requires metal ions as the central cofactors, and the receptor proteins in the cells or on the cell membrane can act ask a key to repress or activate gene regulation by binding or releasing metal ions (Hennecke, 1990). For instance, a protein called “Fur” can be activated by metal to act as a repressor by binding to a specific recognition sequence on the DNA that overlaps the promoter sequences so as to stop the transcription of target gene; on the other side, protein “NifA” and “Fnr” could both enhance transcription with metals (Hennecke, 1990). Last but not least, oxidational reactions and light-responsive regulations in the cell may also demand the metal ions. Li and Korshin (2002) concluded that “the metal binding functionalities in NOM are closely associated with halogen attack sites”. And the metal binding sites are very common in proteins, the analysis of protein with metal could be the key to reflect the metal induction on DBP precursors, and the bacterial part could be a new way to explore DBP formation.

The cellular effects for DBPs production may due to the bacterial responses to environmental pressure, Chu et al. 2016, found that antibiotic chloramphenicol and its analogues could be precursors

of dichloroacetamide and other DBPs, Wang and Helbling, 2016, also reported the directly relations between antibiotics and DBPs. So that antibiotics should obsess a potential mechanism for DBPs formation. Therefore biofilm resistance to antibiotic may generate DBP precursors.

Overall, it has been found that bacteria are very sensitive to the bioavailability of several metals. Many metals are used directly for energetics, but more universally, metals are required for functions that are more fundamental to functionality and cell maintenance. The binding of metals to proteins is often required for the ability for those proteins to simply fold appropriately and work. Altogether, the concentrations of metal ions in drinking water treatment systems are at sufficiently low levels that changes in these concentrations affects regulatory processes and/or provides key nutrients for many physiological functions. The dynamics of the production of DBP precursors and the degradation of DBPs in water treatment systems are largely unknown but important to developing a more comprehensive understanding of DBP exposures.

In this study, I hypothesize that biological metabolisms could affect DBP precursor formations. The differences between chemical and biological reaction of DBP production will provide a mechanistic understanding for the sources of precursors under which DBP formation varies. This study will help determine (1) DBP formation from bacteria, (2) possible ways to predict and reduce the formation of DBPs from bacteria, and (3) bacterial response to environmental pressure in biofilm. The formation of DBPs during chlorination due to biological effects will be studied, and also the protein expression will be tested so as to study the potential mechanisms and further to develop possible methods to predict DBP formation. The study of the formation of DBPs could be benefited not only for better water treatment, but also for studies of bio-resistance, bio-metabolism and artificial biochemical compounds.

CHAPTER II

LITERATURE REVIEW

2.1 Disinfectants and disinfection by-products

Chlorine is the most intensively used chemical disinfectant since it is cost-effective and efficient in killing or inactivating bacteria and pathogens. Other powerful oxidizing agents are also used in disinfecting and treating drinking water, such as UV light, chloramine and ozone. UV and ozone, even though both of them may yield less DBPs, disappear very fast in the water before distribution to consumers; however, persistent residuals of disinfectants are important for continued protection of the water supply against subsequent contamination in the distribution system (Drinking Water and Health: Volume 2, 1980). During the disinfection process, disinfectants themselves may react with naturally present fulvic or humic acids, amino acids, and other natural organic matter, as well as iodide and bromide ions, producing a range of disinfection byproducts (DBPs) that also affect human health (Pan et al., 2017). DBPs include a wide range of types, such as trihalomethanes (THMs), haloacetic acids (HAAs), bromate, and chlorite (which are regulated in the US), and so-called "emerging" DBPs like halonitromethanes, halo-acetonitriles, halo-amides, halo-furanones, iodo-acetic acid, iodo-THMs (iodo-trihalomethanes), nitrosamines, and more others not yet been detected (Richardson et al, 2007). Most of DBPs are toxic and carcinogenic (Boorman, 1999; Bove et al., 2002; Richardson et al., 2007, 2010). Dealing with DBPs is a long-lasting problem in drinking water and wastewater treatment systems. Health effects of DBP exposure have been studied since the 1970s, and Villanueva et al, (2015) has written a

recent comprehensive overview of the health impacts by DBPs. This paper showed that the majority of DBPs are from public drinking water supplies and swimming pool treatment, with more than 600 types identified, and they are widespread and harmful to skins and lungs. The type of DBPs is correlated with how disinfectants are used; for example, trihalomethanes and haloacetic acids are formed after chlorination, bromated DBPs increased by ozone, and nitro-DBPs are produced by chloramine. The reaction between precursors in raw water and disinfectants for water treatment is key for determination of DBP production, but this field still requires further study.

Control of DBPs are often done and proposed by removing precursors prior to disinfection, but a fundamental shortcoming of this is that DBPs also form in water distribution networks when organic matter (such as biofilms) release and act as precursors that react with chlorine or chloramine residual. The presence of DBP precursors in source water can also be attributed to water pollution caused by human activities. The NOM, which is often composed mainly of humic substances, is known to be a major DBP precursor in surface water (Chang et al., 2001; Hua and Reckhow, 2007). Numerous studies have been conducted on NOM to determine its characteristics and DBP yield during water disinfection (Xie, 2003).

NOM contains several groups with varying functionalities like aromatics, carboxylic acids, amino acids, proteins and carbohydrates (Bond et al., 2011). “Humic substances” are the largest, most hydrophobic and highly charged of the NOM groups, which are the main sources of THMs and HAAS precursors (Bond et al., 2011). Carboxylic acids are assumed to be smaller and more hydrophilic than “humic substances”, of which β-dicarbonyl species are important sources of precursors for TTHM and HAA5 (Bond et al., 2011). Amino acids and proteins are particularly important constituents of NOM in waters with high algal activity, and their concentrations are correlated to nitrogenous DBPs (N-DBPs) formation (Richardson et al., 2007). A new development on bacterial matters is extracellular polymeric substance (EPS), secreted by biofilm, which comprise more than 80% of hydrated biofilm (Zhikang Wang et al., 2012, 2013). Furthermore, l-

aspartic acid and l-asparagine are known to be reactive HAA precursors (Reckhow and Kim, 2008). Glucose, arabinose, and mannose are representative carbohydrates in aquatic environments, and have been found to form significant THM levels at pH 8 (Bond et al., 2011). It is well accepted that materials deposited on distribution, pipes, such as corrosion products and biofilm, not only consume a significant amount of disinfectants but also harbor organic DBP precursors. Since water distribution systems are continuously fed with unremoved NOM and biofilm formation is ubiquitous in water distribution systems, high concentrations of organic DBP precursors may accumulate on biofilm surfaces as well as in its internal structures and provide a reservoir of organic material for subsequent DBP formation.

Even though NOM is reported to be a major factor of DBP formation, and the limitation of NOM can significantly decrease DBPs, the studies of NOM cannot well explain the formation of DBPs, Some researchers have switched to study the role of bacterial matter during the process (Yang et al., 2011). Recently, biological studies discovered that cells have correlations with DBP formation, such as absorption of NOM (Wang et al., 2012), and DBP formation from the reaction between extracellular polymeric substances (EPS) (Wang et al., 2013) and algal extracellular organic matter (EOM) (Yang et al., 2011). Simultaneously, the influences from metal ions also have been studied, for example, addition of cupric ions at 0.02 mM increased the formation of THMs and DHAAs by 74–83% and 90–100%, respectively, but decreased the formation of THAAs by 26–27% (Zhao et al., 2016). And NOM oxidation during ozonation and TiO_2–O_3 caused changes in their structure in the direction of an increased proportion of the hydrophilic fraction (up to 70%), which has the most reactive THM and HAN precursors (Molnar et al., 2012). These findings well-elucidated the different aspects of the formation of DBPs. Based on their results we now understand that the bacterial effects and metal ion influences are also factors for DBPs formation as NOM, but the mechanisms, especially with regard to metal ion influences, are not exactly clear

2.2 Biofilm and bacterial matters

Since Williams et al., 1998, found that DBPs concentration were changing with the season that tended to be higher in summer but lower in winter, temperature and bacterial activation had been taken as an issue. The biggest bacterial matter in water treatment is biofilm. A biofilm is any group of microorganisms in which cells stick to each other and often also to a surface (López, 2010). These adherent cells become embedded within a slimy extracellular matrix that is composed of EPS. The EPS components are produced by the cells within the biofilm and are typically a polymeric conglomeration of extracellular DNA, proteins, and polysaccharides (Hall-Stoodley L, 2004). Because they have three-dimensional structure and represent a community lifestyle for microorganisms, biofilms are frequently described metaphorically as "cities for microbes" (Flemming et al., 2007; Watnick, 2000). Microbes form a biofilm in response to many factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional cues, or in some cases, by exposure of planktonic cells to sub-inhibitory concentrations of antibiotics. When a cell switches to the biofilm mode of growth, it undergoes a phenotypic shift in behavior in which large suites of genes are differentially regulated. Bacteria in biofilms resist killing by antibiotics, biocides, and disinfectants in comparison to free-floating cells (Xu et al., 2000). Because of the resistance mounted to antimicrobial agents in biofilms, existing methods for measuring bacterial killing, which use planktonic cell cultures, are poorly suited to biofilm applications. New biofilm-based methods are needed that allow for rapid and repeatable measurement of biofilm disinfection. Such methods will serve as enabling technology for the discovery of new anti-biofilm agents.

Control of DBPs are often done and proposed by removing precursors prior to disinfection, but a fundamental shortcoming of this is that DBPs also form in water treatment systems when organic matter (such as biofilms) release and act as precursors that react with chlorine residuals. In oligotrophic systems, such as drinking water treatment systems, bacterial metabolic processes are

highly sensitive to regular trace metal elements and some more major ions such as magnesium. Concentrations of these elements vary over orders of magnitude in water treatment systems due to variations in water sources and treatment methods and are at levels that affect microbiological physiology (Deborde, 2007). Because different bacteria biomass components and metabolites act as unique DBP precursors, physiological changes likely affect the relative abundances of DBP precursors. In recent studies, DBP yields from both total bacteria cells with distinct capsular EPS composition and the extracted EPS as well as from single species biofilm were investigated (Kimberly et al., 2016). DBP yield results indicated that bacterial EPS facilitated DBP formation upon chlorination. However, due to the complex chemical compositions of EPS, the relative contributions of biomolecules in EPS to DBP formation were not elucidated.

In many biofilm control applications, the desired endpoint of control is to remove the biofilm, not just disinfect it. Removing the biofilm is an environmentally friendly or "green" approach in that it obviates the requirement for inherently toxic disinfectants. Removal is aimed directly at obtaining a clean (biofilm-free) surface, which is the ultimate objective of a cleaning agent (Pitts, 2003). A strategy based on biofilm removal presents a novel commercial opportunity in that is represents a departure from the traditional emphasis on disinfectants that kill microorganisms in the biofilm. However, DBP formation by biofilms are currently not well understood. Because a lot of bacterial organic matter is contained in biofilm, biofilm is very hard to analyze. A new development on bacterial organic matter is EPS, secreted by biofilm, which comprise more than 80% of hydrated biofilm. Biofilm EPS are composed of a significant concentration of organic carbon and nitrogen rich compounds. Analogously, recent studies investigating the DBP yields of algal extracellular organic matter (EOM), which are reported to possess similar chemical composition as biofilm EPS, reported a wide range of DBP yields (Plummer 1998; Li et al., 2012; Xing et al., 2018). Thus, biofilm EPS may possess similar chemical composition and reactivity to DBP precursors and significantly increase disinfectant demand.

Considering the persistence of biofilm and current operational responses to biofilm outbreak in water utilities, which include increasing the concentration of residual disinfectants to eradicate biofilm, biofilm and its EPS may be a significant contributor to DBP formation in water treatment systems. In recent studies, DBP yields from both total bacteria cells with distinct capsular EPS composition, extracted EPS, and single species biofilm were investigated (Wang et al., 2012, 2013). DBP yield results indicated that bacterial EPS facilitated DBP formation upon chlorination. However, due to the complex chemical compositions of EPS, the relative contributions of biomolecules in EPS to DBP formation were not elucidated.

2.3 Metal requirements for bacteria life

Chandrangsu et al. (2017) elucidates how metal ions are essential for many reactions, but excess metals can be toxic. In bacteria, metal limitation activates pathways that are involved in the import and mobilization of metals, whereas excess metals induce efflux and storage. The resulting transcriptional response to metal stress takes place in a stepwise manner and is reinforced by post-transcriptional regulatory systems. Metal limitation and intoxication by the host are evolutionarily ancient strategies for limiting bacterial growth. Matthew D. R et al., 2012, addressed that iron, calcium, and manganese are accumulated by *S. typhimurium* during lag phase, while levels of cobalt, nickel, and sodium showed distinct growth-phase-specific patterns.

Metals induce large physiological changes in bacteria. Simply using microscopes, magnesium was long known to induce observable physiological changes in bacteria. Many metals are used directly for energetics, but more universally, metals are required for functions that are more fundamental to functionality and cell maintenance. Many types of interactions exist between proteins and metals such as indirect interaction with hydrophobic ligands, supramolecular clathrate compounds, and coordination interactions with the functional groups of amino acids (Makoto and Katsuyuki, 1996; Biros et al., 2007). Divalent metals such as magnesium and manganese affect RNA-based regulatory processes that controls multiple aspects of physiology

including activation of selective amino acid production. The binding of metals to proteins is often required for the ability for those proteins to simply fold appropriately and work; magnesium is needed for the functionality of chemotaxis proteins, and zinc is needed to activate regulatory proteins that control the production of other metal-dependent proteins, for example. Iron is the metabolic center of many enzymes and affects cellular regulation. Cobalt has been found key for dechlorination enzymes and many cobalt-containing ligands have been characterized.

Molybdenum is incorporated into organometallic cofactors that allows the metal to catalytically cycle between oxidation states, molybdenum-bearing enzymes are by far the most common bacterial catalysts for breaking the chemical bond in atmospheric molecular nitrogen in the process of biological nitrogen fixation. At least 50 molybdenum enzymes are now known in bacteria, plants, and animals, although only bacterial and cyanobacterial enzymes are involved in nitrogen fixation (Fay, 1992; Bothe et al., 2010). These nitrogenases contain molybdenum in a form different from other molybdenum enzymes, which all contain fully oxidized molybdenum in a molybdenum cofactor (Santos et al., 2004; Lancaster et al., 2011). These various molybdenum cofactor enzymes are vital to the organisms, and molybdenum is an essential element for life in all higher eukaryote organisms, though not in all bacteria. Magnesium ions interact with polyphosphate compounds such as ATP, DNA, and RNA. Hundreds of enzymes require magnesium ions to function.

Magnesium compounds are used medicinally as common laxatives, antacids, and to stabilize abnormal nerve excitation or blood vessel spasm in such conditions as eclampsia. Magnesium is an essential element in biological systems. Magnesium occurs typically as the Mg^{2+} ion. It is an essential mineral nutrient for life and is present in every cell type in every organism. For example, ATP (adenosine triphosphate), the main source of energy in cells, requiring the binding to a magnesium ion in order to active its function. So magnesium is essential to keep the stability of polyphosphate compounds like ATP in the cells, including the synthesis

of DNA and RNA (Conaway, 1988), because enzymes need the presence of magnesium ions along with catalytic action.

The uptake and control of even non-toxic or low-toxic metals in bacteria are tightly controlled and bacteria will metal scavenge and concentrate available magnesium, manganese, iron, nickel, copper, zinc, and molybdenum when provided in media at ranges of above 0.1 mM for Mg^{2+} and about 0.1-10 μM for the other metals, ranges typical in water treatment systems (Leentvaar and Rebhun, 1982; Singh et al., 2010; Ali et al., 2013). This indicates that these concentrations of metals are generally nutritionally deficient and bacterial physiologically is sensitive to their availability. Zinc, which is sometimes used in distribution pipe coatings, is especially microbiologically important and accumulated in cells up to a concentration of 200 μM. Interesting, galvanized zinc biofilms have increased levels of DBPs over polyvinyl pipe biofilms, but the role of zinc on this was not determined. Simply using microscopes, magnesium was long known to induce observable physiological changes in bacteria (*Van Nostrand's Scientific Encyclopedia*. 2006; Lusk et al., 1968). Transition metals are required trace elements for all forms of life. Due to their unique inorganic and redox properties, transition metals serve as cofactors for enzymes and other proteins (Palmer and Skaar, 2016). Studies of transition metal quotas of *Escherichia coli*, for instance, reveal that individual bacteria concentrate Zn^{2+} and Fe by several orders of magnitude relative to the concentration in a typical growth medium until they achieve a quota of about 2×10^5 atoms per cell, which is equivalent to a total concentration of about 0.1 mM (Outten and O'Halloran, 2001). Metals such as Cu^{2+} and Mn^{2+} are maintained in the 10 to 100 μM range. Other metal elements are also concentrated by the *E. coli* cell to a narrow, fixed, total concentration as follows: K and Mg, 10^8 atoms per cell, >10 mM; Ca, Zn, and Fe, 10^5 atoms per cell, ∼0.1 mM; Cu, Mn, Mo, and Se, 10^4 atoms per cell, ∼10 μM; Co and Ni, low abundance (Finney and O'Halloran, 2003). In addition, metalloprotein, which is a kind of protein that contains a metal ion cofactor, is important in biological function and play important roles on the maintenance of cells' structure. Harding et al.,

2009, found that metals may be present in over one-third of the whole proteins in nature. Even though some heavy metals may be inhibitors for metabolism like nickel is restricted to some enzymes, but a lot of metals are very essential for many proteins, Mg actives DNA polymerases in enzymes such as ATPase and polymerize actin in photosynthetic cyanobacteria, Molybdenum is important cofactor in sulphite oxidases, nitrogenase and nitrate reductases, and Manganese is obiligated in some oxygen-evolving photosynthetic reactions by bacteria (Waldaron and Robinson, 2009). And many metal-binding proteins require combination of more than one metal, for example, iron plus nickel, iron plus copper, and iron plus Moco6. Molybdenum can be replaced by vanadium in nitrogenases, and tungsten can be used in aldehyde oxidoreductase, formate dehydrogenase and acetylhydratase, in a few selected organisms, mostly Archaea (Silva and Williams, 2001).

2.4 Bacteria and metals in water treatment

In oligotrophic systems, such as drinking water treatment systems, bacterial metabolic processes are highly sensitive to trace elements (metals) and some more major ions such as magnesium. Concentrations of these elements vary over orders of magnitude in water treatment systems due to variations in water sources and treatment methods and are at levels that affect microbiological physiology. Because different bacteria biomass components and metabolites act as unique DBP precursors, physiological changes likely affect the relative abundances of DBP precursors. Additionally, since degradation processes are dependent on trace metals, the biodegradation of DBPs in water treatment systems is also likely sensitive to ranges of trace metal concentrations.

In drinking water treatment systems, bacterial metabolic processes are highly sensitive to metal elements such as magnesium. These metal elements could be captured by bacterial absorption, and through the chemical and biological reactions in the water and the treatment, they could affect microbiological physiology (Deborde, 2007). *Enterobacter bacteria* and *Bacillus*, these two common bacteria in environment were isolated from the nature and identified as prevalent

microorganisms in water treatment plant. *Enterobacter bacteria* is the most widely studied prokaryotic model organism, and is an important species in the fields of biotechnology and microbiology, because it have a long history of laboratory culture and ease of manipulation. *E. coli* plays an important role in modern biological engineering and industrial microbiology. Most of *Enterobacter* strains do not cause disease, but virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis, and Crohn's disease. Common signs and symptoms include severe abdominal cramps, diarrhea, hemorrhagic colitis, vomiting, and sometimes fever. In rarer cases, virulent strains are also responsible for bowel necrosis (tissue death) and perforation without progressing to hemolytic uremic syndrome, peritonitis, mastitis, septicemia, and gram-negative pneumonia. Very young children are more susceptible to develop severe illness, such as hemolytic uremic syndrome, however, healthy individuals of all ages are at risk to the severe consequences that may arise as a result of being infected with *E. coli* (Kaper 2004). *Bacillus* species are ubiquitous in nature, but also occur in extreme environments such as high pH (*B. alcalophilus*), high temperature (*B. thermophilus*), or high salt (*B. halodurans*) (Slonczewski & Foster 2011). *B. subtilis* is one of the well-studied bacteria, no matter in molecular or cellular biology. (Graumann 2012)

This study covers three important metal elements for bacteria metabolism: molybdenum (Mo), magnesium (Mg), manganese (Mn), and 1 frequent used metal element in water treatment: Aluminum (Al). Mo is a typical transition mental in essential of the molybdoproteins and enzymes in nearly all life from bacteria to human beings. Fe-Mo cofactor in nitrogenase and Mo cofactor in molybdoproteins are both of the most prevalent complexes of Mo, the biological molybdate transporter family, MOT, might be involved in the uptake of Mo from extracellular matters. Elimination of molybdenum occurs via the kidney and usually is complete within several weeks (Barceloux and Barceloux, 1999). In water, Mo may be released from fertilizers and metal alloys, and Mo is also rapidly excreted from urine by human (Wlillard, 1979), therefore Mo could be very common in raw water and able to induce the metabolism of bacteria. The other essential metal

element, Mg, is one of the most important metal for bacteria, it has many diverse biological roles, such as essential cofactor in ATP-mediated enzymatic reactions and signaling molecule that activates important virulence systems (Groisman et al., 2013). CorA, MgtE and MgtA (magnesium transporter A)/MgtB are three major classes of *S. typhimurium* and *E. coli* (Papp-Wallace et al., 2008), they can regulate the transportation (both intracellular and extracellular) of Mg and the expression of genes that is required for bacteria to adapt environments (Snavely et al., 1991). Because the Mo is also popularly used in human life, the disposals of food and drugs contribute to the Mg compounds in the water. The third important metal, Manganese (Mn), which is an essential cofactor for all forms of life, help cells to response to oxidative stress (Juttukonda and Skaar, 2015). Manganese enzymes are particularly essential in detoxification of superoxide free radicals in organisms that must deal with elemental oxygen. Manganese also functions in the oxygen-evolving complex of photosynthetic plants. While the element is a required trace mineral for all known living organisms, it also acts as a neurotoxin in larger amounts. Especially through inhalation, it can cause manganism, a condition in mammals leading to neurological damage that is sometimes irreversible. Waterborne manganese has a greater bioavailability than dietary manganese. According to results from a 2010 study (Bouchard, et al., 2011), higher levels of exposure to manganese in drinking water are associated with increased intellectual impairment and reduced intelligence quotients in school-age children. Potassium permaganate ($KMnO_4$), which is an active disinfectant that have strong inhibition on bacteria may cause Mn to be a factor on DBP formation. Al is often use in coagulation during drinking water treatment, but the Al is also needed for bacteria, the ability of aluminum to substitute for magnesium in biological systems is derived from a high association constant with diverse ligands. Inhibitory aluminum concentrations increased porphyria synthesis and excretion in *Arthrobacter aurescens* (Scharf et al. 1994), aluminum treatment also caused a significant decrease in intracellular cells.

By understanding how bacterial communities present in drinking water networks affect DBP concentrations as a result of trace metal elements, we have the potential to transform our approach to DBP reduction in drinking water. By investigating the links between regular trace metals, bacteria, and DBP production and formation, the discovery of elements that reduce bacterial DBP precursors and increase bacterial DBP degradation will develop the proof of concept needed to manipulate biofilms in water treatment systems for DBP control. This research will investigate a critical gap in how humans are exposed to DBPs and microbial responses with regards to regular trace metals which will be important to the broader field of biofilm as well. By understanding how regular trace metals impact these bacterial communities and thus affect DBP concentrations, we will be able to award the potential on better decision making processes that could limit DBP exposures.

2.5 DBP removal and antibiotics resistance in water treatment

Many of the efforts had been put into practice since DBP issues being focused. The preliminary strategy is to remove the precursors (especially the organic matters) in the raw water. Enhanced coagulation (EC), powdered activated carbon (PAC), and granulated activated carbon (GAC) are most effective methods (Uyak et al., 2007). However, they are all costly and also frequent backwashing (once a week) to control biological growth on the filter (Kristiana et al., 2011). Ozone and ultraviolet (UV) are alternative choices for primary disinfection because they produce very few DBPs. But ozone cannot remove organics that may cause DBP formation after secondary disinfection and it has low efficiency on treating water with high pesticide and also react with bromide to form Br-DBPs. In addition, ozone cost a lot on the maintenance. UV is another expensive disinfectant, however, as well as ozone, UV cannot sustain in the water after disinfection process, there could be potential infection during water distribution in the pipeline. Hu et al., 2018. Try to figure out a good combination of disinfection processes (pre-oxidation, conventional treatment, and advanced treatment) that can generate much lower DBPs. The authors had set up a

series of experiments combined with different disinfectants (chlorine and chloramine), pre-oxidations (Blank, $KMnO_4$, O_3, K_2FeO_4, and ClO_2) and advanced treatments (O_3-GAC, GAC, and Blank), finding that the best performance in removing chloroform (CF) and dichloroacetonitrile (DCAN) precursors can be achieved when coupling micro-ozone pre-treatment with CSF and O_3-GAC. The process of pre-oxidations may have a negative impact on chloropicrin (TCNM) and chloral hydrate formation, but it could have a positive effect on DCAN. At the aspect of the toxicity, pre-oxidations with O_3 and O_3-GAC had a best performance on the control of overall cytotoxicity of DBPs, but for the control of overall genotoxicity, the ClO_2 pretreatment showed best results, whether or not GAC or O_3-GAC was involved.

More and more studies have proven DBPs are toxic to human, and new toxic DBPs were found time after time. Water suppliers focused more DBP issues and national drinking water regulations need to be improved especially in California and Massachusetts (Richardson et al., 2007). It is well accepted that materials deposited on distribution, pipes, such as corrosion products and biofilm, not only consume a significant amount of disinfectants but also harbor organic DBP precursors. Since water distribution systems are continuously fed with unremoved natural organic matter (NOM) and biofilm formation is ubiquitous in water distribution systems, high concentrations of organic DBP precursors may accumulate on biofilm surfaces as well as in its internal structures and provide a reservoir of organic material for subsequent DBP formation.

Antibiotic resistance in biofilm has close relationship with DBPs, Lv et al. 2014 found the exposure to mutagenic DBPs can lead to increase of antibiotic resistance in *pseudomonas aeruginosa*, and Li et al. 2016 reported DBPs induce antibiotic resistance of *E.coli*. And as described in Chapter 1, antibiotic drug could be potential DBP precursor, too. So the antibiotic resistance of biofilm might be used for biomarker for DBPs. Ciprofloxacin is a member of the fluoroquinolone (FQs) class of antibiotics (Chin, 1984) that is commonly prescribed for the treatment of a wide variety of infections (Almalki, 2017). This wide use of ciprofloxacin has led

to the selection of bacterial pathogen strains that demonstrate resistance to this broad-spectrum antibiotic (Hooper and Jacoby 2015 Mechanisms of drug resistance: quinolone resistance, Ann.NY Acad. Sci.). Ciprofloxacin is frequently detected in sewage due to its incomplete uptake and metabolism in patients (Heberer, 2002; Daughton and Ternes, 1999; He and Blaney, 2015; Johnson et al., 2015), and has been reported in domestic and hospital wastewater treatment plant effluents at concentrations ranging from 6 to 11,000 ng L^{-1} (Ferreira, 2016).

The CIP biodegradation efficiency depends on many factors, extracellular polymeric substances (EPS), which is an important microorganism product for organic micropollutants removal (Sheng, 2010) was reported to play a key role in CIP bio-adsorption, the pH and iron salt dosing impacted sorption of ciprofloxacin onto activated sludge under aerobic and anoxic conditions (Polesel, 2015), And the sludge residence time (SRT) and hydraulic residence time (HRT) caused a negative impact on the performance of biodegradation of pharmaceuticals (Philip and Bhallamudi, 2016). Simultaneously, some paper work on exploring the bacterial resistance of CIP and inducing related genes so as to enhanced the tolerance and then benefit the treatment with sludge (Marti, 2014; Anssour, 2016; Vinué, 2016), but the biodegradation capabilities of those microorganism are quit uncertain, further studies are needed to understand complex interactions between CIP and biomass. The activated sludge process is a typical wastewater treatment process for treating sewage or industrial wastewaters using aeration and a biological floc composed of bacteria and protozoa, it takes advantage of aerobic micro-organisms that can digest organic matter in sewage. CIP biodegradation in activated sludge could benefit both of wastewater treatment in WWTP and pre-treatment in industrial factories, and some studies had begun to focus on it (Ferreira et al., 2016; Davids et al., 2017; Kang et al., 2018; Min et al., 2018). These uncertainties would be further complicated by the expected increased CIP flux entering WWTPs in the event of an anthrax release.

CHAPTER III

EFFECT OF METALS ON DBP FORMATION POTENTIAL IN ISOLATES

3.1 Process

To determine the effects of metals on DBP precursor formation, two bacteria isolates had been isolated, screened, and characterized for their contribution to high-levels of DBPs precursors, then determined the influence of metal ions, including magnesium, molybdenum, manganese and aluminum on DBP precursors from bacteria isolates. *Enterobacter cloacae* and *Bacillus* was chosen in this study because their bacterial structures and genomes have already been well established. These bacteria were cultured with recommended media: Lysogeny Broth (LB), which is good for bacteria growth and have detectable NOM in the solution. Solutions were prepared using sterilized LB media and separated to two parts: one incubated with each strain and another one without any strain (control). While the water resources may contain some metals ions and the bacterial requirement of metal ion range from 0.0001-10 mM, a reasonable range (0.01-1 mM) and similar concentration of metals had been set up in the experiment. Because metals are key co-

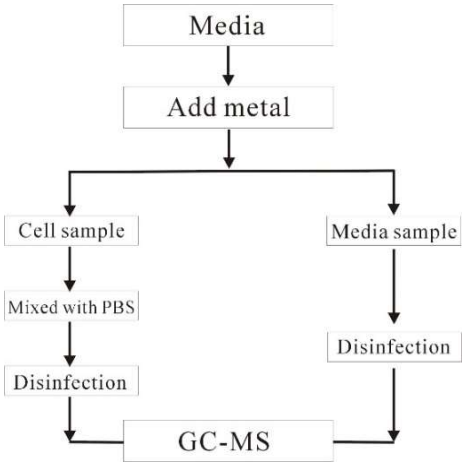


Figure 3.1 Process of experiment

factors for bacterial metabolic processes, it is expected that changes in metal concentrations will influence on the yield of bacterial products.

3.2 Materials and methods

Reagent grade or higher chemicals were prepared and used for the experiments. DI water (18 MU, cm) was provided by a Water Purification System (PALL). The disinfectant, NaOCl stock solution (20 mg/L) was prepared by diluting 5% sodium hypochlorite solution (Allied Signal). Peptone, yeast extract, arga, sodium chloride, sodium thiosulfate and metal salts were all purchased from Sigma Aldrich.

3.2.1 Bacteria isolation

Water samples were collected with sterilized Teflon tubes from the surface water in Theta Pond at Oklahoma State University (36°07'11.5"N+97°04'14.3"W). After dilution, water samples were incubated on the culture dish with solid LB culture (1% tryptone (w/v), 0.5% yeast extract (w/v), 0.1% NaCl (w/v) and 1.5% agar, pH=7) in 25 °C incubator for seven days, next, seven different colonies with different appearances were selected and collected by transferring into sterilized 2 mL DNA collecting tubes (DNA soil extraction kit, MoBio) with autoclaved tweezer. 16S rRNA sequences of the strains were identified by Meta-transcriptomical data which is analyzed by using the OSU HPCC. Transcripts encoding putative proteins was identified, annotated, and deposited into public databases (NCBI). Sanger sequencing at the OSU DNA/Protein core facility from a universal PCR amplification of total DNA extract using the PowerSoil Total DNA Extraction Kit (MoBio). According to sequence results (Fig 3.2), two well-known strains (*Enterobacter cloacae* and *Bacillus*) were selected.

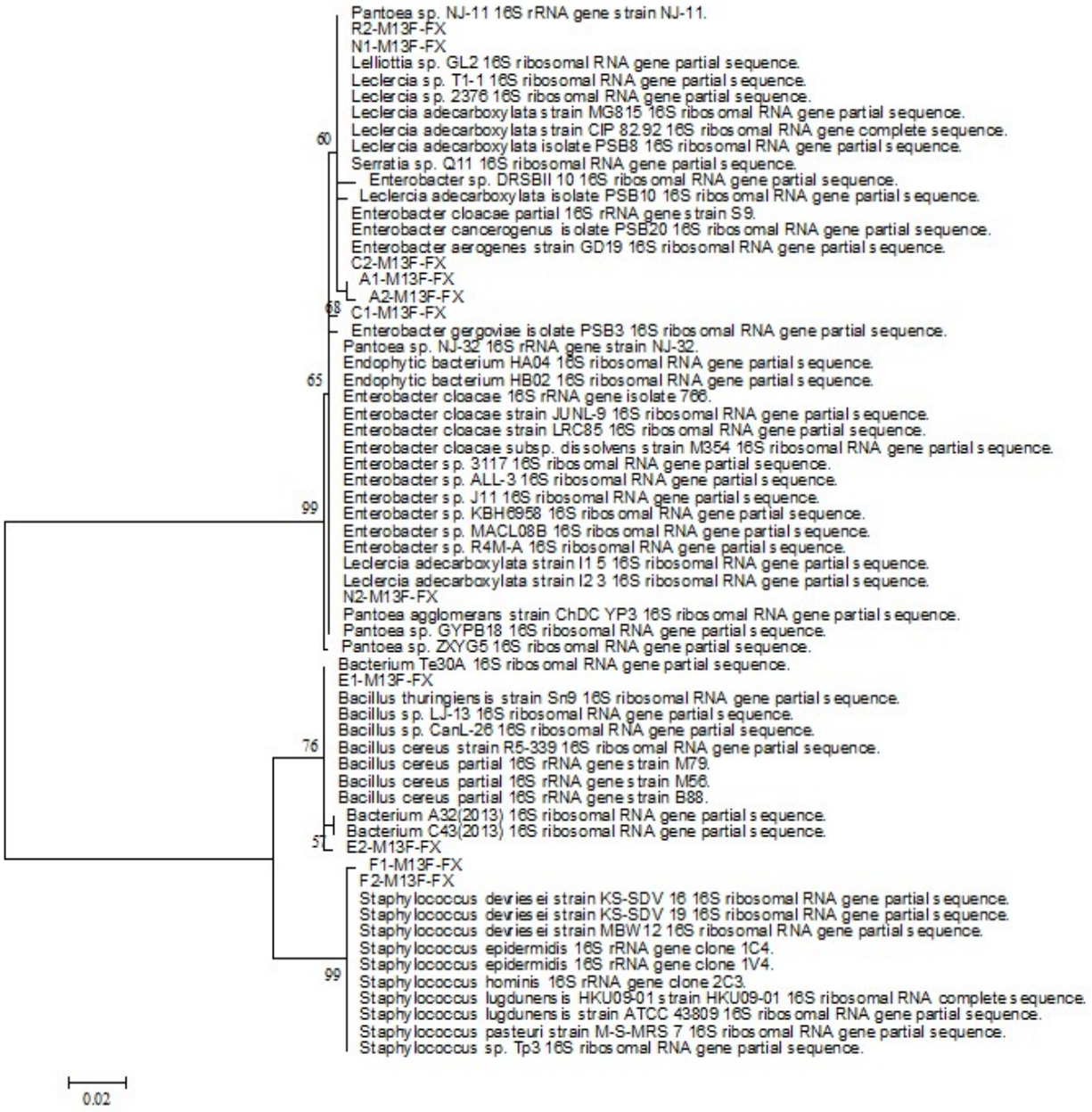


Figure 3.2 DNA sequencing analysis of isolated strains from water samples

3.2.2 Bacterial growth rate test and metal ion addition

The bacterial growth rate was estimated by OD600 test, which was carried out by the measurement of ultraviolet (UV) absorbance at 600 nm with a 96-well cuvette by using a spectrophotometer (Thermoscientific, USA). Isolated *Enterobacter cloacae* and *Bacillus* from

colonies were first incubated in 100 mL modified liquid media LB culture (1% tryptone (w/v) and 0.5% yeast extract (w/v), without NaCl) to avoid potential chloride residues in the media), after 3 days, 200μL of samples were collected from the media and dropped into 96-well cuvette to test OD600 and estimated cell populations. Then 1mL of 10^4 CFU (colony forming unit)/ml of both strains were added into the 100mL liquid with triplicates, the metal additions were achieved by preparing 0.01 uM, 0.1 uM and 1 uM of Mg^{2+} ($MgSO_4$), Mo^{6+} (Na_2MoSO_4), Mn^{2+} ($MnSO_4$), Al^{3+} ($Al_2(SO_4)_3$) into the same liquid media respectively before incubation.

3.2.3 Separation of intracellular and extracellular matters and media

Both bacteria were cultured for 8 h to log growth phase with metals. 1mL sample was collected from each group, and all of the samples were centrifuged to separate into two parts: intracellular and extracellular : Cell preparation was referred to the method reported by Wang and Zhang (2010), Cells were cultivated with LB media under 30 °C for 8 h, and then were harvested by centrifugation with 10,000 rpm for 5 min. The suspensions were collected to form extracellular matter; and the pellets were washed with PBS solution (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 and 0.24 g/L KH_2PO_4, pH adjusted to 7.0-7.4) three times and lastly resuspended with 0.5 mL PBS to form a cell intracellular suspension.

3.2.4 Disinfection

The disinfection process was achieved by adding 0.5 mL of 20 mg/L NaOCl stock solution to 0.5 mL of both of intracellular and extracellular samples separately (1:1) in 2 mL serum bottles in the dark for 72 h. After it, 1 mL disinfected samples for each bottles were transferred to clean 10 mL serum bottles and diluted with 4 mL HPLC grade water (Agilent, USA), then 1 g of oven dried sodium sulfate was added to quench the residual free chlorine, and 3 mL of MTBE was used to extract the disinfection by-products from the sample with shaking for exactly 4 minutes to let settle and separate and be tested on the gas chromatograph (GC).

3.2.5 GC method

Analyses of DBPs were carried out with a gas chromatograph (Agilent 7890B) with an electron capture detector (ECD). Samples were extracted in MTBE and taken by 10 µL silicon injector, and 2 µL of extracted samples injected through ECD column. Based on our method modified by USEPA method 551.1, the column used was a DB-1 fused silica capillary column (30m x 0.32mmI.D. with 1µm film thickness). The GC temperature program consisted of an initial temperature of 35 °C for 9 min, ramping to 40 °C at 5 °C/min and holding for 5 min, ramping to 120 °C at 20 °C/min and holding for 10min , lastly ramping to 150 °C at 10 °C/min and holding for 5 min.

DBP calibration standards were applied with Chlorinated Disinfection By-Products Mix (Lot#TS150901003, SPEXCerti Prep, USA), which contains 15 typical DBPs in 2000 mg/L. Our DBP samples were analyzed according to the calculations with this standard curve.

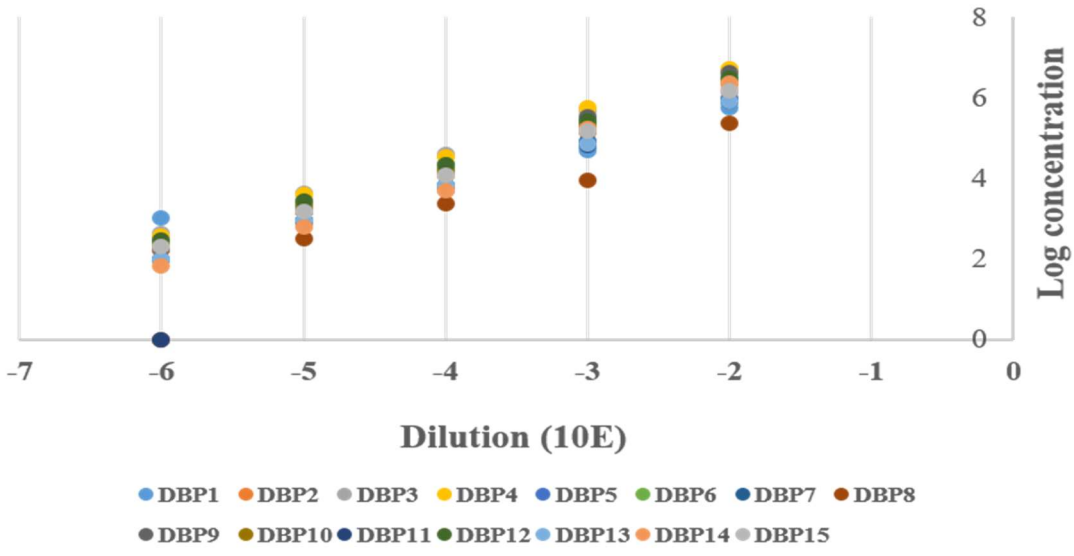


Figure 3.3 Standard Curve regression

Table 3-1. Standard Curves for 15 DBPs

		Standard Curve	
	Group	Equation (log)	R value
A	Chloroform	Y = 0.692x + 6.836	0.9301
B	1,1,1-Trichloroethane	Y = 0.986x + 8.096	0.9991
C	Carbon tetrachloride	Y = 1.006x + 8.654	0.9996
D	Trichloroacetonitrile	Y = 1.041x + 8.786	0.9982
E	1,1-Dichloroacetone	Y = 1.011x + 8.396	0.9991
F	Dichloroacetonitrile	Y = 0.994x + 8.370	0.9992
G	Bromodichloromethane	Y = 0.995x + 7.906	0.9986
H	Trichloroethene	Y = 0.989x + 8.180	0.9987
I	Chloropicrin	Y = 1.110x + 8.788	0.9952
J	Dibromochloromethane	Y = 1.003x + 8.324	0.9985
K	1,2-DIbromoethane	Y = 1.372x + 8.964	0.9329
L	Tetrachloroethene	Y = 0.997x + 8.412	0.9987
M	Bromoform	Y = 0.974x + 7.800	0.9983
N	Dibromoacetonitrile	Y = 1.153x + 8.590	0.9901
O	1,2-Dibromo-3-chloropropane	Y = 0.968x + 8.040	0.9977

3.3 Results and Discussion

To probe the potential effects on cell growth by metals, the growth rate of *Enterobacter cloacae* and *Bacillus* with four metals were measured by OD600 test. With this method, colony forming unit (CFU) was detected for each combination. Fig 3.4 and Fig 3.5 show that slight differences observed with metals since the bacteria began to grow (4 h to 12 h), and the CFU in stationary phase (48 h) seemingly to be increased with metals. But both of two strains' growth rates might be not affected much by the metals at last, instead, some delays or increases were occurred during each phase with different metals. *Bacillus* had more CFU after 24 h and also had more variations under the influences of metal ions. Metal may not change the growth rate of bacteria, or the concentrations of metals used in this experiment were relatively mild to the microorganisms. However there are few reports suggesting the metals could improve bacterial growth rate. In contrast, certain levels of heavy metals can inhibit the growth of microorganisms (Sadler and Trudinger, 1967; Anwar et al., 2007; Gadd 2010).

Zamyadi et al. (2011), reported that the DBP concentrations in environmental bloom conditions with very high cell numbers were over the guideline values. And as introduced above, NOM is the sources of DBPs and bacterial matters are important parts of NOM, so the cell numbers (represented by CFU) may have direct relationship with the final DBP productions. However, cell number is just partially link to the cell activity, the yields of proteins and enzymes and other bacterial matters inside/outside cells could be also changed by metal ions that may not present with cell numbers. And it should be pointed out that some seeming changes still showed up at 8 h and those after 12 h, such a phenomenon may result from the correlation between DNA/protein and metal ions.

The data proved that the concentrations from 0.01 mM to 1 mM of these four metals do not inhibit the growth of bacteria, which could help to better observe bacterial mechanisms related to DBP formation.

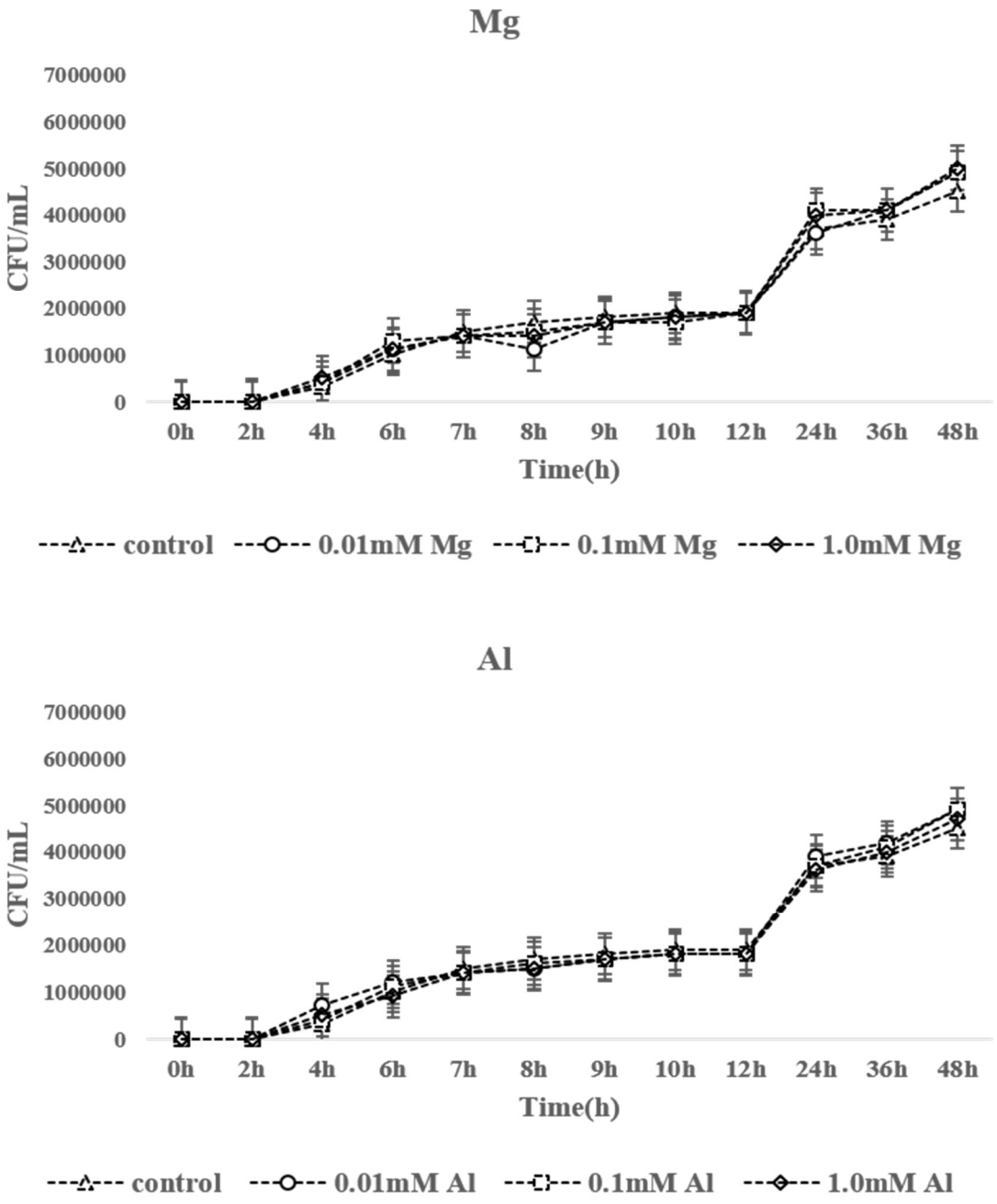
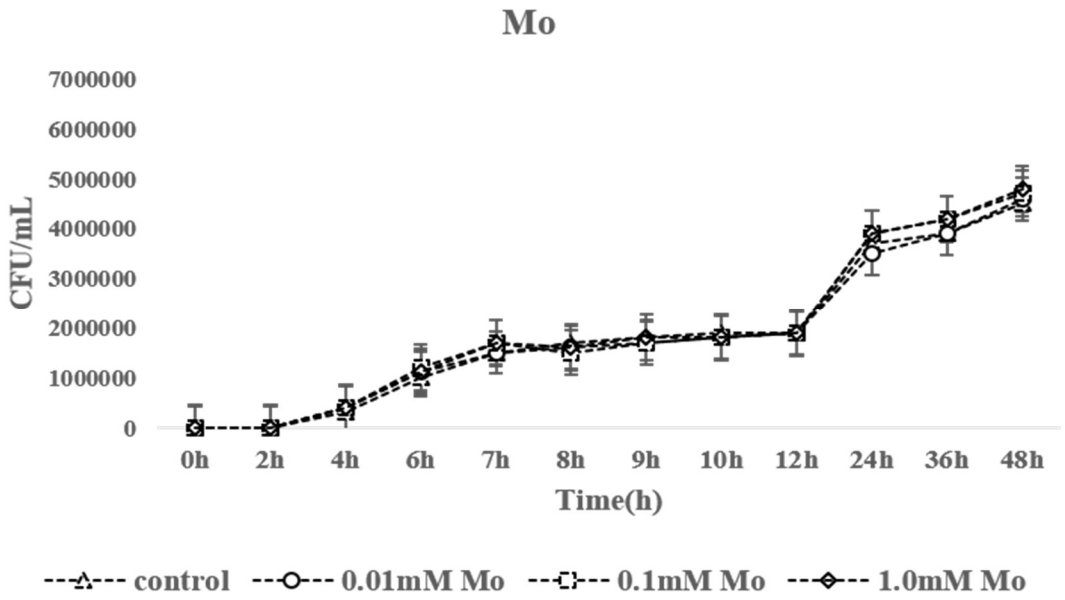


Figure 3.4 Growth rate of *Enterobacter cloacae* with different magnesium and aluminum



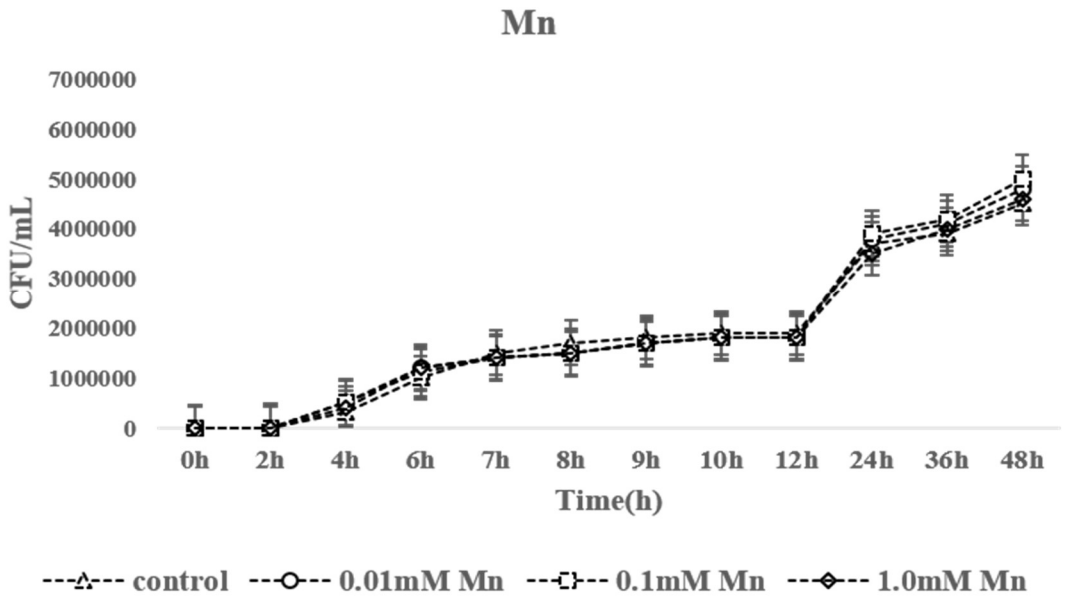


Figure 3.5 Growth rate of *Enterobacter cloacae* with molybdenum and manganese

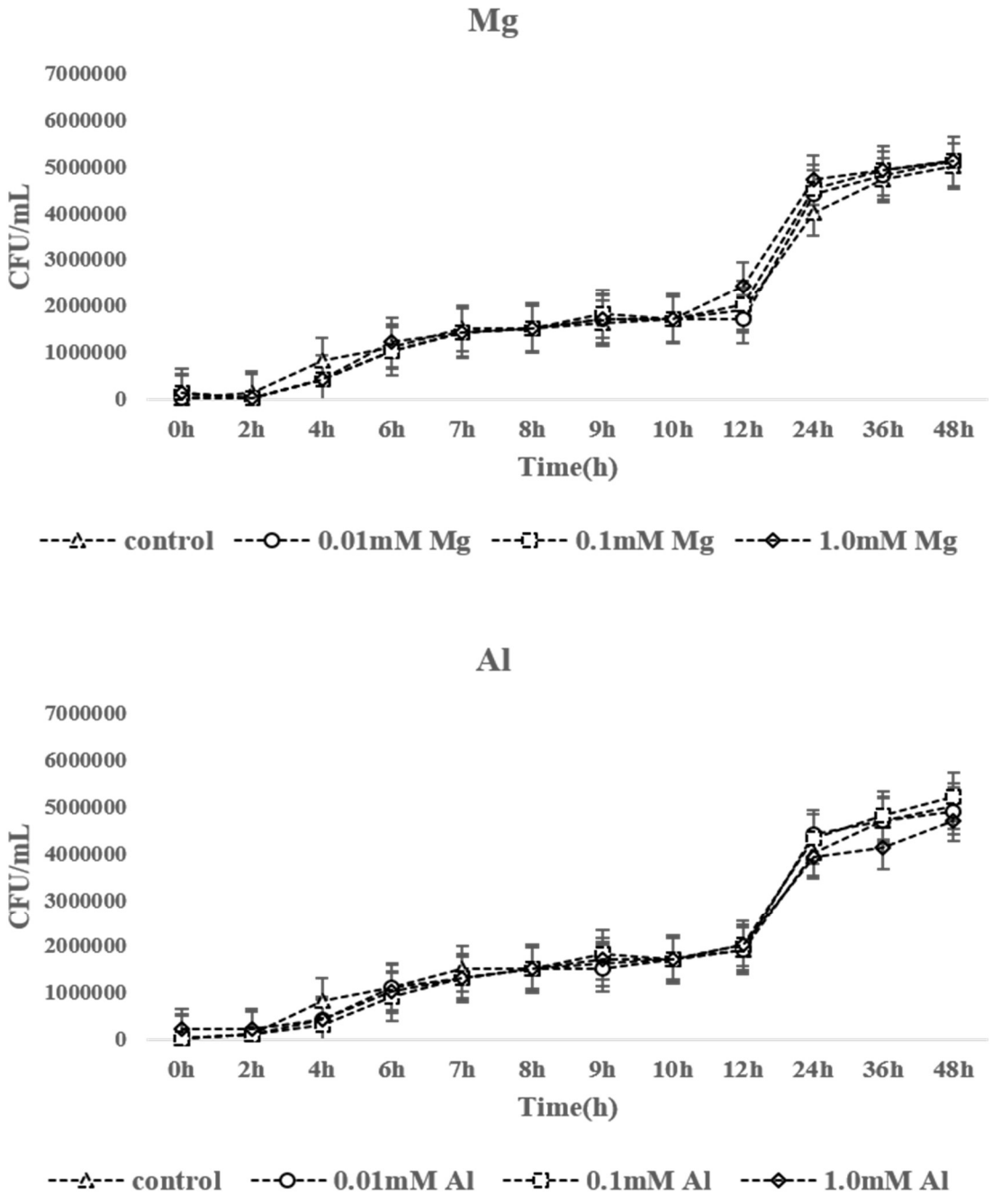


Figure 3.6 Growth rate of *Bacillus* with magnesium and aluminum

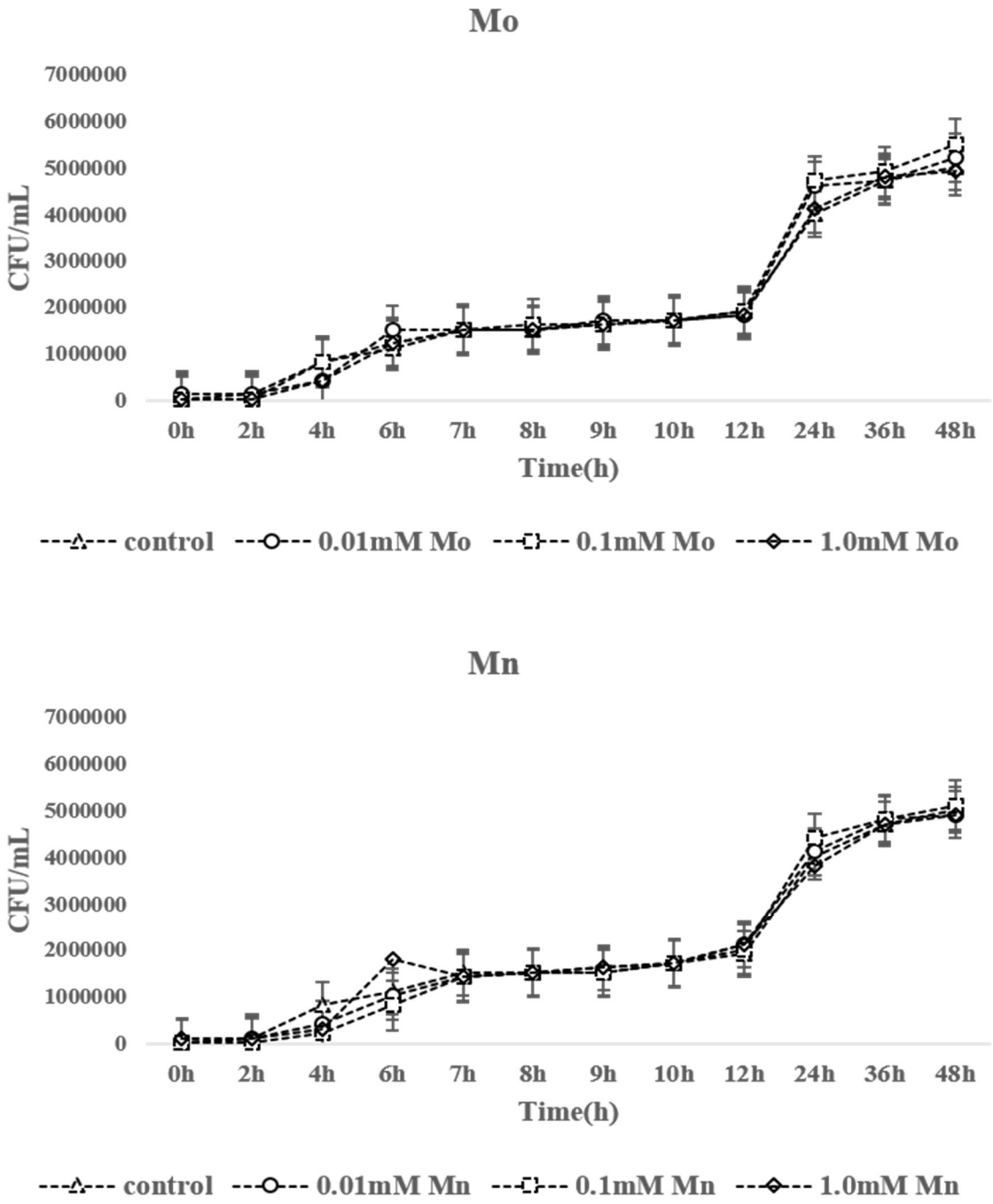


Figure 3.7 Growth rate of *Bacillus* with molybdenum and manganese

DBP yielding test was achieved by evaluating 15 common types of DBPs (EPA method 551.1) through GC method; the result of DBPs yields are shown on the tables and figures below, DBPs from *Enterobacter cloacae* cells in Table 3-2, 3-3, 3-4 and 3-5 showed that most of the DBPs were formed in intracellular parts, chloroform was the highest DBP of all in our study, and only 1,1,1-trichloroethane, 1,1-dichloroacetone, bromodichloromethane and trichloroethane formed extracellularly. Especially, bromodichloromethane only generated out of cells, while most of 1,1,1-trichloroethane occurred extracellularly rather than intracellularly. 1,1,1-trichloroethane (B), 1,1-dichloroacetone (E), bromodichloroethane (G) and trichloroethene (H) are the only four that were generated extracellularly. But B and G were totally dominated by extracellular parts. And interestingly, bromodichloromethane only formed with Mg^{2+} and Mo^{6+}, while the other bromide DBP: dibromochloromethane (J) also had obviously higher levels with these two metals, and Al^{3+} and Mn^{2+} got a lot more of Bromoform (M). The other 9 DBPs only formed inside the cells.

For *Enterobacter cloacae*, the group with 0.1μM of Mn^{2+} has the highest level of chloroform (1.6 μg/mL), and 1 μM of Al^{3+} and 0.01 μM of Al^{3+} has the least (0.28 μg/mL and 0.33 μg/mL, respectively), while all of the others with metals produced 0.5-0.6 μg/mL of chloroform, comparing with the control (0.43 μg/mL). Al^{3+} may potentially suppress the formation of chloroform, in contrast, Mo^{6+}, Mg^{2+} and Mn^{2+} increased its yield. The highest level of chloroform generated inside *Bacillus* cells was under 0.01 μM of Al^{3+}, with 2.7 μg/mL of chloroform; this group increased more than the others (also around 0.5-0.6 μg/mL) and the highest one in *Enterobacter cloacae*. On the other hand, bromide, which is a compound from media are available in spaces of both intracellular and extracellular. That means the precursors of intracellular bromide DBPs may be able to travel through the membrane.

This result indicated that the type of DBPs may depend on the certain organic matters. The organic matter of cells are primarily proteins, ligands, fats and polymers; however, fat and ligands are able to penetrate cell membrane while proteins and polymers are selective by the membrane.

Table 3-2. Total **DBP formation potential and percent by mass among the 15 DBPs measured when additional magnesium was added vs control in washed *Enterobacter cloacae* cells (show in average and standards deviations for 3 replicates).**

	Control		Additional Magnesium 0.01µM		0.1µM		1µM	
Total (µg/mL)	0.788 ± 0.050		0.879 ± 0.131		0.960 ± 0.096		0.854 ± 0.135	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	0.742 ± 0.028	0.046 ± 0.022	0.820 ± 0.119	0.059 ± 0.012	0.894 ± 0.086	0.066 ± 0.009	0.786 ± 0.128	0.068 ± 0.007
Chloroform	0.435 ± 0.000	0.012 ± 0.000	0.534 ± 0.036	0.015 ± 0.004	0.593 ± 0.013	0.020 ± 0.003	0.510 ± 0.060	0.020 ± 0.003
1,1,1-Trichloroethane	0.000 ± 0.000	0.005 ± 0.006	0.001 ± 0.001	0.009 ± 0.001	0.001 ± 0.000	0.009 ± 0.001	0.001 ± 0.000	0.008 ± 0.000
Carbon Tetrachloride	0.009 ± 0.000	N/A	0.004 ± 0.001	N/A	0.004 ± 0.001	N/A	0.005 ± 0.000	N/A
Trichloroacetonitrile	0.016 ± 0.000	N/A	0.005 ± 0.002	N/A	0.005 ± 0.003	N/A	0.007 ± 0.000	N/A
1,1-Dichloroacetone	0.071 ± 0.001	0.020 ± 0.002	0.044 ± 0.013	0.022 ± 0.003	0.037 ± 0.009	0.023 ± 0.004	0.044 ± 0.007	0.026 ± 0.002
Dichloroacetonitrile	0.027 ± 0.000	0.001 ± 0.001	0.043 ± 0.013	N/A	0.062 ± 0.029	N/A	0.038 ± 0.012	N/A
Bromodichloromethane	N/A	0.002 ± 0.003	N/A	0.003 ± 0.002	N/A	0.004 ± 0.000	N/A	0.004 ± 0.001
Trichloroethene	0.024 ± 0.000	0.006 ± 0.009	0.025 ± 0.002	0.010 ± 0.001	0.027 ± 0.002	0.010 ± 0.002	0.027 ± 0.005	0.010 ± 0.001
Chloropicrin	0015 ± 0.000	N/A	0.013 ± 0.002	N/A	0.016 ± 0.008	N/A	0.013 ± 0.006	N/A
Dibromochloromethane	0.002 ± 0.000	N/A	0.004 ± 0.002	N/A	0.005 ± 0.003	N/A	0.003 ± 0.001	N/A
1,2-Dibromoethane	0.089 ± 0.026	N/A	0.096 ± 0.036	N/A	0.104 ± 0.008	N/A	0.092 ± 0.030	N/A
Tetrachloroethene	0.004 ± 0.000	N/A	0.002 ± 0.002	N/A	0.002 ± 0.002	N/A	0.002 ± 0.002	N/A
Bromoform	0.020 ± 0.000	0.001 ± 0.001	0.021 ± 0.001	N/A	0.020 ± 0.003	N/A	0.020 ± 0.004	N/A
Dibromoacetonitrile	N/A	N/A	0.006 ± 0.002	N/A	0.003 ± 0.002	N/A	0.003 ± 0.002	N/A
1,2-Dibromo-3-chloropropane	0.031 ± 0.000	N/A	0.021 ± 0.000	N/A	0.016 ± 0.003	N/A	0.019 ± 0.002	N/A

Table 3-3. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional molybdenum was added vs control in washed *Enterobacter cloacae* cells (show in average and standards deviations for 3 replicates).

	Control		Additional Molybdenum 0.01µM		0.1µM		1µM	
Total (µg/mL)	0.788 ± 0.050		0.970 ± 0.106		0.940 ± 0.112		0.904 ± 0.121	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	0.742 ± 0.028	0.046 ± 0.022	0.820 ± 0.096	0.059 ± 0.010	0.894 ± 0.101	0.066 ± 0.010	0.786 ± 0.108	0.068 ± 0.012
Chloroform	0.435 ± 0.000	0.012 ± 0.000	0.665 ± 0.010	0.012 ± 0.002	0.599 ± 0.008	0.014 ± 0.004	0.535 ± 0.013	0.010 ± 0.000
1,1,1-Trichloroethane	0.000 ± 0.000	0.005 ± 0.006	0.001 ± 0.096	0.008 ± 0.001	0.001 ± 0.001	0.008 ± 0.000	0.001 ± 0.001	0.008 ± 0.000
Carbon Tetrachloride	0.009 ± 0.000	N/A	0.003 ± 0.000	N/A	0.004 ± 0.001	N/A	0.006 ± 0.001	0.000 ± 0.000
Trichloroacetonitrile	0.016 ± 0.000	N/A	0.005 ± 0.002	N/A	0.003 ± 0.001	N/A	0.005 ± 0.001	N/A
1,1-Dichloroacetone	0.071 ± 0.001	0.020 ± 0.002	0.034 ± 0.009	0.015 ± 0.005	0.032 ± 0.002	0.017 ± 0.006	0.049 ± 0.007	0.017 ± 0.009
Dichloroacetonitrile	0.027 ± 0.000	0.001 ± 0.001	0.041 ± 0.014	N/A	0.062 ± 0.035	N/A	0.036 ± 0.002	N/A
Bromodichloromethane	N/A	0.002 ± 0.003	N/A	0.002 ± 0.001	N/A	0.002 ± 0.000	N/A	0.002 ± 0.001
Trichloroethene	0.024 ± 0.000	0.006 ± 0.009	0.023 ± 0.005	0.010	0.028 ± 0.004	0.011 ± 0.000	0.039 ± 0.012	0.008 ± 0.001
Chloropicrin	0015 ± 0.000	N/A	0.016 ± 0.005	N/A	0.012 ± 0.002	N/A	0.014 ± 0.003	N/A
Dibromochloromethane	0.002 ± 0.000	N/A	0.003 ± 0.002	N/A	0.005 ± 0.003	N/A	0.003 ± 0.000	N/A
1,2-Dibromoethane	0.089 ± 0.026	N/A	0.095 ± 0.035	N/A	0.092 ± 0.022	N/A	0.085 ± 0.028	N/A
Tetrachloroethene	0.004 ± 0.000	N/A	0.002 ± 0.002	N/A	0.001 ± 0.002	N/A	0.003 ± 0.001	N/A
Bromoform	0.020 ± 0.000	0.001 ± 0.001	0.020 ± 0.009	N/A	0.031 ± 0.015	N/A	0.053 ± 0.029	N/A
Dibromoacetonitrile	N/A	N/A	0.003 ± 0.002	N/A	0.005 ± 0.004	N/A	0.012 ± 0.009	N/A
1,2-Dibromo-3-chloropropane	0.031 ± 0.000	N/A	0.011 ± 0.001	N/A	0.012 ± 0.002	N/A	0.018 ± 0.004	N/A

Table 3-4. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional aluminum was added vs control in washed *Enterobacter cloacae* cells (show in average and standards deviations for 3 replicates).

	Control		Additional Aluminum 0.01µM		0.1µM		1µM	
Total (µg/mL)	0.788 ± 0.050		0.647 ± 0.118		0.956 ± 0.072		0.575 ± 0.270	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	0.742 ± 0.028	0.046 ± 0.022	0.590 ± 0.111	0.057 ± 0.007	0.909 ± 0.065	0.047 ± 0.007	0.521 ± 0.260	0.054 ± 0.010
Chloroform	0.435 ± 0.000	0.012 ± 0.000	0.330 ± 0.029	0.022 ± 0.001	0.652 ± 0.014	0.017 ± 0.005	0.277 ± 0.149	0.017 ± 0.004
1,1,1-Trichloroethane	0.000 ± 0.000	0.005 ± 0.006	0.001 ± 0.000	0.010 ± 0.001	0.001 ± 0.000	0.009 ± 0.001	0.019 ± 0.023	0.011 ± 0.001
Carbon Tetrachloride	0.009 ± 0.000	N/A	0.008 ± 0.003	N/A	0.009 ± 0.001	N/A	0.008 ± 0.002	0.000 ± 0.000
Trichloroacetonitrile	0.016 ± 0.000	N/A	0.010 ± 0.005	N/A	0.009 ± 0.001	N/A	0.006 ± 0.005	N/A
1,1-Dichloroacetone	0.071 ± 0.001	0.020 ± 0.002	0.056 ± 0.032	0.015 ± 0.004	0.046 ± 0.003	0.010 ± 0.001	0.041 ± 0.024	0.013 ± 0.001
Dichloroacetonitrile	0.027 ± 0.000	0.001 ± 0.001	0.019 ± 0.007	N/A	0.018 ± 0.003	N/A	0.010 ± 0.009	0.001 ± 0.001
Bromodichloromethane	N/A	0.002 ± 0.003	N/A	N/A	N/A	N/A	N/A	N/A
Trichloroethene	0.024 ± 0.000	0.006 ± 0.009	0.019 ± 0.002	0.010 ± 0.001	0.019 ± 0.002	0.010 ± 0.000	0.018 ± 0.007	0.012 ± 0.002
Chloropicrin	0015 ± 0.000	N/A	0.016 ± 0.002	N/A	0.016 ± 0.003	N/A	0.013 ± 0.003	N/A
Dibromochloromethane	0.002 ± 0.000	N/A	0.001 ± 0.001	N/A	0.000	N/A	N/A	N/A
1,2-Dibromoethane	0.089 ± 0.026	N/A	0.055 ± 0.018	N/A	0.067 ± 0.018	N/A	0.051 ± 0.016	N/A
Tetrachloroethene	0.004 ± 0.000	N/A	0.003 ± 0.001	N/A	0.005 ± 0.001	N/A	0.004 ± 0.001	N/A
Bromoform	0.020 ± 0.000	0.001 ± 0.001	0.045 ± 0.010	0.001 ± 0.000	0.030 ± 0.010	0.001 ± 0.000	0.034 ± 0.011	0.001 ± 0.001
Dibromoacetonitrile	N/A	N/A	0.006 ± 0.006	N/A	0.006 ± 0.006	N/A	0.005 ± 0.005	N/A
1,2-Dibromo-3-chloropropane	0.031 ± 0.000	N/A	0.023 ± 0.005	N/A	0.031 ± 0.005	N/A	0.034 ± 0.004	N/A

Table 3-5. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional manganese was added vs control in washed *Enterobacter cloacae* cells (show in average and standards deviations for 3 replicates).

	Control		Additional Manganese 0.01µM		0.1µM		1µM	
Total (µg/mL)	0.788 ± 0.050		0.920 ± 0.145		1.969 ± 1.158		0.823 ± 0.224	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	0.742 ± 0.028	0.046 ± 0.022	0.869 ± 0.137	0.051 ± 0.008	1.930 ± 1.152	0.039 ± 0.006	0.778 ± 0.211	0.046 ± 0.013
Chloroform	0.435 ± 0.000	0.012 ± 0.000	0.575 ± 0.073	0.021 ± 0.002	1.600 ± 1.062	0.013 ± 0.003	0.541 ± 0.129	0.014 ± 0.002
1,1,1-Trichloroethane	0.000 ± 0.000	0.005 ± 0.006	0.001 ± 0.000	0.007 ± 0.000	0.001 ± 0.000	0.008 ± 0.001	0.001 ± 0.001	0.008 ± 0.001
Carbon Tetrachloride	0.009 ± 0.000	N/A	0.008 ± 0.002	N/A	0.012 ± 0.005	N/A	0.007 ± 0.002	N/A
Trichloroacetonitrile	0.016 ± 0.000	N/A	0.012 ± 0.005	N/A	0.012 ± 0.001	N/A	0.006 ± 0.003	0.004 ± 0.007
1,1-Dichloroacetone	0.071 ± 0.001	0.020 ± 0.002	0.052 ± 0.005	0.013 ± 0.005	0.058 ± 0.013	0.008 ± 0.001	0.039 ± 0.014	0.009 ± 0.002
Dichloroacetonitrile	0.027 ± 0.000	0.001 ± 0.001	0.018 ± 0.002	0.000 ± 0.000	0.034 ± 0.028	N/A	0.020 ± 0.006	N/A
Bromodichloromethane	N/A	0.002 ± 0.003	N/A	N/A	N/A	N/A	N/A	N/A
Trichloroethene	0.024 ± 0.000	0.006 ± 0.009	0.029 ± 0.003	0.009 ± 0.001	0.023 ± 0.005	0.009 ± 0.001	0.021 ± 0.006	0.010 ± 0.001
Chloropicrin	0015 ± 0.000	N/A	0.015 ± 0.001	N/A	0.019 ± 0.004	N/A	0.016 ± 0.006	N/A
Dibromochloromethane	0.002 ± 0.000	N/A	0.001 ± 0.000	N/A	0.001 ± 0.002	N/A	0.001 ± 0.001	N/A
1,2-Dibromoethane	0.089 ± 0.026	N/A	0.074 ± 0.025	N/A	0.076 ± 0.003	N/A	0.052 ± 0.011	N/A
Tetrachloroethene	0.004 ± 0.000	N/A	0.005 ± 0.001	N/A	0.006 ± 0.002	N/A	0.004 ± 0.001	N/A
Bromoform	0.020 ± 0.000	0.001 ± 0.001	0.045 ± 0.013	0.001 ± 0.000	0.045 ± 0.020	0.000 ± 0.000	0.037 ± 0.015	0.001
Dibromoacetonitrile	N/A	N/A	0.007 ± 0.004	N/A	0.007 ± 0.005	N/A	0.006 ± 0.004	N/A
1,2-Dibromo-3-chloropropane	0.031 ± 0.000	N/A	0.027 ± 0.002	N/A	0.036 ± 0.004	N/A	0.028 ± 0.012	N/A

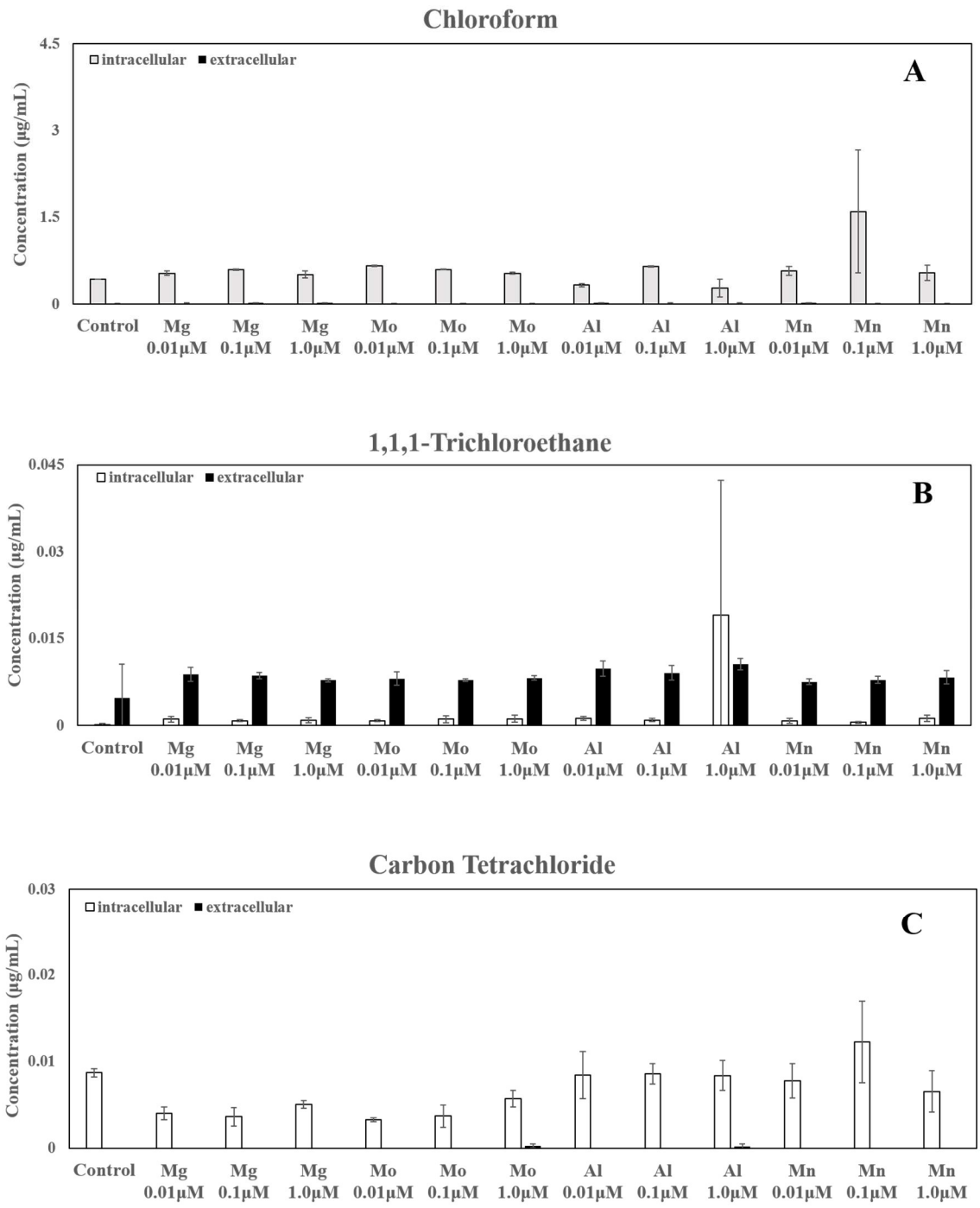


Figure 3.8 Intracellular and Extracellular Production of 15 DBPs (A to C) measured with additional metals in *Enterobacter cloacae*

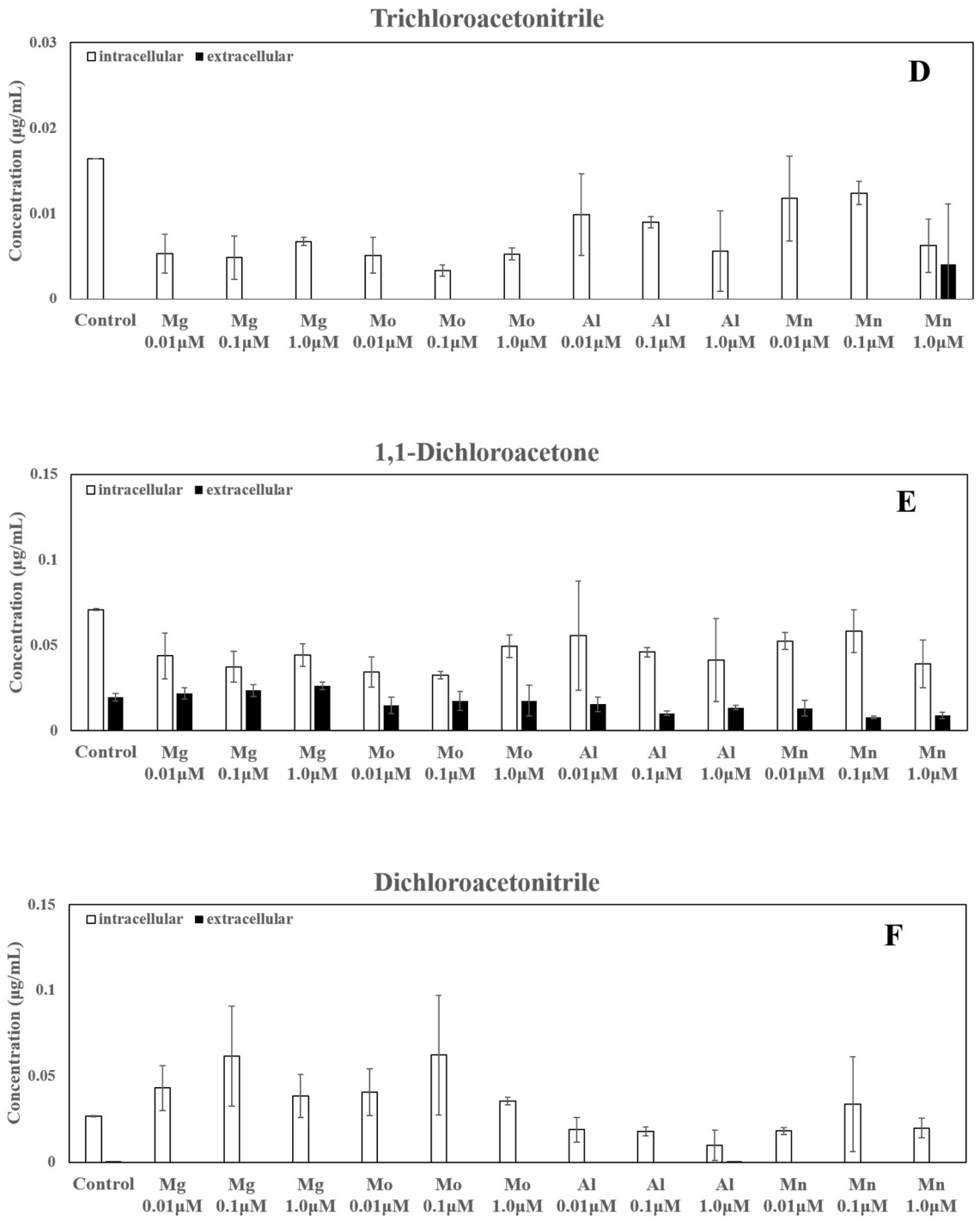


Figure 3.9 Intracellular and Extracellular Production of 15 DBPs (D to F) measured with additional metals in *Enterobacter cloacae*

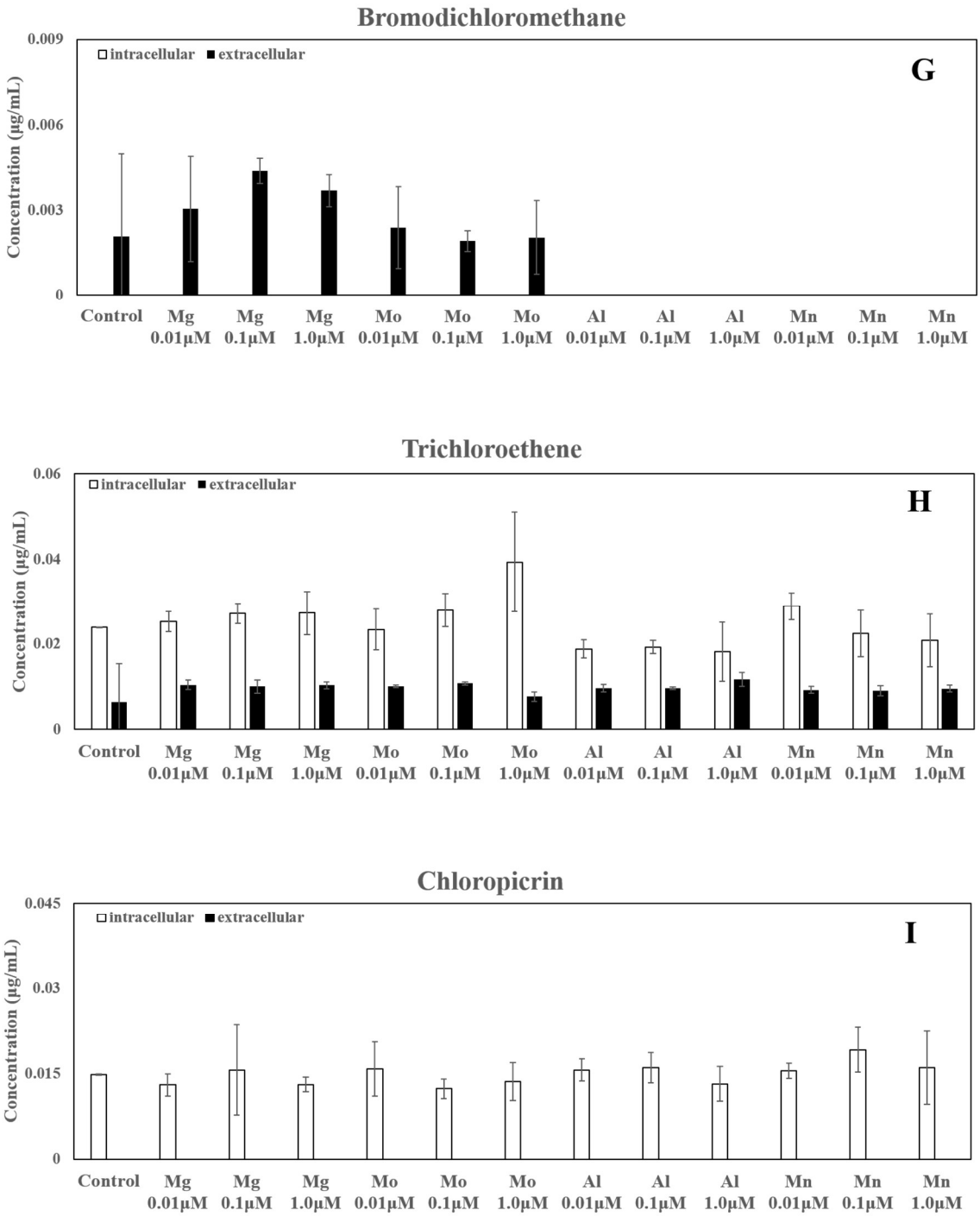


Figure 3.10 Intracellular and Extracellular Production of 15 DBPs (G to I) measured with additional metals in *Enterobacter cloacae*

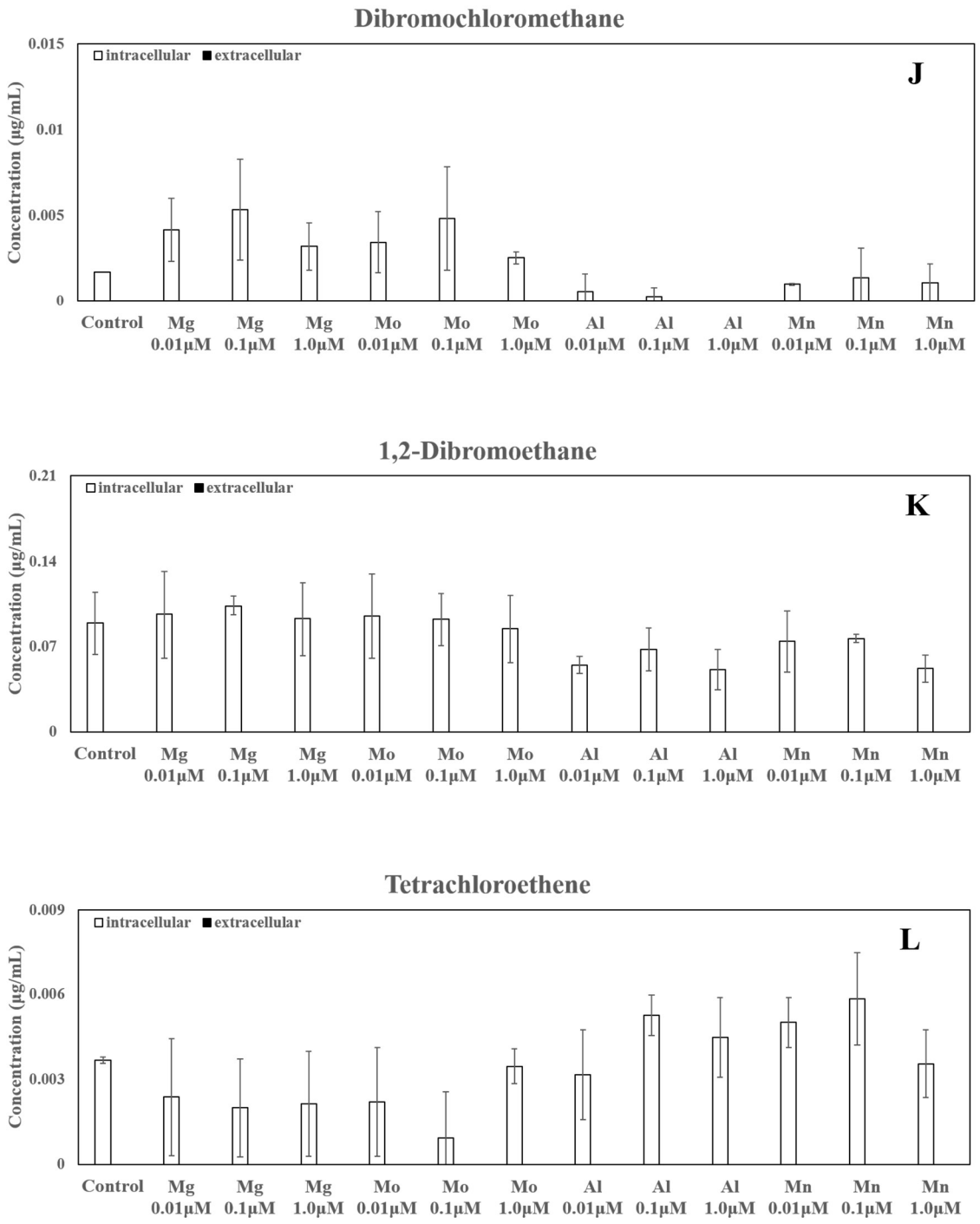


Figure 3.11 Intracellular and Extracellular Production of 15 DBPs (J to L) measured with additional metals in *Enterobacter cloacae*

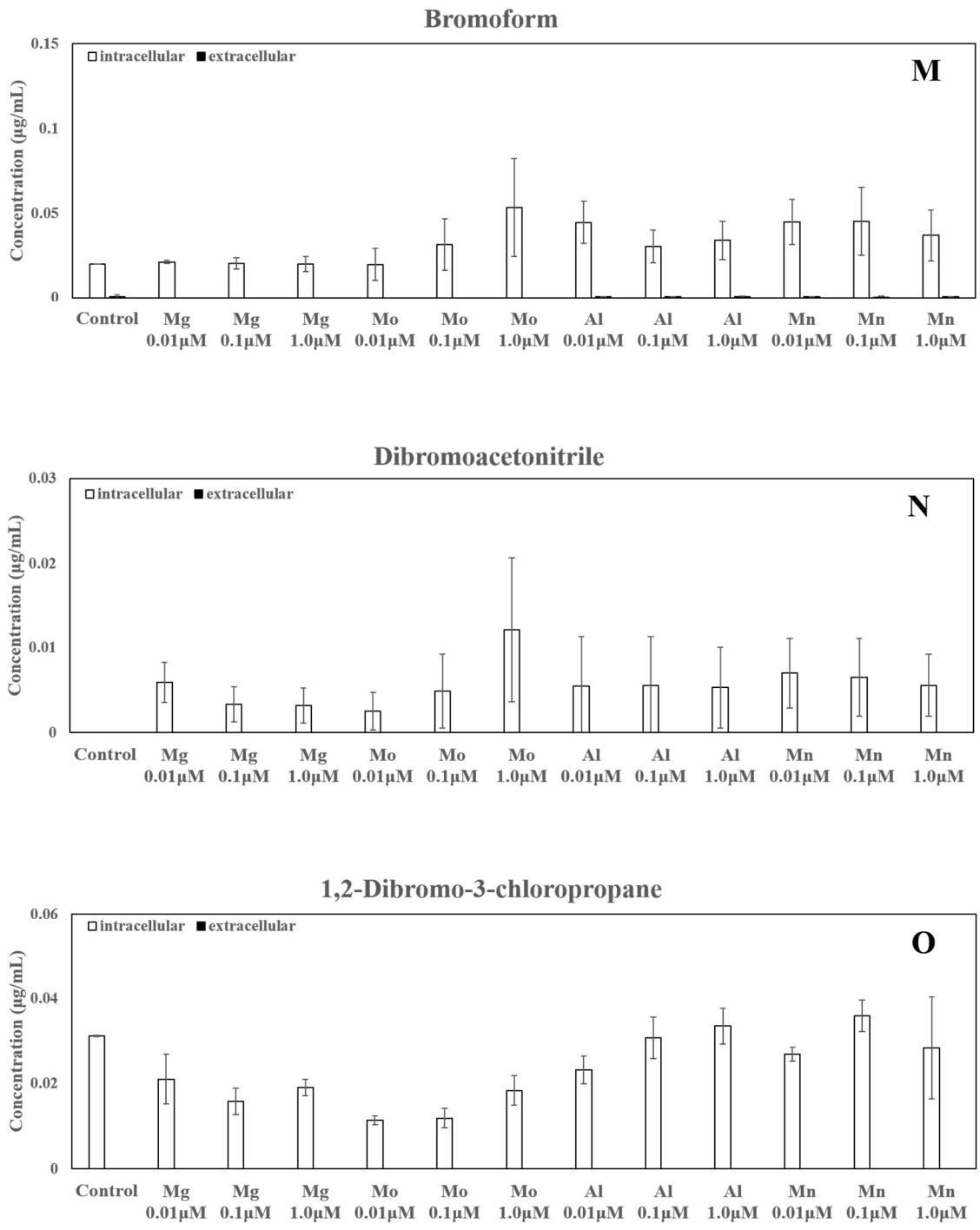


Figure 3.12 Intracellular and Extracellular Production of 15 DBPs (M to O) measured with additional metals in *Enterobacter cloacae*

Bacillus had similar results of *Enterobacter cloacae*. Because most of the DBPs in *Bacillus* were generated inside cells, and varied by metal ions. But *Bacillus* might potentially generate more extracellular DBPs precursors (Table 3-6 to 3-9), and its DBP increased by 0.01 μM Al^{3+} but mainly decreased under the pressure of Mn^{2+}. Conversely, *Enterobacter cloacae* DBPs may favor Mn^{2+} more but decreased by 0.1 μM and 1.0 μM of Al^{3+}. The other metal combinations seemed to decrease in *Bacillus*, with 10% decrease in the total DBPs yield, while *Enterobacter cloacae* had around 17-25% increase with metals. 0.1 μM Mg^{2+}, 0.1 μM Mo^{6+} and 0.1 μM Mn^{2+} were more efficient in changing the DBPs production. And the tendency of DBPs yielding was modified by metal ions (either increase or decrease), indeed, although the changes of DBPs with metals may not be occupied with their concentrations, influences by metals were statically. In addition, the bromide DBPs (Br-DBPs) could be as sensitive to metals as chlorinated DBPs (Cl-DBPs), but certain metals occurred concomitant with a clear modification on Br-DBP or Cl-DBP.

Mo^{6+} and Mg^{2+} showed increase on bromodichloroethane (G) and dibromochloromethane (J) while Al^{3+} and Mn^{2+} decrease them, and Mo^{6+}, Mg^{2+} and Mn^{2+} decreased a lot with bromoform (M), etc. Again, these findings point to metals as a potentially controlling factor in the formation of DBP precursors and perhaps instant DBP. Although the levels of metals used in this study might be low, it has been obviously in making changes on DBP formation. Furthermore, the use of metal factors as the inducer of bacterial matters were effectively. These natural pressures that may therefore increase DBPs in particular environments, not only exit in raw water from the nature, but also occur in the treated water with the purpose of coagulation or decrease hardness as well. Further research is needed, particularly on the effect of biochemical parameters on active cellular genes response to metal ions, and on the wider role of other prevalent organisms in environment, such as those favor bromide chemicals as nutrient, and those play roles in the carbon cycle.

Table 3-6. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Magnesium was added vs control in washed Bacillus cells (show in average and standards deviations for 3 replicates).

	Control		Additional Magnesium					
			0.01µM		0.1µM		1µM	
Total (µg/mL)	1.087 ± 0.073		0.943 ± 0.166		0.942 ± 0.081		0.896 ± 0.132	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	1.030 ± 0.061	0.057 ± 0.011	0.884 ± 0.158	0.059 ± 0.008	0.867 ± 0.072	0.075 ± 0.009	0.825 ± 0.120	0.071 ± 0.012
Chloroform	0.710 ± 0.001	0.015 ± 0.001	0.552 ± 0.057	0.013 ± 0.003	0.577 ± 0.011	0.027 ± 0.000	0.561 ± 0.009	0.018 ± 0.002
1,1,1-Trichloroethane	0.001 ± 0.000	0.011 ± 0.002	0.001 ± 0.000	0.009 ± 0.002	0.001 ± 0.000	0.008 ± 0.000	0.001 ± 0.000	0.008 ± 0.001
Carbon Tetrachloride	0.011 ± 0.003	0.000 ± 0.000	0.005 ± 0.002	N/A	0.005 ± 0.001	N/A	0.005 ± 0.001	N/A
Trichloroacetonitrile	0.013 ± 0.005	N/A	0.006 ± 0.000	N/A	0.006 ± 0.002	N/A	0.008 ± 0.002	N/A
1,1-Dichloroacetone	0.083 ± 0.027	0.018 ± 0.004	0.056 ± 0.012	0.025 ± 0.000	0.049 ± 0.011	0.026 ± 0.005	0.052 ± 0.008	0.031 ± 0.004
Dichloroacetonitrile	0.023 ± 0.000	0.000 ± 0.001	0.049 ± 0.017	N/A	0.034 ± 0.001	N/A	0.036 ± 0.010	N/A
Bromodichloromethane	N/A	0.001 ± 0.002	N/A	0.002 ± 0.001	N/A	0.003 ± 0.002	N/A	0.003 ± 0.003
Trichloroethene	0.029 ± 0.002	0.012 ± 0.001	0.030 ± 0.006	0.010 ± 0.001	0.028 ± 0.003	0.011 ± 0.001	0.030 ± 0.004	0.010 ± 0.001
Chloropicrin	N/A	N/A	0.016 ± 0.002	N/A	0.015 ± 0.001	N/A	0.015 ± 0.002	N/A
Dibromochloromethane	N/A	N/A	0.006 ± 0.004	N/A	0.002 ± 0.000	N/A	0.003 ± 0.002	N/A
1,2-Dibromoethane	0.051 ± 0.009	N/A	0.103 ± 0.026	N/A	0.093 ± 0.028	N/A	0.063 ± 0.054	N/A
Tetrachloroethene	0.003 ± 0.000	N/A	0.001 ± 0.002	N/A	0.003 ± 0.000	N/A	0.002 ± 0.002	N/A
Bromoform	0.083 ± 0.009	N/A	0.027 ± 0.019	N/A	0.031 ± 0.009	N/A	0.027 ± 0.020	0.001 ± 0.001
Dibromoacetonitrile	N/A	N/A	0.009 ± 0.004	N/A	0.006 ± 0.001	N/A	0.002 ± 0.002	N/A
1,2-Dibromo-3-chloropropane	0.022 ± 0.003	N/A	0.021 ± 0.005	N/A	0.018 ± 0.004	N/A	0.021 ± 0.004	N/A

Table 3-7. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Molybdenum was added vs control in washed Bacillus cells (show in average and standards deviations for 3 replicates).

	Control		Additional Molybdenum 0.01µM		0.1µM		1µM	
Total (µg/mL)	1.087 ± 0.073		0.848 ± 0.159		0.974 ± 0.149		1.013 ± 0.078	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	1.030 ± 0.061	0.057 ± 0.011	0.765 ± 0.155	0.083 ± 0.004	0.898 ± 0.138	0.076 ± 0.011	0.949 ± 0.070	0.064 ± 0.008
Chloroform	0.710 ± 0.001	0.015 ± 0.001	0.501 ± 0.009	0.028 ± 0.001	0.600 ± 0.016	0.019 ± 0.003	0.692 ± 0.011	0.013 ± 0.001
1,1,1-Trichloroethane	0.001 ± 0.000	0.011 ± 0.002	0.001 ± 0.000	0.009 ± 0.000	0.001 ± 0.000	0.008 ± 0.000	0.001 ± 0.000	0.009 ± 0.000
Carbon Tetrachloride	0.011 ± 0.003	0.000 ± 0.000	0.005 ± 0.002	N/A	0.005 ± 0.002	N/A	0.004 ± 0.001	N/A
Trichloroacetonitrile	0.013 ± 0.005	N/A	0.006 ± 0.005	N/A	0.004 ± 0.001	N/A	0.004 ± 0.001	N/A
1,1-Dichloroacetone	0.083 ± 0.027	0.018 ± 0.004	0.046 ± 0.015	0.029 ± 0.001	0.040 ± 0.008	0.034 ± 0.007	0.038 ± 0.006	0.029 ± 0.006
Dichloroacetonitrile	0.023 ± 0.000	0.000 ± 0.001	0.042 ± 0.018	N/A	0.067 ± 0.037	N/A	0.045 ± 0.019	N/A
Bromodichloromethane	N/A	0.001 ± 0.002	N/A	0.004 ± 0.001	N/A	0.005 ± 0.000	N/A	0.005 ± 0.000
Trichloroethene	0.029 ± 0.002	0.012 ± 0.001	0.027 ± 0.006	0.012 ± 0.001	0.028 ± 0.001	0.010 ± 0.001	0.025 ± 0.003	0.010 ± 0.001
Chloropicrin	N/A	N/A	0.013 ± 0.001	N/A	0.014 ± 0.001	N/A	0.013 ± 0.002	N/A
Dibromochloromethane	N/A	N/A	0.003 ± 0.002	N/A	0.006 ± 0.004	N/A	0.004 ± 0.002	N/A
1,2-Dibromoethane	0.051 ± 0.009	N/A	0.046 ± 0.042	N/A	0.076 ± 0.045	N/A	0.088 ± 0.019	N/A
Tetrachloroethene	0.003 ± 0.000	N/A	0.001 ± 0.002	N/A	0.001 ± 0.002	N/A	0.001 ± 0.002	N/A
Bromoform	0.083 ± 0.009	N/A	0.046 ± 0.041	N/A	0.036 ± 0.015	N/A	0.018 ± 0.000	N/A
Dibromoacetonitrile	N/A	N/A	0.009 ± 0.009	N/A	0.004 ± 0.004	N/A	0.005 ± 0.002	N/A
1,2-Dibromo-3-chloropropane	0.022 ± 0.003	N/A	0.019 ± 0.004	N/A	0.017 ± 0.001	N/A	0.012 ± 0.002	N/A

Table 3-8. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Aluminum was added vs control in washed Bacillus cells (show in average and standards deviations for 3 replicates)

	Control		Additional Aluminum 0.01µM		0.1µM		1µM	
Total (µg/mL)	1.087 ± 0.073		3.042 ± 1.522		0.912 ± 0.203		0.908 ± 0.102	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	1.030 ± 0.061	0.057 ± 0.011	2.988 ± 1.515	0.055 ± 0.007	0.837 ± 0.130	0.075 ± 0.073	0.857 ± 0.093	0.051 ± 0.008
Chloroform	0.710 ± 0.001	0.015 ± 0.001	2.711 ± 1.284	0.018 ± 0.001	0.515 ± 0.038	0.046 ± 0.060	0.546 ± 0.022	0.018 ± 0.002
1,1,1-Trichloroethane	0.001 ± 0.000	0.011 ± 0.002	0.001 ± 0.000	0.011 ± 0.002	0.001 ± 0.000	0.008 ± 0.001	0.001 ± 0.000	0.008 ± 0.002
Carbon Tetrachloride	0.011 ± 0.003	0.000 ± 0.000	0.011 ± 0.011	N/A	0.009 ± 0.003	N/A	0.008 ± 0.001	N/A
Trichloroacetonitrile	0.013 ± 0.005	N/A	0.009 ± 0.008	N/A	0.008 ± 0.004	N/A	0.009 ± 0.002	N/A
1,1-Dichloroacetone	0.083 ± 0.027	0.018 ± 0.004	0.050 ± 0.029	0.016 ± 0.003	0.058 ± 0.018	0.010 ± 0.005	0.047 ± 0.009	0.012 ± 0.002
Dichloroacetonitrile	0.023 ± 0.000	0.000 ± 0.001	0.016 ± 0.014	N/A	0.018 ± 0.004	0.003 ± 0.005	0.020 ± 0.004	N/A
Bromodichloromethane	N/A	0.001 ± 0.002	N/A	N/A	N/A	N/A	N/A	N/A
Trichloroethene	0.029 ± 0.002	0.012 ± 0.001	0.027 ± 0.016	0.009 ± 0.001	0.024 ± 0.003	0.008 ± 0.002	0.027 ± 0.003	0.011 ± 0.002
Chloropicrin	N/A	N/A	0.015 ± 0.014	N/A	0.020 ± 0.012	N/A	0.015 ± 0.001	N/A
Dibromochloromethane	N/A	N/A	0.001 ± 0.001	N/A	0.000 ± 0.001	N/A	0.001 ± 0.001	N/A
1,2-Dibromoethane	0.051 ± 0.009	N/A	0.051 ± 0.047	N/A	0.065 ± 0.022	N/A	0.075 ± 0.025	N/A
Tetrachloroethene	0.003 ± 0.000	N/A	0.003 ± 0.003	N/A	0.005 ± 0.001	N/A	0.005 ± 0.000	N/A
Bromoform	0.083 ± 0.009	N/A	0.062 ± 0.058	0.001 ± 0.000	0.070 ± 0.019	0.000 ± 0.000	0.060 ± 0.022	0.001
Dibromoacetonitrile	N/A	N/A	0.013 ± 0.015	N/A	0.011 ± 0.005	N/A	0.010 ± 0.002	N/A
1,2-Dibromo-3-chloropropane	0.022 ± 0.003	N/A	0.016 ± 0.015	N/A	0.030 ± 0.002	N/A	0.033 ± 0.001	N/A

Table 3-9. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Manganese was added vs control in washed Bacillus cells (show in average and standards deviations for 3 replicates).

	Control		Additional Manganese					
			0.01µM		0.1µM		1µM	
Total (µg/mL)	1.087 ± 0.073		0.853 ± 0.172		0.851 ± 0.117		0.852 ± 0.141	
	Intracellular	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**	**Intracellular**	**Extracellular**
In total/Ex total	1.030 ± 0.061	0.057 ± 0.011	0.798 ± 0.159	0.054 ± 0.013	0.812 ± 0.108	0.039 ± 0.009	0.810 ± 0.129	0.043 ± 0.012
Chloroform	0.710 ± 0.001	0.015 ± 0.001	0.502 ± 0.033	0.017 ± 0.001	0.539 ± 0.048	0.013 ± 0.003	0.533 ± 0.030	0.016 ± 0.002
1,1,1-Trichloroethane	0.001 ± 0.000	0.011 ± 0.002	0.000 ± 0.000	0.010 ± 0.002	0.001 ± 0.000	0.011 ± 0.004	0.001 ± 0.000	0.010 ± 0.006
Carbon Tetrachloride	0.011 ± 0.003	0.000 ± 0.000	0.007 ± 0.001	N/A	0.008 ± 0.002	N/A	0.008 ± 0.002	N/A
Trichloroacetonitrile	0.013 ± 0.005	N/A	0.009 ± 0.003	N/A	0.009 ± 0.002	N/A	0.010 ± 0.003	N/A
1,1-Dichloroacetone	0.083 ± 0.027	0.018 ± 0.004	0.042 ± 0.002	0.014 ± 0.007	0.046 ± 0.005	0.006 ± 0.001	0.045 ± 0.007	0.007 ± 0.002
Dichloroacetonitrile	0.023 ± 0.000	0.000 ± 0.001	0.017 ± 0.003	N/A	0.018 ± 0.006	N/A	0.017 ± 0.004	N/A
Bromodichloromethane	N/A	0.001 ± 0.002	N/A	N/A	N/A	N/A	N/A	N/A
Trichloroethene	0.029 ± 0.002	0.012 ± 0.001	0.022 ± 0.006	0.012 ± 0.003	0.018 ± 0.006	0.008 ± 0.001	0.019 ± 0.003	0.008 ± 0.001
Chloropicrin	N/A	N/A	0.014 ± 0.001	N/A	0.015 ± 0.001	N/A	0.015 ± 0.002	N/A
Dibromochloromethane	N/A	N/A	0.001 ± 0.000	N/A	0.001 ± 0.002	N/A	0.001 ± 0.001	N/A
1,2-Dibromoethane	0.051 ± 0.009	N/A	0.105 ± 0.082	N/A	0.064 ± 0.005	N/A	0.082 ± 0.042	N/A
Tetrachloroethene	0.003 ± 0.000	N/A	0.004 ± 0.001	N/A	0.004 ± 0.002	N/A	0.004 ± 0.001	N/A
Bromoform	0.083 ± 0.009	N/A	0.039 ± 0.019	0.001 ± 0.000	0.046 ± 0.017	0.001 ± 0.000	0.040 ± 0.028	0.000 ± 0.000
Dibromoacetonitrile	N/A	N/A	0.007 ± 0.006	N/A	0.009 ± 0.007	N/A	0.007 ± 0.004	N/A
1,2-Dibromo-3-chloropropane	0.022 ± 0.003	N/A	0.030 ± 0.003	N/A	0.035 ± 0.005	N/A	0.029 ± 0.002	N/A

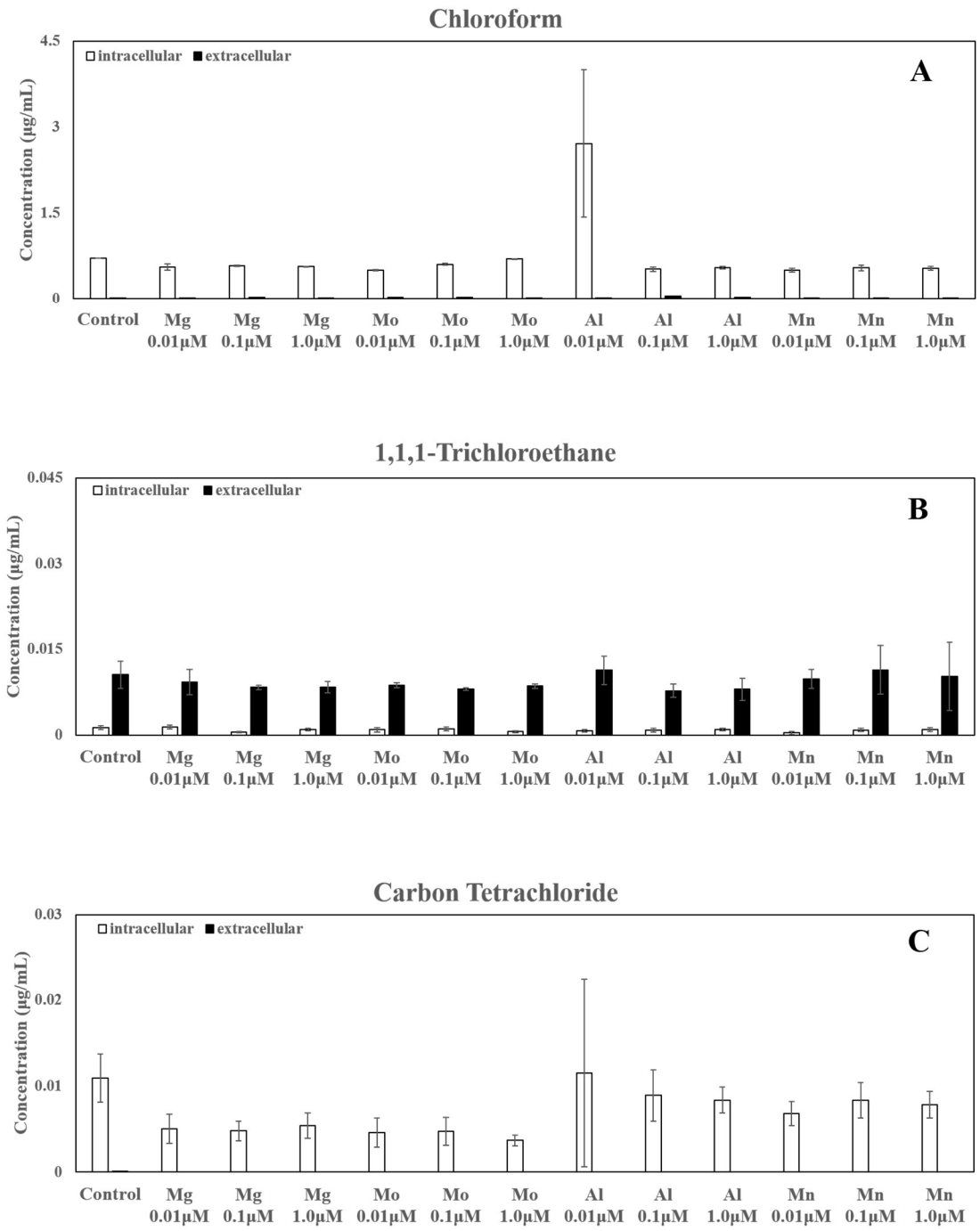


Figure 3.13 Intracellular and Extracellular Production of 15 DBPs (A to C) measured with additional metals in *Bacillus*

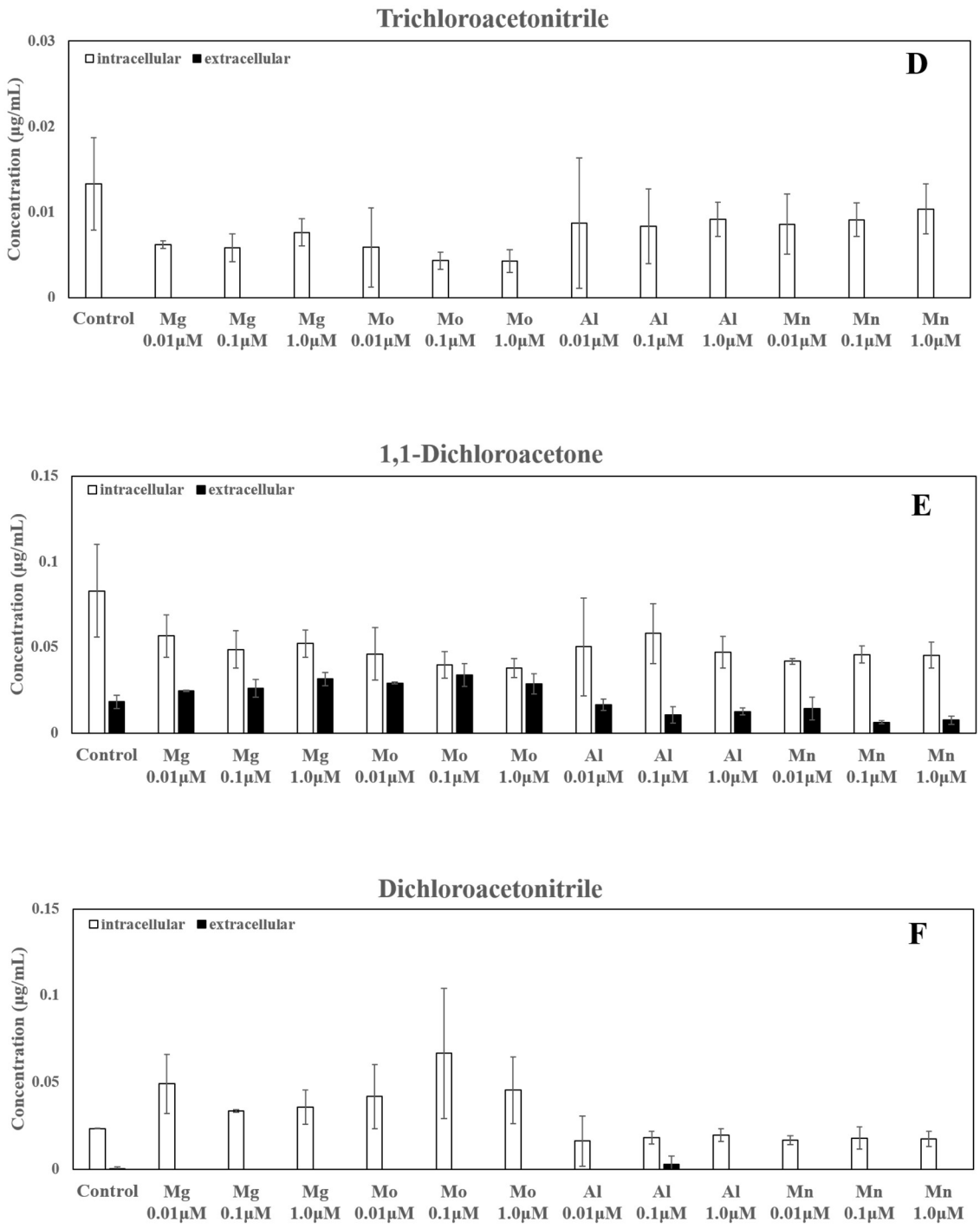


Figure 3.14 Intracellular and Extracellular Production of 15 DBPs (D to F) measured with additional metals in *Bacillus*

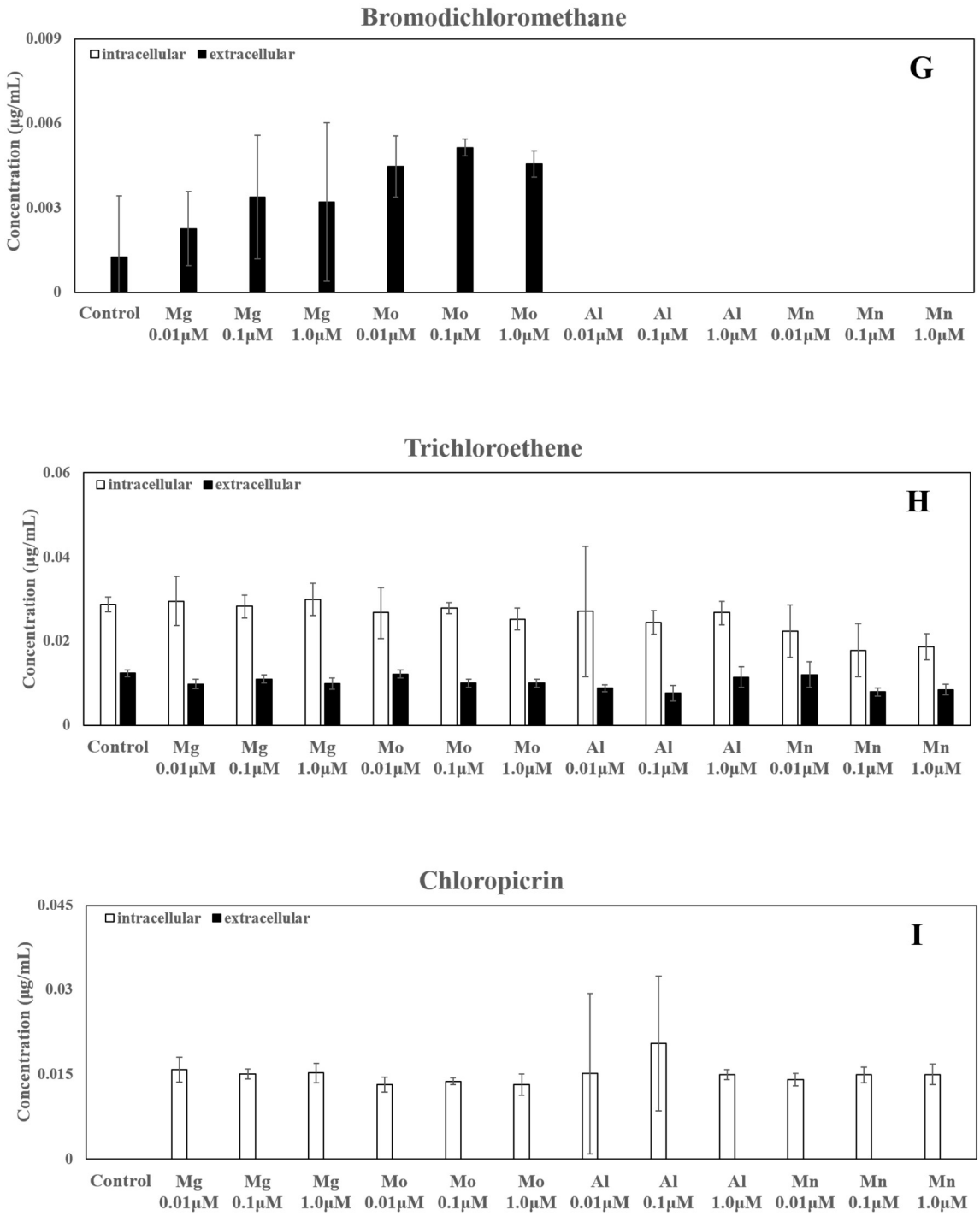


Figure 3.15 Intracellular and Extracellular Production of 15 DBPs (G to I) measured with additional metals in *Bacillus*

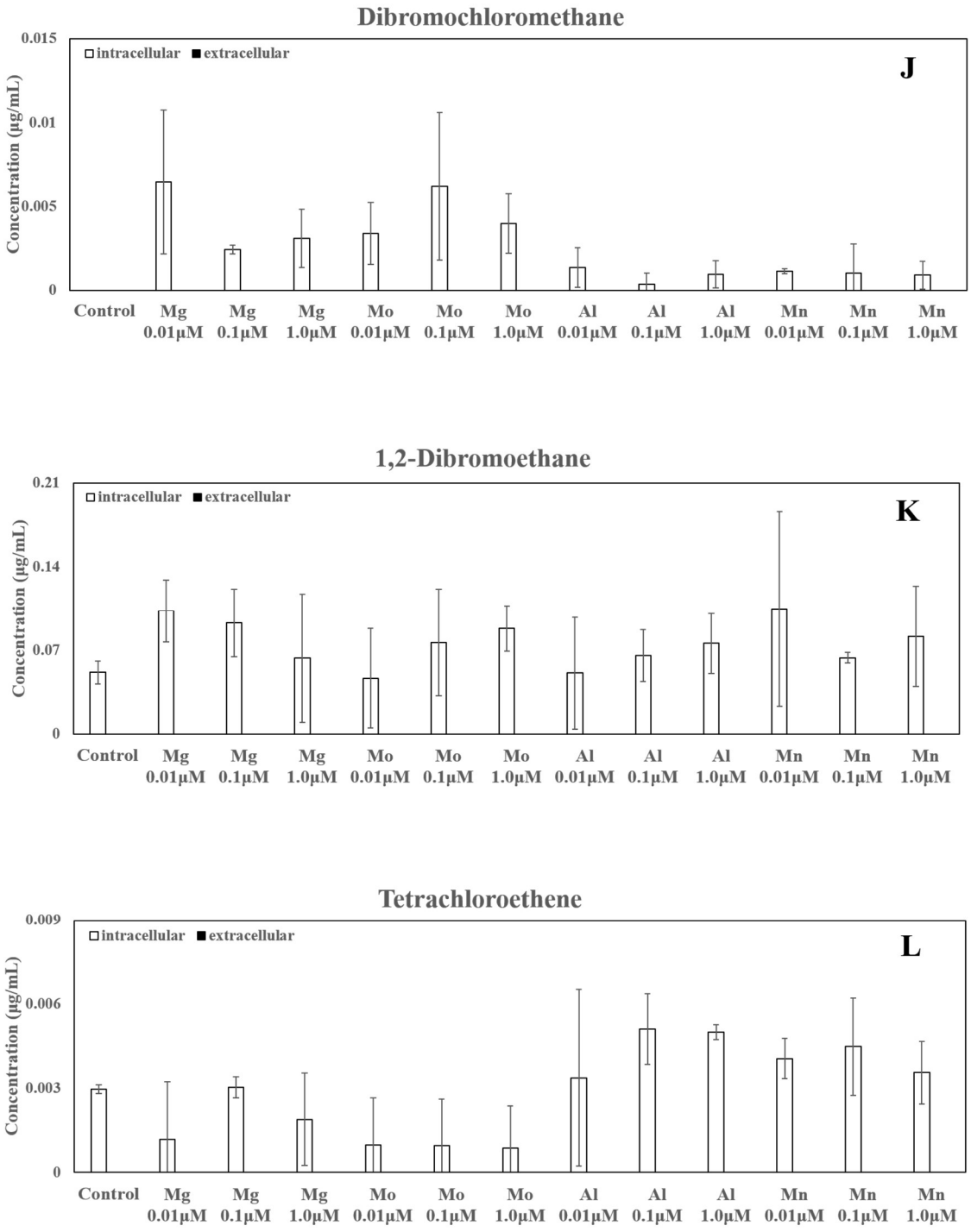


Figure 3.16 Intracellular and Extracellular Production of 15 DBPs (J to L) measured with additional metals in *Bacillus*

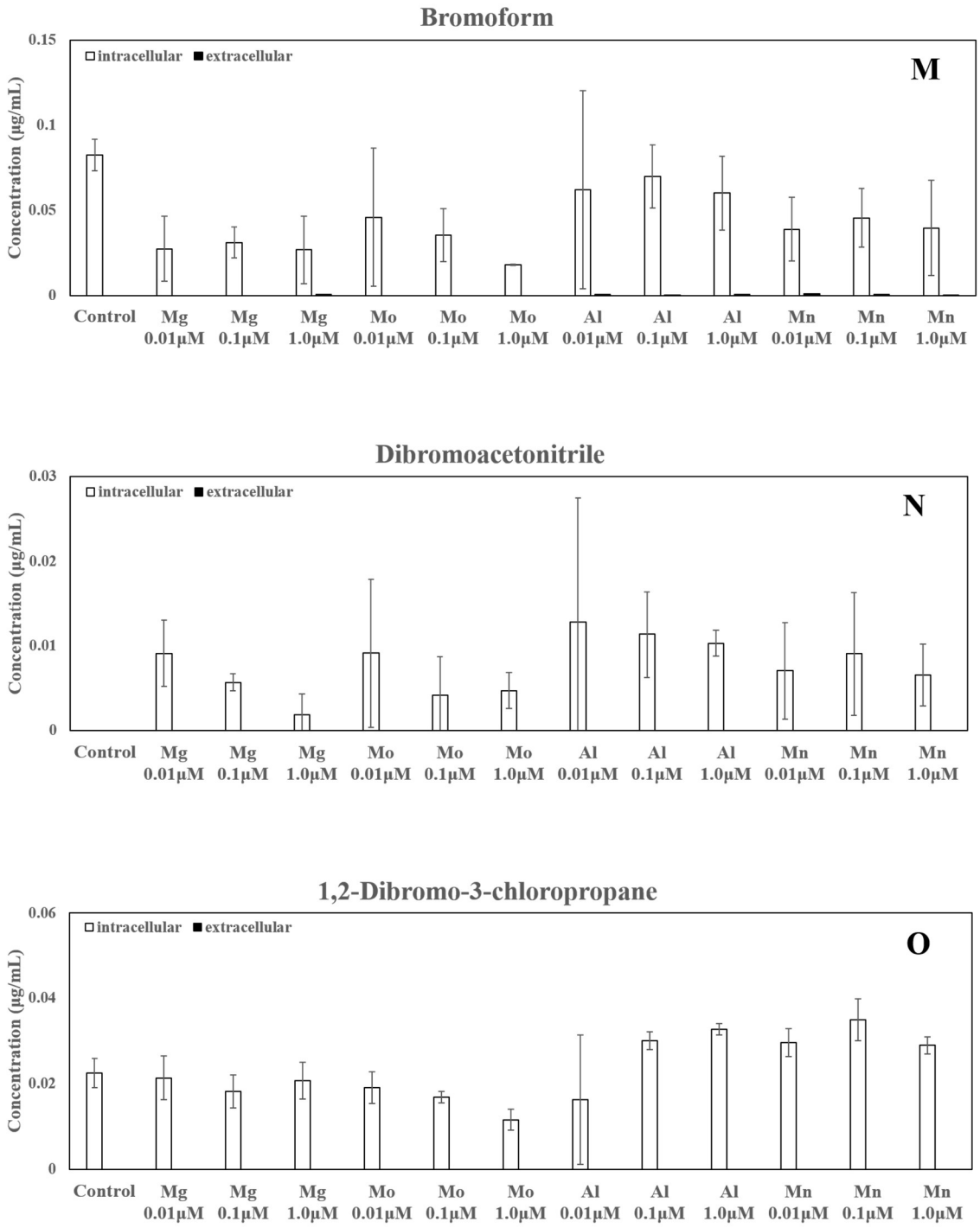


Figure 3.17 Intracellular and Extracellular Production of 15 DBPs (M to O) measured with additional metals in *Bacillus*

The DBPs may contain some NOM from LB media, especially those Br-DBPs because bromide compounds were just from the nutrients in the media. Table 3-10 to 3-13 and Fig 3.18 to Fig 3.22 showed the DBPs from the pure media with the same experiment method of above. Interestingly, only few DBPs were formed in the media, and two Br-DBPs were available: Bromodichloromethane and Bromoform, as well as the data above, Bromodichloromethane just occur with Mo^{6+} and Mg^{2+}. However, both of two Br-DBPs had very low concentrations (< 0.002 μg/mL) from media, so that these could be more likely the slightly chemical reaction between metals and bromide. Unexpectedly, the biggest DBP, chloroform, which took over 60% of total DBPs in *Enterobacter cloacae* and *Bacillus*, was tiny in the media DBPs, that means cell efforts played very important role on chloroform formation during disinfection. On opposite, 1, 1, 1-trichloroethane, still keep similar concentration in media without cells in compare with the extracellular 1, 1, 1-trichloroethane in both of *Enterobacter cloacae* and *Bacillus*, suggesting that 1, 1, 1-trichloroethane may mainly generate from the media NOM. The other similar DBP, trichloroethene, also showed close concentration between groups with cells and groups without cells. But it was different that trichloroethane also occurred intracellularly, so there is a possibility that the precursors of trichloroethane could be both from the NOM of the media and the bacterial cell matters.

The last DBP appeared in media without media (no cell), 1,1-dichloroacetone, was in a low concentration in compare with its levels with cell. These data add up to the hypothesis that bacterial cells and bacterial matters are resources and intermediators of DBPs formation.

Table 3-10. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Magnesium was added vs control in no cells media (show in average and standards deviations for 3 replicates).

	Control	Additional Magnesium		
		0.01μM	0.1μM	1μM
Total (μg/mL)	0.081 ± 0.052	0.207 ± 0.025	0.177 ± 0.006	0.173 ± 0.007
Chloroform	0.007 ± 0.001	0.010± 0.000	0.009 ± 0.000	0.008 ± 0.001
1,1,1-Trichloroethane	0.068 ± 0.049	0.176 ± 0.021	0.150 ± 0.003	0.147 ± 0.004
Carbon Tetrachloride	N/A	N/A	N/A	N/A
Trichloroacetonitrile	N/A	N/A	N/A	N/A
1,1-Dichloroacetone	0.002 ± 0.001	0.007± 0.002	0.004 ± 0.001	0.005 ± 0.000
Dichloroacetonitrile	N/A	N/A	N/A	N/A
Bromodichloromethane	N/A	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Trichloroethene	0.005 ± 0.002	0.012 ± 0.002	0.011 ± 0.000	0.011 ± 0.001
Chloropicrin	N/A	N/A	N/A	N/A
Dibromochloromethane	N/A	N/A	N/A	N/A
1,2-Dibromoethane	N/A	N/A	N/A	N/A
Tetrachloroethene	N/A	N/A	N/A	N/A
Bromoform	N/A	N/A	0.001 ± 0.001	N/A
Dibromoacetonitrile	N/A	N/A	N/A	N/A
1,2-Dibromo-3-chloropropane	N/A	N/A	N/A	N/A

Table 3-11. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Molybdenum was added vs control in in no cells media (show in average and standards deviations for 3 replicates).

	Control	Additional Molybdenum		
		0.01μM	0.1μM	1μM
Total (μg/mL)	0.081 ± 0.052	0.184 ± 0.012	0.192 ± 0.007	0.194 ± 0.013
Chloroform	0.007 ± 0.001	0.013± 0.003	0.009 ± 0.000	0.009 ± 0.000
1,1,1-Trichloroethane	0.068 ± 0.049	0.152 ± 0.008	0.164 ± 0.005	0.162 ± 0.007
Carbon Tetrachloride	N/A	N/A	N/A	N/A
Trichloroacetonitrile	N/A	N/A	N/A	N/A
1,1-Dichloroacetone	0.002 ± 0.001	0.005± 0.000	0.006 ± 0.001	0.007 ± 0.001
Dichloroacetonitrile	N/A	N/A	N/A	N/A
Bromodichloromethane	N/A	0.002 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Trichloroethene	0.005 ± 0.002	0.011 ± 0.000	0.011 ± 0.001	0.011 ± 0.002
Chloropicrin	N/A	N/A	N/A	N/A
Dibromochloromethane	N/A	N/A	N/A	N/A
1,2-Dibromoethane	N/A	N/A	N/A	N/A
Tetrachloroethene	N/A	N/A	N/A	N/A
Bromoform	N/A	0.001 ± 0.001	0.001 ± 0.001	0.002 ± 0.002
Dibromoacetonitrile	N/A	N/A	N/A	N/A
1,2-Dibromo-3-chloropropane	N/A	N/A	N/A	N/A

Table 3-12. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Aluminum was added vs control in in no cells media (show in average and standards deviations for 3 replicates).

	Control	Additional Aluminum		
		0.01μM	0.1μM	1μM
Total (μg/mL)	0.081 ± 0.052	0.053 ± 0.004	0.055 ± 0.012	0.072 ± 0.009
Chloroform	0.007 ± 0.001	0.008± 0.001	0.006 ± 0.003	0.006 ± 0.003
1,1,1-Trichloroethane	0.068 ± 0.049	0.037 ± 0.002	0.040 ± 0.008	0.057 ± 0.005
Carbon Tetrachloride	N/A	N/A	N/A	N/A
Trichloroacetonitrile	N/A	N/A	N/A	N/A
1,1-Dichloroacetone	0.002 ± 0.001	0.002± 0.001	0.002 ± 0.001	0.002 ± 0.000
Dichloroacetonitrile	N/A	N/A	N/A	N/A
Bromodichloromethane	N/A	N/A	N/A	N/A
Trichloroethene	0.005 ± 0.002	0.006 ± 0.000	0.006 ± 0.000	0.007 ± 0.001
Chloropicrin	N/A	N/A	N/A	N/A
Dibromochloromethane	N/A	N/A	N/A	N/A
1,2-Dibromoethane	N/A	N/A	N/A	N/A
Tetrachloroethene	N/A	N/A	N/A	N/A
Bromoform	N/A	N/A	N/A	N/A
Dibromoacetonitrile	N/A	N/A	N/A	N/A
1,2-Dibromo-3-chloropropane	N/A	N/A	N/A	N/A

Table 3-13. Total DBP formation potential and percent by mass among the 15 DBPs measured when additional Manganese was added vs control in in no cells media (show in average and standards deviations for 3 replicates).

	Control	Additional Manganese		
		0.01μM	0.1μM	1μM
Total (μg/mL)	0.081 ± 0.052	0.117 ± 0.085	0.059 ± 0.018	0.061 ± 0.008
Chloroform	0.007 ± 0.001	0.010± 0.000	0.008 ± 0.001	0.004 ± 0.000
1,1,1-Trichloroethane	0.068 ± 0.049	0.099 ± 0.083	0.044 ± 0.016	0.048 ± 0.007
Carbon Tetrachloride	N/A	N/A	N/A	N/A
Trichloroacetonitrile	N/A	N/A	N/A	N/A
1,1-Dichloroacetone	0.002 ± 0.001	0.001± 0.000	0.001 ± 0.000	0.001 ± 0.000
Dichloroacetonitrile	N/A	N/A	N/A	N/A
Bromodichloromethane	N/A	N/A	N/A	N/A
Trichloroethene	0.005 ± 0.002	0.008 ± 0.002	0.007 ± 0.001	0.007 ± 0.001
Chloropicrin	N/A	N/A	N/A	N/A
Dibromochloromethane	N/A	N/A	N/A	N/A
1,2-Dibromoethane	N/A	N/A	N/A	N/A
Tetrachloroethene	N/A	N/A	N/A	N/A
Bromoform	N/A	N/A	N/A	N/A
Dibromoacetonitrile	N/A	N/A	N/A	N/A
1,2-Dibromo-3-chloropropane	N/A	N/A	N/A	N/A

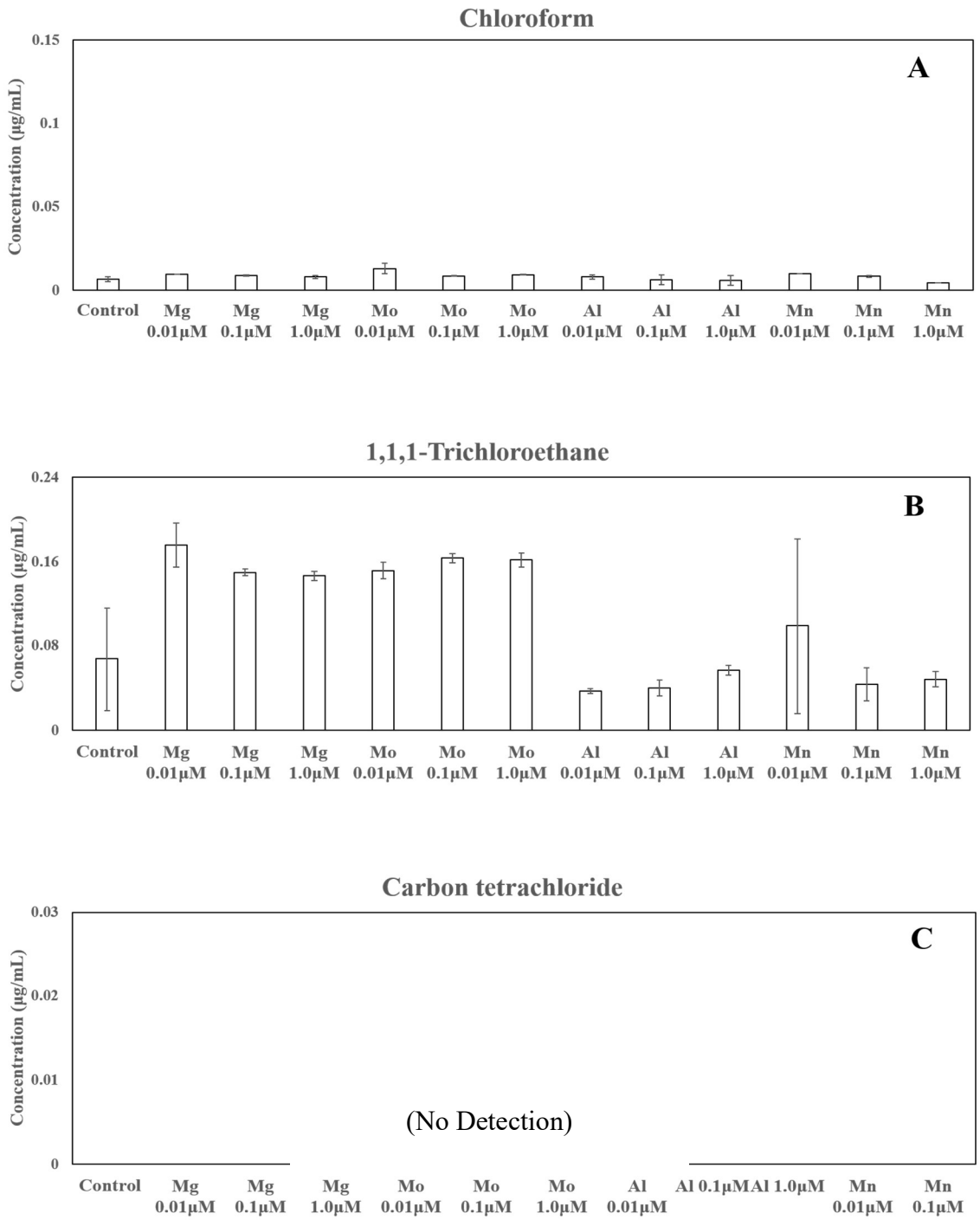


Figure 3.18 Production of 15 DBPs (A to C) measured with additional metals in no cell media

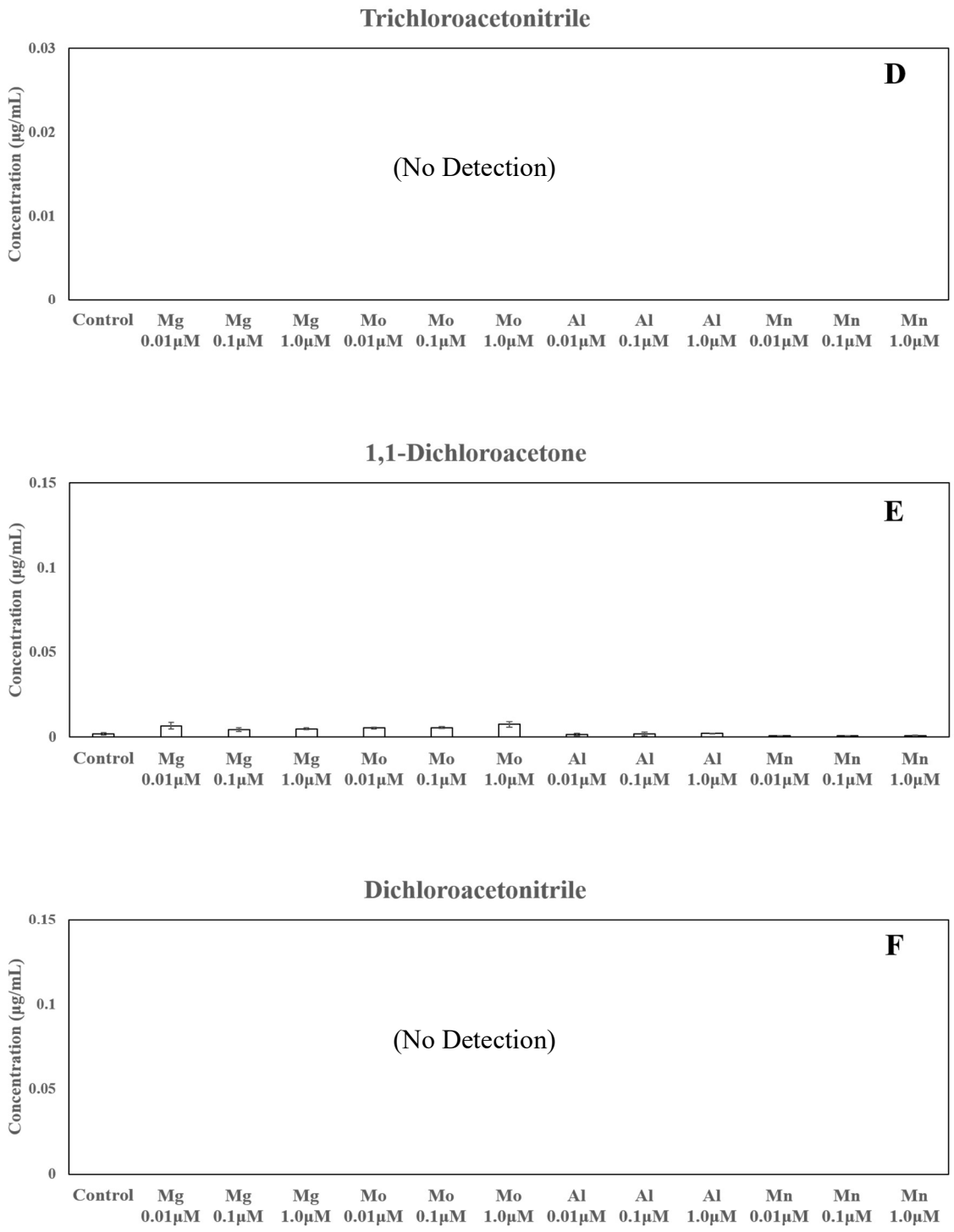


Figure 3.19 Production of 15 DBPs (D to F) measured with additional metals in no cell media

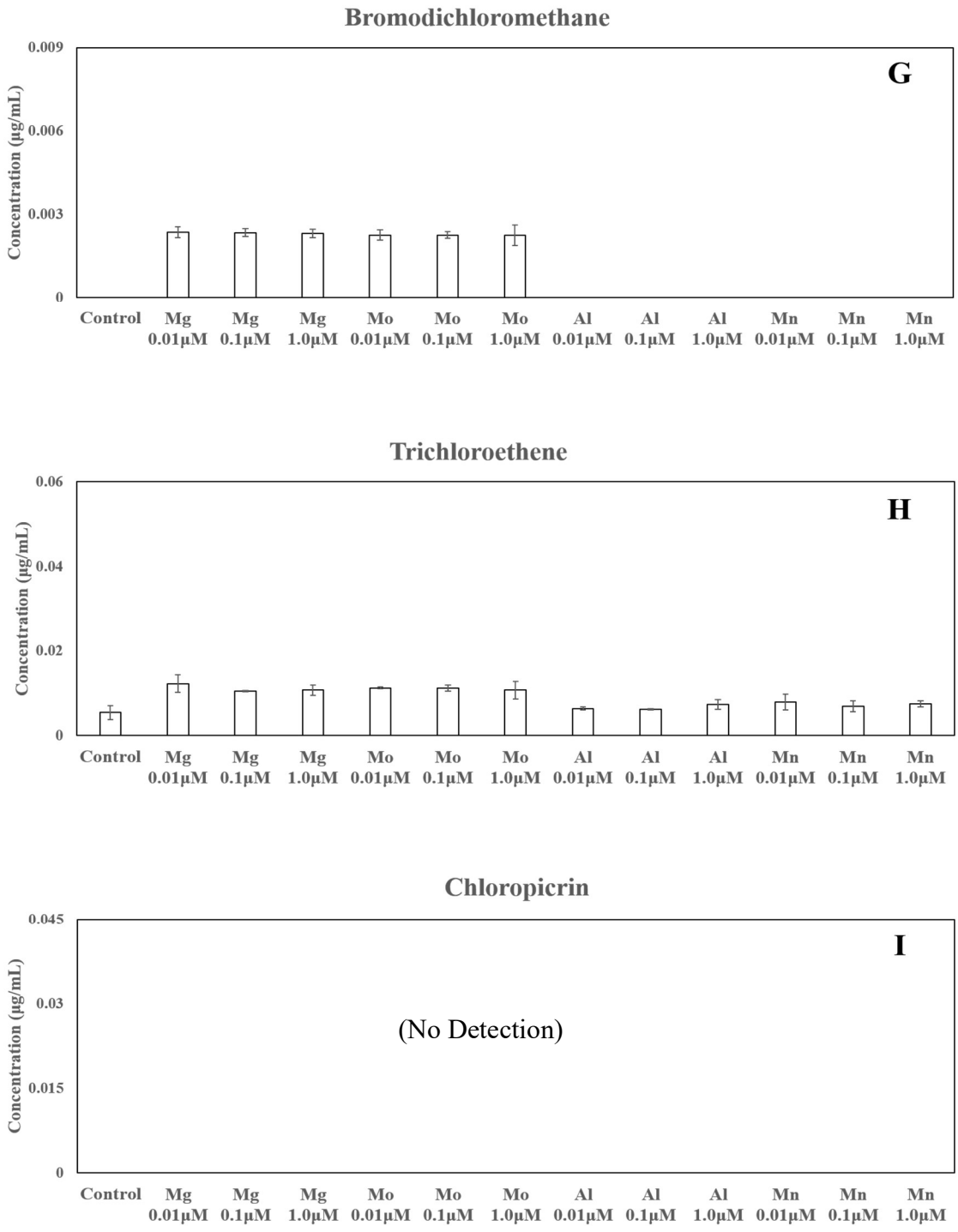


Figure 3.20 Production of 15 DBPs (G to I) measured with additional metals in no cell media

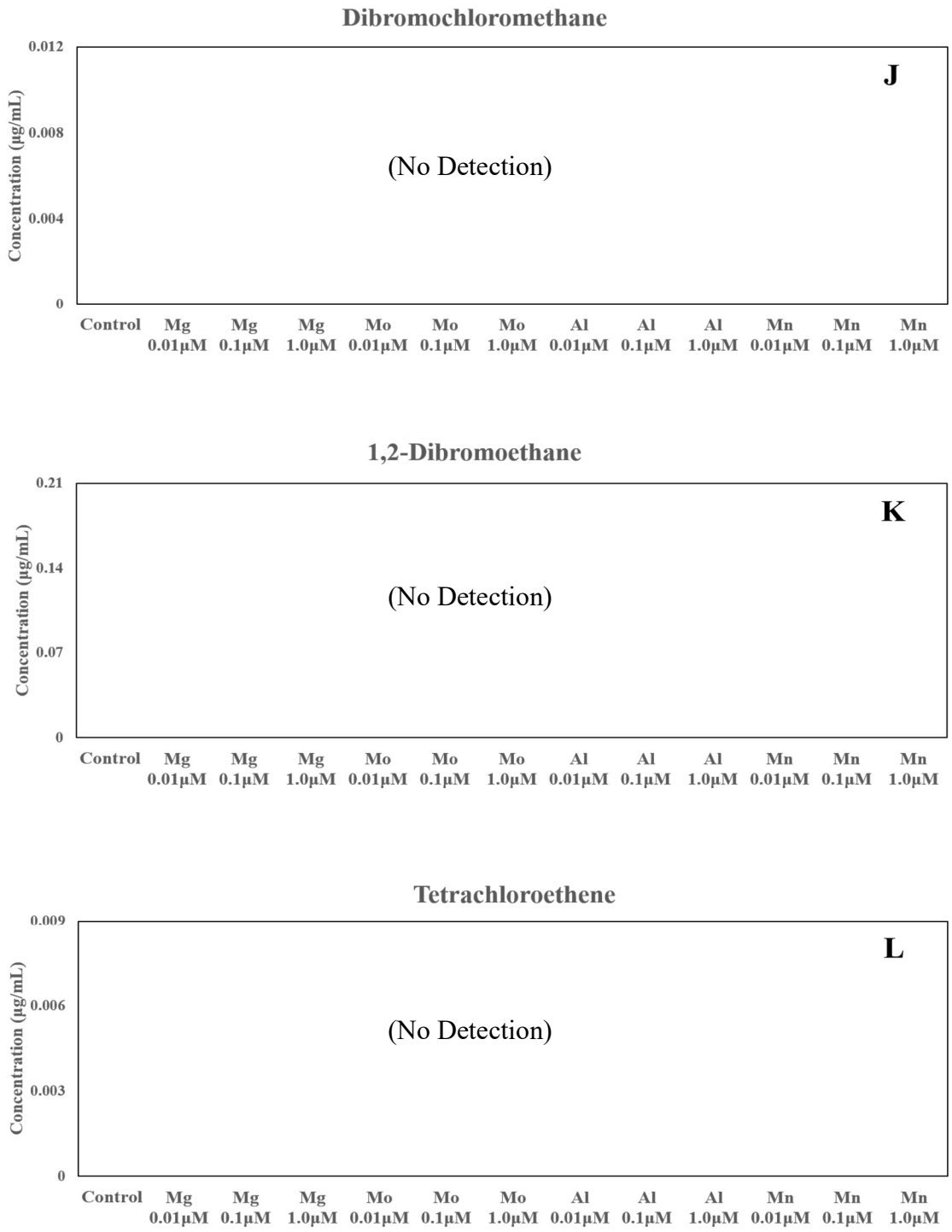


Figure 3.21 Production of 15 DBPs (J to L) measured with additional metals in no cell media

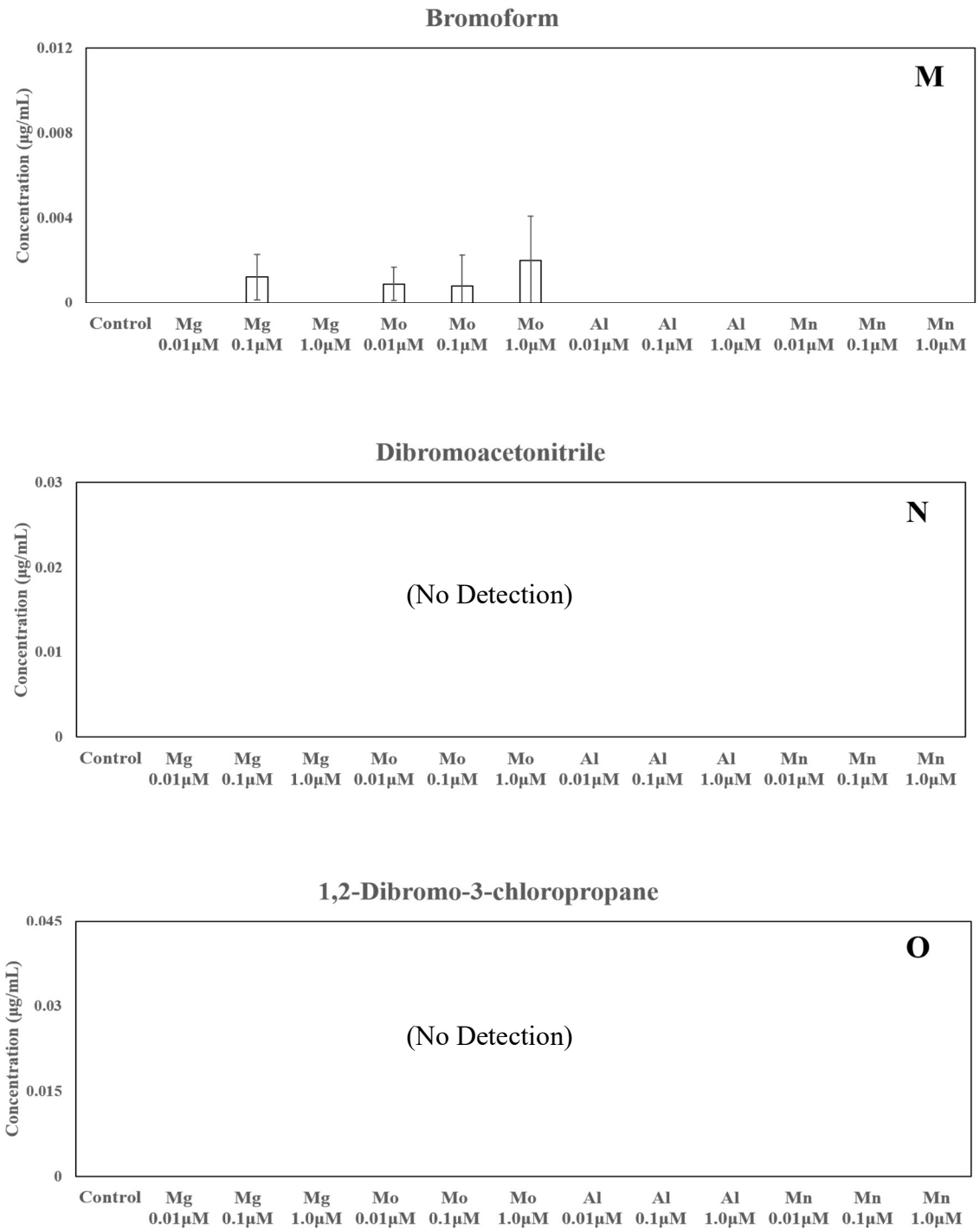


Figure 3.22 Production of 15 DBPs (M to O) measured with additional metals in no cell media

In general, chloroform is the dominated DBP in this study; the rest of 14 DBPs were all below 0.1 μg/mL. The effects of metal ions were significant that made changes on DBP formations and some changes of DBP followed with the increase of the metals. *Enterobacter cloacae* was sensitive to Mo^{6+}, Al^{3+} and Mn^{2+}, especially the 0.01 μM Mo^{6+}, 0.1 μM Al^{3+}, 1.0 μM Al^{3+}, 0.1 μM Mn^{2+} and 1.0 μM Mn^{2+}. Mo^{6+} decreased the ratio of chloroform with its levels increased, and Mg^{2+} did not change much but increase a bit of chloroform while decreased the 1, 1-dichloroacetone. More 1, 1, 1-trichloroethane had been induced by the aluminum in *Enterobacter cloacae*, meaning its precursors may be especially activated by this metal. Overall, intracellular DBPs apparently had greater concentrations than extracellular DBPs, and *Bacillus* yielded more extracellular DBPs. In *Enterobacter cloacae*, 0.1 μM Al^{3+} and 1.0 μM Al^{3+} decreased the intracellular DBPs, Mg^{2+} was obviously increase extracellular DBPs but not as strong on intracellular DBPs. Mo^{6+} may be the best inducer for *Bacillus* extracellular DBP precursors. Even though metal ions may just had slight influence on cell growth and total cellular materials, metals affected the DBPs yielding obviously in comparison with controls.

And the no cell media DBPs results progressively reflected that the reactions or the combinations between metals and cells are very important to DBP formation. There are some studies had reported the metal influences on metals (Navalon et al., 2009; Liu et al., 2011, 2013; Zhao et al., 2016; Sharma et al., 2017), In the study made by Zhao (2016), the effects of calcium, cupric, ferrous and ferric ions on the formation of THMs and HAAs in NOM were compared with the metal efforts on the DBP formation in natural water samples, they found that all of the metals could either increase or decrease the THMs and HAAs effectively, and concluded that the molecular structure and weight of metal complexes could be the reason of metal influences. Other studies suggested that DBP formation is also primarily influenced by disinfectant type, dose, reaction time, temperature, and pH. Chlorine as the most commonly used disinfectant, had been applied to primary disinfection worldwide, but it often produces high amounts of trihalomethanes

(THM) and haloacetic acids (HAA) that could be increased by natural factors. In our study, although metal ions did have many influences on DBP formation, the changes were limited by the availability of cells but not media NOM. Without careful chemical detection, it is hard to tell if the characteristic of metal ions determines the changes of metal ions on DBP formation. Our data supports the assumption that there could be correlations between metals and organic matters (both NOM and BOM) to affect DBP productions.

Another phenomenon hinted in our DBP formation results were quite interesting, extracellular DBPs only took comparative lower ratio than intracellular DBPs for both of *Enterobacter cloacae* and *Bacillus*, but Li, et al. (2011), had reported that the EOM of algae may yield more DBPs than intracellular organic matters, and Yang et al. (2011), reported that algal cells and EOM of Microcystis and Chlorella exhibited a high potential for DBP formation, and yields of total DBPs varied with the algae cultivation age, so that cellular materials contributed a lot to DBP formation. In addition, Zhou et al. (2014), found that intracellular organic matter (IOM) release increased with ferrate (VI) dosage and contributed to the formation of THM and HAA as chlorination by-products. However the extracellular DBPs in our study were not as significant as intracellular DBPs that may be because bacteria are different from algae in many aspects or the structural difference, and we did find some differences between two strains that *Bacillus* yielded more extracellular DBPs, so whether intracellular or extracellular changes may be due to the characteristics of cells and metals.

Zhang (2019), addressed that DBPs have investigated the effects of some common ions in water on DBP formation, where K^+ only reduce the formation of DBPs during chloramination. Ca^{2+} forms less DBPs than Mg^{2+} does during chlorination and chloramination due to the stronger binding effect. Al^{3+} and their hydroxide colloids have a significant effect on DBP formation.

Zamyadi et al. (2012), addressed that the DBP concentrations in environmental bloom conditions with very high cell numbers were over the guideline values. But my observation of the

metals we selected ranged from 0.01mM to 1mM did not reflect obvious correlations between cell number and DBP production, the more essential correlations might hide inside the cells or the products generated from cells under metal pressures.

With the comparison between intracellular DBPs and extracellular DBPs, it is clear that the higher of total DBPs, the more ratio of intracellular DBPs; and the fewer of total DBPs, rewarded more extracellular DBPs. This phenomenon may because of the chloroform which takes most of the ratio and mainly form intracellularly.

The spread ratio of DBPs may help to estimate what kinds of DBP are favored by the metals (more likely to have reaction with metals), the results were shown on the figures below.

The overall DBPs spread ratios were shown on Fig 3.23 and Fig 3.24, The DBP distributions for both strains may prove the metals also impact the DBP spreads, it is seemed like that the more of total DBPs, the less of extracellular DBPs. *Enterobacter cloacae* had high intracellular ratios with 0.1 μM Al^{3+} and 0.1 μM Mn^{2+}, and smaller ratios with 0.01 μM Al^{3+}, 1.0 μM Al^{3+} and 1.0 μM Mg^{2+}. DBP Distribution in *Bacillus* seemed to be strongly affected by 0.01 μM Al^{3+}, but metals may have increased the ratios of dichloroacetonitrile and 1, 2-Dibromoethane while suppress the ratio of chloroform. All of the increase and decrease were coincident with the increase and decrease of total DBPs. But there is a question that why 0.1 μM Mn^{2+} to *Enterobacter cloacae* and 0.01μM Al^{3+} to *Bacillus* were so bizarre? Again from the study made by Zhao et al. (2016), which had observed seemingly changes of metal ions on algae were based on some certain concentration of metals but there was no changes correlated with metal concentrations. And some other studies, like that reported by Gan et al. (2015), which tested the ferrate (VI) impacts on the DBP formation by chlorination, resulting in uneven changes with gradient metal levels (1 and 5 mg/L of Fe yields more than 10 and 20 mg/L of Fe). The concentration of metals could be another factor, and it is not only the link to the chemical capacity, but also relates to the concentration-sensitive bacterial mechanisms, such as the pathway on the cell membrane. If so, the surprising change on certain

metal levels may make sense, but we should expect some discrepancies during the experiment by operation or equipment, for now, more evidences are required.

The DBPs spread with Mg^{2+} were stable, either in *Enterobacter cloacae* or *Bacillus*, the ratios of intracellular DBPs with Mg^{2+} were just in range of 92% to 94%, unlike the intracellular DBPs with Mo^{6+}, which switched from over 95% in *Enterobacter cloacae* to 90% in *Bacillus*, so did those with Al^{3+} and Mn^{2+}. As some other studies had been described above, the characteristics of metals could be a potential factor influencing the extracellular DBP formation.

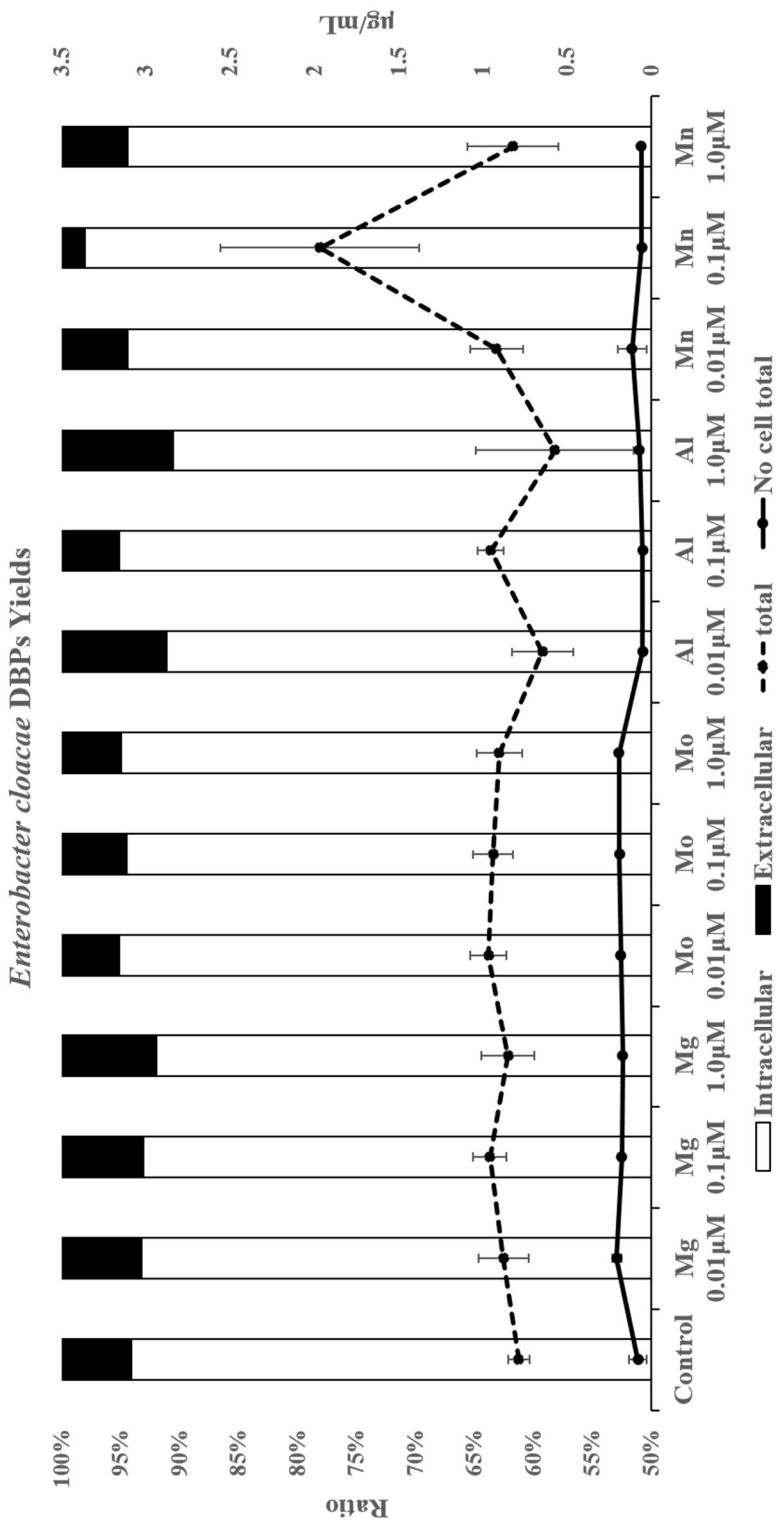


Figure 3.23 Comparison of intracellular DBPs and extracellular DBPs in *Enterobacter cloacae*

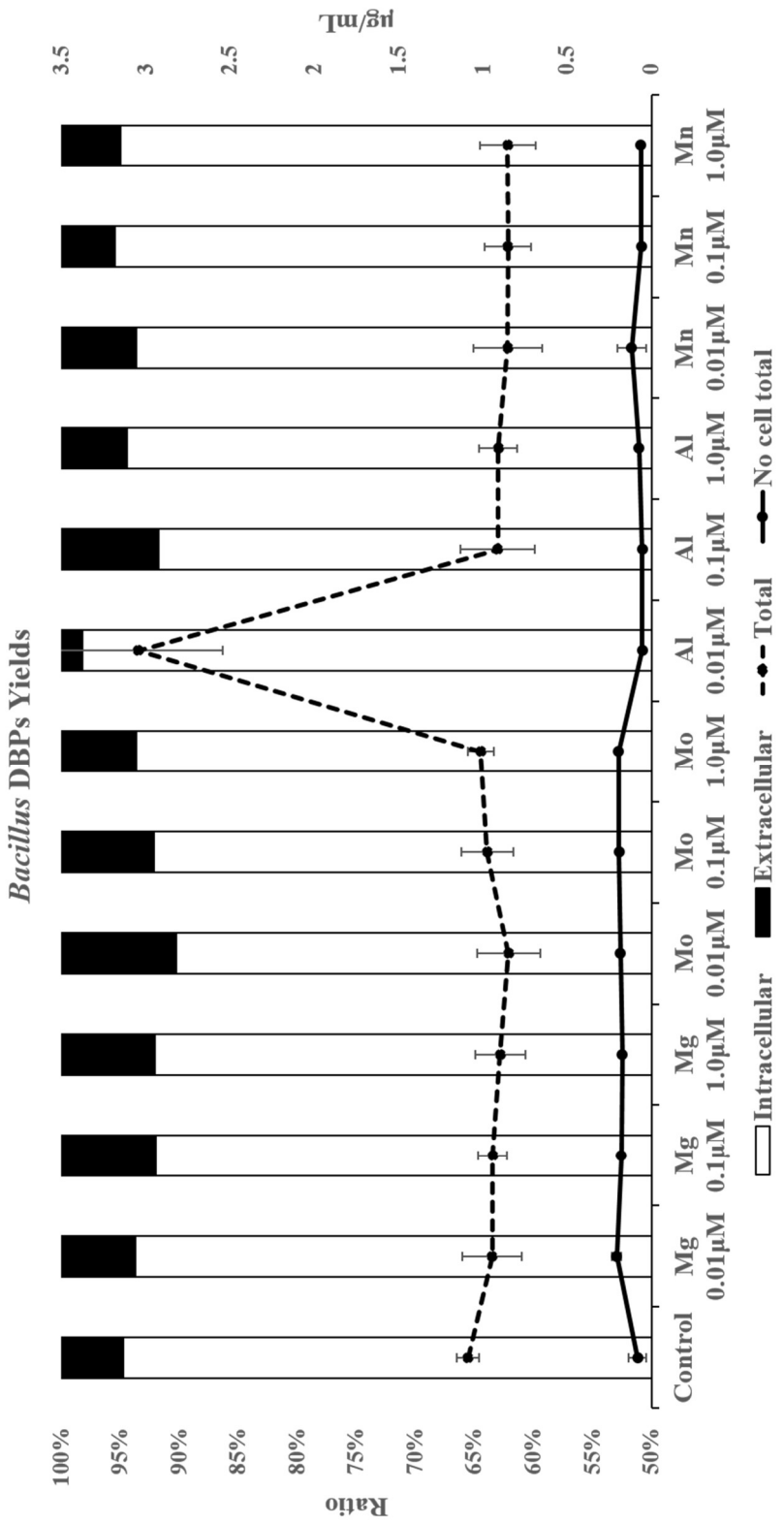


Figure 3.24 Comparison of intracellular DBPs and extracellular DBPs in *Bacillus*

On DBP type's level, there was a dominated DBP, with cells, chloroform is the major DBP; in media without cells, 1,1,1-trichloroethane was prevalent. Chloroform may be more likely to form by cells, and our intracellular data also resulted with much more chloroform than extracellular chloroform, and chloroform, if not, could be the simplest DBPs of all, because it just contains 3 elements: C, H, and Cl, and chloroform is chemically and biologically stable due to its structure. In addition, the NOM could applied profound carbohydrate for chloroform formation during the disinfection. But the higher level of chloroform inside cells may suggest that the chlorine or NaOCl compounds could react with cell matters, then to kill the cells. In a study done by Qi et al., 2016, found that instead of killing algae cells with over dose of chlorine, the lower dose of chlorine that to damage on the algae cells primarily could induce more algae organic matter release that yielded more DBPs through the disinfection, because the highly damaged cells may possess the ability to accumulate organic materials via adsorption. On our point, the disinfection process of chlorine could achieve by denaturing the bacterial materials and proteins with chlorine, and chloroform might be intermediate products. For NOM in no cell media, where 1,1,1-Trichloroethane dominated, just had a little of chloroform formation. It is suspected that the NOM of the media was the chemical state to form 1,1,1-Trichloroethane than chloroform, and these two DBPs have very similar chemical structures.

Above all, major DBPs were correlated to total DBP yielding, because 0.1 μM Al^{3+}, 0.1 μM Mn^{2+} that yielded more DBPs in *Enterobacter cloacae* and 0.01 μM Al^{3+} that yield most DBPs in *Bacillus* still formed much more chloroform than others, while 0.01 μM Al^{3+}, 1.0 μM Al^{3+} with *Enterobacter cloacae* and 0.01 μM Mo^{6+} with *Bacillus* that yielded fewer DBPs had less chloroform ratios. Other DBP also shifted with the total DBP formation, such as bromoform. The media DBPs were undoubtedly dominated by 1,1,1-Trichloroethane.

Under the comparison between *Enterobacter cloacae* and *Bacillus*, intracellular and extracellular DBPs also yielded differently (Fig 3.25 and Fig 3.26), *Bacillus* yielded similar

intracellular DBPs to *Enterobacter cloacae*, but a little more extracellular DBPs (Fig 3.28 and Fig 3.29). *Bacillus* may have more DBP precursors out of cells because of its smooth cell membrane, and 1,1-Dichloroacetone had highest ratio in extracellular parts but the no cell media (Fig 3.27) formed very little of it, it rises an assumption that 1,1-Dichloroacetone may generated by inside the cell but ejected from the cells later on.

The data from this study may help water treatment plants that applying chlorine in disinfection to better adjust their water treatment strategies that decrease the production of DBPs by adding or declining metal ions in the water, and pay attention to special chemical compounds during water treatment. Additionally, the plants use other disinfectants like ozone and UV light may also be benefited by the biofilm and the cellular work in this study with a better understanding of their sensitive of special metal ions/organic matters. Many studies have done a lot of work focusing the big group matter: NOM, but it is still unable to figure out the contributions and catch the points. There is a question that what if the bacteria themselves also play important roles on the DBPS formation? Our results could prove the contribution from proteins which is a kind of NOM in the nature, but more important is for the strategy in water treatment process that the protein inside the cells in the disinfection process could also generate some DBPs that go through distribution pipe for human consumption. And NOM could be the food for bacteria and absorbed or attached, then get into the cells then protein matters cycle back to the environment after cell death.

On the other hand, the proteins and some organic particles release some DBPs after disinfection. So the filtration of NOM could get rid of some proportion of DBPS but the leftovers in the bacteria cells will be brought to the disinfection steps. Not only proteins, but also mall particles could be gathering inside the cells or on the membrane to finally be a part of DBPs. Bacteria are potential big vectors transport DBPs precursors. Another issue is that the bacteria can grow and form biofilms in the pipe before disinfection processes, and the proteins and small organic particles from them are possible DBPs precursors, too. Future strategy of DBPS treatment should

consider to filter as many of cells as possible before disinfection steps. The 3-step disinfection (Li et al., 2017) have less DBPs in first step is because of that did not react with much proteins but only break down the cells, but the following steps also generate some DBPs.

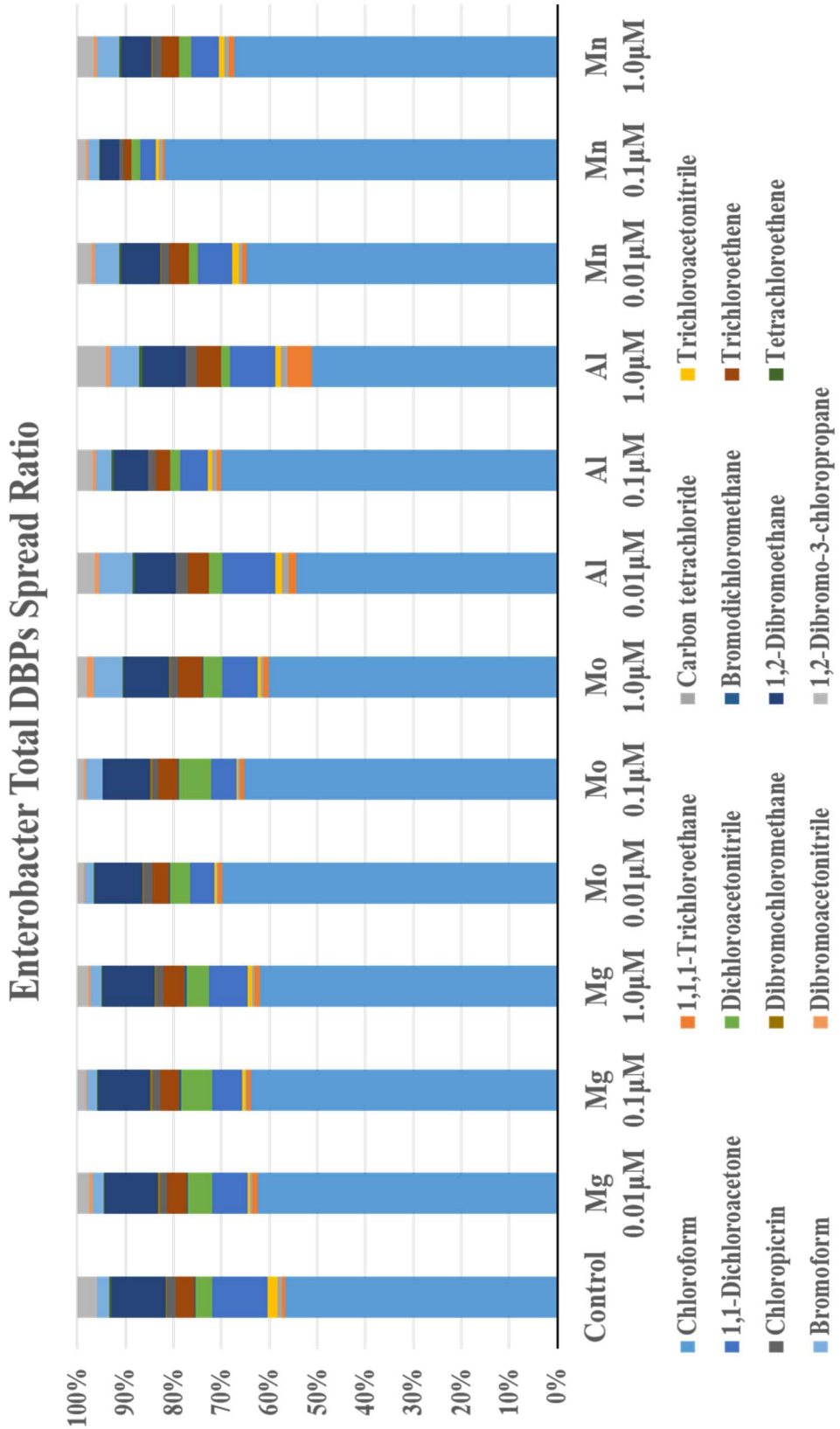


Figure 3.25 Total DBPs spread ratio in *Enterobacter cloacae*

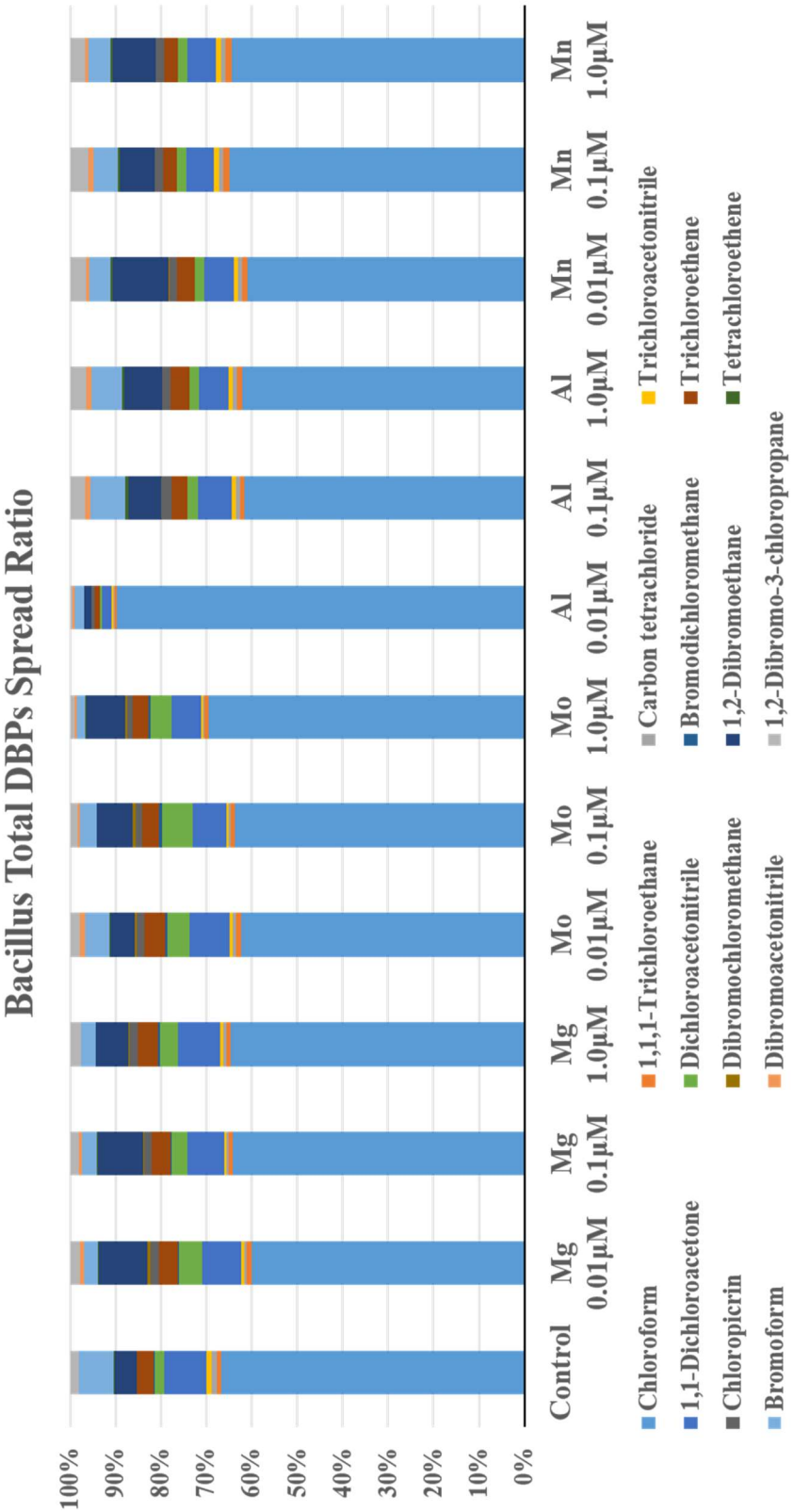


Figure 3.26 Total DBPs spread ratio in *Bacillus*

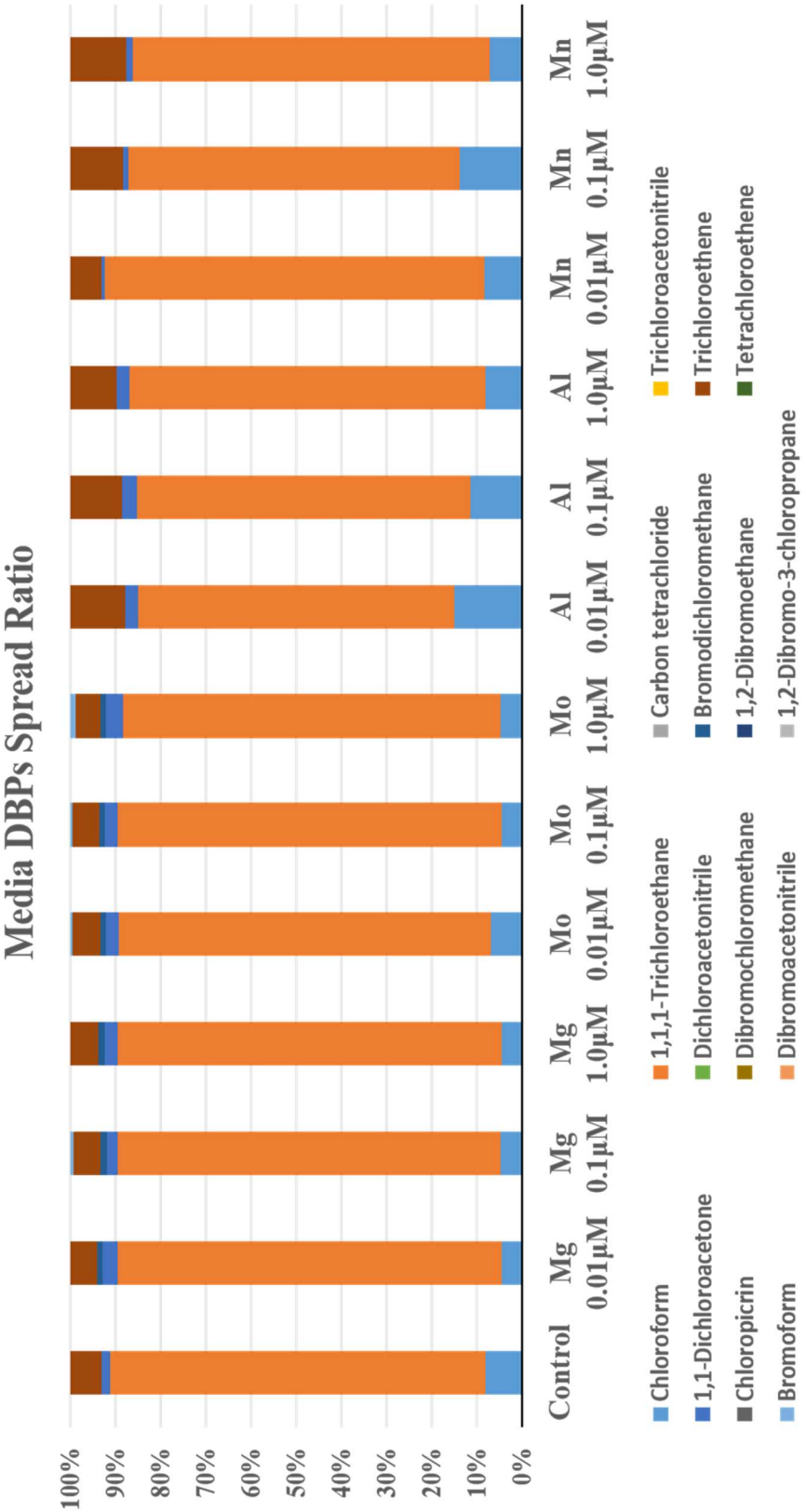


Figure 3.27 Total DBPs spread ratio in no cell media

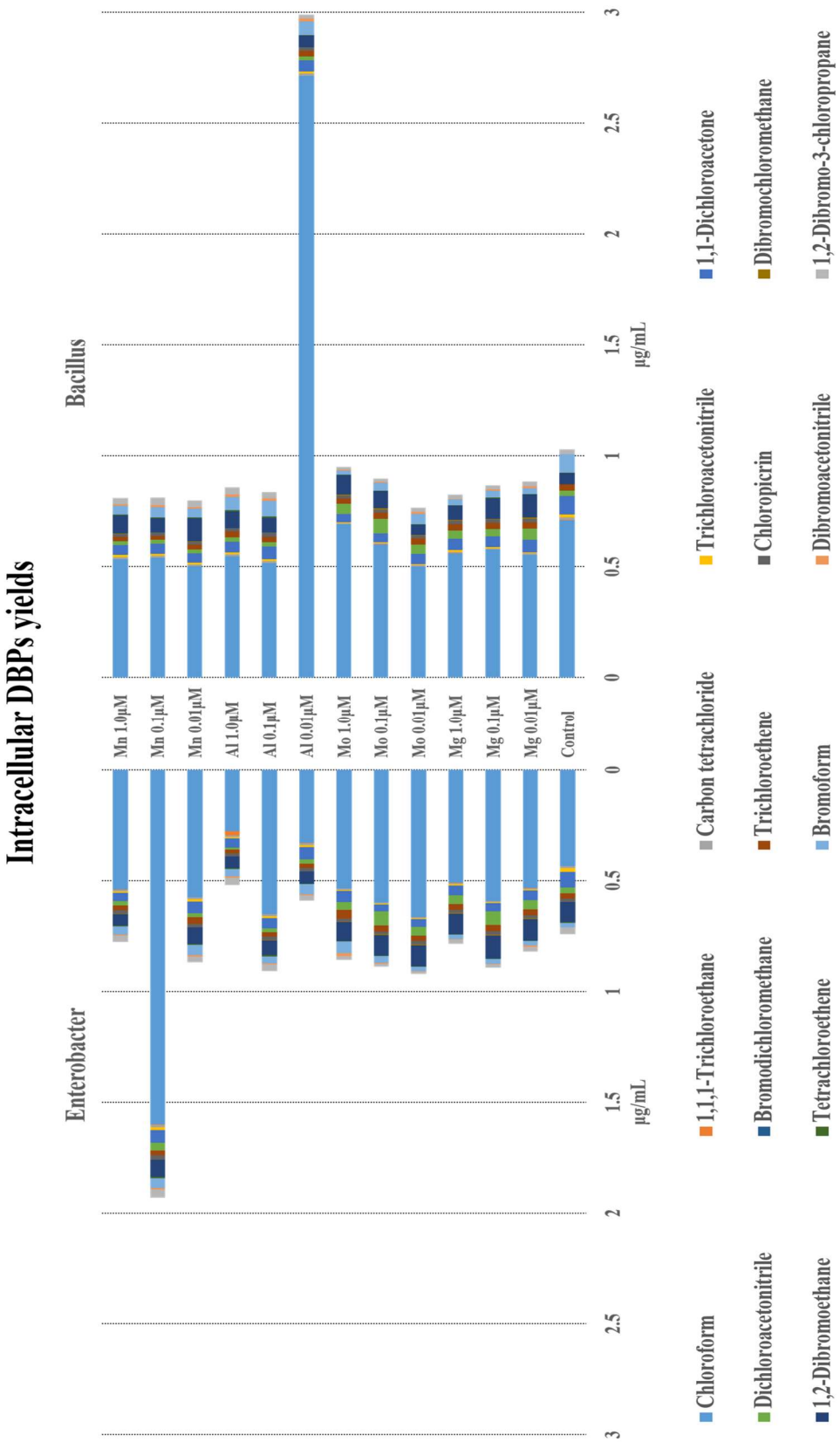


Figure 3.28 DBPs spread ratio in intracellular space of both strains

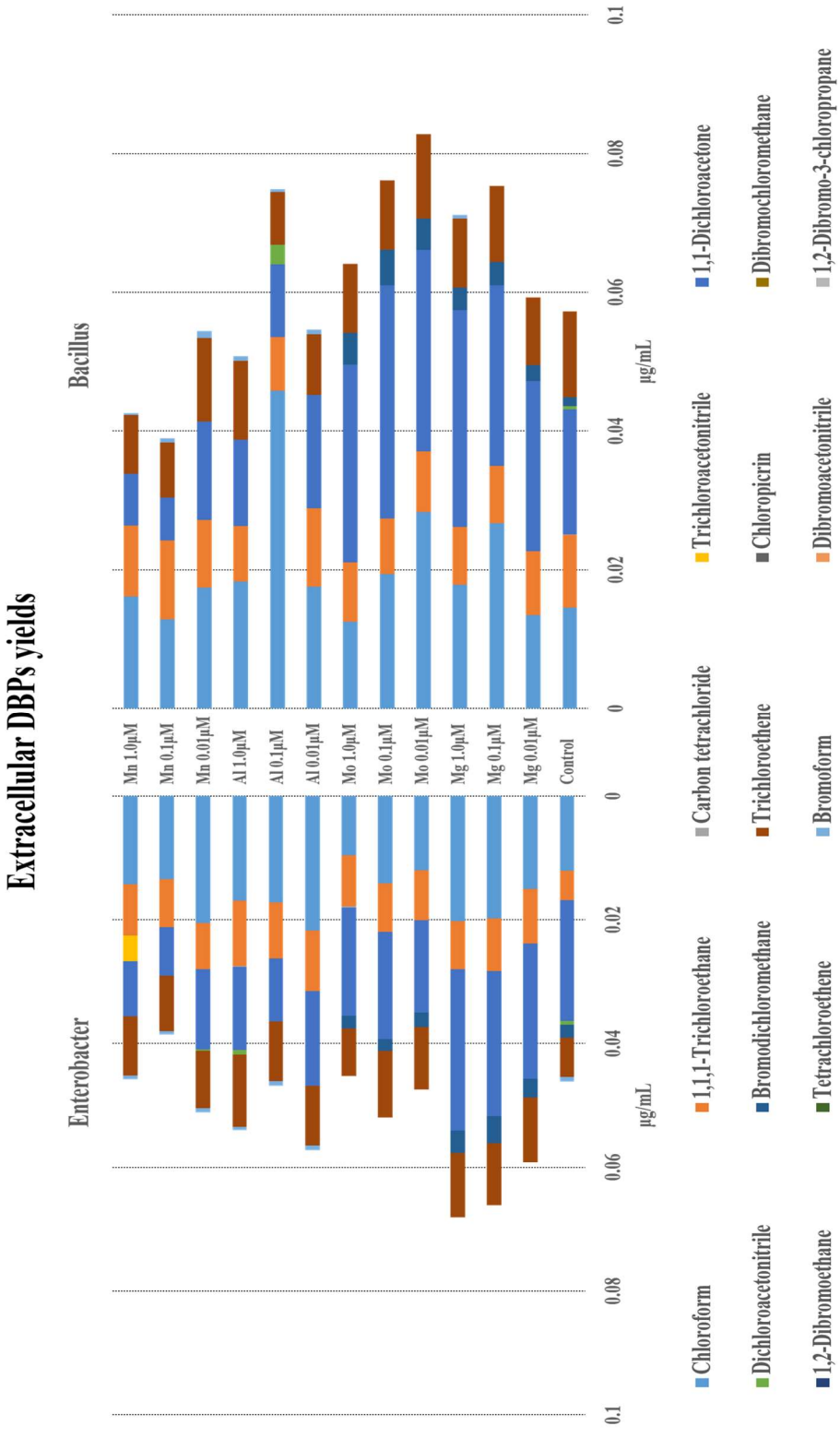


Figure 3.29 DBPs spread ratio in extracellular space of both strains

Health effects of DBP exposure have been studied since the 1970s, Villanueva et al. (2015), wrote an overview of the health impacts by DBPs that have been reported, they used some experimental evidence from the studies of animal, transgenic bacteria, mammal and human cell culture lines to explain the possible mechanisms, which include the DNA damages, protein bindings and epigenetic changes, however, such mechanistic researches are limited because of the complex nature of the DBP mixture. To face the challenge of its complexity on assessment of human exposure, markers have been applied for the DBP detection, for instance, one kind of DBPs: trihalomenthane is a surrogate of total DBP content, and levels in exhaled air and levels in blood are both useful in evaluating short-term exposure. Some markers are developing for DBP detection, especially those can cause caners, because cancers are the most focused outcomes by DBP exposure, such as bladder cancer and colon and rectal cancer, but other than that, fertility and fetal loss could be also the issues caused by DBPs, even though the limitation of the researches show no clear evidence yet, the potential damages from DBPs are still potential risks.

Unlike in drinking water, DBP exposure in swimming pools may be less diverse, the absorption of nitro-DBP, chloramines and trihalomenthane through skin and inhalation could be the major risks during swimming, and they have higher prevalence in the body of swimming pool workers and elite swimmers, but tend to be less in children even though they are more vulnerable than adults. However, it does not explain how DBPs are generated through the disinfection process and how to detect them, the imperfect markers and the single factor of organic matters could not be a good answer for the complexity of DBPs. In water treatment plants, the type of DBPs may depends on the types of cells and small particles that could be transported by the bacteria. However, many DBPs could appear both in or out of cells, in comparison with controls, chloroform seemed to be increased in cells and bromide DBPs may be formed by some organic matters that could release out of cells by the bacteria during the growth and metabolism. EPS is one of the major groups of extracellular organic matters, and EPS can be recognized as one of NOM generated by

bacteria, this phenomenon is supporting the hypothesis that NOM from bacteria could be an important part of the DBP precursors.

The amount of DBP production is expected to be sensitive to bacterial concentrations. The relationship between bacterial organic matters and DBP precursors will likely be complex, with some DBPs decreasing and others increasing as bacterial cell concentrations vary. An important consideration with regards to decision making in the environmental engineering field will likely be the relative biochemistry of the compounds involved in that trade-off. The field of DBP research is a relatively fast-moving field. For example, it was recently found that the emerging DBP dichloroacetamide has been misidentified, and the correct DBP is most likely N-chloro-dichloroacetamide which is reduced to dichloroacetamide in previous analysis due to quenching reagents. New insights into DBPs will continue to occur and many of the approaches above may be altered. The literature will be continuously reviewed, and specific bacterial organic matters, such as glucose, arabinose, and mannose will be considered in later research processes, because they are representative carbohydrates in aquatic environments, and have been found to form significant THM levels at pH 8. It is well accepted that materials deposited on distribution, pipes, such as corrosion products and biofilm, not only consume a significant amount of disinfectants but also harbor organic DBP precursors. At the end of this objective, proliferation and suppression of metal ions to DBP precursors will be developed. Both of them would be critically needed to determine the contributions of metal-bacteria linkage to producing DBP precursors across various environmental systems such as intake structures, source waters, unit processes, etc. Metal study is also useful in research to quickly predicted DBP precursor formation without chemical extraction/analytical analysis methodologies. Importantly, this objective will provide an alternative strategy for water treatment process, because many metal ions, like Al and Fe are often used in the coagulation step, and some metals that precipitate during sedimentation make the potential of affecting bacterial organic matters so as to decrease DBP precursor production, which will likely

lead to insights regarding the production and identities of bacterial based precursors. Some of the trace elements at the highest levels are expected to induce toxicity mechanisms, but most conditions are at sub-toxicity levels. It is expected that a diversity of bacterial organic matters will be affected, as metals will induce transporter genes both for uptake and, at higher concentrations, toxicity responses, as well as affect the number of regulatory processes and catalytic activities involved in precursor production. Analysis of bacterial organic matters will be limited to products of known function, and those which are implicated across many samples (of metals and bacteria) to be detectable.

For the treatment of DBPs, the removal of DBP precursors before disinfection is believed to be a very effective method to deal with DBPs by many researchers. However, with the natural organic matters (NOM) was reported as the dominated precursors of all kinds, a question comes out to scientists: How do different NOM contribute to DBP formation as precursors? To answer it, Li et al. (2017), set up an experiment, which compared with the DBPs contribution between hydrophobic (HPO) NOM and hydrophilic (HPI) NOM. During this comparison, their outcomes are tested under 3 major disinfection methods: chlorination, chloramination and ozonation. Their results showed that HPO is the major precursor (around 60%) to form nitrogenous DBPs and the largest donor of the heterocyclic DBPs, while HPI formed 5-15 times more of ketone and twice more of esters and alcohols than HPO. In addition, the experiment also found that humic acid-like substances and protein-like materials are possible precursors for halogen DBPs and nitrogenous DBPs, respectively. Daiber et al. (2018), studied the DBPs yields in spas (hot tub) waters, trying to reveal the factors that could increase DBP concentrations in pools and spas. After the experiment, the authors found that cyanogen chloride was the most frequent DBP in finished water, while heavily used spa had the highest concentration of dichloromethylamine (a very toxic DBP), thus, some types of DBPs may be generated from the human behaviors. And since the total dispense of DBPs in pool and spa samples were highly complex, it is applicable that controlling the production

of DBPs could begin with reducing the inputs to pools and spas, such as cleaning of spas, exchanging of water in pools, showering that enter pools/spas, and urinating or wearing products in pools/spas. Hladik and Focazio, 2014. Focused on the generation of brominated DBPs from gas commercial wastewater treatment plant (CWT) and publicly owned wastewater treatment plant (POTW). An experiment of comparing the levels of DBPs among 4 sites (from upstream to downstream) in a river in Pennsylvania had been designed by the authors, Michelle et al., the results were quite interesting: 6 types of DBPs in the range of 0.01 to 0.09 ug/L were detected from POTW and the CWT had found a relatively high concentrations of dibromochloronitromethane (DBCNM) (up to 8.5 ug/L) in the outfall site. The DBPs outcomes from POTW and CWT could be a potential problem, because there were not DBPs from the upstream, but the CWT brings DBCNM into the downstream with a final level of 1.2 ug/L. Considering the produced waters (by-product of the extraction of oil and gas from the ground) can produce high levels of DBPs in other POTW and CWT in Pennsylvania, the author though that bromide and phenol might be the precursors of DBPs.

Pramanik et al. (2015), detected if the removal of dissolved organic carbon (DOC) and dissolved organic nitrogen (DON) could better control DBPs production, they had made a comparison between biological filtration and chemical coagulation to find out what factors could help to control DBPs production. During the experiment, effects of biological aerated filter (BAF), sand filter (SF), biological activated carbon (BAC), alum coagulation, and *Moringa oleifera* , coagulation on the removal of DOC and DON from reservoir water were investigated. The DOC removal efficiency was in the following sequence: BAC (56%), BAF (51%), SF (45%), and alum (27%), and *Moringa oleifera* (22%). While the trend for DON removal efficiency was similar to that of DOC, so the better performance by BAC may be attributed to the effective microbial breakdown of organic matter and the adsorption of some molecules onto activated carbon.

However, many reports on DBP studies just use the sample from one single source, which might have specific conditions than many others, multi-sites sampling should be a better idea for

it; and the catalog of the DBPs in the paper were lack of details. Our work evaluated the formation of DBPs from microbially-derived organic matter (pure cultures and strains) during chlorination, studying the impact of metals on DBP yields, and study the biodegradation of DBPs. The focus on DBP fate is important and often overlooked. Most of DBPs inside cells have higher concentration than the matters out of cells. The role of metals on formation of disinfection byproducts have been investigated, bacteria and the bacteria formed biofilm could potentially increasing DBP formation or could promote DBP degradation. The metal ions could help to determine the influence of metal elements (especially trace metals) on production of DBP precursors.

Considering bacterial aspects, the DBPs changes in different strains may also support the idea that bacteria could supply react positions and organic materials for DBP formation. Because *bacillus* have smoother membrane than *Enterobacter* stains, and the enzyme of each one tends to make up of some sites for chemical reactions of chlorination. Another interesting idea asks if the chlorination could occur inside the cells before the breakdown of the membrane. Because chloride may potentially penetrate the membrane so as to induce the emergent response from the genes in the cells, and there are some differences between emergent inside and outside, because emergent proteins could be ejected out of the cells and as a source of DBP precursors, but if the chloride would inhibit such a response inside cells, some precursors might be decreased. On the other hand, some NOM might be reacted with chlorine by the mediation of cellular matters like enzyme and glucose that apply attached sites and energies. It is apparent that bacteria change the DBPs contents, even though the mechanism is yet unknown, the most profound matters, especially the NOM of bacteria are undoubtedly the proteins and small bacterial particles like lipids and sugars. Because the level and category of DBPs in extracted cultures are close to the DBPs in pure cultures, the NOM in culture should not be big concern, instead, that bacterial matters, both organic and inorganic seemed to change DBP levels a lot, making clear differences. The temperature could be

a factor for either bacterial growth or chemical reaction, but given that our controls are also incubated under same temperature, this many not be true case.

Scientists have found that not only human and animals could be poisoned by DBPs, but also bacteria could be killed with them. Under such conditions, it is needed to reveal the mechanisms of DBPs' toxicity to bacteria if we want to understand and control this harmful scenario. Daniel Stalter et al. (2016), showed the possible toxicity pathway of 50 types of DBPs on a popular bacteria, *E. coli*, which exists in human and animals' intestines. The experiment in this paper had done a cellular bioassays, revealing the reactivity towards proteins and peptides. According to the data, an enzyme named NRf2 in bacteria cells, had been proved to be depressed by DBPs with a portion of 98%, and an important protein, named p53, which help bacteria in response to toxicity, also have been activated by DBPs and the induced efficiency is around 68%. And all DBPs reactive towards DNA in the *E. coli* assay and activating p53 also induced oxidative stress, confirming that the oxidative stress is a key mechanism of DBP's toxicity. Based on the results, the authors hypothesized that the toxic mechanism of DBPs is because they can induce indirect genotoxicity through oxidative stress and enzyme inhibitions rather than the direct damage on bacterial DNA. Butterfield et al. (1943) studied percentages of inactivation as functions of time for *E. coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Shigella dysenteriae*, and they found that the primary factors governing the bactericidal efficacy of free available chlorine and combined available chlorine were: 1. the time of contact between the bacteria and the bactericidal agent, which means the longer the time, the more effective the chlorine disinfection process; 2. the temperature of the water in which contact is made, suggested that the lower the temperature, the less effective the chlorine disinfecting activity; and 3. the pH of the water in which contact is made, it is apparently the higher the pH, the less effective chlorination. Thus, the test bacteria will be killed more rapidly at lower pH values and at higher temperatures.

Disinfection does not necessarily kill all microorganisms, especially resistant bacterial spores, instead, it is less effective than sterilization, which is an extreme physical and/or chemical process that kills all types of life. Disinfectants work by destroying the cell wall of microbes or interfering with their metabolism. Disinfectants are used to rapidly kill bacteria. They kill off the bacteria by causing the proteins to become damaged and outer layers of the bacteria cell to rupture. The DNA material subsequently leaks out. With effects on metabolism by disinfectants, bacteria will die by famine and be unable to compose necessary proteins, during such a process, DBPs may be formed by the bacterial matters released by the cells. This is because a disinfectant like chlorine has very powerful oxygenic ability. The damage on membrane of cells would also allow chlorine to react with cellular compounds. The data in the experiment had showed the cells change the DBPs levels, this may validate the idea that bacterial matters could be important DBP precursors. While metal ions that could increase or decrease metabolism of cells also changed the formation of DBPs. Mg^{2+} and Mo^{6+} are quite like prompt factors for bacterial metabolism, the metals may also potentially inhibit the reactions between disinfectants and some organic matters by their spontaneous chemical reactions. Many types of interactions exist between proteins and metals such as indirect interaction with hydrophobic ligands, molecular compounds, and coordination interactions with the functional groups of amino acids.

Bacterial effects on the NOM in water should be another pathway for precursor formation under the metabolism that transfer NOM compounds, in this experiment, the consumption of the media chemicals by bacteria may also contributes precursors even small, a typical reaction in between cells and media is the glucose cycles that compose the ATP by using carbohydrates from the nutrient sources, and the growth of bacteria must use that, too. Such a consumption in water treatment systems may occur during flocculation and in the settling basin where solids with organic matters in water aggregate together as a media for bacteria.

CHAPTER IV

PROTEIN ANALYSIS

4.1 Process

The goal was to analyze the proteomics and transcriptomics during growth with transition and regular trace metals to identify the proteins that may correlate to specific DBP formations. Molecular methods was used to elucidate the mechanisms of degradation and to serve as indicators in water treatment systems, for proteomics, samples from media was collected and prepared with buffers with relatively mild non-ionic detergents (Such as Dithiothreitol, DTT), then denatured to unfold the protein and total proteins were separated by SDS-PAGE under two-dimensional gel electrophoresis. Separated protein was digested and go to mass spectrometer to analyze the protein information.

4.2 Materials and methods

4.2.1 Cell preparation

Cell samples were prepared by the method preparation of whole cell protein extracts reported by Sanchez et al., 2003. Cell pellets were harvested by centrifugation under 13,000 rpm for 15min in 4 °C, then washed twice with 0.9% NaCl, the pellets were resuspended with 1mL 0.9% NaCl in 2mL collecting tubes (MioBio), 1 g of glass beads (2 mm diameter) was added into each tube and vortexed for 4min (30 s in vortex/ 30 s in cold ice) at the maximum setting to extract proteins from the cells. Ultrasonic method (30 min) was used to aid the protein extraction if needed. Later, samples with extracted proteins were moved to new 2 mL collecting tubes and discarded

glass beads, and 1mL of sample buffer (0.0625 M Tris-HCl, 2% SDS and 10% glycerol, pH = 6.8) was added to each tube. After mixing well, samples were heated at 105 °C for 10 min (denaturing), then cooled. All samples and store at -20°C. 1M DTT only add to the samples with 5% (v/v) just before SDS-PAGE.

4.2.2 Protein test by Bio-Rad Protein Assay

1. Prepare dye reagent by diluting 1 part dye reagent concentration with 4 parts DDI water. Filter through a Whatman #1 filter (or equivalent) to remove particulates.

2. Prepare three to five dilutions of a protein standard, which is representative of the protein solution to be tested. The linear range of this microtiter plate assay is 0.05 mg/ml to approximately 0.5 mg/ml. Protein solutions are normally assayed in duplicate or triplicate.

3. Pipet 10 µl of each standard and sample solution into separate microtiter plate wells.

4. Add 200 µl of diluted dye reagent to each well. Mix the sample and reagent thoroughly using a microplate mixer. Alternatively, use a multi-channel pipet to dispense the reagent. Depress the plunger repeatedly to mix the sample and reagent in the well. Replace with clean tips and add reagent to the next set of wells.

5. Incubate at room temperature for at least 5 minutes. Absorbance will increase over time; samples should incubate at room temperature for no more than 1 hour.

6. Measure absorbance at 595 nm.

4.2.3 2D Gel Electrophoresis (SDS-PAGE) for protein analysis

Dip the samples in SDS running buffer and place in clean wells to position the strips gel-side up on the back plate with the + side next to ladder lane, propping the gel up, pipette the gel into the lane, letting it spill over into the ladder lane and add 5 ul ladder into the very bottom of the

ladder lane, add 20 to 40 ul into the very bottom of the rest lanes, then run gels at 100 V for 5 h. Then run at 130 V until the blue line runs off the gels.

4.2.4 Gel staining, imaging and spot picking

Prepare fixing solution (50% methanol, 10% glacial acetic acid), staining solution (0.1% coomassive brilliant blue R-250, 50% methanol, 10% glacial acetic acid), Destaining solution (40% methanol, 10% glacial acetic acid) and Storage solution (5% glacial acetic acid). Wash the gel with MilliQ water and stain with coomassie blue (G-250). Fix gel in fixing solution for 1hr to overnight with gentle agitation, change solution once at first 1hr, stain gel in staining solution for 20min with gentle agitation. Destain gel in destaining solution and replenish the solution several times until background of the gel is fully destained. At last store in storage solution.

4.3 Results and discussion

Protein test showed very clear results that metal ions induced bacteria yielded more proteins than no metal controls after 12 h, but this increase dropped back at 48 h. It just occurred in both strains, but the cells grown without metals had higher protein concentrations before 8h, especially in *Enterobacter cloacae*. In consideration of the outcome that metals did not affect much on the growth rate of the cells, such protein results means that the metals may delay the protein yielding before exponential phase (12-36 h) to stationary phase (48 h). Many of those over-produced proteins could be stress proteins that in response to metal induction by cells, and then lost their stability and naturally lysis later on. Stephani, et al., 2003, had studied the dynamic control of a stress protein levels in *E.coli*, they found that the wild type strains had lost the stability of their stress proteins dramatically in 30 min during stationary phase but maintained their stability during exponential phase; and Agniezka Sokol et al.(2007), found that the mRNA levels of stress-inducible genes of histone H3 might decrease with time. So the sudden protein increase in our data was likely caused by stress proteins, however, the question why did those protein levels not show up before 12 h still requires deeper examination. In addition, it is curious that *Bacillus* had relatively less

impacts with metals, especially with Mo^{6+}, which induced *Bacillus* yield most extracellular DBPs among 4 metals. All of these protein responses by different strains could help to identify special proteins correlating to certain metals.

The change of protein may reflect how bacteria response to metal ions, and indicated if protein may be precursors of DBPs. Stress proteins may be potential candidates but there are some other proteins sensitive to metal ions. Such as a study worked by Hugh et al. (2003), which found *E.coli* could minimize the number of iron requiring proteins through metabolism when iron levels were low, and in converse that increased the protein levels when metal ions were sufficient. There are around 1/4 or 1/3 proteins of all require metal ions (Waldron and Robinson, 2009), and some metal elements used in this experiment may positively induce those proteins by favoring the given metals. Such an association with metal is not only for proteomic functions, but also for the stability of enzymes, Tottey et al. 2008, reported that metal cofactor could enable the structural folding in many enzymes, such as manganese cofactor: MncA which excess competitive metals have the potential to interfere with folding by reducing the folding rate.

On scrutiny of protein yield, both strains had big change in protein levels during 7-9 h, especially by the *Enterobacter cloacae* with Mn^{2+} and *Bacillus* with Al^{3+}, both of them were obviously influenced by the metals. It may suggest that protein yield could reflect the influences of metals on DBP production, but this needs more proof, and the question why did such a rate change so frequently during growth has to be further examined.

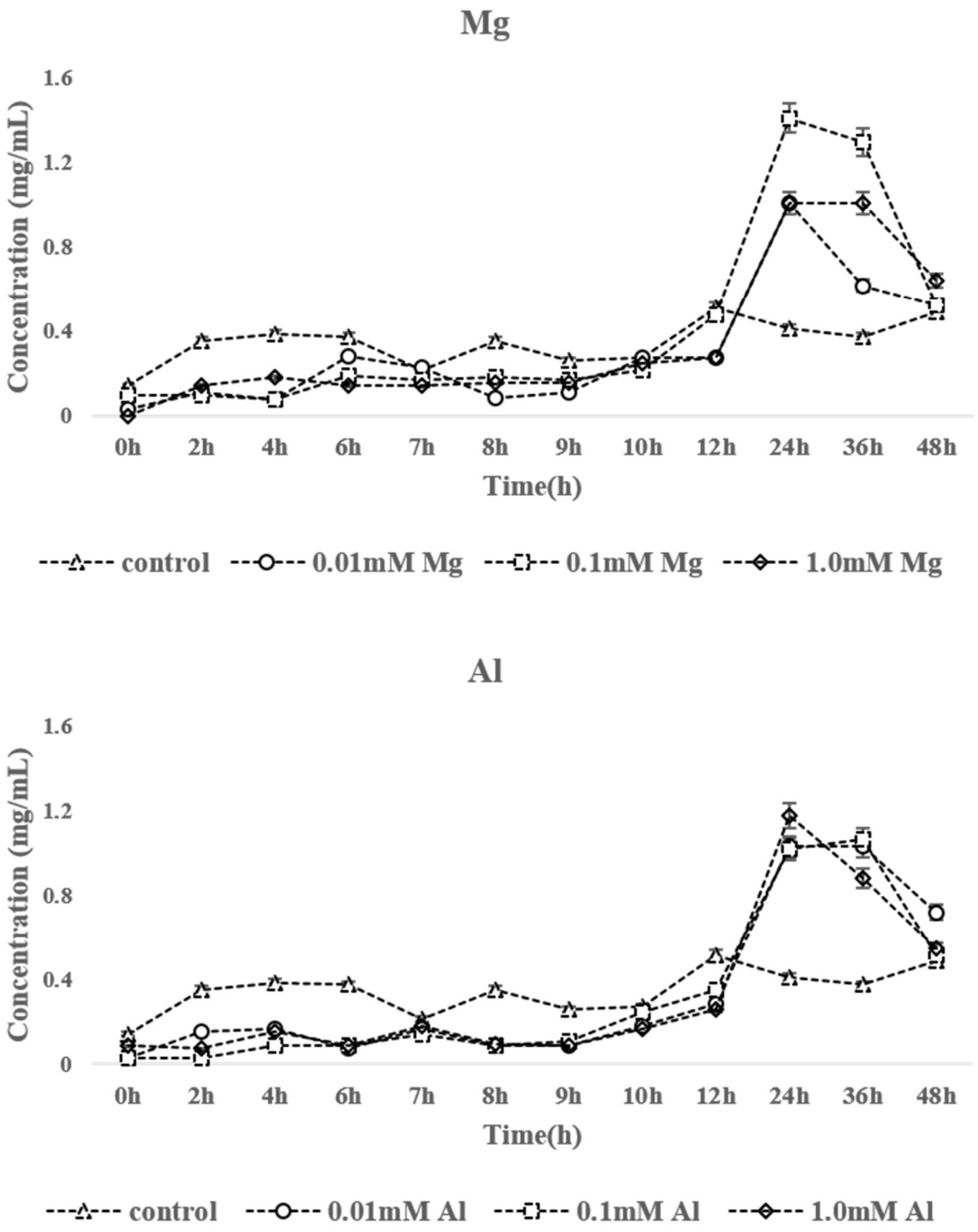


Figure 4.1 Protein level in *Enterobacter cloacae* with time for magnesium and aluminum

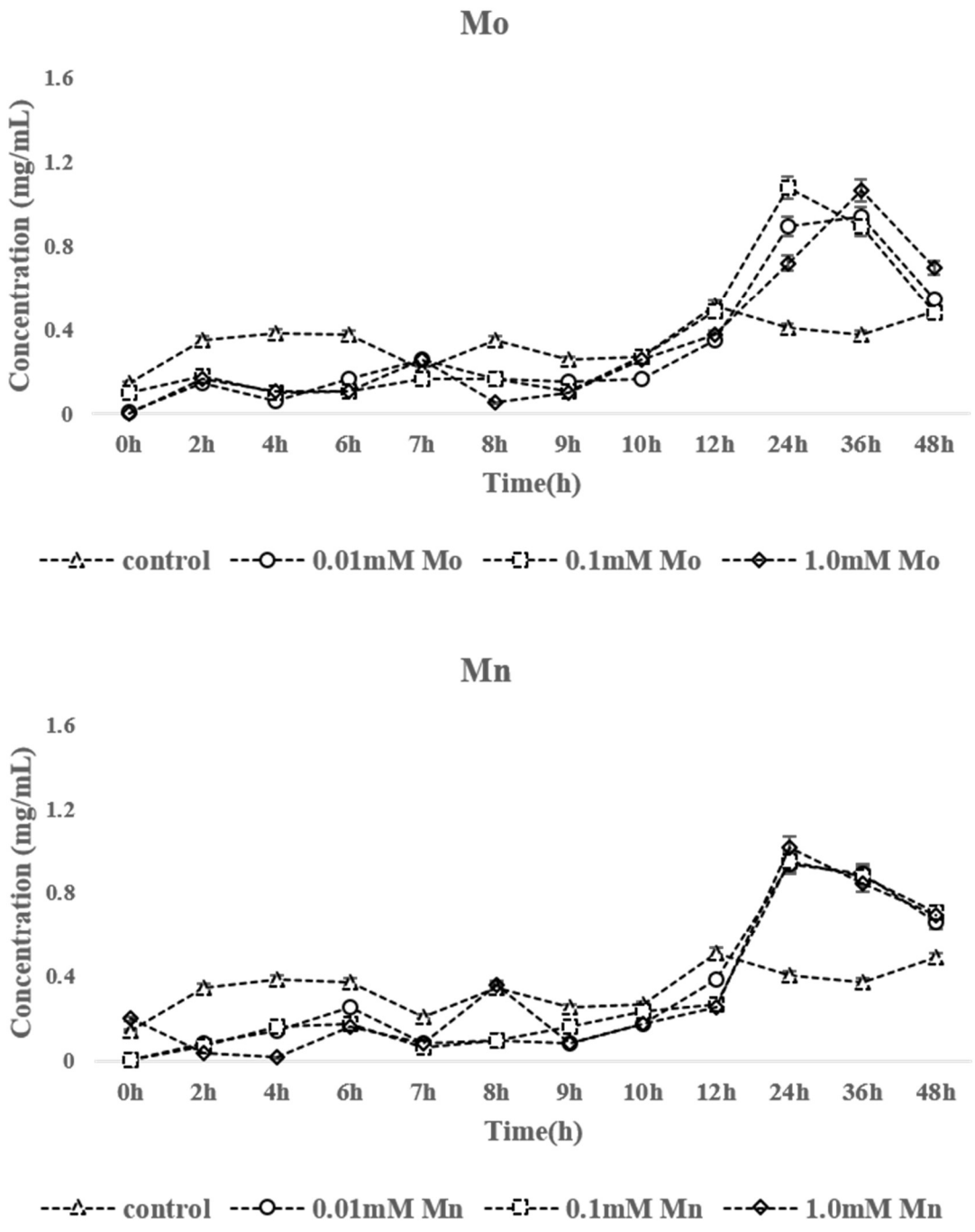
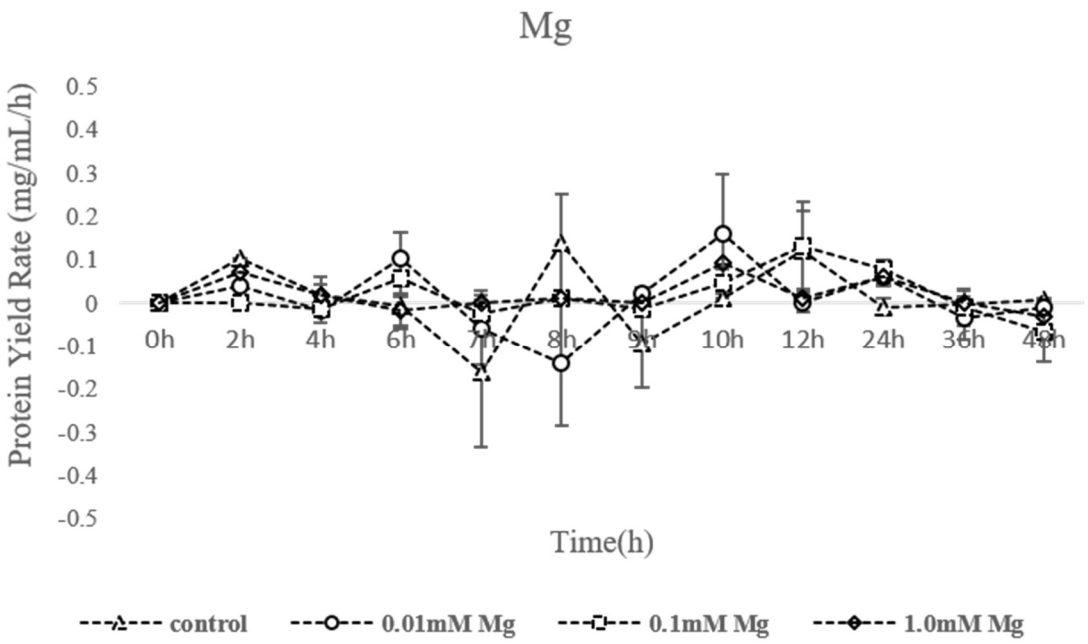


Figure 4.2 Protein level in *Enterobacter cloacae* with time for molybdenum and manganese



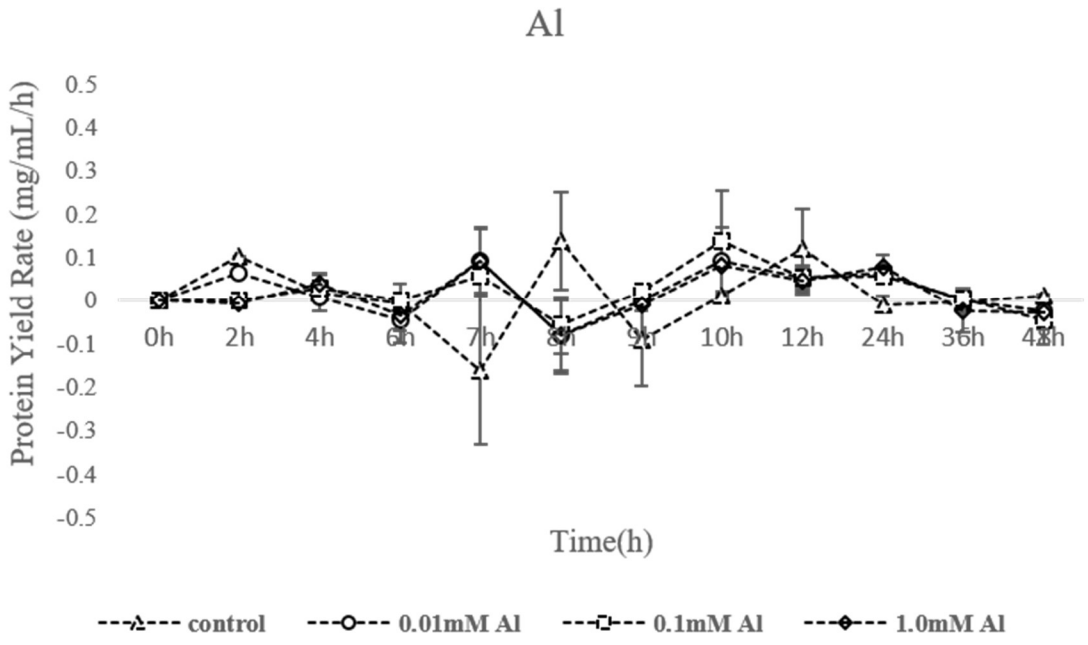


Figure 4.3 Protein yield by *Enterobacter cloacae* with time with magnesium and aluminum

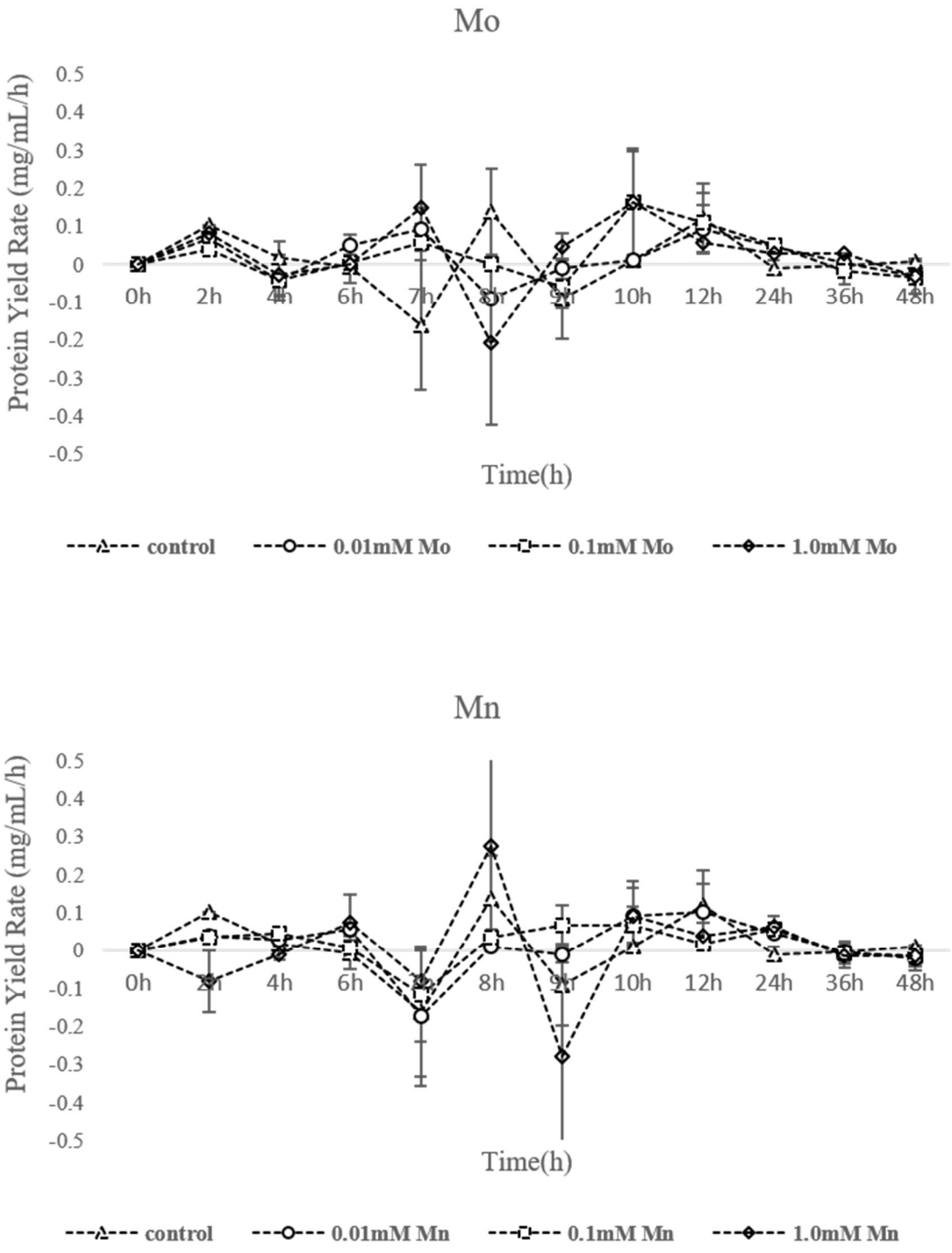


Figure 4.4 Protein yield by *Enterobacter cloacae* with time with molybdenum and manganese

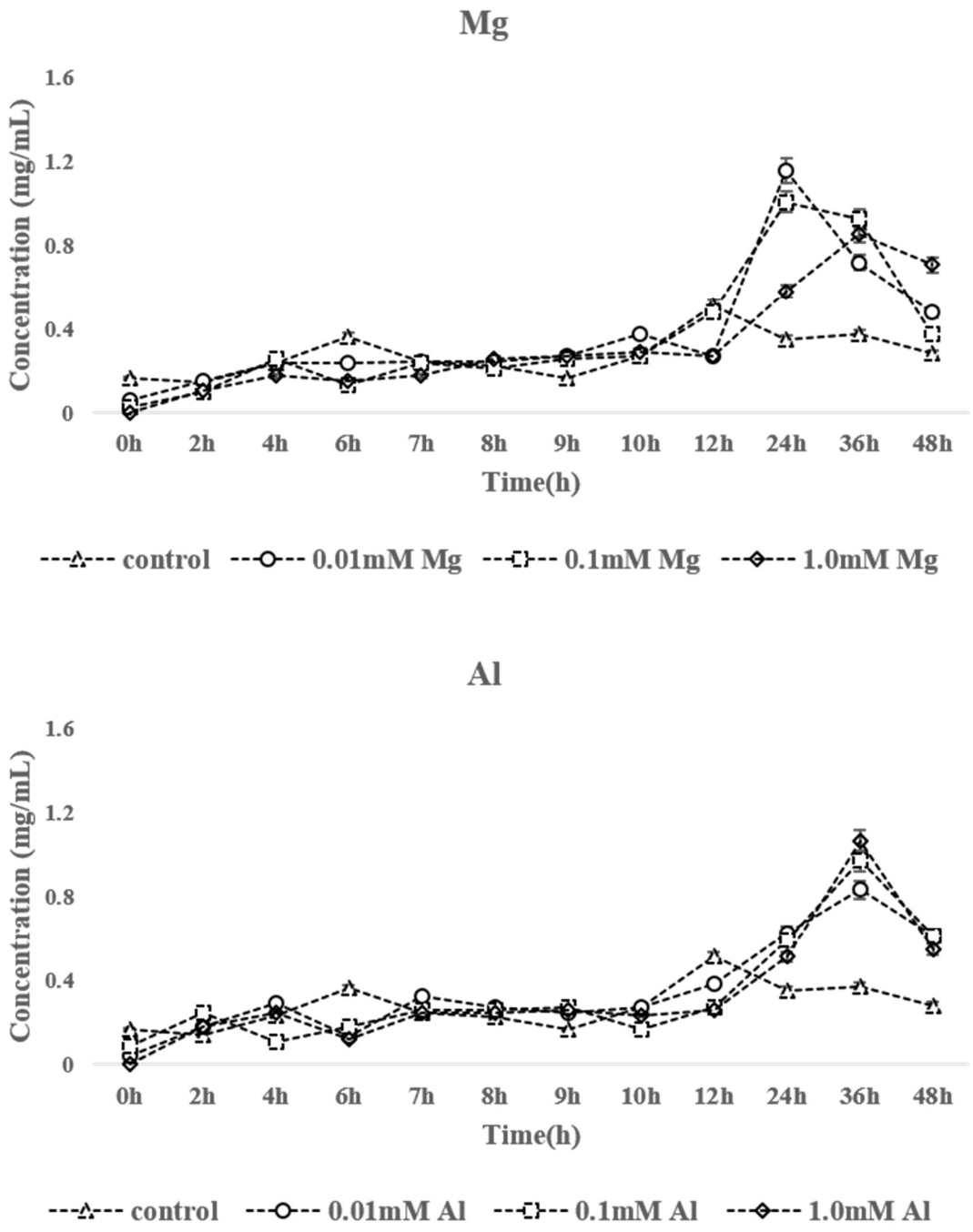


Figure 4.5 Protein level in *Bacillus* with time with additional magnesium and aluminum

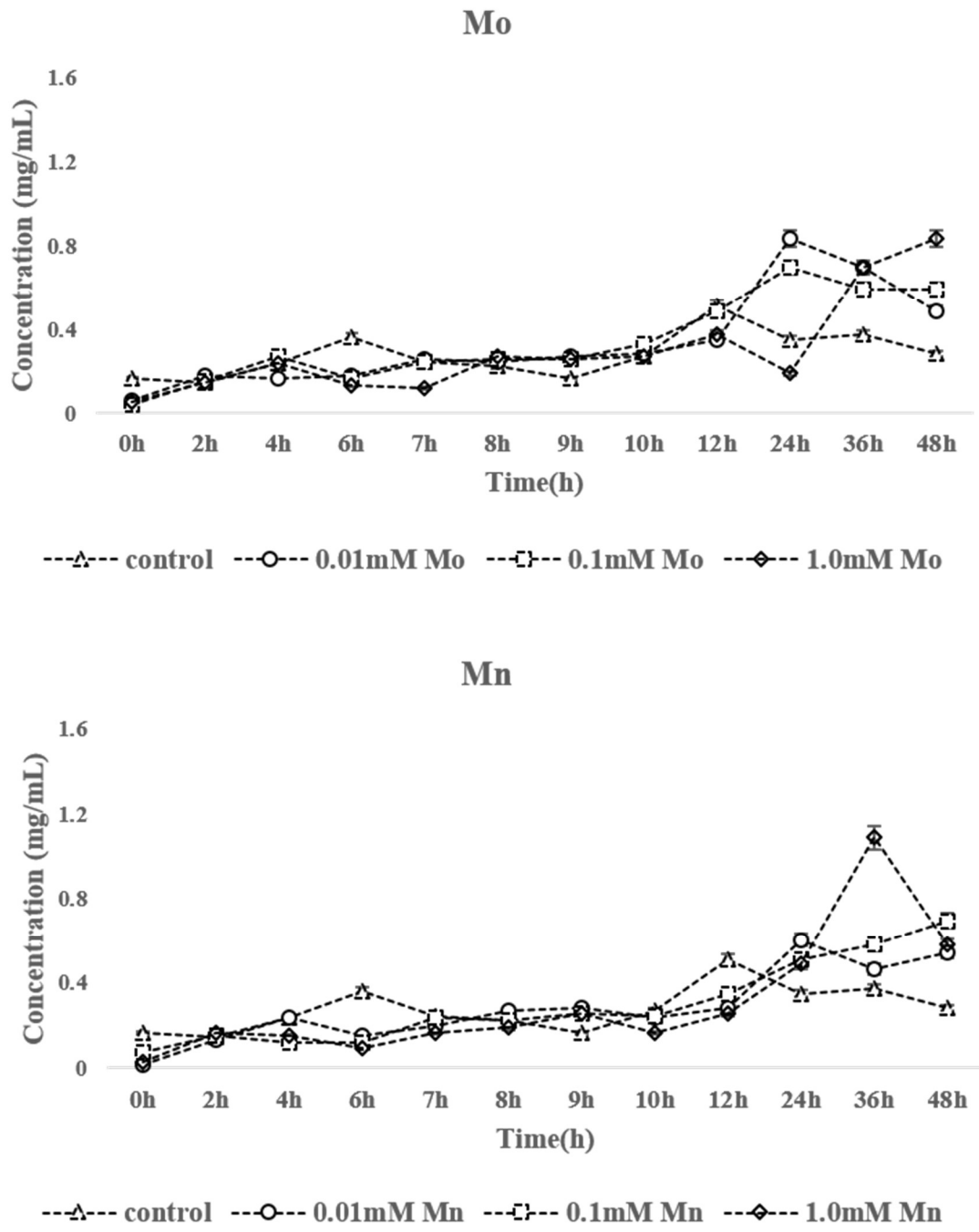


Figure 4.6 Protein level in *Bacillus* with time with additional molybdenum and manganese

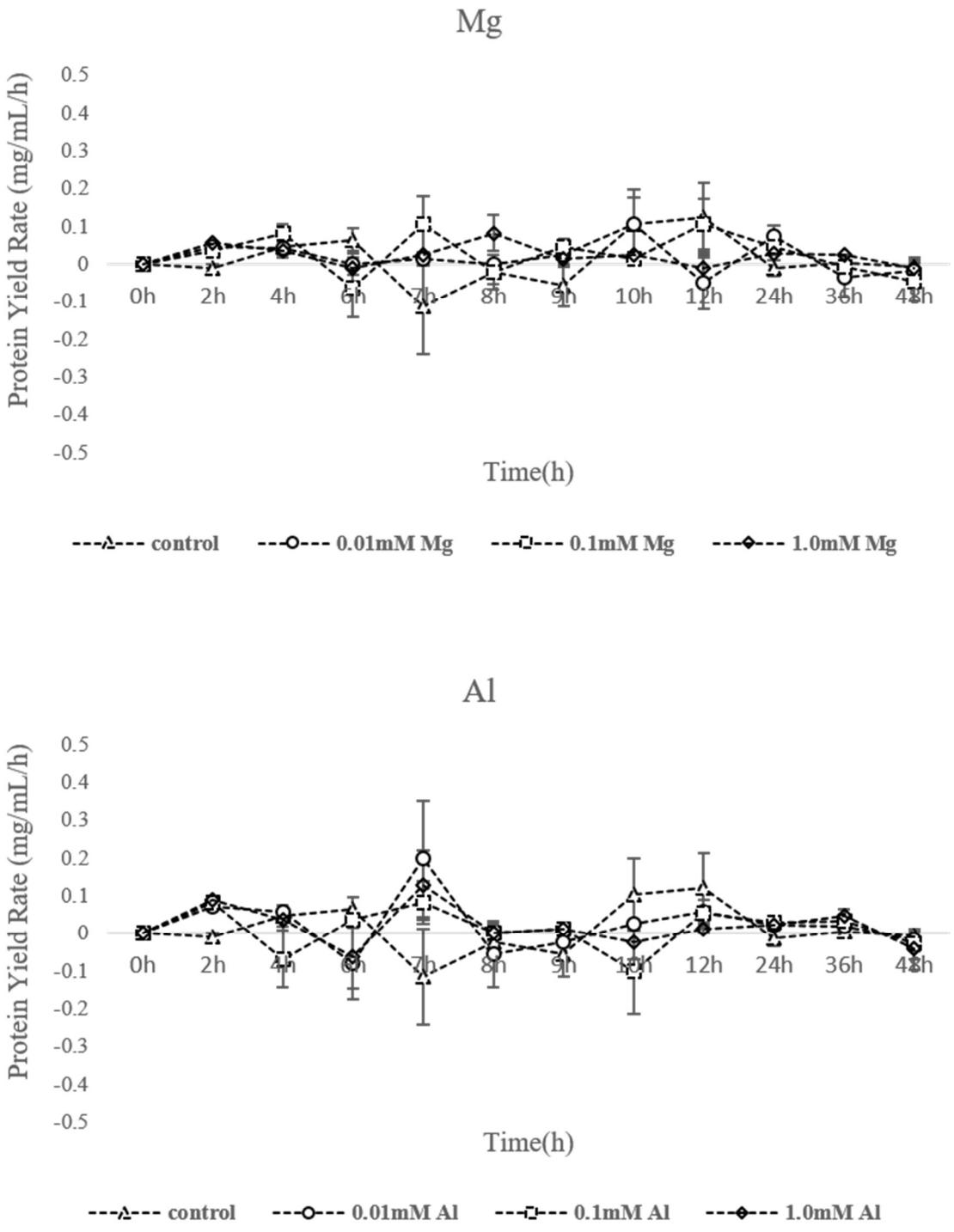


Figure 4.7 Protein yield by *Bacillus* with time with additional magnesium and aluminum

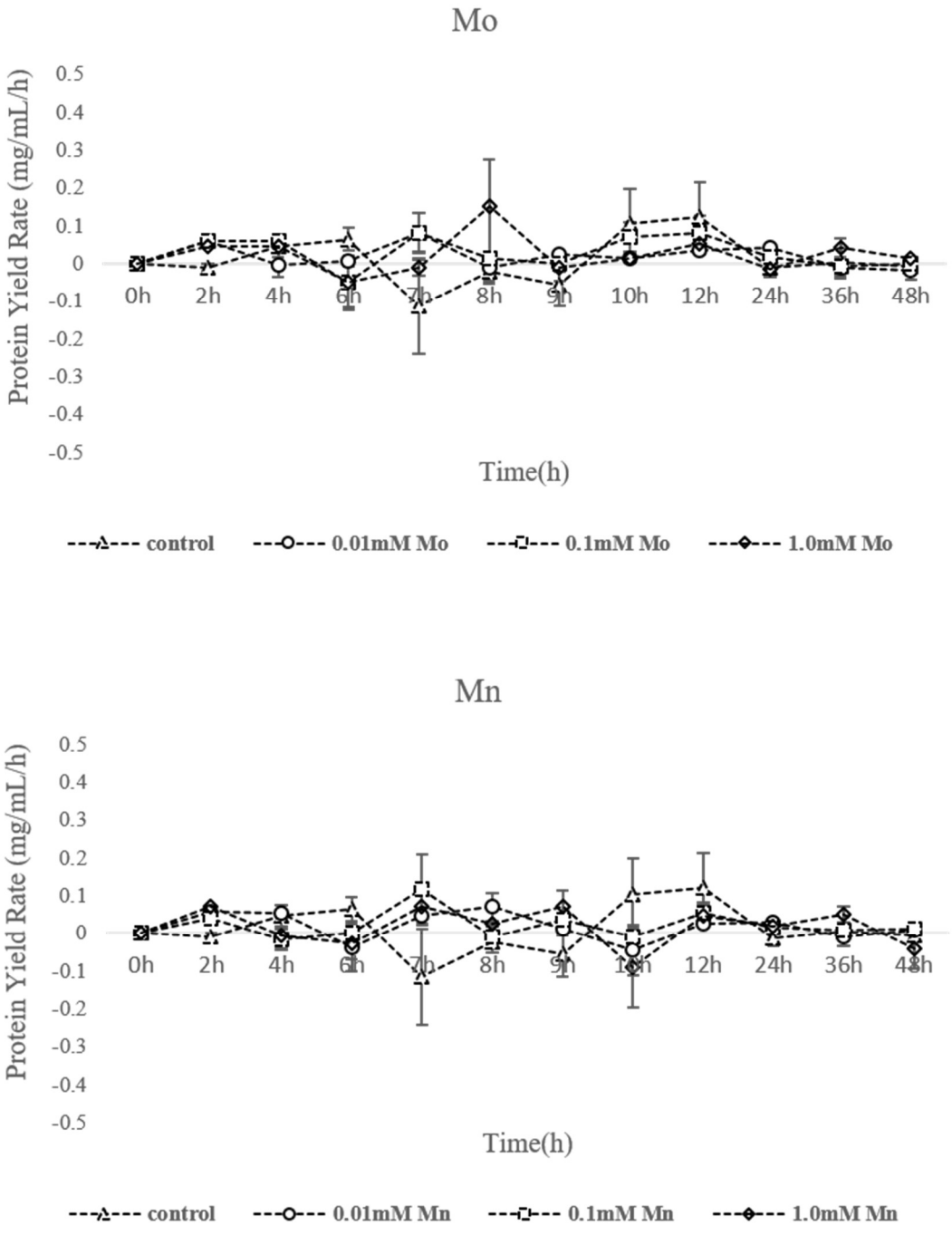


Figure 4.8 Protein yield by *Bacillus* with time with additional molybdenum and manganese

Given that metal elements are very essential for protein functions and activities such as protein catalysis, structural folding and chemical stability, the proteomic responses to available metals in solution should be apparent and varied. A popular category for the roles of metals on protein is the Irving-Williams series, which shows the tendency of affinities for metals that in the order of proteins' preference. This stability of protein complexes formed by divalent first-row transition metal ions generally increase across the period to a maximum stability at copper: (Mg^{2+} and Ca^{2+} (weakest binding) < Mn^{2+} < Fe^{2+} < Co^{2+} < Ni^{2+} < Cu^{2+} > Zn^{2+}) (Irving and Williams, 1953). However, the principle of Irving-Williams series to analyze the competition of metals on campaigning metal binding sites of proteins while two or more metal elements are available in the solution, for single metal's stability on protein, this category may be less useful.

A more related factor for protein stability is pH changes in the solution (Yang and Honig, 1993; Schaefer et al., 1997; Tollinger et al., 2003). Long et al., 2007, studied the aggregation of protein by Al and found that pH value, anion, salts temperature and solvent have obvious effects. Our test of pH change with time was shown in Table 4-1 and 4-2, control and groups with Al^{3+} and Mn^{2+} had their pH increase to above 7.4 after 24 h, while Mo^{6+} and Mg^{2+} can maintain the pH to around 7.0. In consider of the stable performance of Mg^{2+} and Mo^{6+} in previous data. It may conclude that Mo^{6+} and Mg^{2+} did not trigger much cellular response, instead, Mo^{6+} and Mg^{2+} may supply nutrient and energy that cells need than induce resistance of cells. The increase of proteins yielding induced by Mo^{6+} and Mg^{2+} may be different from those yielded with Al^{3+} or Mn^{2+}.

Table 4-1. pH changes in *Enterobacter cloacae* cells (show in average and standards deviations for 3 replicates).

	Additional Metal		
Group	0h	8h	24h
Control	6.88 ± 0.10	6.90 ± 0.18	7.94 ± 0.25
0.01μM Mo	6.70 ± 0.18	6.70 ± 0.31	7.04 ± 0.26
0.1μM Mo	6.86 ± 0.33	7.13 ± 0.26	6.77 ± 0.04
1μM Mo	6.89 ± 0.08	6.67 ± 0.30	6.67 ± 0.13
0.01μM Mg	6.74 ± 0.25	6.76 ± 0.06	6.75 ± 0.03
0.1μM Mg	6.96 ± 0.15	6.88 ± 0.17	6.93 ± 0.07
1μM Mg	6.71 ± 0.04	6.90 ± 0.27	6.57 ± 0.32
0.01μM Al	6.68 ± 0.11	6.35 ± 0.00	7.78 ± 0.42
0.1μM Al	6.80 ± 0.05	6.41 ± 0.01	7.72 ± 0.03
1μM Al	6.68 ± 0.11	6.11 ± 0.20	7.41 ± 0.09
0.01μM Mn	6.74 ± 0.03	6.44 ± 0.04	7.54 ± 0.044
0.1μM Mn	6.81 ± 0.02	6.34 ± 0.12	7.51 ± 0.41
1μM Mn	6.70 ± 0.21	6.36 ± 0.25	7.59 ± 0.30

Table 4-2. pH changes in *Bacillus* cells (show in average and standards deviations for 3 replicates)

	Additional Metal		
Group	0h	8h	24h
Control	6.90 ± 0.31	6.96 ± 0.01	7.87 ± 0.22
0.01μM Mo	6.81 ± 0.16	6.81 ± 0.27	6.92 ± 0.11
0.1μM Mo	6.72 ± 0.24	7.16 ± 0.31	6.85 ± 0.28
1μM Mo	6.64 ± 0.19	7.25 ± 0.53	6.71 ± 0.33
0.01μM Mg	6.82 ± 0.21	6.94 ± 0.29	6.93 ± 0.36
0.1μM Mg	6.84 ± 0.25	6.87 ± 0.16	6.83 ± 0.18
1μM Mg	6.73 ± 0.27	6.75 ± 0.18	6.92 ± 0.01
0.01μM Al	6.76 ± 0.23	6.30 ± 0.28	7.95 ± 0.49
0.1μM Al	6.77 ± 0.33	6.50 ± 0.32	7.87 ± 0.32
1μM Al	6.74 ± 0.35	5.99 ± 0.12	7.38 ± 0.21
0.01μM Mn	6.83 ± 0.03	6.15 ± 0.31	7.61 ± 0.05
0.1μM Mn	6.87 ± 0.07	6.18 ± 0.24	7.97 ± 0.11
1μM Mn	6.82 ± 0.13	6.23 ± 0.41	7.95 ± 0.52

In this study, the major DBPs seemed to be under similar tendency both for *Enterobacter cloacae* and *Bacillus*, Fig 4.9 to Fig 4.12 showed SDS-PAGE results of protein particle density of both strains, it could be seen that protein bands with Mo^{6+} and Mg^{2+} around 150 kd to 250 kd had additional protein band for both strains, and more proteins may be raised by all metals from 10 kd to 37 kd. Groups with Al^{3+} and Mn^{2+} had higher density in the range of 15 kd to 25 kd. *Bacillus* seemed to produce more proteins bands between 37 kd to 50 kd than *Enterobacter cloacae*. And *Enterobacter cloacae* with 0.1 μM Mo^{6+} had a much larger protein band between 37 kd to 50 kd. Overall, most of the protein bands between two strains were similar, even though the structures of *Enterobacter cloacae* and *Bacillus* are quite different. Those unique bands may be caused by metal-induction, supporting an idea that DBPs formation may be dependent on the proteins rather than the structures, however, the membrane and the pore of the cells might block the NOM or EPS from coming out of cells, and this mechanism is indirectly affecting the reaction of the formation of DBPs precursors. The NOM may be also absorbed by the membrane, for example, bromide, which is in the media is partially reacted during bacterial metabolism, and the leftovers and intermediates would continue to react with chloride.

In addition, bacterial matters should not be the only issue, substrates after metabolism such as methanol and polysaccharide are potential intermediate compounds leading to THMs. The mechanism that responded to different metals may enable cells to survive from the pressures of metals so as to yield special proteins that act as precursors of some DBPs that increase following with the metal levels.

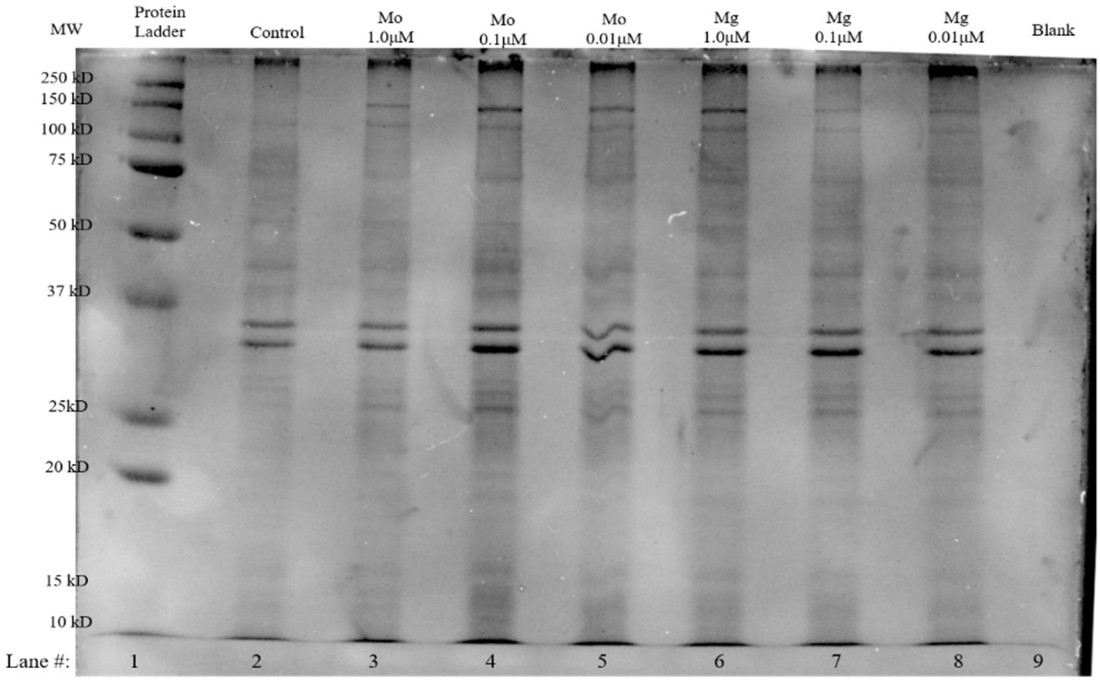


Figure 4.9 *Enterobacter cloacae* Whole cell SDS-PAGE with Mo and Mg

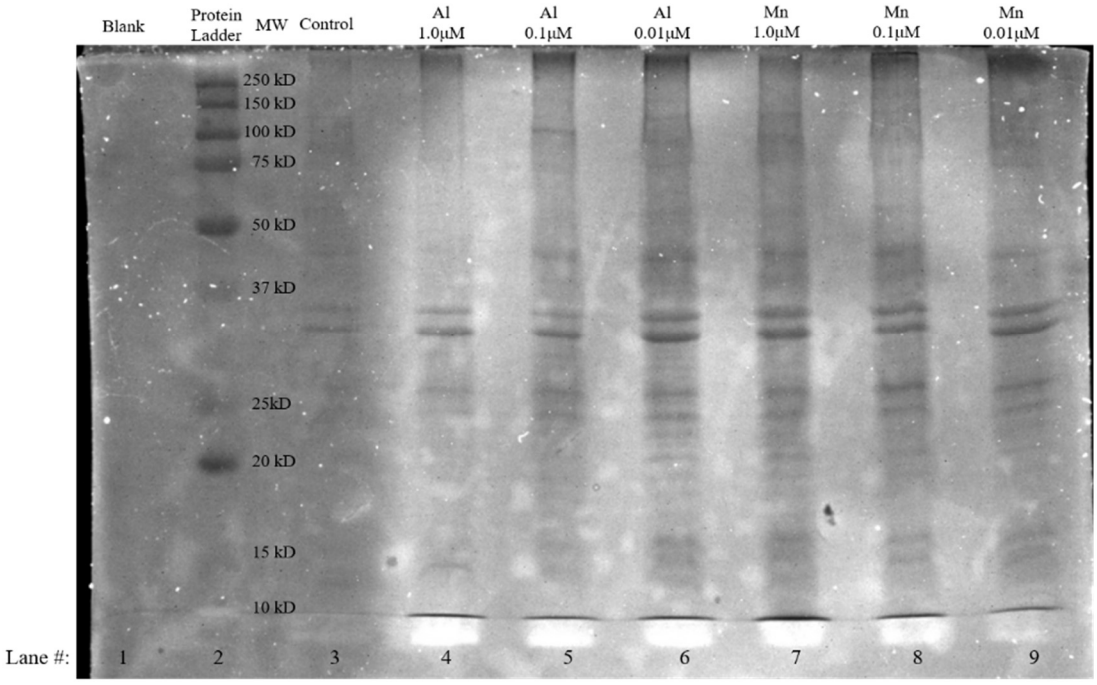


Figure 4.10 *Enterobacter cloacae* Whole cell SDS-PAGE with Al and Mn

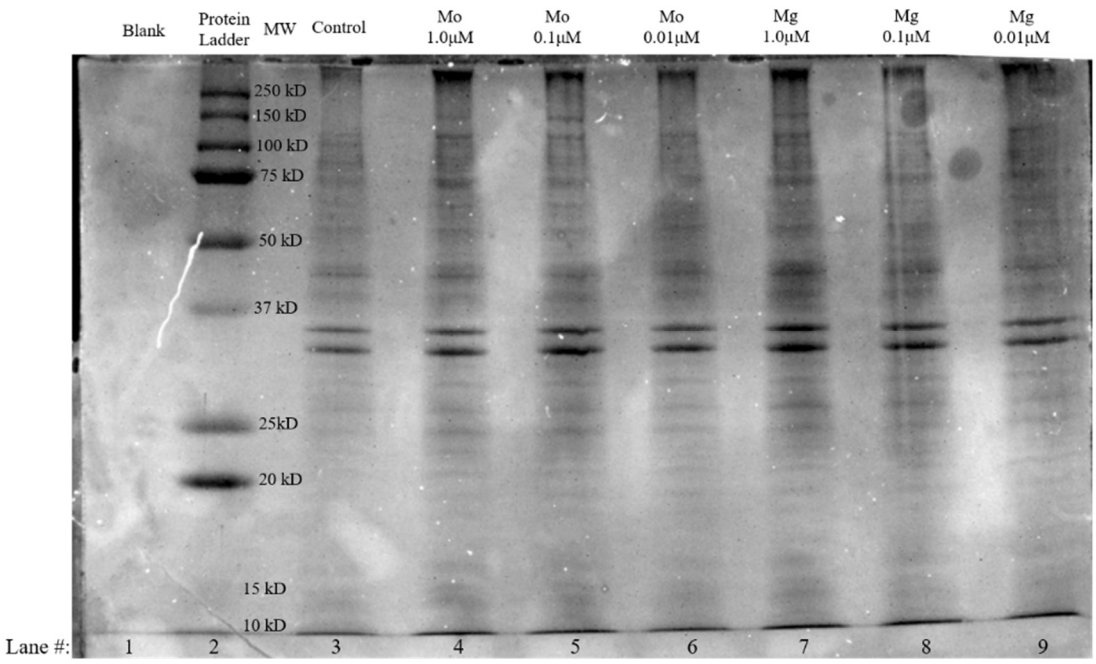


Figure 4.12 *Bacillus* Whole cell SDS-PAGE with Mo and Mg

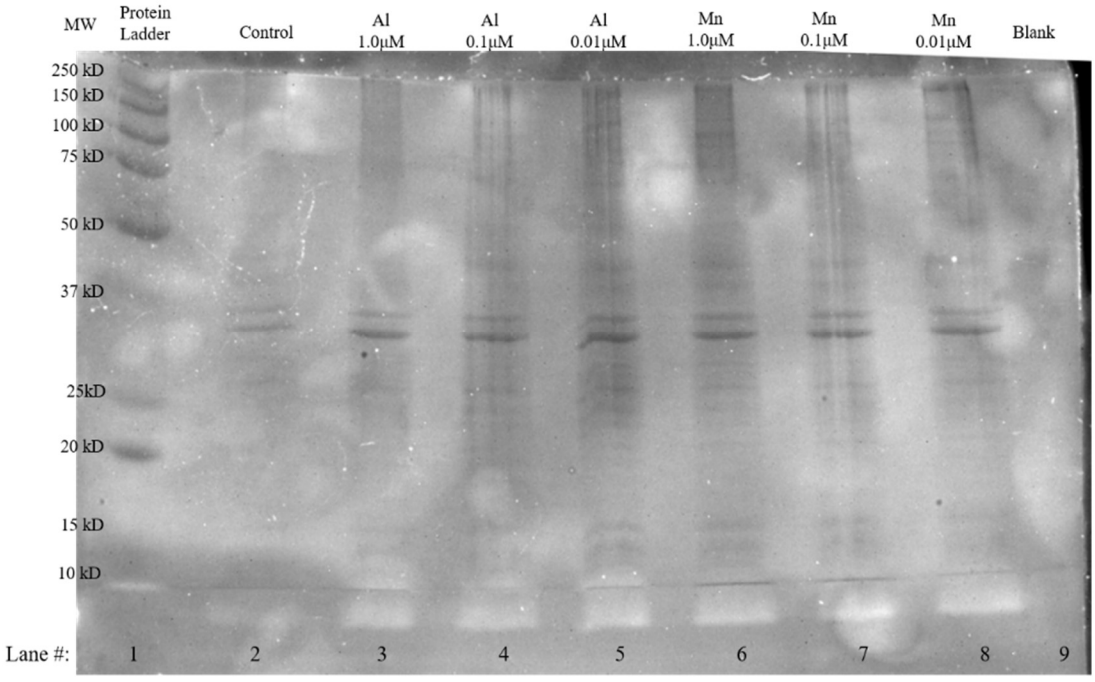


Figure 4.13 *Bacillus* Whole cell SDS-PAGE with Al and Mn

The 4 metal elements had significant influences on the proteins in our experiment, and both strains responded to these metals in a short time. Although all of the metals may similarly increase the protein yields for *Enterobacter cloacae* and *Bacillus*, distinct changes were made among those metals. It is apparently that Al^{3+} and Mn^{2+} could have stronger impacts on protein yields, pH and protein diversity. This might due to the characteristic of metal elements and the levels of them used in this study. With the results, we can confirm that metal elements are required for cells, and also are important and extremely useful in for the study of DBPs formation, because the presence and the level of metal elements should be very crucial to the performance and production of those metal-demanded proteins, which could be potential DBP precursors.

The types of proteins or peptides that react with metals needs to be determined for detecting possible bacterial DBP precursors, and there are some metal-binding proteins have been reported. Research on bacterial metabolism have discovered that many biological activities are conducted by the associations between metal and protein. For example, metal ions align with the ligands of amino acid residues in a protein to achieve its functions, Magnesium is contained by DNA polymerase and hexokinase (Romani, 2013); Molybdenum is essential for nitrate reductase, sulfite oxidase and xanthine oxidase (Ralf, 2013); Manganese is required by arginase and oxygen-evolving complex enzymes (Roth, 2013); and Aluminum, even though have not much reports in bacteria, is an inducer of the protein localization of a cell wall-associated receptor kinase in Arabidopsis (Mayandi et al., 2003). And various metal-binding effects on proteins are necessary to maintain the overall structural stability and the integrity of proteins. Metals might be able to supply anchor points for secondary, quaternary and even tertiary structures of the protein, such as zinc in the zip protein family (Bin et al., 2018), metal ions could be more than selective but mandatory for the cell survive.

But it should be noted that metal effects are not just working on metal-binding sites, additionally, the chemical induction and inhibition by metals is also a critical function of metals in the environment. In water resources and water treatment plants, pollutions entering the water by

human activities often contain high concentration of heavy metals, which could be toxic to aquatic microorganisms because of their interruption on metabolisms. A study focused on the heavy metal-binding proteins from metal-simulated activated sludge culture found that there was a lot of those proteins recovered by copper ion (Sano et al., 2006). Bontidean et al., 2000, analyzed some bacterial metal-resistance proteins that are able to respond to down to 10^{-15} M concentration of Hg, Cu^{2+}, Zn^{2+} and Cd in pure solution, showing a good correlation between the resistant proteins and metals. These results proved that some special proteins have dynamic levels in respect to the presence of metals in the system. With the understanding of this mechanism, high metal concentration in water treatment may cause bacterial responses that generate a lot of cellular DBP precursors, further work is needed to establish this potential relationship for the prediction and detection of DBPs, and it can be also used for the design of biomarkers. But metal-binding is not the only feedback of protein response, the more common situation of metal-protein correlation is defensive reaction, because heavy metals can cause DNA and protein damages with their toxicity and carcinogenesis (Kasprzak, 2002; Bánfalvi, 2011), and reactive oxygen species (ROS) are the major effects by metals that inhibit most cellular processes at various levels of metabolism (Sytar et al., 2013).

In addition to passive activation with metals, protein itself may be able to collect metal ions from the environment, Lundin M et al., 2012, found that the presence of human proteins can promote the level of metals released from chromium metals and stainless steel materials. In microbiology, metal-specific importers and exporters on cell membrane play important roles on the regulation of cellular metabolism, the balance between them may be controlled by metal ions (Nies et al., 2007; Waldron and Robinson, 2009). An outer-membrane protein named ompF in gram negative bacteria may trigger the pathway for metals to get through the membrane (Nikaido and Vaara, 1985), and metal-binding peptides are associated with the vacuolar membrane in yeast to act for heavy metal tolerance (Daniel et al., 1995). Metal-biding peptides on the membrane could

be also used for enhanced bioaccumulation of heavy metal ions (Kotrba et al., 1999), meaning that metal influences on proteins could be firstly begin on the cell membranes.

Former studies suggest that the bacterial cells may contact with metals primarily on the membrane. Proteins and peptides on membrane are mostly importers and exporters, and many of them often coupled with ATP to acquire energy for attracting metal ions, so called ATP-biding cassette transporters (ABC transporters) (Saurin et al., 1999; Jones and George, 2004).While Mg^{2+} is the core metal for ATPase, whether the addition of Mg^{2+} in this study could induce the yielding of ATPase and so as to increase the level of transport proteins might be a good evidence. However, more proteomic and MS test must be done for this hypothesis. Molybdenum as a trace element helps to regulate the iron stores and is involved in the carbohydrate, lipid and urine metabolism. Further work to analysis multi-metal influence on DBPs is waiting ahead.

CHAPTER V

CIPROFLOXACIN IN WASTEWATER TREATMENT ACTIVATED SLUDGE

5.1 Process

Ciprofloxacin was chosen as the antibiotic for this experiment and the biofilm was prepared in activated sludge reactor with continuous flow inlet and outlet. Parameters of the biofilm were controlled under the optimized condition for biofilm. Sludge for biofilm was collected from the distribution system in nearby drinking water or waste water treatment plant or the water treatment plant in OSU, the culture and enrichment of biofilm were made in a set of aerobic bioreactors with nutrients from amended media and continuous oxygen supply, because a lot of bacterial organic matters were contained in biofilm and many common metal ions can accumulate in freshwater biofilm to modify embedded bacterial communities. This study can help to explain the biofilm-metal issues and develop a better strategy to deal with biofilm in water treatment.

5.2 Material and Methods

5.2.1 Development and operation of bench-scale activated sludge bioreactors

Combined activated sludge/sedimentation reactors were built as previously described (Stover and Kincannon 1983; Barber and Veenstra, 1986). The reactors have a 3L activated sludge compartment which is aerated with an aquarium pump and fed a synthetic wastewater influent with a hydrostatic pump. Reactor contents flow under a sluice gate into a 2L sedimentation compartment where effluent flows out a spigot at the top of the water line while solids settle. Contents in the

activated sludge side are well-mixed through aeration such that churning on the bottom of the activated sludge compartment recirculates solids settled from the settling compartment back into the activated sludge side. A synthetic wastewater, named "Syntho", was prepared as described previously (Boeije et al., 1999) and used as influent. The influent was loaded into the activated sludge compartment at a rate of 5 mL min^{-1}. Activated sludge collected from the wastewater treatment plant in Stillwater, Oklahoma, USA was used to seed reactors at the mixed liquor volatile suspended solids (MLVSS) concentration of 1000 mg L^{-1}. MLVSS was measured every 2-4 days and maintained below 1600 mg L^{-1} by periodically removing excess solids. The aeration was controlled by crimping the aeration tube such that dissolved oxygen (DO) was maintained at 2 to 4 mg L^{-1}. The pH of the reactors was maintained between 6.9 and 8.1.

5.2.2 Antibiotic settlement

Ciprofloxacin was purchased from Sigma-Aldrich (St. Louis, MO, USA). After 14 days of stable operation (with chemical oxygen demand (COD) removal of >50%), ciprofloxacin was amended at a concentration of 2.7 mg L^{-1} into the "Syntho" influent for triplicate reactors (referred herein as "ciprofloxacin-challenged reactors"), and another set of triplicate reactors continued to receive only "Syntho" to serve as controls. The concentration of ciprofloxacin is based on the prophylaxis dosage of 1 g $person^{-1}$ day^{-1} that the CDC prescribes to the affected population in the case of a suspected anthrax attack (CDC Fact sheet 2017), divided by an average wastewater usage rate of 370L $person^{-1}$ day^{-1}. This scenario assumes no metabolism in the body and the water resources recovery facility serving an entirely affected population undergoing prophylaxis for anthrax exposure. Reactors were operated for 14 days with ciprofloxacin amended influent.

5.2.3 COD/TOC/MLVSS/DO/pH test

Chemical oxygen demand (COD) was tested every 3 days by the kit of COD2 Mercury-Free COD Reagent (20-1500mg/L), total organic carbon (TOC) was tested every 6 days by the kit of Mid-Range TOC test "N Tube^{TM} Reagent Set (15-150mg/L C) purchase from HACH (Loveland, CO, USA), DO was measured with a YSI 5100 Benchtop DO meter. Mixed Liquor Volatile Suspended Solids (MLVSS) was measured by evaporation of 10 mL of sample at 550 °C for 2H. The pH of the reactors were measured daily with a Seven Compact pH probe.

5.2.4 DNA analysis

Samples were collected in specially cleaned sample container from the sludge every week, and concentrated by centrifuge with 10000rpm for 5min. DNA was from the PowerSoil DNA extraction kit (MoBio, Carlsbad, CA, USA). DNA extract of the original soil inoculum were submitted to Molecular Research LP (Shallowater, TX, USA) for PCR amplification of the V4 variable region of 16S rRNA genes with primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACVSGGGTATCTAAT-3') (Caporaso et al 2011), bTEFAP© Illumina MiSeq 2 × 300 bp barcoded amplicon sequencing, and analysis with a proprietary pipeline (Dowd *et al.*, 2008). The total number of reads varied from 16,931 to 105,497 per sample. OTUs were delineated at a 97% identity level with UCLUST (Edgar, 2010).

5.3 Results and Discussion

The influent media was measured to have a COD of 716.5 ± 10 mg L^{-1} and a TOC of 213 ± 19 mg L^{-1}. After an initial week of stabilization, the COD and TOC in the effluent was consistently measured between 239-394 mg L^{-1} and 40-80 mg L^{-1}, respectively, in the reactors prior to ciprofloxacin amendment (Figure 5.1). After ciprofloxacin amendment in the influent commenced for the ciprofloxacin treated reactors, both the effluent COD and TOC maintained at

stable levels similar to that of the controls. Thus, the loading of ciprofloxacin did not affect the COD or TOC removal efficiencies of the reactors during this time frame. Day 0 denotes start of ciprofloxacin loading. MLVSS, DO and pH (Fig 5.2 to Fig 5.4) also represent similar conditions between ciprofloxacin treated reactor and control, it proved that the biofilm have resistance to ciprofloxacin.

According to our data, the antibiotic effect from ciprofloxacin had only a little influence on the condition of activated sludge, the 2 mg/L ciprofloxacin level used in the study may not cause the death of biomass in the activated sludge, instead, a quick resistance from the bacteria community, especially the *Citrobacter freundii*, was induced by the antibiotic chemical.

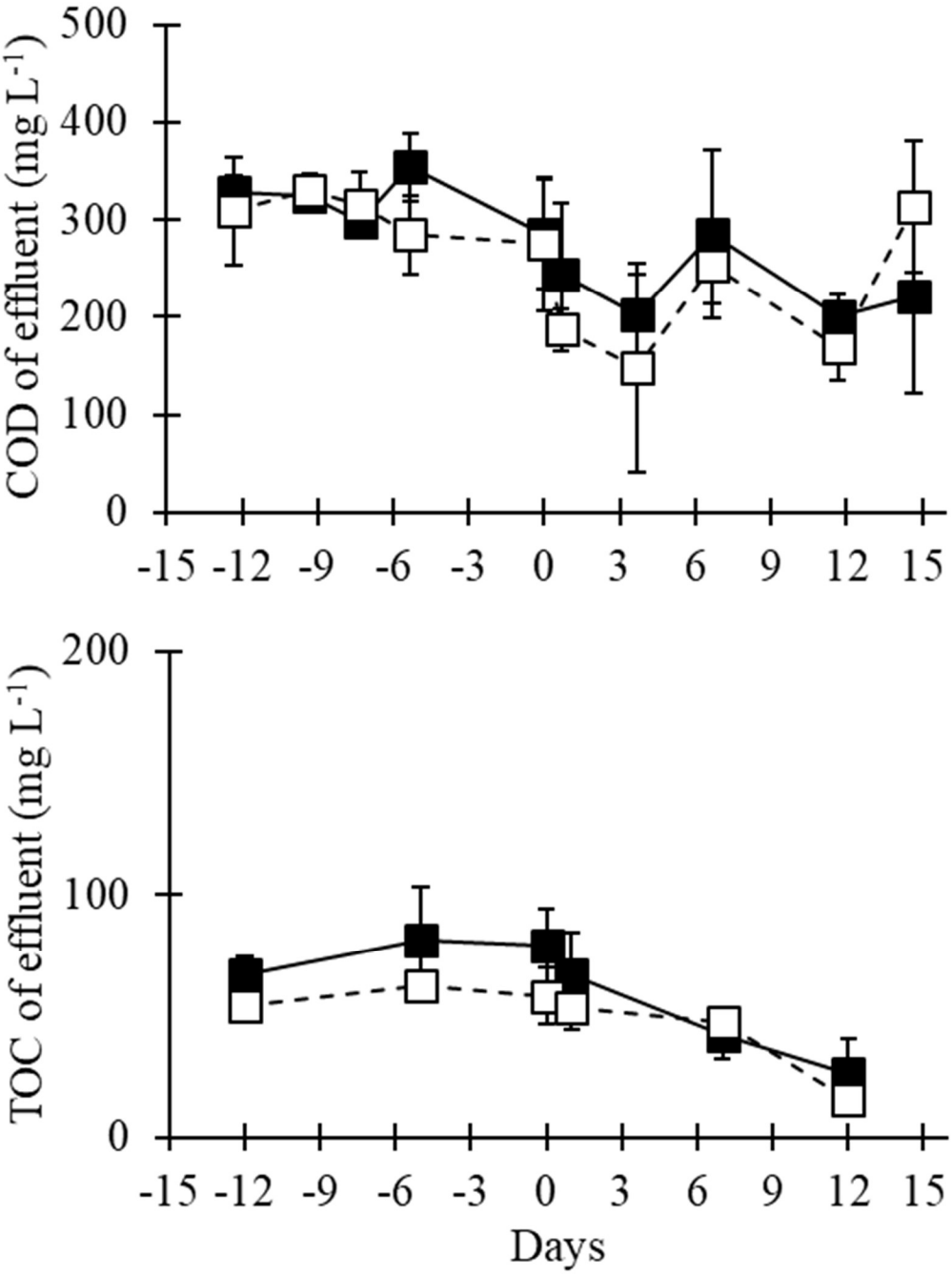


Figure 5.1. COD and TOC of the ciprofloxacin treated reactors (solid squares/lines) and control reactors (open squares, dashed lines) over time. Ciprofloxacin treatment began on Day 0.

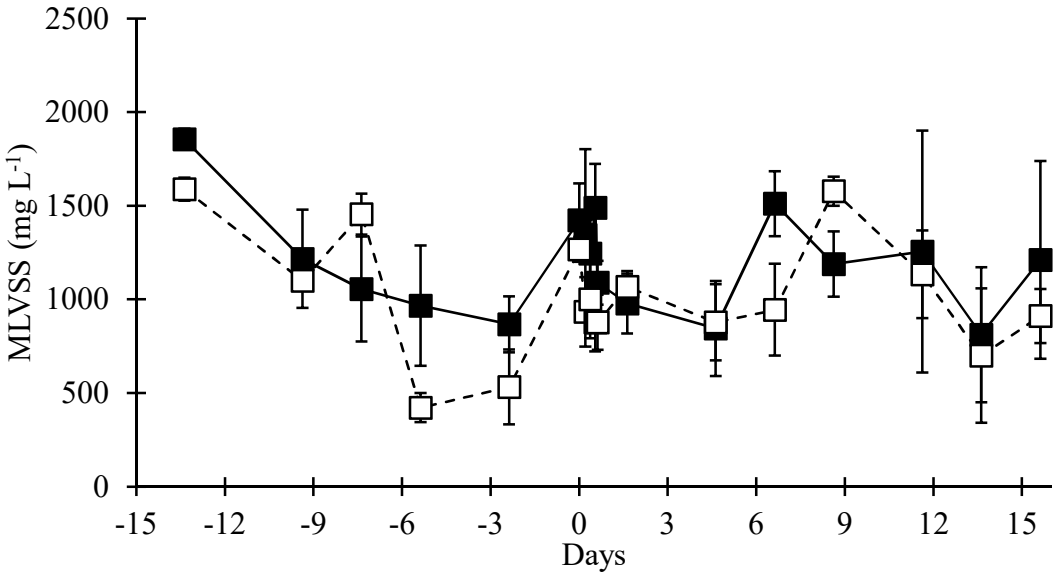


Figure 5.2. Mixed liquor volatile suspended solids (MLVSS) of reactors subject to ciprofloxacin treatment (solid squares/lines) and controls (open squares, dotted lines). Ciprofloxacin treatment began on Day 0.

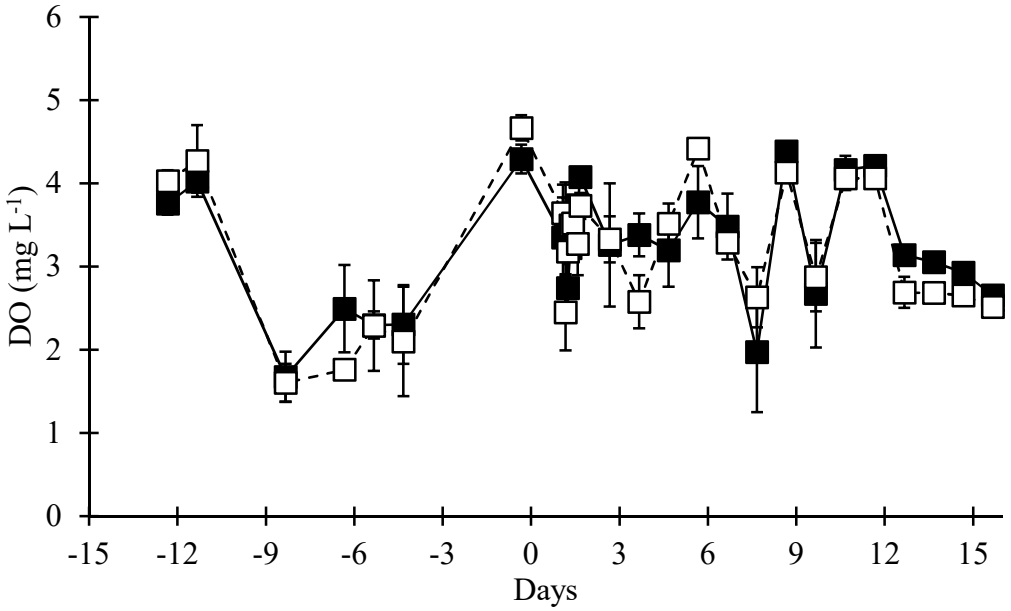


Figure 5.3. Dissolved oxygen (DO) of reactors subject to ciprofloxacin treatment (solid squares/lines) and controls (open squares, dotted lines). Ciprofloxacin treatment began on Day 0.

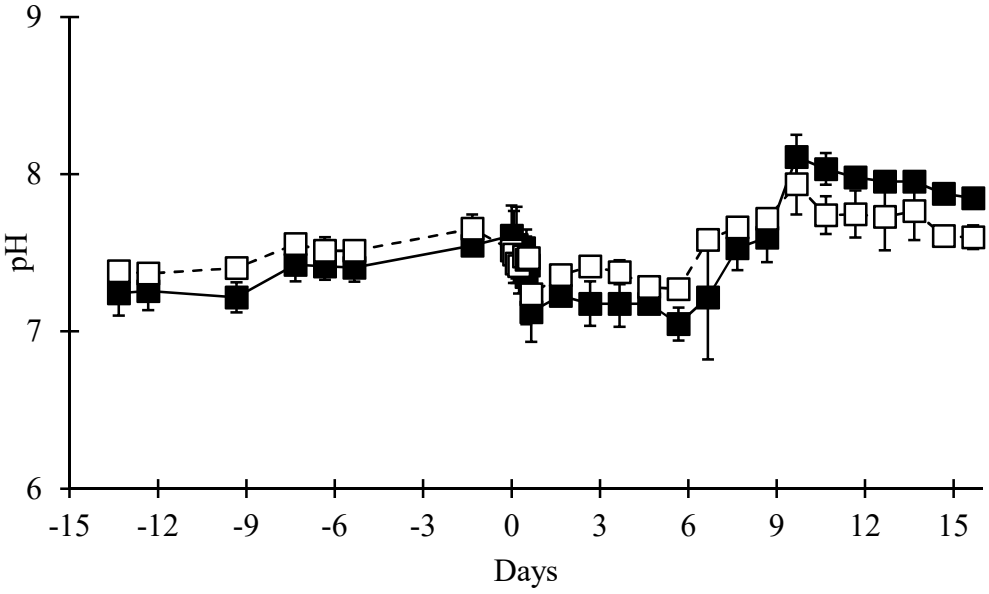


Figure 5.4. The pH of reactors subject to ciprofloxacin treatment (solid squares/lines) and controls (open squares, dotted lines). Ciprofloxacin treatment began on Day 0.

While there was not apparent change in reactor condition, the detection inside biofilm had done for indicating resistant strength. The phyla level that represent the abundance of bacterial community is shown in Table 5-1. phyla level relative read depth for DNA samples analyzed 5 days prior to, and 1, 7, and 14 days following ciprofloxacin introduction in the ciprofloxacin amended reactors and in the control reactors. The phyla *Proteobacteria* and *Bacteroides* combined represented 74% to 87% of the relative read depth in all model reactor samples. By Day 14, four phyla (*Gemmatimonadetes*, *Synergistetes*, *Cyanobacteria*, and *Verrucomicrobia*) were statistically significantly less abundant in the ciprofloxacin-amended reactors (Student's t-test, $p< 0.05$), compared to the control reactors. Of these four phyla, the read depth of *Gemmatimonadetes* demonstrated the greatest reduction (99.5 %) from 1.65 ± 0.8 % in the control reactors to 0.009 ± 0.004 % observed in the in the ciprofloxacin-challenged reactors. The read depths of the *Cyanobacteria*, the *Synergistetes* and the *Verrumicrobia* were 96.4%, 81.1%, and 17.4% less abundant in the ciprofloxacin amended reactors compared to the control reactors, respectively.

Table 5-1. Phylogenetic relative read depths five days before, and at 7 and 14 days after the ciprofloxacin amendment began. The phyla Deferribacteres, Dictyoglomi, Aquificae, Deinococcus-Thermus, Lentisphaera, Caldiserica, and Nitrospirae were all less than 0.01% in every sample and are not shown here.

	Day -5		Day 7		Day 14	
Phylum	Ciprofloxacin treated	Control	Ciprofloxacin treated	Control	Ciprofloxacin treated	Control
Proteobacteria	63.5 ± 5.7	56.4 ± 3.5	57.0 ± 1.6	51.9 ± 2.1	56.0 ± 7.9	53.0 ± 8.2
Bacteroidetes	23.4 ± 7.9	22.3 ± 3.1	22.4 ± 2.0	25.6 ± 2.0	27.8 ± 9.4	22.9 ± 6.9
Firmicutes	4.8 ± 2.2	7.1 ± 2.4	5.3 ± 3.1	3.3 ± 1.4	2.3 ± 0.4	1.8 ± 0.6
Acidobacteria	3.0 ± 0.8	5.5 ± 1.7	8.1 ± 4.6	7.6 ± 1.4	4.8 ± 2.7	6.8 ± 1.7
Verrucomicrobia	1.7 ± 1.1	3.8 ± 4.1	3.4 ± 2.0	6.9 ± 0.6	6.3 ± 0.5	7.7 ± 0.58
Actinobacteria	1.4 ± 0.6	2.4 ± 1.7	1.9 ± 0.5	2.0 ± 0.5	1.7 ± 0.9	3.4 ± 1.4
Fusobacteria	0.82 ± 0.97	0.23 ± 0.12	0.65 ± 0.60	0.19 ± 0.09	0.15 ± 0.11	0.10 ± 0.09
Chlorobi	0.36 ± 0.13	0.45 ± 0.25	0.04 ± 0.03	0.06 ± 0.01	0.018 ± 0.012	0.05 ± 0.06
Planctomycetes	0.22 ± 0.07	0.43 ± 0.33	0.36 ± 0.28	0.66 ± 0.44	0.46 ± 0.41	1.1 ± 0.5
Spirochaetes	0.13 ± 0.18	0.40 ± 0.23	0.43 ± 0.51	0.42 ± 0.20	0.09 ± 0.06	0.5 ± 0.6
Tenericutes	0.21 ± 0.21	0.24 ± 0.36	0.01 ± 0.008	0.04 ± 0.04	0.003 ± 0.002	0.006 ± 0.003
Synergistetes	0.23 ± 0.06	0.37 ± 0.37	0.08 ± 0.06	0.16 ± 0.15	0.02 ± 0.001	0.09 ± 0.02
Chloroflexi	0.10 ± 0.02	0.18 ± 0.10	0.18 ± 0.13	0.27 ± 0.12	0.14 ± 0.13	0.39 ± 0.16
Chlamydiae	0.08 ± 0.04	0.07 ± 0.03	0.10 ± 0.03	0.06 ± 0.01	0.22 ± 0.29	0.07 ± 0.05
Nitrospirae	0.02 ± 0.01	0.08 ± 0.12	0.02 ± 0.02	0.11 ± 0.15	0.02 ± 0.02	0.21 ± 0.20
Cyanobacteria	0.02 ± 0.02	0.03 ± 0.02	0.04 ± 0.02	0.11 ± 0.05	0.006 ± 0.002	0.16 ± 0.07
Gemmatimonadetes	0.002 ± 0.001	0.02 ± 0.02	0.02 ± 0.004	0.47 ± 0.50	0.009 ± 0.004	1.65 ± 0.81
Ignavibacteriae	0.002 ± 0.002	0.007 ± 0.006	0.003 ± 0.002	0.03 ± 0.03	-	0.07 ± 0.12
Candidatus Saccharibacteria	0.001 ± 0.002	0.009 ± 0.012	0.002 ± 0.002	0.03 ± 0.01	0.0004 ± 0.0007	0.01 ± 0.01
Cloacimonetes	0.002 ± 0.003	0.006 ± 0.008	0.01 ± 0.01	0.05 ± 0.07	0.002 ± 0.001	0.03 ± 0.05
Elusimicrobia	0.003 ± 0.005	0.005 ± 0.004	0.01 ± 0.02	0.004 ± 0.004	0.002 ± 0.001	0.003 ± 0.003
Fibrobacteres	-	0.003 ± 0.004	0.01 ± 0.01	0.02 ± 0.01	0.0001 ± 0.0009	0.009 ± 0.01

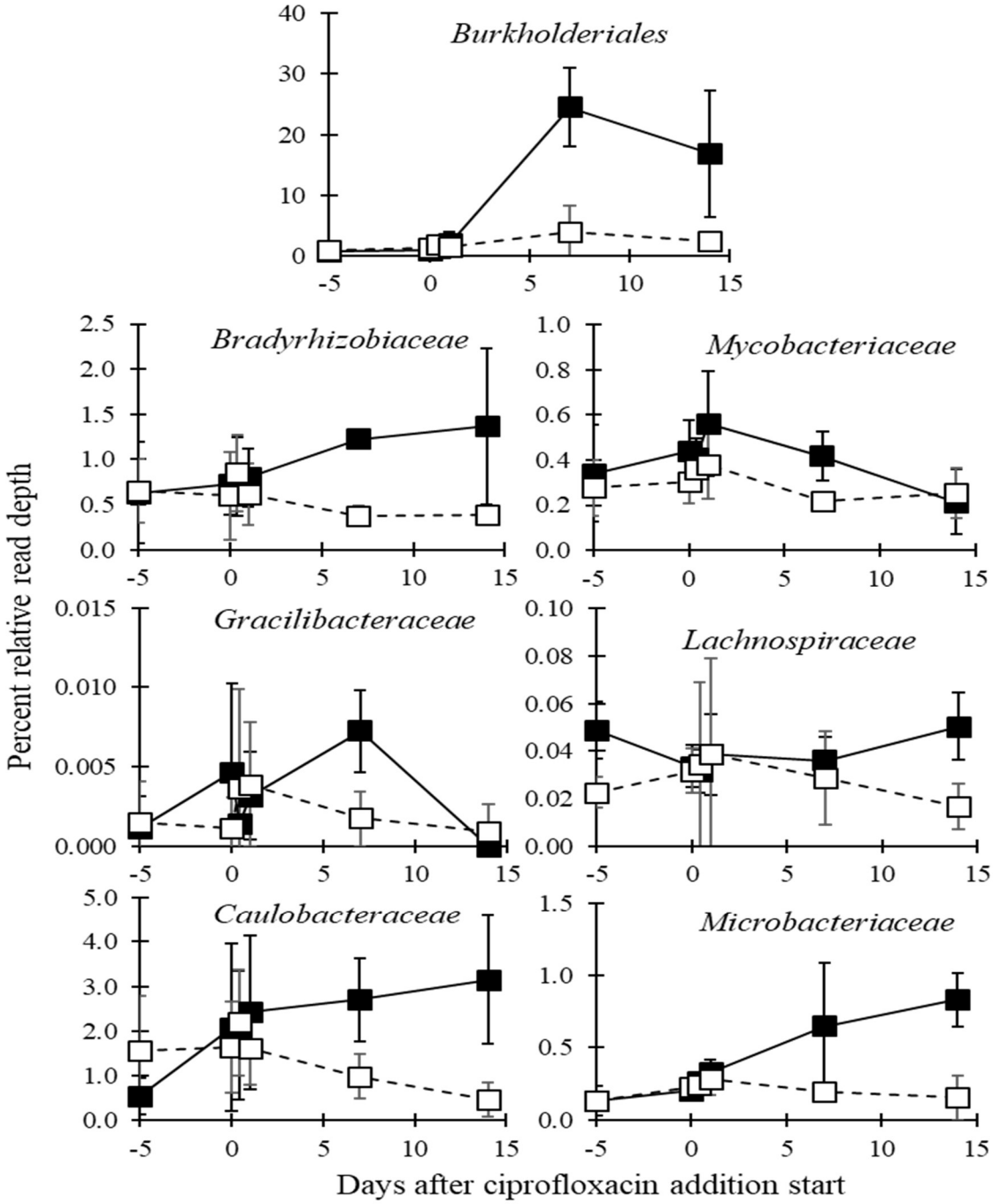


Figure 5.5. The phylogenetic families found to be significantly enriched in the reactors with ciprofloxacin treatment controls (Student T-test, P value ≤ 0.05) on at least one sampling time point based on relative read depth from the 16S rRNA amplicon Illumina sequencing. Reactors with ciprofloxacin amendment are shown as black squares/solid lines, while control reactors are shown with open squares/dotted lines.

On the phyla level, no enrichment from the ciprofloxacin loading was evident, however, many phylogenetic families were enriched (Figure 5.5). By Day 7, ciprofloxacin led to an increase in the read depth for *Burkholderia*, *Bradyrhizobacteraceae*, *Mycobacteriaceae, Gracilibacteraceae*, and *Caulobacteraceae*. Increased read depth for *Burkholderiales*, *Bradyrhizobacteraceae*, and *Caulobacteraceae* was also observed on Day 14. The read depth of the *Microbacteriaceae* and *Lachnospiraceae* were also significantly increased by Day 14.

This result could imply that the adaptable concentration of ciprofloxacin can lead to the enhancement of antibiotic-resistant bacteria, while some studies ended up with conclusions as WWTP supply continuously antibiotic-resistant bacteria or genetic determinants to the environment (Guardabassi et al. 2002; Reinthaler et al. 2003; Tennstedt et al. 2003; Costa et al. 2006; Ferreira da Silva et al. 2006, 2007; Watkinson et al. 2007a; Sabate et al. 2008; Zhang et al. 2009), our findings also suggest to pay attention to antibiotic-resistant bacteria and the control of pharmaceuticals in WWTP. A paper reported that the longer hydraulic retention time (HRT) necessary to achieve higher removal rates may contribute to the establishment of resistant bacterial populations in the treating biomass leading to a progressive increase of the prevalence of antibiotic resistance in the treated effluent (Celia M. Manaia, 2010). However, the HRT could influence the quality of activated sludge, and although the conditional test showed that the property of activated sludge might be unaffected by relatively low level of ciprofloxacin, the bacterial community structure of activated sludge had the increased prevalence of those antibiotic-resistant bacteria like *Citrobacter freundii*, whether such a change is good for biodegradation or not have not yet been revealed, future studies are needed to understand the influence of *Citrobacter* on the activated sludge. Ciprofloxacin amendment selected for both ciprofloxacin- and multidrug- resistant bacterial isolates and these isolates appeared with greater prevalence in ciprofloxacin amended reactors compared to the control. Aside from *Citrobacter freundii*, our study also found *L. adecarboxylata, Escherichia spp., Klebsiella oxytoca, Citrobacter spp.,* and *K. oxytoca* may

develop resistance to ciprofloxacin in the bioreactor. And such a development of resistance by microorganism can cause the resistance to other antibiotics at the same time, our analysis of multi-drug resistance of *Citrobacter freundii* found that the it had also been able to survive in media with some antibiotic, including Amoxicillin (AMC), Ampicillin (AMP), C (Chloramphenicol), Gentamicin, Trimethoprim + Sulfamethoxazole(SXT), Tetracycline(TE), and Ticarcillin(TIC). The resistance to multi-drug may due to the same mechanism share by those drugs, like the targeted proteins or enzymes. Previous researched considered the extended-spectrum β-lactamases (ESBL) is closely associated with the ciprofloxacin resistance in *K. pneumoniae* (David L. Paterson, et al., 2000), and those isolates are usually resistant to penicillins, cephalosporins, aminoglycosides, and TMP-SMZ. Mutation of *gyrA* gene could be another factor that allows the resistance of ciprofloxacin (Laura Vinué et al., 2016; P. Hemarajata et al., 2016), which detected in *Neisseria gonorrhoeae* and *E.coli*. Such a genomic change could be very fast, during our observation, the strains of *Citrobacter freundii* had their multidrug resistant prevalence go up to 95% from 17% only after 72h, while the dosage in this study is considered low (2 mg/L). Higher antibiotic use may encourage high resistance rates, and more resistant genes may be produced and released to the environment (Hande Arslan, 2005), the WWTP face the risk of the prevalence of pharmaceutical resistance bacteria during the wastewater treatment.

The CIP favors resistant strains that with enhanced survival ability or virulence in the environment while kicks normal strains out, this antibiotic can rapidly give rise to vast numbers of resistant progeny (David M. Livermore, 2003). However, the increase of resistant bacteria by the induction of CIP is not the only issue, moreover, some resistant genes could be spread into the environment, because the antibiotic resistance genes (ARGs) are able to travel among different cells (Berendonk et al., 2015), and some developments of resistance may occur due to horizontal gene transfer from donor bacteria, phages, or free DNA to recipient bacteria (Virender K.Sharma et al., 2016). A recent study about the ARGs discharge in urban water treatment plant (UWTP) reported that, the final effluent of UWTP can discharge about 10^9–10^{12} CFU per day, per

inhabitant equivalent, and among these, at least 10^7–10^{10} could have any kind of acquired antibiotic resistance (L.Rizzo, 2013). Transfer of DNA is most often via plasmids, within plasmids, resistance genes are often carried by transposons, which can shuttle determinants between more and less promiscuous plasmids, or into and out of the chromosome (David M. Livermore, 2003), such as plasmid-mediated SHV β-lactamases are derived from the chromosomal β-lactamases of *Klebsiella pneumoniae*; the plasmid-borne AmpC enzymes emerging in *Klebsiella* spp. and *E. coli*are chromosomal escapes from *Citrobacter freundii, Hafnia alvei, Morganella morganii*, and *Enterobacter cloacae* (David M. Livermore, 2003), and this change inside the genome could be permanent, as a kind of so called genetic contamination.

Disinfection could be a good way to get rid of antibiotic resistant bacteria (ARB) and ARGs, however, they increase the cost and energy, for example, more chlorine (More than 30 mg/L) is needed for 90% removal than a typical used amount in WWTP (1-16 mg/L), and the addition of catalysts is required if there is a switch to ozonation (Junsik Oh, 2014); Zhang et al., 2015, addressed that the combined disinfection method, UV + chlorination could mix to make inactivation of antibiotic resistance genes in municipal wastewater effluent; Miranda et al., 2016, also suggested chlorination is very effective to treat antibiotic resistant *E. coli* strain. Under such a tendency, it is anticipated that more chlorine may be used for drinking water/wastewater treatment, and more DBPs should be also yielded with antibiotic resistant biofilm. There could be more complicated mechanism of DBPs production from water treatment, because antibiotics itself could be DBP precursor. Chu et al., 2016 found the zero valent iron could produce dichloroacetamide from chloramphenicol antibiotics even before disinfection. So, it is necessary to consider the DBP production with antibiotics, and microbiological study of antibiotic resistant biofilm may be a key to reveal the possible cellular organic matter contribution to DBPs. My study of combined antibiotic resistant biofilm and DBPs can support such a complicated situation in the future.

CHAPTER VI

RESULTS AND CONCLUSIONS

This study revealed the relationship between cells and DBPs. Two prevalent bacteria and four common metal elements were selected for the experiment. The separation of intracellular organic matter, extracellular organic matters and pure culture organic matters clearly showed the differences among them. For *Enterobacter cloacae*, 9 of 15 DBPs were just formed inside cells while bromodichloromethane only produced out of cells and 1,1,1-trichloroethane, 1,1-dichloroacetone and trichloroethene can be generated with extracellular matter; *Bacillus* had similar DBPs yielding but more extracellular DBP. Pure media experiment proved that extracted NOM can only form chloroform, 1,1,1-trichloroethane, 1,1-dichloroacetone, trichloroethene, bromodichloromethane and bromoform with chlorine disinfection. So most of the extracellular DBP may just come from extracted NOM, but pure media apparently generated more of those DBP than extracellular matter because of the consumption of media NOM by bacteria. With these data we can conclude that fresh cellular matters were major resources of DBP precursors rather than extracted NOM. On the other hand, the metal addition had obviously changed the DBP yielding in compare with control, some DBPs had clearly higher or lower production with metals in compare with control, and *Enterobacter cloacae* with Mn^{2+} and *Bacillus* with Al^{3+} yielded most total DBPs in each group, *Enterobacter cloacae* with Al^{3+} yielded least DBPs, especially with certain metal concentrations. Mg^{2+} and Mo^{6+} had more powerful impacts on Br-DBP than Al^{3+} and Mn^{2+}. These changes also reflected by the DBP spread ratios that intracellular DBPs and chloroform varied in

percentages by metal induction. Additionally, the growth rate of each strain had not been changed much with metals, meaning metals ions did not inhibit the cellular health. Those results imply that the correlation between cells and metals possesses potential mechanism to influence DBP formation.

To answer why DBP formation changed by metal additions while there was no change on cell growth, experiments inside cells was studied by protein analysis. More protein had been yielded with metals, and pH values suggesting that metal did cause increased ion levels in the solution. The 2D gel SDS-PAGE showed the proteomic response to metal ions by bacteria. Big particulate protein around 150kd to 250kd was seen in both strains, and more proteins may be produced by metals from 10kd to 37kd. These proved that proteins had been induced by metals, and metal-binding proteins and metal activated transporters on membrane could be potential DBP precursors.

To extend it to biofilm in water treatment, biofilm resistance to antibiotics had also been studied because former studies had reported the antibiotic can contribute to DBP production, and biofilm is very sensitive to antibiotic than metals under non-toxic concentrations. My study of bacteria effects on DBPs production cooperates the needs of antibiotic resistant biofilm studies, and the research of DBPs under complicated situation in water treatment is definitely necessary for future practices.

In summary, I found bacterial organic matters could be very possible DBP precursors with comparison between vivo bacterial cells and extracted organic matters; and the addition of metals assisted my research to exploit potential cellular mechanism inside cells. Protein analysis should be a key for revealing such a mechanism and also benefit for organic proteomic studies. Last but not the least, my data on antibiotic resistant biofilm validated some aspects of biological response that possibly relate to DBP formation. My study provides a creative and inspired basis for future

DBPs research and treatment. The extension of this study could be benefited for the studies of bio-resistance, bio-metabolism and artificial biochemical compounds.

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