Evaluating the Performance of Sand/Gravel Bioreactors in Treatment of High Strength, High Salinity Wastewater

THESIS

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By

Feng Chen

Graduate Program in Food, Agricultural & Biological Engineering

The Ohio State University

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Master's Examination Committee:

Karen M. Mancl, Advisor

Olli H. Tuovinen

Yebo Li
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Abstract

Many food processors use salt, resulting in high strength, high salt content wastewater. The goal of this study was to determine the impact of salt concentration on the treatment of turkey slaughterhouse wastewater using sand/gravel bioreactors. Six unsaturated sand/gravel columns were intermittently dose treating the wastewater in a single pass. Turkey processing wastewater served as the control and 3 g/L and 6 g/L of table salt were added to wastewater for treatment is duplicate laboratory columns. BOD\textsubscript{5} and NH\textsubscript{3}-N removal was measured during the 74-day experiment. The BOD\textsubscript{5} removal achieved and maintained over 99% after day 21 at all salt levels. The NH\textsubscript{3}-N removal achieved over 99% removal after day 32. The conductivity of the effluent matched the influent indicating that the treatment system did not remove salt. It was concluded from this study that sand/gravel bioreactors were able to treat high strength, high salinity (up to 0.6%) turkey slaughterhouse wastewater.

The biofilm developed on the sand/gravel surface is the functional part of wastewater treatment. In this study, ATP was measured to investigate the impact of salt on the biomass in the treatment system. Sand/gravel samples were collected from each layer of the columns in the wastewater control and wastewater plus 6 g/L salt. The results showed that salt had an initial inhibition on the biomass in the system. System performance decreased as the biomass declined. After the biomass reached a steady level, the
treatment performance was maintained at high level. The study also found that the coarse sand layer was the most active layer treating wastewater.
Acknowledgments

First, my thanks go to my advisor, Dr. Karen Mancl, for her academic guidance throughout the research project. Her knowledge and experience helped me overcome the anxiety at the beginning of this study. I also want to thank Dr. Olli Tuovinen, for his insightful instruction for the ATP test.

Then, I would like to thank Qingyan Min for her assistance during the summer in 2015 when I went back to China. Thanks also to Whitewater Processing Co. for delivering all the materials needed in this research.

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Vita

October 8, 1991……………Born-Zhejiang, China

2010………………………B.S. Biological Engineering, ZJUT

2016 to present ……………Graduate Research Associate, The Ohio State University

Fields of Study

Major Field:  Food, Agricultural & Biological Engineering
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Chapter 1: Introduction

Salt serves as a food additive to enhance flavor and is also a food preservation agent. Many food sectors require a high amount of salt in processing. Pickled vegetable, snack food, cured meat, meat canning and fish processing industries all use a large amount of salt. Saline effluents also rich in organic matter are constantly generated by these industries. For example, olive oil processing discharges a liquid effluent with about 6 g/L of salt (Inan et al., 2004). The chemical oxygen demand (COD) was 191,090 mg/L when using a batch milling process and 54,320 mg/L in the continuous milling process (Vitolo et al., 1999). Omil et al. (1996) treated three different kinds of seafood processing wastewater in a pilot plant. The COD of tuna-cooking effluent, mussel-cooking effluent and fish-meal plant effluent were 29.5 g/L, 18.5 g/L and 89.4 g/L, respectively. The chloride concentration in the three different effluents ranged from 13 g/L to 19 g/L. Biological treatment is often adversely affected by the hypersaline wastewater. Stewart et al. (1962) constructed extended aeration pilot plants to study the effect of elevated salinity on the treatment of synthetic wastewater. They found that under severe changes in salinity a temporary reduction in treatment would occur only when it was combined with a heavy loading rate of organic matter. Rinzema et al. (1988) conducted a lab-scale up-flow anaerobic sludge bed (UASB) study to treat synthetic wastewater using methanogens. They found that at neutral pH 10%, 50% and 100% inhibition of specific
acetoclastic methanogenic activities were observed when sodium concentrations were at 5, 10 and 14 g/L, respectively. To treat these challenging wastewaters, physiochemical treatments are added to biological treatment to reduce the salt content. The main physiochemical technologies are thermal, ion exchange and membrane. Membrane technologies include all engineering methods transferring selected molecules under pressure or a concentration gradient. Reverse osmosis (RO) is a suitable membrane technology to remove salt from wastewater. Water is filtered through a semi-permeable membrane under a pressure greater than the osmotic pressure produced by the dissolved solids in the wastewater. One advantage of using RO over other desalination methods is that dissolved organic matter is also blocked by the membrane, thus 5-day biological oxygen demand (BOD$_5$) decreases. However, high quality influent is required to prevent constant membrane fouling due to suspended solids, colloidal matter and other large organic molecules such as proteins (Lefebvre and Moletta, 2006). Membrane fouling is a process where solute or particles in fluid deposit on a membrane surface or in membrane pores, so that the performance of the membrane declines. Membrane exchange or chemical cleaning is required to maintain the performance of the treatment system, however it will increase the system operating cost.

A membrane bioreactor (MBR) is a combination of a suspended growth bioreactor followed by membrane filtration. Lay et al. (2010) reviewed two types of MBR systems. The elevated salt concentration can greatly impact system performance including physicochemical, microbiological and membrane activities. In high salt concentration, unfavorable physicochemical parameters for oxygen transfer, viscosity, turbidity, and salt precipitation will occur. Since effective pretreatment approaches are not applicable to
MBR systems, salt promoted membrane fouling is a difficult operational problem. For membrane system fouling reduces the achievable flux. Chemical addition is needed to clean the membrane system with extra cost and the production of chemical waste sludge. Sharrer et al. (2007) evaluated the performance of a pilot-scale MBR system to treat biosolids from fish culture and reclaim salty water. The cBOD₅ removal was over 99% at all salt levels, biological treatment of nitrogen through nitrification/denitrification was also sufficient at salinity from 0% to 3.2%. The author also reported that a MBR system to reclaim water flow of 87000 m³/day would cost about $470,000 ($5.40/m³), not including the maintenance cost.

The overall goal of this thesis is to develop and evaluate a new, simple and effective way to treatment wastewater containing organic matter and salt. Physicochemical processes require expensive equipment and continuous maintenance cost. The objectives of this thesis are: (1) determine the impact of salt concentration on fixed media treatment receiving meat processing wastewater; and (2) examine the impact of salt concentration on the microbes in the biofilm of a treatment system.
Chapter 2: Treatment of high strength, high salinity meat processing wastewater using fixed media bioreactors

2.1 Introduction

2.1.1 Characteristics of food related wastewater

The universal need for freshwater is increasing along with the growth of human population. A large portion of freshwater flows into the world’s agriculture, beverage and food industries. Industry freshwater usage was 64,352 ML/d in the United States in 2005 (USGS, 2015). Among all of the food industrial consumption, meat processing takes up 24% of the freshwater, dairy 12% and fruits and vegetables 10% (Bustillo-Lecompte and Mehrvar, 2015). The facilities and processes impact the character of the wastewater. Meat processing wastewater, for example, has high organic matter content, and high levels of fat, oil and grease as compared to domestic sewage as shown in Table 1. Ammonia levels can also be high.
2.1.2 Treatment of high fat wastewater

Common problems in treating meat-processing wastewater lie in the high five-day biochemical oxygen demand (BOD$_5$), fat and ammonia content. Treatment of high fat content wastewater has been studied to develop low-cost and effective onsite methods for the meat processing industry. Jia et al. (2012) used lab-scale SBR to treat high fat content slaughterhouse wastewater. They found the removals of chemical oxygen demand (COD), NH$_4^+$-N and PO$_4^{3-}$-P were 99%, 85% and 99%, respectively after seven-day pre-fermentation.

Kang et al. (2007) in a lab study treated turkey-processing wastewater in layered sand/gravel bioreactors. They found that 94% of total organic carbon (TOC) and 98% of BOD$_5$ was removed in sand/gravel bioreactors at hydraulic loading rates below 132 L m$^{-2}$

<table>
<thead>
<tr>
<th>Source</th>
<th>BOD$_5$ mg/L</th>
<th>COD mg/L</th>
<th>TSS mg/L</th>
<th>N$-$NH$_4^+$ mg/L</th>
<th>DS mg/L</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poultry slaughterhouse</td>
<td>750-1890</td>
<td>3000-4800</td>
<td>300-950</td>
<td>-</td>
<td>-</td>
<td>Rajakumar et al. (2012)</td>
</tr>
<tr>
<td>Poultry processing</td>
<td>660-6400</td>
<td>-</td>
<td>40-3700</td>
<td>9</td>
<td>-</td>
<td>Rusten et al. (1998)</td>
</tr>
<tr>
<td>Turkey processing</td>
<td>706</td>
<td>1552</td>
<td>281</td>
<td>-</td>
<td>-</td>
<td>Sheldon et al. (1990)</td>
</tr>
<tr>
<td>Dairy processing</td>
<td>-</td>
<td>6000</td>
<td>-</td>
<td>151.3</td>
<td>-</td>
<td>Lu et al. (2016)</td>
</tr>
<tr>
<td>Fish processing</td>
<td>1400-1800</td>
<td>3100-3700</td>
<td>-</td>
<td>268-328</td>
<td>25</td>
<td>Aloui et al. (2009)</td>
</tr>
<tr>
<td>Citrus processing</td>
<td>-</td>
<td>2382-4650</td>
<td>0.8-1.5</td>
<td>0.5-11.6</td>
<td>227-704</td>
<td>Jiménez-Tototzintle et al. (2015)</td>
</tr>
<tr>
<td>Bacon processing</td>
<td>8500-27000</td>
<td>9800-16000</td>
<td>5100-7900</td>
<td>-</td>
<td>730-3400</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>Domestic sewage</td>
<td>155-220</td>
<td>275-360</td>
<td>95-126</td>
<td>20-30</td>
<td>-</td>
<td>Li et al. (2015)</td>
</tr>
</tbody>
</table>
d⁻¹. Guar et al. (2010) looked at the role of the coarse sand and gravel layers in the
treatment of turkey processing wastewater. They found the removal of BOD₅ and COD
slightly increased with addition of a 15 cm pea gravel layer cap above a coarse sand layer.
The addition of a pea gravel cap, improved treatment performance. A 10 cm coarse sand
layer was enough to obtain acceptable removal performance. They also examined the
sand particles in the bioreactors using scanning electron microscope (SEM). They
examined the formation of an extensive biofilm on the sand. This confirmed the
hypothesis that microbes were the main actor degrading the fat. Mancl et al. (2015)
presented the results of a 757,000 L/d sand/gravel bioreactor system to renovate turkey
processing wastewater based on the design presented by Kang et al. (2007). The 1.6 ha.
treatment system has met all 30-day discharge requirements of carbonaceous BOD₅
(CBOD₅) of 10 mg/L, TSS of 12 mg/L, ammonia of 1 mg/L in summer and 3 mg/L in
winter. No fat, oil and grease was ever detected in the effluent.
The curing meat to make bacon, sausage and other processed meat products adds
additional salt to the wastewater. Unpublished data from an Ohio bacon processor
reported average levels of 2200 mg/L total dissolved solids (TDS) in the processing
wastewater during eight operation days in 2014.

2.1.3 Impact of salt on organic matter removal in biological treatment systems
Salt has been shown in studies to inhibit microbes by disrupting the metabolism resulting
from plasmolysis that is a process where cells lose water in a hypertonic solution. Many
aerobic and anaerobic wastewater treatment systems operating under saline conditions
have been inoculated with halophilic bacteria to improve the system performance. Aloui
et al. (2009) studied acclimatized activated sludge using a sequential batch reactor (SBR) to treat fish processing wastewater in a lab study. The system removed over 90% of the COD up to 4% salt concentration and 855 mg L\(^{-1}\) d\(^{-1}\) organic loading rate (OLR). When the salinity was over 6% and OLR above 1140 mg L\(^{-1}\) d\(^{-1}\), the remove efficiencies of COD and BOD\(_5\) decreased below 54%. Uygur and Kargi (2004) treated synthetic wastewater using a lab-scale SBR. A mixed microbial community was inoculated before the start of the treatment. They found that percent COD removal decreased from 96% at 0% salt concentration to 32% at 6% salt concentration. The COD removal rate decreased from 151 mg L\(^{-1}\) h\(^{-1}\) to 122 mg L\(^{-1}\) h\(^{-1}\) when salt concentration increased from 0% to 3%.

Khengaoui et al. (2015) used one layered sand filter consisting of 60 cm dune sand to treat synthetic saline wastewater with salinity from 0.065% to 0.265% in a lab study. The authors found that the COD remove efficiencies decreased from 89% at 0.065% salinity to 83% at 0.265%.

2.1.4 Impact of salt on NH\(_3\)-N removal in biological treatment systems

Although ammonia solutions do not cause problems for human and other mammals, ammonia even at dilute concentrations is highly toxic to aquatic animals. For this reason, all kinds of wastewater should be under ammonia limits if it is discharged to water bodies after treatment. Panswad and Anan (1999) conducted a lab-scale study of anaerobic/anoxic/aerobic systems to treat synthetic wastewater with various salinity levels. Two models were set up, a regular culture model and a NaCl-acclimated culture model. They found that the specific ammonia uptake rate (SAUR) in the regular culture model decreased from 4.76 to 2.14 mg NH\(_3\)-N/g mixed liquor suspended solids (MLSS)-
h when the salt concentration increased from 0% to 3%. The SAUR number of NaCl-acclimated model decreased from 3.84 to 2.71 mg NH$_3$-N/g MLSS-h when the salt concentration increased from 0.5% to 3%. Campos et al. (2002) adopted a lab-scale activated sludge unit to study long-term effects of high nitrogen and salt concentration on specific activity and reactor performance. The synthetic wastewater had an ammonia loading rate between 0.5 and 3.3 g N/L. The nitrifying activity losses of 55% and 30% for non-adapted and adapted microbes, respectively was observed when salt concentration increased from 0% to 1.8%. Uygur and Kargi (2004) also studied the effect of salt concentration on the ammonia removal along with the COD removal. They found that NH$_4^+$-N removal efficiencies decreased from 96% at 0% salt concentration to 39% at 6% salt concentration. When salinity increased from 0% to 6%, NH$_4^+$-N removal rate reduced from 9.33 to 3.92 mg L$^{-1}$ h$^{-1}$.

This paper aims to determine the impact of salt concentration on the treatment of salted meat processing wastewater using sand/gravel bioreactors. In particular: (1) examine the removal of organic matter in the presence of salt; (2) determine the impact on ammonia removal at high salt concentrations; (3) document the movement of salt through a bioreactor system.

2.2 Materials and Methods

2.2.1 Wastewater

An artificial salted meat processing wastewater was prepared by combining turkey slaughterhouse wastewater and added table salt. Turkey slaughterhouse wastewater was used as a control, because its treatment has been extensively studied and the impact of
salt on the treatment process could be more easily determined. The raw wastewater was obtained from a turkey slaughterhouse (Whitewater Processing Co. Harrison, OH) and stored at 4°C before further treatment. The wastewater characteristics varied with the normal plant operation and the range of BOD$_5$, ammonia and total dissolved solids is presented in Table 2. Three wastewaters were tested. The turkey processing wastewater was treated as a control. Salt was added to simulate cured meat processing wastewater. Table salt (mainly sodium chloride, trace anticaking agent calcium silicate) was added to the wastewater at normal and high salt levels of 3 g/L and 6 g/L, respectively.

<table>
<thead>
<tr>
<th>Wastewater characteristic (mg/L)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD$_5$</td>
<td>1348±343</td>
</tr>
<tr>
<td>NH$_4^+$-N</td>
<td>37.96±18.68</td>
</tr>
<tr>
<td>Total dissolved solids</td>
<td>859.4±51.9</td>
</tr>
</tbody>
</table>

Table 2. Characteristics of turkey processing wastewater

2.2.2 Column design

A total of six columns were constructed with acrylic tubing. The columns have an interior diameter of 14.5 cm and a 3 mm wall thickness. All sand and gravel was washed and dried to remove fine particles and dust. The columns were filled with four sand layers, 5 cm of pea gravel, 46 cm of fine sand, 15 cm of coarse sand and 15 cm of pea gravel from bottom to top. Acrylic base caps are fitted to all columns with a hole to drain the effluent, as shown in Figure 1. The effective size and uniformity coefficient (Mancl and Tao, 2011) of pea gravel, coarse sand and fine sand were 4.1 mm and 1.8, 1.4 mm and 2.3 and 0.24
mm and 2.6, respectively. Two columns were used for raw wastewater (control), two for wastewater plus 3g/L salt and two for wastewater plus 6g/L salt.

Prior to applying wastewater, the columns were rinsed with tap water. The volume of tap water held in the column at field capacity was 1517 mL. The hydraulic loading rate was 4 cm/d (707 mL/d) per column divided into four equal doses per day applied six hours apart. Salt was dissolved in the wastewater prior to loading the bioreactors. Columns were operated in the Soil Environmental Technology learning lab at Ohio State University at room temperature (21.3°C±1.4°C).

![Diagram of lab-scale sand bioreactor](image)

Figure 1. Diagram and photo of lab-scale sand bioreactor

### 2.2.3 Chemical Analysis

The wastewater strength was measured as BOD$_5$ using the azide modification of the Winkler titration method (APHA, 1998). Ammonia nitrogen was measured using Hach Salicylate Method. Ammonia kit (Hach Cat. No. 58700-40) was used to test the ammonia concentration in the influent and effluent. The BOD$_5$ and ammonia nitrogen analysis was
performed every two days in the first three weeks. Tests were then every four days for the next two weeks, then finally every week for the remainder of the study.

Wastewater conductivity from all the experimental groups was measured by conductivity meter (EC-19101-00, Cole-Parmer Instrument Co.). To convert specific conductance to salt concentration a standard curve was developed as shown in Equation 1. The conductivity is calculated as follows:

\[
\kappa = 1.7391c + 0.348 
\]  

(1)

where

\( \kappa \) = the conductivity of the solution (mS/cm)

\( c \) = the concentration of the total dissolved solids (g/L)

2.3 Results

Sand/gravel bioreactors were operated in the lab for 74 days. Wastewater BOD\textsubscript{5} and ammonia varied from 780 to 1848 mg/L and 8.75 to 68 mg/L, respectively. Wastewater was applied to the unsaturated sand/gravel bioreactors and discharged in a single pass. Wastewater discharged from the bioreactors within ten minutes after application. The bioreactors rested for six hours before the next wastewater dose.

2.3.1 BOD\textsubscript{5} removal

In all bioreactors BOD\textsubscript{5} removal efficiencies started at over 90% on day 1. Then the removal rates of all groups declined to the lowest points on day seven. Peak removal of 99% was achieved by day 21. The BOD\textsubscript{5} removal remained over 99% for the remainder of the experiment, as shown in Figure 2. The trend of all curves was similar.
2.3.2 NH$_3$-N removal

In all bioreactors NH$_3$-N removal efficiencies decreased gradually over 13 days. The control columns with no added salt dropped to 50% removal by day 13. The lowest NH$_3$-N removal was initially from the columns receiving the highest salt concentration where ammonia removal dropped to 22.7% on day 13. The control columns quickly achieved high NH$_3$-N removal of over 99% after day 21. The highest salt concentration delayed the development of ammonia removal to day 32. However, all three treatments achieved high NH$_3$-N removal remained over 99% for the remainder of the experiment, as shown in Figure 3.
2.3.3 Salt concentration in influent and effluent

Because salt is a conservative compound in this experiment, conductivity was measured in both the influent and effluent. As shown in Figure 4, the salt concentration in sand/gravel bioreactor effluent of the wastewater plus 6 g/L salt group increased over the first six days and maintained at a steady level after six days. This indicates initial wastewater dilution from the clean water retained in the moist sand at field capacity.
2.4 Discussion

Salts are conservative compounds that are not commonly removed in biological treatment processes. After day six, the salt concentration of the influent was similar as that of effluent, therefore the salt did not accumulate within the sand bioreactors. Multiple-effect evaporation (MEE) and membrane technologies (ultrafiltration, reverse osmosis) would be required to remove salt from the effluent. Since the dilution of salt concentration in the first six days was confirmed, BOD$_5$ and NH$_3$-N removals in first six days should be corrected accordingly. Using specific conductance to account for the dilution, the adjusted removal rates appear as a smoother curve as shown in Figures 5 and 6. The actual BOD$_5$ removal rates in day one, day three and day five were much lower than previous calculated. The water retained in the sand at field capacity initially diluted the BOD$_5$. NH$_3$-N removal was also affected.

![Calibrated BOD$_5$ removal](image)

Figure 5. Calibrated BOD$_5$ removal
Comparisons of treatment system performance are presented in Table 3 and Table 4. From BOD$_5$ removal results, treatments of the start-up period were significantly different (P < 0.05) from those of steady period. System performance was not significantly different (P < 0.05) among different salt levels in acclimation and steady periods, respectively.

As to the NH$_3$-N removal efficiencies, both wastewater plus 3 g/L salt and wastewater plus 6 g/L salt were significantly different (P < 0.05) from the control groups at the start-up period, while there was no significant difference (P < 0.05) between them. After acclimation, all bioreactors were not significantly different (P < 0.05) in NH$_3$-N removal.
Table 3. Summary statistics of percentage removal of BODs in start-up, steady and whole periods

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Start-up (Day 1-Day 17)</th>
<th>Steady (Day 21-Day 69)</th>
<th>Whole period (Day 1-Day 69)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Mean ± S.D.</td>
<td>Range</td>
<td>Group Mean ± S.D.</td>
</tr>
<tr>
<td>WW</td>
<td>92.8± 11.3 91.3± 6.5</td>
<td>56.9-100 82.2-100</td>
<td>99.9± 99.7± 0.3</td>
</tr>
<tr>
<td>WW + 3g/L salt</td>
<td>82.3± 30.2 86.8± 11.5</td>
<td>31.0-100 61.2-100</td>
<td>99.9± 99.7± 0.2</td>
</tr>
<tr>
<td>WW + 6g/L salt</td>
<td>87.7± 15.5 85.3± 20.5</td>
<td>44.0-100 22.4-100</td>
<td>99.5± 99.4± 0.6</td>
</tr>
</tbody>
</table>

§ Mean values followed by the same letter were not statistically significant (P < 0.05). WW means wastewater.
Table 4. Summary statistics of percentage removal of NH$_3$-N in start-up, steady and whole periods

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Start-up (Day 1- Day 17)</th>
<th>Steady (Day 21- Day 69)</th>
<th>Whole period (Day 1- Day 69)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Mean</td>
<td>Mean ± S.D.</td>
<td>Group Mean</td>
</tr>
<tr>
<td>WW</td>
<td>78.9a§</td>
<td>74.5± 33.5 83.2± 19.6</td>
<td>50-100 50-100</td>
</tr>
<tr>
<td>WW + 3g/L salt</td>
<td>60.2b</td>
<td>52.2± 25.0 68.1± 19.8</td>
<td>19.8-96.5 34.9-99.9</td>
</tr>
<tr>
<td>WW + 6g/L salt</td>
<td>53.8b</td>
<td>54.5± 18.4 53.1± 28.2</td>
<td>31.8-84.3 10.5-88.4</td>
</tr>
</tbody>
</table>

§ Mean values followed by the same letter were not statistically significant (P < 0.05). WW means wastewater.
High salt concentration suppresses biological treatment. Aloui et al. (2009), Uygur and Kargi (2004) and Khengaoui et al. (2015) all found similar results when treating high salt content wastewater with aerobic treatment systems. Panswad and Anan (1999) and Campos et al. (2002) found that high salt concentration inhibits biological treatment processes, reducing their effectiveness. This study confirmed the inhibition from high salt concentration on the biological treatment. However, the sand/gravel bioreactors were able to develop salt resistance within 32 days.

The microbes treating BOD$_5$ are not the same as those treating NH$_3$-N as the latter are more susceptible to high salt concentration. This can be derived from the differences of the delayed removals between BOD$_5$ and NH$_3$-N. The microbial community treating BOD$_5$ was able to develop salt tolerance in a short period of time, while the microbial community treating NH$_3$-N was slower to adjust to the salt concentration.

Future study should test high salinity levels to determine the limits of sand/gravel bioreactors in treatment of high strength, high salt content wastewater. Also examination of the microbial community in the sand/gravel bioreactors during the experiment may reveal salt tolerant microbes and aid in the understanding of the mechanisms of fixed media treatment processes.

2.5 Conclusion

This study investigated the impact of salt on the treatment of high strength, high salinity meat processing wastewater. BOD$_5$ removal was not largely affected by the salt levels up to 0.6%. Sand/gravel bioreactors achieved over 99% BOD$_5$ removal after 21 days and
maintained over 99% removal for the remainder of the experiment at all salt concentrations.

NH$_3$-N removal was susceptible to salt levels. The lowest NH$_3$-N removal rate was from the sand/gravel bioreactors receiving the highest salt concentration of 6 g/L. Sand/gravel bioreactors showed the development of salt resistance after 32 days and maintained over 99% NH$_3$-N removal at all salt concentrations.

Initially high BOD$_5$ and NH$_3$-N removal by the bioreactors was puzzling. However, at field capacity water in the moist sand created significant dilution of the pollutants. The conservative compound, salt, was used to correct for the impact of dilution at system start-up. The impact of dilution was shown in the first six days until almost three times the equivalent wastewater volume of field capacity was applied to the column. After six days the salt concentration in the effluent was similar to that of the influent. Salt did not accumulate in the sand/gravel bioreactors.
Chapter 3: Biofilms for wastewater treatment in high salt content environments

3.1 Introduction

Biofilms form in a range of natural environments. Biofilms are found on sand in aquatic environments, inside pipes in water systems and even on teeth. The development and function of biofilms is the topic of a range of scientific inquiry.

3.1.1 Biofilm formation

A biofilm is a group of microbial cells irreversibly attached to a surface and embedded within a self-produced matrix of extracellular polymeric substance (EPS). Non-cellular particles such as silt, clay particles or minerals can also be found in the biofilm matrix depending on the environment where the biofilm is developed (Donlan, 2002). Substrate effects, hydrodynamics of the aqueous system, medium characters and the properties of cells needed to be considered before obtaining a clear picture of the process of attachment. Characklis et al. (1990) pointed out that the degree of microbial attachment increases as the surface roughness increases. This is because the shear stress in the fluid decreases when it contacts a rough surface area. Rijnaarts et al. (1993) conducted lab-scale batch and column tests to investigate the adhesion and detachment during the transfer of substrata through air-liquid interface. They found that as the velocity increased, the boundary (interface between substrate and fluid) layer decreased. The cells
would undergo increasing turbulence and mixing. Detachment was more likely to happen on larger cells with less motility. Other factors such as temperature, pH, ionic strength, nutrient levels may also influence the rate of microbial attachment to a substrate. Cowan et al. (1991) used a lab-scale recirculating model system to investigate the effect of nutrient concentration on colonization by different species. They found that with the increase in the nutrient concentration the number of attached cells on the tubing walls. The properties of the cells themselves also make a difference in the process of the attachment. The hydrophobicity of the cell surface and the material surface is critical to attachment because it enhances attachment when both of them have similar properties. Fimbriae, one of the cell surface components, contains of a high percentage of hydrophobic amino acid. It overcomes the barrier of the initial electrostatic repulsion between the substrate and the cell and makes the attachment feasible (Corpe et al., 1980).

3.1.2 Biofilm in high salt environments

High salt concentration has an adverse effect on the metabolisms of microbes, and it is also an important factor to consider in the formation of biofilms. Eduok et al. (2016) conducted a lab-scale study to investigate the morphology and chemistry of adhering bacterial biofilm on stainless steel (316 L grade). They observed a more compacted and denser biofilm in 0.75% (w/v) salt concentration media than the control groups with no added salt. The secretion of EPS from biofilms was confirmed to reduce the corrosion rate of the steel. Lee et al. (2008) investigated the development of the communities of marine biofilms formed on three kinds of artificial surfaces (glass, acryl and steel) submerged in seawater. The microbial community also has an influence on the formation
and the changes of the biofilm over time. They found that the microbial communities developed on three kinds of surfaces were mutually different. A succession was also confirmed by detecting pioneer species followed by enrichment of other groups. A pioneer species has low numbers but grow rapidly.

### 3.1.3 Biofilm research methods

Many techniques have been developed to study complex microbial ecosystem. Scanning electron microscope (SEM) is widely used to study the morphology of the biofilms. Eduok et al. (2016) used SEM to obtain fine micrographs of the biofilms on stainless steel. Hall-Stoodley et al. (2004) used a scanning electron micrograph to illustrate the formation of the biofilm on the distal tip of a cardiac pacemaker lead in a patient. Liu et al. (2003) conducted a lab study to assess the biodegradation of animal fat in media filter systems. They used ATP (adenosine triphosphate) method to measure the biomass of the biofilms in the layered media. Temperature gradient gel electrophoresis (DGGE), 16S rDNA sequencing/16S rRNA clone libraries, terminal restriction fragment length polymorphism (T-RFLP), real-time polymerase chain reaction (RT-PCR) and metagenomics are more capable of providing genetic information of the microbial communities in the biofilm. Lee et al. (2008) analyzed the composition of the microbial communities developed on glass, acryl and steel artificial surfaces in a lab study using both T-RFLP and 16S rRNA clone libraries. Mullany et al. (2008) introduced metagenomics for analyzing dental biofilms especially for bacteria that cannot yet be cultivated in the laboratory.
The goal of this research was to examine the development of biofilms in a media bioreactor system receiving high strength, high salt content wastewater with time. The specific objectives were to (1) track the biofilm development with the onset of wastewater treatment capability, and (2) differentiate the biofilm activity by its position in the treatment unit.

3.2 Material and methods

3.2.1 Wastewater

The raw wastewater was obtained from a turkey slaughterhouse (Whitewater Processing Co. Harrison, OH) and stored at 4°C before further treatment. The wastewater characteristics varied with the normal plant operation and the range of BOD\textsubscript{5}, ammonia and total dissolved solids is presented in Table 2. Two wastewaters were tested. The turkey processing wastewater was treated as a control. Salt was added to simulate cured meat processing wastewater. Table salt was added to the wastewater at high salt levels of 6 g/L.

3.2.2 Column design

A total of four columns were constructed with acrylic tubing. The columns have an interior diameter of 14.5 cm and a 3 mm wall thickness. All sand and gravel was washed and dried to remove fine particles and dust. The columns were filled with four sand layers, 5 cm of pea gravel, 46 cm of fine sand, 15 cm of coarse sand and 15 cm of pea gravel from bottom to top. One hole (2 cm in diameter) was made for sampling 3 cm down from the interface of two layers. Acrylic base caps are fitted to all columns with a
hole to drain the effluent, as shown in Figure 7. The effective size and uniformity coefficient (Mancl and Tao, 2011) of pea gravel, coarse sand and fine sand were 4.1 mm and 1.8, 1.4 mm and 2.3 and 0.24 mm and 2.6, respectively. Two columns were used for raw wastewater (control), and two for wastewater plus 6g/L salt.

Figure 7. Diagram and photo of lab-scale bioreactor

3.2.3 ATP method

ATP is the energy currency in every living cell, thus the biomass in the media bioreactors can be representative by the amount of ATP in collected samples. ATP measurement by bioluminescence is fast and sensitive. When firefly luciferase catalyzes the oxidation of D-luciferin ATP is consumed and light is emitted through the following reactions:

\[
\text{MgATP + Luciferase + Firefly Luciferase } \rightarrow \text{Adenyl-luciferin + PP}_i \quad (2)
\]

\[
\text{Adenyl-luciferin + O}_2 \rightarrow \text{Oxyluciferin + AMP + CO}_2 + \text{light} \quad (3)
\]
The light emitted (560-600 nm) can be measured by Appliskan (Thermo Scientific) and converted to the amount of ATP by a standard curve. The detailed procedures were as follows.

From each hole on the wall of the columns, about 2.5 wet gravel/sand samples were collected in 50 mL centrifuge tubes and stored in -20°C freezer before the final measurement. Gravel/sand samples were collected one week, two weeks, three weeks, four weeks and 25 weeks after the system operation started. All glassware used in the experiment was washed by 0.1 M HCl, rinsed by deionized water and dried before the usage.

Preparation of the standard curve: Sigma-Aldrich ATP Bioluminescent Assay Kit (FL-AA) was used for this experiment. The ATP Assay Mix solution, ATP AssayMix Dilution Buffer and ATP Standard solution were prepared with sterile water according to the instruction. The ATP Standard solution was diluted to 10⁻⁶, 10⁻⁷, 10⁻⁸, 10⁻⁹, 10⁻¹⁰ M. 25-fold Assay Mix solution was prepared to detect ATP concentration in the range of 2 × 10⁻¹⁰ to 2 × 10⁻⁷ M. The series of ATP solutions were measured in Appliskan. The standard curve drawn in the experiment was as follows:

\[
\log_{10}^{\text{RLU}} = 0.8075\log_{10}^{\text{C}} + 9.2833
\]  

(4)

where

\[\text{RLU} = \text{relative light units}\]

\[\text{C} = \text{ATP concentration (mole/L)}\]

Preparation of the samples: 25 mL 10-fold TCA-PO₄ extractant (81.6 g trichloroacetic acid, 89.6 g Na₂HPO₄•12 H₂O, per liter) was added to each sample. All samples were mixed on a vortexer for 1 min at 2000 rpm, then centrifuged at 4°C, 8000 rpm for 5 min.
Each 1 mL sample was transferred from centrifuge tubes into 1.5 mL Eppendorf tubes and kept on ice before they were measured in a 96 well plate by Appliskan.

3.3 Results
Gravel/sand samples were collected carefully without disturbing the layers. Samples were immediately stored in a -20°C freezer after they were weighted. All the samples were analyzed once and the average ATP content was listed in Table 5. The average ATP content varied from 128.9 to 986.2 pg/g wet sand in control group, or 21.9 to 130.9 pg/cm² sand surface area. The amount of ATP varied from 151.8 to 684.0 pg/g wet sand in wastewater plus 6 g/L salt, or 15.3 to 90.8 pg/cm² sand surface area. Since biofilms form on the surface of the sand, the results calculated by sand surface area is more meaningful than sand weight. The following figures and discussions are all based on the results calculated by the sand surface area. For control groups, the ATP content in pea gravel layer was significantly different (p < 0.05) from that in coarse sand layers. However, there was no significant difference between pea gravel layer and fine sand layer. In experimental groups, the findings were similar. There were no significant differences in the same layer between wastewater control and wastewater plus 6 g/L salt.
Table 5. Comparison of average ATP content

<table>
<thead>
<tr>
<th>Layer</th>
<th>Wastewater control</th>
<th>Wastewater plus 6 g/L salt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP (pg/g sand)</td>
<td>ATP (pg/cm² sand)</td>
</tr>
<tr>
<td>Pea gravel</td>
<td>128.9±65.4a§</td>
<td>39.7±20.2c</td>
</tr>
<tr>
<td>Coarse sand</td>
<td>986.2±424.8b</td>
<td>130.9±56.4d</td>
</tr>
<tr>
<td>Fine sand</td>
<td>949.4±565.1b</td>
<td>21.9±13.0c</td>
</tr>
</tbody>
</table>

§ Mean values followed by the same letter were not statistically significant (P < 0.05).

The changes of the amount of ATP in the different media layers over time is shown in Figure 8 and Figure 9 for wastewater control and wastewater plus 6 g/L salt, respectively. During the first three weeks the ATP content declined then increased before achieving a steady level after 25 weeks. In both of the control and experimental groups, a minimum amount of ATP was measured at week 3. The ATP content in the coarse sand layer was significantly higher than other two layers.

![Figure 8](image_url)  
**Figure 8.** Changes of ATP content over time in wastewater control
Figure 9. Changes of ATP content over time in wastewater plus 6 g/L salt

Comparison of wastewater control and wastewater plus 6 g/L salt in gravel layer is shown in Figure 10. The ATP content in wastewater control and wastewater plus 6 g/L salt decreased in the first three weeks and then increased until a steady level was achieved at week 25.

Figure 10. ATP content in gravel layer

Comparison of wastewater control and wastewater plus 6 g/L salt in coarse sand layer is shown in Figure 11. The ATP content in the wastewater control is much higher than that
in the wastewater plus 6 g/L salt in the first month. After 25 weeks, the ATP content achieved a steady level.

![ATP content in coarse sand layer](image)

Figure 11. ATP content in coarse sand layer

Comparison of wastewater control and wastewater plus 6 g/L salt in fine sand layer is shown in Figure 12. The ATP content of wastewater control decreased from week 2 to week 4, while the ATP content in wastewater plus 6 g/L salt remained at a constant level. After 25 weeks, the ATP content achieved a steady level.
Comparison of ATP content in wastewater plus 6 g/L salt and system performance of removing NH$_3$-N is shown in Figure 13. The NH$_3$-N removal decreased in the first 9 days and then increased to the maximum at week 4. After four weeks, the removal was maintained over 99%. While the ATP content decreased in the first three weeks and then increased until it achieved a steady level after 25 weeks.
Figure 13. Comparison of ATP content and system performance for wastewater plus 6 g/L salt

3.4 Discussion

Wet weight of sand was used in the experiment to avoid ATP decay in the drying process. There is a difference in the magnitude of the result from this study and the study from Liu et al. (2003). This may be because the luminometer used in these two studies are different. The Monolight 2020 can measure the light emission without an initial delay, while the Appliskan has a 25-30 seconds initial delay. The wastewater used in the two studies were also quite different.

The ATP content biologically represents for the biomass of the living cells in the gravel/sand bioreactors. It does not necessarily mean the active microbes treating the wastewater. Giving the fact that all the microbes survived in this environment need carbon and nitrogen sources provided by the wastewater, the living cells can roughly be considered all active in treating the wastewater.
The ATP content decreased in the first weeks in almost all the groups, and then increased to a steady level. This could reflect a shift from the pioneering species to the follow up species proposed in the Lee et al. (2008) theory. The decline of the ATP may represent the decrease of the pioneering species in sand/gravel filter systems. The population of subsequent enrichment microbes accounts for the increase of ATP content in the fourth week. Alternatively, the decline could be due to the salt inhibition on the microbes in the systems. While reasonable for the wastewater plus 6 g/L salt, is not a sufficient explanation for the wastewater control, which also decreased in the first few weeks.

In the comparison of the ATP content and the system performance, it was observed that the changes of the system performance was ahead of those of the ATP content. This may be because the microbes treating NH$_3$-N were already effective at low biomass before propagating in the system.

The coarse sand layer can be considered the most effective layer in treating the wastewater in this study because the ATP content in this layer is significantly higher and more than double than other two layers. This finding also corresponds to the finding in Liu et al. (2003). From the comparison of Figure 8 and Figure 9, the salt has an initial inhibition effect on the treatment performance but after a long period of acclimation there is no significant difference between wastewater control and wastewater with added salt. This also correspond the finding in Chapter 2, the same system reached its lowest performance between week 2 and week 3 and was able to develop salt resistance after 32 days.

Further study should focus on the microbial communities in the gravel/sand bioreactors, especially the coarse sand layer. It is where the most biological treatment appears to take
place. The information of microbial communities at different periods may also explain the changes of the system performance.

3.5 Conclusion

This study investigated the biofilm development in the gravel/sand bioreactor systems receiving high strength, high salinity wastewater. The biomass content decreased in the first three weeks, and then increased until a steady point was reached after 25 weeks. With the findings in Chapter 2, the relationship between the biomass in the system and the performance of the system is well related. The system performance decreased with the decline of the biomass in the first three weeks. After the acclimation period, the system maintained at high performance and biomass was at a steady level. The largest amount of biomass is in the coarse sand layer throughout the study. This indicates that the main part of the sand/gravel media filter system treating the wastewater is the coarse sand layer. The pore size is suitable for aeration and much sand surface area for the microbes to occupy.
Chapter 4: Overall conclusions

This study evaluated the performance of the gravel/sand media bioreactors in treating high strength, high salinity meat processing wastewater. The main objectives are (1) examine the removal of organic matter in the presence of salt; (2) determine the impact on ammonia removal at high salt concentrations; (3) document the movement of salt through a bioreactor system; (4) track the biofilm development with the onset of wastewater treatment capability; and (5) differentiate the biofilm activity by its position in the treatment unit.

BOD$_5$ removal was not largely affected by the salt levels up to 0.6%. Sand/gravel bioreactors achieved over 99% BOD$_5$ removal after 21 days and maintained over 99% removal for the remainder of the experiment at all salt concentrations.

NH$_3$-N removal was susceptible to salt levels. The lowest NH$_3$-N removal rate was from the sand/gravel bioreactors receiving the highest salt concentration of 6 g/L. Sand/gravel bioreactors showed the development of salt resistance after 32 days and maintained over 99% NH$_3$-N removal at all salt concentrations.

Initially high BOD$_5$ and NH$_3$-N removal by the bioreactors was puzzling. However, at field capacity water in the moist sand created significant dilution of the pollutants. The conservative compound, salt, was used to correct for the impact of dilution at system start-up. The impact of dilution was shown in the first six days until almost three times
the equivalent wastewater volume of field capacity was applied to the column. After six days the salt concentration in the effluent was similar to that of the influent. Salt did not accumulate in the sand/gravel bioreactors.

The biomass content decreased in the first three weeks, and then increased until a steady point was reached after 25 weeks. With the findings in Chapter 2, the relationship between the biomass in the system and the performance of the system is well related. The system performance decreased with the decline of the biomass in the first three weeks. After the acclimation period, the system maintained at high performance and biomass was at a steady level.

The largest amount of biomass is in the coarse sand layer throughout the study. This indicates that the main part of the sand/gravel media filter system treating the wastewater is the coarse sand layer. The pore size is suitable for aeration and much sand surface area for the microbes to occupy.
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