Treatment Process for Removal of Naphthenic Acid from Oil Refinery Wastewater

Martin W. Flatley  
*The University of Western Ontario*

Supervisor  
Argyrios Margaritis  
*The University of Western Ontario*

Graduate Program in Chemical and Biochemical Engineering  
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Abstract

The impetus for this doctoral research project was random toxicity failures in 2010 that occurred during routine 96 hour toxicity tests using rainbow trout (Oncorhynchus mykiss) fingerlings and Daphnia magna; prior to 2010 the toxicity tests had not failed. Results of the toxicity tests showed that the trout were affected while the Daphnia magna were not. Toxicity identification evaluation (TIE) procedures indicated the source of the toxicity was due to naturally occurring weak carboxylic acids which consisted of fatty, naphthenic and aromatic acids with naphthenic acid comprising the highest percentage (> 90 %). During the TIE testing a novel modification of shortening the time of the toxicity test by lowering the pH of the water to pH 6 versus pH 7.2 based on the weak carboxylic acids becoming associated making it easier for the acid to enter the cytoplasm of the fish cells. The lethal concentration of naphthenic acid in water with a pH of 7 that resulted in a 50 % mortality (LC$_{50}$) of the fish during a 96 hour tests was found to be is 2.1 mg·L$^{-1}$ versus 0.4 mg·L$^{-1}$ in less than 24 hours when the water is adjusted to pH 6. Histopathology analysis was performed on the trout to understand the organs effected to help explain the clinical signs of acute toxicity which included dark skin colour, bloated stomach, total loss of equilibrium (TLOE) and mortality. The project then focused on determining if there were any co-factors effecting the toxicity defining the source of naphthenic acid, performing mass balance tests to find the transport mechanism, and treatment of naphthenic acid in a refinery wastewater system prior to the water being discharged into public waterways in this case the St. Clair river. The cofactor testing indicated that wastewater treatment system did not include co-factors and that measured naphthenic acid concentration would predict toxicity. The source tests lead to the development of laboratory extraction of the acids extracted from high total acid number (TAN) crude oil from Athabasca tar sands to use for speciation, characterization and detailed toxicity testing. The testing indicated that the use of a TAN number can be used to determine the acid purity of the purified fractions. The mass balance tests indicated a number of findings which included that the transport mechanism was due to solids and solids stabilized emulsions from the desalting operation that coagulation and flocculation in reduced the concentration of naphthenic acid by a factor of four at the effluent of the wastewater plant. They also showed that the residence time of 8 hours in wastewater treatment was not enough time for the bacteria in wastewater to biodegrade naphthenic acid in view of this a new physical chemical method was used to separate the naphthenic complex emulsion formed by solids, asphaltenes, surfactants by 90 % which is a significant reduction. In view of this original research finding a patent has been submitted to the Canadian Patent office "Processing of Aqueous Waste Streams to Remove Naphthenic Acids" in October 2015.
Acknowledgments

Although it is customary to first thank those who have helped with the academic and technical portions of the dissertation, in this case I will break with tradition to thank my wife Brenda of 32 years and my children Marty and Melanie. For a number of years they have put up with late nights, missed appointments, listening to details about lab experiments and other interruptions to their lives so I could pursue my Doctoral degree in Electrical Engineering and then in Biochemical Engineering. I owe them a great debt.

I would like to thank my supervisor, Professor Argyrios Margaritis, for his enthusiastic support, guidance, and help throughout and my Ph.D. project including proofreading this thesis. Professor Argyrios Margaritis has inspired me by introducing me to the new discipline of biochemical engineering and to go further than I believed I could have gone.

The research involved in this thesis required information for a wide range of disciplines, specialized analysis equipment, and specialized software therefore in many cases consultation was required. I would like to thank external laboratories that helped with specialized analysis including Jamie Cook at Investigative Science in Burlington Ontario with naphthenic acid analysis development, Bob Adamski at Shell Global Solutions in Houston, Clay Ferguson at Pollutech in Sarnia for toxicity testing, Dr Gary Marty at The Department of Fisheries in British Columbia for the assistance with histopathology analysis of fish, Cameron in Houston Texas for their assistance in computational fluid dynamic simulation of a desalter used to process high TAN crude, Alberta Innovates laboratory in Vegeville Alberta for the Orbitrap analysis of purified naphthenic acid fractions and Dr. Greg Gloor at for his assistance with the 16S rRNA analysis.

I am indebted for the help of Jennifer Southcombe, Dr. Terry Ashe and Dr. Stan Kucerovsky, who graciously proofread the thesis during its evolution, their questions and suggestions led to a much improved thesis. In addition, Terry assisted in the Suncor Sarnia Laboratory when I was occupied with my ‘day job’ inside the refinery and added many analytical insights based on his 35 years experience in mass spectroscopy methods. Any missing commas or lingering questions are my responsibility alone.

Lastly, and certainly not least, I would like to thank the management at Suncor Energy Limited in particular Mark Hiseler VP Refining, Pieter Vermeulen Director of Engineering and Marc Mageau VP Supply Chain who have allowed me the chance to pursue both an academic and professional career.
# Contents

**Certificate of Examination** ................................................. i  
**Abstract** ........................................................................ ii  
**Acknowledgments** ........................................................... iii  
**List of Tables** .................................................................. x  
**List of Figures** ................................................................ xi  
**Glossary** ........................................................................... xv  
**Acronyms** .......................................................................... xviii  
**Notation** ............................................................................ xxi  

## 1 Introduction  
1.1 Research Objectives ......................................................... 1  
1.2 Background ...................................................................... 3  
1.2.1 Naphthenic Acid Analysis History ................................. 6  
1.2.2 Naphthenic Acid Structures .......................................... 8  
1.3 Naphthenic Acid in Oil Refineries ..................................... 10  
1.4 Naphthenic Acid Toxicity .................................................. 10  
1.5 Crude Oil Processing and Interaction with Wastewater Treatment .......................................................... 11  
1.5.1 Crude Oil Blending ....................................................... 13  
1.5.2 Crude Oil Desalting ...................................................... 13  
1.5.3 Wastewater Treatment ............................................... 13  
1.6 Naphthenic Acid Corrosion .............................................. 14  

## 2 Literature Review  
2.1 Naphthenic Acid Toxicity .................................................. 16  
2.2 Desalts ........................................................................... 18
2.3 Emulsions and Crude Contaminantes........................................... 18
2.4 Wastewater Treatment................................................................. 19
2.5 Naphthenic Acid Analysis............................................................. 20
2.6 Methods to Control Naphthenic Acid.............................................. 21

3 Wastewater Toxicity Identification.................................................... 22
3.1 Toxicity Identification Evaluation................................................... 22
3.2 Clinical Signs of the Toxicant on Rainbow Trout Fingerlings............... 24
3.3 Toxicity Identification Evaluation Findings...................................... 24
3.3.1 Baseline Toxicity Test.............................................................. 24
3.3.2 Graduated pH Test................................................................. 25
3.3.3 Zeolite Filtration................................................................. 27
3.3.4 Aeration Test................................................................. 27
3.3.5 Filtration Test................................................................. 27
3.3.6 Filtration and $C_{18}$ Solid Phase Extraction Test............................ 28
3.3.7 Filtration, $C_{18}$ Solid Phase Extraction and Methanol Extraction Test...... 28
3.3.8 Methanol Fractionation............................................................ 29
3.3.9 Salt Addition Test............................................................... 30
3.3.10 Carbon Filtration.............................................................. 30
3.3.11 Flocculant Testing.......................................................... 31
3.3.12 Rainbow Trout Recovery....................................................... 32
3.3.13 Summary of TIE Tests.......................................................... 32
3.4 Determination of Naphthenic Acid as the Toxicant............................ 34
3.4.1 Clarifier Effluent............................................................... 36
3.5 Extracted Naphthenic Acid Toxicity............................................... 37
3.6 Acute Toxicological Effects of Naphthenic Acid.................................... 39
3.6.1 Fish Collection................................................................. 40
3.6.2 Sample Preparation.......................................................... 41
3.7 Analysis of TIE Treatments and Fish Exposures to Naphthenic Acid........... 42
3.8 Histopathology Summary............................................................ 49
3.9 Weak Acid Transport Across a Cell Lipid Bilayer.................................. 49
3.10 Microtox................................................................................. 52

4 Wastewater Treatment Analysis............................................................ 55
4.1 Wastewater Treatment Description................................................... 55
4.2 Naphthenic Acid Sources.............................................................. 59
4.3 Naphthenic Acid Mass Balance in an Oil Refinery.................................... 59
4.3.1 First Naphthenic Acid Mass Balance Test..................................... 60
4.3.2 Second Naphthenic Acid Mass Balance Test ........................................ 61
4.3.3 Third Naphthenic Acid Mass Balance Test ........................................ 62
4.3.4 Mass Balance Summary ..................................................................... 63
4.4 Naphthenic Acid Transport Mechanism in a Refinery ............................... 64
4.5 Solids Wetting Agent Addition to Desalter Feed ..................................... 64
4.6 Desalter Brine Treatment ....................................................................... 65
4.7 Naphthenic Acid Toxicity and Biodegradability in an Oil Refinery Wastewater Treatment System ............................................................. 68
4.7.1 Bioreactor Tests .................................................................................. 69
4.8 16S rRNA Genetic Analysis of a Microbial Community in an Oil Refinery Wastewater System ................................................................. 70
4.8.1 Samples .............................................................................................. 72
4.8.2 Steps Used to Analyze 16S rRNA .......................................................... 73
4.8.3 Viable Bacteria Count ......................................................................... 75
4.8.4 DNA Isolation and Extraction ............................................................... 75
4.8.5 16S rRNA Amplification and Sequencing ............................................. 75
4.8.6 Bacteria Taxonomy ............................................................................... 76
4.8.7 Analysis of the 16S rRNA Data .............................................................. 76
5 Desalting of Crude Oil and Emulsions .......................................................... 82
5.1 Desalting .................................................................................................. 82
5.2 Desalter Operation Fundamentals ............................................................. 83
5.3 Desalter Layout ........................................................................................ 84
5.4 Computational Fluid Dynamic Analysis of a Desalter ............................... 86
5.4.1 Process Flow Description ..................................................................... 86
5.4.2 CFD Simulation .................................................................................... 87
5.4.3 Flow Pattern Analysis .......................................................................... 89
5.5 Desalter Process Control .......................................................................... 89
5.6 Desalter Chemical Addition .................................................................... 90
5.7 Issues With Desalting ................................................................................ 92
5.7.1 Viscosity .............................................................................................. 92
5.7.2 Solubility of Water in Crude Oil ............................................................ 92
5.7.3 Internal Electrical Components ............................................................ 93
5.7.4 Oil Conductivity ................................................................................... 93
5.7.5 Solid Contaminants in Crude Feed ...................................................... 94
5.7.6 Differential Density ............................................................................. 94
5.8 Emulsions ................................................................................................ 94
5.8.1 Thermodynamics of Emulsions ............................................................ 95
5.8.2 Solids .................................................................................................. 101
5.8.3 Asphaltenes ......................................................................................... 106
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8.4</td>
<td>Emulsion Separation by Centrifugation</td>
<td>108</td>
</tr>
<tr>
<td>5.9</td>
<td>Water-Oil Partitioning of Naphthenic Acid</td>
<td>108</td>
</tr>
<tr>
<td>5.10</td>
<td>Naphthenic Acid in Desalter Brine</td>
<td>110</td>
</tr>
<tr>
<td>6</td>
<td>Naphthenic Acid Analysis</td>
<td>112</td>
</tr>
<tr>
<td>6.1</td>
<td>Extraction and Separation of Acid Species from Crude Oil for Speciation</td>
<td>112</td>
</tr>
<tr>
<td>6.1.1</td>
<td>Extraction of Naphthenic Acids from Crude Oil</td>
<td>113</td>
</tr>
<tr>
<td>6.1.2</td>
<td>Separation of Extract by Column Chromatography</td>
<td>115</td>
</tr>
<tr>
<td>6.1.3</td>
<td>Collected Acid Fractions</td>
<td>115</td>
</tr>
<tr>
<td>6.1.4</td>
<td>Naphthenic Acid Extraction from Aqueous Samples</td>
<td>118</td>
</tr>
<tr>
<td>6.2</td>
<td>Analytical Test Results</td>
<td>120</td>
</tr>
<tr>
<td>6.2.1</td>
<td>FTIR/ATR</td>
<td>120</td>
</tr>
<tr>
<td>6.2.2</td>
<td>GC/MS</td>
<td>123</td>
</tr>
<tr>
<td>6.2.3</td>
<td>LC/QTOF/MS</td>
<td>129</td>
</tr>
<tr>
<td>6.2.4</td>
<td>Orbitrap&lt;sup&gt;®&lt;/sup&gt;</td>
<td>133</td>
</tr>
<tr>
<td>7</td>
<td>Conclusions and Recommendations</td>
<td>136</td>
</tr>
<tr>
<td>7.1</td>
<td>Conclusions</td>
<td>136</td>
</tr>
<tr>
<td>7.1.1</td>
<td>Transport</td>
<td>136</td>
</tr>
<tr>
<td>7.1.2</td>
<td>Histopathology</td>
<td>137</td>
</tr>
<tr>
<td>7.1.3</td>
<td>TIE</td>
<td>137</td>
</tr>
<tr>
<td>7.1.4</td>
<td>Acute Toxicity Tests</td>
<td>139</td>
</tr>
<tr>
<td>7.1.5</td>
<td>Naphthenic Acid Extraction</td>
<td>139</td>
</tr>
<tr>
<td>7.2</td>
<td>Recommendations</td>
<td>140</td>
</tr>
<tr>
<td>7.2.1</td>
<td>Collaboration with Government and Industrial Peers to Develop Naphthenic Acid Analysis Method</td>
<td>140</td>
</tr>
<tr>
<td>7.2.2</td>
<td>Purify Extracted Acid Samples from High TAN Crude Using Preparative HPLC</td>
<td>140</td>
</tr>
<tr>
<td>7.2.3</td>
<td>Biotreater for Solids Extracted from Coagulant and Flocculant Treatment</td>
<td>141</td>
</tr>
<tr>
<td>Bibliography</td>
<td></td>
<td>142</td>
</tr>
<tr>
<td>Appendices</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A Patent Application</td>
<td></td>
<td>159</td>
</tr>
<tr>
<td>B 16S rRNA Procedure</td>
<td></td>
<td>187</td>
</tr>
</tbody>
</table>
G.2.2 Bacteria Taxonomy Identified in Aeration Basin 1
Bottom Layer ........................................................................................................... 253

G.2.3 Bacteria Taxonomy Identified in Aeration Basin 2
Top Layer ...................................................................................................................... 255

G.2.4 Bacteria Taxonomy Identified in Aeration Basin 2
Bottom Layer .................................................................................................................. 257

G.2.5 Broth used for Petri Dish Samples of Top Layer of Aeration Basin 1 .................. 259

G.2.6 Bacteria Taxonomy Identified in a Petri Dish Colonies from Aeration Basin 1 Top Layer ................................................................. 261

G.2.7 Bacteria Taxonomy Identified in Recycle Sludge from Clarifier 2 ....................... 265

G.2.8 Bacteria Identified in Recycle Sludge from Clarifier 3 Water Effluent .................. 267

G.2.9 Bacteria Taxonomy Identified in the Recycle Sludge from Clarifier 3 .................. 269

G.2.10 Bacteria Taxonomy Identified in Combined Recycle Sludge from Clarifier 2 and 3 ........................................................................................................ 271

G.2.11 Bacteria Taxonomy Identified in Hydrocell® Effluent ........................................ 273

H Test Equipment Specifications ...................................................................................... 275

H.1 FTIR/ATR .................................................................................................................... 275
H.2 GC/MSD ..................................................................................................................... 275
H.3 ICP/OES ....................................................................................................................... 276
H.4 LC/QTOF/MS ............................................................................................................ 276
H.5 SEM/EDX ..................................................................................................................... 276
H.6 XRD ............................................................................................................................. 277
H.7 XRF ............................................................................................................................. 277

I Naphthenic Acid GC/MS Control Parameters ................................................................. 278

J Naphthenic Acid GC/MS Macro .................................................................................... 284

K Naphthenic Acid LC/QTOFMS Control Parameters ...................................................... 310

Index .................................................................................................................................. 312

Vita ................................................................................................................................... 312
List of Tables

2.1 Naphthenic acid properties .................................................. 20

3.1 Fraction volume and toxicity dependence on pH .......................... 30
3.2 Effect of carbon filtering ......................................................... 31
3.3 Summary of trout toxicity responses to TIE treatments .................. 33
3.4 Lesion semi-quantitative scores ................................................ 42
3.5 Histopathology group 1 trout ................................................... 43
3.6 Histopathology group 2 trout ................................................... 43
3.7 Histopathology group 3 trout ................................................... 44
3.8 Lesions only occurring in exposed fish ....................................... 45
3.9 Lesions more common or severe in exposed fish ......................... 45
3.10 Lesions not associated with exposure ...................................... 46
3.11 Lesion scores in fish exposed to clarifier effluent versus dechlorinated water .................................................. 46
3.12 Lesion scores in fish exposed to clarifier effluent with TIE treatments .................................................. 47
3.13 Lesion scores in fish exposed to fractions of naphthenic acid ....... 48

4.1 Mass balance of naphthenic acid test 1 ..................................... 61
4.2 Mass balance of naphthenic acid test 2 ..................................... 62
4.3 Mass balance of naphthenic acid test 3 ..................................... 63
4.4 Naphthenic acid concentration reduction after desalter brine treatment .................................................. 67
4.5 Solid concentration versus size in a sample of desalter brine ............ 68
4.6 OUR reduction due to naphthenic acid introduction to biomass .......... 69
4.7 Sample locations and names for 16S rRNA analysis ...................... 73
4.8 Summary of taxonomy of bacteria identified .................................. 76
4.9 Phylum of bacteria identified .................................................... 77

5.1 Water flow conditions and fluid properties for CFD study ............ 88
5.2 Size distribution analysis ......................................................... 103
List of Figures

1.1 Research project flow diagram ........................................ 4
1.2 Examples of $z$ number definition .................................... 7
1.3 Examples of naphthenic acid structures .............................. 9
1.4 Naphthenic acid with an aromatic and ringed structures ........... 10
1.5 Process flow and instrument diagram in a petroleum refinery ... 12

3.1 The effect of pH on the concentrations of a weak acid on organisms ................................................................. 26
3.2 Naphthenic acid species in the low TAN desalter brine outlet. ..... 35
3.3 Naphthenic acid species in the high TAN desalter brine outlet. ..... 36
3.4 Acute toxicity of wastewater treatment effluent ..................... 37
3.5 Acute toxicity of trout fingerlings when exposed to extracted naphthenic acid fractions at pH 6 ............................. 38
3.6 Acute toxicity of trout fingerlings when exposed to extracted naphthenic acid fractions at pH 6 ............................. 39
3.7 log $K_{OW}$ values for naphthenic acids with $z = -2$ to -12 and C$_0$ to C$_{24}$ . 40
3.8 Acid pKa versus disassociation percent ................................. 50
3.9 Weak organic acid transport across cell membrane .................. 51
3.10 Typical Microtox responses to toxins ................................. 53
3.11 Microtox results with fish toxicity at pH 6 ........................... 54

4.1 Naphthenic acid mass balance sample locations .................... 56
4.2 Clarifier flocculation ....................................................... 58
4.3 Naphthenic acid concentration in clarifier effluent .................. 66
4.4 Cross section of a rod bacteria ........................................... 71
4.5 Ribosomal large and small subunit ...................................... 72
4.6 Location of bacteria samples during WWT steady state operation ... 74
4.7 Scree plot of principal components of bacteria analysis ........... 78
4.8 Biplot of the PCA data from genetic analysis ....................... 79
4.9 Heat plot 16S rRNA analysis of the WWT samples ................ 80
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>Cross section view of aPETRECO™ BILECTRIC™ desalter</td>
<td>84</td>
</tr>
<tr>
<td>5.2</td>
<td>Emulsion at different layers of a desalter under upset conditions</td>
<td>85</td>
</tr>
<tr>
<td>5.3</td>
<td>Cross section of a desalter</td>
<td>87</td>
</tr>
<tr>
<td>5.4</td>
<td>Cut-away view of a desalter</td>
<td>88</td>
</tr>
<tr>
<td>5.5</td>
<td>Stream line of water particles in the desalter</td>
<td>90</td>
</tr>
<tr>
<td>5.6</td>
<td>Desalter water flow path lines</td>
<td>91</td>
</tr>
<tr>
<td>5.7</td>
<td>Visualization of a stable emulsion</td>
<td>96</td>
</tr>
<tr>
<td>5.8</td>
<td>Micropipette deflation of an emulsion showing a rigid film</td>
<td>97</td>
</tr>
<tr>
<td>5.9</td>
<td>Emulsion in the desalter</td>
<td>98</td>
</tr>
<tr>
<td>5.10</td>
<td>Photomicrographs of emulsion</td>
<td>99</td>
</tr>
<tr>
<td>5.11</td>
<td>Typical emulsions found in desalters</td>
<td>100</td>
</tr>
<tr>
<td>5.12</td>
<td>Typical size distribution of emulsions in a desalter</td>
<td>101</td>
</tr>
<tr>
<td>5.13</td>
<td>SEM/EDX analysis of solids in crude</td>
<td>102</td>
</tr>
<tr>
<td>5.14</td>
<td>Box plot of crude solid sizes</td>
<td>103</td>
</tr>
<tr>
<td>5.15</td>
<td>Images from SEM analysis of particle size</td>
<td>104</td>
</tr>
<tr>
<td>5.16</td>
<td>ICP/OES analysis of solids in acid extraction</td>
<td>105</td>
</tr>
<tr>
<td>5.17</td>
<td>Suggested structure of an asphaltene molecule</td>
<td>107</td>
</tr>
<tr>
<td>5.18</td>
<td>Image of asphaltenes from an emulsion</td>
<td>107</td>
</tr>
<tr>
<td>5.19</td>
<td>Emulsion after centrifuging</td>
<td>108</td>
</tr>
<tr>
<td>5.20</td>
<td>Calculated naphthenic acid concentration in brine versus pH</td>
<td>111</td>
</tr>
<tr>
<td>6.1</td>
<td>Acid extraction from crude oil</td>
<td>114</td>
</tr>
<tr>
<td>6.2</td>
<td>Naphthenic acid extract after the dichloromethane shown in Figure 6.1(d) has been evaporated.</td>
<td>114</td>
</tr>
<tr>
<td>6.3</td>
<td>Images of the extracted acid purification steps.</td>
<td>116</td>
</tr>
<tr>
<td>6.4</td>
<td>TLC plate of naphthenic acid extract prior to separation</td>
<td>116</td>
</tr>
<tr>
<td>6.5</td>
<td>Naphthenic acid from desalter brine samples</td>
<td>120</td>
</tr>
<tr>
<td>6.6</td>
<td>FTIR comparison of seven separated acid naphthenic acid fractions</td>
<td>121</td>
</tr>
<tr>
<td>6.7</td>
<td>FTIR spectra of four extracted acid fractions</td>
<td>122</td>
</tr>
<tr>
<td>6.9</td>
<td>Relative intensities of the specific ions due to the derivatizing agent</td>
<td>124</td>
</tr>
<tr>
<td>6.10</td>
<td>Derivatizing agent mass fragments</td>
<td>125</td>
</tr>
<tr>
<td>6.11</td>
<td>Homologous series of extracted naphthenic acid</td>
<td>126</td>
</tr>
<tr>
<td>6.12</td>
<td>GC/MS analysis of extracted naphthenic acid samples</td>
<td>127</td>
</tr>
<tr>
<td>6.13</td>
<td>GC/MS speciation of acid in extracted acid fractions</td>
<td>128</td>
</tr>
<tr>
<td>6.14</td>
<td>Typical GC/MS speciation of organic acids from aqueous wastewater</td>
<td>129</td>
</tr>
<tr>
<td>6.15</td>
<td>Typical LC/QTOF/MS speciation of naphthenic acid</td>
<td>131</td>
</tr>
<tr>
<td>6.16</td>
<td>LC/QTOF/MS speciation of naphthenic acid of clarifier outlet sample</td>
<td>132</td>
</tr>
<tr>
<td>6.17</td>
<td>Orbitrap® analysis of the Hydrocell® outlet sample</td>
<td>133</td>
</tr>
<tr>
<td>6.18</td>
<td>Orbitrap® analysis of extracted acid fractions 1 and 2</td>
<td>134</td>
</tr>
</tbody>
</table>
Glossary

**16S rRNA** is a gene which is a section of prokaryotic (bacteria) DNA found in all bacteria.

**activated sludge** refers to a mass of microorganisms cultivated in the treatment process to break down organic matter into carbon dioxide, water, and other inorganic compounds plural.

**acute** means happening within a short period of time; in the thesis it is meant as mortality occurring with trout fingerlings within $\leq 96$ hours.

**adhesive** forces are the attractive forces between unlike molecules. They are caused by forces acting between two substances, such as mechanical and electrostatic forces.

**atrophy** degeneration of cells which can cause the associated organ to waste away.

**biplot** displays information on both samples and variables of a data matrix graphically. Samples are displayed as points while variables are displayed as vectors.

**caudal** of, at, or near the tail or the posterior end of the body.

**cohesive** forces are the attractive forces exist between molecules of the same substance which cause a tendency in liquids to resist separation. They are caused by intermolecular forces such as hydrogen bonding and Van der Waals forces plural.

**cytoplasm** is the thick solution that fills a cell and is enclosed by the cell membrane. It is mainly composed of water, salts, and proteins plural.

**dendrogram** is a branching diagram that represents the relationships of similarity among a group of entities plural.

**diagenesis** located far from a point of reference, such as an origin or a point of change of sediments or existing sedimentary rock into different sedimentary rock at temperatures and pressures less than that required for the formation of metamorphic rock.
distal anatomically located far from a point of reference, such as an origin or a point of attachment.

enthalpy is the or energy (J) to create a system.

entropy the unavailability of a system's thermal energy for conversion into mechanical work (J.°K).

eosinophilic is the staining of cells after they have been washed with eosin which is a pink dye that stains the cytoplasm of cells, as well as extracellular proteins.

epithelial is the tissue that lines the surfaces and cavities of organs. The cells that compose the surfaces can be different shapes and one or more layers.

epithelium cells that are arranged in one or more layers that form part of a covering or lining of a body surface. The cells usually adhere to one another along their edges and surfaces.

Ethylenediaminetetraacetic acid is a hexadentate ("six-toothed") ligand and chelation agent, i.e. it has the ability to sequester metal ions such as Ca$^{2+}$ and Fe$^{3+}$. After being bound by EDTA, metal ions remain in solution but exhibit diminished reactivity.

fingerling a juvenile fish that can feed on its own with developed scales and working fins.

fry a fish that can feed on its own but has not developed scales and working fins.

Gibbs is a thermodynamic potential that measures the maximum or reversible work that may be performed by a thermodynamic system at a constant temperature and pressure (isothermal, isobaric).

heat map a graphical representation of data where the individual values contained in a matrix are represented as colors. It is used to provide an immediate visual summary of information.

hematopoietic cells are responsible for producing the cells which circulate in the blood. In a teleost those cells are in the head kidney versus bone marrow in mammals.

hepatocellular pertaining to or affecting liver cells.

heteroatom is an atom that replaces a carbon or hydrogen atom in an organic compound. Heteroatoms include sulphur, nitrogen and halogens.
hydropic a general histopathological finding in which cells absorb much water, indicating chemical/toxic injury or early autolysis. Autolysis is the destruction of a cell through the action of its own enzymes.

hypervariable region Bacterial 16S ribosomal RNA (rRNA) genes contain nine hypervariable regions termed V1 through V9 that demonstrate considerable sequence diversity among different bacteria. Species-specific sequences within a given hypervariable region constitute useful targets for diagnostic assays and other scientific investigations. No single region can differentiate among all bacteria.

karyorrhexis is the stage of cellular necrosis in which the fragments of the nucleus and its chromatin (protein, DNA, RNA) are distributed irregularly throughout the cytoplasm.

lesion is any abnormality in the tissue of an organism, usually caused by disease, trauma or toxic compounds.

macrophage a large white blood cell, occurring principally in connective tissue and in the bloodstream, that ingests foreign particles and infectious microorganisms by phagocytosis. Phagocytosis is the method that a cell uses to engulf a particle by surrounding it in cytoplasm.

muscular tunics are the muscular layers of a tubular structure; for most of the gastrointestinal tract, it consists of an outer longitudinal layer of muscle and an inner circular layer.

necrosis is a form of traumatic cell death that results from acute cellular injury. Necrosis is caused by factors external to the cell or tissue, such as infection, toxins, or trauma which result in the unregulated digestion of cell components by enzymes.

octanols are alcohols with eight carbons used as a surrogate for aquatic tissue. The chemical formula is C₈H₁₈O.

operculum the gill cover of fishes.

osmoregulation is the process of maintaining an internal balance of salt and water in a fish’s body. A fish can be considered to be a collection of fluids floating in a fluid environment, with only a thin skin to separate the two.

OTU is an operational taxonomic unit used as a species distinction in microbiology that uses a percent similarity threshold set by a researcher of a primer compared to a known 16S rRNA sequence.
**proximal** anatomically located close to a point of reference, such as an origin or a point of attachment.

**renal** is defined as of, relating to, or in the region of the kidneys.

**renal tubule** is the portion of the nephron containing the filtered tubular fluid. After passing through the renal tubule, the filtrate continues to the collecting duct system. Its function is to regulate the concentration of water and soluble substances such as sodium salts by filtering the blood, reabsorbing what is needed and excreting the remainder as urine.

**teleost** is a term that describes fish which are ray-finned with highly mobile mouth parts and symmetrical tails.

**vacuole** a membrane-bound organelle which is present in all animal and bacterial cells. Vacuoles are enclosed compartments which are filled with water containing inorganic and organic molecules including enzymes in solution.
Acronyms

**ASTM** American Society for Testing and Materials.

**BDH** biliary ductular hyperplasia, liver.

**BHM** Brain hemorrhage.

**CBE** Cerebellar edema.

**DBE** Double bond equivalence.

**DCM** Dichloromethane CH₂Cl₂.

**EDTA** Ethylenediaminetetraacetic acid C₁₀H₁₆N₂O₈.

**EPA** Environmental Protection Agency (US).

**EPT** Erythrophagocytosis.

**FAB** Fast atom bombardment which is a form of ionization used in mass spectrometry.

**FHL** Focal leukocytic hepatitis.

**FTIR/ATR** Fourier transform infrared spectroscopy using attenuated total reflectance.

**GC/MS** Gas chromatography/mass spectrometry.

**GSD** Gastric gland single cell degeneration and necrosis.

**GWV** Gastric wall vacuolation.

**HHD** Hepatocellular hydropic degeneration.

**HPLC** High precision liquid chromatography.
IBO  Intestine bacterial overgrowth.
ICK  Interstitial cell karyorrhexis.
ICP/OES Ion coupled plasma optical emission spectroscopy.
ISD  Intestinal epithelial cell single cell degeneration and necrosis.
ITP  Renal tubular intraluminal protein casts.
LC/QTOF/MS Liquid chromatography quadrupole time-of-flight mass spectrometry.
LHD  Gill lamellar epithelial hydropic degeneration.
MLSS Mixed liquor suspended solids.
MOE  Ministry of the Environment (Ontario).
NAN  Naphthenic acid number, determined by FTIR analysis.
PBN  Pseudobranch single cell necrosis.
pHi Initial pH of a sample. For the samples in this report pHi was pH 7.1 to 7.2.
REH  Renal endothelial cell hydropic degeneration.
RHA  Renal hematopoietic cell atrophy.
RIH  Renal interstitial hydropic degeneration/single cell necrosis.
RIK  Renal interstitial cell karyorrhexis.
RTH  Renal tubular hydropic degeneration.
SCN  Single cell hepatocellular necrosis.
SEM/EDX Scanning electron microscopy with energy dispersive X-ray spectroscopy.
SPE  Solid phase extraction.
SWA  Solids wetting agent.
TAN  Total acid number, percent of organic acid in crude oil mg(KOH)·g−1(oil).
TEP  Renal tubular epithelial protein droplets.
TIE  Toxicity Identification Evaluation.

TLC  Thin layer chromatography.

TLOE  Total loss of equilibrium.

TRE  Toxicity reduction evaluation.

VAC  Vacuole in hepatocellular cytoplasm.

WWT  Wastewater treatment.

XRD  X-ray diffraction.

XRF  X-ray fluorescence.

YSI  Yolk sacculitis.

YSM  Yolk sac macrophage.
Notation

[A-] molar concentration of the disassociated form of a weak acid (mol·L⁻¹).

atm standard atmosphere (101.325 kPa).

C carbon atom.

C_{total} total number of carbon atoms.

°C degrees Celsius.

Da dalton (1 g·mol⁻¹ also known as unified atomic mass unit (u)).

d day.

F force (N).

H hydrogen atom.

H_{total} total number of hydrogen atoms.

Hh_{total} total number of hydrogen and halogen atoms.

[H⁺] hydrogen ion concentration (mol·L⁻¹).

[HA] molar concentration of the undisassociated form of a weak acid (mol·L⁻¹).

[HA]₀ initial molar concentration of the undisassociated and disassociated form of a weak acid in oil phase (mol·L⁻¹).

[HA]_w molar concentration of the undisassociated form of a weak acid in water phase (mol·L⁻¹).

J Joule (N·m), (kg·m²·s⁻²), (Pa·m³), (W·s) sort.

Kₐ acid partition coefficient.
**K<sub>ow</sub>** octanol-water partition coefficient.

°K degrees Kelvin.

L liter.

**log K<sub>ow</sub>** log<sub>10</sub> of the partition coefficient K<sub>ow</sub>.

**LC<sub>50</sub>** the lethal concentration (LC) of a toxicant to produce a mortality rate ≥ 50 % for a given organism sort.

**LT<sub>50</sub>** the calculated lethal time (LT) of a toxicant which a specific concentration of a chemical is expected to produce a mortality rate ≥ 50 % for a given organism sort.

M molar solution (moles·L<sup>-1</sup>).

m meter.

mol mole, the mass of a substance containing the same number of fundamental units as there are atoms in exactly 12.000 g of $^{12}$C.

mDa milli dalton.

mg $10^{-3}$ gram.

mL $10^{-3}$ liter.

mm millimeter ($10^{-3}$ m).

N<sub>total</sub> total number of nitrogen atoms.

O oxygen atom.

P permeability of molecules across a cell lipid bilayer (cm·s<sup>-1</sup>).

pKa log<sub>10</sub> of the acid disassociation constant Ka.

S sulphur atom.

T temperature (°K).

$V_{oil}$ volume of water (L).

$V_{water}$ volume of water (L).
$D$ diffusion coefficient (cm$^2$·s$^{-1}$).

d diameter (m).

$K$ partition coefficient.

$L$ length (m).

$n$ number of carbon atoms.

$\nu$ velocity (m·s$^{-1}$) with direction stated.

$y$ number of nitrogen atoms.

$z$, deficient hydrogen atoms in a structure where $z$ is an integer value equal to 0, -2, -4, -6....

$\gamma$ surface tension (N·m$^{-1}$).

$\Delta\rho_{ow}$ differential density between two fluids with densities expressed as (g·L$^{-1}$).

$\Delta A$ area change (m$^2$).

$\Delta H$ change in enthalpy (J).

$\Delta S$ change in entropy (J·°K$^{-1}$).

$\Delta W$ work required (J).

$\Delta x$ cell membrane thickness (cm).

$\mu L$ $10^{-6}$ liter.
Chapter 1

Introduction

This chapter covers the research objectives and scope of the Ph.D. thesis, and includes the following: a background on naphthenic acid, sources of naphthenic acid in oil refineries and naphthenic acid toxicity of wastewater treatment effluent.

1.1 Research Objectives

The main motivation of this research was based on random failures of rainbow trout fingerling acute toxicity tests in water effluent from the wastewater treatment plant of an oil refinery. A failure is defined as a $\geq 50\%$ mortality rate over the 96 hour duration of the test [1]. A test consists of exposing 10 trout fingerlings to a sample of the wastewater system clarifier effluent in an aquarium and monitoring the mortality rate. An attempt was made to find a correlation between the desalting unit brine effluent, the treatment of the refinery’s wastewater and the wastewater effluent residual toxicity, and to use these results to improve the quality of the wastewater treatment unit.

In addition to trout fingerlings (*Oncorhynchus mykiss*), water fleas (*Daphnia magna*) were tested for acute toxicity at the same time in separate vessels [2].
The oil refinery water effluent into the river, which includes once-through cooling water used in heat exchangers, did not show any toxic effects to aquatic organisms such as trout fingerlings and water fleas.

The water sample gathering and many of the analyses were done on-site at a refinery to provide the opportunity to look at the problem holistically. Samples that required specialized testing such as fish toxicity, histopathology, and bacteria RNA analysis that could not be done at the refinery were sent to external labs with specific requests.

The following sections itemize objectives of the research reported in this thesis:

a) Review the literature on naphthenic acid toxicity, sources, analysis and effects on wastewater treatment which are discussed in each of the chapters below. The sources led to a review of desalting crude and emulsions which is discussed in Chapters 2 and 5

b) Find the root cause of acute toxicity. This was accomplished by following the United States Environmental Protection Agency (EPA) Toxicity Identification Evaluation (TIE) protocol. This is discussed in Chapter 3.

c) The determination of the parameters of the acute toxicity of naphthenic acid on trout fingerlings is discussed in Chapters 3 and 4. The parameters investigated were:

   (i) physiological effects,
   
   (ii) the 50 % lethal concentration \( LC_{50} \) of naphthenic acid,
   
   (iii) whether the acute toxicity of trout fingerlings was affected by other material in wastewater treatment using Toxicity Identification Evaluation protocol,
   
   (iv) which organs were affected, determined using histopathology.
d) The analysis of a refinery wastewater treatment (WWT) system is discussed in Chapter 4. The analysis included the following:

(i) mass balance of naphthenic acid,
(ii) ability to treat naphthenic acid,
(iii) analysis of bacteria population present in wastewater.

e) The transport mechanism between the desalting operation and the wastewater treatment system is discussed in Chapter 5.

f) The following naphthenic acid extraction and analysis methods are discussed in Chapter 6:

(i) extract naphthenic acid from high Total Acid Number (TAN) crude,
(ii) extract naphthenic acid from wastewater treatment aqueous samples,
(iii) analytical instrument methods.

g) Based on the findings from objectives d), e) and f) lab and field testing of methods to minimize naphthenic acid concentration in a desalting operation prior to wastewater influent were performed. The results of the testing resulted in a patent application which is discussed in Chapter 7 and Appendix A.

Figure 1.1 is a summary of the steps and milestones of the research in this thesis.

1.2 Background

Crude oil is a complex mixture of organic compounds, predominately composed of hydrocarbons, and a number of other compounds which are considered contaminants. The contaminants consist of compounds, such as organic and inorganic sulfur species, chloride and nitrogen compounds, trace metals, trace heavy minerals and naphthenic
Figure 1.1 – The flow chart shows the doctoral thesis research milestones outlined in Section 1.1.

- Acute toxicity of trout fingerlings detected during routine toxicity testing of wastewater treatment (WWT) effluent
- WWT effluent tested using EPA toxicity identification evaluation (TIE) method
- Toxicant identified as naphthenic acid (NA) using GC/MS

- Determine sources of NA in WWT feed streams by testing
- Identified desalter processing high TAN crude as being the source of NA
- Determine mass balance of NA in WWT feed streams
- Determine transport mechanism of NA from desalters to WWT

- Carbon filters installed at wastewater treatment effluent.
- Analyze organic compounds on carbon and effectiveness of NA removal
- Test toxicity of extracted acid.
- Document physiological symptoms of chronic fish and NA toxicity
- Histopathology of fish to find lesions formed by NA to try and determine entry methods
- Determine acute toxicity of NA concentrations in WWT effluent samples and acute toxicity limits of extracted acid from high TAN crude.

- Speculate NA from WWT samples
  - Develop robust methods for extraction and analysis of NA from oil and water.
  - Extract, separate and analyze acid fractions from oil to perform toxicity tests
  - Test biodegradation of NA fractions in bioreactors using microbes from WWT
  - Perform 16s rRNA analysis of WWT microbes

- Test methods to reduce NA from desalter and WWT
- Patent methods to remove NA from desalter brine
- Propose Capital project to install equipment to maintain NA concentrations below acute toxicity levels
Contaminants can cause a number of problems including emulsion formation and toxicity when desalting the crude oil.

One group of compounds present in crude oil is naphthenic acids. The source of naphthenic acid in crude oil is due to the heavy biodegradation of the crude oil deposit over millennia by anaerobic bacteria \(^5\). This occurs during diagenesis while the oil is in a geological formation at temperatures less than 80 °C; which is also true of all organic acids in crude oil. The McMurray formation in Alberta originated under the latter conditions.

The chemical composition of naphthenic acid is extremely complex; a great variety of structures and compositions fall within the classification of naphthenic acid. In general, naphthenic acid is characterized by a carboxylic acid functional group attached to a hydrocarbon molecule, and a generalized chemical formula of \( \text{R(CH}_2\text{)}_n\text{COOH} \) can be applied, where \( \text{R} \) is a cyclopentane or cyclohexane ring, and \( n \) is typically greater than 12 \(^6\) \(^7\). The distribution of naphthenic acid molecular weights and species can be used as biomarkers for oil deposits.

Total Acid Number (TAN) is defined as the number of milligrams of KOH required to neutralize the acidity of one gram of oil and is a commonly accepted criterion for the oil acidity \(^8\) \(^9\). Based on this measurement, oils with a TAN greater than 1.0 are classified as highly acidic. High TAN crude oils are commonly encountered in California, Venezuela, the North Sea, Western Africa, India, China, Russia and Alberta. \(^10\) \(^11\) \(^12\). The high TAN requires an increased metallurgy cost for refinery processing equipment and corrosion inhibition. Consequently, crude oils with high naphthenic acid content are considered to be of poor quality and are marketed at a lower price.

A multitude of other organic acidic compounds are also present, including fatty and aromatic acids, and the chemistry of petroleum related naphthenic acid has yet to be completely agreed to. The presence of naphthenic acid compounds contributes to the acidity of crude oils. The percent content naphthenic acid by volume is sometimes
referred to as a Naphthenic Acid Number (NAN).

The removal of naphthenic acid compounds from heavy crude oil is one of the most important processes in heavy oil upgrading. Current industrial practices depend on either dilution or caustic washing methods to reduce the TAN of heavy crude oils. However, neither of these approaches is entirely satisfactory. For instance, blending a high TAN crude oil with a low TAN crude oil may reduce the naphthenic acid content to an acceptable level, but the acidic compounds remain and the value of the low TAN oil is diminished. Caustic treatment can substantially remove naphthenic acid, but the process generates significant volumes of wastewater and emulsions that are problematic to treat. In particular, once an emulsion is formed, it is very difficult to remove it [3].

1.2.1 Naphthenic Acid Analysis History

The term naphthenic acid was first used in 1874 by C. Hell and E. Medinger [13] to describe the observation of the acidity in naphthenic-based crude during studies of the acid fractions of oil from the Wallachia oil fields in Russia. The acid was extracted from the crude before processing using a procedure of washing the crude with a potassium hydroxide (KOH) solution. One of the acids, \( C_{11}H_{20}O_2 \) was isolated.

The acid extraction procedure used is the basis of the American Society for Testing and Materials (ASTM) TAN test which measures the acidic species in crude oil as mg (KOH)·g\(^{-1}\) (oil) [14]. A similar method of extraction was used to obtain naphthenic acid standards from crude oil for the research, as described in Section 6.1.

In 1888 Markownikow and Oglobin isolated an additional acid \( C_{10}H_{18}O_2 \) from the Baku region oil samples. Further studies by Markownikow led him to the following formula for the general structure of the acid

\[
C_nH_{2n+2}O_2
\]

where \( n \) is the number of carbon atoms and \( z \) indicates the number of deficient
hydrogen atoms in the structure that would form a saturated structure and can be calculated using Equation 1.1:

\[ z = (C_{\text{total}} \cdot 2) - H_{\text{total}} \]  

(1.1)

where \( C_{\text{total}} \) is the total number of carbon atoms and \( H_{\text{total}} \) is the total number of hydrogen atoms. The \( z \) nomenclature is convenient to use when grouping carboxylic acids \([15]\). Figure 1.2 shows several examples of the \( z \) values for various carboxylic acids that are grouped in the term naphthenic acid.

![Molecular structures](image)

Figure 1.2 – Example of \( z \) numbers for various carboxylic acids that are grouped in the term naphthenic acid. The \( z \) numbers were calculated using Formula 1.1.

In 1891 a researcher by the name of O. Aschan stated he was going to discontinue his work with the so-called naphthenic acid but considered this work anything but complete. Analysis of the naphthenic acid from crude used for this project is discussed in Chapter 6.
1.2.1.1 Double Bond Equivalence

Another term used to group structures is double bond equivalence, DBE, which is a measure of unsaturation and is shown in Equation 1.2:

\[
DBE = \frac{-(z - 2)}{2}
\]  

(1.2)

Where \( z \) can be calculated from Formula 1.1. If there is a nitrogen heteroatom in the structure then Equation 1.2 becomes

\[
DBE = \frac{-(z - y - 2)}{2}
\]  

(1.3)

Where \( y \) is the number of nitrogen atoms. If the heteroatom is sulphur or oxygen then \( y \) is 0 and Equation 1.3 is equivalent to Equation 1.2. If the heteroatom is a halogen Equation 1.3 becomes

\[
DBE = C_{\text{total}} - \frac{H_{\text{total}}}{2} + \frac{N_{\text{total}}}{2} + 1
\]  

(1.4)

where \( H_{\text{total}} \) is the total number of hydrogen + halogen atoms and \( N_{\text{total}} \) is the total number of nitrogen atoms. DBE is always a positive value.

1.2.2 Naphthenic Acid Structures

Naphthenic acid is a term used in the petrochemical industry that refers to a broad class of carboxylic acids that occur naturally in crude oil. Naphthenic acids are classified as organic acid surfactant compounds composed of alkyl-substituted cycloaliphatic carboxylic acids and to a lesser extent acyclic aliphatic acids. Molecular weights of naphthenic acid range from about 120 to greater than 700 daltons (Da). Naphthenic acid has the general structure RCOOH where R can be any number of aliphatic, cyclohexyl or aromatic groups [16, 17].
For example a naphthenic acid with a linear branched hydrocarbon chain has a $z = 0$, with one ring $z = -2$, with two rings $z = -4$ and with three rings $z = -6$ etc. The carboxylic acid group is most often found attached to a side chain rather than bonded to the cycloaliphatic ring in the naphthenic acid with $z = -2$. Examples are shown in Figures 1.3 and 1.4.

**Figure 1.3** – Examples of naphthenic acid structures with various $z$ values; as the $z$ number increases the number of cycloaliphatic structures also increases. The radicals can contain both hydrocarbon and/or heteroatoms; heteroatoms can also replace carbon atoms in the ring structures.
The acid structures shown in Figures 1.2 and 1.3 indicate that the term ‘naphthenic acid’ is not an accurate term to describe the organic acids found in crude because the organic acids in addition to cycloaliphatic acid contain fatty and aromatic acids, examples of which are shown in Figure 1.4.

Figure 1.4 – Examples of naphthenic acid with aromatic and ringed structures.

1.3 Naphthenic Acid in Oil Refineries

The first step in crude oil refining is washing of the crude oil with water to remove salts and other water soluble contaminants in a vessel called a desalter. The washing of acidic crude oils with a low or high TAN value results in naphthenic acid partitioning from the oil into desalter brine. The desalter brine is joined with other water streams to be treated in the wastewater unit. Mass balances of the naphthenic acid in a refinery wastewater system are discussed in Section 4.3.

1.4 Naphthenic Acid Toxicity

In addition to being toxic to mammals, naphthenic acid is an acutely toxic substance to aquatic lifeforms such as, rainbow trout fingerlings (Oncorhynchus mykiss) and water fleas (Daphnia magna).

The standard test in Canada for acute toxicity of an industrial effluent, including oil refineries, is to collect a sample of the effluent and expose 10 rainbow trout fingerlings.
for 96 hours at full effluent concentration [18, 19]. The tests are done on a prescribed basis and the results must be reported to the governing agency; in the case of Ontario, that is the Ministry of the Environment (MOE). If the acute lethality of the effluent is ≥ to 50 % the test is a failure and further analysis must be done to determine the cause and the company is subject to fines and orders.

The analytical method employed in this research project to determine the toxicant and the lethal concentration, LC$_{50}$, is the U.S. EPA Toxicity Identification Evaluation (TIE).

The refinery studied in this doctoral thesis research experienced random failures of the acute toxicity test as defined by the MOE. Naphthenic acid was determined to be the toxicant by following the TIE protocol.

The naphthenic acid compounds found in the crude oil of this research project had an average pKa of 5.1, they are a weak acid and solubility in water is a strong function of pH. They also have very low volatility with a Henry’s constant of approximately $8.56 \times 10^{-6}$ atm·m$^3$·mol$^{-1}$. Naphthenic acids are amphipathic compounds that have surfactant-like properties and will accumulate at aqueous/nonaqueous interfaces, such as between water and oil.

### 1.5 Crude Oil Processing and Interaction with Wastewater Treatment

Crude oil reserves contain a large number of contaminants such as salts, solids, organic acids and naturally occurring or man-made surfactants. The contaminants must be minimized prior to the crude being processed to prevent corrosion and equipment fouling. Figure 1.5 shows a simplified process flow diagram which starts from crude oil storage tanks; the following sections describe the flow diagram.
Figure 1.5 – Simplified process flow and instrument diagram (PID) in a petroleum refinery which includes crude oil storage and supply, desalting of crude oil and wastewater treatment facilities.
1.5.1 Crude Oil Blending

To efficiently produce refined products such as gasoline, diesel fuel, solvents and asphalt, crude oils with various properties are blended together before the crude oil is processed by the refinery process units. The crude oils are shipped to the refinery and are placed in storage tanks 1, 2 and 3 prior to blending.

In Figure 1.5, Tanks 1, 2 and 3 contain various types of crude oil, such as high TAN crude. Pumps P1, 2 and 3 in addition to flow control, valves V1, 2 and 3 are used to blend various ratios of the crude into Tank 4 which is used as a feed tank. Pump P4 and control valve V4 are used to control the flow rate to the desalter.

Between the desalter and V4, is a heat exchanger, E1 that is used to heat the crude prior to desalting to lower the viscosity and make desalting more efficient. Temperatures of the crude are normally in the range of 120 °C.

1.5.2 Crude Oil Desalting

Recycled water from various process units, termed “wash water”, is pumped via P5 at a defined rate, and mixed with the crude from E1 at V6 before entering the desalter. The oil and water form an emulsion which coalesces in the desalter. To give the emulsion time to coalesce the water/oil interface level is maintained by the valve V7 which controls the rate of the water removed from the desalter. The water, now termed brine due to its salt content of approximately 200 mg·L\(^{-1}\), is cooled by exchanger E2 to approximately 30 °C before entering the wastewater treatment system.

Oil exits the top of the desalter to be further treated in the refinery process units.

1.5.3 Wastewater Treatment

After cooling, the brine effluent from the desalter is combined with other wastewater streams and sent to primary treatment to remove free (undissolved) oil and solids.
prior to biological treatment to remove oil and other biodegradable material. The primary treatment typically consists of the cyclone separator, equalization tank and primary separation. Other streams such as rain water and cleaning water, which have the possibility of containing hydrocarbon, also require treatment; they are shown in Figure 1.5 as the contaminated sewer with primary separation.

The effluent from the primary treatment vessels is treated by bacteria in aeration basin(s) to remove dissolved hydrocarbons, nitrogen and sulphur compounds. The oxygen required to convert the substances to CO$_2$, H$_2$O, N$_2$ and S in the aeration basin is maintained by an air blower B1.

The effluent from the aeration basins is water with minimal contaminants. Clusters of bacteria and solids, termed floc, are passed to clarifier(s) which allow the floc to disengage from the water.

Depending on the risk of organic toxicants a carbon bed may be required to absorb the toxicants.

The settling pond is used to remove any remaining suspended solids from the wastewater treatment system prior to discharge of the effluent into public waterways such as a river. The settling pond can also be placed prior to the carbon bed.

Since the outlet of the desalter is directly coupled to the wastewater system, upsets in the desalting operation will have a direct impact on wastewater. The impacts can be toxicity or excessive air demand in the aeration basins.

1.6 Naphthenic Acid Corrosion

Many compounds in crude cause corrosion in refinery equipment: they include sulphur compounds, salts (in the form of chlorides), nitrogen compounds and naphthenic acid. Processing of crude with a TAN > 0.5 can be considered to be potentially corrosive and the corrosion due to naphthenic acid is of most concern in crude distillation towers in
the temperature range of 220 °C to 400 °C. Naphthenic acid corrosion rates can vary with temperature, z number, carbon number, velocity, local turbulence and wall shear stress. Methods to minimize the effects of naphthenic acid corrosion include blending high TAN crude with lower TAN crude, injection of corrosion inhibitors and materials of construction which include high chromium and molybdenum stainless steels such as 316SS and 317SS [20].
Chapter 2

Literature Review

This chapter includes a state-of-the art literature review that relates to the doctoral thesis research shown in Sections 2.1 through 2.6.

2.1 Naphthenic Acid Toxicity

This dissertation is concerned with acute aquatic toxicity of trout fingerlings (*Oncorhynchus mykiss*). This has been reviewed in [21, 22, 23, 24] at one pH value. Toxicity monitoring of trout fingerlings during this research also considered the development and testing of toxicity tests at pH 6. The reason is that naphthenic acid is a weak acid and therefore, the lower pH would increase the un-ionized form of naphthenic acid to increase toxicity.

A previous study of purified fractions of extracted naphthenic acid [25] used Microtox as the target organism. This study found that Microtox is not an accurate indicator of toxicity of clarifier effluent.

A number of studies have been done on the acute toxicity of naphthenic acid [26, 27, 28, 29] with most using commercial naphthenic acid from speciality chemical companies such as Sigma-Aldrich (Merck KGaA) and Fluka.
No published histopathology studies have been done of the acute toxicity of refinery wastewater effluent at pH and pH 6. Physical biodegradation mechanisms for naphthenic acids have not been well investigated, but microbial biodegradation does occur, although primarily for naphthenic acid with carbon numbers < 21 [30].

A summary of naphthenic acid properties that could cause human hazards was completed in 2012 for oil sands process water [31]. The study found that direct contact with surface water via ingestion and contact present a plausible pathway for human exposure. Due to the low surface water to air partitioning, inhalation was very low risk due to the low concentrations. The concentration range in the Athabasca River was < 3 mg·L⁻¹ to 100 mg·L⁻¹ which the authors considered to be very dilute.

Henry’s Law constant for three model naphthenic acid compounds studied in 2009 were 2.0·10⁻⁶, 9.3·10⁻⁶ and 2.9·10⁻⁸ atm·m³·mol⁻¹. In comparison Henry’s constant for benzene is estimated to be 2.7 and 3.0·10⁻³ atm·m³·mol⁻¹ [32].

To determine if there could be a pathway for human exposure by consumption of fish, the octanol-water coefficient $K_{OW}$ was reviewed, as octanol is used as a surrogate for aquatic biological tissue [33]. $K_{OW}$ is defined as the ratio of the solubility of a compound in octanols to its solubility in water. The higher the value of $K_{OW}$, the more non-polar the compound becomes which makes it easier to pass through a cell’s bi-lipid layer. The value log $K_{OW}$ which is termed lipophilicity, is used as a relative indicator of the tendency of an organic compound to be absorbed by non-polar material such as cell walls and non-polar solvents. The log $K_{OW}$ values are inversely related to aqueous solubility and are directly proportional to molecular weight. The $K_{OW}$ value for naphthenic acid was found to be between 3 and 30 depending on structure and molecular weight [32, 34].

Very few 96-hour bioassay histopathology reports of juvenile rainbow trout have been published [35, 36], and no reports have described the effects of processing high TAN crude on refinery wastewater systems.
2.2 Desalters

Desalting of crude oil is the first refinery unit process and the main purpose is to remove salts, solids and contaminants that can cause corrosion and fouling in equipment downstream of the desalter [37, 38, 39, 40]. To accomplish the removal of the contaminants, water is added to the incoming crude which forms an emulsion that is dehydrated in a desalter. Details of the desalting process are discussed in Chapter 5.

2.3 Emulsions and Crude Contaminantes

Emulsions are formed during the desalting operation. Under ideal conditions the emulsions coalesce in the desalter and a sharp interface between the oil and water layers is created. A number of naturally occurring materials such as asphaltenes, resins [41, 42] and solids, can cause the emulsions to be stable. Stable emulsions carried out of the desalter with the brine can cause problems with toxicity and wastewater treatment, due to high chemical oxygen demand (COD) loading [43].

The formation of emulsions in desalters is discussed in a series of papers [44, 45, 46, 47, 48].

The role of solids in the stabilization of emulsions was first described in 1903 [49] and further explained in 1907 [50]. The roles of metallic compounds and surfactants are described in [51]. Solids, such as kaolinite clay, can act as absorption sites [52, 53] and can transport naphthenic acid from the oil to the water phase.

Naphthenic acid has been identified as one the compounds that can act as a surfactant to stabilize emulsions depending on pH and the availability of metals to form soaps [16]. Experiments done during the research portion of this dissertation found pH to be one of the key variables in emulsion formation [54].
2.4 Wastewater Treatment

The most commonly used process to treat oil-refinery wastewater is a bottom aerated activated sludge bioreactor with primary oil and solids separation (CAS). The CAS bioreactor process removes hydrocarbon, nitrogen and sulphur compounds from water used in the refinery. Activated sludge systems are standard units to treat industrial waste and are described in detail in a number of sources [55, 56, 57]. Newer designs conserve space and increase efficiency of biological removal. There is a trend toward membrane bioreactors which use a conventional activated sludge bioreactor [58].

High level discussion of bacteria types such as those involved in floc formation and the oxidization of various compounds are described in a number of texts [59, 60, 61]. A detailed understanding of the bacterial diversity in a CAS requires the use of 16S rRNA analysis which can reveal the bacteria population size and taxonomy to the species level. There is a large body of work being published concerning the indention and characterization of the bacteria in wastewater treatment systems using 16S rRNA sequencing [62, 63, 64]. The steps required to prepare samples for 16S rRNA analysis are described [65] in the London Regional Genetic Center procedure [66] which is included in Appendix B; both documents were used for the 16S rRNA analysis in this thesis.

To reveal metabolic pathways or novel genes of the bacteria requires more information than the 16S rRNA gene; a field of research termed metagenomics which uses whole DNA sequencing is used [67] for this purpose.

The fate of naphthenic acid with concentrations between 4.2 and 11.6 mg·L$^{-1}$ in a refinery wastewater treatment system has been described in [68]. The findings indicated the bacteria in the CAS was not inhibited with naphthenic acid concentrations up to 400 mg·L$^{-1}$ and to have any significant reduction i.e. >50 % of naphthenic acid required >90 day incubation period. A separate study [69] reported a much lower naphthenic acid concentration of 130 µg·L$^{-1}$ with a 78 % reduction with a CAS with a retention time of 16 hours. In neither case were trout toxicity studies or 16S rRNA studies done.
2.5 Naphthenic Acid Analysis

Naphthenic acid for many analytical laboratory studies is extracted from kerosene/jet fuel, diesel and gas oil fractions that have been processed to remove the acid. Laboratory supply companies such as Sigma-Aldrich and Fisher Scientific supply the acids commercially.

As discussed in Section 1.2.1, research into naphthenic acid has been ongoing since 1874 [13]. The two common methods of extracting the naphthenic acid from water and oil for analysis are liquid/liquid extraction using DCM as a solvent and Solid Phase Extraction (SPE) [70].

One of the specifications of crude oil is the total acid number or TAN [14, 71] which gives an indication of the concentration of naphthenic acid. This is a standard test used to ensure that the corrosion limits of the installed metallurgy in the processing equipment are adequate to withstand the crude corrosive properties. Table 2.1 shows the physical properties of naphthenic acid.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colour</td>
<td>Pale yellow, dark amber, yellowish brown, black</td>
</tr>
<tr>
<td>Odour</td>
<td>Primarily imparted by the presence of phenol and sulphur impurities; musty hydrocarbon odour.</td>
</tr>
<tr>
<td>State</td>
<td>Viscous liquid</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>Between 140 and 450 amu</td>
</tr>
<tr>
<td>Solubility</td>
<td>&lt; 50 mg·L(^{-1})</td>
</tr>
<tr>
<td>Density</td>
<td>Between 9.7 and 0.99 g·cm(^{-3})</td>
</tr>
<tr>
<td>Refractive index</td>
<td>Approximately 1.5</td>
</tr>
<tr>
<td>pKa</td>
<td>Between 5 and 6</td>
</tr>
<tr>
<td>(\log K_{ow})</td>
<td>Approximately 4 at pH 1</td>
</tr>
<tr>
<td></td>
<td>Approximately 2.4 at pH 7</td>
</tr>
<tr>
<td></td>
<td>Approximately 2 at pH 10</td>
</tr>
<tr>
<td>Boiling point</td>
<td>250 °C to 350 °C</td>
</tr>
</tbody>
</table>

There are two methods used to extract naphthenic acid from an oil or aqueous
sample; liquid/liquid extraction or solid phase extraction (SPE). A number of analytical techniques are available to analyze the structure and speciation of extracted naphthenic acids, including Fourier transform infrared spectroscopy using attenuated total reflectance (FTIR/ATR), Gas chromatography/mass spectrometry (GC/MS), Liquid chromatography quadrupole time-of-flight mass spectrometry (LC/QTOF/MS) and Orbitrap® [72].

In 2012 Environment Canada embarked on an interlaboratory study with 15 participating public and private laboratories [73] to determine the current status of methods for naphthenic acid analysis; the methods included FTIR, GC/MS, LC/QTOF and Orbitrap®. Each lab was provided seven samples of distilled water, spiked with known naphthenic acid concentrations between 0.05 and 50 mg·L⁻¹. The results showed the correlation coefficient (r²) ranged from 0.5769 to 0.9975; the main difference appeared to be sample preparation, as a number of sample analysis methods had r² values greater than 0.91. This was followed up in 2014 with another 14 labs with 10 naphthenic acid concentrations between 0.387 and 17.4 mg·L⁻¹ [74]. The results indicated that the correlation coefficient (r²) for naphthenic acid concentrations ≤ 0.6 mg·L⁻¹ they ranged from 0.0 to 1.0 and for concentrations > 0.6 mg·L⁻¹ they ranged from 0.83 to 1.0, further indication that extraction methods were critical as the analysis methods varied.

2.6 Methods to Control Naphthenic Acid

There have been a number of papers published describing the biodegradation of naphthenic acid in wastewater produced from oil processing [23, 75, 76, 77, 78, 79]. Typically the biological treatment involves large sediment ponds such as those in Fort McMurray, Alberta or activated sludge with residence times of 90 days.

Other tests have included solvent extraction and ultraviolet (UV) light sources.
Chapter 3

Wastewater Toxicity Identification

Toxicity of refinery wastewater is routinely assessed using standard 96-hour bioassays with juvenile rainbow trout fingerlings (*Oncorhynchus mykiss*). The goal for wastewater treatment is to produce an effluent that causes no significant differences in fish mortality between those exposed to treated wastewater and unexposed fish used as a control [26, 80, 81]. When wastewaters cause significant fish mortality during toxicity testing, the toxic fraction(s) must be identified and removed. To determine the contaminant(s) of concern or the effluent characteristic associated with a toxicity response, the U.S. EPA has developed a formal *phased* TIE protocol [82, 83, 84] to perform effluent Toxicity Reduction Evaluations (TRE). The ecotoxicity testing was performed with rainbow trout and water fleas with neat clarifier effluent samples. A paper titled “Naphthenic Acid Toxicity Studies of Rainbow Trout Fingerlings” based on this chapter is in preparation.

3.1 Toxicity Identification Evaluation

The objective of the TRE is to assess the most appropriate measures necessary to maintain effluent toxicity at acceptable levels. The three phases of the U.S. EPA
developed TIE and TRE program are:

a) Toxicant Characterization. Tests that can tentatively categorize the toxicants as cationic metals, non-polar organics, oxidants, substances whose toxicity is pH dependent, as well as development of physical/chemical characteristics of the toxicant, such as filterability, degradability, volatility and solubility [82].

b) Toxicant Identification. The major objective of Phase 2 is to identify the suspected toxicants by further isolation of non-toxic compounds from those associated with toxicity. Currently the protocol has techniques for identifying nonpolar organics, Ethylenediaminetetraacetic acid (EDTA) chelatable metals, ammonia and surfactants [83].

c) Toxicant Confirmation Procedures. Contains methods generic to all toxicants that when collectively combined, the results provide a weight of evidence that the toxicant has been identified [83].

Once the toxicant has been isolated by the TIE and TRE programs, an investigation can be completed to determine the internal source of the toxicant and the most effective method to augment or enhance a water treatment system to reduce toxicant levels.

Though the EPA has developed specific protocols for TIE/TRE investigations, the protocols were used merely as a guide for evaluating toxicity problems. Only those TIE steps considered to be relevant for the facility were utilized, in some cases leading to further TIE steps and/or more atypical exploratory or definitive type TIE sample studies.
3.2 Clinical Signs of the Toxicant on Rainbow Trout Fingerlings

The clinical signs of the toxicant on the fish were the following: distended stomach, darkened body, total loss of equilibrium (TLOE), and lethality which is typical of narcosis [81].

Where a sample resulted in lethality, it was preceded by a total loss of equilibrium (TLOE) and an apparent depression in the fish's respiration rate.

3.3 Toxicity Identification Evaluation Findings

To understand the relationships and associated process data between specific wastewater quality variables and rainbow trout toxicity response, a Phase I TIE was performed, utilizing the retained volumes of known toxic samples. This demonstrated that the acute toxicity occurred with the rainbow trout fingerlings with no effect on water fleas.

TIE investigation included specific detailed laboratory toxicity test sample manipulations to categorize and define specific characteristics of the toxicant of concern. It should be noted that due to sample volume restrictions it was sometimes necessary to reduce the volume used, the number of fish tested, and to adjust fish loading densities to allow completion of the investigative TIE protocol.

An overview of the TIE steps and results are discussed in the following sections.

3.3.1 Baseline Toxicity Test

Results of the baseline test were used as a benchmark, to evaluate the effects of the TIE sample manipulations and any toxicity change attributable to sample storage time.

Baseline assessments using retained process effluent samples were completed
without any TIE manipulation to ensure that toxicity was retained and not degraded with the increased storage time, to facilitate validation of the TIE investigation and to provide a method to assess TIE results for changes in toxicity response.

Baseline toxicity tests found little change in toxicant response with increased sample hold times, providing a method of comparison for changes in toxicity response to the parallel TIE sample manipulation toxicity results.

During completion of the original compliance and subsequent baseline toxicity tests, some of the rainbow trout were observed to experience a TLOE and reduced respiration rate, i.e. slowed gill opercula movement, which precedes death. This is a unique characteristic of the toxicant response suggesting a potential neurological type toxicant typically found to be an organic compound. [81]

### 3.3.2 Graduated pH Test

This test determines whether the effluent toxicity can be attributed to compounds whose toxicity is pH dependent. The toxicity depends on the toxicant’s chemical form that is toxic such as ionized versus un-ionized.

Toxicity for ammonia, specific metals and some organic compounds can be affected by pH differences or changes. In the case of ammonia, as pH is increased the toxicity increases due to a shift to a more un-ionized from the ionized form.

The graduated pH toxicity test was completed at pH 6.0, 6.5, 7.0 (close to pH$i$), 8.0 and 8.5. Results showed an increased potency of the toxicant at a slightly reduced pH (i.e. pH 6.0 and 6.5) with 100 % mortality occurring within 24 to 48 hours compared to the baseline toxicity tests, which showed a reduced toxicity response in the same timeframe.

These test results indicated that the toxicant is pH sensitive, exhibiting increased toxicity response or potency with a slight decrease in sample pH. This is the opposite of what we would expect if ammonia was the source of toxicity.
A graph based on 90 experiments with various responses to weak acids from tests on microbes, fish and mammals is shown in Figure 3.1. This is of similar magnitude to the effect seen with pH 6 and pH\textsubscript{i} in the naphthenic acid tests with trout fingerlings.

**Figure 3.1** – The effect of pH on the concentration of a weak organic acid required to give a standard response from a test organism. The continuous line shows the total concentration of the acid and the broken line the corresponding concentration of undissociated acid molecules.

Figure 3.1 gives an important insight into the effect of pH on the toxicity of weak acids. If the pH of a solution containing a weak acid increases above the pKa of the acid, the acid becomes increasingly associated (ionized) and cannot pass through the lipid bilayer of cells of the test organism due to the negative charge of the acid.
3.3.3 Zeolite Filtration

Filtration of the effluent sample through a zeolite resin column, along with the graduated pH test results, provide an indication of whether the source of toxicity is related to the presence of ammonia or other ionized/un-ionized contaminants which could be filtered by zeolite and are affected by small changes in pH.

Zeolite filtration indicated that only a marginal reduction in toxicity response was achievable.

3.3.4 Aeration Test

Aeration of an effluent sample at pH 3, pH$_i$, or pH 11 may result in a loss of toxicity caused by substances that are oxidizable (e.g. chlorine), spargeable (i.e. volatile), or sublatable (e.g. surfactants).

The aeration TIE manipulation was completed at pH 3, pH$_i$ and pH 11, with the sample readjusted back to pH$_i$ for completion of the toxicity test. Toxicity results showed an enhanced toxicant response for the pH 3 aeration manipulation and no real reduction in toxicant response for the pH$_i$ and pH 11 aeration manipulations. If toxicity were attributable to ammonia, the expectation would be for the toxicant to be sparged off at pH 11, with a subsequent reduction in toxicity response, which was not the case.

3.3.5 Filtration Test

Passing of the effluent sample through a 1.5 µm filter paper provides information as to whether the toxicity can be trapped on the filter paper. It does not provide any specific information with regard to the class of the toxicant but does indicate in what phase (i.e. solid, precipitate or dissolved) the toxicant can be found. Filtration at pH 3, pH$_i$ and pH 11 provides information on any characteristic change that might occur to the toxicant with changing pH.
Filtration of the samples at only pH\textsubscript{i} was completed as a preparation step for filtration through a solid phase extraction column. The toxicity of the process effluent samples was not removed by filtration, suggesting that the toxicant exists in a dissolved form at pH\textsubscript{i} or on particles smaller than 1.5 \textmu m.

3.3.6 Filtration and C\textsubscript{18} Solid Phase Extraction Test

An aliquot of the filtered effluent sample was passed through an octydecylsilyl (C\textsubscript{18}) solid phase extraction (SPE) cartridge to determine if non-polar organics, which can be removed by this column, were associated with the occurrence of toxicity. The SPE cartridge may also extract metal and metal complexes as well as some surfactants.

If the toxicity is associated with organics, the compound(s) can usually be extracted from the column's sorbent material (within the SPE cartridge) using a polar solvent such as methanol. Further identification of the toxicant is then possible by submitting the methanol elutriate for chemical characterization (e.g. GC/MS analysis).

Filtration and C\textsubscript{18} SPE at pH 3, pH\textsubscript{i} and pH 9 provides information on any characteristic change that might occur to the toxicant with changing pH. SPE column filtration was completed at pH\textsubscript{i} of the retained process effluent samples. The samples showed that the toxicity was removed suggesting that the toxicant was retained on the SPE column.

3.3.7 Filtration, C\textsubscript{18} Solid Phase Extraction and Methanol Extraction Test

Once it was confirmed that the toxicant can be trapped on the SPE cartridge and removed with methanol, a test was completed to confirm that the toxicant could be extracted from the SPE column by washing the cartridge with 100\% methanol or various fractions of methanol. The methanol fraction desorbed material was added to rainbow
trout control water (dechlorinated lab water) to determine by toxicity testing if the toxicant was recovered within the methanol eluent.

In some cases, various methanol concentrations can also be utilized. By using various methanol concentrations (i.e. 50, 75, 80, 90 and 100 % methanol) in increasing solvent strength, compounds not associated with the occurrence of toxicity are eliminated, and only that methanol concentration/fraction in which the compound resulting in toxicity is recovered is investigated further with GC/MS.

The results indicated that the toxicant of concern could be recovered in the 100 % methanol eluent. It should also be noted that a TLOE was observed prior to occurrence of mortality, a sublethal characteristic associated with completion of the majority of the compliance/baseline toxicity tests. The methanol elution toxicity test suggested that the toxicant is likely a non-polar organic which is typically a paraffin, a cycloparaffin or some aromatic at pH and is readily dissolved in a 100 % methanol solution given its recovery from the SPE column.

### 3.3.8 Methanol Fractionation

In an attempt to isolate a methanol fraction of interest, four fractions of the 90 % and 100 % methanol elutriates were prepared. To prepare the sub-fractions for fish toxicity testing, the 90 % methanol sample was first concentrated three fold under a nitrogen stream. The concentrated sample was injected into a High Performance Liquid Chromatography (HPLC) instrument equipped with a 25 cm C\textsubscript{18} column (1 cm diameter preparative column) until the sample was consumed.

The mobile phase was methanol/water in a 3:1 ratio delivered at a flow rate of 2 mL·min\textsuperscript{-1}. Fractions were collected from the start of the run, with fraction 1 used as a control, through to the elution of the last peak. In this case there were three discrete peaks, or humps that were collected. Two of the fractions, 2 and 3, showed pH dependent behaviour, meaning that their position in the chromatogram shifted when
the pH of the mobile phase was changed from alkaline to acidic. The peaks moved to a position later in the run in an acidic mobile phase, indicating that they had become less polar with acidification. This would be consistent with an organic acid.

The fractions were then rotary evaporated to remove the methanol. Table 3.1 lists the fraction volumes, which were essentially water with a trace of methanol, and toxicity dependence on pH.

Table 3.1 – Fraction volume and toxicity dependence on pH.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (mL)</th>
<th>pH dependent behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>no as fraction was a blank</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>yes in 90% methanol elutriate</td>
</tr>
</tbody>
</table>

3.3.9 Salt Addition Test

There was some speculation that the addition of salt as sodium chloride might have some mitigative effect on the rainbow trout toxicity response, given preliminary analytical results. Sodium chloride was added to 100% volume process effluent samples at varying concentrations of 50, 250, 500, 1,000 and 10,000 mg·L⁻¹.

All trial salt concentrations demonstrated 100% mortality with the exception of the 50 mg·L⁻¹ concentration which showed 66% mortality and 33% TLOE. The addition of salt did not have any obvious mitigative effect on the toxicant response.

3.3.10 Carbon Filtration

Activated carbon test trials were done with the process effluent samples. The concept was that the toxicant of concern could be absorbed by this catch-all type filter media, particularly useful for organic type contaminants. Testing was also completed as a
preliminary step to see if activated carbon could be utilized as a mitigative strategy to control effluent toxicity. Table 3.2 lists the results of testing samples of known toxic clarifier effluent using a carbon filter with a five minute hydraulic retention time.

Table 3.2 – Effect of carbon filtering at various pH levels versus no carbon filtration.

<table>
<thead>
<tr>
<th>Sample treatment</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH(i) no treatment</td>
<td>0</td>
</tr>
<tr>
<td>pH adjusted to 6.5</td>
<td>100</td>
</tr>
<tr>
<td>carbon filtered at pH(i)</td>
<td>0</td>
</tr>
<tr>
<td>pH adjusted to 6.5, carbon filtered</td>
<td>0</td>
</tr>
<tr>
<td>carbon filtered at pH(i) and then adjusted to pH6</td>
<td>0</td>
</tr>
</tbody>
</table>

The results listed in Table 3.2 show the elimination of rainbow trout toxicity using a 5 minute filtration hydraulic retention time for toxicity tests completed at pH\(i\) and post pH adjusted to 6.5. A blend of 50 % carbon filtered process effluent combined with 50 % non-carbon filtered process effluent did not demonstrate toxicity when the test was completed at pH\(i\) but did demonstrate some level of acute and sublethal (TLOE) toxicity when post pH was adjusted to pH 6.5.

The TIE test trials demonstrated that carbon filtration would provide a useful mitigative strategy.

### 3.3.11 Flocculant Testing

It was speculated that the flocculent used to assist with setting the biomass in the clarifiers could be a source of toxicity to rainbow trout. A solution of mixed liquor from the aeration basin was sampled and allowed to settle with the supernatant decanted off for completion of the rainbow trout acute lethality toxicity test.

Both the process effluent and aeration basin sample were non-lethal (0 % mortality) to rainbow trout at pH\(i\). These results suggested flocculent chemical additive is not a source of toxicity within the process effluent.
The lack of toxicity at pH\textsubscript{i} and the occurrence of toxicity at pH 6.5 within the aeration basin sample provide further evidence that the toxicant of concern is likely not the chemical flocculent added to the clarifier and suggests the toxicant source is likely originating upstream of the wastewater treatment plant.

### 3.3.12 Rainbow Trout Recovery

At pH 6.5 rainbow trout within the process effluent sample showed 100% TLOE within 24 hours of exposure. An experiment was completed to see if the toxicant response could be reversed once the fish showed a TLOE. Affected fish were removed from the test vessel containing process effluent that had been pH adjusted to 6.5 and placed in clean dechlorinated water to see if they would recover from exposure. The experiment proved effective as the affected fish showed complete recovery, with zero mortality and zero TLOE observed by the end of the 96 hour exposure period.

The results demonstrated that once fish exposure to toxicant ceased there was complete recovery, suggesting that toxicant effects do not persist and that the toxicant has an anesthetic characteristic to it. This has also been seen in studies of other toxins [86].

### 3.3.13 Summary of TIE Tests

Table 3.3 summarizes the toxicant characteristics. The results show that the toxicant is organic, the toxicity effect is greater when the pH is lowered and the toxicant is polar at pH\textsubscript{i} and above, which indicates a weak organic acid.
Table 3.3 – Summary of trout toxicity responses to TIE treatment of refinery wastewater samples.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Range</th>
<th>Toxicity Response</th>
<th>Conclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH Adjustment</td>
<td>pH 6.0, 6.5 from pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Increase</td>
<td>pH sensitive, possibly a weak acid</td>
</tr>
<tr>
<td></td>
<td>pH 8.0, 8.5 from pH&lt;sub&gt;i&lt;/sub&gt;</td>
<td>No change</td>
<td>toxicant is not ammonia or an amine</td>
</tr>
<tr>
<td>Aeration</td>
<td></td>
<td>No change</td>
<td>compound is not volatile</td>
</tr>
<tr>
<td>Filtration</td>
<td>1.5 µm filter</td>
<td>No change</td>
<td>toxicant is in dissolved form or particles smaller than 1.5 µm</td>
</tr>
<tr>
<td>C&lt;sub&gt;18&lt;/sub&gt; SPE</td>
<td>Filtration</td>
<td>Decrease</td>
<td>toxicant is organic and extractable with a polar solvent</td>
</tr>
<tr>
<td>Methanol Fractionation of C&lt;sub&gt;18&lt;/sub&gt; SPE</td>
<td>Increase polarity of solvent i.e. methanol:DCM ratio</td>
<td>Increase</td>
<td>toxicant is organic and slightly polar at pH&lt;sub&gt;i&lt;/sub&gt; which indicates a weak acid</td>
</tr>
<tr>
<td>Carbon filtration</td>
<td></td>
<td>Decrease</td>
<td>toxicant is organic</td>
</tr>
<tr>
<td>Zeolite filtration</td>
<td></td>
<td>No change</td>
<td>toxicant is not ammonia, nor an amine nor ionized</td>
</tr>
</tbody>
</table>
3.4 Determination of Naphthenic Acid as the Toxicant

Given that the toxicant of concern was likely a weak organic acid as discussed in Section 3.3.13, the TIE tests were then supplemented by detailed fingerprint of the acid content in the brine from two desalters using Investigative Science Incorporated in Burlington, Ontario and Shell Global Solutions in Houston, Texas. One desalter processed crude with a low TAN of 0.5 mg KOH·L\(^{-1}\) coded crude A, the other processed crude with a high TAN greater than 2 mg KOH·L\(^{-1}\) termed crude B.

To conduct the molecular species breakdown, the following steps were used for extracting naphthenic acid from the wastewater clarifier effluent water [87]:

a) A fifty milliliter (50 mL) aliquot of the sample was taken and the pH of the sample adjusted to less than 3 using 1 N hydrochloric acid (HCl).

b) The pH adjusted sample was then shaken with 12 mL of dichloromethane and the dichloromethane portion was loaded onto an amine solid phase extraction cartridge that had been previously rinsed with dichloromethane.

c) The dichloromethane extraction of the water sample was repeated twice more and the dichloromethane fractions were loaded onto the solid phase extraction cartridge.

d) The cartridge was rinsed with a final aliquot of dichloromethane.

e) The polar fraction was then eluted from the solid phase extraction cartridge using acetone and methanol and collected into a weighed vial. The solvent was then removed and a final dry weight was obtained.

f) The dried samples were then submitted for quantization by infrared spectroscopy followed by Fast Atom Bombardment (FAB) mass spectrometry to determine the
molecular weight distribution. All calculations were based on the 50 mL starting sample assuming the density of water to be 1 g·mL$^{-1}$.

Figure 3.2 shows that the mass spectra from the low TAN desalter brine can be filtered into classes. The brine contains a significant saturate fraction with acid species near 255 and 283 amu as well as mono-cyclic, bi-cyclic, and tri-cyclic acids. The mono-cyclic, bi-cyclic, and tri-cyclic distributions appear to be bimodal for this sample.

![Mass Spectra](image)

**Figure 3.2** – Naphthenic acid species in the low TAN desalter brine outlet.

The high TAN desalter effluent has a significantly smaller saturate fraction peaking near 480 amu, and is dominated by mono-cyclic, bi-cyclic, and tri-cyclic acids. The mono-cyclic, bi-cyclic, tri-cyclic, and tetra-cyclic distributions appear to be unimodal for this sample as shown in Figure 3.3.
3.4.1 Clarifier Effluent

When the cause of the fish toxicity was determined to be naphthenic acid, wastewater effluent testing was increased to once per week; at the same time samples were taken at various locations in wastewater for naphthenic acid analysis to determine the trends and for naphthenic acid compared to the changes in the process which included the desalter.

Figure 3.4 shows the variation in the naphthenic acid concentration in the wastewater effluent and the toxicity levels for both pH\text{I} and pH 6.

The variations in naphthenic acid concentration were later found to be due to the addition of coagulant and flocculent in wastewater treatment which aid in removing solids that transport naphthenic acid and the effects of emulsions and solids from the desalter processing high TAN crude. The details are discussed in Chapters 4 and 5.
3.5 Extracted Naphthenic Acid Toxicity

The naphthenic acid from a high TAN crude was extracted and fractionated into four parts using the procedure discussed in Section 6.1. A series of toxicity tests was performed using trout fingerlings, based on these findings: the LC$_{50}$ values shown in Figure 3.4, the TIE pH test results identified in Section 3.3.2 showing an increased toxicity at pH 6.5 and 6, and the effects of toxicity versus pH and pKa shown in Figure 3.1. The tests involved spiking dechlorinated water with various concentrations of the four extracted naphthenic acid fractions in water at pH$_i$ and pH 6.
The toxicity results showed that fractions 1 and 4 were not toxic at concentrations > 20 mg·L⁻¹ which is approximately eight times the highest concentration of naphthenic acid found in the WWT effluent. Fractions 2 and 3 were toxic at approximately 2 mg·L⁻¹ at pHᵢ and approximately 0.4 mg·L⁻¹ and 0.2 mg·L⁻¹ respectively at pH 6.

Figures 3.5 and 3.6 show the toxicity versus time and concentration of the naphthenic acid fraction.

**Figure 3.5** – Acute toxicity of trout fingerlings when exposed to various concentrations of Fractions 2 and 3 of the extracted naphthenic acid at pHᵢ.

Figures 3.5 shows that fractions 2 and 3 are acutely toxic at concentrations between 2.0 and 4.0 mg·L⁻¹ with fraction 3 exhibiting acute toxicity in a shorter period of time. The naphthenic acid concentrations and mortality are similar to the toxicity levels shown in Figure 3.4.
Figure 3.6 – Acute toxicity of trout fingerlings when exposed to various concentrations of Fractions 2 and 3 of the extracted naphthenic acid at pH 6.

Figure 3.6 in comparison to Figure 3.5 shows that fractions 2 and 3 are acutely toxic at levels approximately 10 times less at pH 6 than at pH$i$. This follows the trend in Figure 3.1. In WWT effluent the difference in toxicity between pH 6 and pH$i$ is approximately five times less.

### 3.6 Acute Toxicological Effects of Naphthenic Acid

To identify the mechanism of toxicity for both lethal and sublethal effects identified by TIE on purified naphthenic acid extracted from high TAN crude, it was decided that a histopathology study should be completed. Histopathology is the study of disease at the cellular level and is probably the most powerful method for determining the effect of toxin exposure on cellular structure [88].

The term disease [89] is used to describe an incorrectly functioning organ, or system of the body, which can be caused by a number of factors including toxicity. Histopathology examinations were performed on the trout fingerlings to determine the
organs and cells that were affected, the severity and nature of the effect, e.g. cell injury, cellular response, or cell death, and whether any background abnormalities were present in the fish before the exposure began which might have affected test results [90].

Various studies show that compounds with $\log K_{OW}$ values between 3 to 4 enter the fish through the gills, and that compounds with $\log K_{OW} > 6$ enter the fish by the dietary route; naphthenic acid $\log K_{OW} > 6$ values vary between 3 to 30 depending on molecular weight [31, 91]. In the case of TIE tests, the fish are not fed. However they do swallow water and histopathology does show that the digestive tract is affected. An example of $\log K_{OW}$ values is shown in Figure 3.7.

![Graph](image)

**Figure 3.7** – $\log K_{OW}$ values for naphthenic acids with $z = -2$ to -12 and $C_0$ to $C_{24}$ [91].

### 3.6.1 Fish Collection

The Ministry of the Environment (MOE) requires toxicity testing of the effluent from the clarifier of the wastewater treatment as part of a certificate of approval (CofA) to operate the plant. Accredited laboratories are required do the testing and the samples in this study were sent to Pollutech in Point Edward, Ontario for trout toxicity tests.
as described in Sections 3.1 and 3.5. In all cases the trout fingerlings were euthanized using recognized protocols [92]. The caudal fin and the operculum were then removed and the ventral body wall slit and preserved in 10 % neutral buffered formalin for shipment to Dr. Gary Marty, a fish pathologist with the Animal Health Center at the British Columbia Ministry of Agriculture in Abbotsford, British Columbia, who performed sample preparation and histopathology. Based on TIE testing discussed in Sections 3.1 through 3.4, three samples of rainbow trout fingerlings were sent to Dr. Marty for analysis. Three histopathology reports were issued between 2013 and 2014 (2013-3505RBT, 2014-0726RBT and 2014-2336RBT) which are summarized in this section. Clinical details and images of the lesions are listed in Appendix C.

### 3.6.2 Sample Preparation

After the fish were received at the Animal Health Center, they were decalcified in 10 % EDTA solution for approximately 46 hours, transected mid-sagittally, and then processed into paraffin.

The fish were embedded so that each slide contained two halves of a single fish including a single section of the caudal peduncle and caudal fin. To ensure broad organ coverage in the sections from all fish, a section was cut from the face of each paraffin block, and then two additional deeper sections were cut at intervals between 200 to 250 µm.

Sections of all organs on the slides were assessed for microscopic features using published methods for systematic histopathology [93, 94]. Table 3.4 shows the scoring for the severity of the lesions.

The data in Table 3.4 was used to score the assessed microscopic features which included three quality control measures, five measures of physiologic condition, and 15 to 17 lesions depending on the test, as outlined in Section 3.7. Mean scores were calculated for each scored item and each exposure group. Trends in mean lesion score
Table 3.4 – Lesion semi-quantitative scores.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0</td>
</tr>
<tr>
<td>small amounts/mild</td>
<td>1</td>
</tr>
<tr>
<td>moderate</td>
<td>2</td>
</tr>
<tr>
<td>abundant/severe</td>
<td>3</td>
</tr>
</tbody>
</table>

differences were identified.

Good examples of lesions were also identified. Due to differences in preservation, plane of section, and section quality, good examples could not be identified for all lesion scores. Details of each scored lesion for each fish are included in Tables 3.11, 3.12 and 3.13.

Organs not listed in the tables had no significant lesions.

3.7 Analysis of TIE Treatments and Fish Exposures to Naphthenic Acid

A total of three groups of rainbow trout were examined using histopathology. Two groups were from the TIE testing of fish exposed to clarifier effluent as described in Chapter 3, and one group was exposed to purified naphthenic acid fractions extracted from high TAN crude. The procedure for extraction and purification is discussed in Sections 6.1.1 and 6.1.2. Details of the groups are listed below.

A total of 90 fish were used to study the treatments listed in Table 3.5; 45 were controls and 45 were exposed to the clarifier effluent with the listed TIE treatments. The purpose of this study was to determine how the histopathology changed for a given TIE treatment.
Table 3.5 – Histopathology Group 1, trout exposed to TIE tests of clarifier effluent.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Changes</th>
<th>Number of fish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exposed</td>
</tr>
<tr>
<td>pH</td>
<td>pH(_i) no adjustment</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>adjustment to pH 3 then pH 6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>adjustment to pH 11 then pH 6</td>
<td>3</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>4 mL(\cdot)L(^{-1}) adjusted to pH 6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8 mL(\cdot)L(^{-1}) adjusted to pH 6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>16 mL(\cdot)L(^{-1}) adjusted to pH 6</td>
<td>3</td>
</tr>
<tr>
<td>1.5 µm</td>
<td>filtered and adjusted to pH 6</td>
<td>5</td>
</tr>
<tr>
<td>aeration</td>
<td>aerated and adjusted to pH 6</td>
<td>5</td>
</tr>
</tbody>
</table>

Histopathology study 2 used 3 fish as controls and 21 fish exposed to TIE treatments listed in Table 3.6. The purpose of this study was to confirm the treatments in Group 1 and to determine if metals in the clarifier effluent were involved by using Ethylenediaminetetraacetic acid (EDTA) which is used to chelate metals.

Table 3.6 – Histopathology Group 2, trout exposed to TIE tests of clarifier effluent.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Changes</th>
<th>Number of fish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Exposed</td>
</tr>
<tr>
<td>pH</td>
<td>pH(_i) no adjustment</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>adjustment to pH 6</td>
<td>3</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>4 mL(\cdot)L(^{-1}) adjusted to pH 6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8 mL(\cdot)L(^{-1}) adjusted to pH 6</td>
<td>3</td>
</tr>
<tr>
<td>EDTA</td>
<td>4 mL(\cdot)L(^{-1}) adjusted to pH 6</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>8 mL(\cdot)L(^{-1}) adjusted to pH 6</td>
<td>3</td>
</tr>
<tr>
<td>1.5 µm</td>
<td>filtered and adjusted to pH 6</td>
<td>3</td>
</tr>
</tbody>
</table>

The purpose of study 3 was to determine if the results of fish exposed to toxic
concentrations of extracted naphthenic acid fractions at pH 6 were the same as those found at pH$i$. Nine fish were used as controls and 27 fish were exposed to three fractions and two concentrations which are listed in Table 3.7.

**Table 3.7 –** Histopathology Group 3, trout exposed to extracted acid fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentration (mL·L$^{-1}$)</th>
<th>Number of fish</th>
<th>Exposed</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.2</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.2</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.2</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>3</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

The organs and associated lesions found in rainbow trout fingerlings that were exposed to naphthenic acid were grouped into three categories; those found only in fish exposed to naphthenic acid, lesions more common or severe in exposed fish, and the lesions that could not be associated with naphthenic acid exposure. Tables 3.8, 3.9, and 3.10 list the organs and lesions of the three categories. A detailed description of the lesions is found in Appendix C.

Tables 3.11, 3.12 and 3.13 are the summary of the lesions and the mean of the scores found in the three lesion categories. The values with a background colour in **green** are the average of the lesions found in control fish while those in **light red** are values that are statistically greater than the controls.

The lesions in Tables 3.11, 3.12 and 3.13 show that there are multiple pathways for naphthenic acid into the various organs, including ingestion of solid particles and diffusion through the gills and skin.
### Table 3.8 – Lesions only occurring in exposed fish.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>cerebellar edema (CBE)</td>
</tr>
<tr>
<td>Gills</td>
<td>lamellar epithelial hydropic degeneration (LHD)</td>
</tr>
<tr>
<td></td>
<td>pseudobranch single cell necrosis (PBN)</td>
</tr>
<tr>
<td>Kidney</td>
<td>tubular intraluminal protein casts (ITP)</td>
</tr>
<tr>
<td></td>
<td>endothelial cell hydropic degeneration (REH)</td>
</tr>
<tr>
<td></td>
<td>renal hematopoietic cell atrophy (RHA)</td>
</tr>
<tr>
<td></td>
<td>renal tubular epithelial protein droplets (TEP)</td>
</tr>
<tr>
<td>Liver</td>
<td>hepatocellular hydropic degeneration (HHD)</td>
</tr>
<tr>
<td></td>
<td>single cell hepatocellular necrosis (SCN)</td>
</tr>
<tr>
<td>Stomach</td>
<td>gastric wall vacuolation (GWV)</td>
</tr>
</tbody>
</table>

### Table 3.9 – Lesions more common or severe in exposed fish.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kidney</td>
<td>renal interstitial hydropic degeneration/single cell necrosis (RIH)</td>
</tr>
<tr>
<td></td>
<td>renal interstitial cell karyorrhexis (RIK)</td>
</tr>
<tr>
<td></td>
<td>interstitial cell karyorrhexis (ICK)</td>
</tr>
<tr>
<td>Kidney and Spleen</td>
<td>erythrophagocytosis (EPT)</td>
</tr>
<tr>
<td>Stomach</td>
<td>gastric gland single cell degeneration and necrosis (GSD)</td>
</tr>
<tr>
<td>Intestine</td>
<td>intestinal epithelial cell single cell degeneration and necrosis (ISD)</td>
</tr>
</tbody>
</table>
Table 3.10 – Lesions not associated with exposure.

<table>
<thead>
<tr>
<th>Organ</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>hemorrhage (BHM)</td>
</tr>
<tr>
<td>Intestine</td>
<td>intestinal bacterial overgrowth (IBO)</td>
</tr>
<tr>
<td>Kidney</td>
<td>renal tubular hydropic degeneration (RTH)</td>
</tr>
<tr>
<td>Liver</td>
<td>biliary ductular hyperplasia (BDH)</td>
</tr>
<tr>
<td></td>
<td>focal leukocytic hepatitis (FHL)</td>
</tr>
<tr>
<td></td>
<td>vacuoles in hepatocellular cytoplasm (VAC)</td>
</tr>
<tr>
<td>Yolk</td>
<td>yolk sac macrophage (YSM)</td>
</tr>
<tr>
<td></td>
<td>yolk sacculitis (YSI)</td>
</tr>
</tbody>
</table>

Table 3.11 – Lesion scores found with fish exposed to carbon filter inlet at pH\textsubscript{i} and pH 6 and control fish in dechlorinated water at pH\textsubscript{i} and pH 6. This was the first histopathology analysis.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Dechlor</th>
<th>Carbon in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH\textsubscript{i}</td>
<td>pH 6</td>
</tr>
<tr>
<td>VAC</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>FHL</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>BDH</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>GSD</td>
<td>0.3</td>
<td>0.0</td>
</tr>
<tr>
<td>IBO</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>TEP</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RIH</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RIK</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>ITP</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>RHA</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>REH</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>EPT</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>YSI</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>CBE</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>BHM</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>
Table 3.12 – The following lesion scores were found in test two which consisted of various TIE treatments to determine if there were co-factors to naphthenic acid toxicity. This was the second histopathology analysis. D = too decomposed for analysis.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>Dechlor</th>
<th>Carbon In</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>8 ml·L⁻¹</th>
<th>16 ml·L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH⁶ to pH⁶</td>
<td>pH⁶ to pH⁶</td>
<td>Na₂S₂O₃ pH⁶ filter</td>
<td>pH⁶ filter</td>
<td>EDTA pH⁶</td>
<td>EDTA pH⁶</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VAC</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.7</td>
<td>0.3</td>
<td>0.5</td>
<td>0.3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>FHL</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>GSD</td>
<td>0.3</td>
<td>1.0</td>
<td>1.3</td>
<td>1.5</td>
<td>1.0</td>
<td>1.0</td>
<td>2.0</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td>GWV</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>IBO</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>TEP</td>
<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>1.7</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>RHA</td>
<td>0.0</td>
<td>0.7</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>0.5</td>
<td>1.3</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>REH</td>
<td>0.0</td>
<td>2.3</td>
<td>1.7</td>
<td>2.0</td>
<td>2.0</td>
<td>1.5</td>
<td>2.0</td>
<td>2.3</td>
<td></td>
</tr>
<tr>
<td>EPT</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>CBE</td>
<td>0.0</td>
<td>2.0</td>
<td>0.0</td>
<td>1.7</td>
<td>2.0</td>
<td>1.0</td>
<td>2.3</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>BHM</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>LHD</td>
<td>0.0</td>
<td>D</td>
<td>1.3</td>
<td>D</td>
<td>D</td>
<td>1.0</td>
<td>0.7</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>PBN</td>
<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
<td>0.0</td>
<td>0.3</td>
<td>0.5</td>
<td>1.0</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>YSM</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.7</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>
Table 3.13 – The following lesion scores were found in test three which consisted of purified fractions 1, 2 and 3 of extracted naphthenic acid from high TAN crude. The methanol fraction (MeOH) was used as a control. This was the third histopathology analysis.

<table>
<thead>
<tr>
<th>Lesion</th>
<th>MeOH (mL·L⁻¹)</th>
<th>Fraction 1 (mL·L⁻¹)</th>
<th>Fraction 2 (mL·L⁻¹)</th>
<th>Fraction 3 (mL·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VAC</td>
<td>0.0 0.0</td>
<td>0.7 1.0 1.0</td>
<td>1.0 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>HHD</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>1.7 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>SCN</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>1.3 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>GSD</td>
<td>0.3 0.3</td>
<td>0.3 0.7 0.3</td>
<td>1.3 0.5 0.7</td>
<td>1.0 0.0 0.0</td>
</tr>
<tr>
<td>GWV</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>1.0 0.0 0.7</td>
<td>1.7 0.0 0.0</td>
</tr>
<tr>
<td>IBO</td>
<td>0.0 0.3</td>
<td>0.0 0.3 0.0</td>
<td>0.0 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>ISD</td>
<td>1.0 1.0</td>
<td>0.7 1.0 1.0</td>
<td>1.3 1.0 0.7</td>
<td>1.3 D D D D</td>
</tr>
<tr>
<td>TEP</td>
<td>0.3 0.0</td>
<td>0.0 0.0 0.0</td>
<td>1.3 0.0 0.3</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>ICK</td>
<td>0.0 0.3</td>
<td>0.7 0.3 0.0</td>
<td>0.3 0.3 2.0</td>
<td>2.0 1.7 1.7</td>
</tr>
<tr>
<td>RHA</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>1.0 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>REH</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>2.3 0.3 0.3</td>
<td>1.0 0.0 D D D D</td>
</tr>
<tr>
<td>RTH</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>0.3 0.0 0.0</td>
<td>0.3 0.0 0.0</td>
</tr>
<tr>
<td>EPT</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>0.7 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
</tr>
<tr>
<td>CBE</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>1.3 1.0 0.3</td>
<td>1.0 0.7 0.7</td>
</tr>
<tr>
<td>BHM</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>0.3 0.0 0.0</td>
</tr>
<tr>
<td>LHD</td>
<td>0.0 0.0</td>
<td>0.0 0.0 0.0</td>
<td>0.0 D 0.5 0.3 D D D D</td>
<td></td>
</tr>
<tr>
<td>YSM</td>
<td>0.7 1.0</td>
<td>0.0 0.7 0.7</td>
<td>0.3 0.7 0.3</td>
<td>0.0 0.7 0.7</td>
</tr>
</tbody>
</table>
3.8 Histopathology Summary

The significant lesions in the fish from all three groups were the result of exposure to naphthenic acid in the water, which affected the gills and organs associated with the bloodstream and digestive system. Toxin(s) may have been absorbed from the water through the gills, stomach or skin. For all of these lesions the affected organs were able to recover when the fish were removed from the water with naphthenic acid as found in earlier TIE experiments discussed in Section 3.3.12.

3.9 Weak Acid Transport Across a Cell Lipid Bilayer

Overton’s Rule defines the permeability \( P \) (cm·s\(^{-1}\)) of any solute across a cell lipid bilayer as

\[
P = \frac{K \cdot D}{\Delta x} \tag{3.1}
\]

where \( K \) is the partition coefficient of the solute into the hydrocarbon core of the lipid bilayer, \( D \) (cm\(^2\)·s\(^{-1}\)) is the diffusion coefficient of the solute into the hydrocarbon core of the lipid bilayer, and \( \Delta x \) (cm) is the thickness of the cell membrane. The diffusion coefficient \( D \) is a measure of the rate of entry into the cytoplasm depending on the molecular weight or size of a solute. A low value of \( K \) describes a molecule like water that is not soluble in a lipid; a weak acid has a variable value for \( K \) depending on pH.

To explain a cell’s absorption of a weak acid such as naphthenic acid, \[25\] the Henderson-Hasselbach equation can be used to determine how the value of \( K \) in Equation 3.1 changes with pH. The Henderson-Hasselbach equation is given as:
\[ \text{pH} = \text{pKa} + \log_{10} \left( \frac{[A^-]}{[HA]} \right) \]  

where pKa is the \( \log_{10} \) of the acid disassociation constant \( K_a \), \([A^-]\) is the molar concentration (mol·L\(^{-1}\)) of the disassociated form of a weak acid and \([HA]\) is the molar concentration (mol·L\(^{-1}\)) of the associated form of a weak acid. A graph of various weak acid pKa values in the region of 5.1 versus the disassociated acid percentages is shown in Figure 3.8.

Figure 3.8 shows that the concentration of disassociated acid is a strong function of pH for a weak carboxylic acid. This effect is seen as an increase in acute fish toxicity when the pH is lowered to 6 from pH\(_i\).

Figure 3.8 – Acid pKa between 4.9 and 5.3 versus disassociated acid percentage for a given pH value.

Figure 3.9 illustrates the effect of pH on the ability of a weak organic acid to
be transported through the lipid bilayer of a cell. The possible mechanisms that can interrupt the internal working of a cell are shown.

Figure 3.9 – An overview of organic acid toxicity and tolerance mechanisms of a lipid bilayer of an *E. coli* bacteria. Diffusion of undisassociated acid molecules occurs freely in an acidic medium but is limited at neutral or basic pH as shown in Figure 3.1. The toxic effects associated with organic acids are the result of both anion specific effects on metabolism as well as increased internal proton concentrations. Effects on internal pH are mitigated by transport of protons out of the membrane, and consumption of protons by decarboxylation reactions [96].

Similar methods of weak acid transport have been described for eukaryote cells [97, 98, 99].
3.10 Microtox

Although trout fingerlings will show the effects of acute naphthenic acid toxicity at pH 6 at a faster rate than at pH$i$, that test can take up to 96 hours to show the mortality. Analytical techniques to determine naphthenic acid concentrations that could be used to predict toxicity of the wastewater effluent take hours to perform and interpret.

A method that uses a bioluminescent bacteria, *Vibrio fischeri*, to determine toxicity is called Microtox [100]. In this test if the bacteria light production falls off after being exposed to a water sample it shows that toxicants may be present. An increase in light output indicates a nutrient is in the sample. Examples of the bioluminescent response to toxicity [101] are shown in Figure 3.10.

In Figure 3.10 the red trend lines with the label 'conc. A' have a higher concentration of a given toxicant than the blue trend line labeled 'conc. B'.

The advantage of Microtox is that it is a rapid and simple test, although the results must be correlated with the toxicity effects of the organism under study; in the case of this study the organism is trout fingerlings. A series of tests with refinery wastewater samples versus fish toxicity tests at pH 6 and pH$i$ showed the Microtox test [102] does not differentiate acute toxicity effects of naphthenic acid on trout fingerlings. This data is presented in Figure 3.11.

The toxicity identification evaluation (TIE) showed that organic acid primarily composed of naphthenic acid was the cause of acute toxicity of the wastewater effluent. This work was required to clearly identify the toxicant and any co-factors as the matrix of material in the wastewater effluent can include organic and inorganic recalcitrant material. The ecotoxicity testing showed that water fleas were not effected to the same extent as the trout fingerlings.
Figure 3.10 – The images above are typical bacterial responses to toxins in the Microtox test. (a) phenol. (b) organic compounds. (c) metals. Copied from various figures in [101].
Figure 3.11 – Microtox results with fish toxicity at pH 6 which show no differentiation between controls and naphthenic acid concentrations that are toxic to fish.
Chapter 4

Wastewater Treatment Analysis

The wastewater treatment system of a refinery was analyzed to determine the sources of naphthenic acid, the mass balance of naphthenic acids at various locations in the system, and biodegradability within the residence time of the system, which is 8 hours. The bacteria population 16S rRNA hypervariable region V4 was analyzed for future reference in designing a biotreatment system for oil coated solids which is discussed in Chapter 7. Two papers “Naphthenic Acid Analysis of an Oil Refinery Wastewater Treatment Samples” and “Study of Microbial Community in an Oil Refinery Wastewater System using 16S rRNA Hypervariable Region V4 Analysis” are being prepared based on information in this chapter.

4.1 Wastewater Treatment Description

Refining crude oil requires that water be available for cooling, steam generation, and removing water soluble contaminants from crude in order to minimize fouling and corrosion. Contaminants include amine, which is used in upstream operations to scavenge H$_2$S, and solids which are typically minerals and salts. A block diagram of the equipment required to treat the water prior to discharge to public waterways is
shown in Figure 4.1.

**Figure 4.1** – Sample locations in the refinery wastewater treatment system for naphthenic acid mass balance study. Sample locations are identified in Tables 4.1, 4.2 and 4.3.

These varied uses of water result in some water streams that are in intimate contact with hydrocarbons, and other streams that have minimal or no contact with hydrocarbon sources. This means that there are typically two sewer streams in a refinery, one that is high in contaminants and the other that is low in contaminants. Typically the sewers are separated for safety due to flammable and toxic gases such as $\text{H}_2\text{S}$ and sizing of separation equipment. For this thesis the term process sewer will refer to water that has been in contact with hydrocarbons and contaminated sewer will refer to water that has had minimal contact with hydrocarbon. Typical hydrocarbon concentrations and speciation are listed in Appendix D.

Both sewer streams are first treated by primary separation equipment to remove
undissolved oil and any solids before the water is biologically treated. The primary separation for the process sewer requires a more involved process than the contaminated sewer as there is a higher probability of undissolved oil and solids, primarily from the desalter. The cyclone separator removes solids and oil from the process sewer water; the oil and solids are routed to a sump and the water effluent is pumped via P3 to an equalization tank.

The equalization tank is used to allow further separation of the water from the cyclone separator and to act as a surge tank. The effluent is then sent to a Hydrocoell® which is an induced gas floatation separator that uses nitrogen to create bubbles that adhere to the solids which are then skimmed off the top.

The activated sludge is the biological part of the wastewater system, used for the treatment of liquid wastes in the refinery. The sludge is held in two aeration basins, which are connected in series, and consists of floc that contains bacteria of several taxonomic groups, solids, and hydrocarbons not dissolved in the water. A given floc concentration expressed as g·L\(^{-1}\) is maintained in the aeration basins to allow for biodegradation of hydrocarbons, sulphur, and nitrogen compounds. This is done by allowing the floc from the effluent of the aeration basins to settle in clarifiers and recirculating that material back to the aeration basins. Figure 4.2 shows the mixture of microbes, solids and hydrocarbons within the floc.

The mass of floc in the aeration basins is termed mixed liquor suspended solids (MLSS). The mass of MLSS is determined by filtering a known volume of mixed liquor suspended solids through a 1.5 \(\mu\)m glass filter. The volatile portion of the floc is termed mixed liquor volatile suspended solids (MLVSS) which is determined by burning the MLSS sample and filtering at 500 °C for 30 minutes. The MLVSS is considered to be the mass of the microbes with include bacteria (prokaryotes), protozoa (eukaryotes) and metazoa (multi-cellular eukaryotes) in the aeration basins.

During heavy rain falls water must be emptied from storage tank dykes, ditches
Figure 4.2 – Bacteria and solids in clarifier effluent. (a) Solids and unstained bacteria and (b) Gram stained bacteria from clarifier effluent.

and process units to prevent flooding. Since that water cannot be treated without overwhelming the aeration basins it is stored in an impounding basin and treated at a later date.
The clarifiers are designed to allow the floc from the aeration basins to gravity separate from the treated water. The clarifier effluent is then passed through carbon filters to remove naphthenic acid.

The electrical equivalent circuit of the wastewater treatment system is shown in Appendix E.

4.2 Naphthenic Acid Sources

The primary source of naphthenic acid in a refinery wastewater treatment system is from brine created during the desalting operation as discussed in Chapter 5. The purpose of a desalter is to mix water with crude oil to remove salts and solids. The mixing operation is performed with a mixing valve which forms an emulsion due to the shear force, oil, water, solids and naturally occurring or manmade surfactants. Emulsions $> 10 \mu m$ in diameter should coalesce with water, salts and solids exiting the bottom of the desalter while the oil is taken off of the top of the desalter. If the emulsions are less than this diameter or are stable (do not coalesce), they can cause oil to be carried with the brine which increases the naphthenic acid concentration in the wastewater treatment system.

4.3 Naphthenic Acid Mass Balance in an Oil Refinery Wastewater Treatment System

Sample volumes of 2 L were collected at various locations within the wastewater treatment system to determine the naphthenic acid mass balance. This was done to determine the sources of naphthenic acid and to account for the naphthenic acid mass reduction between the desalters and the clarifier effluent in the wastewater system that could not be explained by dilution. At the same time a hydrocarbon mass balance was used to understand in further detail the effects of hydrocarbon distribution versus
oxygen demand and any correlation between naphthenic acid and the hydrocarbon concentrations. The hydrocarbon analysis is shown in Appendix D.

The analysis of the samples for naphthenic acid concentration is shown in Section 6.1.4. The liquid flows were determined at specific points in WWT, with either installed flow transmitters used for operations personnel to monitor and control the process, or a temporary clamp-on ultrasonic flow meter to obtain the required data.

The mass balance of naphthenic acid in the wastewater treatment system was done on three occasions at selected points in the wastewater treatment of the refinery. The data from these tests are shown in Tables 4.1, 4.2 and 4.3. The locations are shown in Figure 4.1.

4.3.1 First Naphthenic Acid Mass Balance Test

The first naphthenic acid mass balance test is shown in Table 4.1 and was done to determine possible sources of naphthenic acid. While the main source of naphthenic acid was from the desalter brine, due to processing high TAN crude, other water sources come in contact with the crude. One suspected source was water at the bottom of the storage tanks, which is routed to the contaminated sewer for treatment in the wastewater treatment plant. The results of the first mass balance test showed that there were no sources of naphthenic acid from the contaminated sewer (Wemco® influent/effluent).

The variance of naphthenic acid mass between the desalter brine and the Hydrocell® is due to solids, oil and emulsion separation in the cyclone separator and equalization tanks. This is seen in the 58% reduction of mass between the influent and effluent of the Hydrocell®. The reduction is due to the skimming of oil/solids out of the Hydrocell®.

The two aeration basins show a higher mass of naphthenic acid than can be explained by the Hydrocell® effluent, i.e. the mass in the aeration basins is approximately 670% higher than in the Hydrocell®. This is caused by the volume of
Table 4.1 – First mass balance of naphthenic acid on April 30 2014 in a refinery wastewater treatment system. *ND* refers to a sample with a naphthenic acid concentration < 0.1 mg·L\(^{-1}\). The water flow and mass flow values have been normalized.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water Flow (m(^3)·d(^{-1}))</th>
<th>Naphthenic acid Concentration (mg·L(^{-1}))</th>
<th>Mass flow (kg·d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desalter effluent</td>
<td>0.025</td>
<td>14.9</td>
<td>0.282</td>
</tr>
<tr>
<td>Hydrocell® influent</td>
<td>0.220</td>
<td>1.5</td>
<td>0.238</td>
</tr>
<tr>
<td>Hydrocell® effluent</td>
<td>0.220</td>
<td>0.9</td>
<td>0.143</td>
</tr>
<tr>
<td>Wemco® influent</td>
<td>0.577</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td>Wemco® effluent</td>
<td>0.577</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td>Aeration basin 1 effluent</td>
<td>0.794</td>
<td>1.7</td>
<td>1.000</td>
</tr>
<tr>
<td>Aeration basin 2 effluent</td>
<td>0.975</td>
<td>1.3</td>
<td>0.924</td>
</tr>
<tr>
<td>Clarifier effluent</td>
<td>0.971</td>
<td>0.2</td>
<td>0.182</td>
</tr>
<tr>
<td>Carbon filter effluent</td>
<td>0.376</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td>Carbon filter backwash</td>
<td>0.085</td>
<td>0.4</td>
<td>0.022</td>
</tr>
<tr>
<td>North impounding rework</td>
<td>0.170</td>
<td>0.2</td>
<td>0.032</td>
</tr>
<tr>
<td>Process effluent</td>
<td>1.000</td>
<td>ND</td>
<td>0.000</td>
</tr>
</tbody>
</table>

solids and undissolved free oil that is entrained in the floc from the desalter over time. Floc in the aeration basin contains naphthenic acid bound in solids and emulsions. The solids and mass are slowly taken out of the system by the wasted activated sludge (WAS) stream between the clarifiers and the aeration basins.

4.3.2 Second Naphthenic Acid Mass Balance Test

As with the first naphthenic acid mass balance test no sources of naphthenic acid were found in the contaminated sewer. However, there was an indication of another source of the acid between the desalter outlet and the Hydrocell® which is used for the process sewer primary separation. Table 4.2 shows the results of that test.

This test was carried out to determine the reason for the higher mass of naphthenic acid in the Hydrocell® in comparison to the desalter effluent. The cause was determined to be carry over of the solids and oil from the equalization tank, shown in Figure 4.1.
Table 4.2 – Second mass balance of naphthenic acid on September 9 2014 in a refinery wastewater treatment system. *ND* refers to a sample with a naphthenic acid concentration < 0.1 mg·L\(^{-1}\). The water flow and mass flow values have been normalized.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water Flow (m(^3)·d(^{-1}))</th>
<th>Naphthenic acid Concentration (mg·L(^{-1}))</th>
<th>Mass flow (kg·d(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desalter effluent</td>
<td>0.029</td>
<td>15.0</td>
<td>0.125</td>
</tr>
<tr>
<td>Hydrocell(^{\circledR}) influent</td>
<td>0.245</td>
<td>2.7</td>
<td>0.194</td>
</tr>
<tr>
<td>Hydrocell(^{\circledR}) effluent</td>
<td>0.245</td>
<td>2.6</td>
<td>0.189</td>
</tr>
<tr>
<td>Wemco(^{\circledR}) effluent</td>
<td>0.637</td>
<td><em>ND</em></td>
<td>0.000</td>
</tr>
<tr>
<td>Aeration basin 1 effluent</td>
<td>0.882</td>
<td>3.8</td>
<td>1.000</td>
</tr>
<tr>
<td>Aeration basin 2 effluent</td>
<td>1.000</td>
<td>2.9</td>
<td>0.843</td>
</tr>
<tr>
<td>Clarifier effluent</td>
<td>1.000</td>
<td>0.5</td>
<td>0.084</td>
</tr>
<tr>
<td>Clarifier recycle stream</td>
<td>0.132</td>
<td>3.2</td>
<td>0.125</td>
</tr>
<tr>
<td>Carbon filter effluent</td>
<td>0.490</td>
<td>0.2</td>
<td>0.028</td>
</tr>
<tr>
<td>Carbon backwash</td>
<td>0.076</td>
<td>0.4</td>
<td>0.148</td>
</tr>
<tr>
<td>North impounding rework</td>
<td>0.119</td>
<td>0.1</td>
<td>0.005</td>
</tr>
<tr>
<td>Process effluent</td>
<td>1.000</td>
<td>0.2</td>
<td>0.023</td>
</tr>
</tbody>
</table>

The equalization tank has a large volume of solids that rise above the inlet line from P3 and the outlet line to the Hydrocell\(^{\circledR}\). This resulted in channeling of the flow through the tank, and the channels are subject to collapse, leading to entrainment of oil-coated solids, emulsion and oil.

### 4.3.3 Third Naphthenic Acid Mass Balance Test

The third naphthenic acid mass balance test was done during a period of time when:

a) there was a low solids concentration of < 60 mg·L\(^{-1}\) in the crude feed to the desalter,

b) the cyclone separator was experiencing poor solids separation due to a plugged solids outlet line,

c) the Hydrocell\(^{\circledR}\) flocculent injection was out of service.
Table 4.3 shows the results of that test.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Water Flow (m³·d⁻¹)</th>
<th>Naphthenic acid Concentration (mg·L⁻¹)</th>
<th>Mass flow (kg·d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desalter effluent</td>
<td>0.031</td>
<td>4.3</td>
<td>1.000</td>
</tr>
<tr>
<td>P3 outlet</td>
<td>0.255</td>
<td>5.4</td>
<td>0.994</td>
</tr>
<tr>
<td>Hydrocell® influent</td>
<td>0.275</td>
<td>2.1</td>
<td>0.422</td>
</tr>
<tr>
<td>Hydrocell® effluent</td>
<td>0.275</td>
<td>3.1</td>
<td>0.610</td>
</tr>
<tr>
<td>Wemco® effluent</td>
<td>0.666</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td>Aeration basin 2 effluent</td>
<td>1.000</td>
<td>1.2</td>
<td>0.896</td>
</tr>
<tr>
<td>Clarifier effluent</td>
<td>1.000</td>
<td>0.2</td>
<td>0.136</td>
</tr>
<tr>
<td>Carbon filter effluent</td>
<td>0.648</td>
<td>ND</td>
<td>0.000</td>
</tr>
<tr>
<td>Process effluent</td>
<td>1.000</td>
<td>ND</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The third test indicated that at low solids concentrations in the crude feed to the desalter, there was a lower naphthenic acid concentration in the desalter effluent. Further testing has shown that solids removal from the desalter brine is critical to lower naphthenic acid concentration. The increase of naphthenic acid concentration between the Hydrocell® outlet versus the inlet was due to solids and oil from the inside walls of the Hydrocell® being sloughed off. The concentration drop across the clarifier, i.e. aeration basin 2 effluent to clarifier effluent, is due to flocculent injection which binds the floc together to minimize solids to the clarifier effluent.

4.3.4 Mass Balance Summary

The three naphthenic acid mass balance tests indicated that there was variation across the wastewater treatment system, i.e. there were not fixed ratios between the naphthenic acid mass from the desalter outlet and the clarifier effluent. Further
investigation indicated that the naphthenic acid mass from the desalter varied between 5.4 and 15.5 kg·d$^{-1}$. The cause of the variation was found to be varying concentrations of oil coated solids and emulsions contained in the desalter brine under-carry. This was determined by visually monitoring the colour and turbidity of the desalter brine in clear 1 L sample bottles and comparing those observations to the naphthenic acid concentration determined in the laboratory.

The laboratory test for measuring the naphthenic acid concentration is described in Section 4.6.

### 4.4 Naphthenic Acid Transport Mechanism in a Refinery

Although there are a number of variables such as brine pH and percentage of high TAN crude in the desalter feed that can cause naphthenic acid concentration to increase in the desalter brine, the primary transport mechanisms are oil coated solids, containing naphthenic acid on the oil film, and stable emulsions; both of which are carried in the desalter brine. This relationship was suspected during analysis of the three naphthenic acid mass balance tests described in Section 4.3. To study this relationship various concentrations and types of coagulant and flocculent were added to samples of desalter effluent in laboratory experiments that showed the strong effect of both additives; the results are discussed in Section 4.6.

### 4.5 Solids Wetting Agent Addition to Desalter Feed

Solids wetting agents (SWA) are designed to water-wet oil-coated solids making them less likely to stabilize emulsions, and thereby reducing the probability that they would carry naphthenic acids into wastewater, having been removed prior to the aeration basin.
Based on the finding that the primary transport mechanisms of naphthenic acid concentration in desalter brine were via solids and emulsions, field tests of SWA were performed. The test period was from July 2015 through December 2015, and the tests were performed using an injection rate for SWA of 9 - 15 $\mu$L per liter of crude oil. The injection site was ahead of the desalter shown in Figure 4.1.

To ensure that the SWA would not be a source of toxicity in the WWT plant at the proposed injection rates, a laboratory test was performed by adding SWA at a concentration of 20 $\mu$L$^{-1}$ into a 10 L bioreactor containing samples of influent to the aeration basins and microbes from the aeration basins. After a period of time the effluent from the bioreactors was used for a rainbow trout toxicity test. The results indicated that the SWA had an LC$_{50}$ of 40 $\mu$L$^{-1}$ which is above the maximum injection rate of 15 $\mu$L$^{-1}$ per m$^3$ of crude oil.

The removal of water-wetted solids from the desalter brine takes place in the cyclone separator, equalization tank and primary separation shown in Figure 4.1 and discussed in section. Figure 4.3 on the next page shows the effect of SWA addition.

### 4.6 Desalter Brine Treatment

During the processing of high TAN crude solids, stabilized emulsions can be formed and carried out with the brine to the wastewater treatment system; Chapter 5 describes this in detail. The solids and emulsions can cause naphthenic acid toxicity of the wastewater effluent as retention times in a refinery wastewater system do not allow for biodegradation of naphthenic acid. The pre-treatment of the brine with standard coagulant and flocculent chemistry can decrease the naphthenic acid concentration by 80%.
Figure 4.3 – Naphthenic acid concentration in clarifier effluent before and after solids wetting agent addition. The concentration after the addition of the solids wetting agent is at a much lower and relatively constant value.

Table 4.4 lists the results of laboratory experiments to test the effects of coagulant and flocculent on desalter brine to lower the naphthenic acid concentration by minimizing the concentration of emulsions and solids. The laboratory work is supported by the mass balance tests described in Section 4.3.

Table 4.5 on page 68 lists the solid particle size versus concentration of brine from a desalter processing high TAN crude. The solids that are greater than 1.22 µm are normally bound in oil due to viscosity while the particles below this value are part of the emulsion. The brine volume is the maximum volume that would pass through a given filter without plugging.
Table 4.4 – Naphthenic acid concentration reduction after treating desalter brine with various combinations of coagulants and flocculents. C1 and C2 are commercial metallic coagulants. F1, F2 and F3 are commercial polymer based flocculents.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FeCl$_3$ (1295 mg·L$^{-1}$)</th>
<th>NaOH (30%)</th>
<th>C1 (µL/L)</th>
<th>C2 (µL/L)</th>
<th>F1 (µL/L)</th>
<th>F2 (µL/L)</th>
<th>F3 (µL/L)</th>
<th>Naphthenic acid concentration (mg·L$^{-1}$)</th>
<th>Reduction of naphthenic acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>2.7</td>
<td>86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>3.0</td>
<td>85</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>3.7</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>3.8</td>
<td>81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>3.5</td>
<td>82</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>Y</td>
<td>2.1</td>
<td>89</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>2.5</td>
<td>2.5</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>5</td>
<td>6.1</td>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>2.5</td>
<td>2.9</td>
<td>86</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Y</td>
<td>7.5</td>
<td>2.5</td>
<td>88</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4.5 – Solid concentration versus size in a sample of desalter brine.

<table>
<thead>
<tr>
<th>Filter size (µm)</th>
<th>Solids (mg·L⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>647</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>1.22</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>7</td>
</tr>
<tr>
<td>0.45</td>
<td>11</td>
</tr>
<tr>
<td>0.22</td>
<td>2</td>
</tr>
<tr>
<td>0.1</td>
<td>52</td>
</tr>
<tr>
<td>0.05</td>
<td>10</td>
</tr>
<tr>
<td>0.025</td>
<td>2</td>
</tr>
</tbody>
</table>

4.7 Naphthenic Acid Toxicity and Biodegradability in an Oil Refinery Wastewater Treatment System

The disassociation constant (pKa) of naphthenic acid is approximately 5.1 which by definition makes it a weak acid and therefore acute toxicity is a strong function of the water pH as described in Section 3.3.2.

Naphthenic acid is biodegradable by some species of aerobic microbes within 7 to 204 days [68]. However, the refinery waste water treatment residence time is 8 hours. Therefore biodegradation does not occur to any appreciable extent. This has been proven in a number of bioreactors that were spiked with naphthenic acid concentrations derived from high [TAN] crude samples.
4.7.1 Bioreactor Tests

Seven 10 L bioreactors containing biomass from the aeration basins and wastewater from a refinery were spiked with known toxic concentrations of naphthenic acid and operated for seven days to determine if:

a) the microbes in the bioreactors would be affected; based on monitoring the oxygen uptake rate (OUR),

b) the naphthenic acid concentrations would be biodegraded to an extent that they would no longer be toxic to trout fingerlings.

Methanol was used as a carrier for the naphthenic acid in water as it is not toxic to trout in low concentrations.

Table 4.6 – OUR reduction due to naphthenic acid introduction to biomass from the refinery wastewater treatment aeration basins.

<table>
<thead>
<tr>
<th>Test</th>
<th>Naphthenic Acid (mg·L⁻¹)</th>
<th>Methanol (mL)</th>
<th>Average OUR (mg·L⁻¹·h⁻¹)</th>
<th>OUR Reduction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test 1</td>
<td>0</td>
<td>0</td>
<td>18.92</td>
<td>n.a.</td>
</tr>
<tr>
<td>Test 2</td>
<td>0</td>
<td>0.071</td>
<td>21.29</td>
<td>-12.53</td>
</tr>
<tr>
<td>Test 3</td>
<td>0</td>
<td>1.415</td>
<td>20.26</td>
<td>-7.07</td>
</tr>
<tr>
<td>Test 4</td>
<td>1</td>
<td>0</td>
<td>20.54</td>
<td>-8.58</td>
</tr>
<tr>
<td>Test 5</td>
<td>5</td>
<td>0</td>
<td>21.54</td>
<td>-13.86</td>
</tr>
<tr>
<td>Test 6</td>
<td>10</td>
<td>0</td>
<td>21.31</td>
<td>-12.65</td>
</tr>
<tr>
<td>Test 7</td>
<td>20</td>
<td>0</td>
<td>20.80</td>
<td>-9.94</td>
</tr>
</tbody>
</table>

Based on the OUR reduction for this range of naphthenic acid concentration, it was determined that there is no effect on the bacteria with the introduction of naphthenic acid between 1 and 20 mg·L⁻¹.

For all naphthenic acid concentrations listed in Table 4.6, there was no reduction in fish toxicity and a minor reduction in naphthenic acid concentration after the seven day period. In view of the fact that the residence time of wastewater in the aeration
basins and clarifiers is eight hours, it can be concluded that there is no reduction of naphthenic acid concentration in the refinery wastewater system due to biodegradation.

4.8 16S rRNA Genetic Analysis of a Microbial Community in an Oil Refinery Wastewater System

This study was done to determine the taxonomy of the bacteria in a refinery wastewater system exposed to low concentrations of naphthenic acid during steady-state and upset conditions. The study involved examination of the hypervariable region \[ V4 \] of the 16S rRNA gene. There are nine hypervariable regions (V1 - V9) in 16S rRNA which demonstrate considerable diversity among different bacteria which allows the accurate determination of the bacteria taxonomy in a sample such as wastewater. These hypervariable regions are separated by sections of conserved regions that are used as universal primers for sequencing.

The meaning of 16S rRNA follows. The 16S term is the number of Svelberg units of the ribosome, determined by where the portion of the rRNA settles during high speed centrifuging, and referred to as the sedimentation rate or coefficient. The abbreviation rRNA is ribosomal ribonucleic acid which binds with various proteins to form ribosomes. Ribosomes are used in cells for building proteins which have a wide range of functions including messaging, enzyme production, structural development and the creation of antibodies.

The analysis was performed by next generation sequencing and 16S rRNA libraries. Sequencing and phylogenetic analyses from the 16S rRNA library revealed a high diversity of taxa for the total bacterial community.

Because of their abundance, most of the bacterial species living on earth have
not been identified. DNA hybridization and 16S rRNA gene sequencing are the most common techniques used to identify the bacteria present in a sample such as wastewater. Figure 4.4 shows a cross section of the components of a rod bacteria cell.

Figure 4.4 – Cross section of a rod bacteria [103].

The nucleoid contains the DNA which is circular and coiled to conserve space. Plasmid are small sections of DNA that can assist in antibody rejection or assist with a changing environment. The pili allow bacteria to attach to surfaces or other bacteria. The cytoplasm is a gel like material that is primarily water and salt, having a number of functions including transport and storage of material used by the cell. The plasma membrane is permeable to certain ions and organic molecules and therefore controls the movement of substances in and out of the bacteria. The cell wall and capsule add structure to the bacteria and protect if from the environment. The ribosome was discussed earlier in this section.

The 16S rRNA gene sequence was chosen for analysis as it shows a high degree of conservation among species of bacteria. This is assumed to result from the importance of the 16S rRNA as a critical component of bacteria function. Although the absolute
rate of change in the 16S rRNA gene sequence is not known, it does mark evolutionary
distance and relatedness of organisms. Figure 4.5 shows the structure of the large and
small subunits of a ribosome.

Figure 4.5 – Ribosomal subunits, with RNA in orange and yellow and proteins in blue [104].

A 16S rRNA analysis was performed to determine the diversity of the bacteria
in the wastewater treatment system, and to allow identification of the bacteria in
bioreactor tests if they proved effective in minimizing concentrated naphthenic acid.
A recent publication has isolated an acid-degrading and metal-resistant bacterium
Cupriavidus gilardii CR3 that can metabolize naphthenic acid [105].

4.8.1 Samples

Table 4.7 on the next page lists the names and locations of the samples that were
collected in the wastewater treatment system. A block diagram of the locations and
abbreviated names is shown in Figure 4.6 on page 74 which represents a conventional
activated sludge (CAS) wastewater treatment unit in an oil refinery.

All samples were refrigerated to 4 °C during transportation and storage before
further processing at the Biochemical Engineering laboratories, Department of
Chemical and Biochemical Engineering, University of Western Ontario.
**Table 4.7** – Sample locations and names for 16S rRNA analysis.

<table>
<thead>
<tr>
<th>Description</th>
<th>Sample names</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocell® effluent</td>
<td>Hcos1,2,3</td>
</tr>
<tr>
<td>Top layer of aeration basin 1 (AB1)</td>
<td>Ab1s1,2,3</td>
</tr>
<tr>
<td>Bottom layer of aeration basin 1</td>
<td>Ab1bots1,2,3</td>
</tr>
<tr>
<td>Top layer of aeration basin 2</td>
<td>Ab2s1,2,3</td>
</tr>
<tr>
<td>Petri dish sample 1 from top layer of AB1</td>
<td>Ba1s1,2,3</td>
</tr>
<tr>
<td>Bottom layer of aeration basin 1</td>
<td>Ab2bots1,2,3</td>
</tr>
<tr>
<td>Petri dish sample 2 from top layer of AB1</td>
<td>Ba2s1,2,3</td>
</tr>
<tr>
<td>Clarifier 3 effluent</td>
<td>C3os1,2,3</td>
</tr>
<tr>
<td>Recycle sludge from clarifier 2 (C2)</td>
<td>C2fos1,2,3</td>
</tr>
<tr>
<td>Recycle sludge from clarifier 3 (C3)</td>
<td>C3fos1,2,3</td>
</tr>
<tr>
<td>Combined recycle sludge from the C2 and C3 to AB1</td>
<td>C23toAb1s1,2,3</td>
</tr>
</tbody>
</table>

### 4.8.2 Steps Used to Analyze 16S rRNA

There are a set of prescribed steps to analyze the 16S rRNA which are:

a) isolate the bacteria,

b) lysis the bacteria to remove the cell contents,

c) separate the cell contents by ultra centrifugation,

d) extract and purify the rRNA,

e) amplify the 16S rRNA gene,

f) sequence a portion of the 16S rRNA gene, in this case hypervariable region 4 (V4) was chosen,

g) compare the sequenced gene with a library to obtain taxonomy.
Figure 4.6 – Block diagram of refinery wastewater treatment system with sample locations for 16S rRNA analysis during steady state operation.

Hydrocell Process Sewer (high dissolved hydrocarbon concentration)

Aeration basin 1

Ab1s1, Ab1s2, Ab1s3 (org 2,26,50)

Ab1bots1, Ab1bots2, Ab1bots3 (org 3,27,51)

Ab2s1, Ab2s2, Ab2s3 (org 6,30,54)

Ab2bots1, Ab2bots2, Ab2bots3 (org 7,31,55)

Clarifier 2

Clariﬁer 3

C3os1, C3os2, C3os3 (org 10,34,58)

C2f0s1, C2f0s2, C2f0s3 (org 8,32,56)

C3fos1, C3fos2, C3fos3 (org 9,33,57)

Water from top of clarifier to carbon filters then to river

FLOC (microbes and solids) from bottom of clarifier to AB1.

Nomenclature

Note: All samples are technical replicates

Hcosx: Hydrocell out

Ab1sx: Aeration basin 1 top

Ab1botsx: Aeration basin 1 bottom

Ab2sx: Aeration basin 1 top

Ab2botsx: Aeration basin 2 bottom

Ba1x: Petri dish bacteria morphology 1

Ba2x: Petri dish bacteria morphology 2

C3osx: Clarifier 3 water effluent

C2f0sx: Clarifier 2 flocculent effluent

C3f0sx: Clarifier 3 flocculent effluent

C2toAb1sx: Combined clarifier flocculent effluent

Contaminated Sewer (low dissolved hydrocarbon concentration)

Sanitary Sewer (from refinery buildings)

C23toAb11, C23toAb12, C23toAb13 (org 4,28,52)

Note: All samples are technical replicates
4.8.3 Viable Bacteria Count

Prior to DNA extraction, approximate concentrations of the microbial cultures were determined using a microscope and imaging software, to ensure sample volumes of 500 $\mu$L of supernatant, containing sufficient DNA concentrations of at least $5 \cdot 10^9$ cells.

4.8.4 DNA Isolation and Extraction

Separation of the bacteria from the supernatant of the samples was performed by centrifuging at 15,000 g for 30 minutes at a temperature of 4 °C. The resultant pellet was resuspended in 500 $\mu$L phosphate buffered saline (PBS) (0.1M, pH 7.4).

The genomic DNA was extracted with PowerSoil® DNA Isolation Kits from MO BIO Laboratories. The DNA extraction was carried out following the protocol for the kit.

4.8.5 16S rRNA Amplification and Sequencing

The total genomic DNA of the samples was processed according to the protocol for Illumina MiSeq Microbiome (V4) PCR and bar coded for Multiplex Microbiota using Illumina instruments developed by Dr. Gregory Gloor, professor in the Department of Microbiology of the University of Western Ontario. The protocol is listed in Appendix B. The method is also described on the Earth microbiome project Internet site [http://www.earthmicrobiome.org/emp-standard-protocols/16s/](http://www.earthmicrobiome.org/emp-standard-protocols/16s/).

The sequencing of the 16S rRNA gene hypervariable region V4 using an Illumina MiSeq Next-Generation was performed at the London Regional Genomics Centre, Robarts Research Institute, London, Ontario.
4.8.6 Bacteria Taxonomy

The 16S rRNA sequences were compared with the available RNA sequences of the NCBI GenBank database using the program BLAST to obtain the bacteria taxonomy major ranks, kingdom (which was bacteria), phylum, class, order, family, genus and species. The sequences were clustered into bins called Operational Taxonomic Units (OTU) based on their similarity to each other [106].

A total of 252 OTUs representing different bacteria were identified. Table 4.8 lists the phylum ranks which is the lowest rank where all 252 bacteria were identified.

<table>
<thead>
<tr>
<th>Taxonomy rank</th>
<th>Number identified</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phylum</td>
<td>252</td>
<td>100.0</td>
</tr>
<tr>
<td>Class</td>
<td>250</td>
<td>99.2</td>
</tr>
<tr>
<td>Order</td>
<td>221</td>
<td>87.7</td>
</tr>
<tr>
<td>Family</td>
<td>155</td>
<td>61.5</td>
</tr>
<tr>
<td>Genus</td>
<td>70</td>
<td>27.8</td>
</tr>
<tr>
<td>Species</td>
<td>6</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Table 4.8 shows that searches of databases do not always yield full taxonomy detail for a given bacteria. There are a number of reasons for this, including the fact that not all bacteria found in wastewater treatment systems have been identified, the search was limited to the hypervariable region V4, there can be local genetic changes in bacteria as well as errors in databases. Table 4.9 on the next page lists the bacteria by phylum rank from the taxonomy summary in Table 4.8.

4.8.7 Analysis of the 16S rRNA Data

The 16S rRNA analysis generated data that included the OTU count and taxonomy for three technical replicates for each wastewater treatment sample listed in Table 4.7 on page 73; this produced a 252 x 34 matrix which is too large to visualize the relationships...
Table 4.9 – Phylum of bacteria identified.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Number identified</th>
<th>Percentage of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteobacteria</td>
<td>138</td>
<td>54.8</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>35</td>
<td>13.9</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td>20</td>
<td>7.9</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>19</td>
<td>7.5</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>14</td>
<td>5.6</td>
</tr>
<tr>
<td>Chlorobi</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>4</td>
<td>1.6</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>Elusimicrobia</td>
<td>3</td>
<td>1.2</td>
</tr>
<tr>
<td>OD1</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>Armatimonadetes</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>BHI80-139</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Chlamydiae</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Fusobacteria</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Nitrospirae</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>Spirochaetes</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>TM6</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>WPS-2</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>252</strong></td>
<td><strong>100.0</strong></td>
</tr>
</tbody>
</table>

between the samples. Principal component analysis (PCA) is a technique used to analyze the data from the wastewater treatment samples.

PCA involves a mathematical procedure that transforms a number of possibly correlated variables into a smaller number of uncorrelated variables called principal components. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. A scree plot which displays the variance against the number of the principal components is shown in Figure 4.7 [107].

The result is a new set of variables that represent linear combinations of the original
Figure 4.7 – The plot shows the Scree plot of the principal components found by PCA of the OTU file data from the 16S rRNA analysis. In this case the first two principal components account for 65.3% of the variance in the data; this value can be found in Section G.1.

Variables that are uncorrelated and reflect the most important structure of the data. Values for each sample projected onto these loadings are then calculated and called scores. A biplot graph is used to display the data [107].

The program used for the PCA analysis is RStudio which is a free open source integrated development environment (IDE) for R which is statistical computing and graphics programming language. Appendix F contains the R code used for the analysis in this section.

Figure 4.8 is the biplot from the PCA analysis, and indicates there are three basic groupings:

a) Top left quadrant - clarifiers and aeration basins that contain aerobic bacteria,

b) Top right quadrant - aeration basin bottom samples and clarifier return to the aeration basins that contain anoxic and anaerobic bacteria,
c) Bottom half - Hydrocell® and petri dishes that contain a large population of a single bacteria.

Figure 4.8 – Biplot of the PCA data from genetic analysis showing relationships between the samples and the identified bacteria.

Figure 4.9 shows the dendrogram and heat map plots of the bacteria identified by 16S rRNA analysis. The dendrogram above the heat map is a branching diagram.
that represents the relationships of similarity among a group of entities, in this case the OTUs, found in the individual wastewater treatment samples.

Figure 4.9 – A heat plot of the samples showing a dendrogram at the top which has boxes drawn around the most closely related bacteria. The heat diagram below shows the percentage of bacteria type found in the samples. This style of graph and the PCA plots are used to visualize the large data sets. The y axis on the dendrogram diagram indicates how closely related the samples are genetically and the y axis on the heat diagram indicates the relative abundances of the bacteria.
The vertical lines in the dendrogram are termed clades and the termination of a clade is called a leaf. The arrangement of the clades shows which leaves are most similar to each other, which is highlighted in Figure 4.9 by red boxes. The height of the branches indicates how similar or different they are to each other: the greater the height, the greater the difference. It is interesting to note that the similarities seen in Figure 4.9 are also seen in the dendrogram.

The y axis of the heat map in Figure 4.9 represents the fraction of the OTUs found in a particular sample. A legend was not included with the diagram for clarity due to the number of OTUs. The taxonomy details and legends for each sample shown in Figure 4.9 are presented in Appendix G in Figures G.1 through G.11.
Chapter 5

Desalting of Crude Oil and Emulsions

Emulsions are formed during the desalting of crude oil which is the first step in the oil refining process. The emulsions can cause upsets and toxicity in the wastewater system by the transport of free oil and oil coated solids. The purpose of desalting is to remove salts and particulate matter in the crude to minimize corrosion and fouling of downstream equipment such as distillation columns, heat exchangers and piping. There has been a recent emphasis on this technology within refining since crude feed changes towards heavy crude oil have become common due to lower supply costs [44, 115, 116, 108]. A paper titled “Desalter Brine Treatment of Athabasca Oil Sands Crude Oil to Minimize Naphthenic Acid Concentration in an Oil Refinery” based on the information discussed in this chapter is being prepared.

5.1 Desalting

The following sections describe issues that have been experienced when desalting heavy crude oil. A fundamental understanding of the material is critical as process upsets in
the desalter can lead to:

a) Wastewater treatment process upset due to oil and/or naphthenic acid under-carry.

   (i) acute toxicity effects on aquatic life,

   (ii) acute effects on the microbe population in the aerobic digesters,

   (iii) oil sheens in public waterways.

b) Equipment damage due to brine over carry.

   (i) flashing in the crude heater,

   (ii) lifting of trays in the crude tower,

   (iii) exchanger damage due to flashing.

5.2 Desalter Operation Fundamentals

Separation within the desalter vessel is based on gravity, and can be described by a modification of Stokes’ Law. Equation \[5.1\] shows that the rate of separation depends upon the drop size of the dispersed phase, the differential density of the phases, and the viscosity of the continuous phase.

\[
v = \frac{1.8 \times 10^{-6} \Delta \rho \text{ow} d^2}{\mu_o}
\]  \hspace{1cm} (5.1)

where $v$ is the downward velocity of the water droplet relative to the oil, (m·s$^{-1}$), $d$ is the diameter of the water droplet (\(\mu m\)), $\Delta \rho \text{ow}$ differential density between the oil and water (g·L$^{-1}$) and $\mu_o$ is the dynamic viscosity of the oil in centipoise (cP).

Since heavy oils are close to the density of water, the driving force for separation is small. At elevated temperatures, this differential density becomes even smaller.
5.3 Desalter Layout

A cross-section of a typical desalter is shown in Figure 5.1.

![Cross-sectional diagram of a desalter vessel](image)

**Figure 5.1** – Cross sectional of a PETRECO® BILECTRIC® desalter vessel [109].

The oil-water mixture enters a mix valve to allow intimate contact between the crude oil and water to dissolve salts and entrain solids into the water phase and form a water in oil emulsion. The emulsion enters through the distributor inlet and exits into the vessel through the three distributor outlets. At the distributor outlet is a series of three high voltage electrical grids which are isolated from the desalter ground by a series of insulators; the grids are used to coalesce the wash water that is entrained in the crude. There are three single phase transformers that supply the grid through bushings located at the top of the desalter. The oil exits through the top of the vessel and the water exits through multiple outlets across the length of the vessel. The six outlet pipes are also used for the mud wash system which removes solids from the desalter.
vessel. There are other nozzles on the vessel, used for maintenance entry. Location of the oil/water interface is determined by a level transmitter/controller (LC) which is mounted on the top of the desalter. Samples of the brine, emulsion or oil layers can obtained by moving the "swing arm" in an arc; Figure 5.2 shows samples from the internal of a desalter using the swing arm during an upset condition.

![Figure 5.2](image)

**Figure 5.2** – Emulsion at different layers of a desalter under upset conditions.

The samples shown in Figure 5.2 were taken during a desalter upset at various heights, from the sample outlet to the 900 cm level which is just above the emulsion phase shown in Figure 5.1. Sample 1 is the sample outlet, where under normal desalter operation the colour is dependent on the make-up of the water sources that mix with the crude, and ranges from clear to milky white due to micron size emulsion particles. Samples 2 and 3 are taken at the 15 cm and 30 cm levels, and during normal operation they are the same colour as sample 1. However, in this case they are dark due to an oil in water emulsion which is composed of oil and solids. Sample 4 is taken at the 60 cm level and is typically 50 % water and 50 % oil, in the upset condition the oil has not coalesced. Sample 5 is from the 90 cm level and is oil with no trace of water.
5.4 Computational Fluid Dynamic Analysis of a Desalter

When the desalter being used to process high TAN crude oil was identified as the source of naphthenic acid in the refinery wastewater treatment system, the desalter vendor, Cameron, in Houston, Texas, was asked to perform a computational fluid dynamic study of the desalter based on operation conditions and the crude oil mix being processed. This section summarizes the findings.

A bilelectric desalter vessel used to process a normalized flow rate of 350 m$^3$·d$^{-1}$ of water and 7000 m$^3$·d$^{-1}$ of oil was utilized as a computational fluid dynamics (CFD) model. Section 5.3 describes and illustrates the vessel internals. The CFD calculations were performed to understand the following:

1. Analyze the flow pattern and distribution across the water phase of the vessel volume.

2. Conduct particle tracking and histogram analysis of the water particles exiting through all six water outlets to understand the effectiveness of the water volume for flow separation.

5.4.1 Process Flow Description

Uniform flow distribution inside the fluid zone plays an important role for superior separation. The existence of flow channeling, recirculation zones and non-uniformities can be detrimental to the flow separation. The goal of this study was to determine the characteristics of the water flow distributions and path lines of the six outlet nozzles distributed along the length of the vessel.

Figure 5.3 shows the vessel internals along with the expected water flow vectors after exiting the three distributor outlets. The oil-water mixture enters the distributor
through stream 1 (located at the bottom of the vessel) and exits into the vessel through the three distributor outlets. It is reasonable to assume that most of the water would separate from the oil and travel in the downward direction. As the water travels across the rag layer, uniform water velocity is expected across the whole cross-section of the vessel. The emulsion layer is located beneath the interface level; it is typically 0.3 m in depth based on field testing. Hence, for the CFD modeling, the vessel volume below the emulsion layer is considered. The flow through the distributor manifold is ignored and a uniform flow across the inlet cross-section is assumed.

![Figure 5.3](image)

**Figure 5.3** – Cross section of a desalter vessel which is typically filled with brine, showing the expected flow vector and the rag layer.

### 5.4.2 CFD Simulation

For any CFD simulation, the geometric model that closely represents the flow domain is first created. For this study, the brine section of the vessel encompassing below the interface line is considered as shown in Figure 5.4. GAMBIT 2.4.3 software is used to create the geometric model and to mesh the fluid volume. To create the mesh, the geometric model that represents the fluid domain is subdivided into many small cells.

The CFD simulation was performed using FLUENT 12.1 (ANSYS Inc.) software.
Figure 5.4 – Cut-away view of a desalter vessel showing inlet and outlet piping.

The flow conditions used for the CFD calculations are shown in Table 5.1. As discussed in Section 5.4.1, only the brine volume is modeled for the CFD calculations and the single phase modeling scheme is used. The flow is considered to be turbulent and the RNG-\(k-\varepsilon\) turbulence model was used. A uniform velocity inlet boundary condition is used for the inlet boundary and the velocity outlet boundary condition is used for the six outlet boundaries. After the completion of the flow calculations, the water particle tracking calculations are performed using the discrete phase modeling (DPM) feature, available in FLUENT 12.1 software.

Table 5.1 – Water flow conditions and fluid properties for CFD study.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Density</td>
<td>956.8 kg\cdot m^{-3}</td>
</tr>
<tr>
<td>Viscosity at operating temperature</td>
<td>0.21 cP</td>
</tr>
<tr>
<td>Flow rate</td>
<td>400 m^{3}\cdot d^{-1}</td>
</tr>
<tr>
<td>Operating pressure</td>
<td>100 kPa</td>
</tr>
<tr>
<td>Operating temperature</td>
<td>130 °C</td>
</tr>
</tbody>
</table>
5.4.3 Flow Pattern Analysis

For the current study with multiple water outlets, uniform velocity beneath the rag layer is assumed, as discussed in Section 5.4.1. The gravitational force of $9.81 \text{ m-sec}^{-2}$ is considered in a vertically downward direction.

The water flow path lines entering from the inlet boundary are shown in Figure 5.5. The flow path lines in Figure 5.5b clearly show six localized flow regions, one surrounding each water outlet. Due to geometric symmetry, the flow distributions surrounding the two end outlets (1 and 6) show similar flow patterns. The flow path lines surrounding the four middle outlets also show very similar flow patterns to each other. In between the localized flow regions, small stagnation zones were observed near the bottom wall of the vessel. However, the flow patterns in each localized region show fairly uniform flow distributions.

Figure 5.5a shows the isometric view of the water flow path lines colored by the injection location (particle ID) of the water particles.

Figure 5.6 shows the flow path lines colored by residence time of the water particles. Results indicate that very few water particles exceed a residence time greater than approximately 200 min (12,000 sec). Typically, flow regions with particle residence time much greater than the theoretical residence time (vessel volume / inlet flow rate = 7340 sec) indicate the existence of recirculation zones or dead zones.

5.5 Desalter Process Control

The oil level of a desalter must be maintained to allow sufficient residence time for the emulsion to dehydrate. This is made difficult due to the fact that there is a varying emulsion layer in the desalter from the mixing of water and crude at the inlet to the desalter. Maintaining the oil level is typically accomplished with capacitance probes or
conductivity, as conventional level measurement instruments such as displacement and differential pressure fail to sense the changes in oil level, due to the changes in emulsion layer and hence density.

5.6 Desalter Chemical Addition

Chemical treatment is used in desalters to dehydrate emulsions formed during the mixing of water and oil to remove salts and solids. Typically a demulsifier is used to destabilize the emulsions allowing them time to flocculate and then coalesce before reaching the brine or oil outlet of the desalter. The required demulsifier injection rate to dehydrate the emulsion varies, due to variations of the crude feed properties such as solids, asphaltene and both natural and man-made emulsifiers. Suspended solids and semi-soluble hydrocarbon fractions can cause demulsifier treatment to be ineffective. Often, wetting agents prove useful for separating the suspended solids.
Figure 5.6 – Water flow path lines are colored by the residence time in seconds (a) side view, (b) bottom view, (c) top view.

from the oil and forcing them into the water layer, as described in Section 4.5 on page 64. In this project we propose to use biological surfactants produced by the aerobic bacteria that act as demulsifiers. Biological demulsifiers are totally biodegradable and environmentally friendly, compared to chemical demulsifiers used in oil industry today.
5.7  Issues With Desalting

During the processing of crude with varying density, solids concentration and surfactant concentration, a number of issues can occur which can cause emulsion and oil under-carry with the brine and entrained water in the crude outlet. This is due to properties of the emulsion surface which do not allow coalescence in the desalter.

Emulsion in the brine, called under-carry, can cause a number of issues in the wastewater treatment system such as high oxygen demand due to high molecular weight hydrocarbons in the emulsion surface, toxicity due to naphthenic acid carried with the emulsion and solids depositing in the aeration basins if they are not separated.

Entrained emulsion in the oil phase can cause issues downstream in crude heaters and crude towers due to flashing of the water phase of the emulsion.

5.7.1  Viscosity

Increased viscosity leads to slower emulsion velocity due to Stokes’ Law as shown in Equation 5.1. To minimize viscosity the desalter is operated at elevated temperatures of 100 to 120 °C.

5.7.2  Solubility of Water in Crude Oil

Water solubility in crude oil increases with temperature. In general the solubility approaches 0.4 % by volume at 150 °C. In a desalting process, the water that dissolves in the oil carries no salt with it; however, the loss of water from highly saline water droplets may result in the precipitation of crystalline salt. The surface of the salt crystals acts as any other interface and tends to collect insoluble or semi-soluble contaminants, which in turn make the crystal difficult to contact with the wash water. Injection of a portion of the wash water into the crude prior to heating can help alleviate this problem.

A more difficult issue involves attainment of water-in-oil specifications. Process
samples are collected through coolers that lower the temperature to prevent loss of volatile liquids. The cooling precipitates some of the soluble water, thereby increasing the reported water content of the oil. Since the desalter can only separate water that exists as a separate phase, this precipitated water represents a component that cannot be treated under conditions existing within the process vessel. The interpretation of true separate phase water content under process conditions is further complicated by incomplete equilibrium in both the dissolution process and the precipitation reaction.

5.7.3 Internal Electrical Components

A desalter uses high voltage internal grids, which are typically > 10 keV, to assist with dehydrating emulsions. As part of that assembly insulators are used to isolate the high voltage components from the desalter metal components which are at a 0 V potential. The insulator material of construction is selected for properties that resist fouling, such as Teflon or other fluorocarbons; fouling can lead to the formation of carbon tracks on the insulator which effectively forms a short circuit and a reduction of desalting efficiency. However, the materials that are the most effective in resisting fouling have temperature limitations due to their mechanical properties. As discussed in this section temperature reduction affects oil viscosity.

5.7.4 Oil Conductivity

Crude oil becomes more conductive with increased temperature. This conductivity increases the electrical power requirements, necessitating larger power supplies and also limiting the sustainable magnitude of the electrostatic field.
5.7.5 Solid Contaminants in Crude Feed

Solids of the oil bearing deposits are composed of sand and silt and corrosion products as discussed in Section 5.8.2. The solids are found both in the bottom of the vessel and in the water/oil interface zone where they contribute to stabilized or slowly resolving emulsion layers; this is sometimes referred to as the ‘rag layer’. During normal operations this layer is a few centimeters in depth, however it can expand in depth due to contaminants in the crude feed to the desalter. As the depth increases it affects emulsion dehydration and can cause oil under-carry in the brine layer or water over-carry to downstream equipment. To minimize increases in the water/oil interface zone, chemical injection is increased or crude feed is varied and in some cases oil refineries have added a separate line on the desalter to remove the interface zone. Periodic removal of the solids that form on the bottom of the desalter is accomplished by agitating the water layer sufficiently to mix the interface sludge into the water layer and then removing it by taking a portion of the brine outlet and recycling it though a distribution header at the bottom of the desalter as shown in Figure 5.1. This is referred to as ‘mud washing’.

5.7.6 Differential Density

As the density of the crude increases, the density difference with respect to water decreases, leading to poor separation between the water and oil which can increase as heavier crude oil feeds are processed. This is discussed in Section 5.2.

5.8 Emulsions

There are a number of locations in the refinery where an emulsion can be formed due to the presence of oil, water, surfactants and solids with a desalter being the primary location. An emulsion is a mixture of two immiscible fluids; examples of emulsions can
be seen in Figures 5.9 and 5.10.

### 5.8.1 Thermodynamics of Emulsions

The energies to form and break an emulsion are considered in this section.

Surface tension $\gamma$ (N·m$^{-1}$) is the property of like molecules such as water sticking together due to cohesive forces. It is defined as

$$\gamma = \frac{F}{L} \quad (5.2)$$

where $F$ is force (N) and $L$ is length (m).

When two different liquids such as oil and water are in contact there are adhesive forces that form interfacial tensions, which are the tensions between the liquid phase of one substance and either a liquid or gas phase of another substance. In the case of desalting the interaction occurs at the surfaces of the wash water and the crude oil. Therefore Equation 5.2 can be considered to be interfacial tension in the case of the tension between two liquids in contact.

From Equation 5.2 the mechanical work $\Delta W$ (J) required to increase a surface area of a system is defined as

$$\Delta W = \gamma \cdot \Delta A \quad (5.3)$$

where $\Delta A$ is the area change (m$^2$).

The change of Gibbs energy (J) of a system at constant temperature and pressure is defined as

$$\Delta G = \Delta H - T \cdot \Delta S \quad (5.4)$$

where $\Delta H$ is the change in enthalpy (J) in the system, $T$ is the temperature (°K) and $\Delta S$ is the change in entropy (J·°K$^{-1}$) in the system. Since $\Delta W$ and $\Delta H$ have the
same units Equation 5.4 can be changed to

$$\Delta G = \gamma \cdot \Delta A - T \cdot \Delta S$$  \hspace{1cm} (5.5)

When two immiscible liquids such as crude oil and water are added together without mechanical force such as the shear force imparted by a pump, an interface develops between the two liquids due to adhesive and cohesive forces and they will not form an emulsion. When free energy is imparted to the crude oil and water mixture, an emulsion will form; this is the purpose of a mixing valve on a desalter.

Due to the positive Gibbs free energy in the system introduced by the shearing action, the emulsion under ideal conditions would flocculate and then coalesce to bring the Gibbs energy to zero. However due to solids, surfactants and large organic molecules such as asphaltenes, a rigid film can form. Figures 5.7 and 5.8 illustrate the effects that solids and polar hydrocarbons such as resins and asphaltenes have on emulsions, causing steric hindrance and thereby inhibiting the flocculation and coalescence of the emulsion particles.

![Figure 5.7](image)

**Figure 5.7** – A visualization of the stabilizing effect of asphaltene and solids on water droplets. [118]. The labeled particles in the illustrations were found in samples from the desalter crude oil feed and listed in Table 5.2.
Figure 5.8 – Deflation of a water droplet by withdrawing fluid back into the water-filled micropipette. The surrounding continuous phase contained 0.1 % bitumen diluted in heptol. As the surface area of the droplet was compressed, a rigid adsorbed layer is evident. [119].

Depending on the size and characteristics of the solids, asphaltene concentration, natural surfactants and upstream or pipeline additives, there is the possibility of emulsion under-carry into the brine outlet of the desalter which is part of the feed to the wastewater treatment system. Since the emulsions in a desalter are typically 10 \( \mu \text{m} \cdot \text{L}^{-1} \) a microscope is required to view them. Figures 5.9 and 5.10 show typical desalter emulsions.

Figure 5.9 shows a series of emulsion formations from a desalter. In Figure 5.9(a) the center and right sample bottle contents appear to be the same. However, they have a different composition of oil, sludge, emulsion, water and solids. The water sample shade changed from clear to black at random times as loading progressed. Figure 5.9(b) gives a better indication of the sample composition. Oil, water and asphaltenes/solids can be seen on the slides. The different colors on the slides also give indication that basic sediment and water (BS&W) will vary between samples.
Figure 5.9 – The samples were taken from a desalter during normal operation and an upset. Figure (a) shows two different phases of material from the desalter, the left sample is primarily water, the middle and right samples are emulsion. Figure (b) shows four microscope slides with emulsions with different concentrations of water, oil and solids.
Figure 5.10 shows photomicrographs of the structure of the emulsion. Comparing Figures 5.10(b), 5.10(c) a definite change in the emulsion structure between September 8 2011 and September 9 2011 can be seen. This change is not apparent when viewing the original samples shown in Figure 5.9(a).

Figure 5.10 – The photomicrographs above were taken from the samples shown in Figure 5.9. Figure (a) is a particle of emulsified oil at 400x. This can give the water various shades of brown. Figure (b) is the September 8 sample at 100x. Figure (c) is the September 9 sample at 100x. Figure (d) is the September 9 sample at 400x. The scale at 400x is approximately 500 µm x 500 µm.
The emulsions also contain naphthenic acid as they are slightly polar and are part of both the coating on the solids and the asphaltene on the surface of the emulsion. The solids are oil wetted clay or heavy minerals from the mining or drilling operations upstream with particle size distribution of 10 nm to 10 µm; the lower range stabilizes the emulsion. The clay particles are typically Kalonite or Illite while the heavy minerals are varied [120]. An example of emulsions and solids in a desalter is shown in Figure 5.11.

Figure 5.11 – Typical emulsions found in desalters. The emulsion particle labelled ‘microemulsified water’ is approximately 10 µm in diameter. A distribution of particle and emulsion sizes in the brine is shown in Figure 5.12.

Emulsion particles with size distribution of 0.1 to 10 µm and Zeta potentials of -15 to -40 mV were determined with particle size and Zeta potential analyzers from Malvern. Figure 5.12 shows the distribution of emulsion particles from the Malvern analyzer.
5.8.2 Solids

Solids in crude oil originate from the extraction process and can help to stabilize emulsions. This was first discovered by Pickering in 1923 and solids stabilized emulsions are referred to as Pickering emulsions. Elemental analysis of the solids in this study was done by scanning electron microscopy with dispersive x-ray spectrometer SEM/EDX, ion plasma chromatography using an optical emission spectrometer ICP/OES, x-ray florescence XRF, and compositional analysis by x-ray diffraction XRD.

Figure 5.13 shows an image from a SEM/EDX analysis indicating the elements that are present in a sample of solids that have been extracted from an emulsion by washing the sample with dichloromethane (DCM) and vacuum filtering with a 0.45 µm filter element.

The minimum detection limit of EDX is typically 0.1 % of a sample mass whereas ICP/OES is in the low range of µg·kg⁻¹ depending on the element [121].
Figure 5.13 – SEM/EDX analysis of the solids from a 25 mL sample of crude oil that was vacuum filtered with a 0.45 µm filter paper. Typical solid concentrations range from 80 to 350 mg·L$^{-1}$. XRD analysis indicates clays and heavy minerals.
The particle size distribution of filtered solids of a sample of desalter crude oil feed is shown as a box plot in Figure 5.14. Table 5.2 lists the statistics of the solids size distribution.

**Figure 5.14** – The box plot shows the particle size distribution of solids found in the crude sample listed in Table 5.2. The statistics are listed in Table 5.2.

**Table 5.2** – SEM/EDX size analysis of solids contained in a desalter crude feed sample. The concentrations of the compounds vary depending on crude type and shipment.

<table>
<thead>
<tr>
<th>Statistic</th>
<th>Value (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>1.178</td>
</tr>
<tr>
<td>Medium</td>
<td>0.430</td>
</tr>
<tr>
<td>Maximum</td>
<td>13.343</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>1.020</td>
</tr>
<tr>
<td>Quartile 1</td>
<td>0.157</td>
</tr>
<tr>
<td>Minimum</td>
<td>0.026</td>
</tr>
</tbody>
</table>
Figure 5.15 shows SEM images of the particles < 1 µm that were the basis for the statistics shown in Figure 5.14 and Table 5.2.

**Figure 5.15** – The images show the size distribution of the solids of approximately 1 µm and smaller in the crude oil feed to a desalter.

The particle sizes shown in Figure 5.15 have large surface areas that have exposed metals in the lattice structure which can have a positive charge due to cations based on the oxidation state of the metal in the compound matrix listed in Table 5.3 on page 106. Weak acids are anions and will be attracted to the edge of the particles.
Figure 5.16 shows the metal distribution of an emulsion sample from ICP/OES analysis. This corresponds to the compounds shown in Table 5.3.

**Figure 5.16** – Concentration of metals by ICP/OES of the bulk extract. Elements that show no value are below the minimum detection limit (MDL) for the method. The microwave digestion method for this analysis uses nitric acid not hydrofluoric acid and therefore the silicon value is low.
Table 5.3 – XRD analysis of solids contained in a desalter crude feed samples. The concentrations of the compounds vary depending on crude type and shipment.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Concentration (% mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kaolinite</td>
<td>AlSiO(OH)</td>
<td>18</td>
</tr>
<tr>
<td>Quartz</td>
<td>SiO₂</td>
<td>14</td>
</tr>
<tr>
<td>Goethite</td>
<td>FeO(OH)</td>
<td>12</td>
</tr>
<tr>
<td>Illite</td>
<td>KAlSiO(OH)</td>
<td>9</td>
</tr>
<tr>
<td>Siderite</td>
<td>FeCO₃</td>
<td>9</td>
</tr>
<tr>
<td>Pyrite</td>
<td>FeS₂</td>
<td>6</td>
</tr>
<tr>
<td>Anatase</td>
<td>TiO₂</td>
<td>6</td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>(NH₄)₂SO₄</td>
<td>5</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>Ca₅(PO₄)₃(OH)</td>
<td>4</td>
</tr>
<tr>
<td>Magnetite</td>
<td>Fe₃O₄</td>
<td>3</td>
</tr>
<tr>
<td>Calcite</td>
<td>CaCO₃</td>
<td>3</td>
</tr>
<tr>
<td>Microcline</td>
<td>KAlSi₃O₈</td>
<td>2</td>
</tr>
<tr>
<td>Albite</td>
<td>NaAlSi₃O₈</td>
<td>2</td>
</tr>
<tr>
<td>Clinochlore</td>
<td>(Mg,Fe,Al)₆(Si,Al)₄O₁₀(OH)₁₂</td>
<td>2</td>
</tr>
<tr>
<td>Dolomite</td>
<td>CaMg(CO₃)₂</td>
<td>2</td>
</tr>
<tr>
<td>Halite</td>
<td>NaCl</td>
<td>2</td>
</tr>
<tr>
<td>Cuprite</td>
<td>Cu₂O</td>
<td>1</td>
</tr>
</tbody>
</table>

5.8.3 Asphaltenes

Asphaltenes are present in all heavy oils and bitumens from oil sands and to a lesser extent in crude extracted from conventional wells. The material is defined by solubility, and the components that dissolve in toluene and precipitate in n-alkane solvents are asphaltenes. For analytical purposes, the concentration of asphaltene in a crude oil is defined by precipitation with either n-pentane C⁵ or n-heptane C⁷ [122].

Figure 5.17 illustrates a theoretical structure of an asphaltene particle and Figure 5.18 shows images from testing for asphaltenes in an emulsion sample using toluene and n-heptane.
Figure 5.17 – Suggested structure of an asphaltene molecule from Chart 3 of [123]. Note the vanadium porphyrin in the structure. Nickel porphyrins are also found in asphaltenes and the ratio of vanadium to nickel in an asphaltene sample can be used to identify the crude source. The formula for the structure is $C_{318}H_{395}N_6O_6S_8V$.

Figure 5.18 – Image of asphaltenes from an emulsion that was partially separated in a centrifuge. (a) The left hand vial is the result of mixing 15 mL of n-heptane and 0.5 mL of oil from the emulsion, the right hand vial is the result of mixing 15 mL of toluene with 0.5 mL of the oil. Asphaltenes can been seen in the n-heptane solution. (b) Photomicrograph of asphaltene conglomeration from the left hand vial shown in (a).
5.8.4 Emulsion Separation by Centrifugation

Due to the different densities of oil, brine, asphaltenes and solids it is possible to do a rough separation of the emulsions discussed in Section 5.8 by using a heated centrifuge designed for 100 mL API graduated centrifuge tube. The centrifuge time is variable as it is hard to visually estimate how long it will take the phases to separate. Figure 5.19 shows a separation of an emulsion after 30 minutes of centrifuging.

The layers in Figure 5.19 from top to bottom are oil, emulsion that has not separated, brine and solids that are both water and oil wetted.

![Figure 5.19](a) sample viewed in fluorescent light (b) sample illuminated with 365 nm light to show better contrast and definition between the layers due to electronic excitation of the emulsion components.

5.9 Water-Oil Partitioning of Naphthenic Acid

Disassociation of naphthenic acid in an aqueous phase is determined by the disassociation constant, $pK_a$, of the naphthenic acid. It has been reported that $pK_a = 4.9$ for $C_{10-16}$ naphthenic acid containing one to three saturated rings. The $pK_a$ value from this study was 5.1.
Assuming that the acid in crude oil contains both fatty acid and naphthenic acid, then depending on pH, the acid will be in disassociated and undisassociated forms. At higher pH the acid will form micelles in the water phase and reverse micelles in the oil phase.

\[ [HA]_o \rightleftharpoons [HA]_w \tag{5.6} \]

where \([HA]_o\) and \([HA]_w\) are the molar concentration (mol·L\(^{-1}\)) of the associated form of a weak acid in the water and oil phase respectfully. The molar concentration of acid that will partition to the aqueous phase can be defined as

\[ [HA]_w \rightleftharpoons [A^-] + [H^+] \tag{5.7} \]

where \([A^-]\) is the molar concentration of the disassociated form of a weak acid (mol·L\(^{-1}\)) and \([H^+]\) is is the molar concentration (mol·L\(^{-1}\)) of hydrogen ions due to the pH of the water.

From Equations 5.7 the partition coefficient \(K_{wo}\) of the acid between the water and the oil phases can be defined as

\[ K_{wo} = \frac{[HA]_w}{[HA]_o} \tag{5.8} \]

Equations 5.6 and 5.8 can be combined to define \(K_a\) the acid partition coefficient as

\[ K_a = \frac{[A^-] \cdot [H^+]}{[HA]_o} \tag{5.9} \]

Equation 5.10 describes the total concentration of undisassociated and disassociated forms of naphthenic acid in an aqueous phase.
\[ [HA]_{\text{w,tot}} = \frac{[HA]_{\text{init,oil}} \left( \frac{V_{\text{oil}}}{V_{\text{water}}} \right)}{1 - \frac{1}{1 + K_{\text{wo}}(V_{\text{water}}/V_{\text{oil}})(1+K_{\alpha}/[H^+]})} \]  

(5.10)

where \([HA]_o\) and \([HA]_w\) are the initial naphthenic acid molar concentrations (mol·L\(^{-1}\)) of the undissociated and dissociated in crude oil and water respectively, water-oil volume ratio \(V_{\text{water}}/V_{\text{oil}}\), acid dissociation constant \(K_{\alpha}\) and acid water-oil partition constant \(K_{\text{wo}}\) (mol·L\(^{-1}\)).

Since naphthenic acid refers to a broad class of compounds, the water/oil partition constant can vary by more than two orders of magnitude. Table 5.4 summarizes literature reported partition constants for naphthenic acid as a function of carbon number and number of hydrocarbon rings [24].

### Table 5.4 – Partitioning constants for crude oil naphthenic acid with different number of rings [24].

<table>
<thead>
<tr>
<th>Number of Carbons</th>
<th>Partition Coefficient ((K_{\text{OW}}, \text{mol·L}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One Ring</td>
</tr>
<tr>
<td>10</td>
<td>6.0 × 10(^{-3})</td>
</tr>
<tr>
<td>11</td>
<td>3.0 × 10(^{-3})</td>
</tr>
<tr>
<td>12</td>
<td>6.6 × 10(^{-4})</td>
</tr>
<tr>
<td>13</td>
<td>4.2 × 10(^{-4})</td>
</tr>
<tr>
<td>14</td>
<td>7.7 × 10(^{-5})</td>
</tr>
<tr>
<td>15</td>
<td>2.3 × 10(^{-5})</td>
</tr>
<tr>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

### 5.10 Naphthenic Acid in Desalter Brine

The fraction of naphthenic acid in the crude oil, related to desalter effluent, is calculated as a function of pH using Equation 5.10. The parameter values used in Equation 5.10
were \( V_{\text{water}}/V_{\text{oil}} = 0.05 \) and \( K_a = 4.9^{-10} \). Equation 5.10 was used to calculate water/oil partition constant \( K_{\text{OW}} \) as a function of naphthenic acid carbon number. Figure 5.20 shows that over the range from pH 3 to pH 11 the fraction of naphthenic acid that partitions to the desalter effluent brine is a strong function of desalter effluent brine pH. Greater fractions of naphthenic acid partition to the desalter brine as desalter effluent brine pH increases.

Figure 5.20 – The graph shows the strong effect of pH of the brine versus naphthenic acid concentration at the desalter effluent.
Chapter 6

Naphthenic Acid Analysis

As the TAN value increases within crude oil, the concentration of organic acids increases, including naphthenic acid, which can be toxic to various fish species. To study the acids in the crude oil the acids must be first extracted using liquid/liquid extraction and then analyzed. The extract contains a number of hydrocarbon types combined with the acids which can be fractionated for toxicity studies. This chapter discussed extraction and analytical methods used to prepare this thesis for oil and aqueous samples in complex matrices. A paper titled “Extraction and Speciation of Acid Species in Crude Oil from Athabasca Oil Sand” is being prepared based on the information discussed in this chapter.

6.1 Extraction and Speciation of Acid Species from Crude Oil for Speciation

The study of the acid species contained in crude oil requires liquid/liquid extraction and purification, described in Sections 6.1.1 and 6.1.2.

The acidic extract contains a number of neutral hydrocarbon species and contaminants such as inorganic solids that must be removed.
6.1.1 Extraction of Naphthenic Acids from Crude Oil

The following procedure was used to extract the organic acid from high TAN crude to allow for analysis, standard development and toxicity tests:

1. In a separatory funnel, 1 L of crude oil and 300 mL of 5 % sodium hydroxide (1:1 methanol:water) were shaken for 5 minutes.

2. After shaking, 500 mL of deionized water was added and the layers were allowed to separate.

3. The aqueous layer was collected and steps 2 and 3 were repeated.

4. The combined orange coloured aqueous layer was extracted twice with 250 mL of n-hexane to remove the hydrocarbons.

5. The solution was then acidified with 1 N hydrochloric acid to pH 2.

6. The solution became a milky yellow mixture that was extracted three times with 300 mL DCM.

7. The DCM solution was then dried with sodium sulphate.

8. The evaporation of DCM led to 740 mg of a brown residue that was purified with column chromatography on silica gel.

The ratios of caustic, methanol and water listed above were determined by trial and error to minimize the emulsion formation when caustic water was added to crude oil. The various steps above are shown in Figures 6.1 and 6.2.
Figure 6.1 – Acid extraction from crude oil. (a) crude oil with base, (b) crude oil with base and water, (c) aqueous phase with n-hexane wash to remove the hydrocarbons from the water; this phase turns pale yellow when acidified and (d) after acidification during extraction, the bottom layer is dichloromethane.

Figure 6.2 – Naphthenic acid extract after the dichloromethane shown in Figure 6.1(d) has been evaporated.
6.1.2 Separation of Extract by Column Chromatography

The brown residue from the extracted naphthenic acid was divided into two aliquots to allow two individual treatments to be used to separate the residue by column chromatography using the following procedure.

1. A glass column was used with dimensions of $3 \times 60$ cm packed with 100 g of 200 mesh silica gel with 1:4 DCM:hexane solution.

2. The extracted material from the crude mass was dissolved in the mobile phase and loaded onto the column.

3. The eluotropic series of solvents were n-hexane, DCM, ethyl acetate and methanol.

4. The treatments listed in tables 6.1 and 6.2 give the solvent ratios to produce four and seven fractions respectively from the extracted material. The number of fractions is somewhat arbitrary and was limited by practical considerations such as time and lab resources. Figure 6.3 shows the fractionation column and colours of the silica gel at various points of the purification process.

5. Thin layer chromatography (TLC) plates were used to periodically visually estimate the purity of each of the above steps. Figure 6.4 shows an example of a TLC plate used to estimate the purity of the naphthenic acid fractions.

6.1.3 Collected Acid Fractions

Two separation tests of the naphthenic acid extract were performed, the first provided four fractions and the second provided seven fractions. The solvents used for the separations and the TAN of each fraction from the separations are shown in Tables 6.1 and 6.2.
Figure 6.3 – Various views of the purification steps. (a) initial column loading of 760 mg of material extracted from crude oil (b) after DCM solvent had eluted, (c) a mixture of ethyl acetate and DCM eluting through the column and (d) after methanol had eluted through the column.

Figure 6.4 – Thin layer chromatography (TLC) plate of naphthenic acid extraction compared to four separate standards.
Table 6.1 – Percent mass and total percent mass recovery of four crude acid fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovery (%)</th>
<th>Solvent</th>
<th>TAN (mg KOH/g oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.4</td>
<td>1:4 DCM:n-hexane</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>24.2</td>
<td>DCM</td>
<td>90.0</td>
</tr>
<tr>
<td>3</td>
<td>46.4</td>
<td>9:1 DCM:Ethyl Acetate</td>
<td>151.0</td>
</tr>
<tr>
<td>4</td>
<td>6.9</td>
<td>3:1 DCM:Ethyl Acetate</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Total 80.9

Table 6.1 indicates that fractions 2 and 3 have high TAN values compared to fractions 1 and 4. The TAN values were found to indicate the relative toxicity of a given fraction. Toxicity tests with rainbow trout described in Section 3.5 showed that fraction 3 had the highest toxicity and fraction 2 was lower while fractions 1 and 4 were not toxic at approximately eight times the concentration of fractions 2 and 3.

Table 6.2 – Percent mass and total percent mass recovery of seven crude acid fractions.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Recovery (%)</th>
<th>Solvent</th>
<th>TAN (mg KOH/g oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.4</td>
<td>1:4 DCM:n-hexane</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>9.2</td>
<td>from 1:4 DCM:n-hexane to DCM</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>15.1</td>
<td>DCM</td>
<td>50.0</td>
</tr>
<tr>
<td>4</td>
<td>24.3</td>
<td>9:1 DCM:Ethyl Acetate</td>
<td>129.0</td>
</tr>
<tr>
<td>5</td>
<td>15.2</td>
<td>from 9:1 DCM:Ethyl Acetate to Ethyl Acetate</td>
<td>85.0</td>
</tr>
<tr>
<td>6</td>
<td>6.9</td>
<td>Ethyl Acetate to Methanol</td>
<td>20.0</td>
</tr>
<tr>
<td>7</td>
<td>16.6</td>
<td>Methanol</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Total 90.7

A second separation was done to determine if there was a solvent mixture that could be used to remove a fraction of the naphthenic acid extract that would render the remaining fractions less toxic. Table 6.2 indicates that fractions 4 and 5 have TAN values close to fractions 2 and 3 from the first separation test. Initial toxicity tests have
shown that fractions with a TAN less than 20 are not toxic to trout at concentrations seen in oil refinery wastewater treatment systems.

### 6.1.4 Naphthenic Acid Extraction from Aqueous Samples

The procedure below is used for the extraction of naphthenic acid from aqueous samples such as those found in a oil refinery wastewater system. Prior to analysis all glassware should be pre-rinsed with DCM. All surfaces contacting DCM have to be glass or Teflon to avoid dissolution of organic contaminants. Any visible oil in the sample is to be removed with a Pasteur pipette. In cases where the sample contains dispersed oil, it may be centrifuged to separate the layers.

1. Mark the water level on the sample bottle with tape or a permanent marker.

2. Measure the pH of water prior to acidification/naphthenic acid isolation using pH paper.

3. Pour 500 mL of water sample at room temperature into 1 L separatory funnel.

4. Acidify to pH 2.5 by adding appropriate volume of sulfuric acid. The exact volume of acid depends on initial sample pH.

5. Add 200 mL of DCM and shake the mixture for 2 min.

6. Allow to separate into an upper aqueous phase and a lower solvent phase.

7. Pass the solvent phase through sodium sulphate seated on a glass wool plug in a conical funnel and collect the DCM in a 250 mL round bottom flask.

8. Add 100 mL of DCM to the aqueous phase and repeat the organic extraction process.

9. Concentrate the combined solvent phase by evaporating in a rotary evaporator at 30 °C to dryness.
10. Add 75 mL of 0.1 N sodium hydroxide (pH = 13) to the organic extract to accomplish dissolution.

11. Adjust pH to 10.0 using sulfuric acid. Monitor pH with pH paper.

12. Filter the mixture through a 0.45 µm PTFE syringe filter into 125 mL separatory funnel.

13. Adjust the final filtrate to pH 2.5 using sulfuric acid and pH paper.

14. Extract with 2:1 sample to solvent volume of DCM. Use the same extraction procedure as described above: shake for 2 min., allow to settle, collect the solvent layer into 250 mL round bottom flask by passing through sodium sulphate.

15. Repeat extraction.

16. Rotavap the combined solvent phase at 30 °C to approximately 2-3 mL and transfer extract into 7 mL vial.

17. Store samples in the fridge prior to further analysis.

To determine the recovery efficiency of the extraction steps above, the aqueous phase was retained and extracted another two times. Each extract was saved and analyzed separately to determine the recovery efficiency. It was critical that spiked blanks be periodically checked for extraction efficiency to ensure the consumables and techniques used were valid.

The efficiency was found to be 98 % based on recovered weights. Figure 6.5 shows examples of the naphthenic acid extraction of desalter brine.
Figure 6.5 – Examples of extraction of two desalter brine samples at step 6. The bottom layer is the dichloromethane solvent that is coloured by the hydrocarbon in the sample.

6.2 Analytical Test Results

The naphthenic acid extracted from crude oil and the wastewater treatment system was analyzed and quantified using a number of analyzer types including FTIR/ATR, CG/MS located at the Suncor Sarnia Refinery Laboratory, LC/QTOF/MS at the Suncor Mississauga Lab and Orbitrap® located at Alberta Innovates in Vegeville, Alberta. The equipment details are listed in Appendix H.

Examples of the analysis results are shown in Sections 6.2.1, 6.2.2, 6.2.3 and 6.2.4.

6.2.1 FTIR/ATR

Extracted naphthenic acid fractions from both oil and water samples were analyzed by FTIR/ATR to determine extracted purity by monitoring the carboxylic acid peak at 1700 cm\(^{-1}\) and the fingerprint region from 1500 to 750 cm\(^{-1}\). Figures 6.6, 6.7 and 6.8 show the results of the crude-oil extractions; the wastewater samples are similar to the examples shown.
The FTIR/ATR analysis was performed at Suncor Sarnia.

Figure 6.6 – Comparison of (a) the seven separated fractions extracted and (b) the Sigma-Aldrich naphthenic acid standard. There are differences in the carboxylic acid C=O at 1700 cm\(^{-1}\) and the aromatic C-H out of plane vibrations in the 900 to 650 cm\(^{-1}\) region.
Figure 6.7 – FTIR analysis of (a) carboxylic acid C=O stretch, note how the peak at 1700 cm$^{-1}$ decreases to a minimum at fraction 4 and then increases suggesting a change in the concentration of carboxylic acid groups. (b) C-H out of plane vibrations of the aromatic ring, note the decrease as the fraction number increases which suggests increasing aromaticity.
Figure 6.8 – FTIR spectra of Fractions 1 through 4 extracted from a high TAN crude. The value of the transmittance % of wavenumber 1700 cm$^{-1}$ shows the carboxylic acid concentration variation between the fractions. This spectra is typical for any naphthenic acid sample analyzed.

6.2.2 GC/MS

Analysis of extracted naphthenic acid samples by GC/MS allowed the acids to be grouped by $z$ number, carbon number and concentration. The three values allowed the data from a sample to be displayed in a three dimensional bar plot which gives insight on the acid speciation. The GC/MS analysis was conducted using the method described by W. P. St. John et al. in 1998 [87] which uses derivitization of the sample to provide better sensitivity. Derivitization is a common chemical method of measurement of oxygenated organic compounds containing active hydrogen atoms [126]. In addition polar functional groups cause a number of issues in chromatography including low volatility, decomposition of the column or injection port thereby leading to non-reproducible results. [127] [128].

The GC/MS accuracy is approximately 300 mDa which does not allow the exact mass of a molecule to be determined. In addition the EI has a high energy and the molecule becomes fragmented, so molecular ions are a very small portion of the detector
signal.

The GC/MS method is listed in Appendix I and the macro to produce data for the three dimensional plots in this section is listed in Appendix J.

The GC/MS analysis was performed at Suncor in Sarnia, Ontario.

Figure 6.9 shows the abundance of ions 75, 117 and 129 which are due to the fragmentation of the derivitization agent and hence they identify the location of acids during the analysis. The fragment of the derivitization agent that remains has a mass of 58 Da and must be removed from the molecular weight to obtain the correct formula for the acid.

![Graph showing ion abundance over time](image)

**Figure 6.9** – This graph shows the relative intensities of the specific ions with masses 75, 117 and 129 Da due to the fracturing of the derivatizing agent. The combinations of the ion concentrations are used to produce the carbon and z series. The formulas for the masses are shown in Figure 6.10.

Figure 6.10 shows the structure of the ion fragments 75, 117 and 129.
Figure 6.10 – Three of the mass fragments from the derivatizing agent used to identify the location of carboxylic acid compounds including naphthenic acid shown in Figure 6.9.

Figure 6.11 shows the $z$ number of the acid series and the homologous series. The actual molecular weight of the acid requires subtraction of 58 Da due to the remaining derivitization agent fragment attached to the acid and the addition of 1 Da to account for the displaced hydrogen due to the addition of the derivitization agent.

As an example for the peak of 357.2 Da the actual molecular weight is equal to

$$357.2 \text{ Da} - 58 \text{ Da} + 1\text{Da} = 300.2 \text{ Da}$$

Figure 6.12 shows the difference in ion abundance in two samples from the wastewater treatment system which can be related to the naphthenic acid concentration. The shape of the ion abundance shown in Figure 6.12(a) is also referred to as a ‘humpogram’ which is caused by the unresolved oil matrix in the sample.

Figure 6.13(a) is a 3D plot of the $z$ series versus carbon numbers and the relative intensity of extracted naphthenic acid from a high TAN crude oil. Figure 6.13(b) on page 128 shows the percent of each $z$ series contained in the purified fractions of extracted acid from a high TAN crude oil.
Figure 6.11 – The lines indicate the $z$ value and the homologous series of the peak at 12 minutes shown in Figure 6.9. The homologous series differs by 14 Da or a methyl group ($\text{CH}_2$). This is shown as a change of one carbon number in Figures 6.13 and 6.14.
Figure 6.12 – GC/MS analysis of extracted naphthenic acid samples. (a) is from a desalter brine outlet and (b) is from the Wemco®. As can be inferred by the ion concentration the desalter brine sample has a much greater concentration of naphthenic acid than the Wemco® outlet. In this case the concentration in the desalter is approximately 15 mg·L⁻¹ and the concentration in the Wemco® is 0.1 mg·L⁻¹. The fatty acids are used as markers in both (a) and (b).
Figure 6.13 – GC/MS speciation of fatty and naphthenic acids in fractions listed in Table 6.2. (a) speciation of Fraction 5; the other fractions change in carbon number, the higher the fraction number the larger the carbon number distribution start and stop (b) relative mass % of acid in Fractions 1 through 7.
Figure 6.14 is a 3D plot of the $z$ series versus carbon numbers and the relative intensity of extracted naphthenic acid from an aqueous sample of the Hydrocell\textsuperscript{®} effluent which is one of the wastewater streams to the aeration basins.

**Figure 6.14** – Typical GC/MS speciation of phenolics, naphthenic acids, and fatty acids from wastewater treatment aqueous samples showing carbon number and $z$ series.

### 6.2.3 LC/QTOF/MS

The primary advantage of a LC/QTOF/MS over a GC/MS is that the mass resolution is approximately an order of magnitude greater than a GC/MS which allows the exact mass of a compound to be determined within 4 mDa. Another advantage is that the ionization method employed for the LC/QTOF is electrospray ionization (ESI) that has less energy than the electron impact (EI) ionization. In a GC/MS the molecular ion is fragmented due to the high energy electron impact ionization method. In addition ESI has less energy than EI therefore fragmentation is not as much of a concern and the
derivitization used for naphthenic acid analysis in GC/MS is not required.

Figures 6.15 and 6.16 show the results of LC/QTOF/MS analysis of samples of an aeration basin influent and a clarifier effluent respectively. Tables 6.3 and 6.4 list the percentage of heteroatom type in the two samples.

Figures 6.15 and 6.16 on page 132 show 3D plots of the naphthenic acid z series, carbon number and relative intensities of two typical wastewater aqueous samples. Due to the more accurate mass determination two heteroatom groups O and SO are seen, although they contribute little to the overall acid concentration as shown in Tables 6.3 on the following page and 6.4 on page 132.

The distribution of naphthenic acid in both the aeration basin influent and the clarifier effluent are indistinguishable as biodegradation of naphthenic acid does not occur during the residence time of the wastewater in the aeration basin as discussed in Section 4.7.1 on page 69.

The LC/QTOF/MS analysis was performed at Suncor in Mississauga, Ontario and Sarnia, Ontario. Appendix K lists the LC/QTOF/MS parameters used for the naphthenic acid analysis.
Figure 6.15 – Typical LC/QTOF/MS speciation of naphthenic acids and fatty acids showing carbon number, z series and associated heteroatom in the aeration basin influent sample of wastewater treatment. Table 6.3 lists the percentages of heteroatom type and z number in the sample.

Table 6.3 – Heteroatom percentage in the aeration basin influent by z value shown in Figure 6.16

<table>
<thead>
<tr>
<th>z number</th>
<th>Heteroatom (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O₂</td>
</tr>
<tr>
<td>0 to -34</td>
<td>99.69</td>
</tr>
<tr>
<td>-8 to -34</td>
<td></td>
</tr>
<tr>
<td>-2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.16 – Typical LC/QTOF/MS speciation of naphthenic acids and fatty acids showing carbon number, \( z \) series and associated heteroatom in the clarifier outlet sample of wastewater treatment. Table 6.4 lists the percentages of heteroatom type and \( z \) number in the sample.

<table>
<thead>
<tr>
<th>( z ) number</th>
<th>Heteroatom (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to -34</td>
<td>99.29</td>
</tr>
<tr>
<td>-8 to -34</td>
<td>0.66</td>
</tr>
<tr>
<td>-2</td>
<td>0.05</td>
</tr>
</tbody>
</table>
6.2.4 Orbitrap®

An Orbitrap® mass spectrometer resolution is approximately 2 mDa which is a higher resolution than a LC/QTOF/MS [29]. In addition the ion trap can be used to concentrate molecular ions prior to them being released to the detector. The Orbitrap® can be connected to a liquid chromatography column for sample separation prior to analysis.

The Orbitrap® analysis of purified naphthenic acid fractions was performed at the Environment and Carbon Management Division of Alberta Innovates in Vegeville, Alberta. Figures 6.17 on the next page and 6.19 on page 135 show the results of the analysis.

![Graph showing percent response vs. heteroatom group]

Figure 6.17 – Orbitrap® analysis of the Hydrocell® outlet sample showing primarily O₂ heteratoms of the extracted naphthenic acid.
Figure 6.18 – Orbitrap® analysis of (a) Fraction 1 (b) Fraction 2. Note the predominance of the O$_2$ series. Based on the response Fraction 1 is predominately hydrocarbon and Fraction 2 is predominately naphthenic acid.
Figure 6.19 – Orbitrap® analysis of two fractions from high TAN crude (a) Fraction 3 (b) Fraction 4. Note the predominance of the O₂ series in Fraction 3. Based on the response and heteroatom groups Fraction 3 is predominately naphthenic acid and Fraction 4 contains heavier aromatic species such as asphaltenes.
Chapter 7

Conclusions and Recommendations

This chapter summarizes the conclusions reached based on laboratory analysis and field results. Included are recommendations for future work based on the conclusions.

7.1 Conclusions

A summary of the conclusions reached in this thesis are discussed in Sections 7.1.1 through 7.1.5.

7.1.1 Transport

The presence of naphthenic acid in the refinery wastewater treatment system was found to be primarily due to the surface layers on very small emulsions and/or solids which were an average of 1 µm or less in diameter. Due to naphthenic acid being a weak organic acid the effect of the toxicity for a given concentration increases as the pH of the desalter brine increases.

When the pH of desalter brine is < 7 it increases the naphthenic acid toxicity in wastewater in two ways

1. A stable emulsion formation is created. As pH increases the naphthenic acid
becomes disassociated (i.e. more polar), and combines with other polar molecules such as asphaltenes and solids to form a stable emulsion.

2. Since naphthenic is a weak acid its concentration in the water phase will increase. The increased concentration in turn increases the acute toxicity effects on the fish.

7.1.2 Histopathology

Exposure of the fish to fractions 2 and 3 of extracted naphthenic acid at concentrations between 0.2 to 0.4 mg·L$^{-1}$, or clarifier effluent with naphthenic acid concentrations $> 2.0$ mg·L$^{-1}$, resulted in significant cellular changes or lesions in the brain, kidney, and stomach.

7.1.3 TIE

The key findings of the TIE testing, and analytical investigation and associated predictions of naphthenic acid concentrations concerning the occurrence of toxicity to rainbow trout of water samples were:

1. Naphthenic acid concentrations were found to range between 4.5 and 6 mg·L$^{-1}$ for process effluent samples that demonstrated toxicity to rainbow trout.

2. Naphthenic acid concentrations were found to range between 0.37 to 0.96 mg·L$^{-1}$ for those process effluent samples that did not demonstrate acute lethality to rainbow trout.

3. Naphthenic acid found within the desalter brine and therefore process effluent were comprised of higher molecular weight with more carbon and cyclic naphthenic acid compounds compared to the available Sigma-Aldrich naphthenic acid reference standard.
4. The predicated 96 hour LT$_{50}$ time estimate for naphthenic acid found within the process effluent samples shows a toxicity range between 1.86 and 5.35 mg·L$^{-1}$, for an average of 3.41 +/- 1.4 mg·L$^{-1}$.

5. The predicated 96 hour LC$_{50}$ concentration estimate for naphthenic acid found within the process effluent samples shows a toxicity range between 3.7 and 5.5 mg·L$^{-1}$, for an average of 4.5 +/- 0.9 mg·L$^{-1}$.

6. A review of published literature indicates that acute LC$_{50}$ fish toxicity for naphthenic acid should occur in the range of 3 to 4.5 +/- 0.9 mg·L$^{-1}$.

7. The naphthenic acid reference standard rainbow trout 96 hour LC$_{50}$ was determined to be 1.62 mg·L$^{-1}$.

8. From the naphthenic acid reference standard toxicity test no acute or sublethal toxicity (i.e. TLOE) was detected at the 1.15 mg·L$^{-1}$ nominal test concentration (No Observed Effect Concentration) and 100 % mortality occurred at 2.3 mg·L$^{-1}$ nominal test concentration (Lowest Observed Effect Concentration).

9. Variables such as sample pH, the molecular weights of the naphthenic acids, and the number of cyclic groups that comprise the naphthenic acid molecules can likely have an influence on the degree of toxicity response.

10. The detection of naphthenic acid within the process effluent at concentrations greater than literature toxicity values, as well as the predicted LT$_{50}$/LC$_{50}$ effect concentrations and naphthenic acid reference standard rainbow trout LC$_{50}$ toxicity test, provide proof that naphthenic acids were the source of toxicity within the process effluent. This is further confirmed by the observation that a TLOE sublethal response was observed during completion of the rainbow trout reference standard toxicity test. This is characteristic of the toxicant of concern as observed during completion of the process effluent toxicity tests.
7.1.4 Acute Toxicity Tests

Acute toxicity tests at pH 6 are approximately six times more sensitive than those at pH\(_i\) (i.e. the concentrations are approximately one order of magnitude different).

1. If pH 6 is chosen to be the controlling limit at the process effluent point monitored for Ministry of the Environment Ontario compliance of LC\(_{50}\) testing, the maximum naphthenic acid concentration should change to 0.1 mg·L\(^{-1}\).

2. If pH\(_i\) is chosen to be the controlling limit at the process effluent point monitored for MOE compliance of LC\(_{50}\) testing, the maximum naphthenic acid concentration should change to 1.0 mg·L\(^{-1}\).

With an 80 % filtration capacity of the total flow from the clarifiers and a maximum naphthenic acid concentration of 4 mg·L\(^{-1}\) at the carbon filter there is a high probability of seeing mortality at pH\(_i\) for the process effluent. The LC\(_{50}\) test would fail at a concentration of approximately 8 mg·L\(^{-1}\) at the carbon inlet. The concentration of naphthenic acid has approached 4 mg·L\(^{-1}\) during an equalization upset and has been over 5 mg·L\(^{-1}\) in the past.

Since there is no sand pre-filter before the carbon filters, the media must be changed on a frequent basis, even more so during an upset, due to pin floc carry over from the clarifiers and loading on the carbon bed. During the time that the filter is out of service the risk of failure becomes higher as less material is treated by the carbon filter.

7.1.5 Naphthenic Acid Extraction

1. The method shown in Section 6.1 has been proven successful in the extraction of acid compounds, including naphthenic acid, from crude.

2. Analysis by LC/QTOF and Orbitrap\(^\text{®}\) indicates a number of organic acid types including fatty acid, and naphthenic acid in the wastewater system.
3. Analysis results indicate that a quadrupole GC/MS has enough resolution for routine acid analysis.

4. Further analysis methods are required to determine purity of extract fractions which will allow standards to be produced.

7.2 Recommendations

7.2.1 Collaborate with Government and Industrial Peers to Develop Naphthenic Acid Analysis Method

Currently there is no agreed to method for naphthenic acid analysis that Government organizations can use to enforce discharge limits. There are currently interlaboratory studies being planned for 2016 to continue work done in 2012 and 2014 [73, 74]. Suncor will be a participant in those studies by doing analysis and suppling extracted naphthenic acid from high TAN crude to be used as a standard.

7.2.2 Purify Extracted Acid Samples from High TAN Crude Using Preparative HPLC for Toxicity and Characterization Studies

Based on the column chromatography discussed in Section 6.1.2 of this thesis, preparative HPLC can be used to separate fractions from the high TAN extracted acid samples. Preparative HPLC allows the for use of multiple solvent pumps to expose the extracted acid loaded into a column to a linear gradient starting at non-polar to highly polar solvents. The extract is then collected into a series of vials which can be used for characterization and toxicity studies.
7.2.3 Biotreater for Solids Extracted from Coagulant and Flocculant Treatment

Develop procedures and methods for extracting solids from the coagulant and flocculent process using a centrifuge described in the patent application. This will allow the solids and oil to be biologically treated to remove naphthenic acids over an extended time period prior to disposal.

Continue the research of biological methods for the removal of naphthenic acid that are more efficient such as recirculating packed bed and immobilized bed bioreactors [130, 131] and cultivation of specific bacteria [131, 132]. The research in this thesis shows that the residence time of an aeration basin in the wastewater treatment system of an oil refinery is not a viable option for biodegradation.

Analyze the microbe population during testing of the biological methods by 16S rRNA and metagenomics methods.
Bibliography


[49] W. Ramsden. Separartions of Solids in the Surface-layers of Solutions and ‘Suspensions’ (Observations on Surface-Membranes, Bubbles, Emulsions, and


[83] EPA. *Methods for Aquatic Toxicity Identification Evaluations Phase II Toxicity


Appendix A

Processing of Aqueous Waste Streams to Remove Naphthenic Acids

Author: Martin Flatley, Suncor
Co-author: Argyrios Margaritis, University of Western Ontario
Date patent application submitted: October 4, 2015
PROCESSING OF AQUEOUS WASTE STREAMS TO REMOVE NAPHTHENIC ACIDS

TECHNICAL FIELD

[0001] The following relates to the treatment of aqueous waste streams, particularly waste streams resulting from the processing of hydrocarbons containing naphthenic acids.

BACKGROUND

[0002] Naphthenic acids are a mixture of organic carboxylic acids that arise from biodegradation of crude oil deposits by anaerobic bacteria. The naphthenic acid content of a hydrocarbon sample is generally measured indirectly by determining the total acid number (TAN), or acidity of the oil, which is determined by measuring the amount of potassium hydroxide in milligrams that is needed to neutralize acids in one gram of oil.

[0003] Hydrocarbon samples having high total acid number (TAN) are associated with a higher naphthenic acid content, with naphthenic acids accounting for a significant portion of the TAN. Naphthenic acids are often grouped or characterized by carbon number and z number (hydrogen deficiency). The molecular weight distribution of naphthenic acids in an oil sample can be used as biomarkers for the source of the oil, with the naphthenic acid characterization varying with the age and method of formation of the deposit.

[0004] In general, heavy oil samples are associated with a greater concentration of naphthenic acids. Hydrocarbons that are mined and extracted from an oil sands reservoir, or are produced in situ (i.e., via wells) from an oil sands reservoir, are generally recognized as having a higher naphthenic acid concentration than conventional crudes.

[0005] As naphthenic acids are somewhat soluble in water, the processing of heavy oil has been shown to result in waste water and process water having elevated levels of naphthenic acids. Therefore, methods for treating refinery and other hydrocarbon processing waste waters have been developed that typically include an activated sludge or biotreatment step, followed by a chemical treatment and solids removal step prior to discharge of the treated waste water as effluent or recycling as process water.

[0006] If high concentrations of naphthenic acids were present in waste water released to the environment, the naphthenic acids could be toxic to fish, producing
effects such as distended stomach, dark colour, loss of equilibrium, and mortality. Accordingly, naphthenic acid levels in treated waste water must be kept below levels that may be considered toxic to the environment, and any solids or liquid streams containing significant concentrations of naphthenic acids must be disposed of as hazardous waste.

**SUMMARY**

[0007] A method is provided for treating aqueous waste streams, particularly waste streams resulting from the processing of hydrocarbons containing naphthenic acids. The method provides for the early destabilization of emulsions within the aqueous waste stream, and subsequent physical separation of a solids stream from a waste water stream. The solids stream and waste water stream can be independently treated to efficiently remove naphthenic acids.

[0008] In a first aspect, there is provided a method for treating an aqueous waste stream containing naphthenic acids and oil-wet solids, the method comprising:

- pre-treating the aqueous waste stream with one or more additives to promote demulsification, flocculation, coagulation, or agglomeration of the oil-wet solids;
- separating the pre-treated aqueous waste stream into a naphthenic acid-concentrated solids stream and a waste water stream; and
- processing the naphthenic acid-concentrated solids stream to remove naphthenic acids from the solids stream.

[0009] In an embodiment, the naphthenic acid-concentrated solids stream comprises an emulsion of oil, water, and oil-wet solids. The aqueous stream can include solids-stabilized emulsion droplets.

[0010] The naphthenic acid-concentrated solids stream can have a higher naphthenic acid content than the waste water stream. Further, the naphthenic acid-concentrated solids stream can have a higher naphthenic acid concentration than either the aqueous stream or the waste water stream.

[0011] In one embodiment, the step of processing the naphthenic acid-concentrated solids stream comprises a biotreatment step to remove naphthenic acids from the solids stream by microbial digestion. The biotreatment step can include incubation of the naphthenic acid-concentrated solids stream in a bioreactor in the presence of microbes,
with a residence time that is at least 7 days, at least 28 days, or any suitable length of
time to obtain a target reduction in naphthenic acid concentration.

[0012] In one embodiment, the bioreactor is an aeration vessel.

[0013] In some embodiments, the bioreactor includes water and microbial nutrients,
and the naphthenic acid concentration of the bioreactor can be monitored during
incubation. Further, one or more operating conditions of the biotreatment step can be
monitored and adjusted to produce a biotreatment effluent having a naphthenic acid
concentration that is below a target concentration. The operating condition of the
biotreatment step that is adjusted can be temperature, pH, residence time, microbial
nutrient concentration, water content, microbial density, or any other adjustable
parameter that can impact the biotreatment process, and thereby control the naphthenic
acid concentration in the bioreactor or of the biotreatment effluent.

[0014] In some embodiments, the biotreated effluent can be a biotreated aqueous
stream, and in some implementations the biotreatment effluent can be a biotreated
solids stream. In any implementation, the biotreatment effluent can include both solids
and liquids. In various implementations, the target naphthenic acid concentration in the
biotreatment effluent can be 2ppm or less, 0.5ppm or less, 0.1ppm or less, or any
suitable target concentration.

[0015] In an embodiment, the one or more additives are selected by testing the
aqueous waste stream to determine a suitable additive or combination of additives for
separation of the oil-wet solids from the waste water. The method can further include
periodically testing the aqueous waste stream to adjust the additive selection or dosage
over time to adapt to changes in the aqueous waste stream composition over time
during operation. In an embodiment, the testing step includes testing the aqueous waste
stream with various additives or combinations of additives using a jar test. The testing
can include zeta potential monitoring, turbidity testing, pH testing, or measurement of
total suspended solids in the feed.

[0016] In an embodiment, the waste water stream or biotreatment effluent is further
processed by conventional waste water treatment methods to remove naphthenic acids
or other contaminants suspended or dissolved in the waste water or biotreatment
effluent prior to reuse, release, or disposal. In some embodiments, the waste water
stream or biotreatment effluent is further processed by:
- a biotreatment step comprising incubation with microbes in a bioreactor;
- a clarification step comprising gravity separation;
- a filtration step; and
- a final separation step comprising gravity separation to collect a clarified liquid effluent.

[0017] In various embodiments, the clarified liquid effluent can have a naphthenic acid concentration of less than 2 ppm, less than 0.5 ppm, less than 0.1 ppm, or any other suitable target concentration.

[0018] In any embodiment, the aqueous waste stream can be a brine stream obtained from a desalter. The desalter can be a desalter used in a hydrocarbon refinery process. In one embodiment, the desalter is used in upgrading bitumen or heavy oil.

[0019] In any of the above embodiments, the aqueous waste stream can be a waste stream that results from the processing of high TAN hydrocarbons. The high TAN hydrocarbons can be hydrocarbons produced or extracted from an oil sands reservoir. In one embodiment, the hydrocarbons have been extracted from mined oil sands ore. In one embodiment, the hydrocarbons have been produced from wells in an oil sands reservoir.

[0020] In some embodiments, the extracted hydrocarbons have been processed using naphtha as a diluent. In some embodiments, the extracted hydrocarbons have been processed using a paraffinic diluent.

[0021] In any of the above embodiments, the one or more additives can be flocculants, coagulants, or pH modifying agents. In one embodiment, the additives are selected from the group consisting of: iron (ferric) chloride, aluminum chloride, sodium hydroxide, an amine polymer, and a polyacrylamide polymer.

[0022] In accordance with an embodiment, the naphthenic acid-concentrated solids stream can include at least 80% of the naphthenic acid content of the aqueous waste stream.

[0023] In any of the above embodiments, the step of separating the pre-treated aqueous waste stream can include gravity separation of oil-wet solids from the pre-treated aqueous stream. In a specific embodiment, the step of separating can include phase separation in a clarifier vessel or centrifuge.
In a second aspect, there is provided a method for processing desalter brine emulsion containing oil-wet solids, the method comprising:

- pre-treating the desalter brine with one or more additives to promote destabilization of the desalter brine emulsion and agglomeration of the oil-wet solids, the one or more additives comprising iron (ferric) chloride, aluminum chloride, sodium hydroxide, an amine polymer, or a polyacrylamide polymer;
- separating the desalter brine into a solids stream and a waste water stream; and
- processing the combined waste water stream via a conventional water treatment process.

In an embodiment, the desalter brine emulsion comprises naphthenic acids.

The method can further comprise processing the solids stream in a biotreatment step to remove naphthenic acids from the solids stream by microbial digestion. The biotreatment step can involve incubation of the solids stream in a bioreactor in the presence of microbes, with a residence time that is at least 7 days, at least 28 days, or any suitable length of time to obtain a target reduction in naphthenic acid concentration.

In one embodiment, naphthenic acid concentration of the bioreactor contents is monitored during incubation. One or more operating condition of the biotreatment step can be monitored and adjusted to produce a biotreatment effluent having a naphthenic acid concentration that is below a target concentration. In some embodiments, the operating condition that is monitored and adjusted is temperature, pH, residence time, microbial nutrient concentration, water content, or microbial density.

In an embodiment, the one or more additives are selected by testing the desalter brine emulsion to determine a suitable additive or combination of additives for use in destabilization of the desalter brine emulsion. In one embodiment, the step of testing the desalter brine emulsion includes testing with various additives or combinations of additives using a jar test to observe settling of solids.

In an embodiment, the step of testing the desalter brine stream comprises zeta potential monitoring, turbidity testing, pH testing, or measurement of total suspended solids in the feed.
[0030] In an embodiment, the method further involves processing the waste water stream using conventional waste water treatment methods to remove naphthenic acids or other contaminants suspended or dissolved in the waste water.

BRIEF DESCRIPTION OF THE DRAWINGS

[0031] Various aspects and implementations will now be described by way of example only with reference to the appended drawings wherein:

[0032] FIG. 1 is a process flow diagram of a conventional refinery waste water treatment process;

[0033] FIG 2 is a process flow diagram for treatment of an aqueous waste stream;

[0034] FIG. 3 is a schematic diagram of a process for treating an aqueous waste stream, in one embodiment;

[0035] FIG. 4 is a schematic diagram of a process for solids treatment, in one embodiment; and

[0036] FIG. 5 is a process flow diagram of a treatment process for handling pre-treated waste water.

DETAILED DESCRIPTION

[0037] A method is provided for the treatment of aqueous waste streams resulting from the processing of hydrocarbons that contain naphthenic acids, for example hydrocarbons extracted or produced from an oil sands reservoir. Naphthenic acids can be removed from these waste streams by removing oil-wet solids from the waste stream prior to conventional waste water processing, and the oil-wet solids can further be independently remediated to reduce naphthenic acid concentration.

[0038] More specifically, it has been determined that naphthenic acids remain associated with oil-wet solids that adhere to emulsion droplets carried within the aqueous stream and persist throughout conventional water treatment processes, with naphthenic acids levels persisting in the treated effluent as a result. The presently described process removes the oil-wet solids (and the naphthenic acids associated with the oil-wet solids) upstream of conventional water treatment steps, by pre-treatment with
one or more additives to promote demulsification, flocculation, coagulation, or agglomeration of the oil-wet solids. Subsequent phase separation provides a naphthenic acid-concentrated solids stream, and a waste water stream that is reduced in naphthenic acid content. The waste water stream, reduced in naphthenic acid content, can then be processed using conventional water treatment methods to produce an effluent with a naphthenic acid content that is below target levels. Further, the solids stream can be processed independently to remove naphthenic acids, for example by biotreatment under conditions that can be optimized for microbial digestion of naphthenic acids. Using the methods described herein, a target degree of remediation can be independently achieved in both the solids stream and the waste water stream prior to recycling, release, or other disposal.

**Naphthenic Acids in Conventional Waste Water Treatment**

[0039] Given the water-intensive nature of hydrocarbon recovery and processing methods, the focus of waste water treatment has been to obtain an effluent that is sufficient for recycling as process water, or is of acceptable quality for release to the environment. Existing processes for naphthenic acid removal have therefore been directed to the remediation of the water phase. In such processes, waste solids are typically removed late in the water treatment process by physical separation such as in clarifiers or settling ponds.

[0040] It has been determined by the present inventor that the primary transport mechanism of naphthenic acid in aqueous streams is by association with oil-wet solids, which persist throughout conventional treatment methods. Specifically, the oil-wet solids, and thereby the naphthenic acids, are carried at the interface of, and serve to stabilize, emulsion droplets. The emulsion may further contain high concentrations of dissolved organic carbon, which negatively impacts the waste water treatment process. Passing the oil-wet solids, naphthenic acids, and dissolved organic carbon through a conventional waste water treatment process, as part of a high volume waste water treatment process, is inefficient. Notably, in conventional waste water treatment processes, early phase separation steps are passive and do not serve to destabilize emulsions. As a result, the emulsion droplets and oil-wet solids (and naphthenic acids) become diluted in the treatment process but are not efficiently removed, allowing naphthenic acids to persist in the waste water treatment effluent.
Analysis of various waste streams resulting from hydrocarbon processing shows that naphthenic acids are highly concentrated in brine streams, and that desalter brine is the major source of naphthenic acids in aqueous refinery waste streams. In hydrocarbon processing, the desalter is used to mix water with crude oil in order to transport salts and contaminants from the oil phase to the water phase, to protect downstream oil processing equipment from corrosion and solid deposition. In operation of the desalter, the mixing of oil with process water creates an emulsion within the desalter, while oil is removed from the top. An emulsion of water, salts, and solids forms within the desalter, and naphthenic acids tend to associate with the emulsion, as the naphthenic acids are slightly polar and become associated with the asphaltene and other hydrocarbons coating the solid particles on the surface of the emulsion. Further, as the pH of the desalter brine increases, the stability of the emulsion increases due to the increased association of the naphthenic acids with other polar molecules such as asphaltenes.

The desalter brine is therefore composed of an emulsion of oil, water, and solids high in naphthenic acid content. With increased pH, the remainder of the naphthenic acid that is not associated with the emulsion partitions into the (polar) water phase. When naphthenic acids persist in the emulsion and also in the water phase throughout conventional water treatment process, conventional water treatment methods cannot keep pace with the rate of waste water production during hydrocarbon processing (as described further below) and are therefore unable to produce a suitably remediated solid and liquid waste stream. As the liquid waste stream can be recycled for use in continued hydrocarbon processing, efforts to remediate the liquid stream have been emphasized, leading to difficulties in disposing of the unremediated solid waste streams.

Figure 1 provides a diagram of a conventional refinery process 100 for treating waste water. Aqueous waste streams 101 from the refinery, such as desalter brine, are combined and processed together through a Separation or Settling step 110, with oil 102 and solids 103 being recovered, and water 104 added as may be necessary. The Separation or Settling step 110 may include cyclone separation, settling tanks, a dissolved air flotation vessel, and other passive or active separation steps. The effluent from the Separation or Settling step 110 is then subject to a Biotreatment step 120, for example in an aeration basin. In the Biotreatment step 120, the feed is exposed to
microbes that naturally metabolize organic matter such as hydrocarbons, and the products of microbial digestion (assuming conditions are appropriate for complete biodegradation) are carbon dioxide and water. However, the residence time for the Biotreatment step 120 of a conventional waste water treatment process is approximately 8 hours due to the large volumes of waste water to be treated. This rate is insufficient to achieve biodegradation of large naphthenic acid compounds in the Biotreatment step 120. Studies have shown that microbial digestion of large naphthenic acid compounds requires at least several days of incubation for suitable decomposition by microbial digestion, as the microbes initially metabolize smaller hydrocarbon compounds before digestion of the larger naphthenic acids. However, despite incomplete bioremediation, the residence time of the Biotreatment step 120 cannot be increased in conventional systems due to the large volumes of aqueous waste water that must be processed. Additional steps are therefore required after biotreatment to further assist in naphthenic acid removal from the waste water.

Following biotreatment, a Flocculation / Coagulation step 130 is present to allow agglomeration and coagulation of remaining solids, and a Settling step 140 provides filtration and/or settling to allow collection of a clarified waste water effluent 105 that may be released to the environment, recycled, or otherwise disposed of. Solids 106 are collected for disposal.

As conventional water treatment methods fail to destabilize emulsion droplets early in the treatment process, oil-wet solids remain associated with the emulsion droplets and are not removed during the early settling phases. This results in naphthenic acid content persisting in the late stages of the conventional water treatment process, as there is insufficient time to provide adequate separation and bioremediation of the oil-wet solids.

**Method for Treatment of Aqueous Streams**

The presently described process provides for the pre-treatment of specific aqueous streams (such as refinery desalter brine streams) that are likely to be concentrated in oil-wet solids and/or that otherwise contain solids-stabilized emulsions. The pre-treatment step destabilizes the emulsion so that the oil-wet solids can be removed, providing a pre-treated aqueous waste water stream that is significantly reduced in naphthenic acid content. The pre-treated waste water stream can then be
combined with other waste water sources and treated by conventional water treatment methods prior to release or recycling, and the oil-wet solids can be independently treated to remove or reduce the naphthenic acid content to an acceptable level for solids disposal.

[0047] Figure 2 depicts a method 200 for treatment of an aqueous stream to remove naphthenic acids and oil-wet solids, in one general implementation. An aqueous waste stream 201, resulting from the processing of heavy (or high TAN) hydrocarbons and containing oil-wet solids and naphthenic acids, is initially mixed with additives 202 in an “additive treatment” step 210 to promote demulsification, agglomeration, coagulation, coalescence, and/or flocculation of solids, particularly oil-wet solids. Disruption of the solids-stabilized emulsion enables the agglomeration and settling of the solids, with associated naphthenic acids. Appropriate additives 202 for demulsification, agglomeration, coagulation, and flocculation will be apparent to those skilled in the art, as will methods and operational conditions for use of same.

[0048] In some implementations, the additives can include coagulants such as metal salts, for example ferric chloride and aluminum chloride, can be used to neutralize the negatively charged colloidal particles in the emulsion, based on the zeta potential (charge) of the emulsion, promoting coagulation of solids. Acid and base compounds, as well as amine polymers, can be used to adjust and/or buffer the pH for optimization of emulsion-associated naphthenic acid partitioning to the solids phase. For example, the additives can include suitable acid and base additives such as ferric chloride, aluminum chloride, and sodium hydroxide. Anionic polymeric flocculants such as polyacrylamides can be used to agglomerate the solids particles to aid separation of the oil-wet solids and naphthenic acids from the aqueous stream.

[0049] Suitable additives can be selected or combined from the following: multivalent cations such as aluminum, iron, calcium, or magnesium and their salts, such as iron (ferric) chloride and aluminum chloride, acids and bases such as metal chlorides and sodium hydroxide, and polymeric flocculants such as polyacrylamides.

[0050] In order to determine suitable additives and suitable dosages of each additive for use with a particular waste water source, samples of the feed stream can be tested with various combinations of additives, for example using the jar test, to determine a suitable combination of additives for solids removal. Further, for continual
optimization of the additive dosage based on feed variability, the feed can be sampled periodically or tested continually. Such tests and measurements can include zeta potential monitoring, turbidity testing, pH testing, and measurement of total suspended solids in the feed.

[0051] A subsequent “separation” step 220 results in the collection of a pre-treated solids stream 203 and a pre-treated waste water stream 204. The pre-treated waste water stream 204 is reduced in naphthenic acid content and can be combined with other waste water streams and further processed using conventional waste water treatment steps for recycling or release to the environment. In experimental testing, the concentration of naphthenic acids in the pre-treated waste water stream 204 was reduced by up to 90% when compared to starting naphthenic acid concentration in the waste stream 201. Accordingly, pre-treatment with additives and separation to remove the oil-wet solids significantly reduces the naphthenic acid content (pre-treated waste water 204) prior to processing through conventional waste water treatment methods.

[0052] The pre-treated solids stream 203 is concentrated in naphthenic acid content and is further treated in a “naphthenic acid removal” step 230, which includes a biotreatment step for microbial digestion of hydrocarbons from the solids stream. The naphthenic acid removal step 230 for the pre-treated solids 203 is not dependent on the method or rate of handling of the pre-treated waste water 204. Accordingly, the naphthenic acid removal step 230 can be handled in a flexible manner based on a desired remediation of the pre-treated solids stream 203. For example, biotreatment of the pre-treated solids 203 can be processed in batches and subject to long term microbial digestion, with each batch processed for several days or weeks to allow complete or substantial naphthenic acid digestion to achieve a desired target naphthenic acid concentration in the solids stream 206, with recovery of produced waste water 205. In some implementations, the naphthenic acid removal step 230 includes a biotreatment step in which microbial digestion occurs for at least 7 days, at least 28 days, or more than 28 days. The disadvantage in conventional systems of having limited residence time, as discussed above, is therefore averted.

[0053] The naphthenic acid removal step 230 for solids can include the use of PAC (powdered activated carbon), batch reactors, or membrane bioreactors. Further process steps may include sand filtration, activated carbon treatment, and chemical oxidation.
[0054] Waste water 204, 205, recovered from the separation step 220 or naphthenic acid removal step 230, respectively, can be collected and stored for processing through conventional waste water treatment methods 240, to remove naphthenic acids and other contaminants suspended or dissolved in the aqueous phase.

[0055] Aqueous streams that can be treated in accordance with the herein-described process can include, for example, desalter brine, spent caustic, sour water, refinery process water (for example water used for steam generation or as cooling water) or any other aqueous stream that is believed to contain oil-wet solids and/or naphthenic acids. Preferably, the aqueous streams result from a heavy hydrocarbon extraction, treatment, upgrading, refining, or other processing methods. The aqueous feed streams can be generated at the same or at separate geographic locations, and two or more such streams may be combined, depending on the process configuration, and treated together.

[0056] With reference to Figure 3, a method 300 and process configuration for pre-treatment of desalter brine is shown, in one implementation. A hydrocarbon feed 301 and water 302 are mixed and processed in a desalter 310 to produce a desalted oil feed 303 and desalter brine 304. The desalter brine 304 includes oil-wet solids, naphthenic acids, and other contaminants removed from the hydrocarbon feed 301 by processing in the desalter 310. The desalter brine 304 is then pre-treated by mixing with additives such as coagulants, flocculants, and acid-base regulating chemicals in the additive mixing vessel 320. In one implementation, each additive is mixed into the feed in a separate compartment of the mixing vessel as the feed progresses through each compartment.

[0057] Any flocculated solids, emulsified oil, oil-wet solids, or other agglomerated or coagulated solids that form during mixing with the additives are recovered, while the remaining aqueous phase is further separated in a clarifier 330. The solids/emulsion stream (containing oil, water, and solids in various forms) is further separated by a centrifuge 350. A holding tank 340 provides surge capacity to allow batch processing by the centrifuge 350. At each pre-treatment step a clarified water stream 305 is recovered and sent to a waste water holding tank 360, while the solids/emulsion streams recovered from each pre-treatment settling step are collected in holding tank 340 and ultimately processed by the centrifuge 350.
The pre-treatment process results in the recovery of three waste product streams: oil 303, which can be directed to oil processing steps; waste water 305, which can be sent to waste water holding tank 360, and pre-treatment solids 306. The pre-treatment solids can be disposed of directly; however in a preferred embodiment, the pre-treatment solids are further processed to remove naphthenic acids therefrom prior to solids disposal. Waste water holding tank 360 can be used to supply a conventional water treatment process to remove naphthenic acids and other contaminants suspended or dissolved in the aqueous phase prior to release or recycling.

Further Processing of Pre-treated Solids

With reference to Figure 4, a biotreatment step is shown for bioremediation of oil-wet solids. Oil-wet solids 401 (such as solids 306 recovered from desalter brine using the method shown in Figure 2), can be fed to a bioreactor 400 that is stocked with microbes 402 and supplemented with water 403 and microbial nutrients 404. Appropriate temperature and aeration conditions are provided during biotreatment to facilitate microbial digestion of hydrocarbons. Bioremediated solids 405 and waste water 406 can be produced from the bioreactor continually or in batches, depending on the preferred mode of operation of the bioreactor. The hydrocarbon and/or naphthenic acid content is measured periodically or continually and the bioreactor conditions are modified as needed. Controlled operation of the bioreactor can be based on a preferred rate of bioremediation, a preferred incubation or residence time in the bioreactor, or based on a target naphthenic acid reduction or concentration in the waste water 406 or bioremediated solids stream 405. Various adjustments to the biotreatment conditions may be made to facilitate this control, such as monitoring and adjusting the temperature, pH, residence time, microbial nutrient concentration, water content, or microbial density in the bioreactor.

Monitoring and adjusting the operational conditions of the solids biotreatment step based on any desired endpoint is possible due to the early recovery of solids by pre-treatment of the desalter brine or other aqueous waste water feed stream. In prior systems where solids are not separated from the aqueous stream early in the process, any bioremediation process is driven by the rate of waste water feed to the system, which does not provide sufficient time for controlled bioremediation, particularly when naphthenic acids are associated with oil-wet solids. Independent handling of a pre-
treated waste water stream and a pre-treated solids treatment provides flexibility to optimize the clarification and decontamination of each stream as desired.

[0061] The pre-treated water stream may be processed using conventional waste water treatment methods. In Figure 5, a waste water treatment process 500 for handling pre-treated waste water (for example waste water stream 406 from the process shown in Figure 4, waste water 305 shown in Figure 3, or waste water 204, 205, from the process shown in Figure 2) is shown. The waste water feed 501 is first subject to a "separation" step 510. The separation step 510 can include one or more passive or active physical separation methods, such as cyclonic separation, settling in an equalization tank, processing in a dissolved air flotation vessel, or by other suitable separation methods. The resulting water phase (overflow) is processed through a "biotreatment" step 520, which can include one or more aeration basins or bioreactors containing hydrocarbon-digesting microbes, under conditions suitable for microbial digestion of suspended hydrocarbons in the aqueous phase. The biotreatment effluent is clarified by addition of coagulants, flocculants, or other additives, that aid in the settling of suspended particles from the water phase. The effluent from this "clarification" step 530 can be passed through a "carbon filtration" step 540 prior to a final "settling" step 550 that can include retention of the filtrate in a settling pond or vessel. The overflow effluent 502 from the final settling step is of suitable quality for release to the environment or for recycling as process water. Suitable monitoring methods may be incorporated into the waste water treatment process to ensure the water effluent 502 is of suitable quality to meet regulatory standards or other quality or safety specifications. A target naphthenic acid concentration may be desired in order to dispose of the water effluent 502 in a desired manner. For example, naphthenic acid concentration of over 2ppm in refinery waste water treatment effluent has been associated with acute toxicity in fish when the effluent is released to the environment. Accordingly, based on the proposed means of disposal of the effluent, a target naphthenic acid concentration of less than 2ppm, less than 1ppm, less than 0.5ppm, or a target naphthenic acid concentration of 0.1ppm may be desired.

[0062] Numerous specific details are set forth in order to provide a thorough understanding of the examples described herein. However, it will be understood by those of ordinary skill in the art that the examples described herein may be practiced without these specific details. In other instances, well-known methods, procedures and
components have not been described in detail so as not to obscure the examples described herein. The description is not to be considered as limiting the scope of the examples described herein.

[0063] The examples and corresponding figures mentioned herein are provided for illustrative purposes only, and different configurations and terminology can be used without departing from the principles expressed herein. The steps or operations in the flow charts and diagrams described herein are provided for example only. Variations to these steps or operations may be possible without departing from the principles discussed above. In some instances, process steps can be added, deleted, modified, or re-arranged without departing from these principles.

[0064] Although the above principles have been described with reference to certain specific examples, various modifications thereof will be apparent to those skilled in the art as outlined in the appended claims.
CLAIMS:

1. A method for treating an aqueous waste stream containing naphthenic acids and oil-wet solids, the method comprising:
   - pre-treating the aqueous waste stream with one or more additives to promote demulsification, flocculation, coagulation, or agglomeration of the oil-wet solids;
   - separating the pre-treated aqueous waste stream into a naphthenic acid-concentrated solids stream and a waste water stream; and
   - processing the naphthenic acid-concentrated solids stream to remove naphthenic acids from the solids stream.

2. The method as in claim 1, wherein the naphthenic acid-concentrated solids stream comprises an emulsion of oil, water, and oil-wet solids.

3. The method as in claim 1 or 2, wherein the naphthenic acid-concentrated solids stream has a higher naphthenic acid content than the waste water stream.

4. The method as in claim 1 or 2, where in the naphthenic acid-concentrated solids stream has a higher naphthenic acid concentration than either the aqueous stream or the waste water stream.

5. The method as in any one of claims 1 through 3, wherein the aqueous stream comprises solids-stabilized emulsion droplets.

6. The method as in any one of claims 1 through 5, wherein the step of processing the naphthenic acid-concentrated solids stream comprises a biotreatment step to remove naphthenic acids from the solids stream by microbial digestion.

7. The method as in claim 6, wherein the biotreatment step comprises incubation of the naphthenic acid-concentrated solids stream in a bioreactor in the presence of microbes, with a residence time that is at least 7 days.

8. The method as in claim 6, wherein the biotreatment step comprises incubation of the naphthenic acid-concentrated solids stream in a bioreactor in the presence of microbes, with a residence time that is at least 28 days.

9. The method as in claim 7 or 8, wherein the bioreactor further comprises water and microbial nutrients.
10. The method as in any one of claims 7 through 9, wherein the naphthenic acid concentration of the bioreactor is monitored during incubation.

11. The method as in any one of claims 6 through 10, wherein one or more operating conditions of the biotreatment step is monitored and adjusted to produce a biotreatment effluent having a naphthenic acid concentration that is below a target concentration.

12. The method as in claim 11, wherein the operating condition of the biotreatment step that is adjusted is temperature, pH, residence time, microbial nutrient concentration, water content, or microbial density.

13. The method as in claim 11 or 12, wherein the biotreatment effluent is a biotreated aqueous stream.

14. The method as in claim 11 or 12, wherein the biotreatment effluent is a biotreated solids stream.

15. The method as in claim 11 or 12, wherein the biotreatment effluent comprises solids and liquids.

16. The method as in any one of claims 11 through 15, wherein the target concentration of naphthenic acid in the biotreatment effluent is 2ppm or less.

17. The method as in any one of claims 11 through 15, wherein the target concentration of naphthenic acid in the biotreatment effluent is 0.5ppm or less.

18. The method as in any one of claims 11 through 15, wherein the target concentration of naphthenic acid in the biotreatment effluent is 0.1ppm or less.

19. The method as in any one of claims 7 through 18, wherein the bioreactor is an aeration vessel.

20. The method as in any one of claims 1 through 19, wherein the one or more additives are selected by testing the aqueous waste stream to determine a suitable additive or combination of additives for separation of the oil-wet solids from the waste water.

21. The method as in any one of claims 1 through 19, further comprising periodically testing the aqueous waste stream to adjust the additive selection or dosage over time to adapt to changes in the aqueous waste stream composition.
22. The method as in claim 20 or 21, wherein testing the aqueous waste stream comprises testing with various additives or combinations of additives using a jar test to observe settling of solids.

23. The method as in any one of claims 20 through 22, wherein the step of testing the aqueous waste water stream comprises zeta potential monitoring, turbidity testing, pH testing, or measurement of total suspended solids in the feed.

24. The method as in any one of claims 1 through 23, wherein the waste water stream or biotreatment effluent is further processed by conventional waste water treatment methods to remove naphthenic acids or other contaminants suspended or dissolved in the waste water or biotreatment effluent prior to reuse, release, or disposal.

25. The method as in any one of claims 1 through 23, wherein the waste water stream or biotreatment effluent is further processed by:

- a biotreatment step comprising incubation with microbes in a bioreactor;

- a clarification step comprising gravity separation;

- a filtration step; and

- a final separation step comprising gravity separation to collect a clarified liquid effluent.

26. The method as in claim 25, wherein the clarified liquid effluent has a naphthenic acid concentration of less than 2 ppm.

27. The method as in claim 25, wherein the clarified liquid effluent has a naphthenic acid or hydrocarbon concentration of less than 0.5 ppm.

28. The method as in claim 25, wherein the clarified liquid effluent has a naphthenic acid or hydrocarbon concentration of less than 0.1 ppm.

29. The method as in any one of claims 1 through 28, wherein the aqueous waste stream is a brine stream obtained from a desalter.

30. The method as in claim 29, wherein the desalter is used in a hydrocarbon refinery process.

31. The method as in claim 29, wherein the desalter is used in a process for upgrading bitumen or heavy oil.
32. The method as in any one of claims 1 through 28, wherein the aqueous waste stream results from the processing of high TAN hydrocarbons.

33. The method as in claim 32, wherein the high TAN hydrocarbons have been produced or extracted from an oil sands reservoir.

34. The method as in claim 32, wherein the hydrocarbons have been extracted from mined oil sands ore.

35. The method as in claim 33 or 34, wherein the extracted hydrocarbons have been processed using naphtha as a diluent.

36. The method as in claim 33 or 34, wherein the extracted hydrocarbons have been processed using a paraffinic diluent.

37. The method as in claim 32, wherein the hydrocarbons have been produced from wells producing from an oil sands reservoir.

38. The method as in any one of claims 1 through 37, wherein the one or more additives are flocculants, coagulants, or pH modifying agents.

39. The method as in any one of claims 1 through 38, wherein the one or more additives are selected from the group consisting of: iron (ferric) chloride, aluminum chloride, sodium hydroxide, an amine polymer, and a polyacrylamide polymer.

40. The method as in claim 1, wherein the naphthenic acid-concentrated solids stream comprises at least 80% of the naphthenic acid content of the aqueous waste stream.

41. The method as in any one of claims 1 through 40, wherein the step of separating the pre-treated aqueous waste stream comprises gravity separation of oil-wet solids from the pre-treated aqueous stream.

42. The method as in any one of claims 1 through 41, wherein the step of separating the pre-treated aqueous waste stream comprises phase separation in a clarifier vessel or centrifuge.

43. A method for processing desalter brine emulsion containing oil-wet solids, the method comprising:

   - pre-treating the desalter brine with one or more additives to promote destabilization of the desalter brine emulsion and agglomeration of the oil-wet solids, the
one or more additives comprising iron (ferric) chloride, aluminum chloride, sodium hydroxide, an amine polymer, or a polyacrylamide polymer;

- separating the desalter brine into a solids stream and a waste water stream;

and

- processing the combined waste water stream via a conventional water treatment process.

44. The method as in claim 43, wherein the desalter brine emulsion comprises naphthenic acids.

45. The method as in claim 43 or 44, further comprising processing the solids stream in a biotreatment step to remove naphthenic acids from the solids stream by microbial digestion.

46. The method as in claim 45, wherein the biotreatment step comprises incubation of the solids stream in a bioreactor in the presence of microbes, with a residence time that is at least 7 days.

47. The method as in claim 45, wherein the biotreatment step comprises incubation of the solids stream in a bioreactor in the presence of microbes, with a residence time that is at least 28 days.

48. The method as in claim 46 or 47, wherein naphthenic acid concentration of the bioreactor contents is monitored during incubation.

49. The method as in any one of claims 45-48, wherein one or more operating condition of the biotreatment step is monitored and adjusted to produce a biotreatment effluent having a naphthenic acid concentration that is below a target concentration.

50. The method as in claim 49, wherein the operating condition that is monitored and adjusted is temperature, pH, residence time, microbial nutrient concentration, water content, or microbial density.

51. The method as in any one of claims 43-49, wherein the one or more additives are selected by testing the desalter brine emulsion to determine a suitable additive or combination of additives for use in destabilization of the desalter brine emulsion.
52. The method as in claim 51, wherein the step of testing the desalter brine emulsion comprises testing with various additives or combinations of additives using a jar test to observe settling of solids.

53. The method as in claim 51 or 52, wherein the step of testing the desalter brine stream comprises zeta potential monitoring, turbidity testing, pH testing, or measurement of total suspended solids in the feed.

54. The method as in any one of claims 43 through 53, further comprising processing the waste water stream using conventional waste water treatment methods to remove naphthenic acids or other contaminants suspended or dissolved in the waste water.
ABSTRACT

A method is provided for the treatment of waste streams resulting from the processing of hydrocarbons that contain naphthenic acids, for example desalter brine resulting from the extraction or production of hydrocarbons from an oil sands reservoir. Naphthenic acids can be removed from these streams by removing oil-wet solids from the waste stream prior to conventional waste water processing, and the oil-wet solids can further be independently remediated to reduce naphthenic acid concentration for disposal.
Fig. 1
Fig. 3

Hydrocarbon feed 301

Water 302

Desalter 310

Brine 304

Additives

Mixing vessel 320

Oil 303

Clarifier 330

Solids/emulsion

Centrifuge 350

Holding Tank 340

Water 305

Solids 306

Tank 360
Fig. 4

Solids

Microbes

Water

Nutrients

Bioreactor

Waste Water

Bioremediated Solids
Appendix B

16S rRNA Procedure
1. Background

1.1. MiSeq. The MiSeq instruments use the first 12 positions in the read for normalization, and appear to require significant complexity in order to separate spots efficiently. The primers used for the MiSeq are composed of 4 random nucleotides at the 5’ end followed by 8mer barcodes that are sequence composition balanced with a minimum edit distance of 3. Primers for the V6 and V4 rRNA gene variable regions are given at the end of this document. With this strategy, we have successfully run as few as 4 multiplexed samples with 5% or less ΦX174 spiked in. We have not had success with 12mer barcodes containing equal nucleotide compositions in the absence of the 4 random nucleotides at the 5’ end. We have not tested shorter or longer segments of random sequence. Figure 1 shows a schematic of the primer and barcode structure.

```
Left-Illumina-adaptor nnnnccaaggtt Left-primer
Right-Illumina-adaptor nnnnccaaggtt Right-primer
```

Figure 1. Structure of the barcoded amplification primers. The 5’ end of each primer contains the left- or right-side Illumina adaptor (black), this is followed by four degenerate nucleotides (dark blue), then by the 8-mer barcode (red) and finally the amplification primer (light blue).

2. Protocol

The protocol below is for primers that contain Illumina adapter sequences attached to the 5’ end as given in the primer sequences.

*Important: do not size select the library without knowing the exact range of amplicon sizes. We do not size select our amplimers prior to loading on an Illumina MiSeq.*
2.1. Amplification 1: Taq is GoTaq hot start 2X colorless master mix from Promega (Catalogue numbers M5131, M5132, M5133).

Primer sequences for the rRNA V4 and V6 gene fragments are given at the end of this file. It is possible to replace these primer sequences with others specific to any desired amplicon. We have used this strategy to amplify single gene sequences from plasmids with high quality reads. Primer stock solutions for long-term storage are made to 200 pMole/µl (200 µM) in deionized water and stored at -80°C. Prior to use, they are diluted to 3.2 pMole/µl in deionized water by adding 3.2 µl of concentrated stock to 197 µl of deionized water. The diluted stock is stable for several freeze-thaw cycles and several months at -20°C. PCR reactions are assembled as follows:

1. 50 µl light mineral oil
2. 1 µl of input DNA
3. 10 µl of primer A at 3.2 pMole/µl
4. 10 µl of primer B at 3.2 pMole/µl
5. Heat 85°C prior to adding the GoTaq
6. Add 20 µl of GoTaq Master mix, heat to 95°C for 3 minutes to activate the GoTaq

Cycling conditions are 1 minute each at 95°C, 55°C and 72°C, for the V6 primer. An annealing temperature of 52°C is used for the V4 primers.

We normally cycle for 25 cycles to reduce chimera formation and partial products. A test amplification should be conducted to ensure that plateau is reached with this number of cycles: in general 25 cycles is more than sufficient. Aliquots of random samples should be run on agarose gels to ensure that the reactions proceeded as planned.

2.2. Quantitation and pooling: The most reliable method is to quantitate using the Qubit dsDNA kit. In this case the amplified product must be greater than 5X the negative control amplifications to be used. It is preferable to include two negative control reactions, one that was cycled, and one that was not. Both should have substantially the same reading. If the cycled negative control more than 25% greater than the non-cycled negative control, then steps to determine sources of contamination must be taken. The negative control readings are subtracted from each QuBit reading. Samples are pooled using their corrected relative concentration. The easiest way to do this is to add 1ul of the most concentrated sample and scale the volumes up for the other samples as needed. Pooled samples are mixed thoroughly, and then an aliquot (50-100 ul) is purified on a PCR cleanup column (Promega, Qiagen and Stratagene kits have been used successfully).

In the case of primers without these adapters the second amplification is not done immediately, instead the pooled, purified library has the Illumina adapters added by ligation using the Illumina paired-end protocol starting at the 3'A addition step. These primers are then amplified using the second stage amplification given below. This has been done at the Centre for Applied Genomics (Toronto).

\footnote{McMurrough et. al. Control of catalytic efficiency by a coevolving network of catalytic and noncatalytic residues. PNAS June 10, 2014 vol. 111 no. 23 E2376-E2383}
2.3. **Amplification 2**: The purified pooled library is diluted 100 fold in water and amplified with primers:

**OLJ139**:

5'ATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGA3'

**OLJ140**:

5'CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAAC3'

Here the amplification is performed for 10 cycles using the same conditions as above, except the annealing temperature is increased to 60 degrees. If no discernible band is found it is acceptable to increase the number of cycles to 15.

2.4. **Sequencing instructions**: Samples are purified using the preferred PCR cleanup kit, quantitated vs. a negative control and sent to the genome centre for sequencing. Tell them that the library is already made and size selection is not required. They will want to know length of the amplimer and the attached adaptors.

For V6 amplimers, ask for a paired-end run with 2x100 cycles.

For V4 amplimers, ask for a paired-end run with 2x200 cycles using the 600 cycle kit.

3. **Computational biology methods**

Current source is cjelli/git/miseq_bin.git. Working copy is in cjelli/Groups/LRGC/miseq_bin

3.1. **Requirements**. You will need an OS X or linux machine with 32 Gb of RAM for the later steps.

1. bash and awk
2. Pandaseq
3. USEARCH: http://drive5.com/usearch/  #the latest 32 bit free version is fine

The directory structure on the machine that the scripts expect is below (the analysis directory will be created):

1. Illumina_bin - location of all scripts and programs. you must know where this is and set it in the workflow.sh script
2. reads - contains the raw fastq and the overlapped fastq made by pandaseq
3. data_something - contains all intermediary data as outlined below, usually something is the variable region, or person’s name, or experiment
4. the samples.txt file must be in the same directory as the reads directory and workflow.sh
5. analysis_something - contains the final read tables and OTU fasta files

The samples.txt file contains information about the sample IDs and the barcodes used. The format is tabbed, plain text, Unicode UTF-8 encoding.

<table>
<thead>
<tr>
<th>BC_L</th>
<th>BC_R</th>
<th>sample</th>
<th>Lpri</th>
<th>Rpri</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccttggaa</td>
<td>ccaaggtt</td>
<td>Extraction Control</td>
<td>V4L5</td>
<td>V5R1</td>
<td>expt1</td>
</tr>
<tr>
<td>ccttggaa</td>
<td>aaggttec</td>
<td>KG04_01</td>
<td>V4L5</td>
<td>V5R2</td>
<td>expt2</td>
</tr>
</tbody>
</table>
Step 1: Download and de-compress the MiSeq reads. This is best done from the Illumina Basespace site, ask for access when you do your run. Place the reads into the reads/directory. Reads are compressed with 7Zip: from the command line:

```
7z e filename
```

Step 2: Overlap the reads with pandaseq. An example command for this with a minimum overlap of 30 nucleotides is below. This command is appropriate for the V4 amplimers:

```
pandaseq -g log.txt -T 8 -f L001_R1_001.fastq -r L001_R2_001.fastq -o 30 -w ps_overlapped30.fastq -F &
```

Step 3: Run the workflow pipeline:

```
./workflow.sh name cluster_pid variable_region
```

```
3.2. What is happening behind the scenes:
extract out the barcodes and primers associated with a particular samples.txt file. Output is a tabbed format file with the fields: read ID, sampleID primer sequence primer barcode q-score

```
$BIN/process_miseq_reads.pl $BIN samples.txt reads/overlapped.fastq $primer 8 0 $name T > $rekeyedtabbedfile
```

make a fasta file of all identical sequences (ISU), and an index of those sequences

```
$BIN/group_gt1.pl $rekeyedtabbedfile $name
```

cluster at 97% identity using usearch (i.e., make OTU), also performs chimera filter singleton reads are excluded

```
$BIN/usearch7.0.1090_i86osx32 -cluster_otus ...
$BIN/usearch7.0.1090_i86osx32 -usearch_global ...
```

regenerate the tabbed reads file with each read tagged as to its OTU and ISU group membership

```
$BIN/map_otu_isu_read_us7.pl $c95file $reads_in_groups_file $rekeyedtabbedfile > $mappedfile
```

make two tables of counts in the analysis directory for OSU and ISU sequences gather the seed sequences for each OTU. Transpose the dataset for ease of import into QIIME

```
$BIN/get_tag_pair_counts_ps.pl $mappedfile $CUTOFF $name
$BIN/get_seed_otus_uc7.pl $c95file $groups_fa_file analysis_$name/OTU_tag_mapped.txt
> analysis_$name/OTU_seed_seqs.fa
Rsscript $BIN/OTU_to_QIIME.R analysis_$name
Use mother (must be installed separately) to annotate the OTU sequences against the silva database

$MOTHUR "#classify.seqs(fasta=analysis_$name/OTU_seed_seqs.fa, template=$TEMPLATE, taxonomy=$TAXONOMY, cutoff=70, probs=T, outputdir=analysis_$name, processors=4)"

$BIN/add_taxonomy_mothur.pl $TAX_FILE analysis_$name/td_OTU_tag_mapped.txt > analysis_$name/td_OTU_tagMapped_lineage.txt
4. V6 barcodes, sets of 4 are balanced

V6L11 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnccaaaggttCWACGCGARcGAACCTTACC
V6L12 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnaaggttccCWACGCGARGAAGACCTTACC
V6L13 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnnctttccaaCWACGCGARGAAGACCTTACC
V6L14 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnntttcaaagCWACGCGARGAAGACCTTACC
V6L15 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnntttggaaCWACGCGARGAAGACCTTACC
V6L16 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnntttgaaccCWACGCGARGAAGACCTTACC
V6L17 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnnggaaaccttCWACGCGARGAAGACCTTACC
V6L18 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnaccctttggCWACGCGARGAAGACCTTACC
V6L19 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnntttcttcgCWACGCGARGAAGACCTTACC
V6L110 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnnggaaatccatCWACGCGARGAAGACCTTACC
V6L111 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnncttttaggtcCWACGCGARGAAGACCTTACC
V6L112 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnnttccaaagCWACGCGARGAAGACCTTACC
V6L113 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnntttggcttcgCWACGCGARGAAGACCTTACC
V6L114 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnncaattggatCWACGCGARGAAGACCTTACC
V6L115 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnntttaccttgCWACGCGARGAAGACCTTACC
V6L116 ACACCTTTTCCCTACACGACGCTCTTCCGATCTnnnnaccgaacaCWACGCGARGAAGACCTTACC

V6R11 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnccaaaggttACRACACGAGCTGAGC
V6R12 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnaaggttccACRACACGAGCTGAGC
V6R13 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnctttccaaACRACACGAGCTGAGC
V6R14 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnntttcaaagACRACACGAGCTGAGC
V6R15 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnntttggaaACRACACGAGCTGAGC
V6R16 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnntttgaaccACRACACGAGCTGAGC
V6R17 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnggaaatccatACRACACGAGCTGAGC
V6R18 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnttttaggtcACRACACGAGCTGAGC
V6R19 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnntttccaaagACRACACGAGCTGAGC
V6R110 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnntttttccatACRACACGAGCTGAGC
V6R111 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnttttaggtcACRACACGAGCTGAGC
V6R112 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnggaaatccatACRACACGAGCTGAGC
V6R113 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnttttaggtcACRACACGAGCTGAGC
V6R114 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnttttaggtcACRACACGAGCTGAGC
V6R115 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnttttaggtcACRACACGAGCTGAGC
V6R116 CGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCTnnnnnttttaggtcACRACACGAGCTGAGC
5. V4 Earth Microbiome barcodes, sets of 4 are balanced

V4L1 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNCcaggttTGGCCAGCMGCCGCGGTAA
V4L2 ACACTCTTTCCCTACAGACGCTCTTCCGATCTGNNNNaggttcGTGCCAGCMGCCGCGGTAA
V4L3 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGggtccaaGTGCCAGCMGCCGCGGTAA
V4L4 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNNctccaggtTGGCCAGCMGCCGCGGTAA
V4L5 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNCctcggGggtccaaGTGCCAGCMGCCGCGGTAA
V4L6 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNMcaggtttccGTGCCAGCMGCCGCGGTAA
V4L7 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgtTGGCCAGCMGCCGCGGTAA
V4L8 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggtttccGTGCCAGCMGCCGCGGTAA
V4L9 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNCcaggttcgTGGCCAGCMGCCGCGGTAA
V4L10 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgTGGCCAGCMGCCGCGGTAA
V4L11 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgTGGCCAGCMGCCGCGGTAA
V4L12 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgTGGCCAGCMGCCGCGGTAA
V4L13 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgTGGCCAGCMGCCGCGGTAA
V4L14 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgTGGCCAGCMGCCGCGGTAA
V4L15 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgTGGCCAGCMGCCGCGGTAA
V4L16 ACACTCTTTCCCTACAGACGCTCTTCCGATCTNNNNGaggttcgTGGCCAGCMGCCGCGGTAA

V5R1 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNCcaggttGGACTACHVGGGTWTCTAAT
V5R2 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R3 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R4 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R5 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R6 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R7 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R8 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R9 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R10 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R11 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R12 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R13 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R14 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R15 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
V5R16 CGGTCTCGGATCTCCTGCTGAACCGCTCTTCCGATCTNNNNNaggtttccGGACTACHVGGGTWTCTAAT
Appendix C

Histopathology Lesions of Trout Exposed to Naphthenic Acid

This Appendix describes the histopathology lesions discussed in Section 3.6

C.1 Trout Anatomy

Figure C.1 is a schematic of the anatomy of a trout. The major organs include those found to be affected by exposure to naphthenic acid.

Sections C.1.1, C.1.2, C.1.3, and C.1.4 provide more information of the main fish organs which are affected by naphthenic acid namely gills, nervous system, kidneys and digestive system.
Figure C.1 – Anatomy of a trout. (a) copied from Figure 24 of [133], (b) internal anatomy of an adult trout copied from Figure 26 of [134].
C.1.1 Gills

The purposes of the gill of a fish are the oxygen transfer from water to blood, osmoregulation, acid-base regulation, and excretion of nitrogenous waste [135]. The gill of a teleost is shown in Figure C.2.

![Fish Gill Diagram](image)

**Figure C.2** – Fish gill showing lamellar structure. From Figure 1 in [135].

Figures C.5 on page 209 and C.6 on page 210 illustrate lesions found on the gill structures shown in Figure C.2.

C.1.2 Nervous System

In the test fish, fluid accumulation in the brain cells (edema) was one of the most significant lesions found in this research. It lesion appeared most severe in the region of the brain that controls movement i.e. the cerebellum. This lesion, cerebellar edema (CBE), is the likely the cause of total loss of equilibrium (TLOE).

A trout brain is divided into three sections having different functions:

1. forebrain (telecephalon) is responsible for the fish’s ability to smell.
2. midbrain (tectum) processes vision, learning, and motor responses.
3. hindbrain (cerebellum) coordinates movement, muscle tone, and balance.
C.1.3 Kidney

The kidney in a teleost is divided into two sections which are the head and posterior. The head kidney is composed of the hemopoietic elements (blood forming) which in combination with the spleen produces white and red blood cells in a process termed hematopoiesis [137]. The posterior kidney maintains the internal salt/water balance (osmotic regulation) in the fish and has a minor role in the elimination of ammonia.

Within freshwater fish, the kidney is responsible for preventing excess solute loss as fish contain more salts within their blood than the water located around their bodies. Due to this concentration gradient, water will naturally diffuse into the fish through osmosis causing large amounts of water to build up inside. Therefore, the kidneys in this type of fish increase the amount of water that passes out in their urine and actively reabsorb the salts that would pass out as well to maintain that balance which results in the production and excretion of large amounts of dilute urine.

In the blood-forming part of the kidney (head kidney), some test fish developed swelling of the lining of small blood vessels (which is endothelial cell hydropic degeneration). Also in the head kidney, fish from most groups (including controls) sometimes had blood-forming cells with fragmented nuclei.
C.1.4 Digestive System

The digestive system of the fish is comprised of the gastrointestinal tract and organs used to process food into energy and nutrients. The gastrointestinal tract is a tubular path that consists of the mouth, esophagus, stomach, intestine and rectum. The organs that assist with the digestion are the liver, gall bladder and pancreas [138]. The digestive system is shown in Figure C.4.

The liver acts as an accessory digestive organ and excretes bile, which is a yellowish-green fluid, through a duct to the gall bladder which aids in the digestion of fats. The liver detoxifies heavy metals and organic compounds to which the fish is exposed [141].

Digestive system lesions photomicrographs are:

a) stomach Figure C.9 on page 213
b) liver Figure C.10 on page 214
c) stomach, liver, and exocrine pancreas Figure C.11 on page 215

C.2 Lesions Occurring Only in Exposed Fish

C.2.1 Brain

Cerebellar Edema (CBE)

CBE is fluid accumulation in the part of the brain that controls movement. Edema is a reversible change resulting from acute cellular damage. Fish with cerebellar edema often have paralysis or TLOE. Cerebellar edema is fairly nonspecific, meaning that it might be a result or the cause of TLOE.
The anatomical region of the cerebellum that is most affected is the band of Purkinje cells (which is between the molecular and granular layers of the cerebellum). Cytoplasm of affected cells is expanded by poorly demarcated clear spaces that provide evidence that the brain has been exposed to toxins. A neurotoxic chemical in the water is the most likely source of the toxin.

CBE only occurred among fish exposed to naphthenic acid. Lesions found in the brain are shown in Figure C.7.
C.2.2 Gills

Gill Lamellar Epithelial Hydropic Degeneration (LHD)

LHD is the reversible swelling of cytoplasmic organelles within the thin lining of cells that allow for gas exchange on the gill lamellae. Swelling of gill lamellar epithelium decreases oxygen exchange, which may in turn lead to hypoxic lesions in other organs. Gill lamellar epithelial hydropic degeneration is evidence of exposure to toxins in the water.

One of the most common causes in cultured fish is chronic exposure to high concentrations of ammonia which was ruled out by TIE analysis. Lamellar epithelial swelling can also develop as a postmortem artifact; therefore, the lesion could not be scored in fish that died before they were sampled. Figures C.5 and C.6 show the lesions found in the gills of exposed fish.

Pseudobranch single cell necrosis (PBN)

PBN is the death of scattered cells within the pseudobranch, a rudimentary gill arch. Necrosis (death) of pseudobranch epithelial cells might be a result of direct toxin exposure, or it might be a result of local hypoxia due to lesions in other organs. In this thesis, the only wastewater group without pseudobranch single cell necrosis had been exposed to the sodium thiosulfate treated water which is due to metals.

C.2.3 Kidney

Renal tubular intraluminal protein casts (ITP)

ITP are usually dull eosinophilic casts in the lumen of kidney tubules that result from glomerular or tubular dysfunction; excess protein leaks through glomeruli (the blood filtering organs of the kidney), or tubules are unable to reabsorb protein. The lesions are consistently mild.

Renal Endothelial Cell Hydropic Degeneration (REH)

REH is a lesion in which the cells lining blood vessels in the kidney swell when
they are injured. This lesion can occur within hours of exposure, and it is reversible when the inciting toxin(s) is removed.

Cytoplasm of affected cells is expanded by fine to large foamy vacuoles that provide evidence that the kidney has been exposed to toxins. Because none of the control fish had this lesion, the most likely source was toxins in the test water. As a less likely differential, it might be a result of circulating oxygen radicals following a period of tissue hypoxia.

Renal Hematopoietic Cell Atrophy (RHA)

RHA is a decrease in the blood-forming cells within the kidney. Potential causes include:

a) acute demand for hematopoietic cells which is an early inflammatory response in which the kidney has released blood cells but not yet responded by producing more blood cells.

b) necrosis of hematopoietic cells without replacement due to infectious disease or toxin exposure.

c) sample location such as the trunk kidney normally has less hematopoietic cells per unit volume than the head kidney.

The primary difference between points b) and c) is postmortem bile digestion of blood-forming cells.

Renal Tubular Hydropic Degeneration (RTH)

RTH is a reversible change in the kidney resulting from acute cellular damage. Cytoplasm of affected cells is expanded by fine to large foamy vacuoles that provide evidence that the kidney has been exposed to toxins. Potential sources of the inciting toxins include the water, or circulating oxygen radicals following a period of hypoxia. When hydropic degeneration can no longer be reversed, affected cells die and become necrotic.
**Renal tubule epithelial Protein Droplets (TEP)**

TEP are bright orange-staining droplets in the cytoplasm of the cells that line tubules of the kidney. In moderate cases, the droplets are sometimes irregularly oval and larger than the nuclei of affected cells. Mild cases tend to have droplets that are spherical and 1 to 2 µm in diameter. A few tubules with small droplets (mild severity) might be a nonspecific response to the stress of exposure, but more abundant and larger droplets (moderate severity) are probably a result of toxin exposure.

Figure [C.8](#) shows samples of the kidney lesions found in the fish exposed to naphthenic acid.

**Single Cell Hepatocellular Necrosis (SCN)**

SCN occurs after single hepatocytes die. Causes of necrosis of individual hepatocytes have not been well defined in fish. Possible differentials include exposure to toxins (endogenous or exogenous), or remodelling of the liver in rapidly growing fish that suddenly go off feed about 24 hours before death.

Apoptosis is the normal way in which hepatocyte numbers are decreased i.e. the hepatocytes are not needed when growing fish stop feeding because few to no nutrients are being absorbed into the blood and entering the liver for processing.

### C.2.4 Liver

**Hepatocellular Hydropic Degeneration (HHD)**

HHD is a reversible change of liver cells resulting from acute cellular damage. Cytoplasm of affected hepatocytes is expanded by fine to large foamy vacuoles that provide evidence that the liver has been exposed to toxins.

Potential sources of the inciting toxins include the water, or circulating oxygen radicals following a period of hypoxia (this can happen as an agonal change with death due to a variety of causes).

Figure [C.10](#) illustrates lesions found in the liver.
C.2.5 Stomach

Gastric Wall Vacuolation (GWV)

GWV is the development of vacuoles which result in distension of the wall of the stomach as a result of fairly uniform interstitial vacuoles that separate the layers of the muscular tunics. This is probably a result of exposure to toxins, possibly through ingestion, but the lesion is otherwise not specific.

C.3 Lesions More Common or Severe in Exposed Fish

C.3.1 Kidney

Renal interstitial cell karyorrhexis (RIK)

RIK is the irreversible fragmentation of nuclei that occurs after cells die. Interstitial cells in the kidney include precursors of red blood cells and white blood cell (hematopoietic cells). Karyorrhexis of renal interstitial cells is evidence of increased cell turnover, possibly as a result of toxin exposure, including as part of an active inflammatory response. Karyorrhexis sometimes occurs after cells with hydropic degeneration die (see Renal interstitial cell hydropic degeneration/single cell necrosis), but in some cases it occurs without cell swelling and, therefore, is scored separately. Renal interstitial cell karyorrhexis occurred in both exposed and control fish, but it was more common in exposed fish and cases of moderate severity occurred only in exposed fish.

Interstitial Cell Karyorrhexis (ICK)

ICK in the kidney is the nuclear fragmentation of blood cell precursors that occurs after affected cells die, as shown in Figure C.8. It is a transient state that does not last much more than 24 hours. Therefore, interstitial cell karyorrhexis is evidence of the
death of blood-forming cells in the kidney within approximately 24 hours of when the fish was sampled. Interstitial cell karyorrhexis is one cause of renal hematopoietic cell atrophy. Because interstitial cell karyorrhexis is also the normal way in which cells die, some mild cases might be within normal limits. Differentials include toxin exposure, or unlikely in this case, infection with a virus or bacteria.

Renal Interstitial Cell Hydropic Degeneration/Single Cell Necrosis (RIH)

(RIH) is a lesion that affects blood cell precursors (i.e., hematopoietic cells) in the kidney. The lesion results from acute cellular damage. The early form, hydropic degeneration, is reversible, but after the cell dies, necrosis is no longer reversible. Cytoplasm of affected cells is uniformly expanded. Cells with hydropic degeneration have fine to large foamy vacuoles, whereas necrotic cells tend to have decomposed into flocculent cellular debris. Both exposed and control fish are affected, but the lesion is more common in exposed fish and cases of moderate severity occur only in exposed fish. In exposed fish, the water is the most likely source of the inciting toxins (e.g., a nephrotoxic chemical). In control fish, the toxins are probably endogenous and part of normal cell turnover.

C.3.2 Spleen and Kidney

Erythrophagocytosis (EPT)

EPT includes macrophages in the spleen or kidney with cytoplasmic pigments that vary from brightly eosinophilic erythrocyte cytoplasm to yellow-brown hemosiderin and lipofuscin, which are common breakdown products of hemoglobin and cells. Erythrophagocytosis is evidence that red blood cells are being removed from the blood stream, usually the result of damaged erythrocyte membranes. This occurs occasionally as part of normal cell turnover, but an increase in prevalence or mean scores is evidence that more red blood cells than normal are being removed from the blood stream.
Differentials include an immune reaction or toxin exposure.

**C.3.3 Stomach**

**Gastric Gland Single Cell Degeneration and Necrosis (GSD)**

Indications of GSD (cell death) include protein droplets in epithelial cell cytoplasm, nuclear fragmentation, and (rarely) sloughing of deeply basophilic necrotic epithelial cells. In most cases, only the first 2 mm of the glandular stomach are affected: a region just distal to the gastro-esophageal junction. Potential causes include toxin exposure and remodeling (apoptosis) as a result of not feeding during the test. Because gastric gland single cell degeneration is also the normal way in which these cells die, some mild cases might be within normal limits. Figure C.9 illustrates the lesion.

**C.3.4 Intestine**

**Intestinal Epithelial Cell Single Cell Degeneration and Necrosis (ISD)**

ISD as shown in Figure C.11 is a lesion characterized by:

a) contracted epithelial cell cytoplasm that is brightly staining (hypereosinophilic)

b) nuclear fragmentation karyorrhexis

In most cases, only the first 2 mm of the intestine and intestinal ceca are affected: a region just distal to the junction of the stomach and intestine. Potential causes include toxin exposure and remodeling (apoptosis) as a result of not eating. Because intestinal epithelial cell single cell degeneration is also the normal way in which these cells die, some mild cases might be within normal limits. In this study, all groups have at least one fish with mild lesions.
C.4 Lesions Not Clearly Associated With Exposure to Naphthenic Acid

C.4.1 Brain

Brain Hemorrhage (BHM)

BHM is characterized by red blood cells in the tissue of the brain which includes the membranes that envelop the brain and spinal cord (meninges) and brain tissue (neuropil). The most common cause of brain hemorrhage is trauma. For fish that are chemically euthanized [92], it is most commonly a result of capture for sampling.

C.4.2 Intestine

Intestine Bacterial Overgrowth (IBO)

IBO in the lumen of the intestinal tract is fairly common in fasting fish. The bacteria might be producing toxins that adversely affect fish health. Bacterial overgrowth could also be the cause or result of decreased intestinal motility and poor digestion. Lack of bacteria in the stomach contents is evidence that the feed was not overgrown when eaten.

This is also known to cause white stringy feces which is seen in all tests using wastewater effluent.

C.4.3 Liver

Biliary Ductular Hyperplasia (BDH)

BDH involves proliferation (hyperplasia) of the cells that line the ducts (epithelial cells) that carry bile in the liver. Hyperplasia of biliary ductular epithelial cells in the liver is evidence of exposure to toxins. The toxins could be produced inside the fish (e.g., bacterial toxins, bile stasis) or come from outside the fish (e.g., from the water).
Vacuoles in Hepatocellular Cytoplasm (VAC)

VAC are nonspecific intracellular spaces that might be a result of accumulation of cytoplasmic lipid, glycogen, or isotonic fluid. Vacuoles in this category tend to be angular, and affected cells have no more than a few of these vacuoles per cell. When vacuole morphology is characteristic of lipid, glycogen, or cell swelling, then more specific diagnoses are used.

Focal hepatitis, leukocytic (FHL)

FHL is an uncommon lesion in cultured salmonoids. The foci probably develop in response to chronic immune stimulation such as focal bacteria, parasites, or other antigens, however the exact cause is rarely determined. The sections have no evidence of hepatic bacteria or parasites. In the three studies hepatitis occurred in only three fish which were all from control groups.

C.4.4 Yolk sac

Although the fish used for the TIE studies were fingerlings approximately 20% of the fish had remnants of yolk sacks which are used as a nutrient source as the fish transitions from alevin to fry.

Yolk Sac Macrophages (YSM)

YSM are phagocytic cells that are fairly commonly associated with involuting yolk sacs in salmon transitioning from alevins to fry. While the fish used for this thesis were fingerlings there were remnants of yolk sacs. In most cases, the process is sterile and probably of little significance.

Yolk Sacculitis (YSI)

YSI involves any combination inflammation of the involuting yolk sac. The amount of inflammation is greater in cases of yolk sacculitis than in normal yolk involution, which often contains small numbers of foamy macrophages that assist with trout transitioning from alevin to fry. The inflammation in the three groups of fish does
Figure C.5 – Photomicrographs of the gill; hematoxylin and eosin stain. (A) The normal gill is composed of a central filament with a series of perpendicular lamellae on each side; lamellae are lined by a thin epithelial layer (between arrowheads). Black box in (A) delineates the area shown at higher magnification in (B). (C) The epithelial layer of this gill is thickened (between arrowheads) and sometimes vacuolated. (D) higher magnification of black box in Figure C.

not include bacteria or fungi; therefore, yolk sacculitis is probably not very significant to the health of the affected fish.
Figure C.6 – Photomicrographs of the pseudobranch; hematoxylin and eosin stain. (A) The normal pseudobranch is composed of curving rows of fused lamellae. (C) high magnification of black box shown in (A). (B) This pseudobranch has several vacuoles that contain shrunken necrotic cells (arrowheads). Black box in (B) delineates the area shown at higher magnification in (D). This fish was exposed to the baseline wastewater that had been treated with 8 mL·L$^{-1}$ EDTA @ pH6.
Figure C.7 – Photomicrographs of the brain. The left side of each image is anterior; the right side, posterior; hematoxylin and eosin stain. (A)/(B). Normal brain (C) black boxes in A and B delineate areas shown at higher magnification in C and D, respectively. The cerebellum is dorsal to the brainstem (B). D through F. (D) Abnormal brain with cerebellar edema; black boxes in D and E delineate areas shown in higher magnification in E and F, respectively. Fluid-filled vacuoles in the Purkinje cell layer separate the peripheral molecular layer (m) and the central granular layer.
**Figure C.8** – Photomicrographs of the head kidney; hematoxylin and eosin stain. (A) The normal head kidney. (B) Higher magnification of black box in Figure A. (C) Many of the hematopoietic cells in this kidney are dead and have fragmented nuclei (D) higher magnification of box shown in Figure (C)
Figure C.9 – Photomicrographs of the stomach; hematoxylin and eosin stain. A and B. The normal stomach wall is composed of dense smooth muscle fibres. Black box in A delineates the area shown at higher magnification in B. C and D. The muscle layers of this stomach are distended due to fluid accumulation (edema,*). Black box in C delineates the area shown at higher magnification in D.
Figure C.10 – Photomicrographs of the liver; hematoxylin and eosin stain. (A) The normal liver is composed of hepatocytes that sometimes contain angular vacuoles (glycogen; *) (B) higher magnification of black box in Figure A (C) Most of the hepatocytes in this liver contain foamy cytoplasmic vacuoles characteristic of hydropic degeneration (arrowheads). A few hepatocytes are necrotic and shrunken (arrow head) (D) higher magnification of black box in figure C.
Figure C.11 – Photomicrographs of the intestine (i), stomach (s), and exocrine pancreas (p); hematoxylin and eosin stain. A - C. The normal proximal intestine (in A) has several ceca (in B) that are lined by tall columnar epithelial cells (in C). Black boxes in A and B delineate areas shown at higher magnification in B and C, respectively. D - F. A few cells in the cecal epithelium of this fish are necrotic and shrunken (arrowheads). Black boxes in D and E delineate areas shown at higher magnification in E and F, respectively.
Appendix D

Wastewater Carbon Loading

D.1 Wastewater Influent and Effluent Carbon Loading Parameters

The carbon loading information in this section are:

a) five day \(\text{BOD}_5\) (mg(O·L\(^{-1}\))

b) chemical oxygen demand (COD (mg(O)\(_2\)·L\(^{-1}\))

c) dissolved carbon concentration (DOC (mg(C)·L\(^{-1}\))

d) total organic carbon (TOC (mg(C)·L\(^{-1}\))

To determine the carbon loading to the aeration basins samples were taken at the effluent of the Wemco® which is termed the contaminantd sewer and the Hydrocell® effluent which is termed the process sewer. The outlet of the wastewater system was sampled at the combined water outlet of the clarifiers. The block diagram of the equipment is shown in Figure 1.5.

Tables 4.1, 4.2 and 4.3 show that the during steady state operation the volumetric flow rate to the aeration basins is composed of 27 % from the process sewer and the
remainder, 73 % is from the contaminantd sewer. Tables D.1, D.2 list the statistics of
the various loading from the two sewer systems while Table D.3 show the statics of the
clarifier effluent which is considered to be the end of the biological treatment.

**Table D.1** – Statistics of carbon loading from Hydrocell® outlet from May 2013 until August 2015.

<table>
<thead>
<tr>
<th>Property</th>
<th>Units</th>
<th>Average</th>
<th>Standard Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD₅</td>
<td>mg(O₂)·L⁻¹</td>
<td>145.7</td>
<td>34.2</td>
<td>9</td>
<td>280</td>
</tr>
<tr>
<td>COD</td>
<td>mg(C)·L⁻¹</td>
<td>511.5</td>
<td>542.4</td>
<td>100</td>
<td>8030</td>
</tr>
<tr>
<td>DOC</td>
<td>mg(C)·L⁻¹</td>
<td>95.3</td>
<td>24.2</td>
<td>49</td>
<td>200</td>
</tr>
<tr>
<td>TOC</td>
<td>mg(C)·L⁻¹</td>
<td>116.0</td>
<td>31.5</td>
<td>69</td>
<td>350</td>
</tr>
</tbody>
</table>

**Table D.2** – Statistics of carbon loading from Wemco® outlet.

<table>
<thead>
<tr>
<th>Property</th>
<th>Units</th>
<th>Average</th>
<th>Standard Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD₅</td>
<td>mg(O₂)·L⁻¹</td>
<td>17.0</td>
<td>11.7</td>
<td>2.0</td>
<td>72</td>
</tr>
<tr>
<td>COD</td>
<td>mg(C)·L⁻¹</td>
<td>52.3</td>
<td>41.3</td>
<td>10.0</td>
<td>280</td>
</tr>
<tr>
<td>DOC</td>
<td>mg(C)·L⁻¹</td>
<td>9.8</td>
<td>6.7</td>
<td>1.3</td>
<td>64</td>
</tr>
<tr>
<td>TOC</td>
<td>mg(C)·L⁻¹</td>
<td>15.4</td>
<td>9.5</td>
<td>4.1</td>
<td>88</td>
</tr>
</tbody>
</table>

**Table D.3** – Statistics carbon loading from clarifier effluent.

<table>
<thead>
<tr>
<th>Property</th>
<th>Units</th>
<th>Average</th>
<th>Standard Deviation</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>BOD₅</td>
<td>mg(O₂)·L⁻¹</td>
<td>2.9</td>
<td>3.6</td>
<td>2.0</td>
<td>7.7</td>
</tr>
<tr>
<td>COD</td>
<td>mg(C)·L⁻¹</td>
<td>27.3</td>
<td>18.1</td>
<td>10.0</td>
<td>3.5</td>
</tr>
<tr>
<td>DOC</td>
<td>mg(C)·L⁻¹</td>
<td>6.5</td>
<td>3.4</td>
<td>0.5</td>
<td>2.0</td>
</tr>
<tr>
<td>TOC</td>
<td>mg(C)·L⁻¹</td>
<td>7.7</td>
<td>3.5</td>
<td>46.0</td>
<td>47.0</td>
</tr>
</tbody>
</table>
The standard deviation of the process sewer in Table D.1 is due to changes in emulsion formation of the desalters while the standard deviation of the contaminantd sewer in Table D.2 is due to processing of rain water in the refinery which contains hydrocarbons from ditches and runoff from the processing units.

Table D.4 lists the removal efficiency of the biological treatment system for the parameters BOD$_5$, COD, DOC and TOC based on a mass balance of the process and contaminantd sewer values listed in Tables D.1 and D.2.

**Table D.4 – The Removal efficiency of the studied oil refinery wastewater treatment system.**

<table>
<thead>
<tr>
<th>Property</th>
<th>Units</th>
<th>Combined influent</th>
<th>Removal Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Deviation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BOD$_5$</td>
<td>mg(O$_2$)·L$^{-1}$</td>
<td>40</td>
<td>94</td>
</tr>
<tr>
<td>COD</td>
<td>mg(C)·L$^{-1}$</td>
<td>140</td>
<td>87</td>
</tr>
<tr>
<td>DOC</td>
<td>mg(C)·L$^{-1}$</td>
<td>26</td>
<td>80</td>
</tr>
<tr>
<td>TOC</td>
<td>mg(C)·L$^{-1}$</td>
<td>32</td>
<td>92</td>
</tr>
</tbody>
</table>

**D.2 Hydrocarbon Distributions in Hydrocell® and Wemco® Effluents**

The hydrocarbon distributions in Tables D.6 and D.7 were sampled at the Hydrocell® and Wemco® outlets for both steady state operations and transients caused by variations in refinery operation. The definition of the terms F1, F2, F3 and F4 used in the later tables are defined by the Canadian Council of Ministers of the Environment (CCME) and listed in Table D.5.
Table D.5 – Hydrocarbon fractions F1 through F4 defined by the Canadian Council of Ministers of the Environment.

<table>
<thead>
<tr>
<th>Property</th>
<th>Carbon number Range</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>C_6 through C_10</td>
<td>volatile petroleum hydrocarbons</td>
</tr>
<tr>
<td>F2</td>
<td>C_{10} through C_{16}</td>
<td>light extractable petroleum hydrocarbons</td>
</tr>
<tr>
<td>F3</td>
<td>C_{16} through C_{34}</td>
<td>heavy extractable petroleum hydrocarbons</td>
</tr>
<tr>
<td>F4</td>
<td>C_{34} through C_{50}</td>
<td>extremely heavy extractable petroleum hydrocarbons</td>
</tr>
</tbody>
</table>

The distributions listed in Tables D.6 and D.7 are the main components for the parameters in Tables D.1 to D.2. The speciation variation of the samples is due to desalter emulsion, rain water processing, tank water bottoms processing and process unit upsets.

Table D.6 – Hydrocarbon distribution in feeds to aeration basin from the process sewer effluent after primary separation.

<table>
<thead>
<tr>
<th>F1 C_6 - C_{10} mg·L^{-1}</th>
<th>F2 C_{10} - C_{16} mg·L^{-1}</th>
<th>F3 C_{16} - C_{34} mg·L^{-1}</th>
<th>F4 C_{34} - C_{50} mg·L^{-1}</th>
</tr>
</thead>
<tbody>
<tr>
<td>11.0</td>
<td>3.1</td>
<td>4.1</td>
<td>0.5</td>
</tr>
<tr>
<td>10.0</td>
<td>3.2</td>
<td>7.7</td>
<td>5.0</td>
</tr>
<tr>
<td>10.0</td>
<td>5.0</td>
<td>12.0</td>
<td>0.6</td>
</tr>
<tr>
<td>10.0</td>
<td>2.5</td>
<td>2.7</td>
<td>0.1</td>
</tr>
<tr>
<td>12.0</td>
<td>15.0</td>
<td>46.0</td>
<td>0.3</td>
</tr>
<tr>
<td>15.0</td>
<td>6.6</td>
<td>7.4</td>
<td>0.2</td>
</tr>
<tr>
<td>14.0</td>
<td>5.2</td>
<td>6.3</td>
<td>0.5</td>
</tr>
<tr>
<td>9.0</td>
<td>4.0</td>
<td>4.1</td>
<td>0.5</td>
</tr>
<tr>
<td>8.0</td>
<td>12.0</td>
<td>36.0</td>
<td>2.3</td>
</tr>
</tbody>
</table>
Table D.7 – Hydrocarbon distribution in feed to aeration basin from the contaminantd sewer effluent after primary separation.

<table>
<thead>
<tr>
<th></th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>F4</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6 - C(_{10}) (\text{mg}\cdot\text{L}^{-1})</td>
<td>C(<em>{10}) - C(</em>{16}) (\text{mg}\cdot\text{L}^{-1})</td>
<td>C(<em>{16}) - C(</em>{34}) (\text{mg}\cdot\text{L}^{-1})</td>
<td>C(<em>{34}) - C(</em>{50}) (\text{mg}\cdot\text{L}^{-1})</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>13.0</td>
<td>20.0</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>1.9</td>
<td>7.5</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>1.3</td>
<td>6.8</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>1.9</td>
<td>10.0</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>2.3</td>
<td>5.5</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.6</td>
<td>4.2</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>1.3</td>
<td>3.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>0.4</td>
<td>0.8</td>
<td>1.1</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>1.1</td>
<td>2.1</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>
Appendix E

Naphthenic Acid Mass Balance

Equivalent Electrical Diagram

The purpose of the circuit shown in Figure E.1 was designed to simulate the wastewater treatment system response to naphthenic acid to gain insight into changes of the naphthenic acid concentration at the clarifier effluent to changes at the aeration basin influent based the desalter naphthenic acid mass rate, the north impounding basin recycle rate, the return activated sludge rate from the clarifier to the aeration basins (RAS) and the wasted activated sludge rate (WAS) which is the removal of sludge from the system.

E.1 Naphthenic Acid Mass Flow rate Input to aeration basin

Based on the naphthenic acid mass balance described in Chapter 4 there are two sources of naphthenic acid to the wastewater treatment system, the desalter brine and the floc/solids recycled from the impounding basin. The two voltage supplies used simulate this are the Noth_impounding_recycle_mass_rate and the
Figure E.1 – Electrical equivalent diagram of naphthenic acid mass balance.
Desalter_effluent_mass_rate. Since the naphthenic acid mass rate from the desalter can be bound in emulsion and solids which is removed in primary separation R16 and R17 are used as a voltage divider to simulate the removal efficiency of primary separation. The efficiency is adjustable by changing the ratio of R17:R15. op-amp U6 is a voltage follower and used to keep a constant impedance output to R6.

The resistors R3, R6 and R7 in combination with U2 R4 and R5 act as a non-inverting summer amplifier where the voltage outlet is equal to Equation E.1

\[
V_{\text{sum}} = \left(1 + \frac{R_5}{R_4}\right) \times \left(\frac{V_a + V_b + V_c}{3}\right)
\]  

(E.1)

Where \(V_{\text{sum}}\) is the voltage output, \(V_a\) is the Noth_impounding_recycle_mass_rate, \(V_b\) is the Desalter_effluent_mass_rate \times the ratio of R17:R15 and \(V_c\) is the Clarifier_recycle_rate.

Given that

\[
\left(\frac{R_5}{R_4}\right) = \left(\frac{20K}{10K}\right) = 2
\]

Equation E.1 can be simplified to Equation E.2

\[
V_{\text{sum}} = \text{Noth_impounding_recycle_mass_rate} + \text{Desalter_effluent_mass_rate} \times \left(\frac{R_{17}}{R_{15}}\right) + \text{Clarifier_recycle_rate} \quad (E.2)
\]

Resistors R12, R13, R14, R16 and U5 form a subtractor circuit with the output, AB_IN expressed in Equation E.3

\[
\text{AB_IN} = V_{\text{sum}} \times \left(\frac{R_{13} + R_{12}}{R_{16} + R_{14}}\right) \frac{R_{16}}{R_{14}} - \text{WAS} \times \left(\frac{R_{12}}{R_{13}}\right) \quad (E.3)
\]
Since $R_{12}=R_{13}=R_{14}=R_{16}$ Equation E.3 is equal to Equation E.4.

$$AB_{\text{IN}} = V_{\text{um}} - \text{WAS}$$ (E.4)

The WAS rate is set by the voltage divider ratio of $R_{11}:R_{10}$. $U_{3}$ is a unity buffer providing a constant impedance source to $R_{2}$ from the voltage out of the subtractor.

Components $R_{2}$, $R_{1}$, $C_{1}$, $D_{1}$, $D_{2}$ and $U_{1}$ form an integrator which is meant to simulate the volume of material in the aeration basin. The voltage output is given in Equation E.5.

$$AB_{\text{2\_out}} = \frac{1}{(R_{1} + R_{2})C_{1}} \times \int_{0}^{t} AB_{\text{IN}}(t)dt$$ (E.5)

$D_{1}$ and $D_{2}$ bias the circuit.

The RAS rate is determined by the ratio of $R_{9}:R_{8}$, and $U_{4}$ is a voltage follower used as a constant impedance source.
Appendix F

R code

F.1 R Code for Biplot and Scree Graphs
The purpose of this R code is to produce a biplot graph and a Scree graph from an OUT file which contains a matrix of rRNA code, sample and taxonomy. The R coding is modified from the 2015 Biochemistry courses 9545Q and 9546Q at UWO.

```r
# load required libraries
library(compositions)
## Loading required package: tensorA
## Attaching package: 'tensorA'
## The following object is masked from 'package:base':
##   norm
## Loading required package: robustbase
## Loading required package: energy
## Loading required package: bayesm
## Welcome to compositions, a package for compositional data analysis.
## Find an intro with "? compositions"
##
## Attaching package: 'compositions'
## The following objects are masked from 'package:stats':
##   cor, cov, dist, var
## The following objects are masked from 'package:base':
##   %*, scale, scale.default

# keep R code and comments in r chunks to page size
library(knitr)
opts_chunk$set(tidy.opts=list(width.cutoff=60))

# define function to plot scree data in graphs from
# http://rstudio-pubs-static.s3.amazonaws.com/27823_dbc155ba6644eae9eb0a6bacz36824f.html
pcaCharts <- function(x) {
    x.var <- x$sdev^2
    x.pvar <- x.var/sum(x.var)
    print("proportions of variance:"
    print(x.pvar)
    par(mfrow = c(2, 2))
}
```r
plot(x.pvar, xlab = "Principal component", ylab = "Proportion of variance explained",
     ylim = c(0, 1), type = "b")
plot(cumsum(x.pvar), xlab = "Principal component", ylab = "Cumulative Proportion of variance explained",
     ylim = c(0, 1), type = "b")
screepplot(x)
screepplot(x, type = "l")
par(mfrow = c(1, 1))
}

# read data the file name would have to change for other data
d1 <- read.table("c:\16srrna\td_OTU_tag_mapped_lineage_1.txt",
                 row.names = 1, header = T, comment.char = "", skip = 1, sep = "\t")
tax <- d1$taxonomy
d1$taxonomy <- NULL

# uniform replacement
d1[d1 == 0] <- 0.5

# use function acomp to compute corrected data
bi <- acomp(d1 + 0.5)

pcx <- princomp(bi)

d1[d1 == 0.5] <- 0

# Biplot PCA data
biplot(pcx, scale = 0, cex = 0.6)
```
# plot Scree data

cpaCharts(pcx)

## [1] "proportions of variance:
##  Comp.1    Comp.2    Comp.3    Comp.4    Comp.5
##  0.37969559  0.27333599  0.09349606  0.07035804  0.05969179
##  Comp.6    Comp.7    Comp.8    Comp.9   Comp.10
##  0.02197386  0.01871135  0.01438405  0.01009472  0.00710157
##  Comp.11   Comp.12   Comp.13   Comp.14   Comp.15
##  0.00618030  0.00527205  0.00449122  0.00398213  0.00391711
##  Comp.16   Comp.17   Comp.18   Comp.19   Comp.20
##  0.00328350  0.00297895  0.00235203  0.00223073  0.00210030
##  Comp.21   Comp.22   Comp.23   Comp.24   Comp.25
##  0.00182975  0.00178579  0.00156965  0.00145667  0.00139819
##  Comp.26   Comp.27   Comp.28   Comp.29   Comp.30
##  0.00130349  0.00108436  0.00102820  0.00086391  0.00080427
##  Comp.31   Comp.32
##  0.00064107  0.00060551
F.2 R Code for Heat and Dendrogram Graphs
The purpose of this R code is to produce a denton diagram and a heat diagram from an OUT file which contains a matrix of rRNA code, sample and taxonomy. The R coding is modified from the 2015 Biochemistry courses 9545Q and 9546Q at UWO.

```r
# keep R code and comments in r chunks to page size
library(knitr)
opts_chunk$set(tidy.opts=list(width.cutoff=60))

clear workspace

ls()

## character(0)

rm(list=ls())
ls()

## character(0)

# column names are samples, row names are OTUs, last column
# is the presumed organism to change analysis between samples
# ensure that the file location is correct

d1 <- read.table("c:\16srrna\td_OTU_tag_mapped_lineage_1.txt", row.names = 1, header = T, skip = 1, comment.char = ",", sep = "\t")

# move your taxonomy information to a new vector in this code
# the taxonomy data is not used for graphing it is something
# that is being worked on April 13 2015

tax <- d1$taxonomy

d1$taxonomy <- NULL

# get proportional abundances

d1.prop <- apply(d1, 2, function(x) { x/sum(x) })
```
# filter so that organisms must be at least x% abundant in
# any sample you can use any abundance, by default the
# abundance is this case is set to 1% in any sample, the
# assumption is any bacteria less than 1 % will not have a
# large effect
abund <- 0.01

d1.abund <- d1[apply(d1.prop, 1, max) > abund,]
taxa.abund <- tax[apply(d1.prop, 1, max) > abund]

# get proportions of the filtered data for plotting below
d1.P <- apply(d1.abund, 2, function(x) {
x/sum(x)
})

# add a prior expectation for 0 count reads, all OTUs have at
# least one column with > 0 reads so our prior expectation is
# that the value of 0 represents a sampling depth problem and
# not
d1.abund[d1.abund == 0] <- 0.5

# apply the centered log ratio function as per Aitchison this
# moves values from a constrained set, to an open set of
# numbers distances between values are represented as
# differences in the ratio between the value and the
# geometric mean value, so a diference of 1 is a 2 fold
# change important #### distances between ratios are linear,
# so we can use euclidian distances
d1.clr <- apply(d1.abund, 2, function(x) {
  log2(x) - mean(log2(x))
})
dist.d1.clr <- dist(t(d1.clr), method = "euclidian")
clust.d1 <- hclust(dist.d1.clr, method = "average")

# remove the prior expectaton in case we want to do other
# explorations on the data
d1[d1 == 0.5] <- 0

# colorscheme
colours <- c("steelblue3", "skyblue1", "indianred1", "mediumpurple1",
  "olivedrab3", "pink", "#FFED6F", "mediumorchid3", "ivory2",
  "tan1", "aquamarine3", "#COCOCO", "royalblue4", "mediumvioletred",
  "#999933", "#666699", "#CC9933", "#006666", "#3399FF", "#993300",
  "#9999CC", "#666666", "#FFC666", "#6699CC", "#663366", "#9999CC",
  "#CCCC99", "#666666", "#FFC666", "#6699CC", "#663366", "#9999CC",
  "#CCCCCC", "#669999", "#CCCCC6", "#CC6600", "#9999FF", "#0066CC",
  "#9999CC", "#999999", "#FFCC00", "#009999", "#FF9900", "#999966",
  "#66CCCC", "#339966", "#CCCC33", "#EDEDED")

# mar=c(1, 3, 2, 1) + 0.1) mar=c(5,4,4,2)+0.1)
# Remove comment from line below to produce a seperate file
# showing dendrogram and heat diagram pdf('martin.pdf')

# Produce the dendogram
par(mfrow = c(2, 1), mar = c(0, 3, 2, 1) + 0.1)
plot(clust.d1, main = NULL)

# The line below will put red boxes around data in the
dendrogram if they are closely related or requires that the
dendrogram be viewed first to determine the number of
# windows
rect.hclust(clust.d1, 6)

# Produce the heatgraph
barplot(d1.P[, clust.d1$order], space = 0, col = colours, las = 2)

dev.off()
F.3 R Code for Heat and Dendrogram Graph
Sample Details
Heat and Dendrogram Graphs for 16S rRNA OTU File

Martin Flatley
April 14, 2016

The purpose of this R code is to produce a denton diagram and a heat diagram from a OUT file which contains a few samples matrix of rRNA code, sample and taxonomy. The R coding is modified from the 2015 Biochemistry courses 9545Q and 9546Q at UWO.

```r
# keep R code and comments in r chunks to page size
library(knitr)
opts_chunk$set(tidy.opts=list(width.cutoff=60))

clear workspace

ls()

## character(0)

rm(list=ls())
ls()

## character(0)

# column names are samples, row names are OTUs, last column
# is the presumed organism to change analysis between samples
# ensure that the file location is correct
d1 <- read.table("c:\16srrna\td_OTU_tag_mapped_lineage_Ab2.txt",
   row.names = 1, header = T, skip = 1, comment.char = ",", sep = "\t")

# move your taxonomy information to a new vector in this code
# the taxonomy data is not used for graphing it is something
# that is being worked on April 13 2015
tax <- d1$taxonomy
d1$taxonomy <- NULL

global abundance

d1.prop <- apply(d1, 2, function(x) {
   x/sum(x)
})

# filter so that organisms must be at least x% abundant in
# any sample you can use any abundance, by default the
# abundance is this case is set to 1% in any sample, the
# assumption is any bacteria less than 1 % will not have a
# large effect
abund <- 0.01

d1.abund <- d1[apply(d1.prop, 1, max) > abund, ]
taxa.abund <- tax[apply(d1.prop, 1, max) > abund]

# get proportions of the filtered data for plotting below
d1.P <- apply(d1.abund, 2, function(x) {
  x/sum(x)
})

# add a prior expectation for 0 count reads, all OTUs have at
# least one column with > 0 reads so our prior expectation is
# that the value of 0 represents a sampling depth problem and
# not
d1.abund[d1.abund == 0] <- 0.5

# apply the centered log ratio function as per Aitchison this
# moves values from a constrained set, to an open set of
# differences in the ratio between the value and the
# geometric mean value, so a difference of 1 is a 2 fold
# change important # distances between ratios are linear,
# so we can use euclidian distances

d1.clr <- apply(d1.abund, 2, function(x) {
  log2(x) - mean(log2(x))
})
dist.d1.clr <- dist(t(d1.clr), method = "euclidian")
clust.d1 <- hclust(dist.d1.clr, method = "average")

# remove the prior expectation in case we want to do other
# explorations on the data
d1[d1 == 0.5] <- 0

# colorscheme
colours <- c("steelblue3", "skyblue1", "indianred1", "mediumpurple1",
"olivedrab3", "pink", "#FFED6F", "mediumorchid3", "ivory2",
"tan1", "aquamarine3", "#C0C0C0", "royalblue4", "mediumvioletred",
"#999933", "#666666", "#3399FF", "#993300",
"#CC9933", "#666666", "#FFCC66", "#6699CC", "#663366", "#9999CC",
"#CC66CC", "#669999", "#CCCC66", "#666600", "#7799FF", "#0066CC",
"#99CC00", "#009999", "#FF9900", "#999966",
"#66CCCC", "#339966", "#CCCC33", "#EDEDED")

# mar=c(1, 3, 2, 1) + 0.1) mar=c(5,4,4,2)+0.1)

# Remove comment from line below to produce a seperate file
# showing dendrogram and heat diagram pdf('martin.pdf')
par(mfrow = c(2, 1), mar = c(0, 6, 2, 3) + 0.5)
plot(clust.d1, main = NULL)
The line below will put red boxes around data in the dendrogram if they are closely related. The number of red boxes requires that the dendrogram be viewed first to determine the number required.

```r
rect.hclust(clust.d1, 2)
```

Produce the heat map. If plotting many graphs leave space at 0 if only a few put space at 1 or greater. The actual number for space will depend on how the graph looks.

```r
par(mar = c(0, 3, 2, 1) + 0.5)
barplot(d1.P[, clust.d1$order], space = 1, col = colours, las = 2)
```

```r
dev.off()
## null device
## 1
```
Appendix G

Bacteria taxonomy of Wastewater Treatment Samples

This appendix contains a listing of the bacteria identified by 16S rRNA analysis is Section 4.8 and a summary of the taxonomy for individual samples listed in Table 4.7 on page 73.

G.1 Taxonomy of all Identified Bacteria in Wastewater Treatment Samples

Table G.1 lists the taxonomy of all 252 bacteria identified by 16S rNA from wastewater treatment samples identified in Section 4.8.1.
G.2 Taxonomy of Bacteria in Samples Listed in Table 4.7

Tables G.2 through G.12 list the taxonomy of the bacteria identified for each sample in Table 4.7 on page 73. The corresponding Figures G.1 through G.11 are the graphs of the data found in the tables.

Three technical replicates were obtained for each sample for quality control purposes. There is a slight variation for the third technical replicate of each sample.
Table G.1 – Bacteria taxonomy of wastewater treatment samples from 16S rRNA analysis.

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<tr>
<th>OTU</th>
<th>Phylum</th>
<th>Class</th>
<th>Order</th>
<th>Family</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
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<td>Firmicutes</td>
<td>Bacilli</td>
<td>Bacillales</td>
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G.2.1 Bacteria Taxonomy Identified in Aeration Basin 1

Top Layer

Table G.2 lists the bacteria found in the top layer of aeration basin 1 in the wastewater treatment unit. One sample was taken and three technical replicates were produced and given sample names names were Ab1s1, Ab1s1 and Ab1s3. Figure G.1 is the heat diagram showing the percentage of each bacteria listed in Table G.2.

The legend used for colour coding Figure G.1 can be found in the first column of Table G.2.

![Heat plot of technical replicates](image)

**Figure G.1** – A heat plot of the technical replicates from the sample of the top layer of aeration basin 1. Table G.2 contains the colour legend and taxonomy for the heat map.
Table G.2 – Bacteria taxonomy identified in aeration basin 1 top layer.

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G.2.2  Bacteria Taxonomy Identified in Aeration Basin 1  
Bottom Layer

Table G.3 lists the bacteria found in the bottom layer of aeration basin 1 in the wastewater treatment unit. One sample was taken and three technical replicates were produced and given sample names names were Ab1bots1, Ab1bots1 and Ab1bots3. Figure G.2 is the heat diagram showing the percentage of each bacteria listed in Table G.3.

The legend used for colour coding Figure G.2 can be found in the first column of Table G.3.

Figure G.2 – A heat plot of the technical replicates from a sample of aeration basin 1 bottom sample. Table G.3 contains the colour legend and taxonomy for the heat map.
## Table G.3 — Bacteria taxonomy identified in aeration basin 1 bottom layer.

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**Notes:**
- **HB2-32-21**
- **Sulfuritalea**
- **Zoogloea**
G.2.3  Bacteria Taxonomy Identified in Aeration Basin 2

Top Layer

Table G.4 lists the bacteria found in the top layer of aeration basin 2 in the wastewater treatment unit. One sample was taken and three technical replicates were produced and given sample names names were Ab2s1, Ab2s1 and Ab2s3. Figure G.3 is the heat diagram showing the percentage of each bacteria listed in Table G.2.

The legend used for colour coding Figure G.3 can be found in the first column of Table G.4.

Figure G.3 – A heat plot of the technical replicates from a sample of aeration basin 2 top layer. Table G.4 contains the colour legend and taxonomy for the heat map.
Table G.4 – Bacteria taxonomy identified in aeration basin 2 top layer.

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G.2.4 Bacteria Taxonomy Identified in Aeration Basin 2
Bottom Layer

Table G.5 lists the bacteria found in the bottom layer of aeration basin 2 in the wastewater treatment unit. One sample was taken and three technical replicates were produced and given sample names: Ab2bots1, Ab2bots1 and Ab2bots3. Figure G.4 is the heat diagram showing the percentage of each bacteria listed in Table G.5.

The legend used for colour coding Figure G.4 can be found in the first column of Table G.5.

Figure G.4 – A heat plot of the technical replicates from a sample of aeration basin 2 bottom layer. Table G.5 contains the colour legend and taxonomy for the heat map.
Table G.5 – Bacteria taxonomy identified in aeration basin 2 bottom layer.

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</table>
G.2.5 Broth used for Petri Dish Samples of Top Layer of Aeration Basin 1

Two predominant aerobic bacteria were isolated from individual colonies from sample Ab1 and cultivated in a Petri dish using an agar which is described in papers to isolate bacteria in naphthenic acid biodegradation studies. The solid medium/nutrient agar consisted of Peptone 5 g L⁻¹, beef extract/yeast extract 3 g L⁻¹, agar 15 g L⁻¹, sodium chloride 5 g L⁻¹, distilled water. In this point the pH was adjusted to neutral (6.8) at 25 °C and sugar (glucose) 0.5 g L⁻¹, urea 0.01 g L⁻¹, sodium phosphate 0.005 g L⁻¹ and Bushnell Haas Broth 50 mL L⁻¹ were added.

The Bushnell Haas Broth contained the following, magnesium sulfate 0.2 g L⁻¹, calcium chloride 0.02 g L⁻¹, monopotassium phosphate 1.0 g L⁻¹, ammonium sulfate 0.5 g L⁻¹, urea 0.38 g L⁻¹, ferric chloride 0.002, sodium phosphate 1.0 g L⁻¹, and manganese sulfate 0.002 g L⁻¹.

The colonies of the two cultured predominant bacteria were described morphologically. Bacteria 1 (Ba1) had opaque, rough white colonies, between 2 and 10 mm diameter, most of them over 4 mm, round with irregular border.

Bacteria 2 (ba2) had shiny, convex, raised, white, well defined circular colonies, less than 2 mm in diameter.

Microscope analyses showed that the samples of Ba1 contained predominantly bacilli with 1.2 to 2.0 µm diameter and 4-5 µm length; endospores and inclusion bodies.

Bacteria 2 (Ba2) had cocci forms with 1- 1.7 µm diameter and spores with 0.4- 0.5 µm diameter.
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G.2.6 Bacteria Taxonomy Identified in a Petri Dish Colonies from Aeration Basin 1 Top Layer

Table [G.6] lists the bacteria identified on a Petri dish colony originating from a sample of aeration basin 1 top layer. Details of the colony and agar used can be found in Section [G.2.5]. One sample of the colony was taken and three technical replicates were produced and given sample names Ba1s1, Ba1s2 and Ba1s3. Figure [G.5] is the heat diagram showing the percentage of each bacteria listed in Table [G.6].

The legend used for colour coding Figure [G.5] can be found in the first column of Table [G.6].

**Figure G.5** – A heat plot of the technical replicates from a cell colony from a Petri dish using bacteria from the top layer of aeration basin. Details can be found in Section [G.2.5]. Table [G.6] contains the colour legend and taxonomy for the heat map.
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<th>Family</th>
<th>Genus</th>
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<td>Bacillaceae</td>
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Table G.6 – Bacteria taxonomy identified in Petri dish culture 1.
Table G.7 lists the bacteria identified on a Petri dish colony originating from a sample of aeration basin 1 top layer. Details of the colony and agar used can be found in Section G.2.5. One sample of the colony was taken and three technical replicates were produced and given sample names Ba2s1, Ba2s2 and Ba2s3. Figure G.6 is the heat diagram showing the percentage of each bacteria listed in Table G.7.

The legend used for colour coding Figure G.6 can be found in the first column of Table G.7.

![Figure G.6](image)

**Figure G.6** – A heat plot of the technical replicates from a cell colony from a Petri dish using bacteria from the top layer of aeration basin. Details can be found in Section G.2.5. Table G.7 contains the colour legend and taxonomy for the heat map.
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G.2.7  Bacteria Taxonomy Identified in Recycle Sludge from Clarifier 2

Table G.8 lists the bacteria found in the recycle of clarifier 2 that is combined with clarifier 3 recycle routed to aeration basin 1 or to a waste stream (WAS) in the wastewater treatment unit. One sample was taken and three technical replicates were produced and given sample names C2fos1, C2fos1 and C2fos3. Figure G.7 is the heat diagram showing the percentage of each bacteria listed in Table G.8.

The legend used for colour coding Figure G.7 can be found in the first column of Table G.8.

![Figure G.7](image)  

**Figure G.7** – A heat plot of the technical replicates from a sample from the bottom outlet of clarifier 2 which is combined with clarifier 3 and used as a feedback to the aeration basin or to waste (WAS). Table G.8 contains the colour legend and taxonomy for the heat map.
Table G.8 – Bacteria taxonomy identified in clarifier 2 recycle sludge.

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</table>
G.2.8 Bacteria Identified in Recycle Sludge from Clarifier 3 Water Effluent

Table G.9 lists the bacteria found in the water effluent of clarifier 3 which is the effluent to the carbon filters shown in Figure 4.6. One sample was taken and three technical replicates were produced and given sample names C3fos1, C3fos1 and C3fos1. Figure G.8 is the heat diagram showing the percentage of each bacteria listed in Table G.11.

The legend used for colour coding Figure G.8 can be found in the first column of Table G.9.

![Figure G.8](image)

**Figure G.8** – A heat plot of the technical replicates from clarifier 3 water effluent. Table G.9 contains the colour legend and taxonomy for the heat map.
Table G.9 – Bacteria taxonomy identified in clarifier 3 water effluent.

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G.2.9  Bacteria Taxonomy Identified in the Recycle Sludge from Clarifier 3

Table G.10 lists the bacteria found in the recycle of clarifier 3 that is routed to aeration basin 1 or to a waste stream (WAS) in the wastewater treatment unit. One sample was taken and three technical replicates were produced and given sample names C3fos1, C3fos1 and C3fos1. Figure G.9 is the heat diagram showing the percentage of each bacteria listed in Table G.11.

The legend used for colour coding Figure G.9 can be found in the first column of Table G.10.

Figure G.9 – A heat plot of the technical replicates from clarifier 3 which is combined with clarifier 2 and used as a feedback to the aeration basin or to waste (WAS). Table G.10 contains the colour legend and taxonomy for the heat map.
Table G.10 – Bacteria taxonomy identified in clarifier 3 recycle sludge.

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G.2.10 Bacteria Taxonomy Identified in Combined Recycle Sludge from Clarifier 2 and 3

Table G.11 lists the bacteria found in the combined recycle of clarifiers 2 and 3 that is routed to aeration basin 1 or to a waste stream (WAS) in the wastewater treatment unit. One sample was taken and three technical replicates were produced and given sample names C23toAb1s1, C23toAb1s1, and C23toAb1s3. Figure G.10 is the heat diagram showing the percentage of each bacteria listed in Table G.11.

The legend used for colour coding Figure G.10 can be found in the first column of Table G.11.

Figure G.10 – A heat plot of the technical replicates from a sample from the outlet of two clarifiers that are used as a feedback to the aeration basin or to wastage (WAS) as shown in figure. Table G.11 contains the colour legend and taxonomy for the heat map.
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Table G.11 – Bacteria taxonomy identified in combined clarifier 2 and 3 recycle sludge to aeration basin 1.
G.2.11 Bacteria Taxonomy Identified in Hydrocell® Effluent

Table G.12 lists the bacteria found in the Hydrocell® effluent that is routed to aeration basin 1. One sample was taken and three technical replicates were produced and given sample names names were C3fos1, C3fos1 and C3fos3. Figure G.11 is the heat diagram showing the percentage of each bacteria listed in Table G.12.

The legend used for colour coding Figure G.11 can be found in the first column of Table G.12.

**Figure G.11** – A heat plot of the technical replicates from a sample from the outlet of the Hydrocell®. Table G.12 contains the colour legend and taxonomy for the heat map.
**Table G.12 – Sample Hco OTU percentages**

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Appendix H

Test Equipment Specifications

H.1 FTIR/ATR

Manufacturer: Thermo Electron Corporation
Model: Nicolet 380
Software: OMNIC 8.3 software, OMNIC Spectra 2.0.391
Accessories: Pike MIRacle™ Single Reflection ATR with Diamond/ZnSe window

H.2 GC/MSD

Gas chromatograph Manufacturer: Agilent
Gas Chromatograph Model: 7890A with flame ionization detector (FID)
Mass spectrometer Manufacturer: Agilent
Mass spectrometer model Model: 5975C single quad pole
Software: Enhanced Chemstation E.02.02.431 with NIST library
Accessories: GC Sampler 80 vial and headspace autosampler
H.3  ICP/OES

Manufacturer: Varian
Model: 720 Axial configuration
Spraychamber: Glass Expansion Twister
Nebulizer: Burgener Teflon Mira-Mist

H.4  LC/QTOF/MS

Liquid Chromatogram Manufacturer: Agilent
Liquid Chromatogram Model: 1200 Series
Time of flight Mass spectrometer manufacturer : Bruker
Time of flight Mass spectrometer manufacturer: MicrOTOF II
Software: Bruker Compass 1.3, including DataAnalysis (Version 4.0 SP 3), micrOTOFcontrol (Version 2.3 Patch 1) for controlling the mass spec. The LC portion is controlled through HyStar (Version 3.2 ).

H.5  SEM/EDX

SEM Manufacturer: Hitatchi
SEM Model: S4500
EDX manufacturer : Quartz Imaging Corporation
EDX Model: Quartz XOne EDS System
Software: Quartz PCI
H.6 XRD

Manufacturer: Rigaku  Model: RTP-300 rotating-anode X-Ray Diffractometer
Parameters: 45 kV and 160 mA

H.7 XRF

Manufacturer: Oxford Instruments
Model: X-Supreme8000
Software: X-Supreme Version 2.0
Appendix I

Naphthenic Acid GC/MS Control Parameters

INSTRUMENT CONTROL PARAMETERS: SARGCMS

C:\MSDCHEM\1\METHODS\NAPACID.M
Sat Jul 11 16:26:20 2015

Control Information
------ -------
Sample Inlet : GC
Injection Source : CTC PAL ALS
Mass Spectrometer : Enabled

CTCPAL METHOD

Injection Volume: 1.00 ul
Syringe Size: 10ul
Cycle File: GC-Inj

CYCLE DETAILS
-------------

278
Air Volume (l): 0
Pre Clean with Solvent 1 (): 5
Pre Clean with Solvent 2 (): 5
Pre Clean with Sample (): 2
  Filling Speed (l/s): 5
  Filling Strokes (): 4
Inject to: GC Inj1
Injection Speed (l/s): 50
  Pre Inject Delay (ms): 500
  Post Inject Delay (ms): 500
Post Clean with Solvent 1 (): 3
Post Clean with Solvent 2 (): 3

No Sample Prep method has been assigned to this method.

Oven
Equilibration Time 0.5 min
Max Temperature 325 degrees C
Slow Fan Disabled
Oven Program
  On
    120 C for 3 min
    then 15 C/min to 320 C for 15.7 min
Run Time 32.033 min

Sample Overlap
Sample overlap is not enabled

Front SS Inlet He
Mode Splitless
Heater On 280 C
Pressure On 27.621 psi
Total Flow On 20.12 mL/min
Septum Purge Flow On 3 mL/min
Gas Saver Off
Purge Flow to Split Vent 15 mL/min at 0.75 min

Back Inlet PCM
Heater Off
Control Mode Pressure
Pressure Off
Temperature Program

80 C for 0 min
then 15 C/min to 390 C for 10 min

Run Time 32.033 min

Cryo On
Cryo Use Temperature 25 C
Fault Detection Off
Timeout Detection On 20 min

Thermal Aux 2 (MSD Transfer Line)
Heater On
Temperature Program On
280 C for 0 min
Run Time 32.033 min

Column #1
Agilent 19091S-433HP-5MS 5% Phenyl Methyl Silox
325 C: 30 m x 250 m x 0.25 m
In: Front SS Inlet He
Out: Aux EPC 3

(Initial) 120 C
Pressure 27.621 psi
Flow 2.12 mL/min
Average Velocity 44.118 cm/sec
Holdup Time 1.1333 min
Pressure Program On
27.621 psi for 0 min
Run Time 32.033 min

Column #2
Agilent 160261510: 1839.44250 fs deactivated .180 mm
450 C: 0.53 m x 180 m x 0 m
In: Aux EPC 3 He
Out: Front Detector FID

(Initial) 120 C
Pressure 3.1595 psi
Flow 2.2714 mL/min
Average Velocity 176.58 cm/sec
Holdup Time 0.0050026 min
Pressure Program On
    3.1595 psi for 0 min
Run Time 32.033 min

Column #3
Agilent 1602615144: 1839.44461
msd inlet fs deactivated
450 C: 1.44 m x 180 m x 0 m
In: Aux EPC 3 He
Out: Vacuum

(Initial) (Initial)
Pressure 3.1595 psi
Flow 2.5916 mL/min
Average Velocity 276.32 cm/sec
Holdup Time 0.0086854 min
Pressure Program On
    3.1595 psi for 0 min
Run Time 32.033 min

Front Detector FID
Heater On 300 C
H2 Flow On 30 mL/min
Air Flow On 400 mL/min
Makeup Flow Off
Const Col + Makeup Off
Flame On
Electrometer On

Aux EPC 1 He
Pressure Program Off
    10 psi for 0 min
Run Time 32.033 min

Aux EPC 2 He
Pressure Program Off
    10 psi for 0 min
Run Time 32.033 min
Aux EPC 3 He: Supplies Column 2

***Excluded from Affecting GC’s Readiness State***

Signals
Signal #1: Front Signal
   Save On
   5 Hz
   Zero @ 0 min

Signal #2: Aux EPC 1,2,3 Pressure #2: Actual
   Save Off
   50 Hz

Signal #3: Test Plot
   Save Off
   50 Hz

Signal #4: Test Plot
   Save Off
   50 Hz

MS ACQUISITION PARAMETERS

General Information
---
Tune File : target.u
Acquisition Mode : Scan

MS Information
--
Solvent Delay : 4.00 min

EMV Mode : Gain Factor
Gain Factor : 2.00
Resulting EM Voltage : 1682

[Scan Parameters]

Low Mass : 33.0
High Mass : 550.0
Threshold : 100
Sample # : 2 A/D Samples 4
Plot 2 low mass : 40.0
Plot 2 high mass : 400.0

282
[MSZones]

MS Source : 230 C maximum 250 C
MS Quad : 150 C maximum 200 C

END OF MS ACQUISITION PARAMETERS

TUNE PARAMETERS for SN: US11384703

Trace Ion Detection is OFF.

EMISSION : 34.610
ENERGY : 69.922
REPELLER : 29.788
IONFOCUS : 90.157
ENTRANCE_LE : 0.000
EMVOLTS : 1776.471

Actual EMV : 1682.35
GAIN FACTOR : 2.08

AMUGAIN : 2950.000
AMUOFFSET : 127.000
FILAMENT : 1.000
DCPOLARITY : 0.000
ENTLENSEFFS : 15.812@ 3 15.812@ 50 18.322@ 69 21.082@131
20.078@219 20.329@0414 20.329@0502 20.329@01049
MASSGAIN : -55.000
MASSOFFSET : -37.000

END OF TUNE PARAMETERS

END OF INSTRUMENT CONTROL PARAMETERS

283
Appendix J

Naphthenic Acid GC/MS Macro

name martin30a

chro,75
mi x,6*60000,25*60000,0,0,0,0
tab res,_datapath$+"6T025.txt"

mi x,10.5*60000,25*60000,0,0,0,0
tab res,_datapath$+"105T025.txt"

open _datapath$+_datafile$+.new for output as #1

m1 = 3.5
m2 = 4.
chro ,159
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,159, m1, m2, peak_area

m1 = 4.5
m2 = 5.0
chro ,173
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,173, m1, m2, peak_area
m1 = 5.5
m2 = 6.1
chro ,187
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,187, m1, m2, peak_area

m1= 6.5
m2=7.1
chro ,201
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,201, m1, m2, peak_area

m1=7.5
m2=8
chro ,215
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,215, m1, m2, peak_area

m1=8.4
m2=9
chro ,229
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,229, m1, m2, peak_area

m1=9.3
m2=9.8
chro ,243
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,243, m1, m2, peak_area

m1=10.
m2=10.6
chro ,257
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,257, m1, m2, peak_area
m1=10.8
m2=11.2
chro ,271
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,271, m1, m2, peak_area

m1=11.5
m2=12.0
chro ,285
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,285, m1, m2, peak_area

m1=12.2
m2=12.6
chro ,299
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,299, m1, m2, peak_area

m1=12.8
m2=13.2
chro ,313
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,313, m1, m2, peak_area

m1=13.5
m2=13.8
chro ,327
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,327, m1, m2, peak_area

m1=14
m2=14.5
chro ,341
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,341, m1, m2, peak_area

m1=14
m2=29.1
chro, 355
mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1,355, m1, m2, peak_area

m1=14
m2=29.1
chro, 369
mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1,369, m1, m2, peak_area

m1=14
m2=29.1
chro, 383
mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1,383, m1, m2, peak_area

m1=14
m2=29.1
chro, 397
mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1,397, m1, m2, peak_area

m1=14
m2=29.1
chro, 411
mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1,411, m1, m2, peak_area

m1=14
m2=29.1
chro, 425
mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1,425, m1, m2, peak_area
m1=14
m2=29.1
chro ,439
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,439, m1, m2, peak_area
m1=14
m2=29.1
chro ,453
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,453, m1, m2, peak_area
m1=14
m2=29.1
chro ,467
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,467, m1, m2, peak_area
m1=14
m2=29.1
chro ,481
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,481, m1, m2, peak_area
m1=14
m2=29.1
chro ,495
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1,495, m1, m2, peak_area
m1=5
m2=10
chro ,185
peaknumber 1, top, x
print  #1,185, m1, m2, peak_area

m1=6.5
m2=10
chro ,199
peaknumber 1, top, x
print  #1, 199, m1, m2, peak_area

m1=6.8
m2=11
chro ,213
peaknumber 1, top, x
print  #1,231, m1, m2, peak_area

m1=7.5
m2=11
chro ,227
peaknumber 1, top, x
print  #1, 227, m1, m2, peak_area

m1=8
m2=11
chro ,241
peaknumber 1, top, x
print  #1, 241, m1, m2, peak_area

m1=9
m2=13
chro ,255
peaknumber 1, top, x
print  #1, 255, m1, m2, peak_area

m1=9.5
m2=13
chro ,269
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 269, m1, m2, peak_area

m1=10
m2=13

chro ,283
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,283, m1, m2, peak_area

m1= 10.5
m2= 14.5

chro ,297
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,297, m1, m2, peak_area

m1= 11.0
m2= 15.0

chro ,311
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,311, m1, m2, peak_area

m1= 11
m2= 17

chro ,325
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,325, m1, m2, peak_area

m1= 11.8
m2= 19.0

chro ,339
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,339, m1, m2, peak_area

m1= 12.0
m2 = 29.1
chro ,353
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,353, m1, m2, peak_area

m1 = 13.0
m2 = 29.1
chro ,367
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 367, m1, m2, peak_area

m1 = 13.0
m2 = 29.1
chro ,381
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 381, m1, m2, peak_area

m1 = 13.0
m2 = 29.1
chro ,395
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 395, m1, m2, peak_area

m1 = 13.0
m2 = 29.1
chro ,409
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 409, m1, m2, peak_area

m1 = 13.0
m2 = 29.1
chro ,423
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 423, m1, m2, peak_area
m1= 13.0
m2= 29.1
chro ,437
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 437, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,451
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 451, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,465
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 465, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,479
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 479, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,493
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 493, m1, m2, peak_area

m1= 6
m2= 7.5
chro ,211
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 211, m1, m2, peak_area
m1= 6.8
m2= 8
chro ,225
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 225, m1, m2, peak_area

m1= 7.
m2= 8.5
chro ,239
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 239, m1, m2, peak_area

m1= 8.0
m2= 11.0
chro ,253
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 253, m1, m2, peak_area

m1= 9.0
m2= 12.0
chro ,267
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 267, m1, m2, peak_area

m1= 10.0
m2= 14
chro ,281
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 281, m1, m2, peak_area

m1= 10
m2= 15
chro ,295
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 295, m1, m2, peak_area

m1= 11.0
m2= 15.5
chro, 309
mx, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 309, m1, m2, peak_area

m1= 11.0
m2= 15.5
chro, 323
mx, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 323, m1, m2, peak_area

m1= 12.0
m2= 16.0
chro, 337
mx, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 337, m1, m2, peak_area

m1= 12.0
m2= 16.5.0
chro, 351
mx, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 351, m1, m2, peak_area

m1= 12.0
m2= 20.0
chro, 365
mx, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 365, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro, 379
mx, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 379, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,393
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1,393, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,407
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 407, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,421
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 421, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,435
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 435, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,449
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 449, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,463
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 463, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,477
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 477, m1, m2, peak_area

m1= 13.5
m2= 29.1
chro ,491
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 491, m1, m2, peak_area

m1= 5
m2= 10.5
chro ,237
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 237, m1, m2, peak_area

m1= 5.5
m2= 10.5
chro ,251
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 251, m1, m2, peak_area

m1= 8.0
m2= 12.0
chro ,265
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 265, m1, m2, peak_area

m1= 10
m2= 13
CHROMATIN

mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 279, m1, m2, peak_area

m1 = 10.5
m2 = 13.8

mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 293, m1, m2, peak_area

m1 = 10.5
m2 = 15.0

mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 307, m1, m2, peak_area

m1 = 11.5
m2 = 15

mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 321, m1, m2, peak_area

m1 = 12.0
m2 = 18.0

mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 335, m1, m2, peak_area

m1 = 12.5
m2 = 21.0

mi x, m1*60000, m2*60000, 0, 0, 0, 0
peaknumber 1, top, x
print #1, 349, m1, m2, peak_area

m1 = 13.0
m2= 25.0
chro ,363
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 363, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,377
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 377, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,391
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 391, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,405
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 405, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,419
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 419, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,433
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 433, m1, m2, peak_area
m1= 13.0
m2= 29.1
chro ,447
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 447, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,461
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 461, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,475
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 475, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,489
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 489, m1, m2, peak_area

m1= 8.0
m2= 13.0
chro ,249
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 249, m1, m2, peak_area

m1= 8.5
m2= 13.5
chro ,263
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 263, m1, m2, peak_area
m1= 10
m2= 15.0
chro ,277
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 277, m1, m2, peak_area

m1= 10.0
m2= 15
chro ,291
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 291, m1, m2, peak_area

m1= 11.0
m2= 18.0
chro ,305
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 305, m1, m2, peak_area

m1= 11.5
m2= 19.0
chro ,319
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 319, m1, m2, peak_area

m1= 12.0
m2= 20.0
chro ,333
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 333, m1, m2, peak_area

m1= 12.0
m2= 20.5
chro ,347
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 347, m1, m2, peak_area

m1= 12.0
m2= 21
Chro ,361
mi x,m1*60000,m2*60000,0,0,0,0,
peaknumber 1, top, x
print #1, 361, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,375
mi x,m1*60000,m2*60000,0,0,0,0,
peaknumber 1, top, x
print #1, 375, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,389
mi x,m1*60000,m2*60000,0,0,0,0,
peaknumber 1, top, x
print #1, 389, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,403
mi x,m1*60000,m2*60000,0,0,0,0,
peaknumber 1, top, x
print #1, 403, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,417
mi x,m1*60000,m2*60000,0,0,0,0,
peaknumber 1, top, x
print #1, 417, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,431
mi x,m1*60000,m2*60000,0,0,0,0,
peaknumber 1, top, x
print  #1, 431, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,445
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 445, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,459
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 459, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,473
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 473, m1, m2, peak_area

m1= 13.0
m2= 29.1
chro ,487
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 487, m1, m2, peak_area

m1= 10.0
m2= 14.0
chro ,289
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print  #1, 289, m1, m2, peak_area

m1= 11.0
m2= 15.0
chro ,303
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 303, m1, m2, peak_area

m1= 11.0
m2= 15.5
chro ,317
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 317, m1, m2, peak_area

m1= 12.0
m2= 16.0
chro ,331
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 331, m1, m2, peak_area

m1= 13.5
m2= 20.1
chro ,345
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 345, m1, m2, peak_area

m1= 13
m2= 29.1
chro ,359
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 359, m1, m2, peak_area

m1= 13
m2= 29.1
chro ,373
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 373, m1, m2, peak_area

m1= 13
m2= 29.1
chro ,387
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 387, m1, m2, peak_area
m1= 13
m2= 29.1
chro ,401
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 401, m1, m2, peak_area
m1= 13
m2= 29.1
chro ,415
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 415, m1, m2, peak_area
m1= 13
m2= 29.1
chro ,429
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 429, m1, m2, peak_area
m1= 13
m2= 29.1
chro ,443
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 443, m1, m2, peak_area
m1= 13
m2= 29.1
chro ,457
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 457, m1, m2, peak_area
m1= 13
m2= 29.1
chro ,471
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 471, m1, m2, peak_area

m1= 13
m2= 29.1
chro ,485
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 485, m1, m2, peak_area

m1= 13
m2= 29.1
chro ,499
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 499, m1, m2, peak_area

m1= 12
m2= 17
chro ,329
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 329, m1, m2, peak_area

m1= 12.5
m2= 17.0
chro ,343
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 343, m1, m2, peak_area

m1= 13.0
m2= 18.0
chro ,357
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 357, m1, m2, peak_area
m1= 13.5
m2= 20.0
chro ,371
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 371, m1, m2, peak_area

m1= 14.0
m2= 29.1
chro ,385
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 385, m1, m2, peak_area

m1= 14.0
m2= 29.1
chro ,399
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 399, m1, m2, peak_area

m1= 14.0
m2= 29.1
chro ,413
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 413, m1, m2, peak_area

m1= 14.0
m2= 29.1
chro ,427
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 427, m1, m2, peak_area

m1= 14.0
m2= 29.1
chro ,441
mi x,m1*60000,m2*60000,0,0,0,0
peaknumber 1, top, x
print #1, 441, m1, m2, peak_area
m1= 14.0
m2= 29.1
chro ,455
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 455, m1, m2, peak_area
m1= 14.0
m2= 29.1
chro ,469
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 469, m1, m2, peak_area
m1= 14.0
m2= 29.1
chro ,483
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 483, m1, m2, peak_area
m1= 14.0
m2= 29.1
chro ,497
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 497, m1, m2, peak_area
m1= 3.5
m2= 5.5
chro ,151
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 151, m1, m2, peak_area
m1= 5
m2= 6
chro ,165
mi x,m1*60000,m2*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 165, m1, m2, peak_area

m1= 6
m2= 8
crho ,179
mi x,m1\*60000,m2\*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 179, m1, m2, peak_area

m1= 6.5
m2= 9.0
crho ,193
mi x,m1\*60000,m2\*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 193, m1, m2, peak_area

m1= 6.0
m2= 9.0
crho ,207
mi x,m1\*60000,m2\*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 207, m1, m2, peak_area

m1= 8.0
m2= 10.0
crho ,221
mi x,m1\*60000,m2\*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 221, m1, m2, peak_area

m1= 8.5
m2= 11.0
crho ,235
mi x,m1\*60000,m2\*60000,0,0,0,0,0
peaknumber 1, top, x
print #1, 235, m1, m2, peak_area

PRINT USING #1, "/ / / / /
close #1

tab header, _datapath$+_datafile$".NEW"
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Appendix K

Naphthenic Acid LC/QTOFMS

Control Parameters

LC parameters:

1. Column : Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HT, 4.6 x 50mm, 1.8 micron, P.N. 927975-902
2. Flow : 0.3 ml-min⁻¹
3. Column temperature : 40 °C.
4. Solvents :
   (a) Solvent A : water w 0.1 % acetic acid
   (b) Solvent B : methanol w 0.1 % acetic acid;

Solvent program :

5. (a) 0 to 3 minutes 70 % solvent B,
   (b) 3 to 5 minutes 100 % solvent B,
(c) 5 to 28 minutes 100 % solvent B,
(d) 28 to 30 minutes 70 % solvent B.

QTOFMS parameters:

1. Ionization mode : Negative mode
2. Mass monitoring : 50-600 m/z
3. Column temperature : 40 °C.
4. Endplate offset : -500 V
5. Capillary : +2500 V
6. Solvent Nebulizer : 3.0 Bar
7. Dry Gas : 8.0 L·min⁻¹
8. Dry Temp : 180 °C.
## VITA

<table>
<thead>
<tr>
<th>NAME</th>
<th>Martin Flatley P.Eng. Ph.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLACE OF BIRTH</td>
<td>Sarnia Ontario</td>
</tr>
<tr>
<td>YEAR OF BIRTH</td>
<td>1957</td>
</tr>
<tr>
<td>POST-SECONDARY EDUCATION</td>
<td>University of Western Ontario</td>
</tr>
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<td>AND DEGREES</td>
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<tr>
<td></td>
<td>1999-2004 Ph.D., Electrical Engineering</td>
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<td></td>
<td>University of Western Ontario</td>
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<td></td>
<td>London, Ontario</td>
</tr>
<tr>
<td></td>
<td>2009-2016 Ph.D., Biochemical Engineering</td>
</tr>
<tr>
<td>RELATED WORK</td>
<td>Instrument Technician</td>
</tr>
<tr>
<td>EXPERIENCE</td>
<td>Eldorado Nuclear</td>
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<tr>
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<td>1975 - 1977</td>
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<td>Avionics Technician</td>
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<td>Moore Aviation</td>
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<td>1977 - 1978</td>
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<td>Electrical Designer</td>
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<td>1980 - 1981</td>
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<td>Instrument Technician</td>
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<td>Suncor Inc.</td>
</tr>
<tr>
<td></td>
<td>1981-1990</td>
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</tbody>
</table>
Instrument Technologist  
Suncor Inc.  
1991-1995

Senior Electrical and Instrument Engineer  
Suncor Inc.  
1995-2001

Teaching Assistant  
University of Western Ontario  
1995 - 2000

Lecturer  
University of Western Ontario  
1997, 2001

Senior Staff Engineer  
Suncor Inc.  
2001 - Present

Full-time employee Suncor Inc.,  
1981 - Present

Manager Crude Emulsion Project  
Canadian Crude Technical Quality Association  
2010 - present

PAPERS PRESENTED IN CONFERENCES

1. M. Flatley and A. Margaritis  
“Extraction and Analysis Naphthenic Acid Concentration in Wastewater Samples”  
65’th Annual Canadian Chemical Engineering Conference, Calgary, AB,  
October 4, 2015.

2. M. Flatley and A. Margaritis  
“Extraction, Separation and Analysis of Acid Species contained in Crude Oil with a Total Acid Number (TAN)  
> 1.0 mg KOH / g oil”  
65’th Annual Canadian Chemical Engineering Conference, Calgary, AB,  
October 4, 2015.
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<td>1. M. Flatley, G. Marty and A. Margaritis</td>
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<td>“Naphthenic Acid Toxicity Studies of Rainbow Trout Fingerlings.”</td>
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<td>“Naphthenic Acid Analysis of Oil Refinery Wastewater Treatment Samples.”</td>
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<td>3. M. Flatley, G. Gloor and A. Margaritis</td>
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<td>“Study of Microbial Community in an Oil Refinery Wastewater System using 16S rRNA Hypervariable Region V4 Analysis.”</td>
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<td>5. M. Flatley and A. Margaritas</td>
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<tr>
<td>“Desalter Brine Treatment of Athabasca Oil Sands Crude Oil to Minimize Naphthenic Acid Concentration in an Oil Refinery.”</td>
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<th>PATENT APPLICATION SUBMITTED</th>
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<tr>
<td>“Processing of Aqueous Waste Streams to Remove Naphthenic Acids”, Canadian Patent Office</td>
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<tr>
<td>Submitted: October 4, 2015</td>
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<td>M. Flatley and A. Margaritis</td>
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