Comparisons of Microbial Communities in a Sequencing Batch Reactor (Cromaglass® Corporation) at Two Time Increments

By:

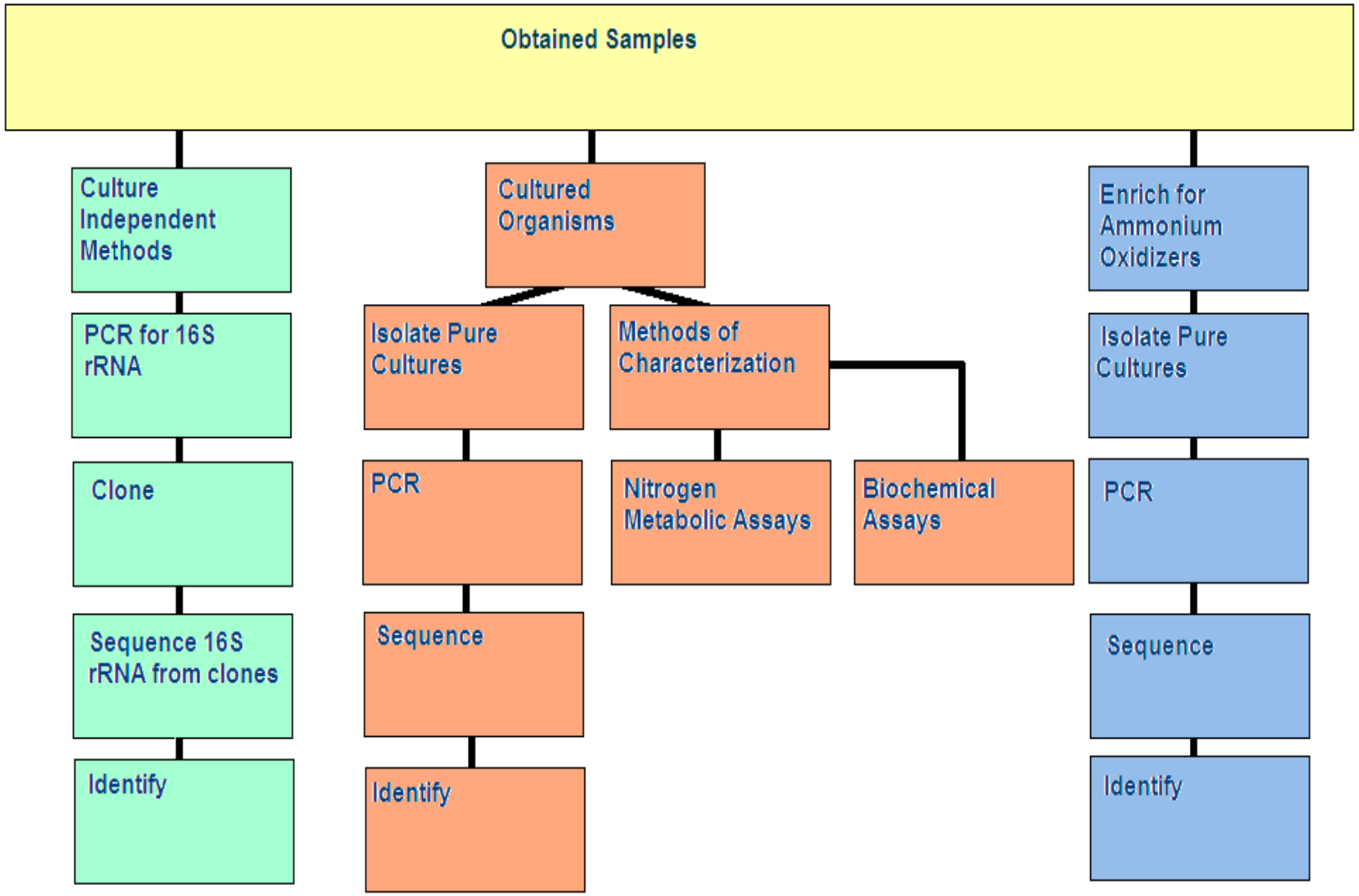
Brittane Miller and Jeff Newman

Lycoming College

Williamsport, Pennsylvania

What is a Sequencing Batch Reactor?

- **GOAL: Decrease the nitrogen levels in treated wastewater effluent**
- **MECHANISM: Increase the biofilm surface area for a microbial community to metabolize nitrogen**
- **Reactor undergoes Aerobic and Anaerobic cycles**
  - **Aerobic – chemolithotrophic oxidation of ammonia and nitrite**
  - **Anaerobic – nitrate reduction and denitrification produce N_2 gas**
- **Provide an improved treatment method for facilities that are not connected to a sewer system**
- My project was to evaluate the microbial community present in the SBR.


Obtained Samples
Culture Independent Methods
PCR for 16S rRNA
Clone
Sequence 16S rRNA from clones
Identify
Cultured Organisms
Isolate Pure Cultures
PCR
Sequence
Identify
Methods of Characterization
Nitrogen Metabolic Assays
Biochemical Assays
Enrich for Ammonium Oxidizers
Isolate Pure Cultures
PCR
Sequence
Identify

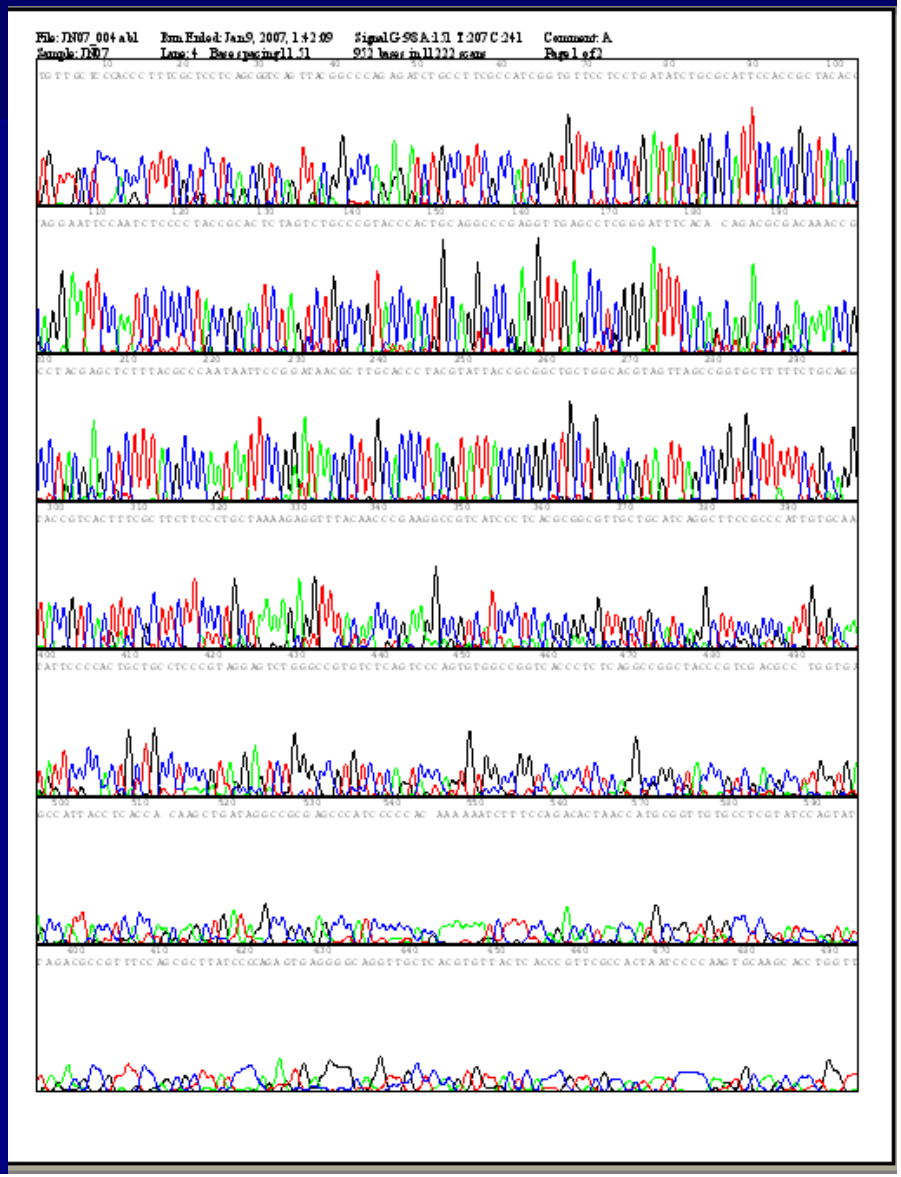
Collecting the Samples

- Removed PVC unit from SBR
- Opened tube and removed “coffee can”
- Obtained biofilm sample by scraping 25 cm^2 area on both the PVC tube and the coffee can
- Place both scrapings in separate sterile screw cap tubes, each containing 25 mL of distilled water
- Take back to lab and analyze

Collecting the Samples

Culture Independent ID's

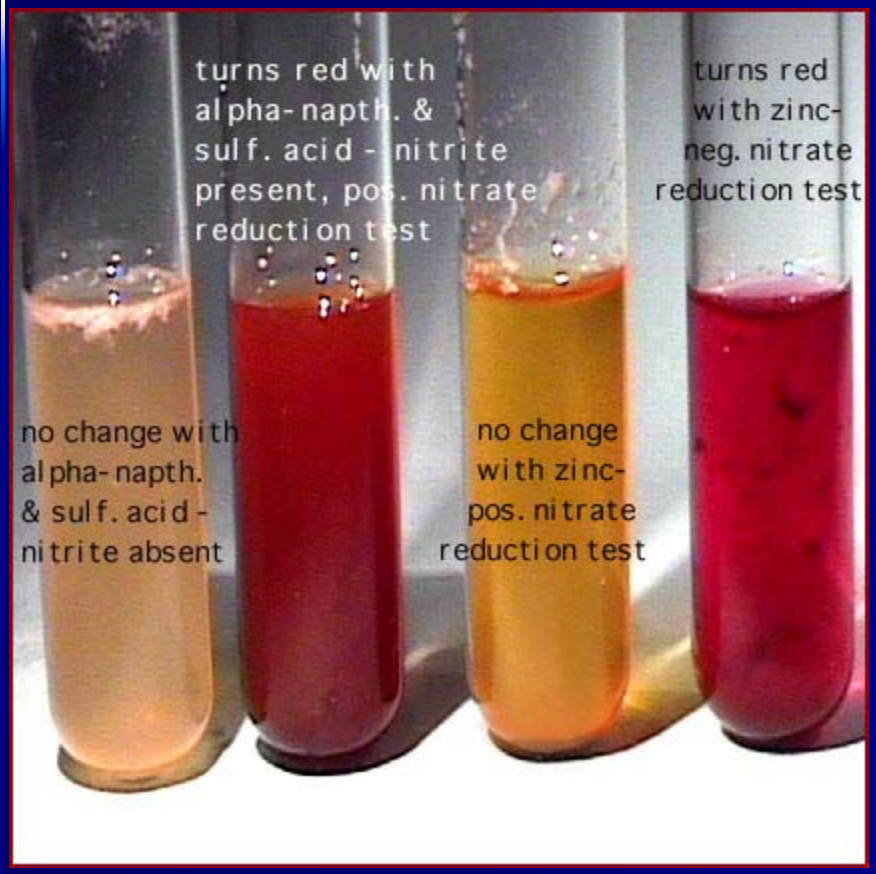
- Only a small percentage, about 10 percent, of microbes can be cultured
- 16S rRNA genes were amplified using PCR
- PCR products were cloned and clones were sequenced
- Source of rRNA gene determined using a BLAST search
- Identifications show the microbial diversity in the SBR at the time the sample was taken
- Most clones showed the greatest similarity to other uncultured organisms



Culture Independent ID's

Name of Closest Matching Relative	Phylogeny	Percent Match
1) *Mycobacterium mucogenicum*	Actinobacteria	>97%
2) Uncultured delta Proteobacteria	delta Proteobacteria	~93%
3) Uncultured Bacterium	TM 7	~93%
4) Unidentified Bacterium	gamma Proteobacteria	93%
5) Uncultured Bacteroidetes	Bacteroidetes	94%
6) Uncultured Xanthomonadaceae	gamma Proteobacteria	95%
7) Uncultured Bacterium	TM 7	94%
8) Uncultured soil bacterium	TM 7	89%
9) Uncultured Bacterium	Bacteroidetes	95%
10) Uncultured Bacterium	Bacteroidetes	99%
11) Uncultured Verrucomicrobia	Verrucomicrobia	~92%
12) *Citrobacter freundii*	gamma Proteobacteria	99%
13) *Citrobacter freundii*	gamma Proteobacteria	96%
14) *Flavobacteriaceae bacterium*	Bacteroidetes	98%
15) *Acinetobacter haemolyticus*	gamma Proteobacteria	95%

Nitrate Reduction and Denitrification Tests



http://www.mc.maricopa.edu/~johnson/labtools/Dbiochem/nit1.jpg

Nitrate reduction tests:

Bacteria grown in medium containing nitrate as the electron acceptor

After addition of sulfanilic acid and α-napthylamine:

Red- positive for nitrite (indicates that nitrate reduction occurred)

Colorless- negative for nitrite and must proceed to next test

Denitrification test:

After the addition of zinc powder:

Still not red- nitrite has been reduced to N_2 gas and denitrification has occurred

Red- nitrate still present - negative for both denitrification and nitrate reduction

Cultured Organism ID's

- Spread 100 µL of diluted samples onto TSA plates
- Incubated plates at 20 - 44°C
- Selected morphologically distinguishable organisms and subcultured onto new TSA and biochemical plates (Endo, EMB, etc…)
- PCR amplified 16S rRNA gene using universal primers
- Gel Purified and sequenced PCR products
- Tested each for Nitrate Reduction and Denitrification

Cultured Organisms ID's (After 2 Weeks of Operation)

- γ-Proteobacteria – Enterobacteriales
  - *Citrobacter freundii*
  - *Enterobacter asburiae*
  - *Klebsiella granulomatis*
  - *Klebsiella oxytoca*
  - *Klebsiella pneumoniae*
  - *Kluyvera ascorbata*
  - *Raoultella ornithinolytica*
  - *Raoultella planticola*
- γ-Proteobacteria - Pseudomonadales
  - *Acinetobacter johnsonii*
  - *Acinetobacter junii novum*
  - *Pseudomonas fluorescens*
  - *Pseudomonas koreensis*
  - *Pseudomonas rhodesiae*
  - *Pseudomonas umsongensis*
- γ-Proteobacteria – Xanthomonadales
  - *Stenotrophomonas nitritireducans*
  - *Stenotrophomonas rhizophila*
- Firmicutes - Bacillales
  - *Exiguobacterium acetylicum*

Organisms Capable of Nitrate Reduction are colored in yellow

Cultured Organisms ID's (After 12 Weeks of Operation)

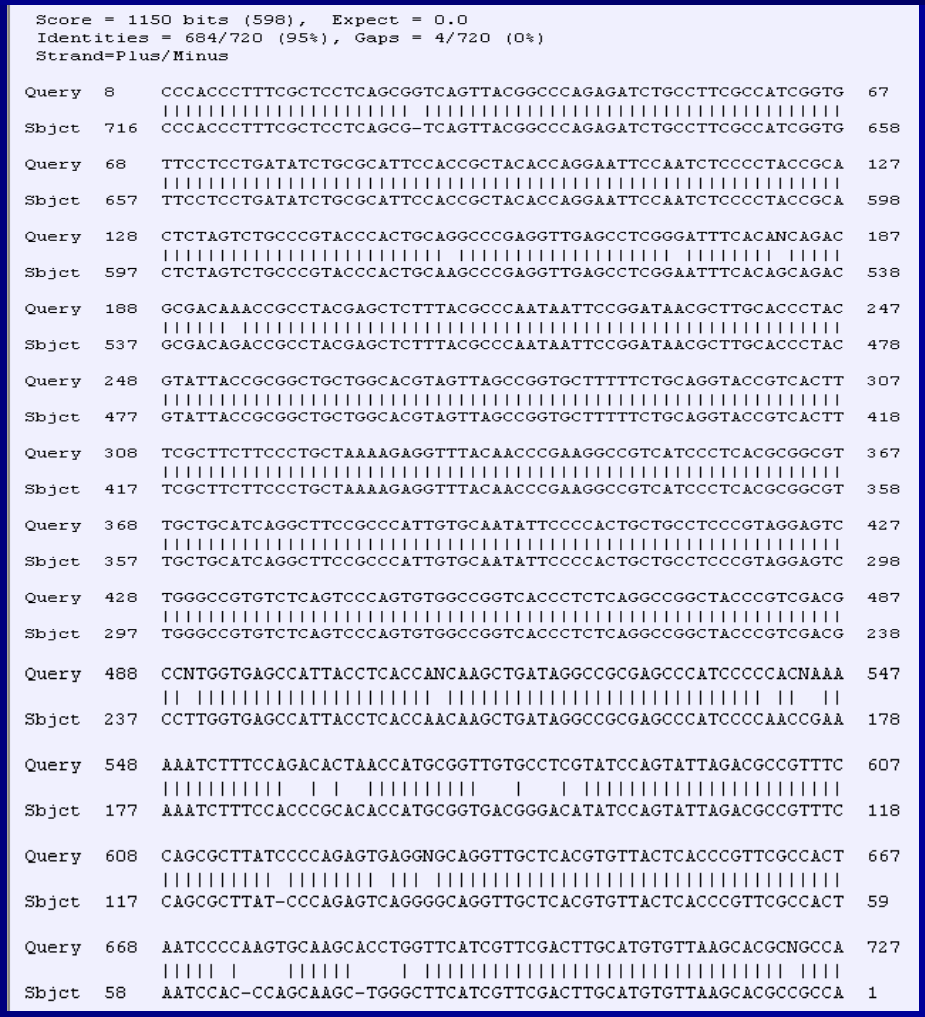
- Gamma Proteobacteria – Enterobacteriales
  - *Klebsiella pneumoniae*
  - *Serratia marcescans*
  - *Raoultella terrigena*
  - *Pantoea anantis*
  - *Pantoea agglomerans*
- Gamma Proteobacteria – Pseudomonadales
  - *Pseudomonas fragi*
  - *Pseudomonas veronii*
  - *Pseudomonas alcaligenes*
- Gamma Proteobacteria – Aeromonadales
  - *Aeromonas media*
  - *Aeromonas hydrophila*
- Beta Proteobacteria – Burkholderiales
  - *Acidovorax defluvii*
  - *Acidovorax temperans*
  - *Comamonas testosteronii*
- Firmicutes – Bacillales
  - *Bacillus pumilis*
  - *Bacillus licheniformis*
- Actinobacteria – Micrococcinae
  - *Microbacterium paraoxydans*
  - *Microbacterium novum*
  - *Microbacterium terregens*
- Actinobacteria –Corynebacterineae
  - *Rhodococcus erythropolis*

Organisms in yellow were capable of Nitrate reduction

Organisms in red were capable of denitrification

Novel Organism Found

- One novel organism was found, in the phylum Actinobacteria and the family Micrococcinae
- The closest published relative is *Microbacterium esteraromaticum*
- At only a 95% match to the closest relative, one can infer that this organism is novel, and it has been temporarily named *Microbacterium novum BLM*



```
 Score = 1150 bits (598),  Expect = 0.0
 Identities = 684/720 (95%), Gaps = 4/720 (0%)
 Strand=Plus/Minus

Query  8    CCCACCCTTTCGCTCCTCAGCGGTCAGTTACGGCCCAGAGATCTGCCTTCGCCATCGGTG  67
            ||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||
Sbjct  716  CCCACCCTTTCGCTCCTCAGCG-TCAGTTACGGCCCAGAGATCTGCCTTCGCCATCGGTG  658

Query  68   TTCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAATCTCCCCTACCGCA  127
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  657  TTCCTCCTGATATCTGCGCATTCCACCGCTACACCAGGAATTCCAATCTCCCCTACCGCA  598

Query  128  CTCTAGTCTGCCCGTACCCACTGCAGGCCCGAGGTTGAGCCTCGGGATTTCACANCAGAC  187
            |||||||||||||||||||||| |||||||||||||||||| |||||||| |||||
Sbjct  597  CTCTAGTCTGCCCGTACCCACTGCAAGCCCGAGGTTGAGCCTCGGAATTTCACAGCAGAC  538

Query  188  GCGACAAACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGATAACGCTTGCACCCTAC  247
            |||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  537  GCGACAGACCGCCTACGAGCTCTTTACGCCCAATAATTCCGGATAACGCTTGCACCCTAC  478

Query  248  GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTTTTCTGCAGGTACCGTCACTT  307
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  477  GTATTACCGCGGCTGCTGGCACGTAGTTAGCCGGTGCTTTTTCTGCAGGTACCGTCACTT  418

Query  308  TCGCTTCTTCCCTGCTAAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGT  367
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  417  TCGCTTCTTCCCTGCTAAAAGAGGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGT  358

Query  368  TGCTGCATCAGGCTTCCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC  427
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  357  TGCTGCATCAGGCTTCCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTC  298

Query  428  TGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCGACG  487
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  297  TGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCACCCTCTCAGGCCGGCTACCCGTCGACG  238

Query  488  CCNTGGTGAGCCATTACCTCACCANCAAGCTGATAGGCCGCGAGCCCATCCCCCACNAAA  547
            || |||||||||||||||||||| |||||||||||||||||||||||||| ||  ||
Sbjct  237  CCTTGGTGAGCCATTACCTCACCAACAAGCTGATAGGCCGCGAGCCCATCCCCAACCGAA  178

Query  548  AAATCTTTCCAGACACTAACCATGCGGTTGTGCCTCGTATCCAGTATTAGACGCCGTTTC  607
            ||||||||||  | |  |||||||||   |   | ||||||||||||||||||||||
Sbjct  177  AAATCTTTCCACCCGCACACCATGCGGTGACGGGACATATCCAGTATTAGACGCCGTTTC  118

Query  608  CAGCGCTTATCCCCAGAGTGAGGNGCAGGTTGCTCACGTGTTACTCACCCGTTCGCCACT  667
            |||||||||| |||||||| ||| ||||||||||||||||||||||||||||||||||||
Sbjct  117  CAGCGCTTAT-CCCAGAGTCAGGGGCAGGTTGCTCACGTGTTACTCACCCGTTCGCCACT  59

Query  668  AATCCCCAAGTGCAAGCACCTGGTTCATCGTTCGACTTGCATGTGTTAAGCACGCNGCCA  727
            ||||| |    ||||||    | ||||||||||||||||||||||||||||| ||||
Sbjct  58   AATCCAC-CCAGCAAGC-TGGGCTTCATCGTTCGACTTGCATGTGTTAAGCACGCCGCCA  1
```

Further Work

- While the project that I have been working on is at it's end, I will hopefully continue to carry on the characterization of *Microbacterium novum*, using methods similar to those discussed in a poster presentation by Kellie Cicconi this weekend
- There is also going to be a continuation of the testing done on the Cromaglass unit under the supervision of Dr. Mel Zimmerman, and it would be possible to monitor the microbial community of the SBR much more thoroughly than I have been able to do and could be carried out within the next year to supplement the data presented in this summary

Conclusions

- Four different orders found in samples obtained after 2 weeks of incubation and 7 different orders found in the samples retrieved after 12 weeks
- While many different organisms were seen in the results of both sequencing runs, the second run clearly, after 12 weeks of operation, shows a more diverse community than after the 2 week interval

Acknowledgements

- Dr. Jeff Newman – my research advisor
- Michael Gerardi at Cromaglass for giving me the idea for the project and allowing me to test the SBR
- Dr. Mel Zimmerman for encouraging me to take the project
- The Lycoming College Biology Faculty for their teachings and support
- PAS for giving me the opportunity to present my work
- My family, fiancé and friends – for listening to me talk about wastewater treatment and pretending to like it
- Dr. John Piper – for his support of student research and his support for this and other scholarly meetings

- This research is being presented at the ASM general meeting in May, 2007 (Toronto, Ontario, Canada)

Use of an On-Site Sequencing Batch Reactor to Satisfy a Total Nitrogen Discharge Limit

Mel Zimmerman (Lycoming College Biology/CWI), Joshua Gliptis and Micheal Gerardi (Cromaglass® Corporation)

Clean Water Institute
CWI
Lycoming College

Cromaglass
Wastewater Treatment Systems