Microbial Indicators of Soil Ecosystem Restoration Following Bioremediation of Oil and Brine Spills

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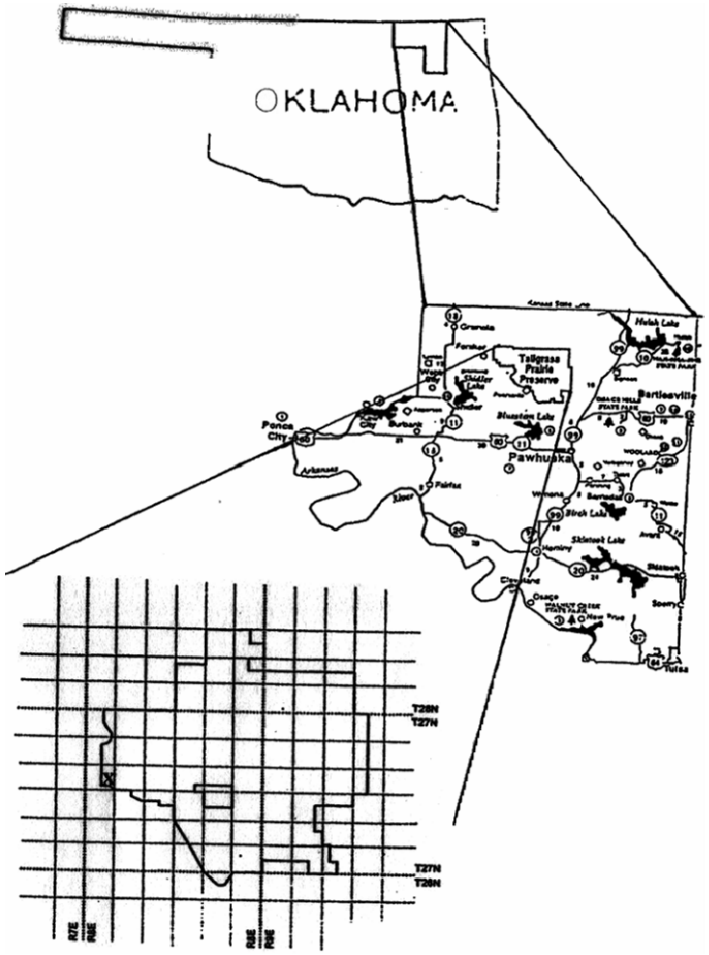
Overview

- “Risk Reduction and Soil Ecosystem Restoration in an Active Oil-Producing Area in an Ecologically Sensitive Setting”
- Two major components
  - Probabilistic Risk Assessment Model
    - Minimize risk in E & P operations
  - Ecologically relevant indicators for remediation endpoints
    - Variety of indicators assessed
    - **Current presentation: microbial indicators**

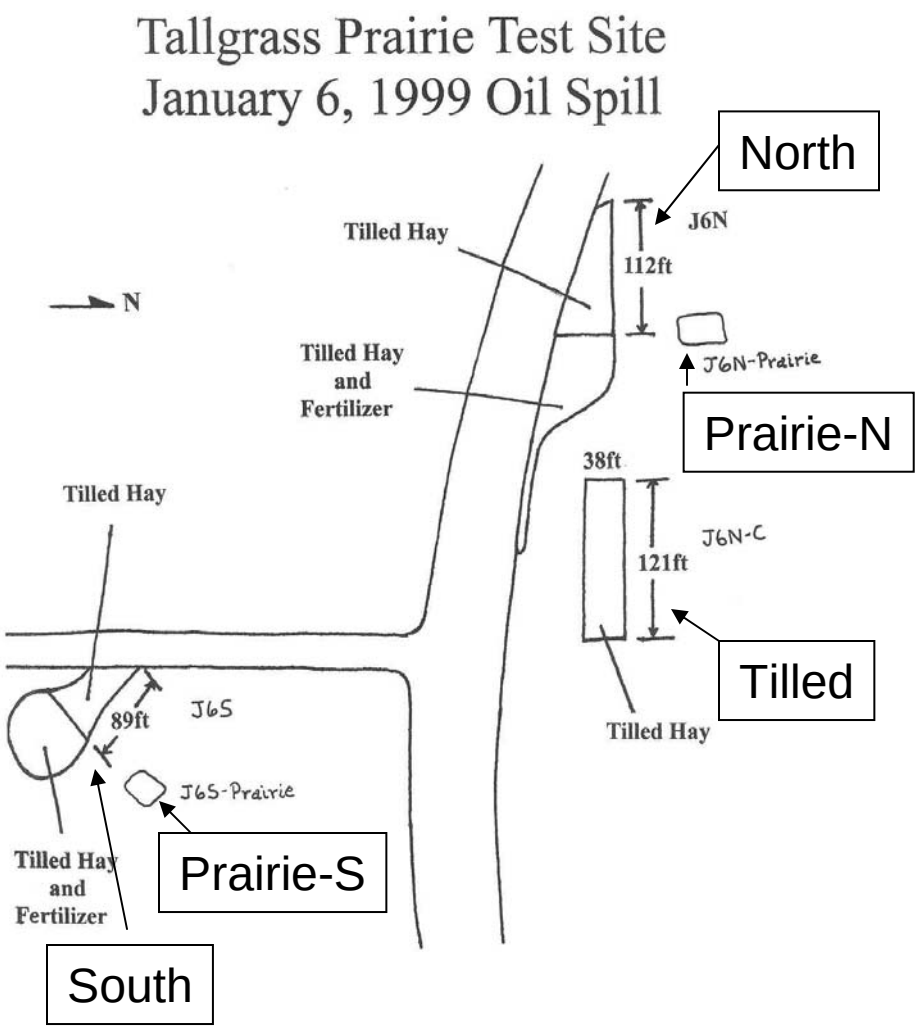
Field Site
Tallgrass Prairie Preserve, Nature Conservancy, Pawhuska, OK



Bison

J6: crude oil contamination

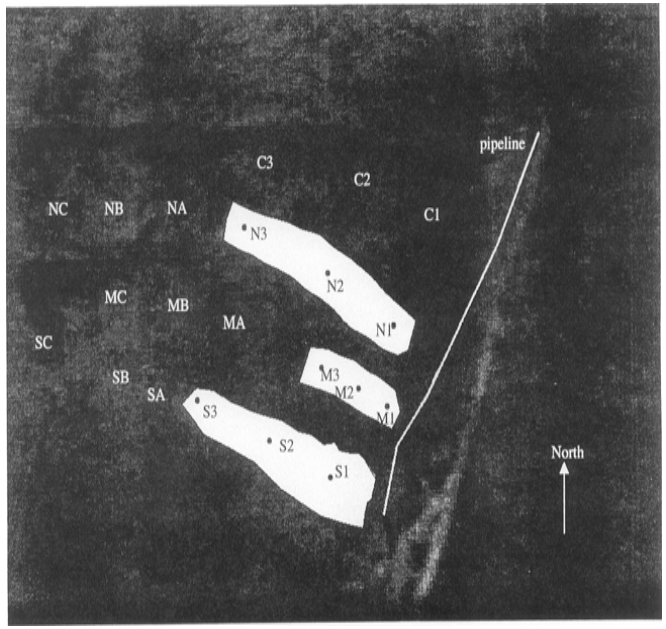
- Pipeline break 1/6/99
  - 70 barrels dewatered crude oil
- Two major areas
  - North: ~10,000 ft^2
    - TPH (3/99): 33,500 mg/kg
  - South: ~ 4800 ft2
    - TPH (3/99): 4800 mg/kg
- Treatments
  - Hay and tilling ("NF"-not fertilized)
  - Hay, tilling, and fertilizer ("F"-fertilized)
- Control areas
  - Uncontaminated, undisturbed ("P"-prairie)
  - Uncontaminated, hay and tilling ("C"-control)



Gibbs #5: Crude oil and brine

- Pipeline break Fall 1999
  - Quantity unknown, 10:1 brine/oil
- Three contaminated areas
  - North ("**N**"): 600 m^2
  - Middle ("**M**"):230 m^2
  - South ("**S**"): 960 m^2
- Treatments: June 2000
  - N: brine interception trench, hay and fertilizer
  - M: interception trench
  - S: hay and fertilizer
- Observation areas downslope: ND, MD, SD
- Control: Uncontaminated, undisturbed ("P"-prairie)

Sampling points



Objectives

- Compare effects of remediation treatments on ecological indicators
  - Does treatment speed recovery?
  - Is treatment cost-effective?
- How can we tell when the ecosystem has recovered?
  - Compare to adjacent uncontaminated sites
- How long does recovery take?
- Which ecological indicators are “best”?

Types of data collected for ecological monitoring

- Replicate soil samples collected 3 x year
  - TPH (total petroleum hydrocarbons): EPA method 418.1
  - Sodium, chloride, conductivity (Gibbs #5)
  - Nutrient analysis
    - Plant available NO_3^- - N, NH_4^+ - N, Phosphorus
    - Total N and Total C
    - N-Mineralization potential
  - Phospholipid fatty acids (PLFA)
  - Nematode #s and functional group diversity
  - Plant biomass and species diversity
  - **Bacterial genetic diversity (16S rRNA and *amoA*)**
    - **DGGE: denaturing gradient gel electrophoresis**
    - **Sequencing cloned PCR products**

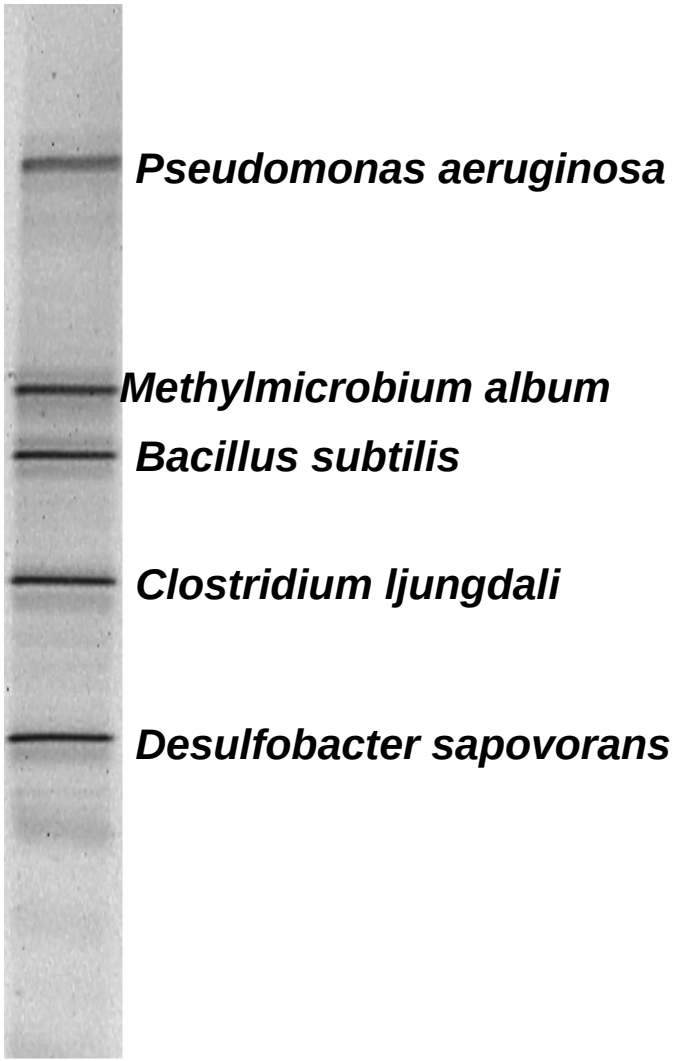
Why use molecular techniques to study microbes?

- Culturable microbes are a fraction of total #s
- Possibly better estimates of microbial diversity
- Microbes from new/unusual environments
- Detect microbes that are difficult to culture
  - This study: bacteria important in nitrogen cycling

DGGE

Denaturing gradient gel electrophoresis

1. Extract DNA from soil
2. Use PCR to amplify 16S rRNA from bacterial DNA present in soil
3. Separate PCR products on a denaturing polyacrylamide gel
4. Position on the gel reflects the nucleotide sequence composition of the PCR product

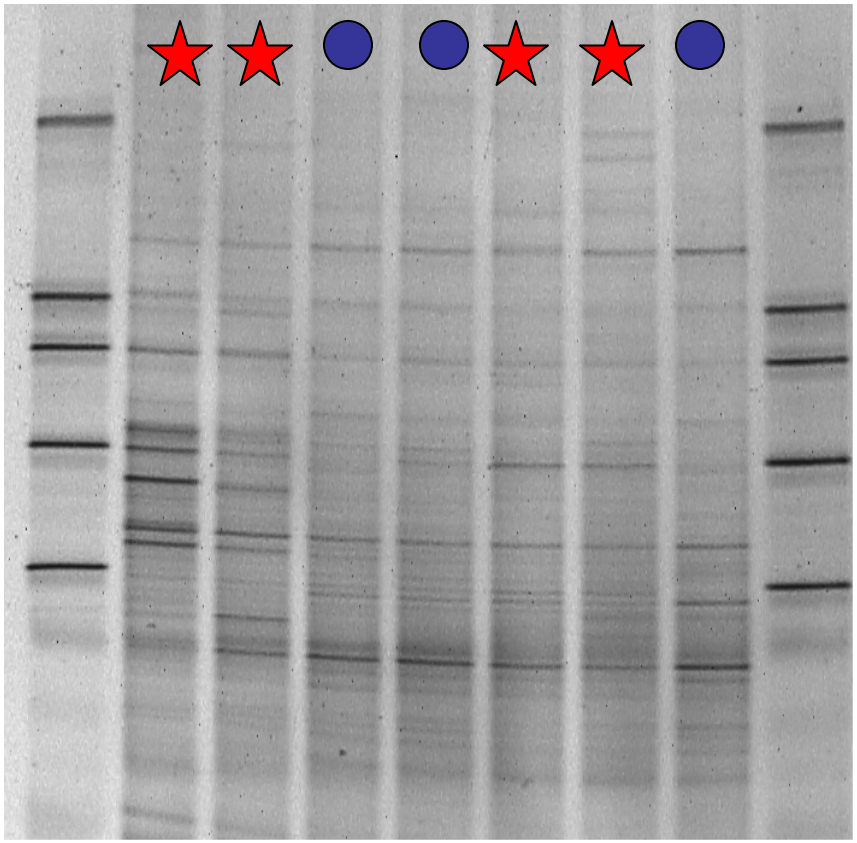


J6, 2 yr. after contamination and treatment

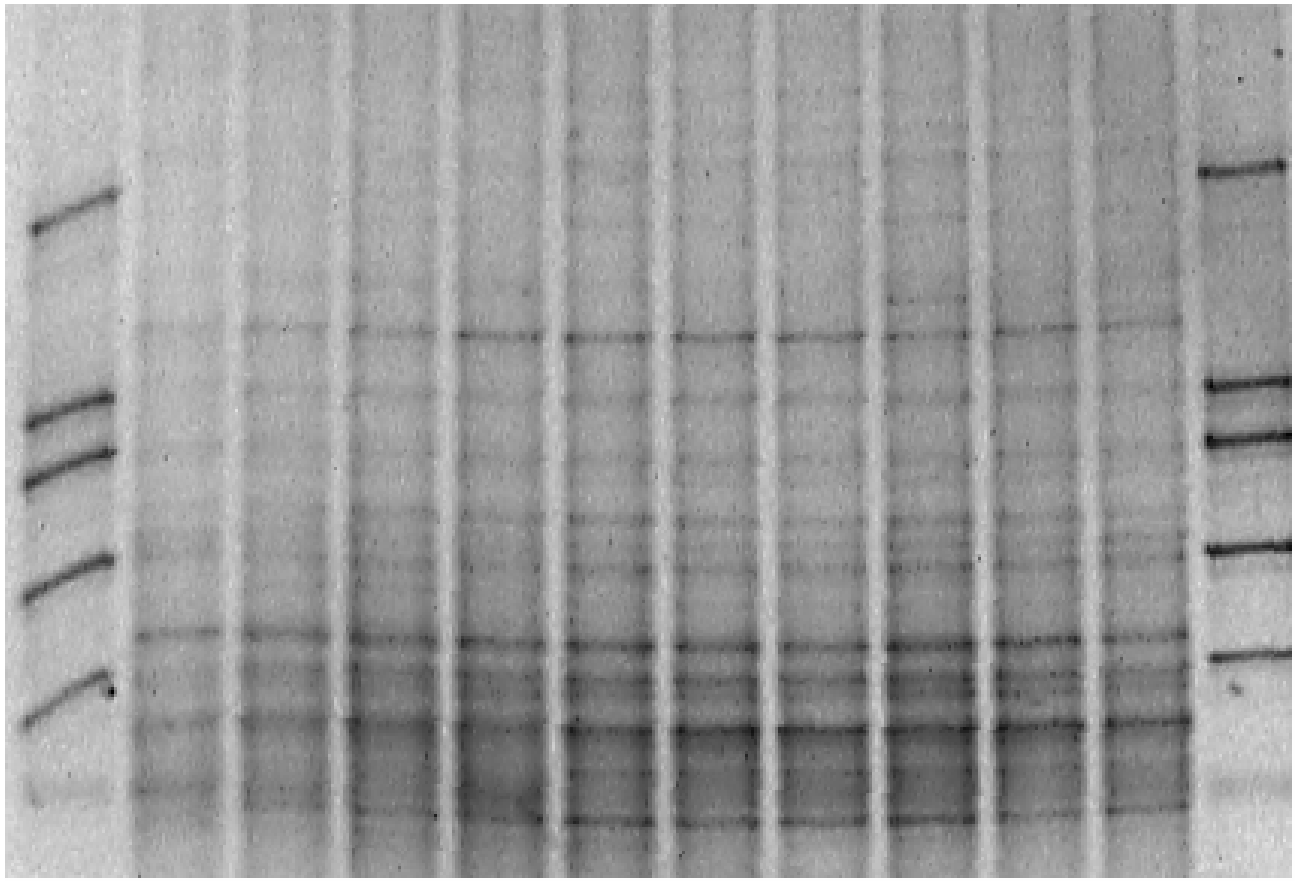
Contaminated sites: ★
Lower diversity
Certain sequences dominant

Most contaminated → least
N-NF, N-F, S-NF, S-F
treated

Uncontaminated sites: ●
Higher diversity
More equal proportions



J6NP: uncontaminated prairie

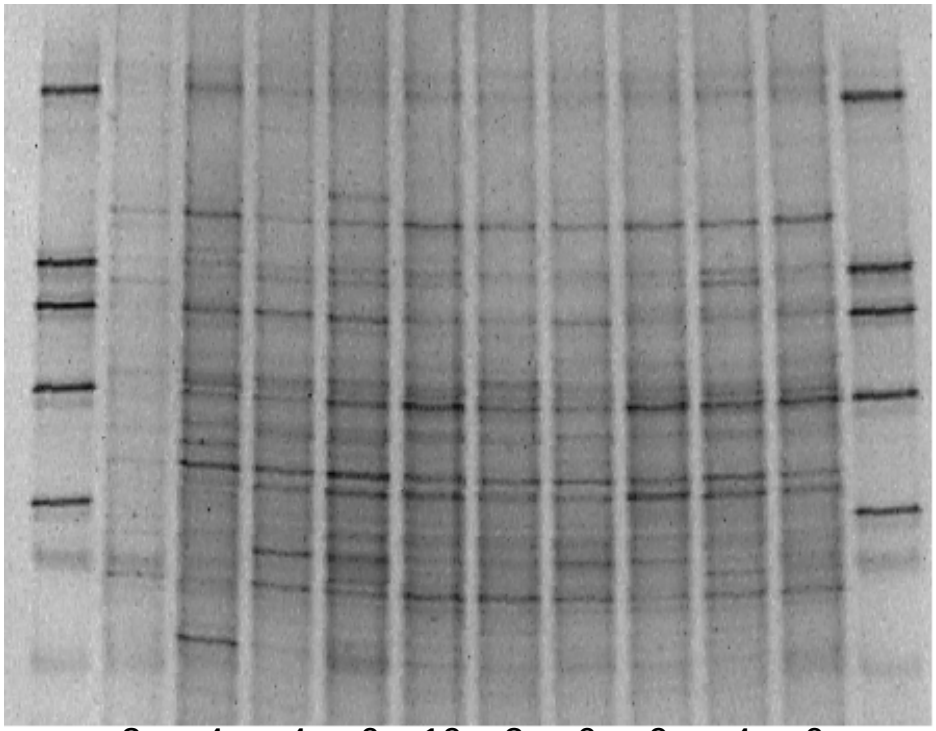


1999

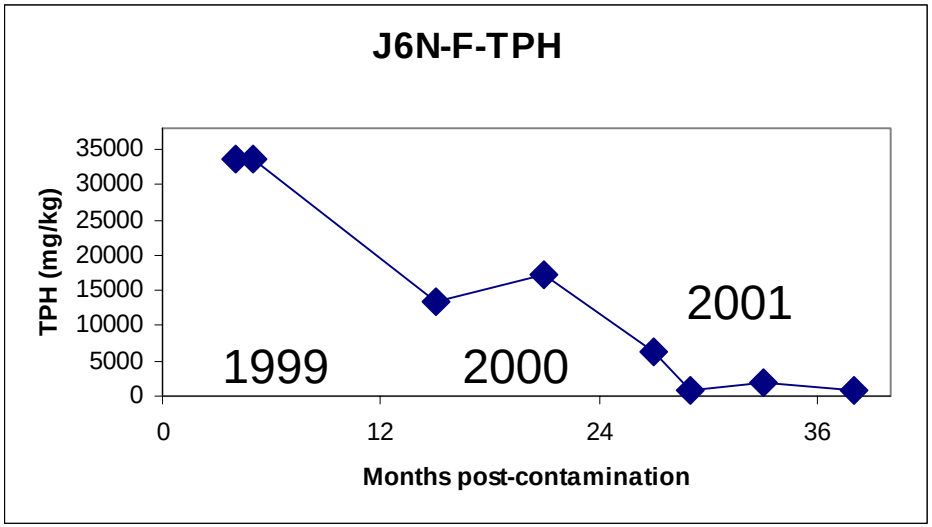
2003

4-year timeline

Uncontaminated sites:
Little obvious change seasonally or yearly



3 4 4 6 10 3 6 9 4 6
’99 ’00 ’01 ’02 ‘03

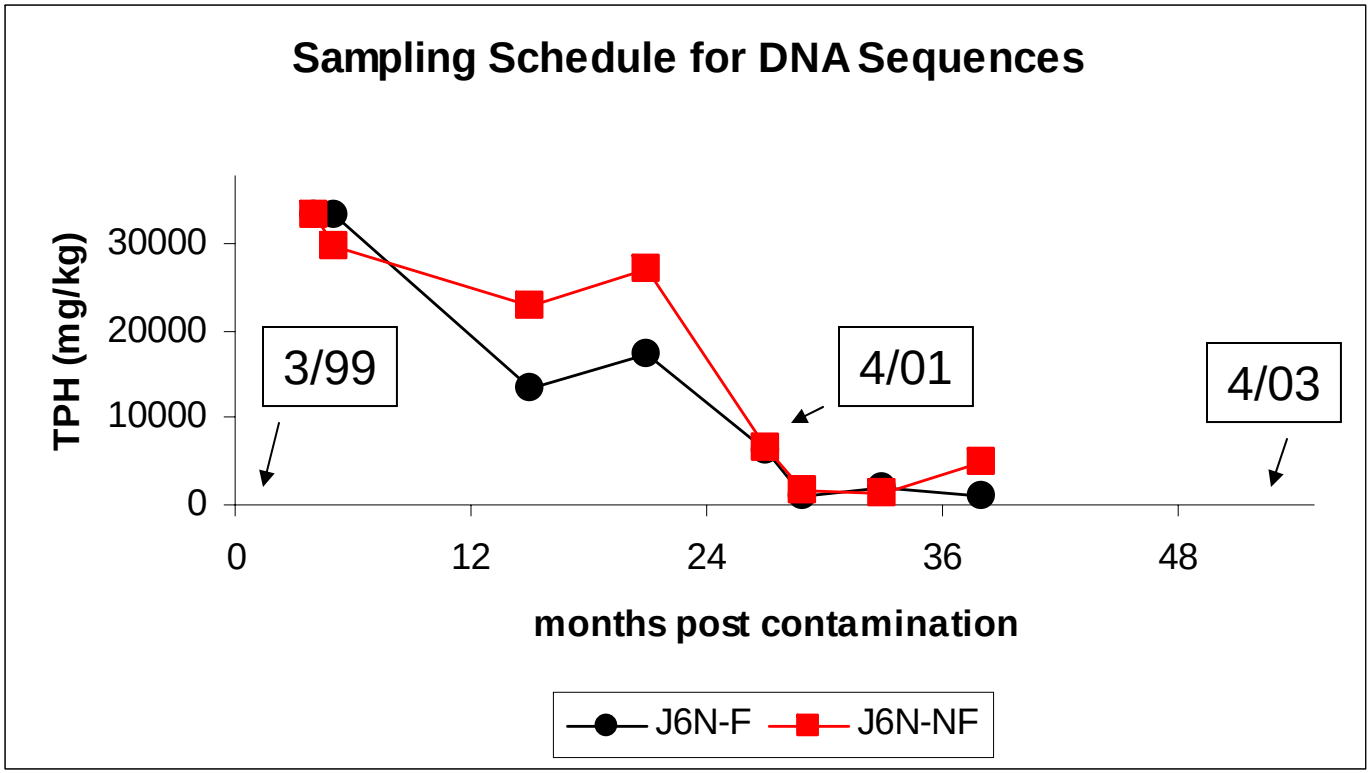


J6N-F

- Contaminated sites
  - Change over the years as remediation takes place
- Ability to detect changes constrained by limitations of PCR and electrophoresis
- Complex communities are difficult to analyze

DNA sequence analysis

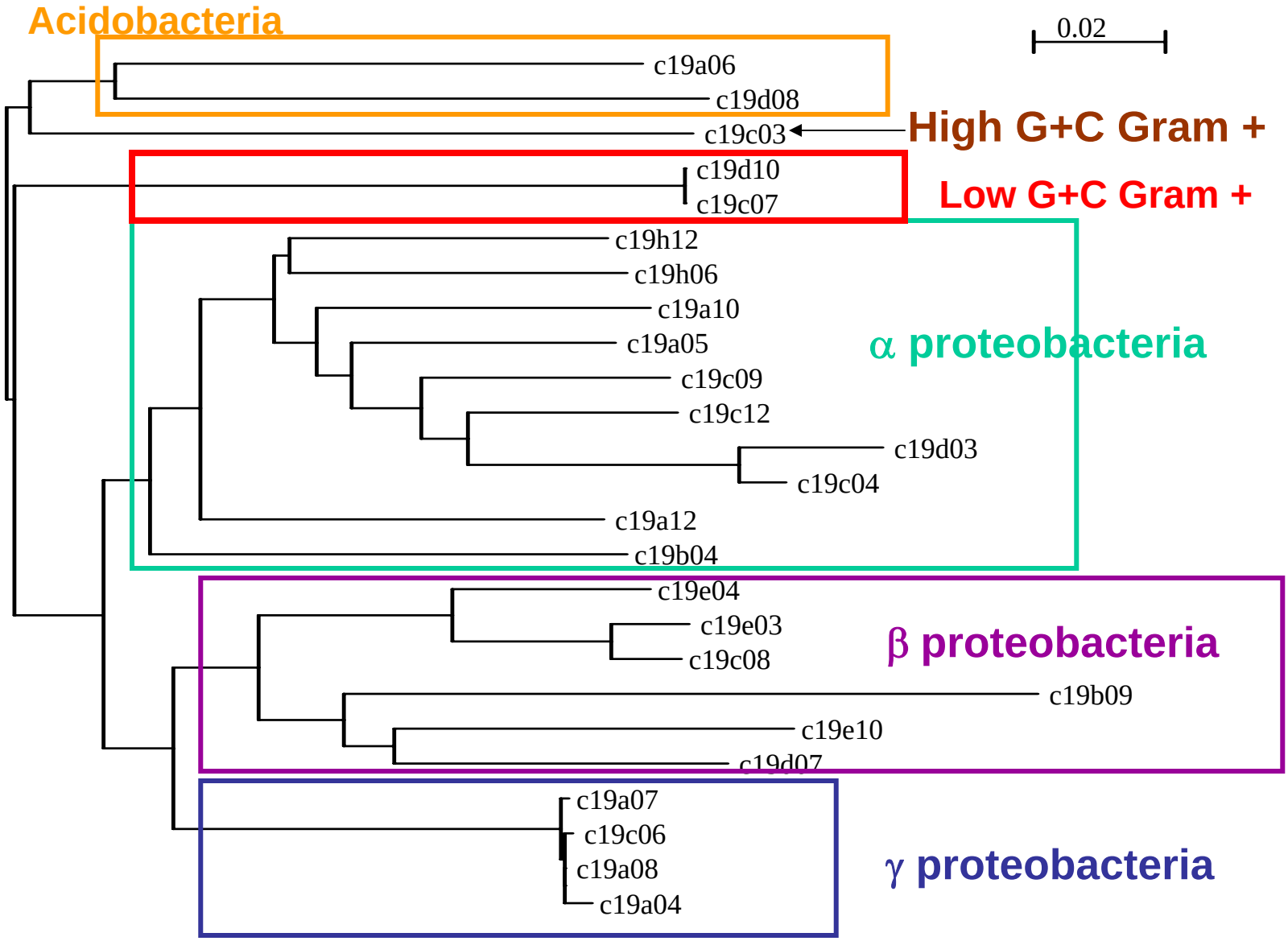
- DNA sequences very informative
  - *But* not necessarily who's most active
- Large 16S rRNA database
- Problems:
  - Determining which species are important
  - Difficulty in inferring functional roles from 16S sequences
  - Obtaining adequate sample sizes
- Our approach:
  - OU's Advanced Center for Genome Technology
  - Dominant community members: 16S rRNA
  - Targeted functional group
    - *amoA* for ammonium oxidizing bacteria
    - Ammonium oxidizing bacteria are important in nitrogen cycling



Schedule	Sites
3/99: Pre-treatment	J6N-NF, J6N-F (contaminated)
4/01: 2 yr treatment	J6C: tilled, not contaminated
4/03: 4 yr treatment	J6NP: uncontaminated prairie

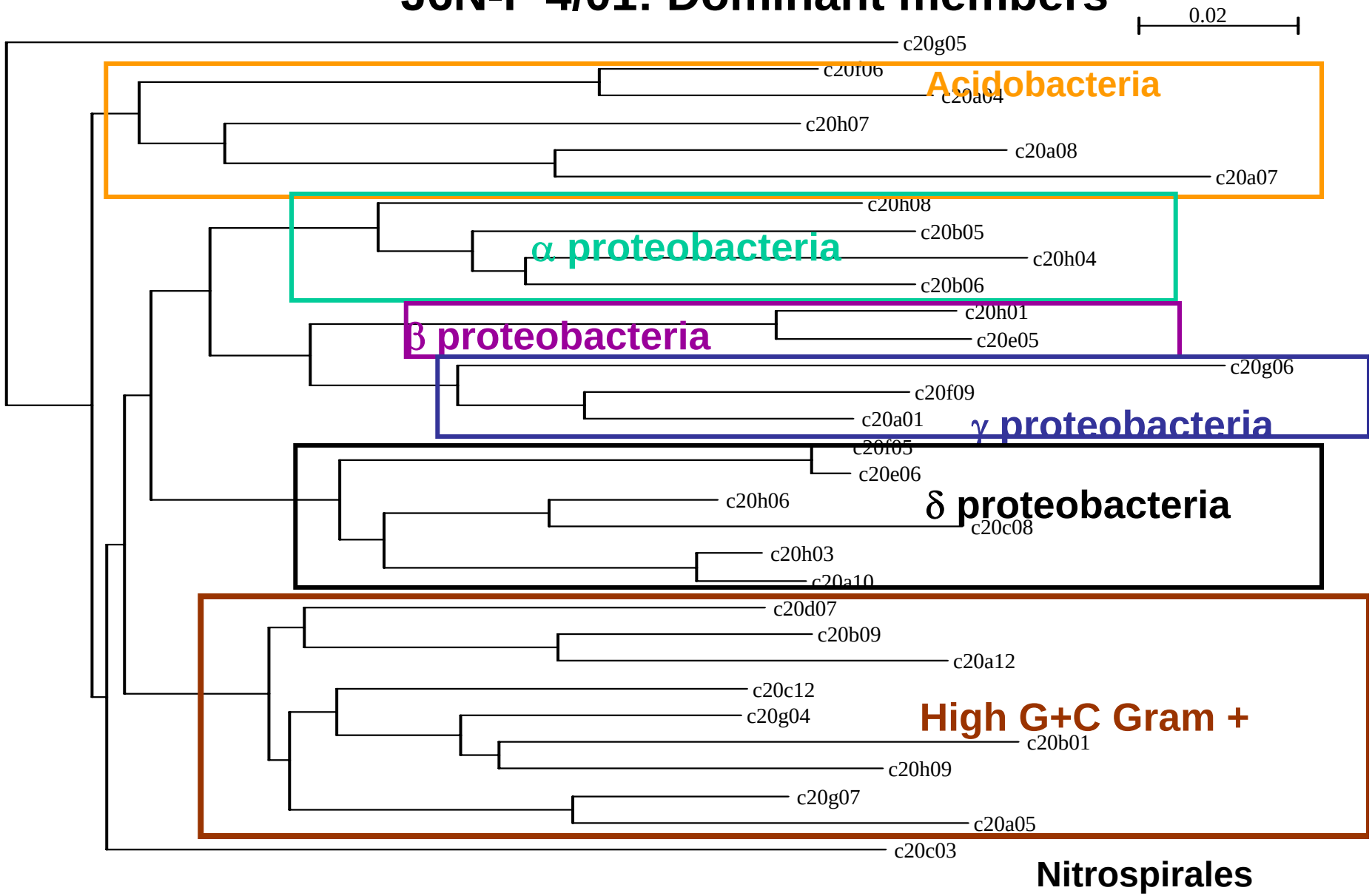
Preliminary data from 4/01: note current TPH levels the same for both contaminated sites

J6N-NF 4/01: Dominant members



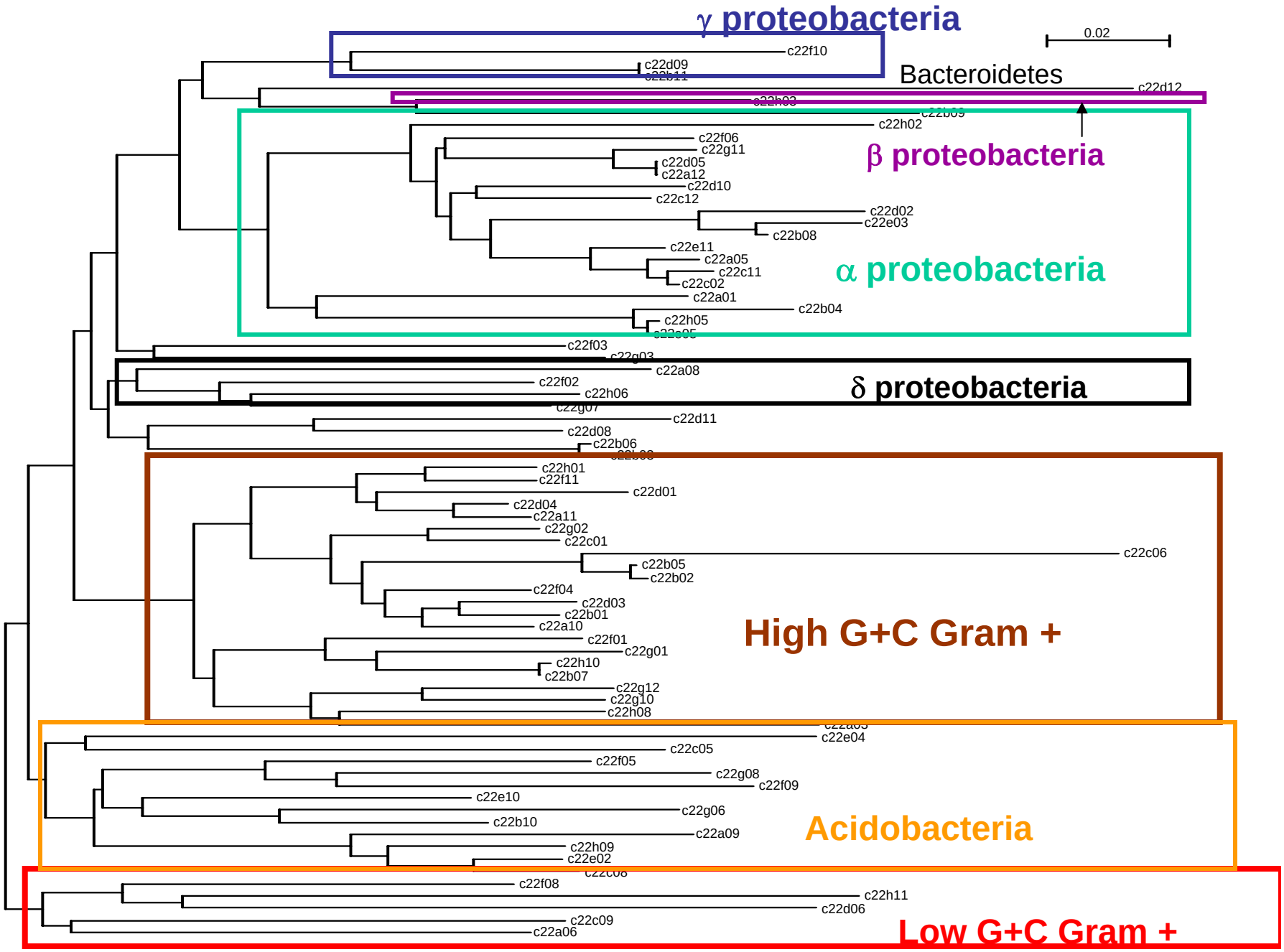
16S rRNA: 25 sequences

J6N-F 4/01: Dominant members

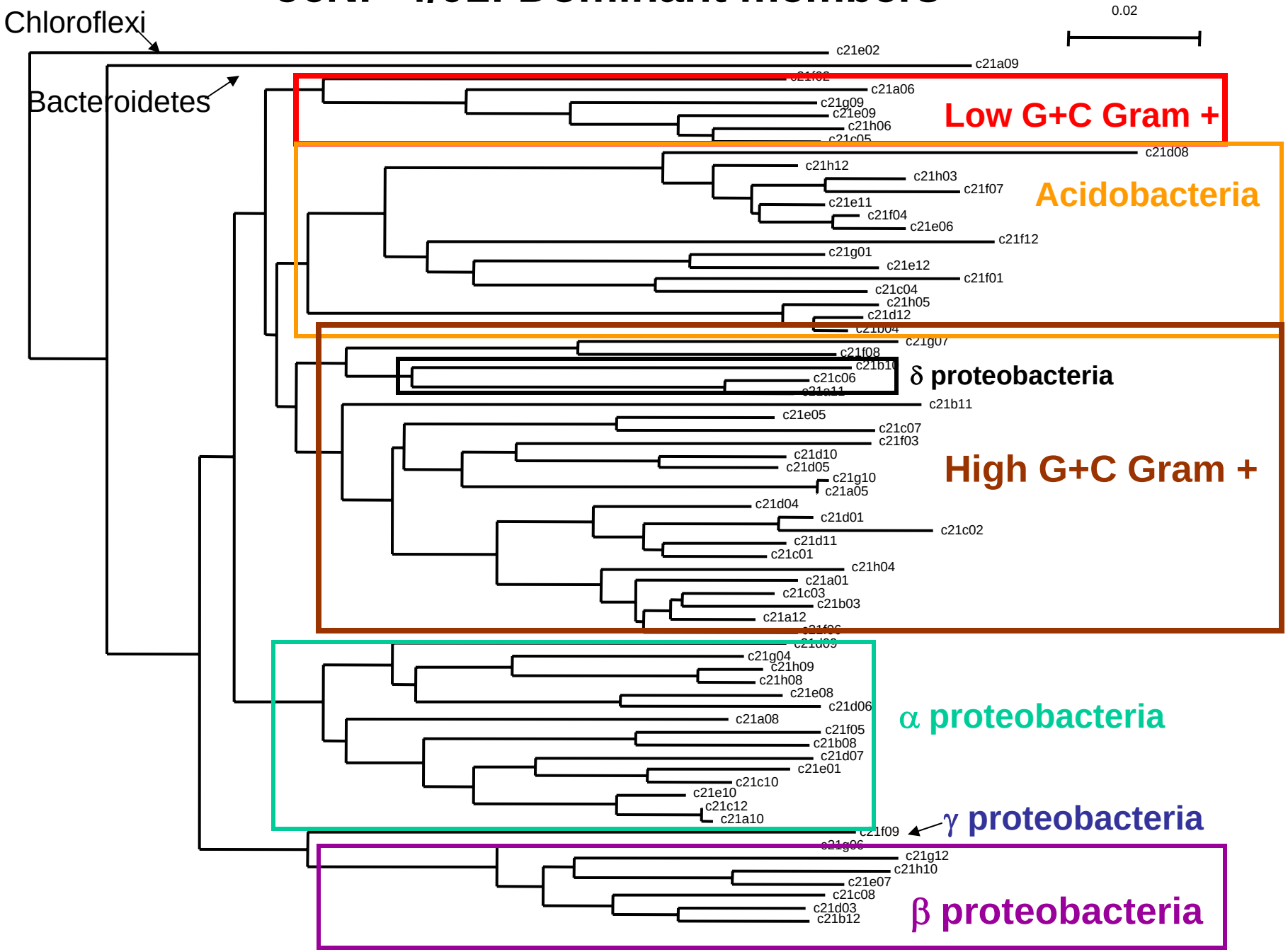


16S rRNA: 31 sequences

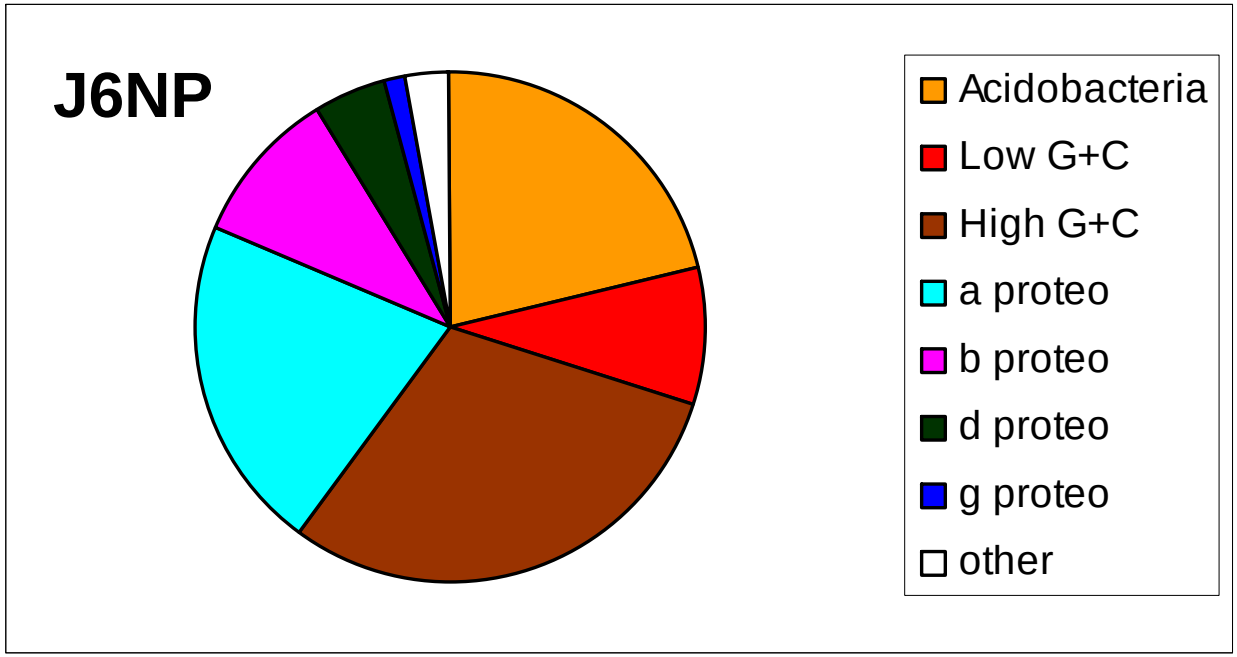
J6C 4/01: Dominant members

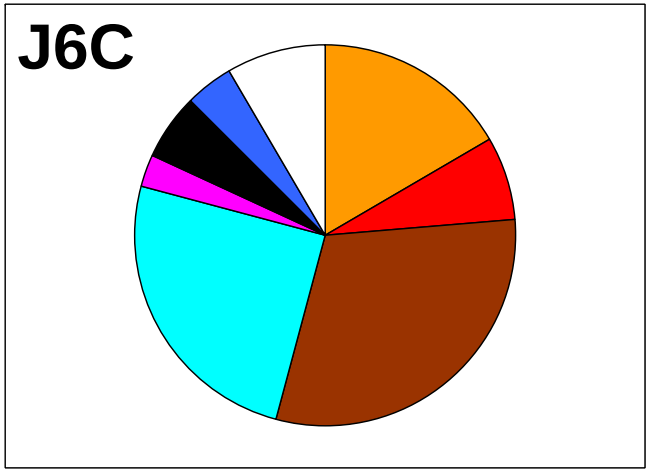


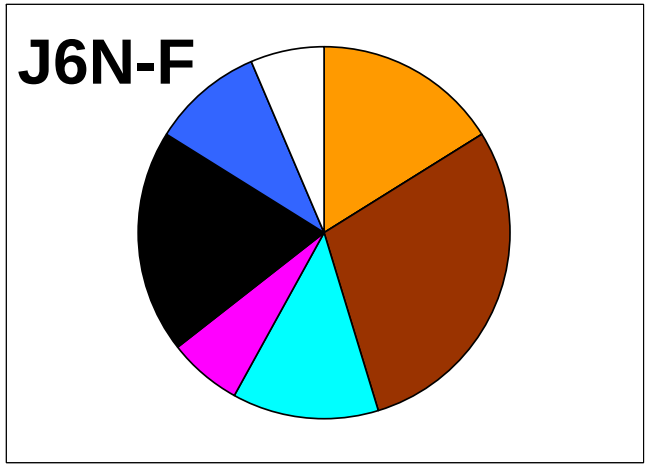
J6NP 4/01: Dominant members

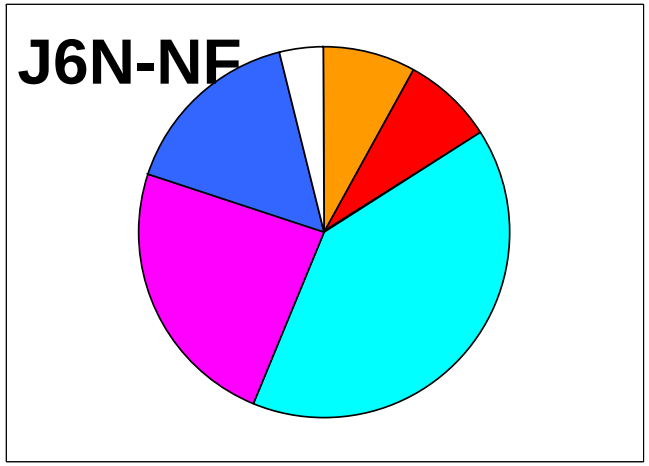


16S sequences for 4/01 samples









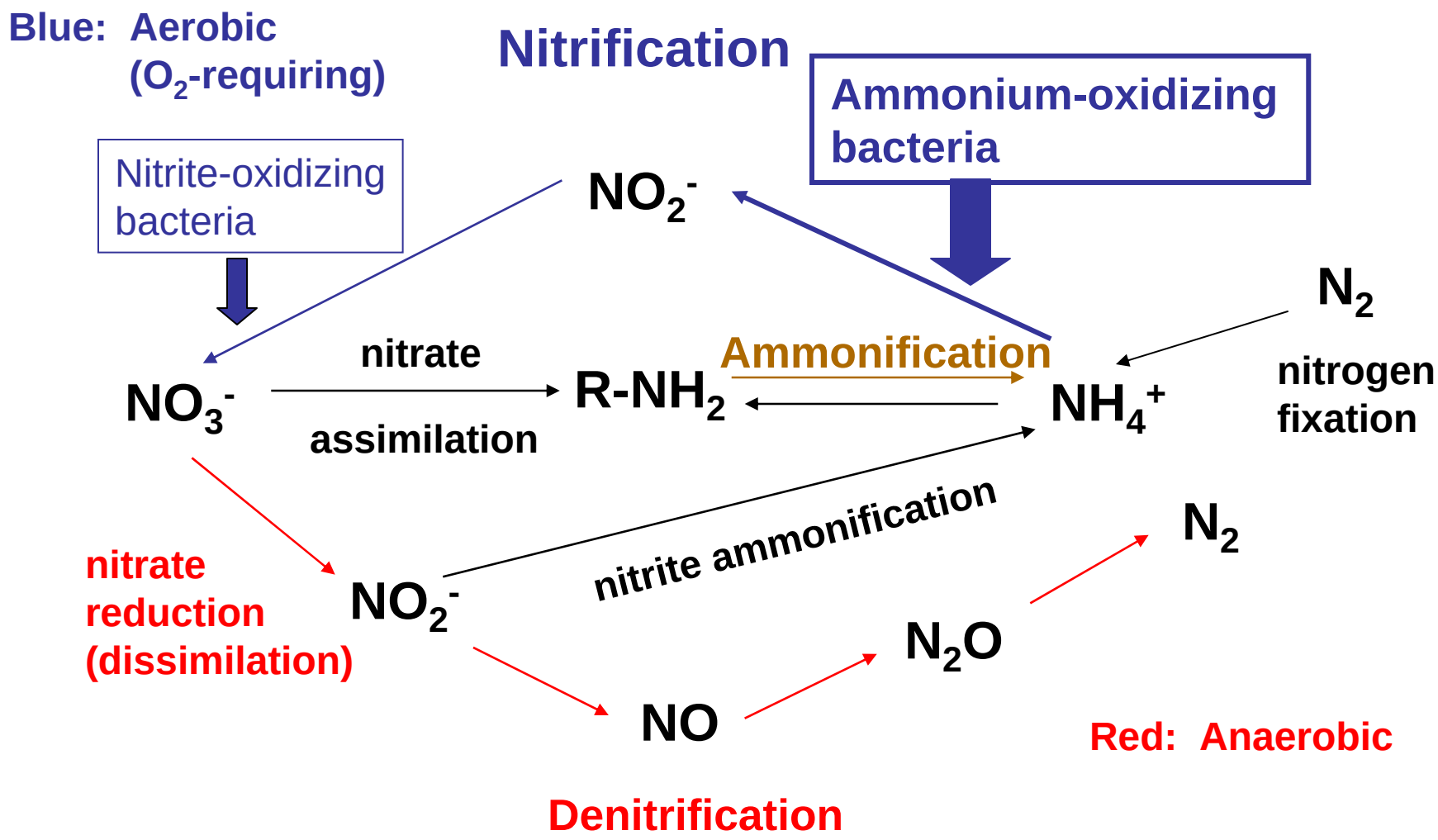
Uncontaminated sites:
High % Acidobacteria and Gram+ Firmicutes (*Bacillus, Clostridium*) and Actinobacteria (*Streptomyces*, actinomycetes)

Targeted Functional Group: Ammonium-0xidizing Bacteria

- Our previous research on the effects of oil plus N on tallgrass prairie soil microorganisms had shown:
- More aerobic heterotrophic, HC-degrading, denitrifying and methanogenic bacteria
- Increase in microbial activity
  - Lower O_2, higher CO_2 and CH_4
- Alteration of soil gas environment
  - More anaerobic sites, heterogeneous
- Alteration of soil pH
  - Affected # nitrifying bacteria and NOx emission
- Plus other effects on nitrogen cycling and availability

Duncan et al. 1999. *Appl. Biochem.& Biotech*. 77-79: 421-434.
Duncan et al. 1998. *Biorem. J*. 1(3):195-208

The Nitrogen Cycle : a link between soil microbes and plants

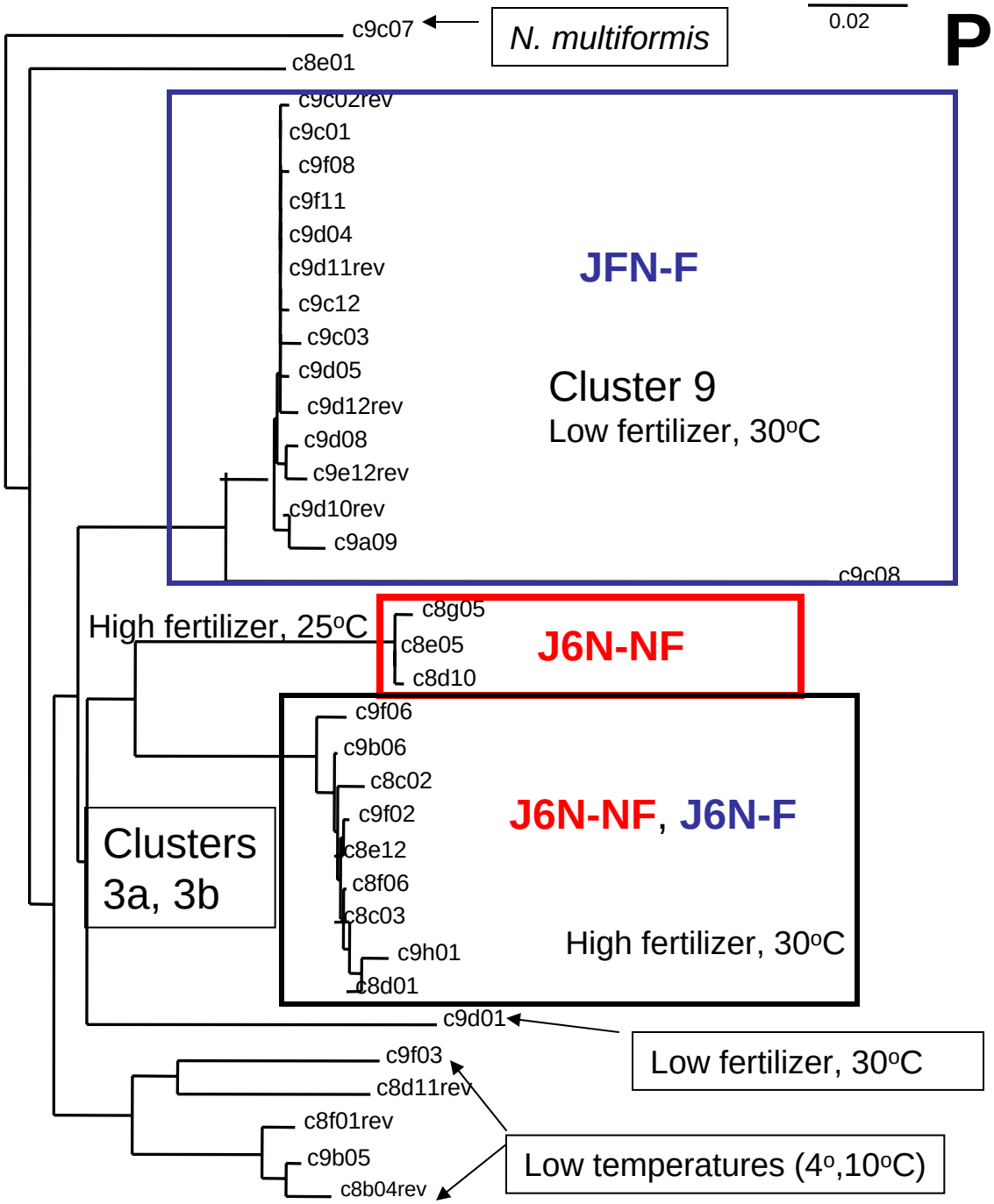


Bacteria perform key steps in nitrogen transformations

Nitrifying Bacteria

- Ammonium-oxidizing bacteria: oxidation of ammonium to nitrite
  - Key enzyme: ammonium monooxygenase (gene for A subunit: ***amoA***)
- Nitrite-oxidizing bacteria: oxidization of nitrite to nitrate
- Both are chemolithotrophs, difficult to culture and SLOW growing
- Standard culture methods underestimate numbers and diversity
  - Detection by molecular techniques gaining favor
- Current study: use PCR to amplify *amoA* from DNA extracted from soil, clone and sequence.

Phylogeny based on *amoA* sequences



DNA extracted from 4/01 J6N-NF, J6N-F

All sequences cluster with *Nitrosospira* spp.

Several different sequence clusters found (3a, 3b, 9)

Similar to sequences from experiments varying N-fertilizer levels and temperature (Avrahami and Conrad, 2003)

J6N-NF: 12, J6N-F: 23

Conclusions

- **Did treatment speed recovery? Yes**
  - J6N-F (fertilized) is more similar to uncontaminated than J6N-NF (not fertilized)
- **Is treatment cost-effective? Factors to consider:**
  - Ultimate use of the land, cost of treatment, effectiveness of treatment
- **Has the microbial community of the contaminated sites completely recovered?**
  - DGGE, PLFA: probably J6S-F and J6S-NF, J6N-F (almost), J6N-NF (not yet)
  - 16S and *amoA* sequencing: in progress
- **How long did it take for the microbial community of this temperate zone grasslands to recover from surface contamination by crude oil?**
  - 3 to 5+ years, depending on degree of contamination and treatment

Which ecological indicators are “best”?

- All show the same trends, but some (e.g. nematodes, plants) designate later stages of recovery
- However, microbial processing of chemical contaminants, restoration of soil structure, and nutrient cycling will be essential for recovery to proceed to the later stages
- Proposed signposts of ecological recovery for terrestrial ecosystems
  - 1. Restoration of microbial community diversity and characteristic functional groups, including N-cycling capacity
  - 2. Nematode #s and diversity
  - 3. Plant biomass and species diversity, if restoration of pre-contamination plant communities is a goal.

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