Proposed Changes in the Protocol for Listing Bioremediation Agents on the NCP Product Schedule

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Oil Spill Bioremediation Product Testing

- History
- Description of Existing Protocol
- Proposed modifications
  (NO DECISIONS HAVE BEEN MADE YET)
  - Supporting data
  - Proposed decision rule
- Conclusions

Historical Perspectives

- In 1990, after Exxon Valdez spill, ORD asked NRMRL to develop objective protocol for testing efficacy of bioremediation products
- In 1991, protocol was developed and peer reviewed by a panel of over 20 experts
- In 1992, protocol was used twice for validation on 20 different products
  - Adopted and published in Federal Register as official testing protocol

Description of Existing Protocol: Generalized Procedure

- 27 identical shake flasks: 9 no-nutrient controls, 9 nutrient controls, and 9 test flasks
  - Triplicate sacrificial flasks for each of 3 sampling events (days 0, 7, and 28)
  - Contents extracted with DCM, analyzed by GC/MS
- Natural seawater used as the test water
- Alaska north slope crude oil weathered at 521 ºF (272 ºC), called ANS 521
- Product added according to recommendation of vendor
- Flasks shaken at room temperature for 28 days

Description of Existing Protocol: Chemical Analysis

- Analytes quantified by GC/MS at each sampling event include:
  - Normal and branched alkanes, n-C_{14} to n-C_{35} plus pristane and phytane
  - Aromatics including 2-, 3-, and 4-fused ring polyaromatics and alkyl-substituted homologs
    - Naphthalenes
    - Phenanthrenes
    - Fluorenes
    - Dibenzothiophenes
    - Napthobenzothiophenes
    - Pyrenes
    - Chrysenes
  - Gravimetric weight loss of oil done prior to GC/MS

Description of Existing Protocol: Microbiological Analysis

- MPN analysis of samples from each sacrificial flask is done at each sampling event
  - The purpose is to confirm that microbial growth took place
  - Procedure includes quantifying alkane and aromatic degraders separately
- Data are not used in deciding pass/fail

Description of Existing Protocol: Statistical Analysis

- The GC/MS data from each sampling event are used in the analysis
  - Analyte concentrations are summed up giving:
    - Total alkanes
    - Total aromatics
- To pass the test, a product must demonstrate a statistically significant difference between the test flasks and the control flasks at Day 28
  - Total alkanes must be lower than the control ($p < 0.05$)
  - Total aromatics must also be lower than the control ($p < 0.05$)
  - Both fractions must be lower, not just one
  - ANOVA used for the comparative analysis

Problems with Existing Protocol

- Reproducibility is inadequate because of the use of natural seawater, which may have different levels of hydrocarbon degraders
- Testing is expensive and many factors measured are not used for pass/fail decisions:
  - GC/MS is the primary tool used in decision-making
  - MPN analysis not needed for pass/fail
  - The Day-7 event not needed for pass/fail
  - The gravimetric oil analysis not needed for pass/fail
  - The nutrient control not used in decision-making

Proposed Modifications

Two different sterile artificial water types are being substituted for natural seawater

- Artificial seawater
- Artificial freshwater

The 7th-day sampling event has been eliminated

The nutrient control has been eliminated

The gravimetric oil measurement has been eliminated

The MPN analysis has been eliminated

A standard inoculum will be provided by EPA for use in the test

The statistical analysis has been greatly simplified

A new decision rule has been proposed for pass/fail rather than relying on a statistical significance test

Proposed Modifications: Exposure Medium

- **Artificial seawater**
  - **The natural seawater would be replaced with a standardized synthetic seawater recipe called GP2**
    - **Ingredients and their concentrations are fully described and easily made**
- **Artificial freshwater**
  - **A synthetic minimal salts freshwater would be used with known ingredients (based on Bushnell-Haas medium)**

Proposed Modifications: Exposure Medium

- With the addition of a freshwater test, a product vendor may decide to test his product only on saltwater, only on freshwater, or both
  - A vendor need only test his product in the appropriate exposure medium if he wants his product approved for use in just that environment
  - If the vendor markets his product for both environments, then he must proceed with testing in both media

Proposed Modifications: Standard Inoculum

- **Purpose: to be used as a positive control to qualify the performing lab, not to compare a product against the EPA inoculum**
  - **The inoculum has a known ability to degrade ANS 521 oil to certain levels**
    - **If the performing lab is unable to demonstrate this ability, it has to repeat the test until it can do so**
  - **For a non-living product (fertilizer, etc.), inoculum is used to test product's ability to stimulate the culture to degrade crude oil to specified levels**

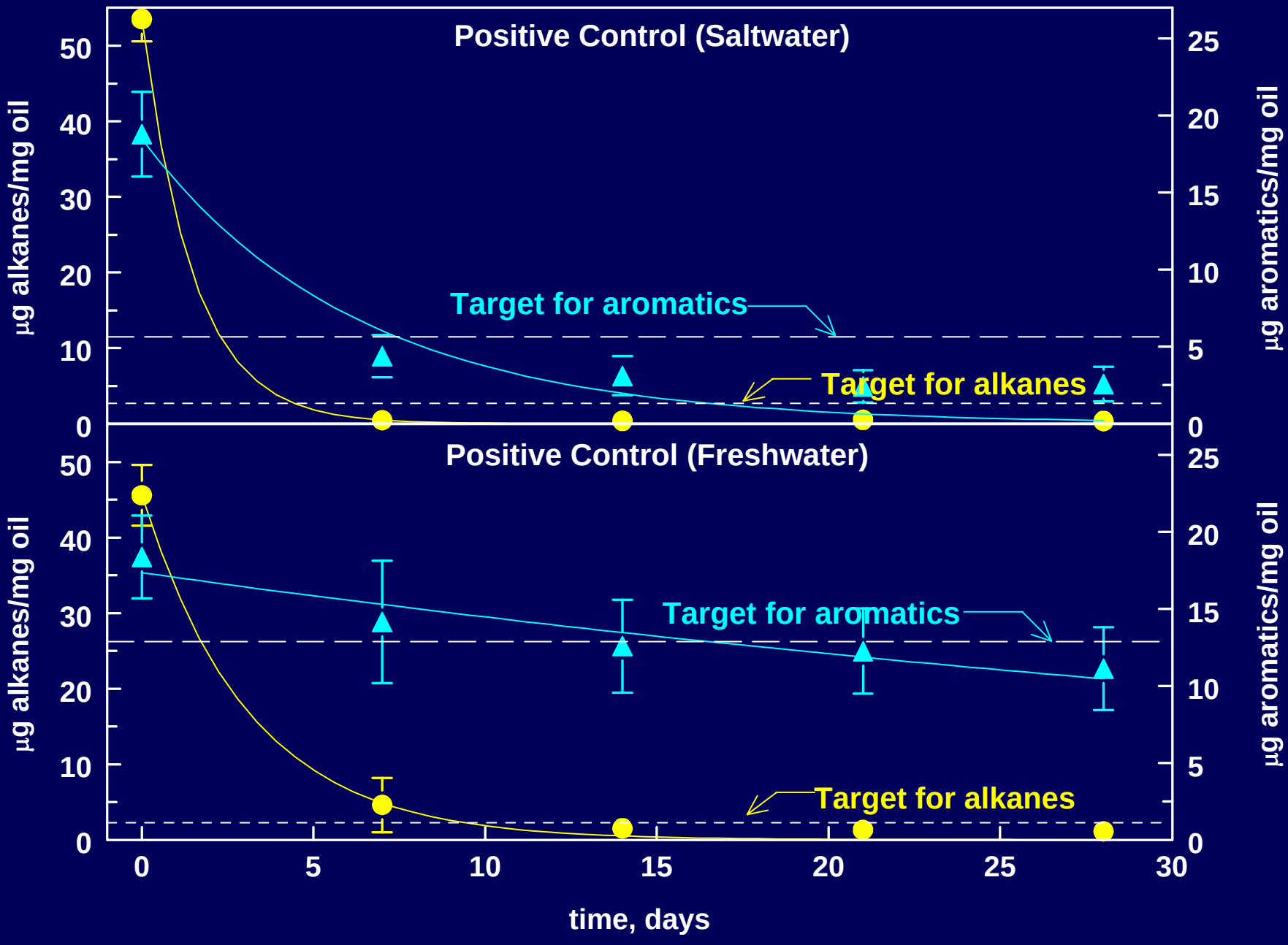
Proposed Modifications: Standard Inoculum

- EPA's standard inoculum is a culture of oil-degrading bacteria isolated from Disk Island in Prince William Sound in 1990
  - It is an excellent degrader of alkanes and aromatics in saltwater and freshwater
  - It is better in saltwater, especially with aromatics

Proposed Modifications: Performance of Standard Inoculum

- In seawater, the standard inoculum is able to degrade alkanes 98.9% and aromatics 79.8% by day 28
  - Reasonable target biodegradation for lab qualification would be 95% and 70%, respectively
- In freshwater, the standard inoculum is able to degrade alkanes by 97.9% and aromatics by 37.8% in 28 days
  - Reasonable target biodegradation for lab qualification would be 95% and 30%, respectively

Required Performance of Standard Inoculum



Proposed Decision Rule for the Product

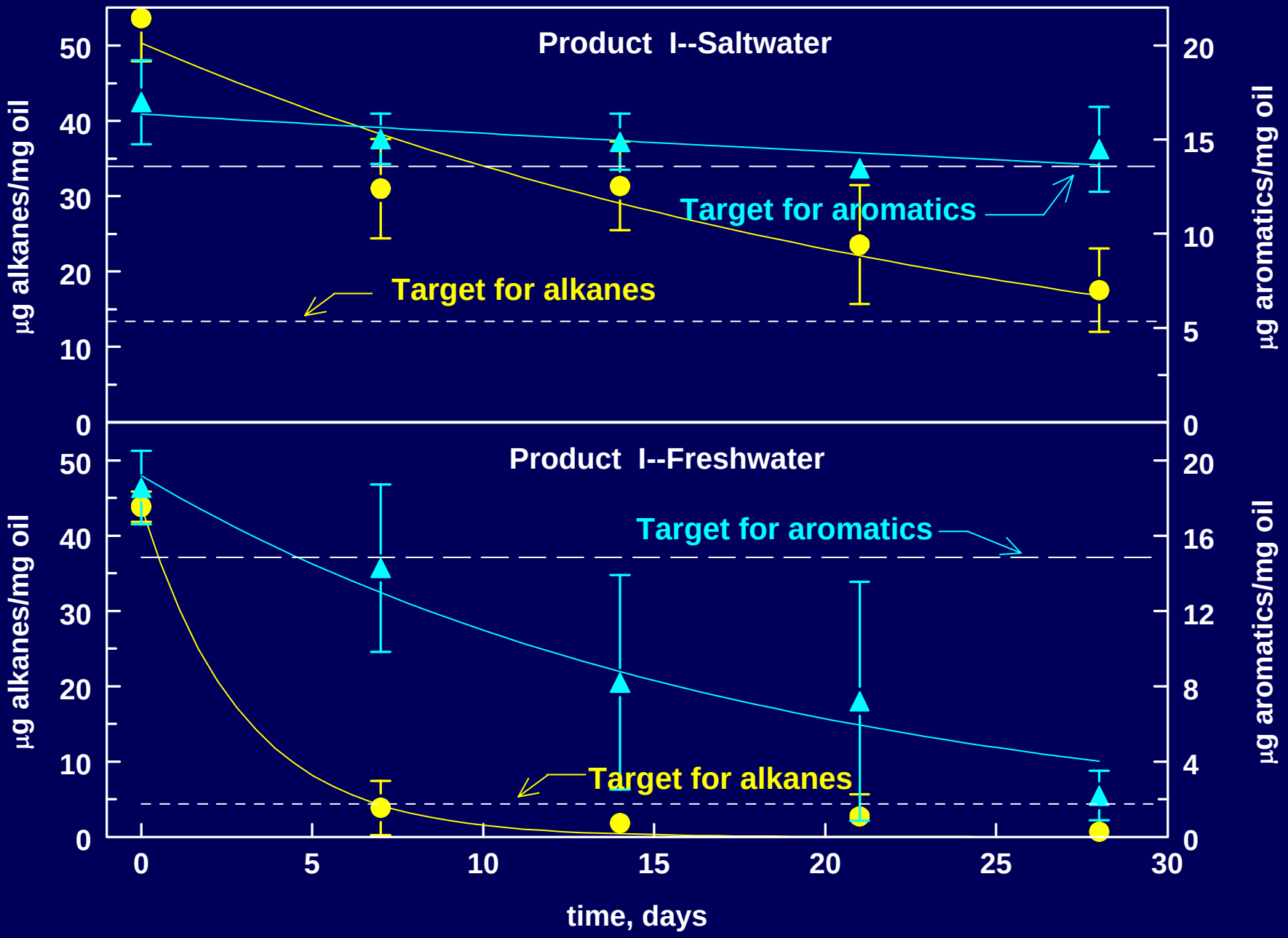
- Product is considered a success if it is able to:
  - Reduce total alkanes by >90% at day 28
  - Reduce the total aromatics by > 20% at day 28, both waters
- Targets based on UCL_{90}, not the mean
- Calculation of the UCL_{90}:

$$UCL_{90} = avg_{28} + \left[(t_{90,df} \times \sigma) \div \sqrt{n}\right]$$

$$\%Reduction = 100 \times \left[1 - (UCL_{90} \div avg_0)\right]$$

where $t_{90,df}$ = 1.885 (from statistical t-tables, 2 degrees of freedom)
n = no. of replicates (3)
σ = standard deviation of the 3 samples

Example of a product that meets one but not the other



Similarity with Canadian Rule

- Environment Canada requires products to meet a mean 80% reduction of total resolvable alkanes and 50% reduction in selected aromatics (which exclude the 4-ring species) in seawater
  - Our requirements (>90% reduction for alkanes and >20% for aromatics) are similar for saltwater: ours are based on the UCL_{90} vs. Canada's mean

Other Proposed Changes

- All products to be treated as standalone items
  - No nutrients will be added by the performing lab; the vendor must supply its own as it would in the field
- Nutrients supplied by a vendor are allowed to be higher than used in the field
  - In closed flasks, nutrient limitations become significant in a short time period

Conclusions

- The new protocol modifications should go a long way to simplifying and streamlining the existing protocol
  - Many unnecessary steps have been eliminated
    - The microbiological analysis
    - The intermediate sampling period
    - The gravimetric oil weight measurement
    - The nutrient control
  - Some steps have been added
    - A positive control to qualify the performing laboratory
    - A standard inoculum to test non-living products
    - A freshwater test in addition to a saltwater test
    - A new decision rule based on performance

Release of Subpart J Rule-Making

- The new bioremediation agent protocol as well as the new dispersant protocol will be published in the Federal Register some time this year
  - Public comment will be sought
  - All products currently listed on the NCP Product Schedule will likely have to re-test