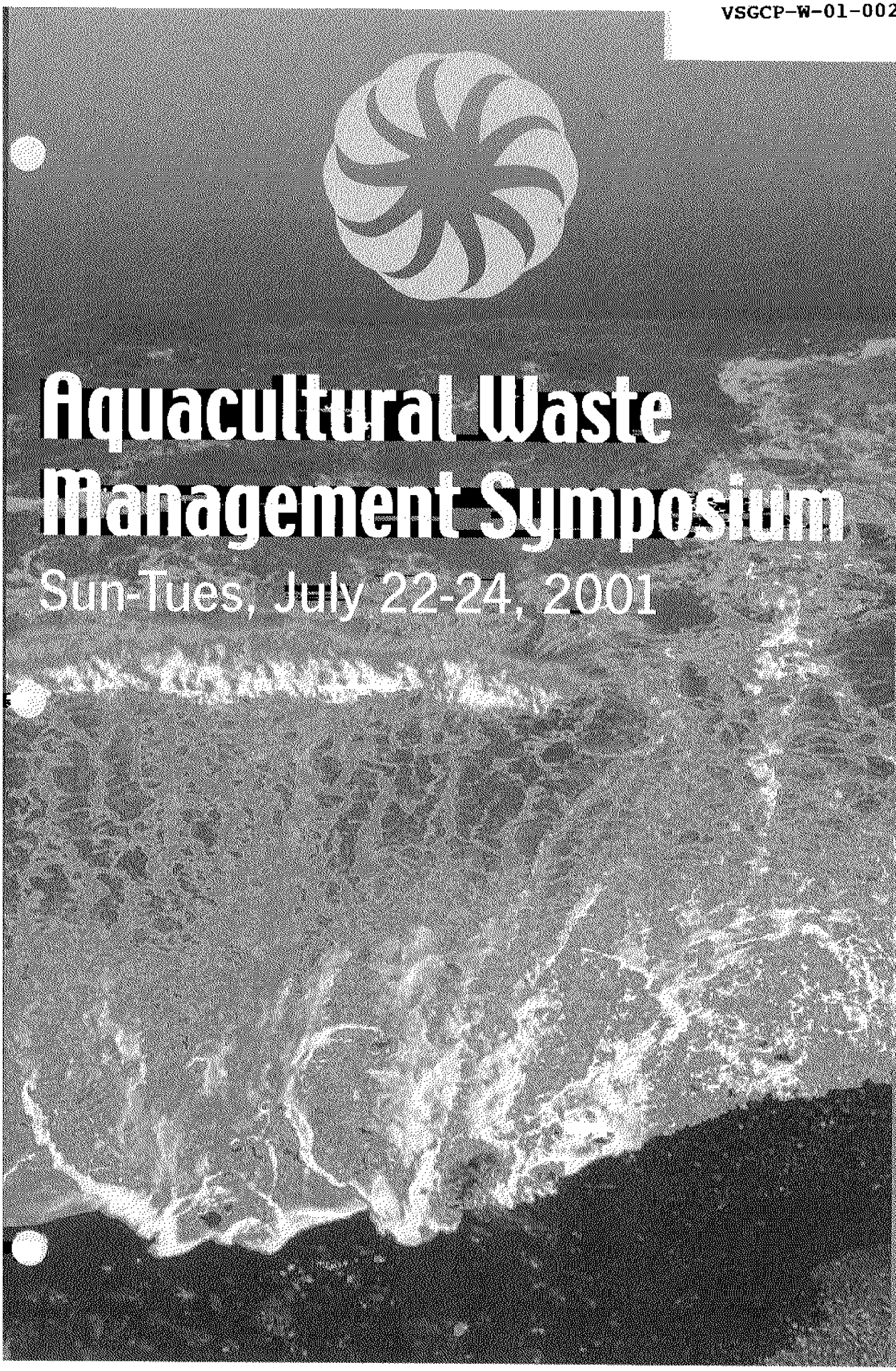

Aquacultural Waste Management Symposium

Sun-Tues, July 22-24, 2001

COTA

NOAA

Sea Grant

Virginia

REACT

Virginia Tech

VIRGINIA POLYTECHNIC INSTITUTE AND STATE UNIVERSITY

Aquaculture Waste Management Symposium

PROCEEDINGS

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Aquaculture Waste Management Symposium Agenda

July 22-24, 2001

Sunday, July 22	
1-5 pm	Tour of Virginia Tech facilities - Lou Helfrich, Virginia Tech
6:30-9 pm	Introduction to aquaculture - Mike Schwarz, Virginia Tech
Monday, July 23	
8:30-8:45 am	Introduction and welcome - Greg Boardman and George Flick, Virginia Tech
8:45-9:15am	JSA aquaculture effluent committee efforts - Max Mayeaux, USDA
9:15-10 am	Update on EPA regulatory process for aquaculture industry - Kristen Strellec, EPA
10-10:30 am	Refreshment break
10:30-noon	Industry perspectives concerning waste management - Jane Walker, Virginia Tech; Kieth Gregg, Harlingen Shrimp Farms; Bill Martin, Blue Ridge
Noon-1:30pm	Lunch provided
1:30-3 pm	Best waste management practices for the shrimp and catfish industries - Claude Boyd, Auburn U.
3-3:30 pm	Refreshment break
3:30-5 pm	Best waste management practices for the trout industry - Harry Westers, Aquaculture Bioengineering
Tuesday, July 24	
8:30-10 am	Best waste management practices for the alligator, crawfish and turtle industries - Greg Lutz, Louisiana State U.
10-10:30 am	Refreshment break
10:30-noon	Best waste management practices for recirculating systems - Steve Summerfelt, Freshwater Institute
Noon-1:30pm	Lunch provided
1:30-2:15 pm	Recent developments in nitrification/denitrification - Tim Hovanec, Aquaria, Inc.
2:15-3 pm	Management of aquacultural waste using aerobic stabilization, reed beds and hydroponic treatments - Steve Van Gorder, Aquamarine Fish Farms, Inc.
3-3:30 pm	Refreshment break
3:30-4:15 pm	Composting fundamentals - Lewis Carr, U. of Maryland
4:15-5 pm	Experiences and performance of actual composting operations - Lewis Carr, U. of Maryland

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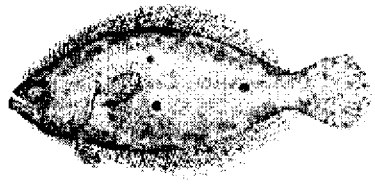
Virginia Tech

Introduction to Aquaculture



Michael H. Schwarz

Aquaculture Specialist

Virginia Seafood Agricultural Research and Extension Center

Introduction to Aquaculture

Michael H. Schwarz
Aquaculture Specialist

Virginia Seafood Agricultural Research and Extension Center
102 S. King Street, Hampton, VA, 23669

Hydroponics- The growing of [illegible] nutrient solutions with or without a medium to [illegible]

Aquaculture- [illegible] cultivation [illegible] and plants

Mariculture- Aquaculture in the marine environment

Polyculture- The growing of more than one crop in a production system

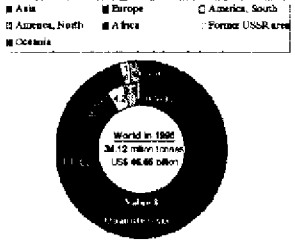
Aquaponics- the cultivation of both plants and animals for the purpose of harvest.

History

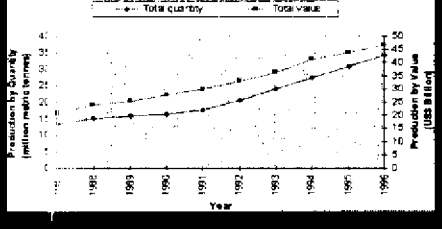
Aquaculture Industry

- Provides over 26% of total world fisheries production
- > 25% of food fish for human consumption derived from aquaculture
- Mariculture fastest growing sector of aquaculture.
- Aquaculture fastest growing sector of agriculture

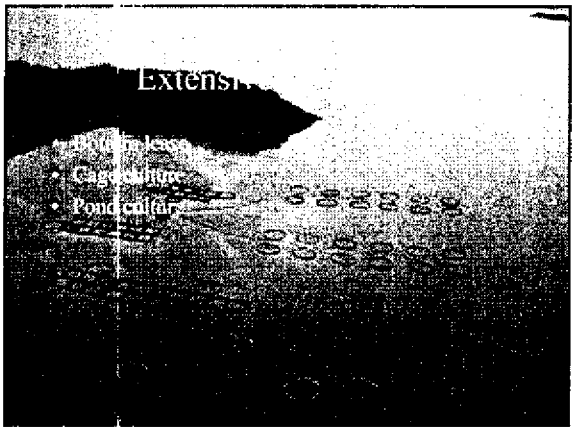
Production

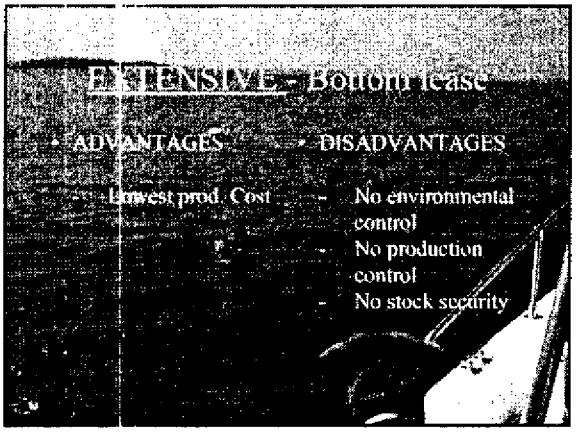


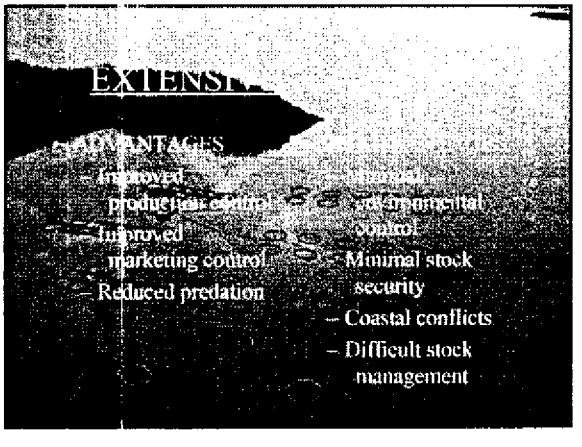
Historic Production



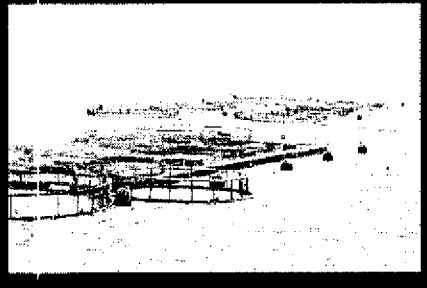
FAO, 1998: 39.4 billion tons valued at USD 52.5 billion


Extensi
• Cage culture
• Pond culture

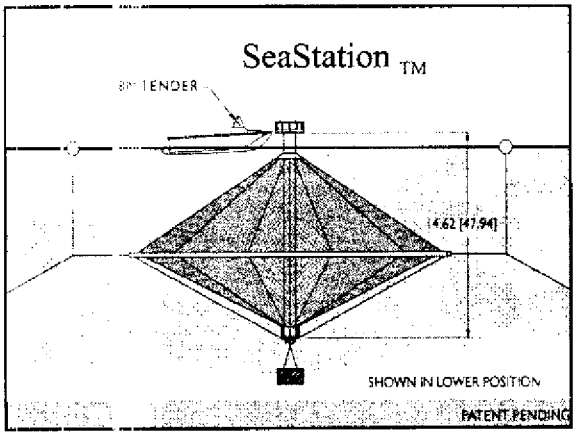

EXTENSIVE – Bottom lease
• ADVANTAGES
– Lowest prod. Cost
• DISADVANTAGES
– No environmental control
– No production control
– No stock security


EXTENSI
ADVANTAGES
– Improved
– Improved
marketing control
– Reduced predation
– Minimal stock security
– Coastal conflicts
– Difficult stock management

Coastal Net Pens



SeaStation TM



SeaStation$_{(TM)}$ (with flounder)

Net Pen Production Rates

- Carrying capacity:

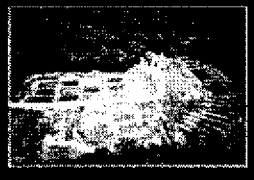
  10 - 20 pounds fish per Ft^3

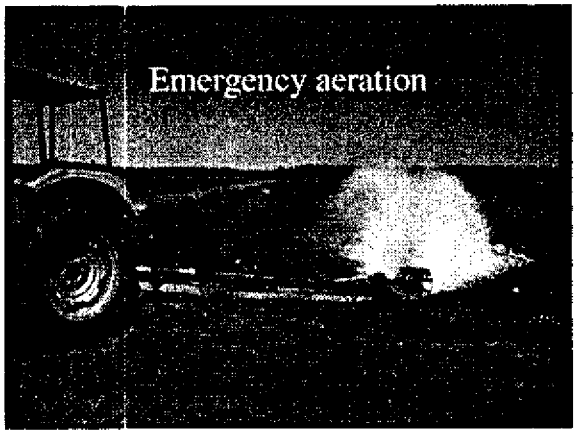
Pond production

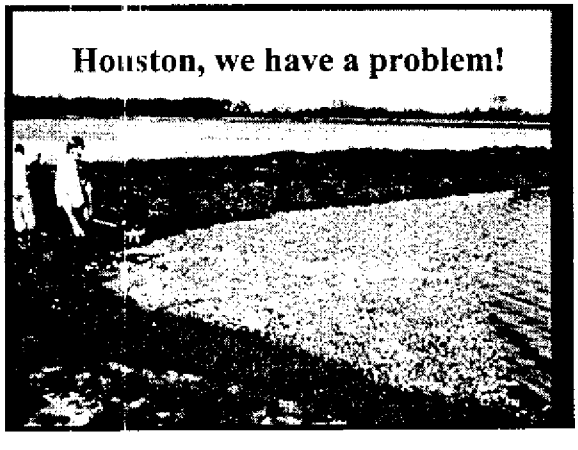
- Moderate production costs
- Moderate production control
- Moderate marketingcontrol
- Minimal environmental control
- Predation
- Point source discharge

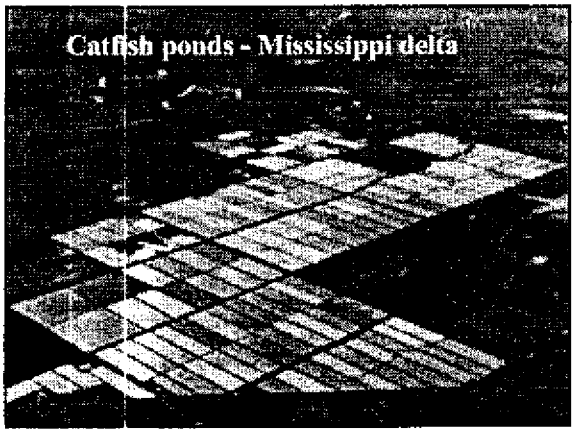
Ponds

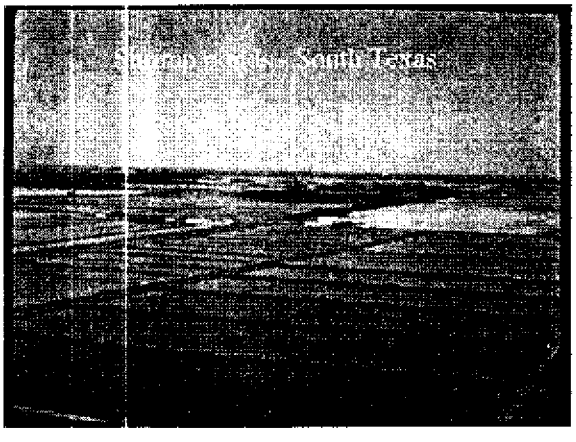
- Ponds are the most common fish production method
- Common food species
  - catfish
  - hybrid striped bass
  - yellow perch
  - tilapia



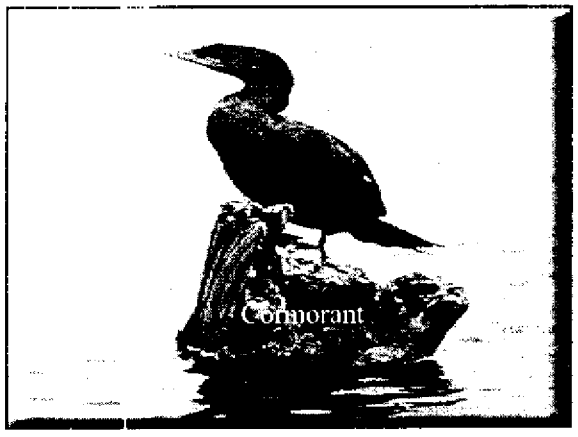




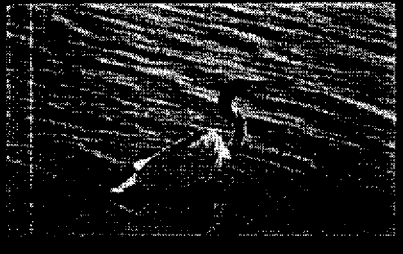

Catfish ponds - Mississippi delta


Shrimp boats - South Texas


Pelican



Great Egret

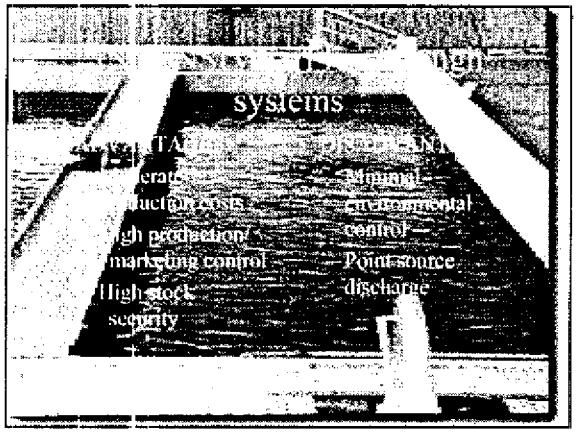


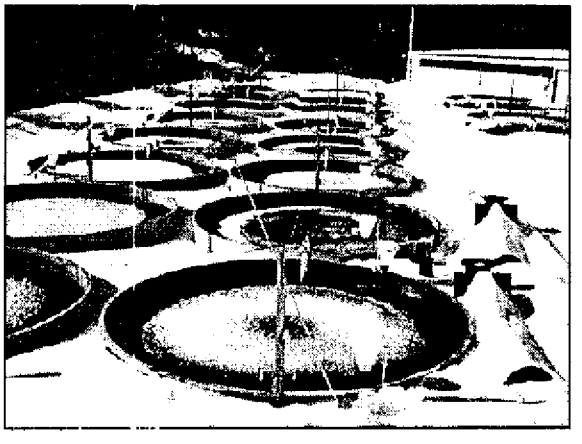
Pond Production Rates

- Carrying capacity:

  1,000 to 10,000 pounds per surface acre


Flow through systems
Recirculating systems


systems
Minimal environmental control
Point source discharge



Flow-through system production rates

- Carrying capacity:

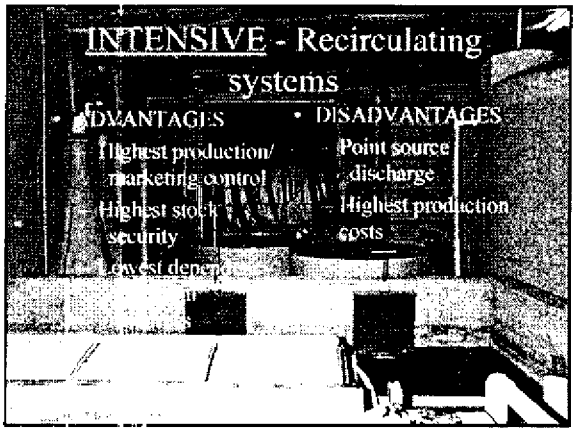
  20,000 to 45,000 pounds /ft^{3}water/sec, (449gpm)

What are RAS?

Systems utilized for the production of aquatic species which recycle in excess of 90% of the system water on a daily basis.

Recirculating Systems

- Site components
  - pump house
  - emergency generator
  - 3 phase electricity
  - bulk feed storage
  - oxygen supply
  - building
- System components
  - oxygen
  - biological filter
  - buffering system
  - heaters/chillers
  - solids filter
  - lighting
  - tanks

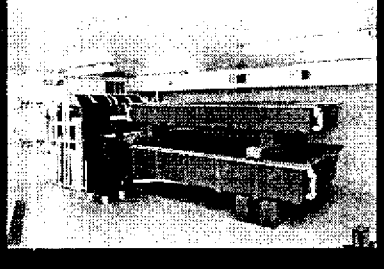


Intensive Recirculating System Production Rates

- Carrying capacity:

  1/4 to 1 pound/gallon of water.

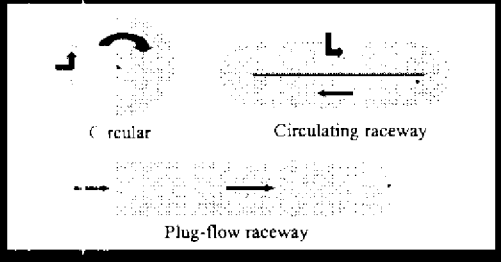
Airdriven System VSAREC



What are the basic components of RAS?

- Culture vessel
- Solids removal
- Biofiltration
- Re-aeration/degassing
- Water movement
- Water sterilization/oxidation

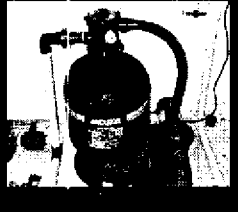
Culture vessels



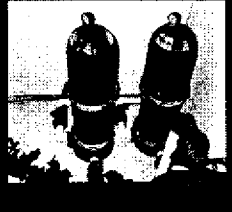
Selection based on species/self cleaning characteristics

Solids removal -

Sand filter

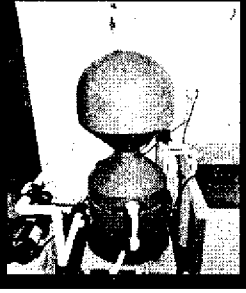


Cartridge filter

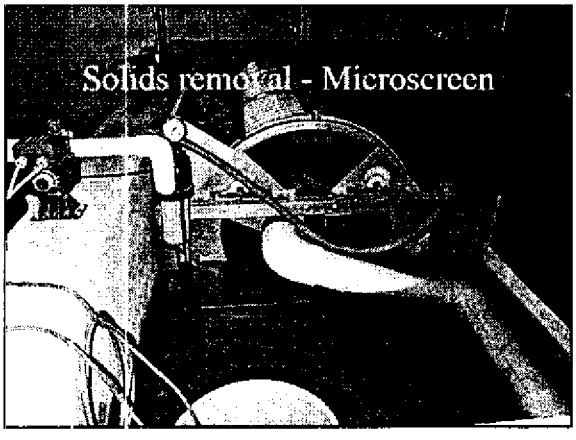


Solids removal - Swirl separation

Solids removal - Bead filter



Solids removal - Microscreen

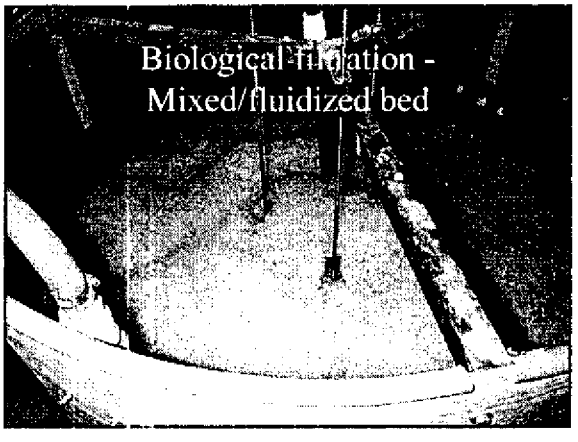


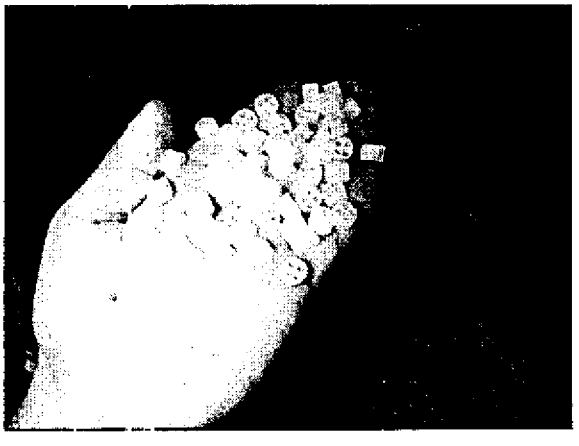
Biological filtration

- Function
  - Nitrification
  - Converts ammonia excreted from fish into nitrite, and nitrite into nitrate

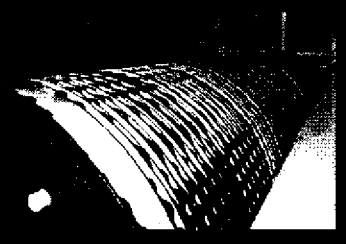
$NH_3 \longrightarrow NO_2^- \longrightarrow NO_3^-$

Biological filtration - Mixed/fluidized bed

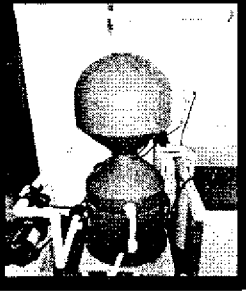




Biological filtration - Rotating biological contactor



Biological filtration - Bead filter



Reaeration/degassing in RAS

O_2 consumption

- Fish respiration
- Nitrification
- Biological oxygen demand
- Chemical oxygen demand

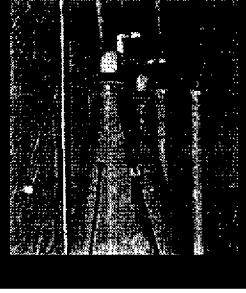
CO_2 production

Oxygen replenishment

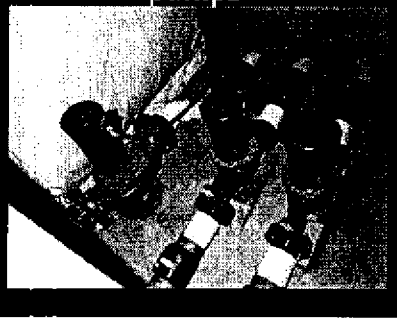
- Sources
  - Aerators
    - agitators (paddlewheels, fountains, etc)
    - blowers
    - venturi pumps
  - Pure oxygen
    - packed towers
    - U-tubes
    - cones

Packed column

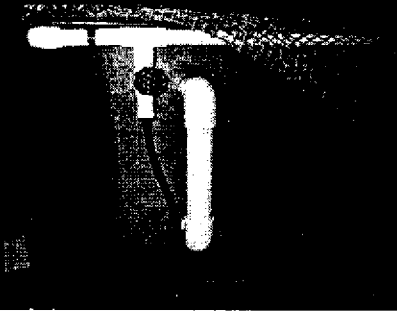
Oxygen injection - downflow contactor



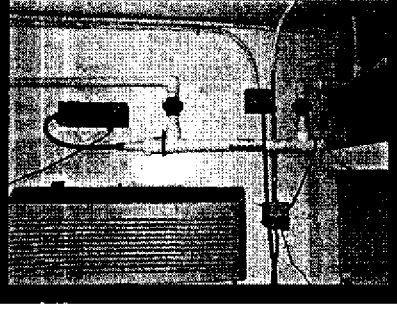
Water circulation - Mechanical pumps

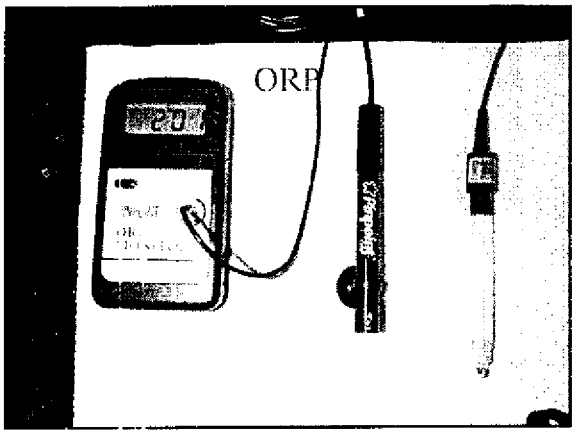


Water circulation - Airlift



Water sterilization - Ultraviolet

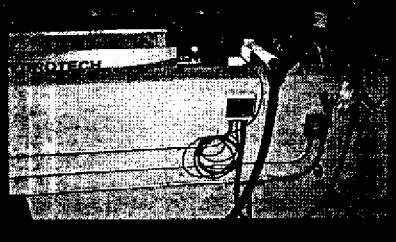




pH Increase

- Rational
  - CO_2 from respiration
  - nitrification is an acidifying process
- Purpose
  - add alkalinity/buffering to water

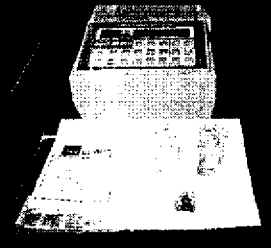
pH Control - sodium hydroxide injection



Water disinfection/oxidation

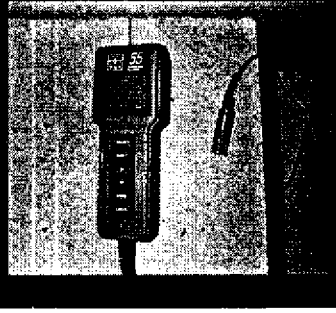
- Ozone, hydrogen peroxide, potassium permanganate.

Water quality monitoring

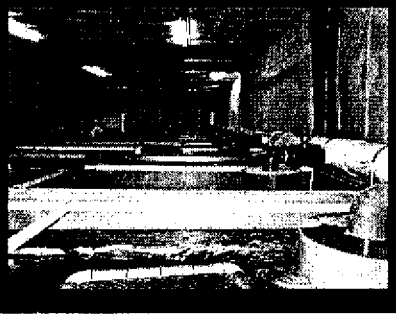


- NH4
- NO2
- NO3
- pH
- Alkalinity
- Dissolved oxygen
- Temperature
- ORP

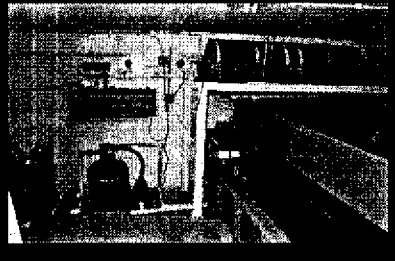
Oxygen Monitoring



Production Troughs



Quarantine Facility VSAREC



What is Ozone?

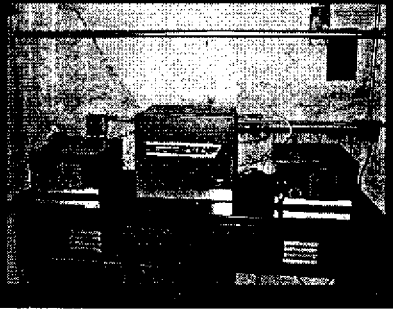
- 0_3
  - Naturally occurring in nature
  - 1/2 life in seawater of several minutes
  - Produces many other oxidizing compounds when reacted with seawater.
- $0_3 = 0_2 + 0$

How can you make ozone?

- O_2 + energy = 0 + 0
  - Energized 0 attaches to existing O_2

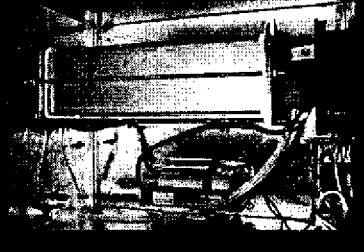
    $O_2 + 0 = O_3$
  - Electrolytic sources
  - Ultraviolet
  - Corona Discharge

Ozone Generation



How can you make ozone? (cont.)

- Corona Discharge



Industrial applications of ozone

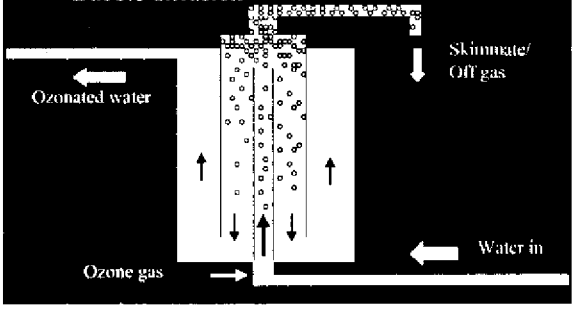
- Drinking water sterilization
- Swimming pool treatment
- Municipal waste-treatment
- Oyster depuration facilities
- Aquaculture

Ozonation benefits in mariculture

- Bacterial, viral and fungal control
- Reductions in TSS, COD, DOC, color and nitrite, elimination of "off-flavor."
- Improved efficiency of solids removal, reduction in biofouling.
- Pretreatment for pesticides/toxins.

How can you apply ozone?

- Bubble diffusion



How can you apply ozone? (cont)

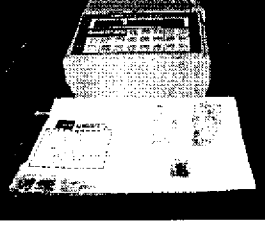
- Foam collection

Ozone application rates/management

- In recirculating aquaculture production systems (<10% makeup water/day), apply 13 to 45g ozone/Kg feed.
- Monitoring
  - HACH Indigo ozone test.
  - Utilization of Oxidation Reduction Potential (ORP) monitoring. Operation parameters 200 - 450 mV.

Ozone application rates/management (cont.)

- Monitoring (cont.)
  - Total chlorine testing (HACH DPD), >0.1ppm.

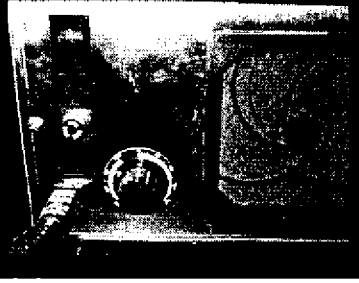


Ozone application management (cont.)

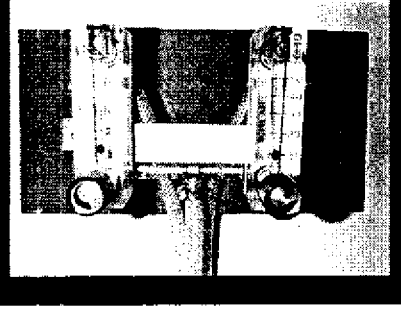
- Intangibles
  - Water appearance
    - Clarity, color, foam presence
  - Water odor
    - Organic, "bleachy"
  - fish behavior
    - Erratic, gasping, UPSIDE DOWN

Ozone application control (cont.)

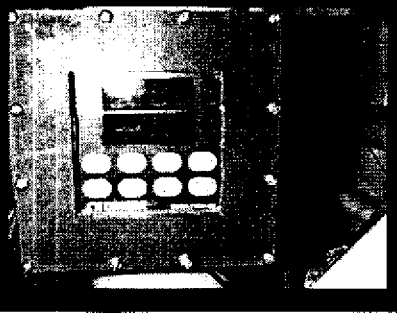
- Control % output of ozone generator



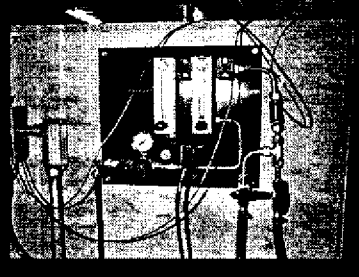
Feed Gas Control



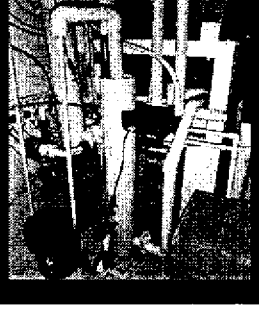
ORP Control



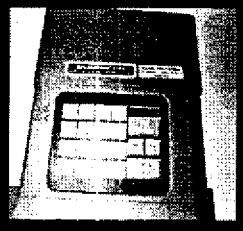
Automated Oxygen Injection



Dissolved Organics Stripping

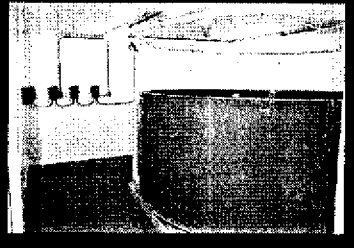


Alarm/Control Points



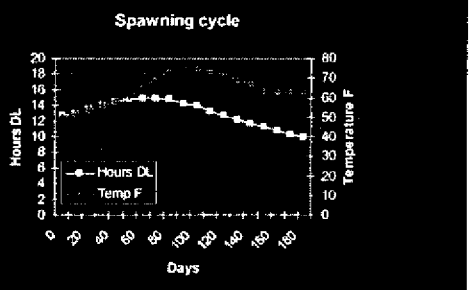
- Temperature
- Oxygen
- Electricity
- pH
- ORP
- Flow rates
- Audio
- Illumination

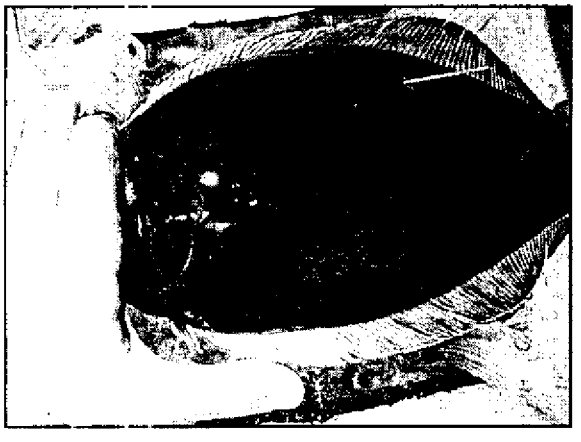
VSAREC hatchery tank



Egg collection

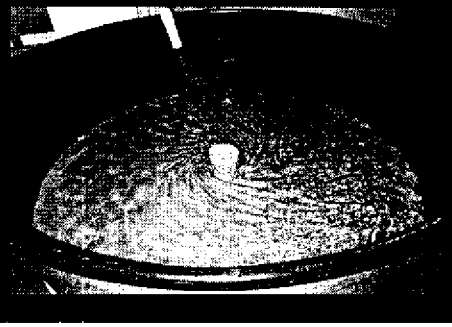
Egg production



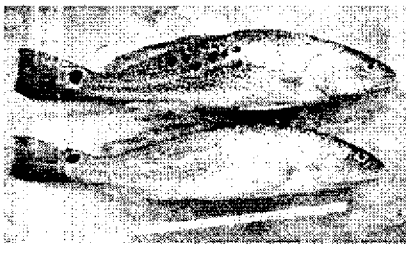


Larval/juvenile production tanks

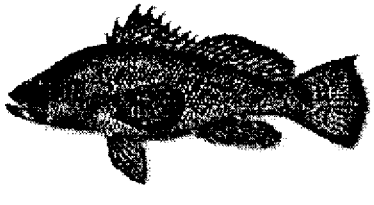
Coloration in larval rearing



Red Drum
(*Sciaenops ocellatus*)

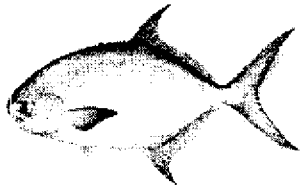


Black Sea Bass
(*Centropristis striata*)

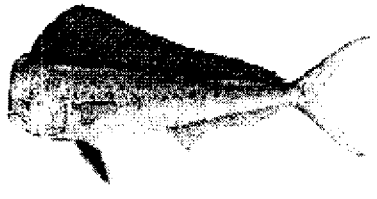


Gulf Flounder
(*Paralichthys albigutta*, *lethostigma*, *dentatus*)

Florida Pompano
(*Trachinotus carolinus*)



Dolphin
(*Coryphaena hippurus*)



Cobia
(*Rachycentron canadum*)

Common Snook
(*Centropomus undecimalis*)

Grouper
(*Epinephelus striatus*)

Role and Function of the Joint Subcommittee on Aquaculture Aquaculture Effluents Task Force: An Update

Presented at the
Aquaculture Waste Management Conference
The Hotel Roanoke and Conference Center
July 22-27, 2001
Roanoke, Virginia

Maxwell H. Mayeaux, Ph.D.

BACKGROUND

It is well known that sustainable capture fisheries have leveled off and that most capture fisheries that are not threatened, overfished, declining, or recovering are at Maximum Sustainable Yield (MSY). The ever-growing population of the Earth and the concomitant increase in demand for high-quality seafood products necessitate that increases in seafood production be provided through aquaculture.

The United Nation's Food and Agriculture Organization (FAO) has tracked aquaculture production world-wide and aquaculture production is an increasing percentage of total fisheries products. As cultured aquatics production is steadily increasing, so to are concerns relative to the environmental impacts of aquaculture farming practices. Several environmental groups have raised issues regarding some production practices used in the aquaculture industry in the U.S. and other countries.

The U.S. Environmental Protection Agency (EPA) developed effluent limitations guidelines (ELGs) for fish hatcheries and fish farms in 1977 but regulations were not promulgated at this time. In January, 1991, the Natural Resources Defense Council (NRDC) won a lawsuit against EPA which led to the development of new or revised guidelines for selected industries. This lawsuit required EPA to propose and take final action on seven effluent guidelines already under development, four effluent guidelines already identified, and eight additional effluent guidelines that had not yet been identified by EPA. In a court-ordered consent decree settlement (January, 1992), EPA was required to promulgate new ELGs or revise old selected industry categories..

EPA asked for public comment on the need for effluent guidelines for aquaculture facilities. The notice made reference to the recent report by the Environmental Defense Fund, which recommended promulgation of ELGs for aquaculture. The Agency received comments arguing either that pollutant discharges are adequately addressed by National Pollutant Discharge Elimination System (NPDES) permits and that ELGs are not needed, or that the permits issued by the various state programs were inconsistent and inadequate. Some comments also provided additional information such as explanations of how aquaculture facilities are regulated under existing permits, comparisons of state permitting policies, and technical papers describing the pollutants generated and treatment processes used in the industry. EPA needed additional

information to determine whether the existing NPDES permit process was adequate to address these discharges and in February, 1999, EPA published in the *Federal Register* their intent to conduct a preliminary study of the aquaculture industry to determine any need for national ELGs.

On January 21, 2000, EPA released the following announcement: "EPA Expands Focus on Nutrient Pollution. EPA's Office of Water is focusing new efforts to help reduce nutrient loadings from commercial agricultural and industrial operations nationwide. Among those efforts is a new activity to develop pollutant controls in the form of nationally applicable discharge standards (known as effluent limitations guidelines and standards) for commercial and public aquaculture operations." The decision to include aquaculture in the development of ELGs was made in January, 2000 under the terms of the 1992 court-ordered consent decree settlement. This settlement agreement established a time line for proposed ELGs by June, 2002 with final rulemaking by June, 2004.

JSA AQUACULTURE EFFLUENTS TASK FORCE

The Federal Joint Subcommittee on Aquaculture (JSA) is a legislatively mandated body whose function is to coordinate Federal activities related to aquaculture in both the public and private sectors. In response to the 1999 *Federal Register* notice announcing the preliminary study, the JSA, under the leadership of USDA and Department of Commerce, formed the Aquaculture Effluents Study Task Force (AETF). The mission of this Task Force is:

To coordinate, facilitate, review, and provide input of science-based information via a broad-based stakeholder and technical expert process to assist EPA in the development of national effluent limitations guidelines and standards for aquaculture facilities in the United States.

Specific objectives of this Task Force are to:

- Create a nationally coordinated and systematic process for collection, analysis, and reporting of science-based data and information.
- Integrate the best available and appropriate science, technology, data, and information into decision-making processes that best serve the nation.
- Provide written scientific and technical reviews and analysis of data and information summaries for different production systems associated with major species in collaboration with EPA.
- Prepare expert, peer-reviewed reporting as scientific and technical guidance for developing final national effluent limitations guidelines and standards.

Specific actions and activities for the AETF include:

- Create a task force consisting of federal agency members and non-federal agency participants who represent diverse stakeholder interests.
- Form technical subgroups composed of persons with recognized expertise, experience, and knowledge of discharges associated with different aquaculture production systems and species

husbandry practices.

- Form project work teams of known experts to address special interest topics as needed.
- Employ an expert peer-review process and science-based protocols for reporting purposes.
- Use the Internet and the World Wide Web for communications and information exchanges.
- Create an Internet home page with information on task force activities and related information.
- Assign specific tasks with time lines for completion, reviews, and follow-up
- Seek funds to support task force activities and accomplishment of objectives

The AETF includes a very diverse membership including representatives from Federal, state, and local government agencies, academia, producer organizations, private aquaculture producers, Land-grant and Sea-grant advisory service, environmental organizations, and professional societies.

Several Technical Subgroups were formed which include individuals from academia, private industry, and federal and state agencies. The overall goal of the technical subgroups is to support a nationally coordinated, systematic process that will identify and report the best-available science, information, and data relating to discharges from diverse aquaculture production systems and husbandry practices. These Technical Subgroups include: Fish Feeds and Nutrition, Catfish Production in Ponds, Trout Production in Flow-through Systems, Salmon Farming in Net Pens, Marine Shrimp Production, Hybrid Striped Bass Production, Baitfish Production in Ponds, Tropical Ornamental Fish Production, Crawfish Production, Alligator Production in Confinement, Molluscan Shellfish Production, Recirculating Aquaculture Systems, Drugs and Chemicals, Aquatic Animal Pathogens, Aquaculture Economics, BMPs, and Data Collection and Analysis.

The Technical Subgroups provided a written listing of literature citations of relevant, science-based information and data to EPA in response to EPA's regulatory data needs. Copies of materials that met the above criteria were included and forwarded to EPA. EPA is using this and other information to develop draft industry profiles summarizing the specific industries EPA is considering to regulate with respect to ELGs.

In September, 2000, the DOC/NOAA sponsored an AETF-organized workshop in Silver Spring, Maryland bringing representatives from all technical subgroups together to provide input to EPA regarding the draft industry profiles under development. This meeting provided a forum allowing the technical subgroup representatives further input relative to the draft industry profiles to assist EPA in making sound decisions based upon the best-available science.

Subsequent to the September meeting, EPA provided responses to frequently asked questions which were placed on the AETF homepage (http://ag.ansc.purdue.edu/aquanic/jsa/effluents/index.html) to educate the public on the AETF and EPA's activities. Additionally, in order to make the whole process more transparent, the AETF homepage includes timely information which can be accessed through the World-Wide Web enabling easy access to the workings and activities of the AETF.

Another effort by EPA to fill in many of the data gaps relative to the aquaculture industry has led to several actions. EPA has been conducting site visits to aquaculture operations both public and private and is currently collecting water quality data on parameters such as nitrogen, phosphorus, suspended solids, etc. They are also evaluating practices and technologies currently in place at these locations that can mitigate potential environmental impacts of aquaculture discharges. EPA also proposed to mail out a screener industry questionnaire which will be used to identify a selected random sample of the industry. Subsequently, an "Information Collection Request (ICR)" is planned to be sent to selected aquacultural producers in an effort to collect information on both public and private aquaculture facilities. The data to be requested may include information such as size of facility, species produced, type of facility, feeding rates, discharge volumes and concentrations, etc. Additionally, since EPA is considering regulatory decisions relative to the economic achievability of ELGs, confidential financial information will be collected from a random sample of facilities which were mailed and completed an initial screener survey. The draft ICR was published in the *Federal Register* for public commentary. Comments were received, changes were made, and the updated ICR sent to OMB for review and is available for public comment. This document can be accessed at EPA's web site.

The process and activities outlined above are certainly proving to be a difficult, complex, and highly emotional process. The AETF's role in this process is to provide a forum in which input and contributions from many individuals, both public and private, can offer a coordinated, systematic approach to contribute to the public good. Although continuous monitoring, clarification, and specific actions need to be taken to keep pace with EPA's timetable, through the AETF, EPA can remain engaged with diverse stakeholders. Additionally, through this process, any reports provided to EPA need to withstand public scrutiny and serve as critical technical guidance in EPA's rulemaking process.

Update and Overview of the Effluent Limitations Guidelines and Standards for the Aquaculture Industry

Aquacultural Waste Management Symposium

July 22-24, 2001

Kristen L. Strellec

U.S. EPA Office of Water

Engineering and Analysis Division

Engineering and Analysis Division Aquaculture Workgroup

◆ Marvin Rubin, Branch Chief	(202) 260-3028
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◆ Kristen Strellec, Co-Project Mngr/Economist	(202) 260-6036
◆ Mike Clipper, Soil Scientist	(202) 260-1278
◆ Karen Maher, Environmental Assessment	(202) 260-3894
◆ Charles White, Statistician	(202) 260-5411
◆ Stephen Sweeney, Attorney	(202) 564-5491

◆ E-mail Address: lastname.firstname@epa.gov

Background

- Aquaculture selected for rulemaking under Consent Decree
  - National Standards never developed for this industry
  - Shift in priorities from toxic metals and organics to siltation, nutrients and pathogens which have been cited as the most prevalent water quality impairments from states
- EPA to develop effluent limitations guidelines and standards
  - National regulations for industrial wastewater discharges
  - Technology-based standards
  - Numerical limitations for specific pollutants and/or Best Management Practices

Overview of the Effluent Guidelines Process

- Define the Industry
- Gather Technical and Economic Data
- Develop Industry Profile
- Develop Technology Options
- Estimate Pollutant Reductions
- Estimate Engineering Cost
- Evaluate Non-Water Quality Environmental Impacts
- Evaluate Economic Achievability
- Determine Achievable Effluent Limitations
- Proposal and Solicitation of Public Comment

Result of Effluent Limitations Guidelines Process

- Determines Best Available Technologies and/or BMPs that are economically achievable
- Establishes "nationally consistent" standards
  - levels the economic "playing field" between states and regions
- Replaces outdated guidance to States with up-to-date standards reflecting the practices and technologies in use today

Possible Rulemaking Outcomes

- No Regulation or Regulation
  - Entire Industry Category
  - Certain Subcategories
  - Certain Size Facilities of a Given Subcategory(ies)
- For No Regulation, options include:
  - EPA issues guidance - BMPs (e.g. voluntary approaches, incentive programs)
  - EPA does not issue guidance

Possible Rulemaking Outcomes (Continued)

- For regulation, options include:
  - EPA establishes numeric limitations
  - EPA develops BMPs
  - EPA develops combination of numeric limitations and BMPs
- Different levels of stringency by industry subcategories
  - levels of stringency are based on a number of factors (e.g., economic impacts, size, production systems, discharge levels, performance of control technology/practices) and can vary for each subcategory. For example:
    - ▲Subcategory A = no regulation
    - ▲Subcategory B = least stringent option considered
    - ▲Subcategory C = more stringent option considered

Technology and Economic Considerations for Effluent Guidelines

Activities Time Schedule for 2001 / 2002

- Revise ICR and Submit to OMB - June 2001
- OMB Review of Survey (60 days) - August 2001
- Revise and Mail Out Screener Survey - August/Sept 2001
- Data Entry - September/October 2001
- SBREFA Process - September thru November 2001
- Mail Out Detailed Survey - November 2001
- Options Selection Process - Dec 2001 thru Feb 2002
- Review/Follow-up/Data Entry of Detailed Survey - Jan/Feb
- OMB Review - March 2002
- Proposal - June 2002

Site Visits and Sampling

- EPA has visited 56 aquaculture facilities
- Facilities visited have been
  - small to large operations
  - various production systems (ponds, raceways, recirculating, and open water systems)
  - various species (catfish, trout, salmon, tilapia, crawfish, hybrid striped bass, alligator, ornamentals, shrimp, molluscan shellfish)
- EPA plans to conduct several site visits in the next few months
- 4-5 Sampling episodes are scheduled for fiscal year 2001

Economic Analysis for Effluent Guidelines

- **Role of Economics in Effluent Guidelines**
  - Assess economic impacts associated with costs of compliance
  - Provide recommendations in terms of "economic achievability" for various control options
  - Conduct Small Business Analysis

Economic Measures for Impacts of Compliance

- Economic Achievability
  - closure analysis
  - job losses
  - financial ratios
- Small Business Impacts
  - consider alternatives to minimize impacts on small entities
  - small entity participation as required by SBREFA
  - outreach to small entities
- Other Community Impacts

Next Steps

- Awaiting OMB approval on survey instruments
- Mail out screener survey upon approval
- Determine if any groups can be excluded from coverage
- Develop statistical approach for detailed survey mail out
- Develop a list of possible treatment options and best management practices
- Review and evaluate data currently available for wastewater characterization
- Gather treatment technology costs
- Develop Economic Impact Methodology

Additional information

- To obtain additional information on the development or progress of the aquaculture guidelines:

  http://www.epa.gov/OST/guide/aquaculture

  http://ag.ansc.purdue.edu/aquanic/jsa/effluents/index.html

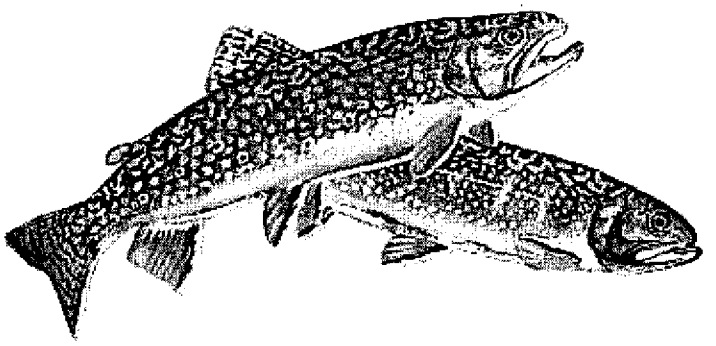
- For technical information:

  Marta Jordan (202) 260-0817

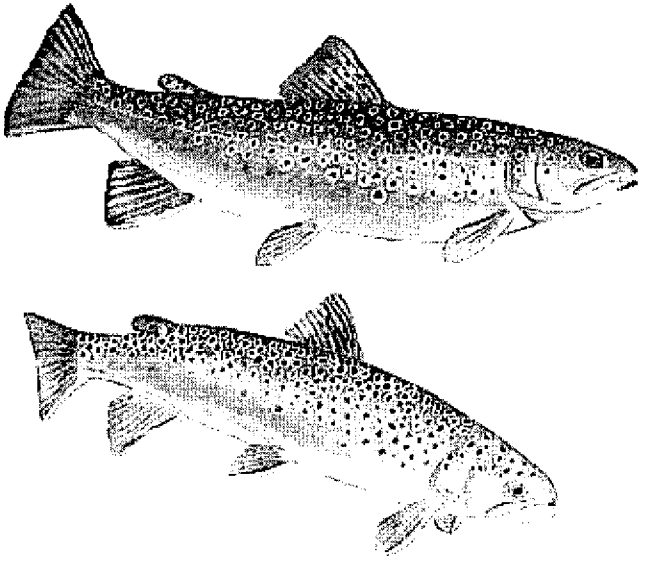
  Mike Clipper (202) 260-1278

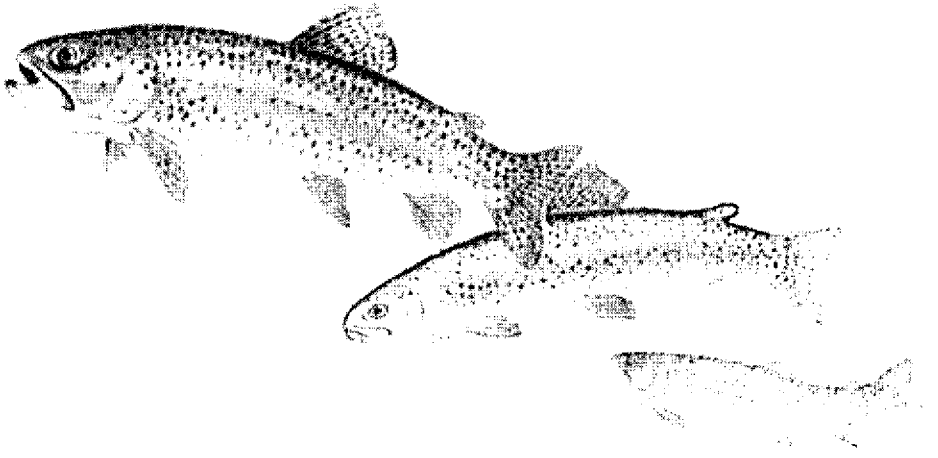
- For economic & technical information:

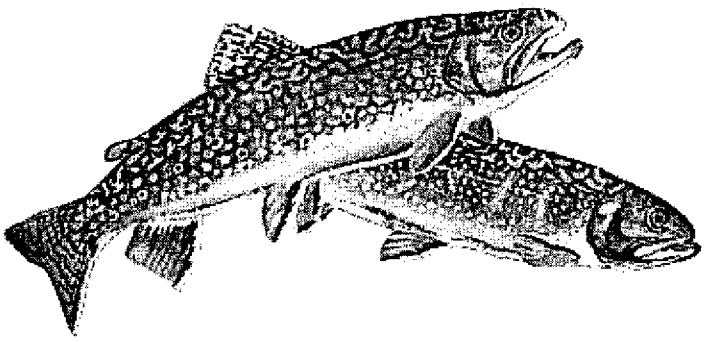
  Kristen Strellec (202) 260-6036



Coping With Water Quality Issues and TMDLs





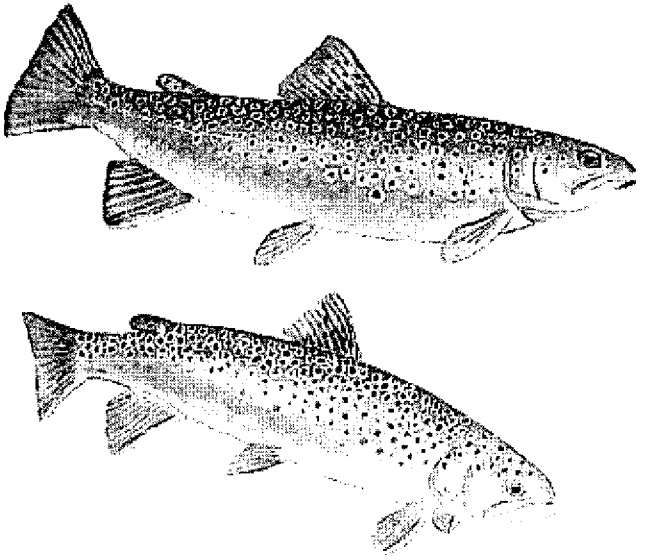


Jane Walker

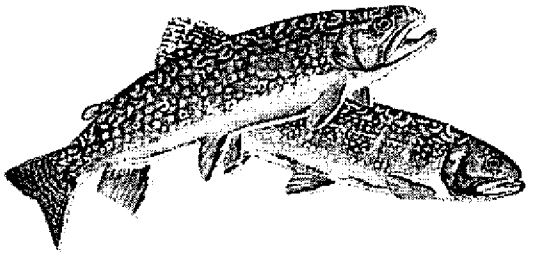
Virginia Water Resources Research Center

Virginia Tech

Blacksburg, VA

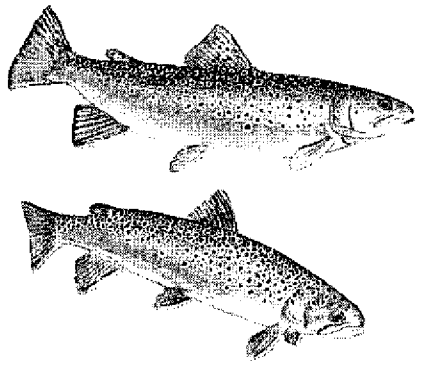


- Fred Benfield, Aquatic Biologist,VT
- Greg Boardman, Environmental Engineer, VT
- Saied Mostaghami, Biological Systems Engineer,VT
- Len Shabman, Resource Economist/VWRRC Director
- Len Smock, Aquatic Biologist, VCU
- Tamim Younos, Environmental Engineer/
  VWRRC Associate Director
- Jason Anderson, Urban Affairs and Planning
  Graduate Student,VT
- Kim Porter, Environmental Science Undergraduate, VT
- Virginia DEQ,Virginia DCR, EPA Region 3



Project Purpose

- Develop Total Maximum Daily Load (TMDL) Assessments for Six Impaired Stream Segments Downstream from Trout Farms.



Background

Spring Fed Streams

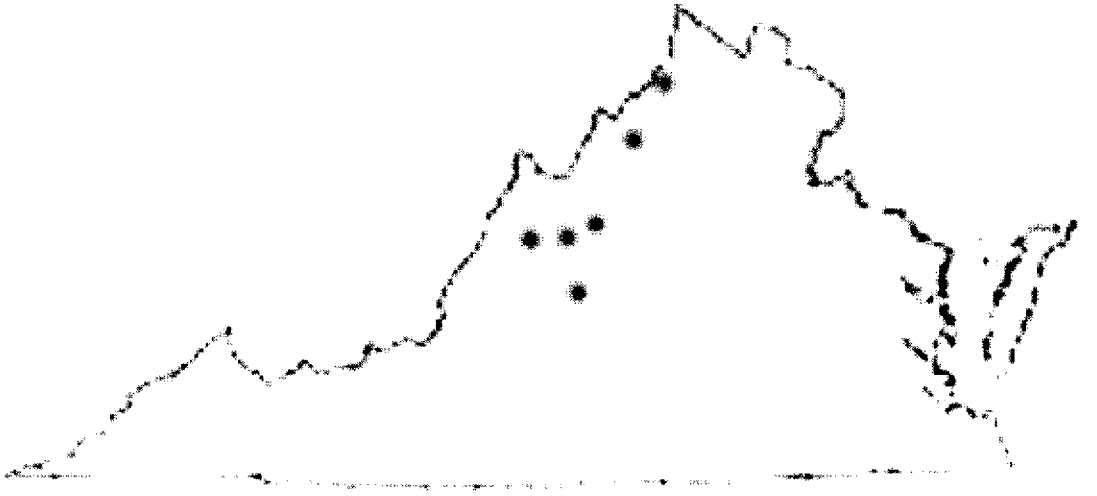
Impairment < 1 mile

Benthic Impairments

Trout Farms

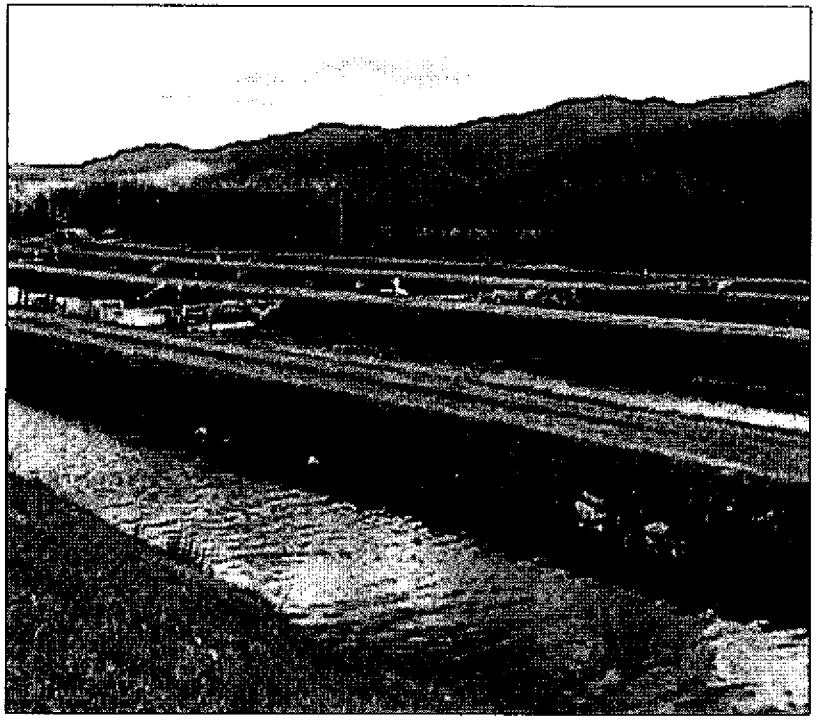
Flow Through Systems

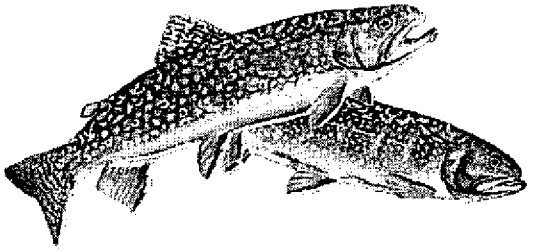
Effluent Permitted by VA DEQ



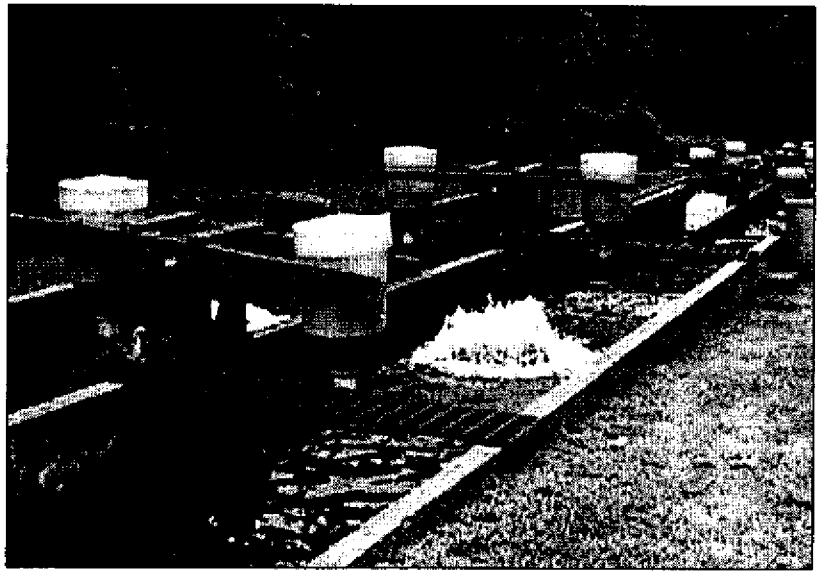
Background

- Spring Size: Small to Large (0.4-10 MGD)
- Farm Size: Small to Large (35,000-240,000 lbs/year harvest)

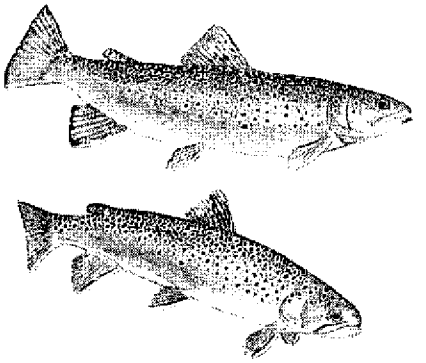




Background



- Concrete Raceways/ Earthen Lined
- Automatic Feeders/ Hand Feeding
- High Energy Fish Feed/ Regular Fish Feed

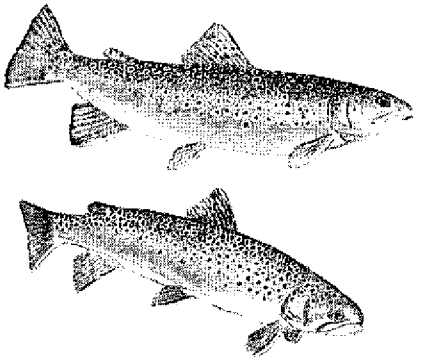


General Permits: (Quarterly Monitoring)
Estimate Daily Maximum Flow (MGD)
TSS (mg/L): Composite Sample
Average ≤ 10 mg/L
Maximum ≤ 15 mg/L
SS (mg/L): Composite Sample
Average ≤ 0.1 mg/L
Maximum ≤ 3.3 mg/L

Individual Permits: (Monthly Monitoring)
Additional Monitoring Required
Ammonia
pH
Dissolved Oxygen
Biological Oxygen Demand

Background

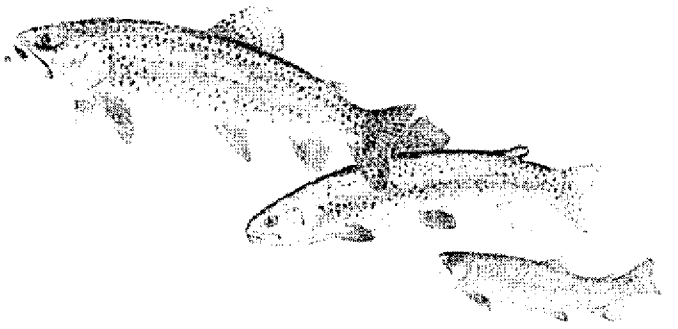
- Effluent Permits: General/Individual
- Monitor: Representative Discharge (feeding, fish harvest, cleaning, etc. Take 1 to 5 Grab Samples/8 Hours)



Background

- Discharge Monitoring Reports Show Compliance with Permits
- Stream Waters Below the Trout Farms Are Designated Impaired

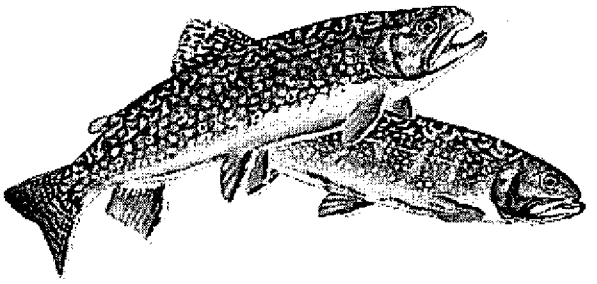
Impaired Waters



- Do not meet State established water quality standards.

Water Quality Standards in Virginia Consist of:

1) Designated Use of the Water (Fishable/Swimmable)
2) Water Quality Criteria to Protect the Designated Use (Numeric or Narrative)
3) Antidegradation Policy

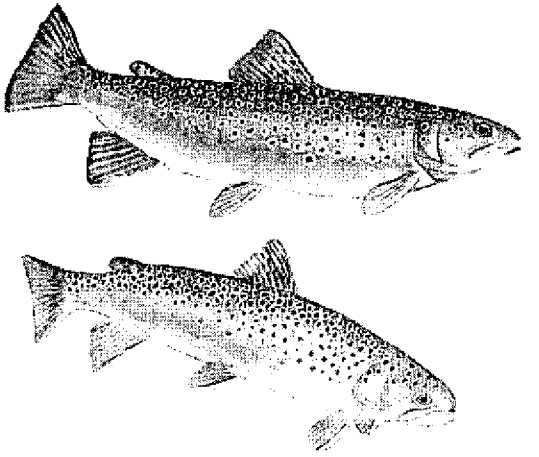


These Six Impaired Stream Segments

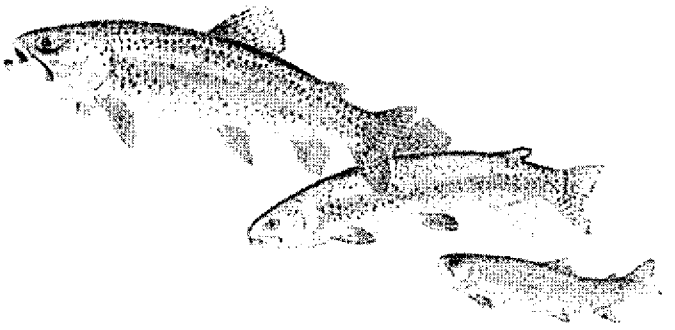
- Do not support the aquatic life use based on the narrative criteria: *All state waters shall be free from substances which interfere with the designated uses or which are harmful to human, animal, plant, or aquatic life.*

Aquatic Life Impairment

- Based on benthic macroinvertebrate assessment of the stream

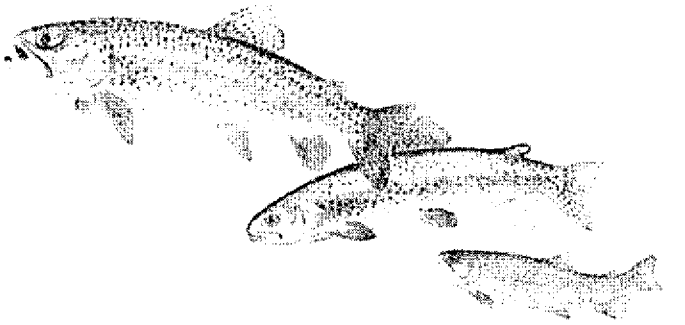


Benthic Macroinvertebrates

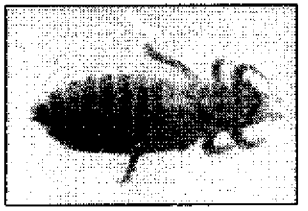


- Benthic organisms refer to those that live on the bottom of a water body.
- Macroinvertebrates are organisms that are visible without a microscope ("macro-") and lack backbones ("invertebrates").

Benthic Macroinvertebrates

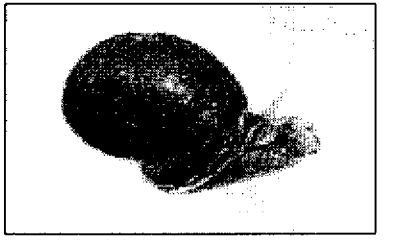


- Include larval or nymph forms of insects (*e.g.*, stoneflies, mayflies, dragonflies, *etc.*), crustaceans (*e.g.*, crayfish), snails, mussels, clams, worms, and leeches.

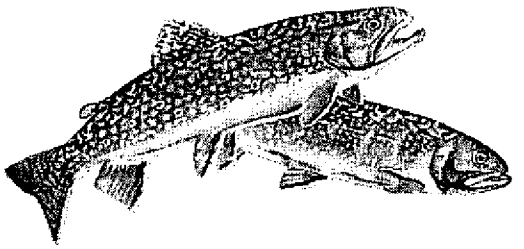


Dragonfly Larvae

Crayfish

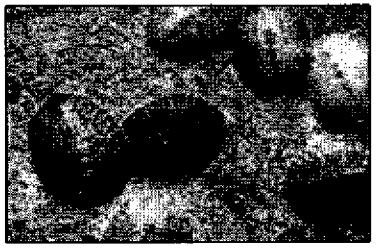


Aquatic Snail



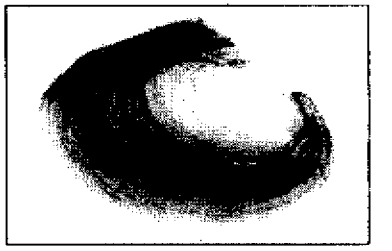
Require Good Water Quality

Stonefly



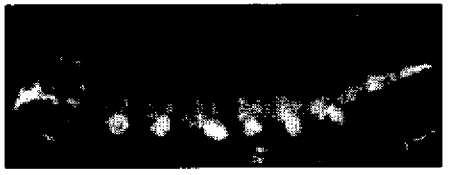
Water Penny Beetle

Mayfly

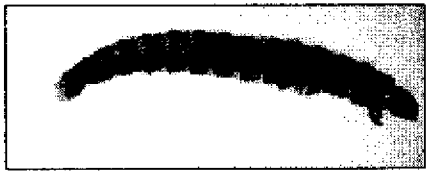


Mussel

Dobsonfly

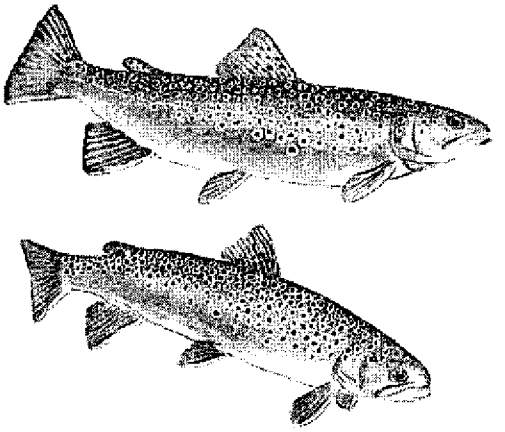


Snipe Fly

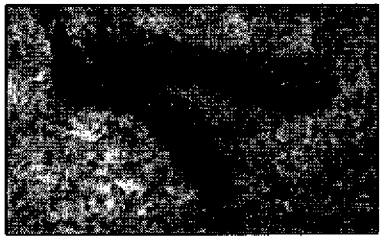


Riffle Beetle

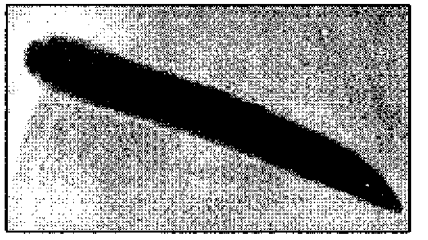
From EPA web site: http://www.epa.gov/eq/atlas/bioindicators/



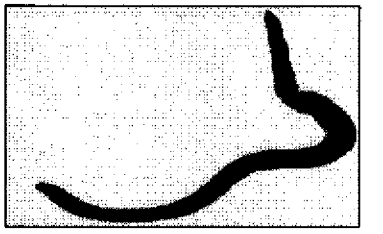
Tolerant of Poor Water Quality



Midge Fly



Leech

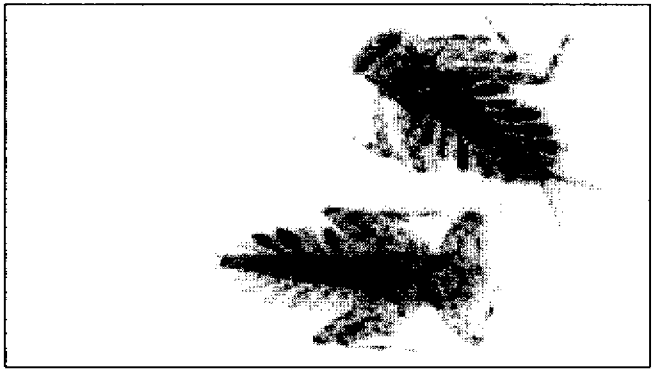


Aquatic Worm

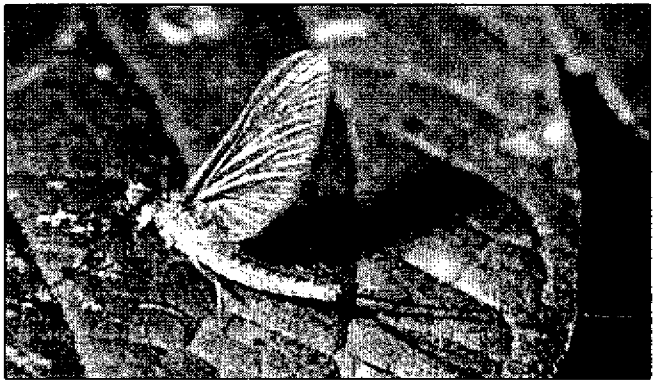
From EPA web site: http://www.epa.gov/eq/atlas/bioindicators/

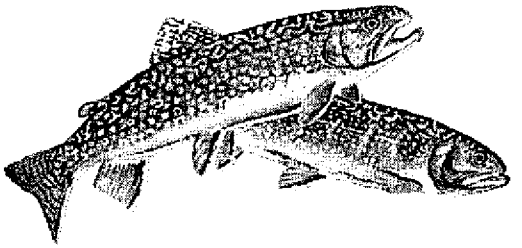
Reasons to Use Benthic Macroinvertebrates

- Primary Source of Food; Healthy Populations Necessary to Support Healthy Aquatic System.



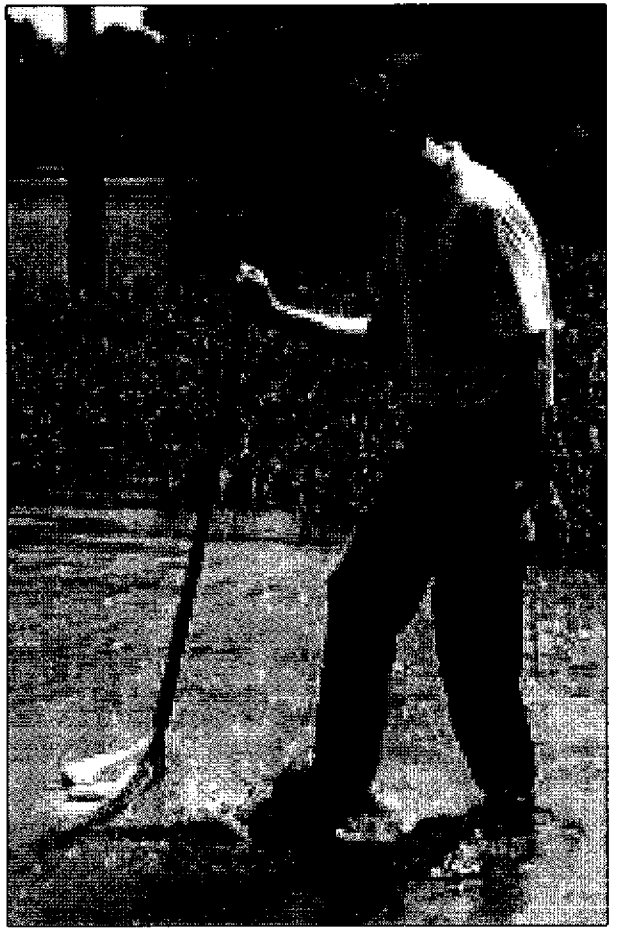
- Indicate Cumulative, Chronic, and Long-term Water Quality Conditions
- Easy to Monitor

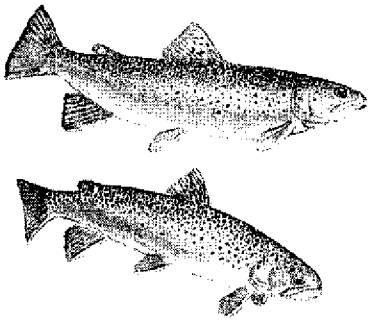




Benthic Macroinvertebrate Monitoring

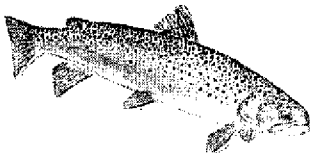
- Determine Benthic Macroinvertebrate Diversity and Abundance
- Compare to Reference Stream





Reference Streams

- Same Ecoregion
  - Limestone Spring
- Similar Flow Size
- Similar Land Use
- Not Impaired

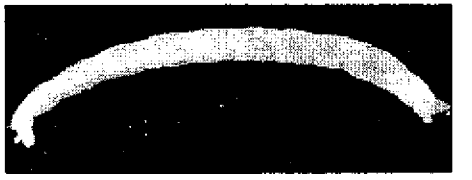


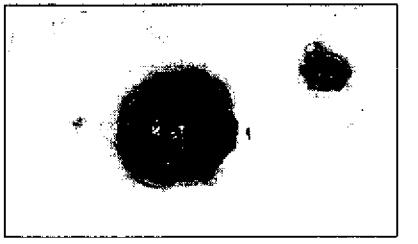
Interpreting Results

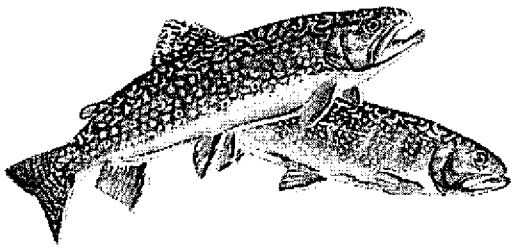
- High Diversity → Good Water Quality; Ecological Balance
- Low Diversity → Water Quality Problem; Ecological Imbalance
  - Few Species & High Abundance → Conditions Favor Tolerant Species

Benthic Monitoring for These Six Stream Segments Indicated

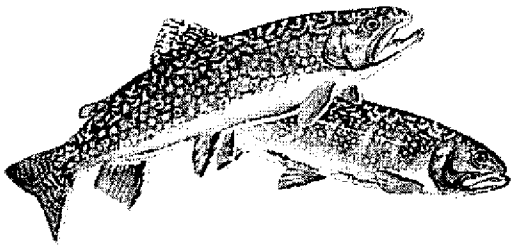
- Low Diversity
- Primarily Pollutant Tolerant Species
  - Sow Bugs
  - Midge Flies
  - Sewer Worms





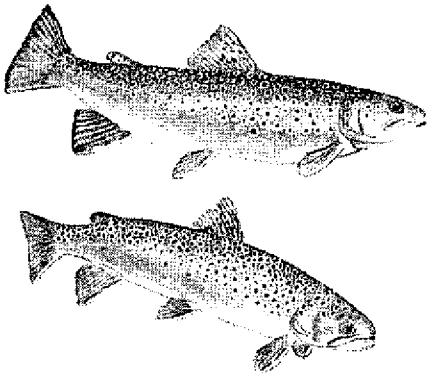


Benthic Macroinvertebrate Monitoring Led to Stream Impairment Listing



A TMDL Is Required for Each Impairment

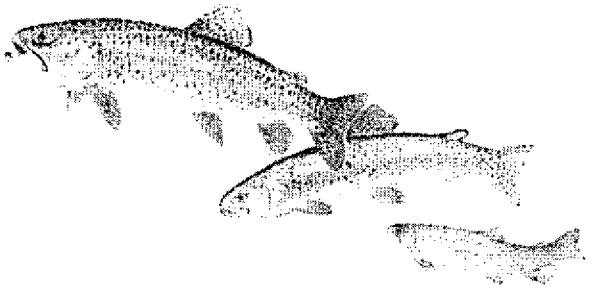
- **Total Maximum Daily Load**—A calculation that estimates all significant sources of a pollutant and the specific amount of the pollutant coming from each source.

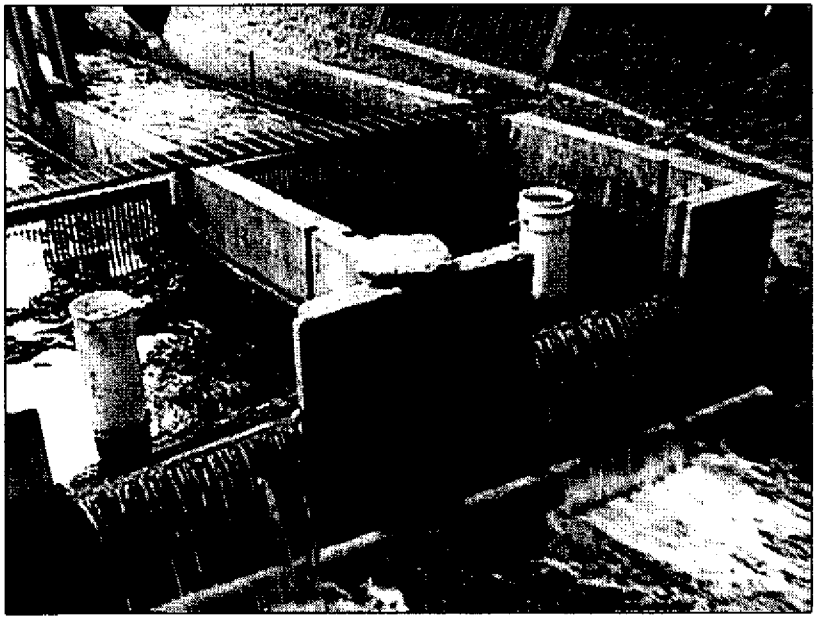


Possible Pollutant Sources

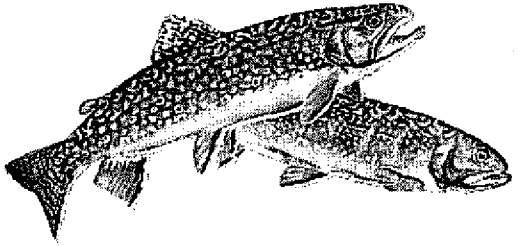
- Point Sources
  - Trout Farm Effluent
- Non-point Sources
  - Runoff

Possible Pollutants From Trout Farms

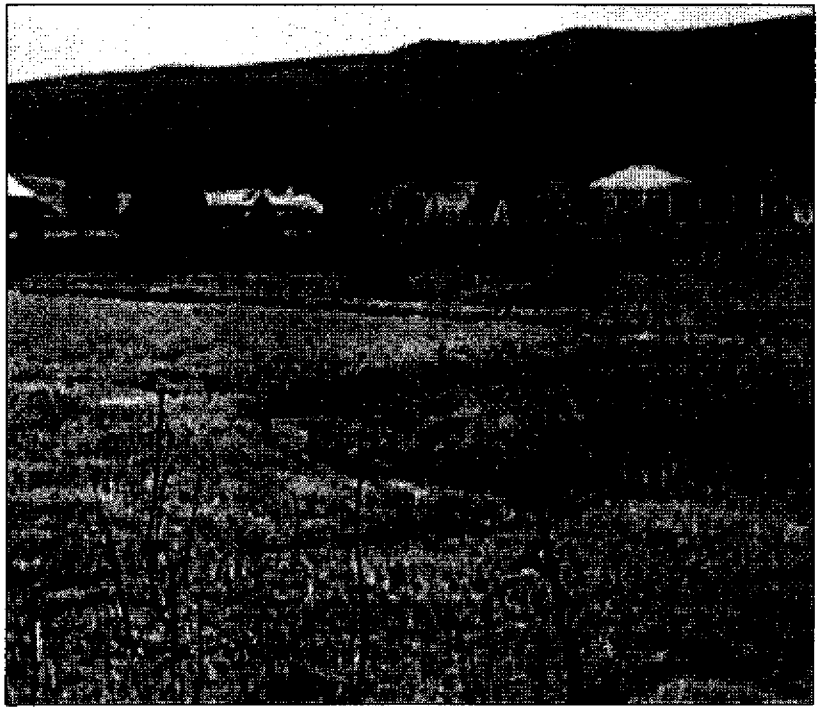




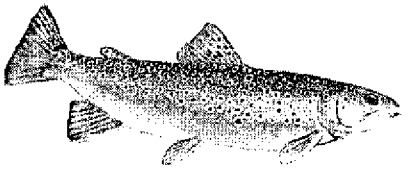
- Unconsumed Feed and Fish Wastes (Solids, Excess Nutrients, Organic Material)
- Therapeutics (Chemical Residues)



Possible Pollutants From Runoff



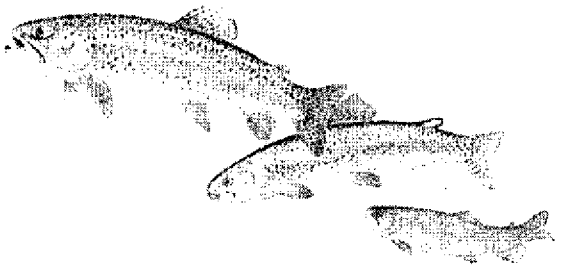
- Sediment
- Excess Nutrients
- Organic Material
- Hazardous Materials (Pesticides, Motor Oil, etc.)



TMDL Process

1) Verify Benthic Impairment
2) Identify Potential Stresses (point and non-point sources of pollution)
   - Fish Population Survey
   - Stream Walk/Land Use Survey
   - Water Monitoring
3) Determine Impact from Each Source
4) Submit TMDL Report to EPA for Approval
5) Develop Implementation Plan

Benthic Macroinvertebrate Survey

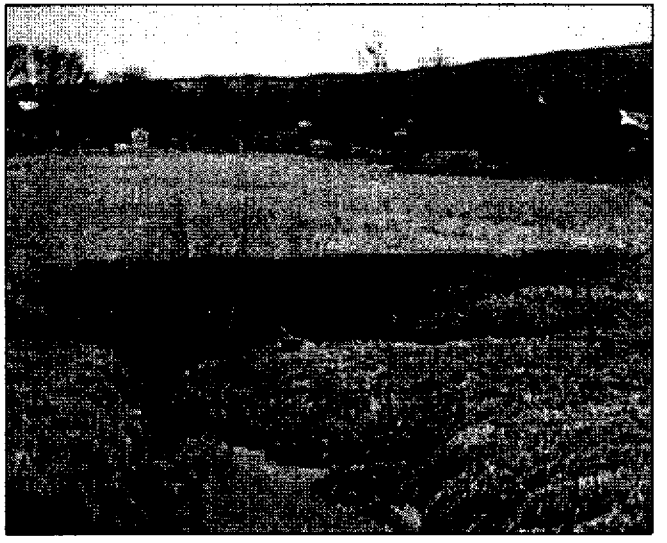


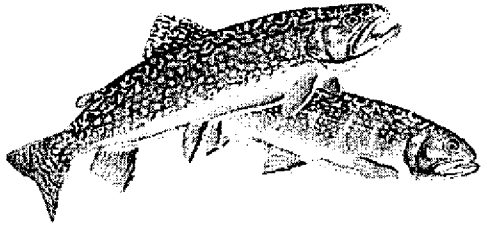
- Determine if stream should still be designated "impaired" based on EPA approved and updated method with more benthic macroinvertebrate samples

Fish Population Survey

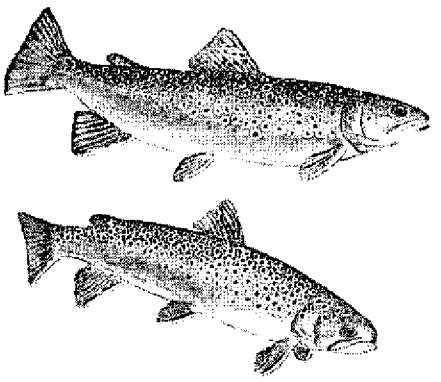
- Fish predation could lower the benthic macroinvertebrate numbers or change the species composition
- Determine fish diversity and abundance
- Compare Impaired Stream with Reference

Stream Walk & Land Use





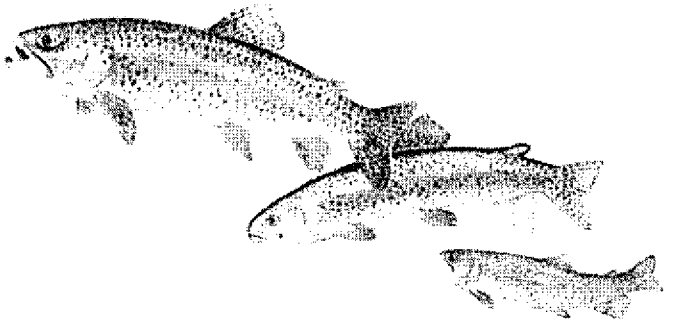
- Land characteristics can indicate possible stresses to macroinvertebrates
- Determine
  - Discharge Sites
  - Stream Bank Erosion
  - Stream Channel Alterations
  - Barriers to Fish Migration
  - Vegetation Coverage
  - Land Use



Water Monitoring

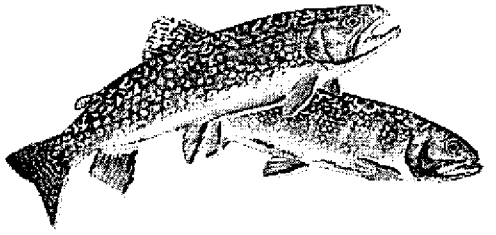
- Water characteristics can indicate stresses to macroinvertebrates
- Determine for Impaired Streams and References
  - Water Flow
  - Alkalinity and Hardness
  - Temperature and pH
  - Dissolved Oxygen
  - Solids
  - Nutrients and Organic Material

Sample Trout Farm Effluent

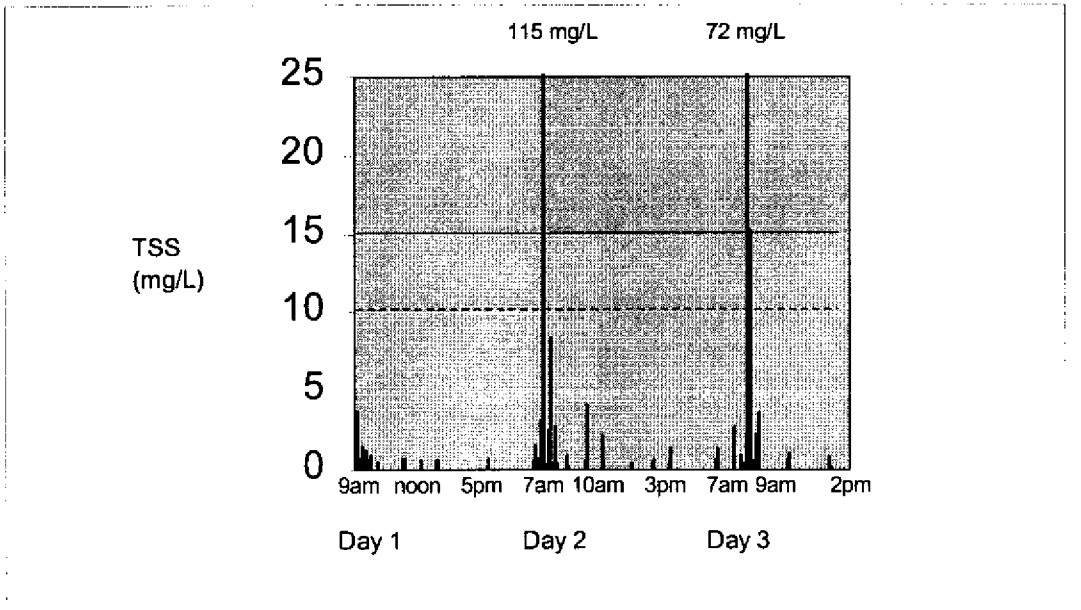


For each trout farm, collect multiple water samples:

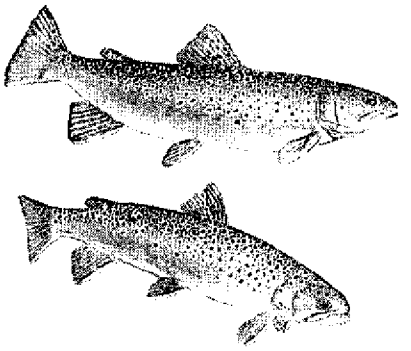
- Upstream of trout farm
- Effluent during low fish activity
- Effluent while feeding fish
- Effluent while cleaning raceways
- Effluent while harvesting fish



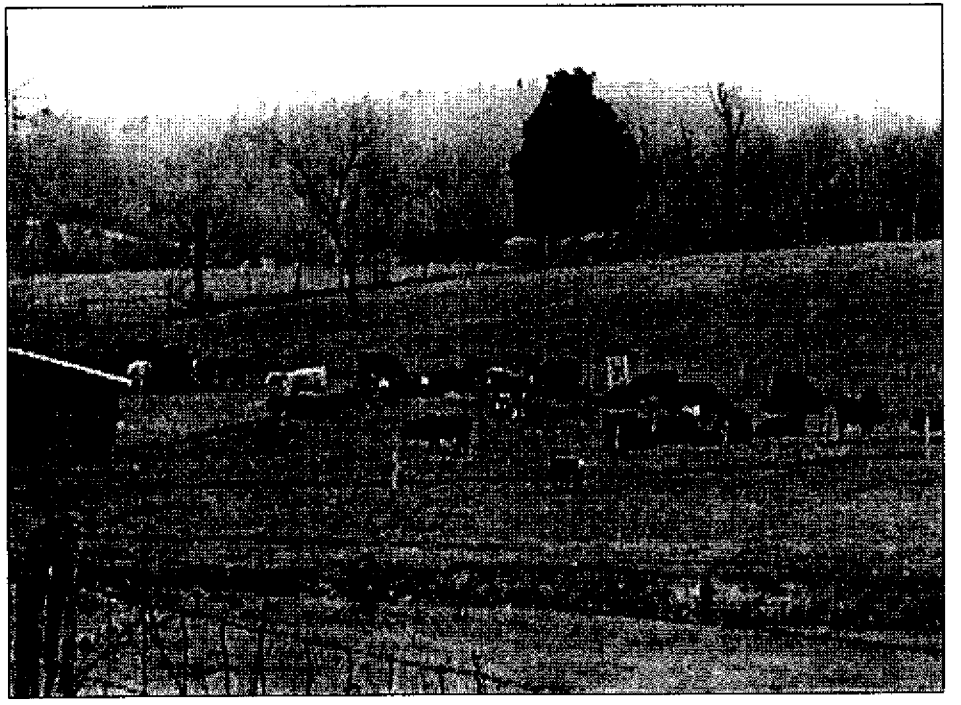
Determine the Load from the Trout Farms



- Use water monitoring results and flow to estimate loads
- Load—The total amount of material (e.g., nitrogen, phosphorus, sediment) carried by a stream (kg/year or lbs/year).



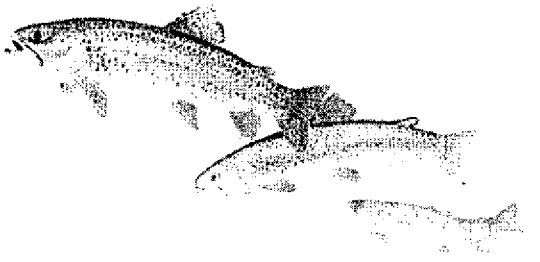
Determine the Load from the Runoff



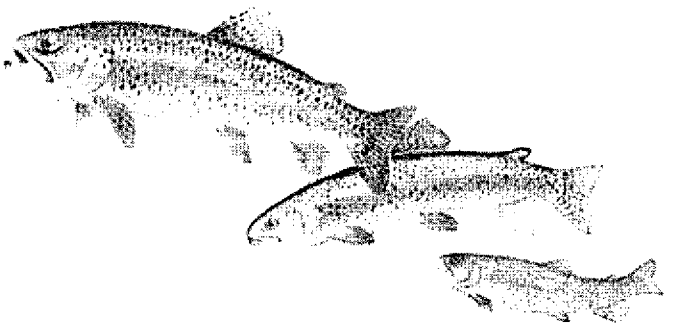
- Use mathematical equations to estimate loads from runoff
  (e.g., Revised Universal Soil Loss Equation)

Continue TMDL Process

- Link Stresses to Benthic Macroinvertebrate Community Conditions
  - Compare Loads of Impaired Streams and Reference Streams
- Determine Changes Needed to Reach Target Condition

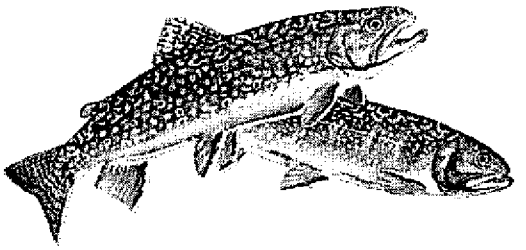


Implementation of Best Management Practices



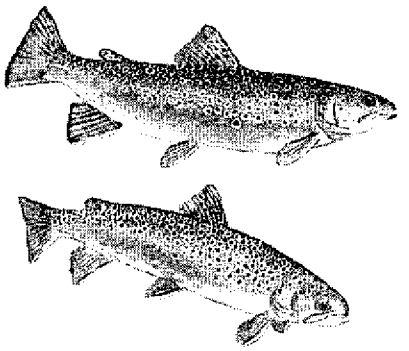
- Trout Farms—
  - Clean raceways often and regularly
  - Use high energy feed, etc.

- Runoff—
  - Vegetated buffer strips
  - Control livestock access to stream, etc.



To Remove Streams From Impairment List

- Additional Benthic Macroinvertebrate Monitoring
- Using an EPA Approved Method
- Comply with the State Standard
- Request EPA Remove the Stream from the Impairment List.
- EPA Makes Final Decision



Possible Outcomes of TMDL Besides Cleaner Stream Water Quality

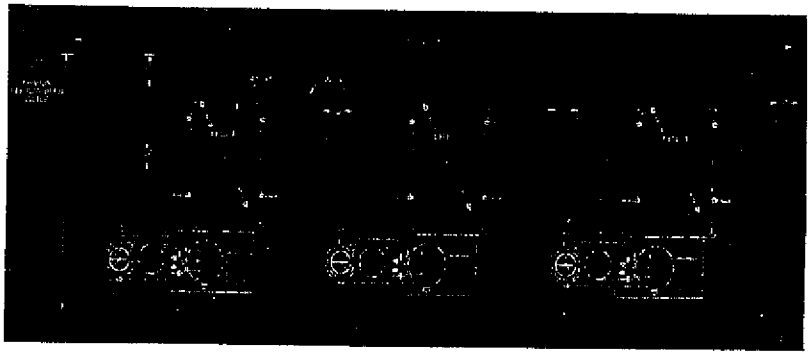
- Cleaner Trout Farm Operation → Healthier Fish
- Some BMPs → Increased Growth Rates of Fish
- Identify Unknown Water Quality Problem so Can Address It (e.g., High CO_2 levels)
- Generate Funds for Cost Share Programs

Best Waste Management Practices for Recirculating Systems

Steven Summerfelt & Brian Vinci
Freshwater Institute
Shepherdstown, WV

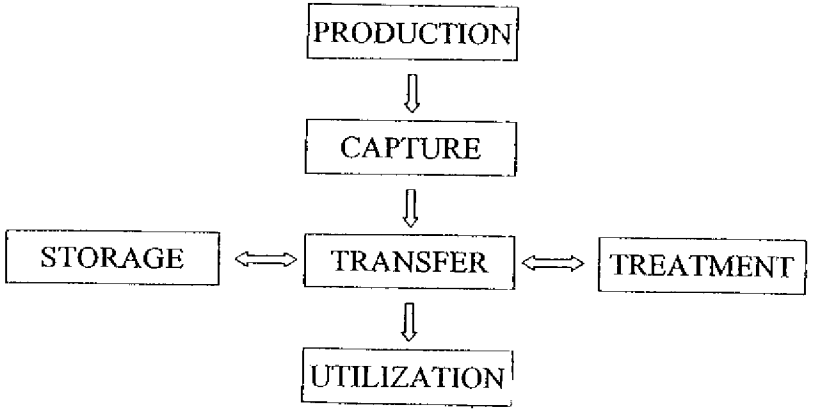
Waste Management in RAS

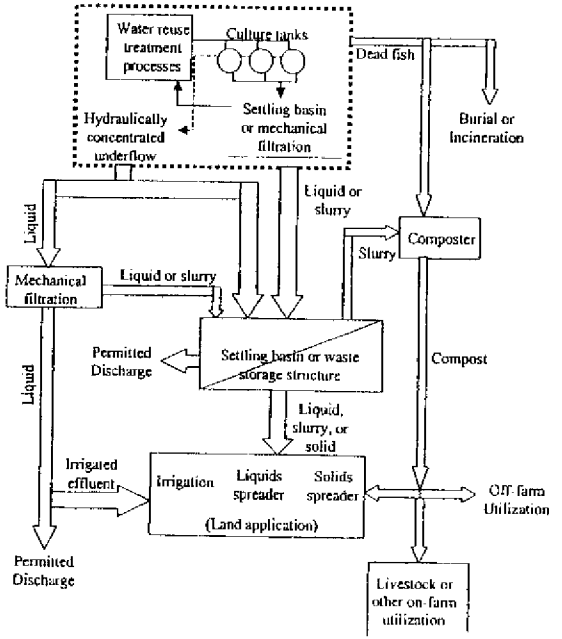
- Complex systems with possibly several different effluents.



(WV Aqua LLC, Man, WV; courtesy of PRAqua Technologies)

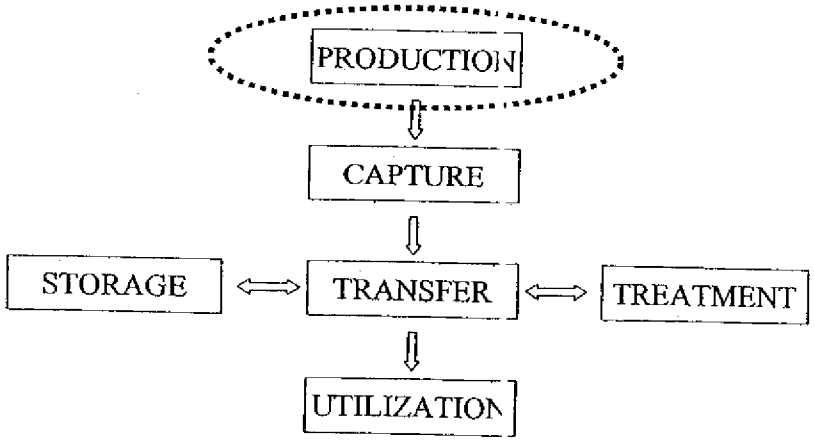
Waste Management Steps





Waste Management in RAS

Fish Waste Production



Fish Waste Production

- Every 1 kg of feed fed produces:

TSS	0.3–0.4 kg
TAN	0.028–0.032 kg
BOD_5	0.1–0.4 kg
TP	0.005–0.011 kg

Water Reuse Concentrates Wastes

- Degree of water reuse controls the waste concentration in a fish farm's combined effluent.

Make-up Flow (L/min)	Flow Reuse (% total)	NO_3-N + tot NH_3-N (mg/L)	TSS in Discharge (mg/L)	Particulate Waste Capture Potential (% of total)
64,000	0.00	0 + 0.6	7	30-60
6,400	90	0 + 5	60	≥ 90
1,600	95	9 + 1	120	≥ 95
640	99	24 + 1	310	≥ 99
160	99.5	96 + 1	1220	> 99
32	99.9	490 + 1	6150	> 99

(all estimates assume steady state)

Water Reuse Concentrates Wastes

- Degree of water reuse:
  - ✓ controls overall ability to capture waste within reuse/recirculating system discharges.

Serial-Reuse Systems

- DILUTE WASTES contained in LARGE FLOWS:
- Effluents are more difficult to treat:
  - ✓treatment efficiency is reduced with dilution
    - for both settling tank and microscreen filters
  - ✓size & cost of treatment process increases with volume

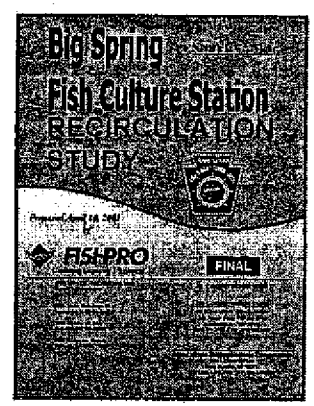
Serial-Reuse Systems

- Environmental regulations can be strict on facilities with large TMDL discharges.


Hatchery to close this fall
800,000 inhabitings... adult fish to be moved

Reuse & Recirc Systems

- Instances appear where serial-reuse systems must be replaced with production systems that discharge less TMDL,
  - ✓i.e., recirculating systems



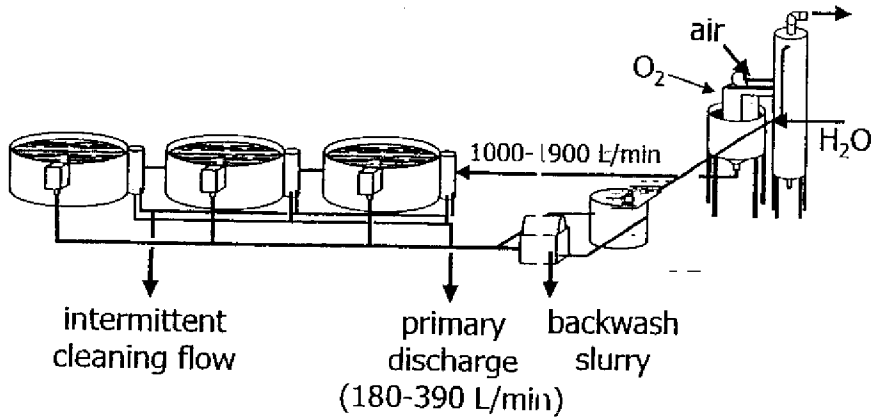
TMDL Control

- Overall waste capture efficiency of culture system
  - ✓depends upon type of reuse systems!

	TSS capture efficiency
serial-reuse raceway systems	25–50%
partial-reuse tank systems	80%
fully-recirculating tank systems	> 97%

Partial-Reuse Systems

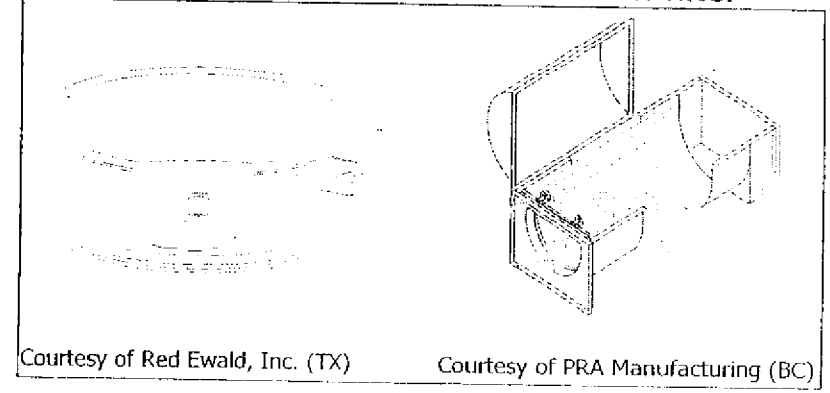
- Freshwater Institute's fingerling system



Courtesy of PRAqua Technologies (BC)

Solids Removal in Partial-Reuse Systems

- 'Cornell-type' dual-drain tank
- Drum filter

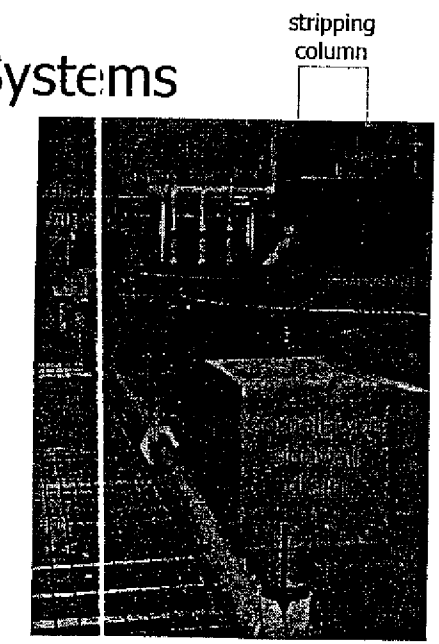


Courtesy of Red Ewald, Inc. (TX)

Courtesy of PRA Manufacturing (BC)

Partial-Reuse Systems

- Solids removal:
  - ✓ sidewall drains to drum filter
  - ✓ bottom drains to standpipe sump
    - discharged from system
    - rapid solids removal (< 5 min)



Partial-Reuse System

- Solids fractionation:

	TSS (mg/L) rainbow trout	TSS (mg/L) arctic charr
Tank inlet flow	1.3 ± 0.1	1.5 ± 0.1
Side-drain flow	2.5 ± 0.2	1.9 ± 0.1
Bottom-drain flow	26.2 ± 2.1	13.1 ± 1.5

Make-up water contained 0.5 ± 0.2 mg/L TSS

Solids Removal

- Treatment processes and system operating strategies should remove solids:
  - ✓rapidly, before they degrade
  - ✓with the least
    - turbulence,
    - mechanical shear, or
    - opportunity for microbial degradation

Water Quality Within RAS

- Water quality within RAS depends upon the efficiency of waste removal (f_{rem}) at each unit process:
  - ✓Solids capture
    - filtration
    - sedimentation
  - ✓Carbon dioxide removal
    - aeration
  - ✓Ammonia removal
    - biofiltration

Water Quality Within RAS

- Efficiency of waste removal at each unit process within recirc system:
  - ✓controls water quality in culture tanks,
  - ✓even when fraction of flow reused is high.

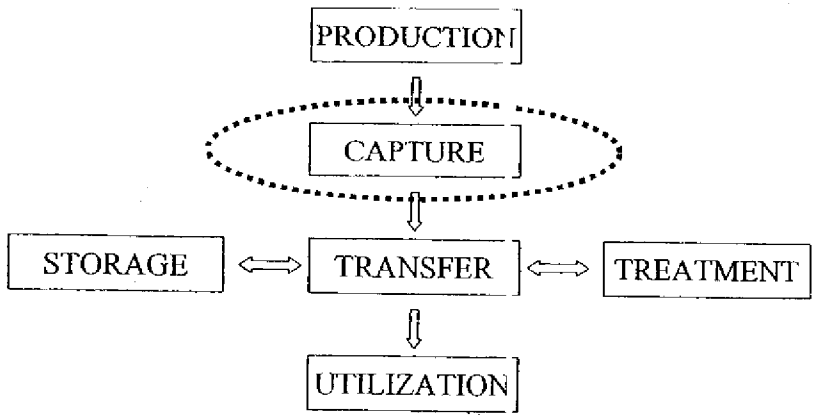
$$\{\text{Waste}\}_{\text{tank}} = \left\{\frac{1}{1 - R + (R \cdot f_{rem})}\right\} \cdot \left\{\frac{P_{waste}}{Q}\right\}$$

$$= \{\text{reuse amplification factor}\} \cdot \{\text{single pass outlet conc.}\}$$

Water Quality Within RAS

- Water quality exiting each culture tank depends on (Liao & Mayo, 1972):
  - ✓waste production rate, P_{waste}
  - ✓waste treatment units removal efficiency, f_{rem}
  - ✓waste flushing rate = fraction of flow reused, R
  - ✓recycle flow rate, Q

Fish Waste Capture



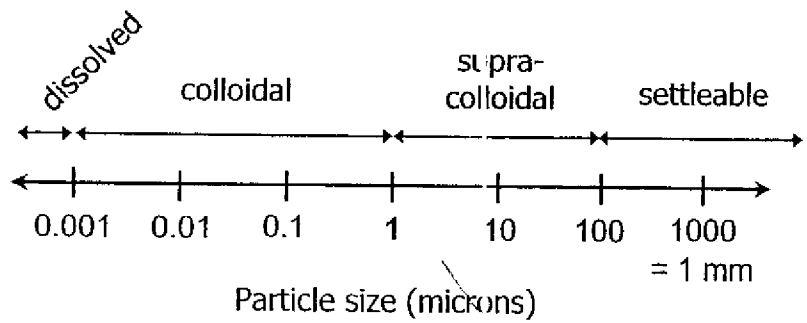
Fish Waste Capture

- RAS will require treatment processes for:
  - ✓Total Suspended Solids (TSS)
  - ✓Settleable Solids
  - ✓Biochemical oxygen demand (BOD_5)
  - ✓Total Phosphorus (TP)

  associated with particulate matter
  - ✓Nitrogen
    - Total Ammonia Nitrogen (TAN)
    - Nitrate Nitrogen (NO_3-N)
  - ✓Pathogens

Particulate Waste Capture

- Depends upon size characterization of waste:



Particulate Control

- Suspended solids adversely impact fish:
  - ✓damage gills;
  - ✓harbor pathogens;
  - ✓breakdown and degrade water quality.
- Suspended solids can mechanically plug:
  - ✓biofilters;
  - ✓aeration columns;
  - ✓orifices, screens, and spray nozzles.

Water Reuse Systems

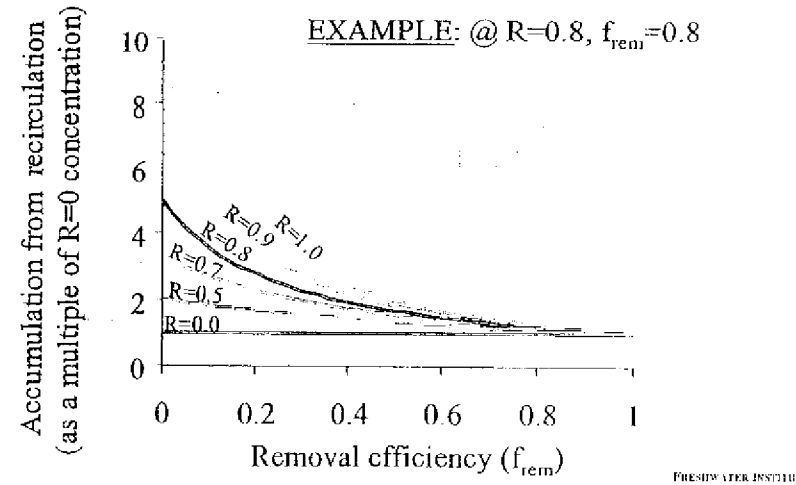
- Accumulation of wastes in culture water:
  - R > 0.99 to 0.999 for warm-water systems
  - R > 0.90 to 0.99 for cold-water systems

$$\text{waste}_{\text{accum}} = \frac{1}{f_{\text{rem}}} \cdot \frac{P_{\text{waste}}}{Q}$$

- ✓ inversely proportional to waste removal efficiency, f_{rem}
- ✓ not dependent on water exchange rate, R

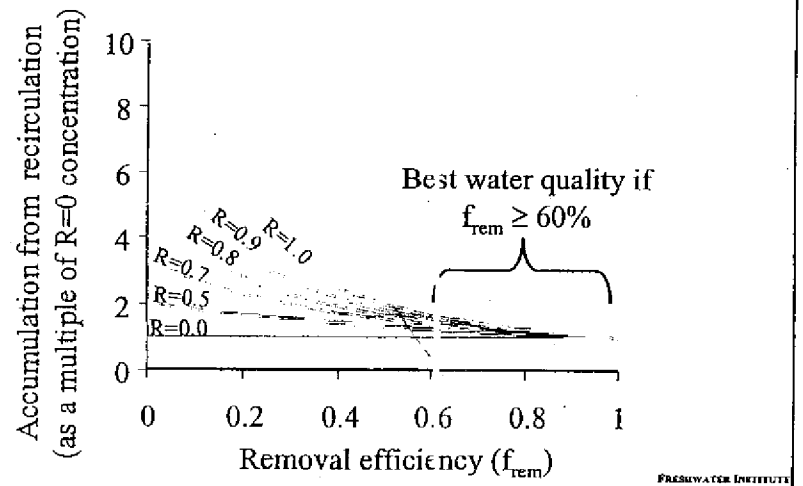
Water Quality Within RAS

- Treatment efficiency (f_{rem}) controls water quality!



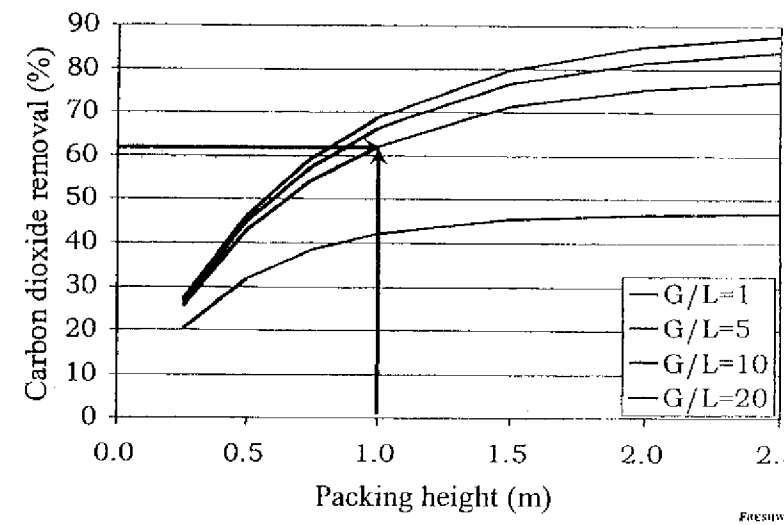
Water Quality Within RAS

- Treatment efficiency (f_{rem}) controls water quality!



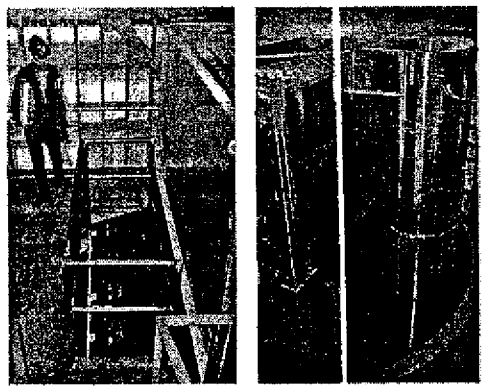
Water Quality Within RAS

- Example: Carbon dioxide removal efficiency.



Water Quality Within RAS

➢Example: Carbon dioxicle removal units.



(Nutreco's Big Tree Creek Hatchery for salmon smolt)

Water Quality Within RAS

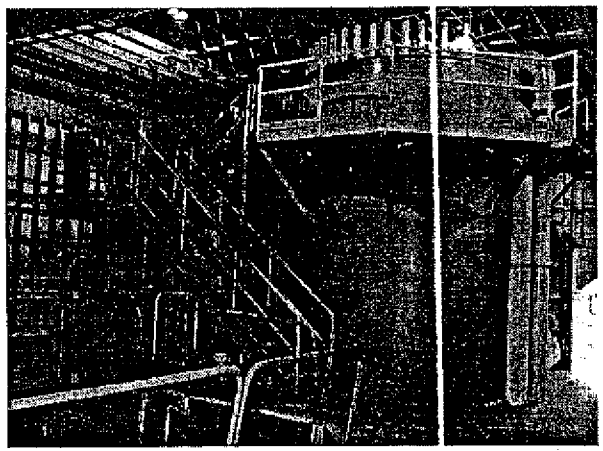
➢Example: TAN removal efficiency.

			TAN Removal Efficiency
			70-90%
			10-20%

(summarized by Timmons & Summerfelt, 1998)

Water Quality Within RAS

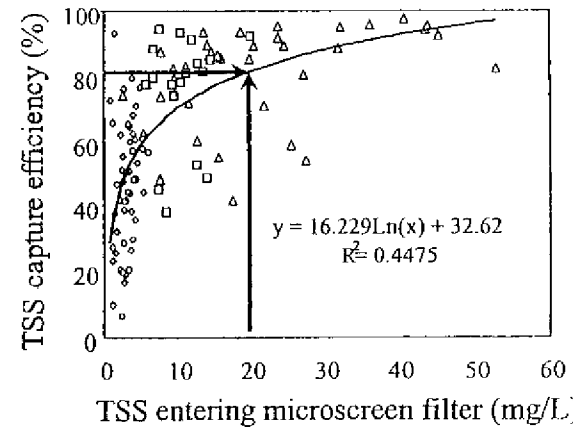
➢Example: TAN removal with f-s biofilters.



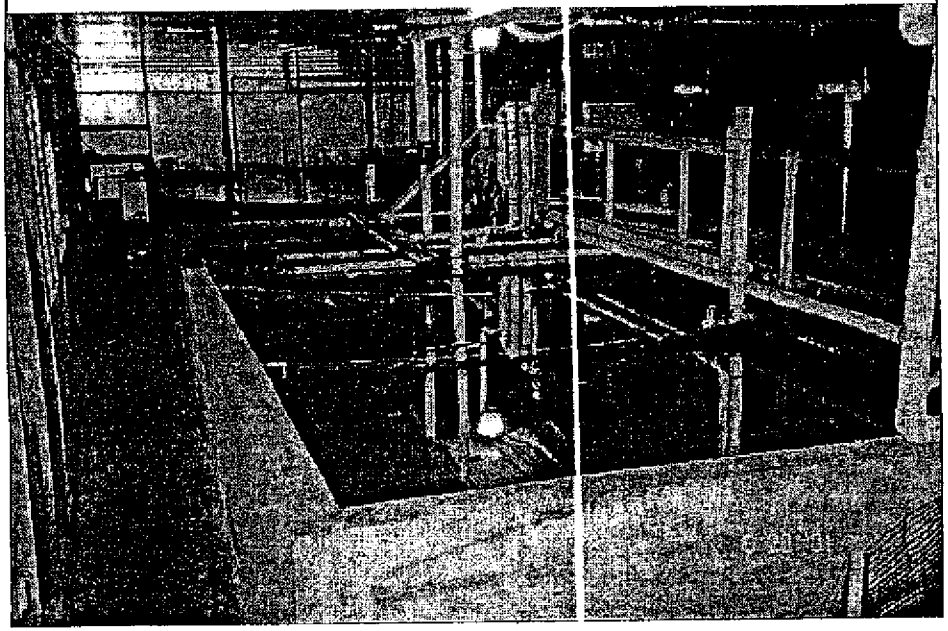
(Nutreco's Big Tree Creek Hatchery for salmon smolt)

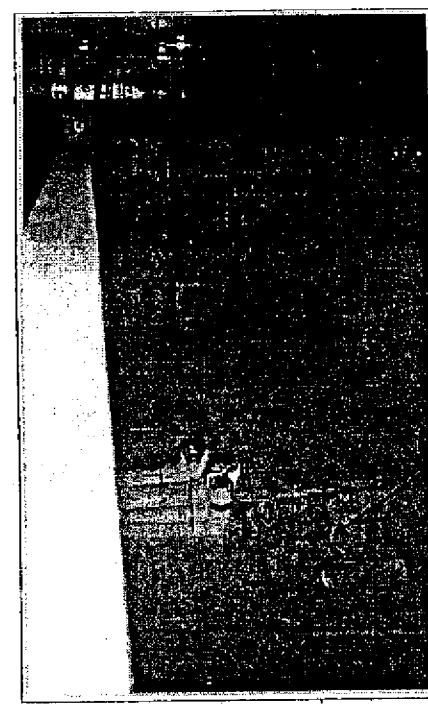
Water Quality Within RAS

➢Example: Solids capture efficiency.



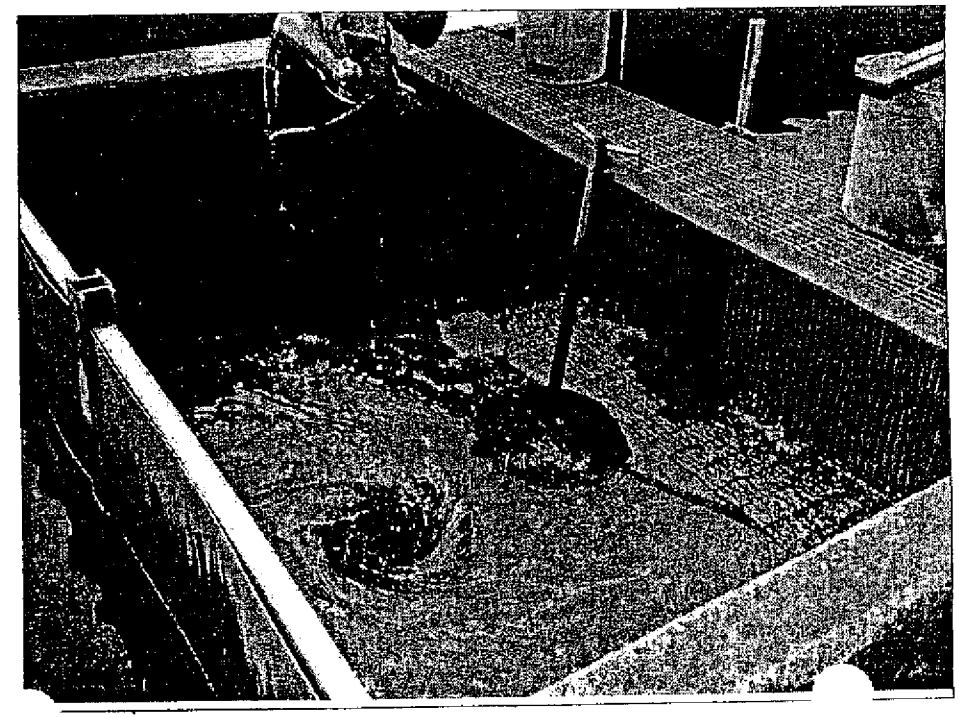
Settling Basins





Settling Basin Solids Removal

Pump pit for pumping solids out

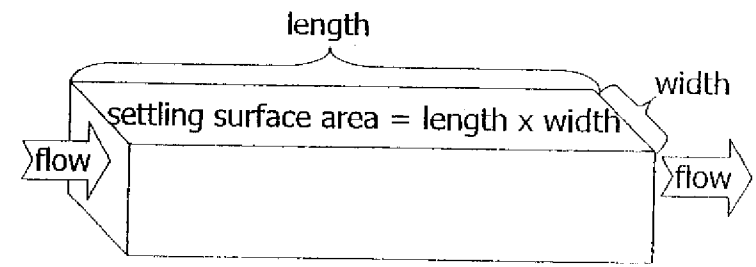


Solids Capture Technologies

- Gravity Separation
  - ✓Settling Basins
    - Quiescent Zones
    - Off-line Settling Basins
    - Tube/Plate Settlers
  - ✓Swirl Separators
- Physical Filtration
  - ✓Microscreen Filters (drum, disc, belt)
  - ✓Granular Media Filters

Settling Basins

- Overflow rates are used for design:



Settling Basins

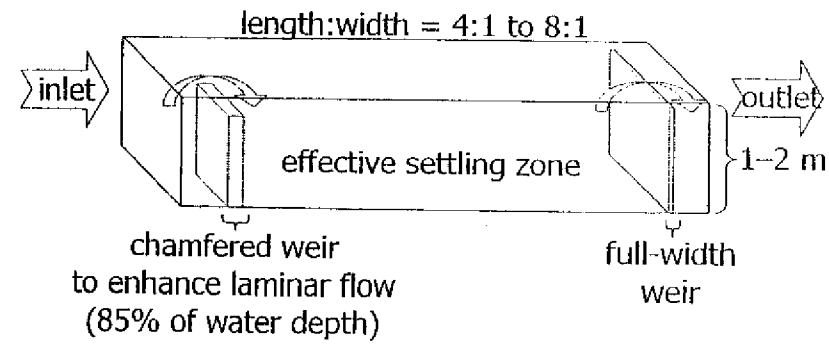
- Design overflow rates:

Type of Basin	Overflow rate ($m^3/m^2 \cdot sec$)
Full-flow settling basin	0.00400
Quiescent zone	0.00940
Off-line settling basin	0.00046

(Idaho Division of Env. Quality)

Settling Basins

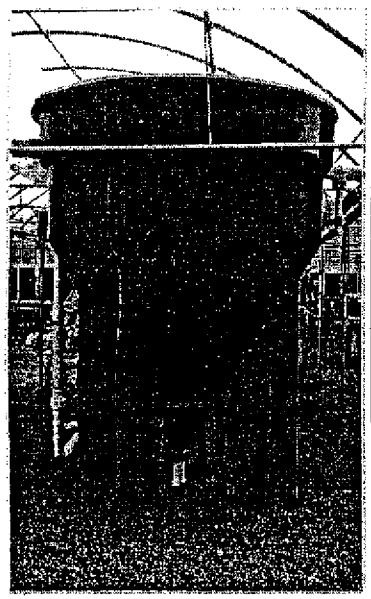
- Design to minimize turbulence:



Off-line Settling Basins

- Designed for solids collection, thickening and storage
- Intermittently loaded from
  - ✓quiescent zone cleaning
  - ✓filter backwashing
  - ✓system cleaning

Off-line Settling Tanks at Freshwater Institute



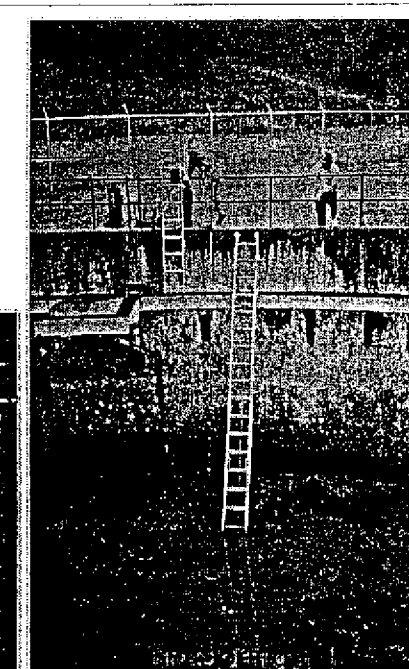
Off-line Settling Tanks

	TSS (mg/L)	TAN (mg/L)
In	5,147 ± 1,411	1.7 ± 0.1
Out	151 ± 24	19.5 ± 2.5

- 97% TSS Removal
- 10% TSS in Manure

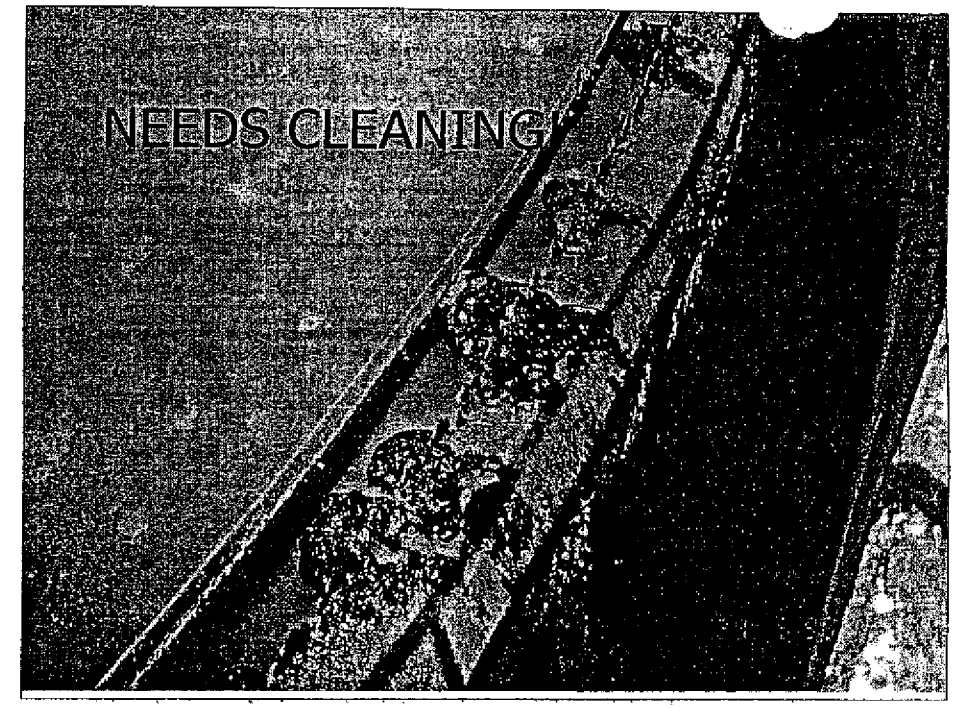
Off-line Settling Basins

LARGE structures with solids storage capacity

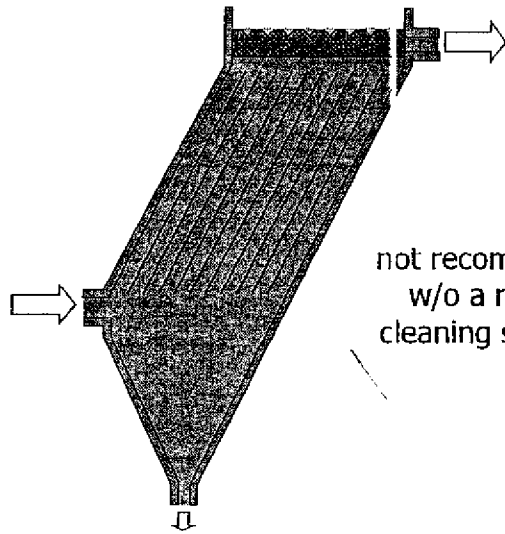


Settling Basins

- Advantages
  - ✓Simple
  - ✓Low maintenance
- Disadvantages
  - ✓requires more space than other alternatives
  - ✓most effective at inlet TSS > 10 mg/L
  - ✓difficult to achieve effluent TSS < 6 mg/L
    - solids storage allows for resuspension and nutrient leaching (Henderson and Bronmage, 1988)



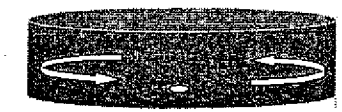
Tube/Plate Settlers



not recommended w/o a regular cleaning schedule

Swirl Separators

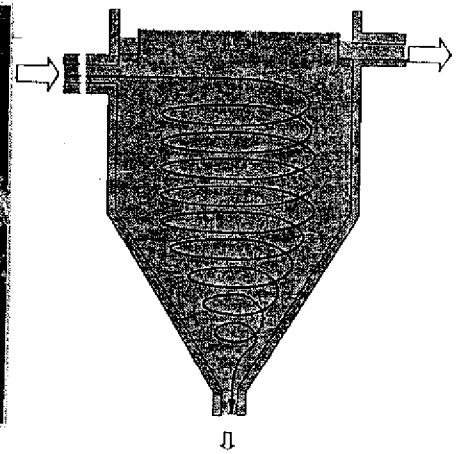
- Swirl Settlers:
  - ✓rotating flow creates secondary radial flow
    - transports settleable solids to bottom center
  - ✓concentrates settleable solids in a small underflow
    - underflow can be 5-10% of total flow
  - ✓low head requirement



primary rotating flow

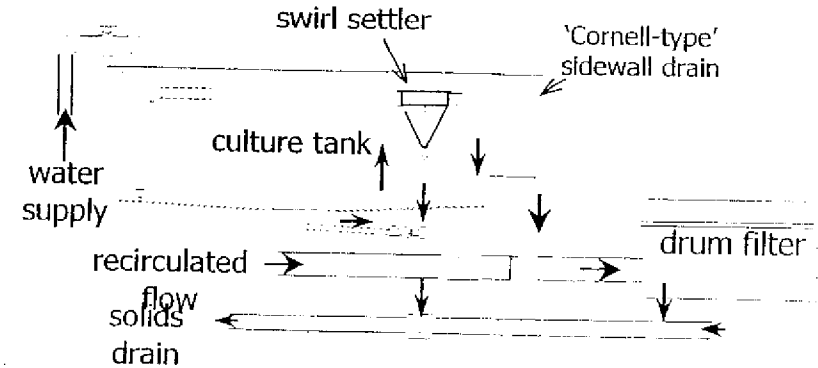
secondary radial flow

Swirl Separators



Swirl Separators

- Treat bottom-drain flow from dual-drain tanks



Swirl Separators

- Advantages
  - ✓do not store solids
    - reducing particulate dissolution and nutrient leaching
  - ✓requires less space than settling basins
- Disadvantages
  - ✓only effective removing
    - solids with specific gravity considerably > water
    - larger particles
  - ✓hydraulics are critical

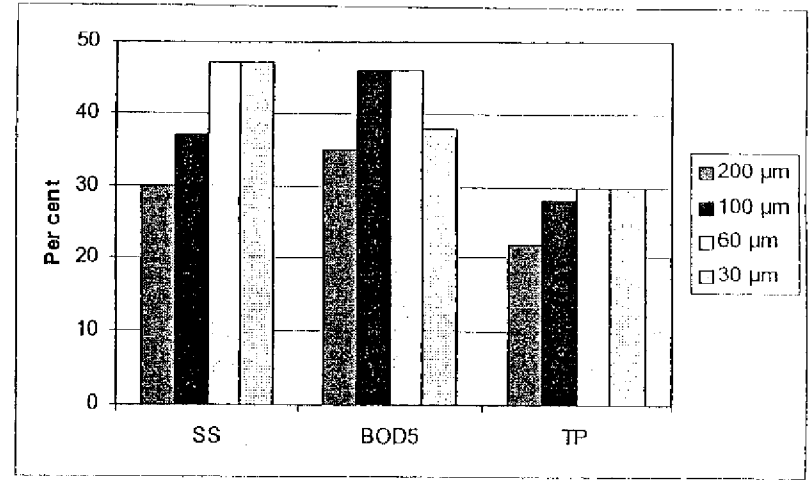
Microscreen Filters

- Sieves that strain water-bound particles
- Frequent backwash removes solids rapidly
- Produces a backwash 0.2 to 2% of the treated flow

Microscreen Filters

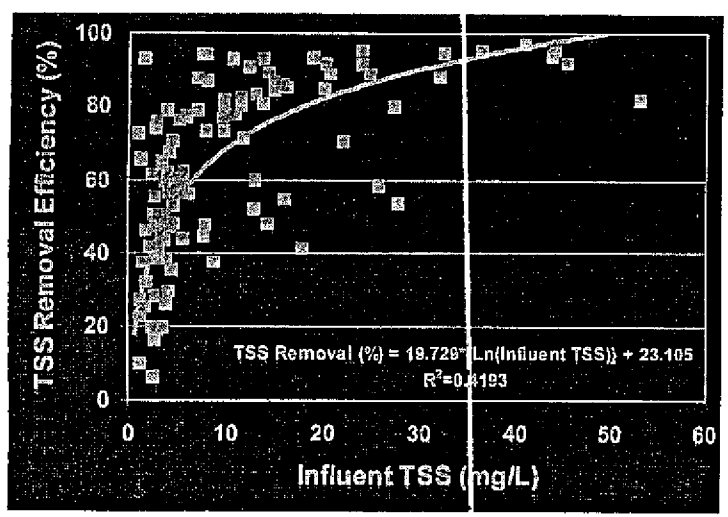
- Microscreen openings range from 20–100 μm
  - ✓ smaller vs. larger openings:
    - smaller removes a little more TSS
    - larger requires less filter area & fewer wash cycles
    - larger requires less pressure wash
    - larger generates less backwash flow
      - more concentrated waste discharged
  - ✓ Several report ~60–100 μm openings provide optimum performance

Microscreen Filters

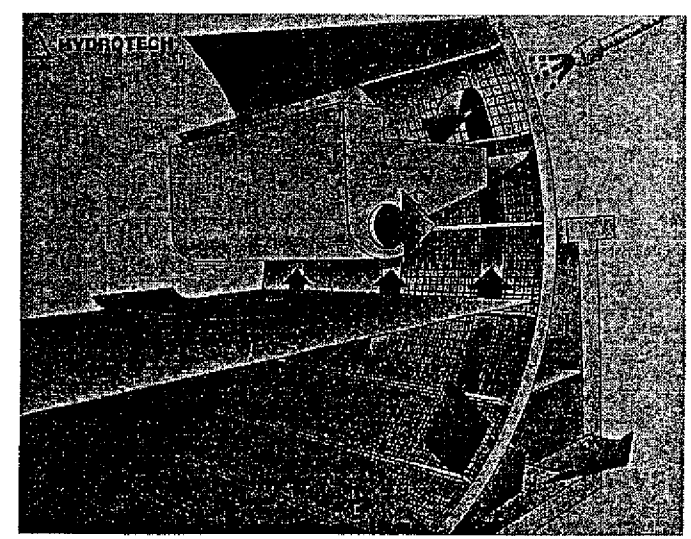


Removal of SS, BOD_5 and TP versus reduced screen mesh size from 200 to 30 μm. Effluent water from two Scottish hatcheries (Kelly, et al. 1997).

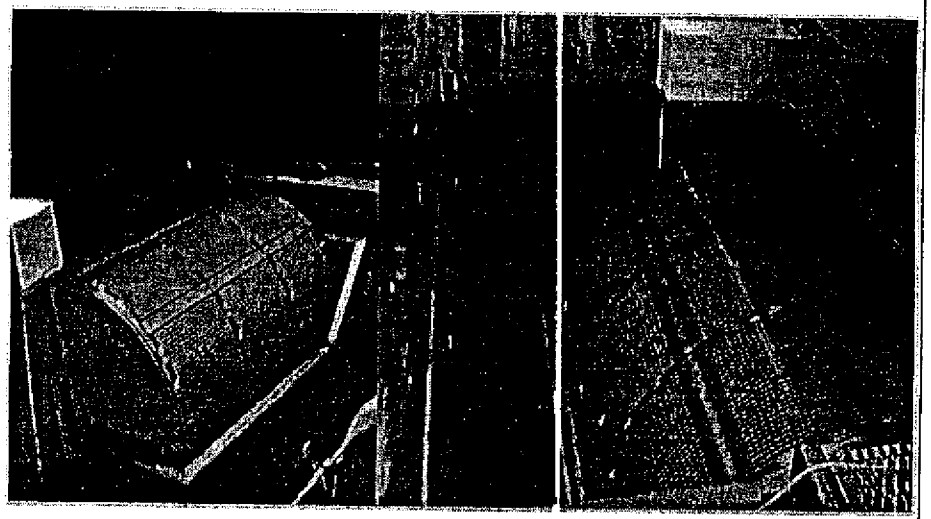
Microscreen Filters



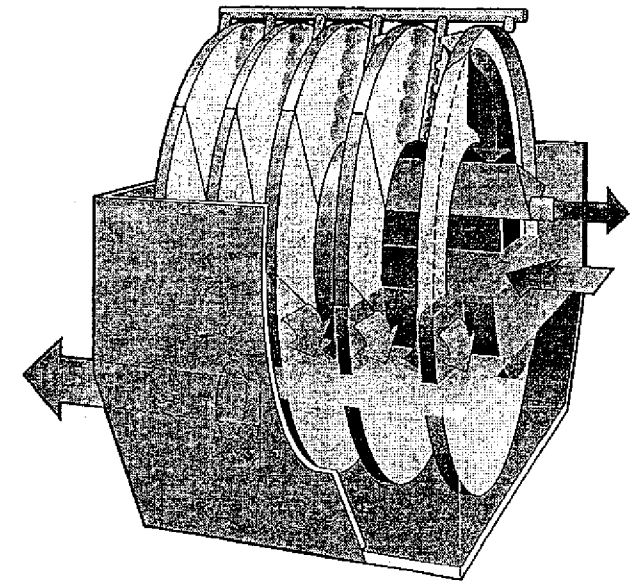
Drum Filters



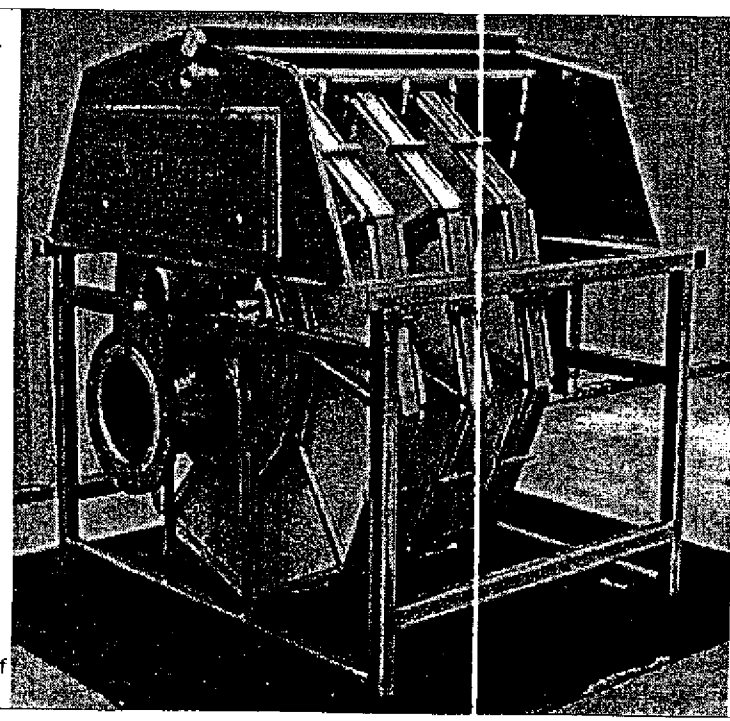
Drum Filters



Disc Filters

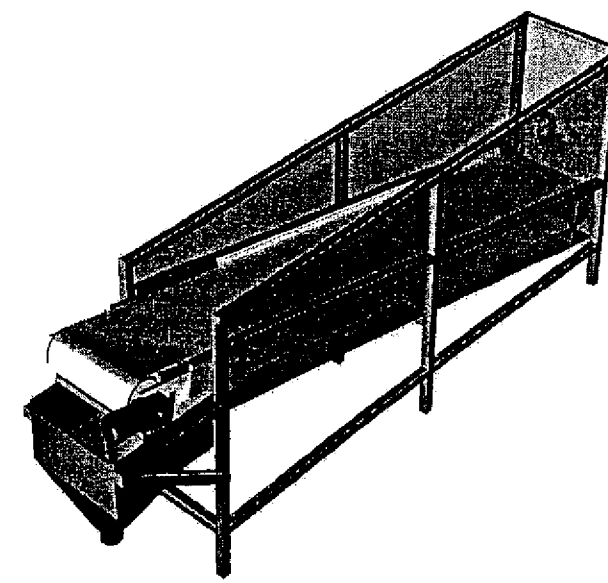


Courtesy of Hydrotech



Courtesy of Hydrotech

Belt Filters



Microscreen Filter Comparison

Filter Type	Removal Rate at 60–100 μm (%)	Costs* (USD/unit)
Drum	SS inlet < 5 mg/L: 31–67 SS inlet > 50 mg/L: 68–94	15,000
Disc	SS inlet < 5 mg/L: 25–68 SS inlet > 50 mg/L: 74–92	8,600
Belt	SS inlet < 5 mg/L: 0–62 SS inlet > 40 mg/L: >89	18,000

*Costs at a unit flow capacity of 10 m^3/min & 100 μm screen

Microscreen Filter Comparison

Filter Type	Advantages	Disadvantages
Drum	Intermittent backwash, reduced backwash volume	
Disc	Lowest capital costs	-High backwash flow volume -Grinding/crushing of bigger particles
Belt	-Gently removes particles -Low maintenance	High capital costs at low flow (< 3–5 m^3/min)

Microscreen Filters

- Advantages
  - ✓large water treatment capacity in small space
    - treat flows from 0.4 m^3/min to 50 m^3/min
  - ✓low pressure drop (< 0.3 m)
  - ✓modular and relatively easy to install
  - ✓rapidly removes solids from bulk flow
    - does not store solids within flow
    - reduces particulate break-down and nutrient leaching
  - ✓removes majority of particles > 40 μm

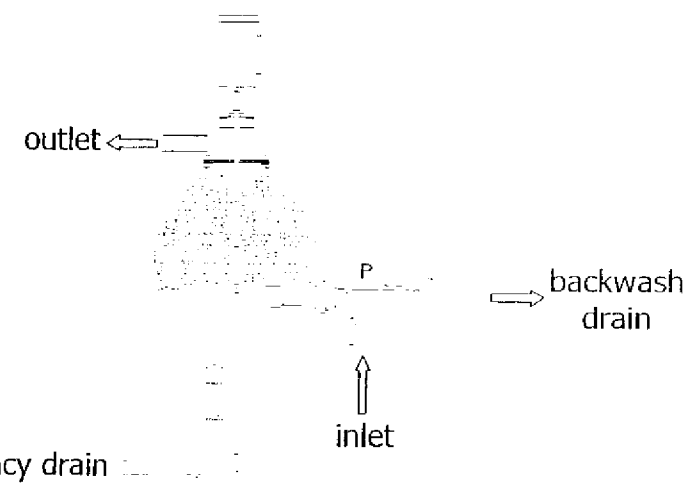
Microscreen Filters

- Disadvantages
  - ✓requires 414–690 kPa pressure wash system
  - ✓mechanical and requires service
    - pressure wash failures
    - screen and gasket maintenance
  - ✓does not capture particles < 20 μm
  - ✓large surges in flow and concentration may cause partial flow-bypass around unit

Granular Media Filters

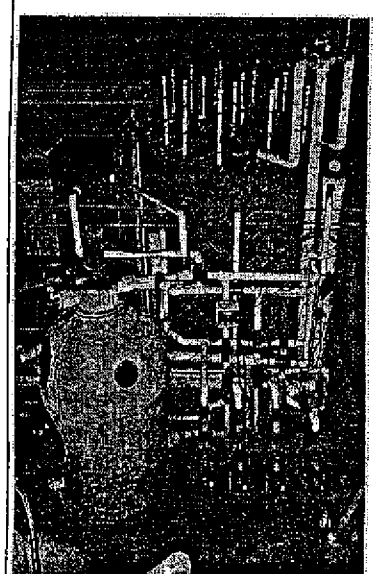
- Gravity sand filters
  - ✓effective at removing fine solids
  - ✓relatively expensive
  - ✓large backwash requirements
  - ✓not often used unless required by effluent regulations

Granular Media Filters



Courtesy of AST (LA)

Bead Filter/Fluidized Bed Combination



Ornamental Fish Hatchery (USA)

Shrimp Hatchery (Ecuador)

Courtesy of AST (LA)

Courtesy of AST (LA)

Super-Size

450 ft^3 Bead Filter

Mora NFH (NM)

Granular Media Filters

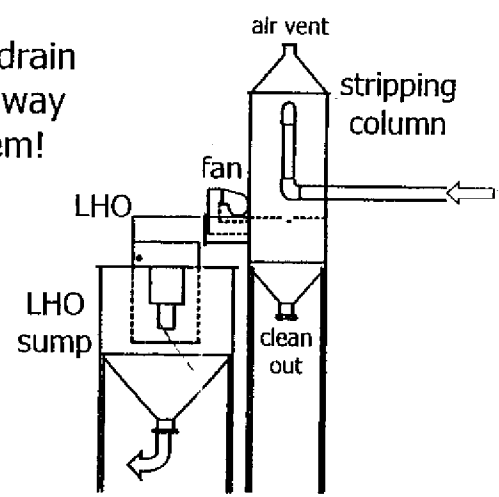
- Pressurized-bead filters -- Advantages
  - ✓effective at removing fine solids
    - plastic beads may have an affinity for fine solids
  - ✓modular and relatively easy to install

Granular Media Filters

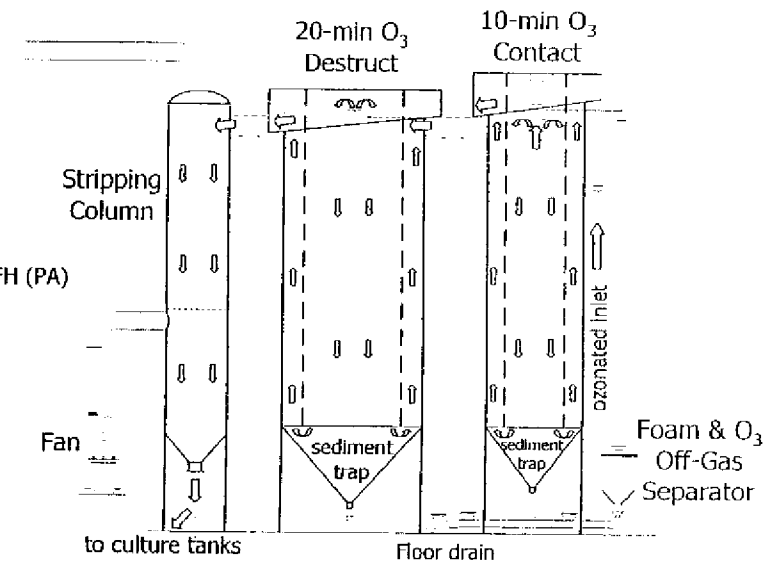
- Pressurized-bead filters -- Disadvantages
  - ✓captured solids are stored in the flow path
    - 30–40% of captured solids can degrade between 24-hr backwash cycles (Chen et al., 1993)
  - ✓pressure drop up to 15 psig
  - ✓Solids subjected to turbulence
  - ✓Filter backwash management can be complex

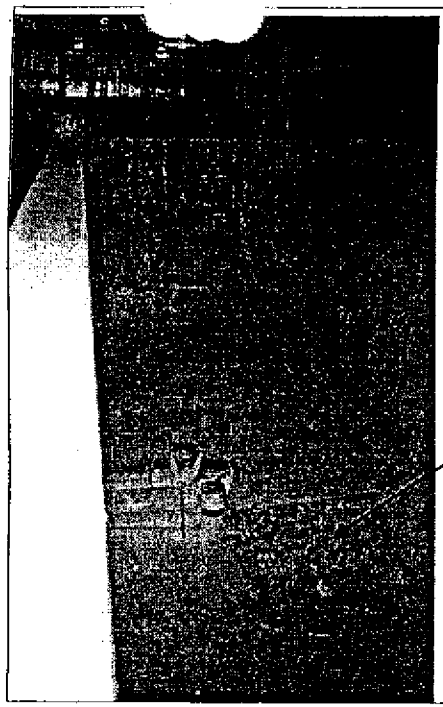
Install Clean-Outs When Possible

- When cleaning, drain pipes & sumps away from reuse system!



Install Clean-Outs When Possible





Instead of clean-outs you can design basins with sloped floors and pump "pits" for pumping solids out

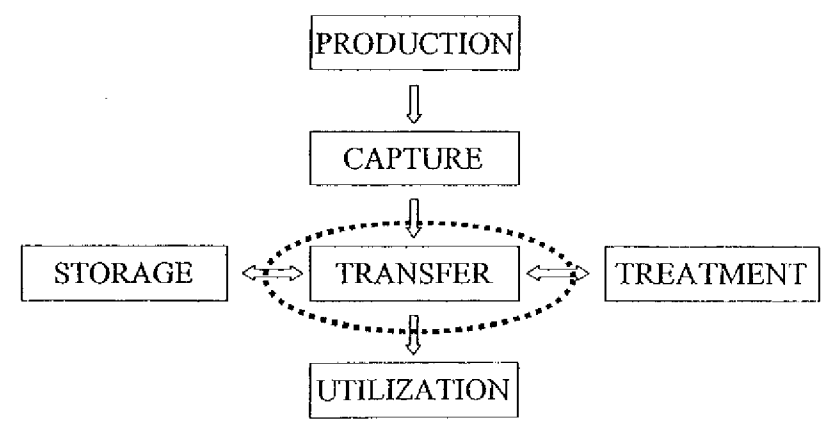
Other Solids Capture Considerations

- Tank, channel, and pipe cleaning routines:
  - ✓produce fluctuations in
    - discharge flowrates
    - consistencies and concentrations of wastes
    - increase TMDL
  - ✓contingencies to contain cleaning flows:
    - divert cleaning flows away from recirculating system
      - e.g., to off-line settling ponds

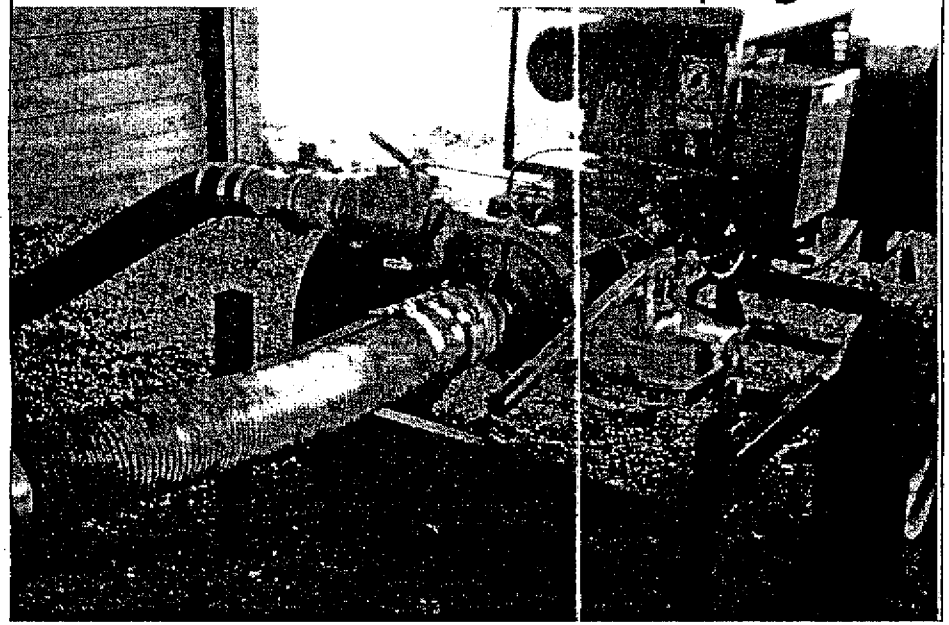
Solids Management

- Treatment processes result in captured solids that must be managed:
  - ✓Transfer
  - ✓Storage
  - ✓Thickening and dewatering
  - ✓Biosolids utilization and disposal
  - ✓Discharge of overflow/backwash
  - ✓Stabilization of solids for pathogen destruction

Solids Transfer

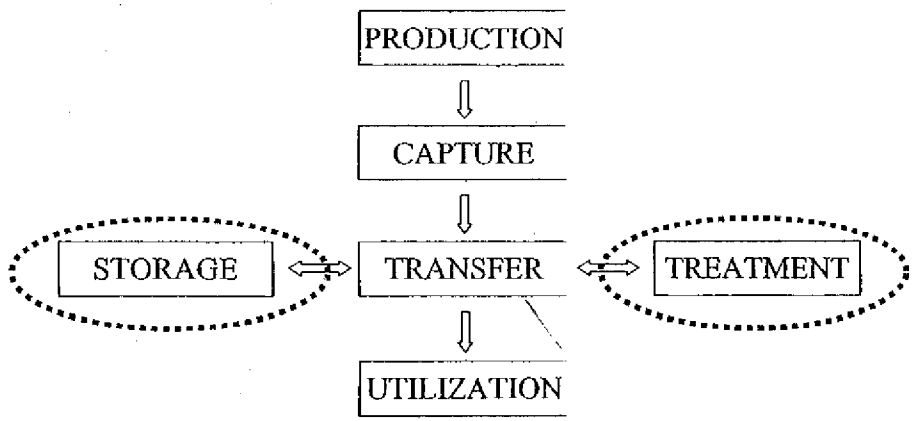


Solids Transfer - Pumping

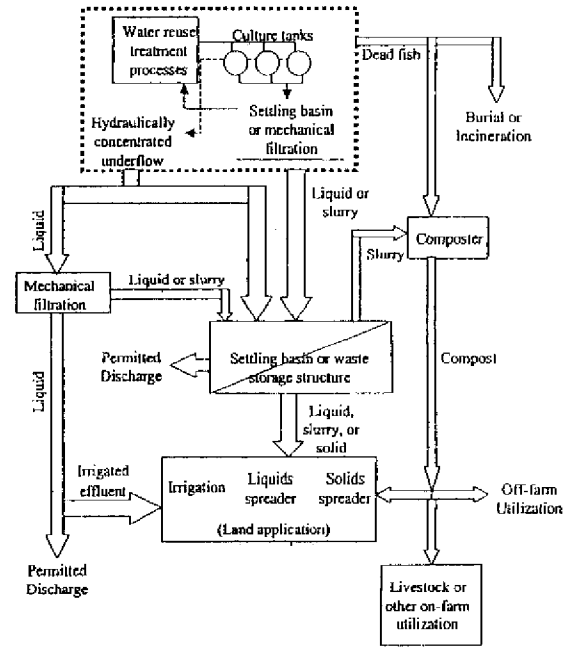


Solids Transfer – Vacuum Tank

Solids Storage & Thickening



Waste Management in RAS



Solids Storage & Thickening

- Captured solids require further dewatering:

	TSS	Reference
Microscreen Filter Backwash	0.01-0.8%	Bergheim and Forsberg, 1993
Quiescent Zone Siphon	3-5%	Idaho DEQ, 1998
Quiescent Zone Siphon	3-5%	Westers, 1991

Solids Thickening

- Solids must be thickened (dewatered) to reduce disposal costs/management.
  - ✓Dewatering reduces sludge volume.
  - ✓Sludge volume for 1,000 lb dry weight solids:
    - 1595 ft^3 1% TSS
    - 319 ft^3 5% TSS
    - 160 ft^3 10% TSS
    - 106 ft^3 15% TSS

Solids Thickening Methods

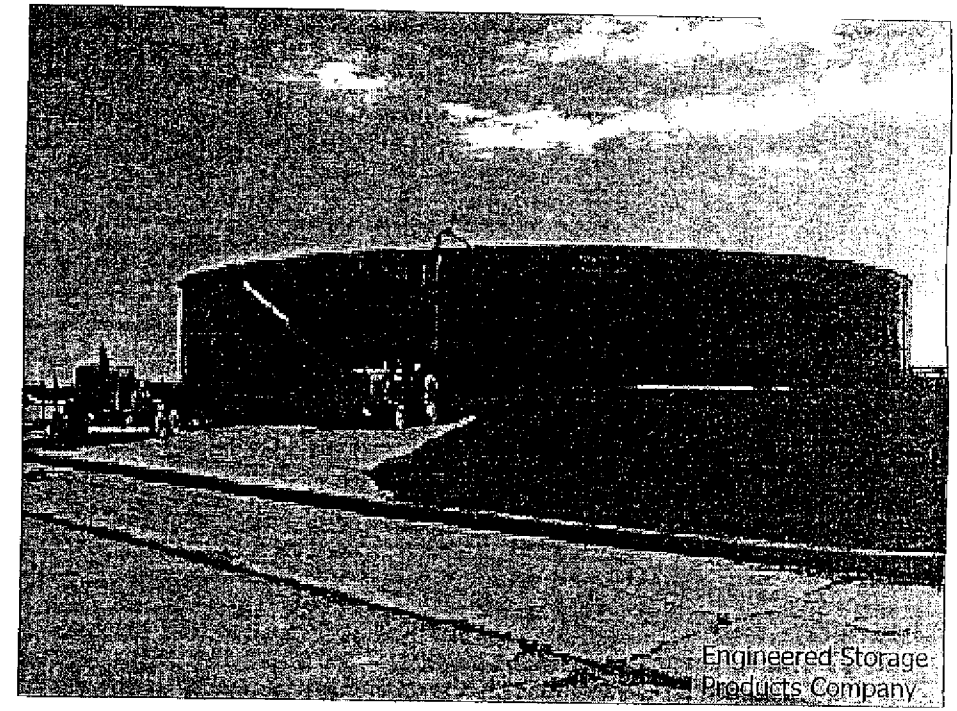
- Processes to thicken clarifier/filter backwash solids:
  - ✓offline settling basins (sludge thickening tanks)
  - ✓reed beds
  - ✓vacuum filters
  - ✓wedgewire beds
  - ✓filter presses
  - ✓centrifuges

Off-line Settling Basins

- Designed for solids collection, thickening and storage
- Intermittently loaded from
  - ✓quiescent zone cleaning
  - ✓filter backwashing
  - ✓system cleaning

Solids Storage – Thickening Tanks

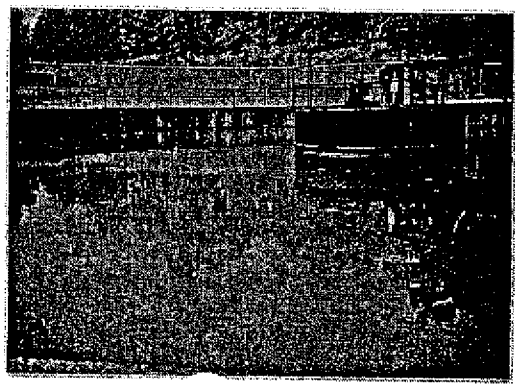
- Slurrystore Tanks (Engineered Storage Products Company)

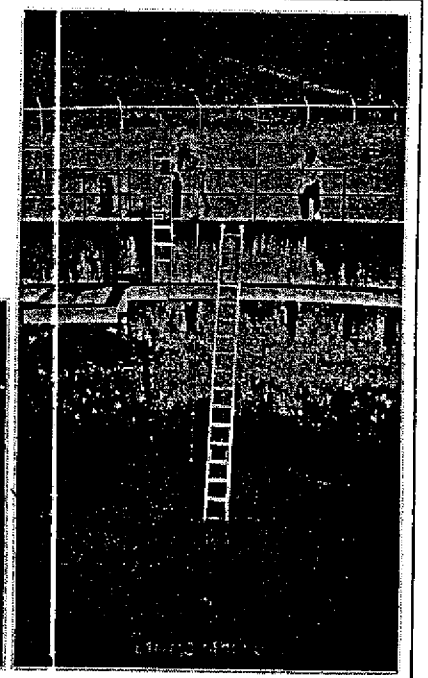


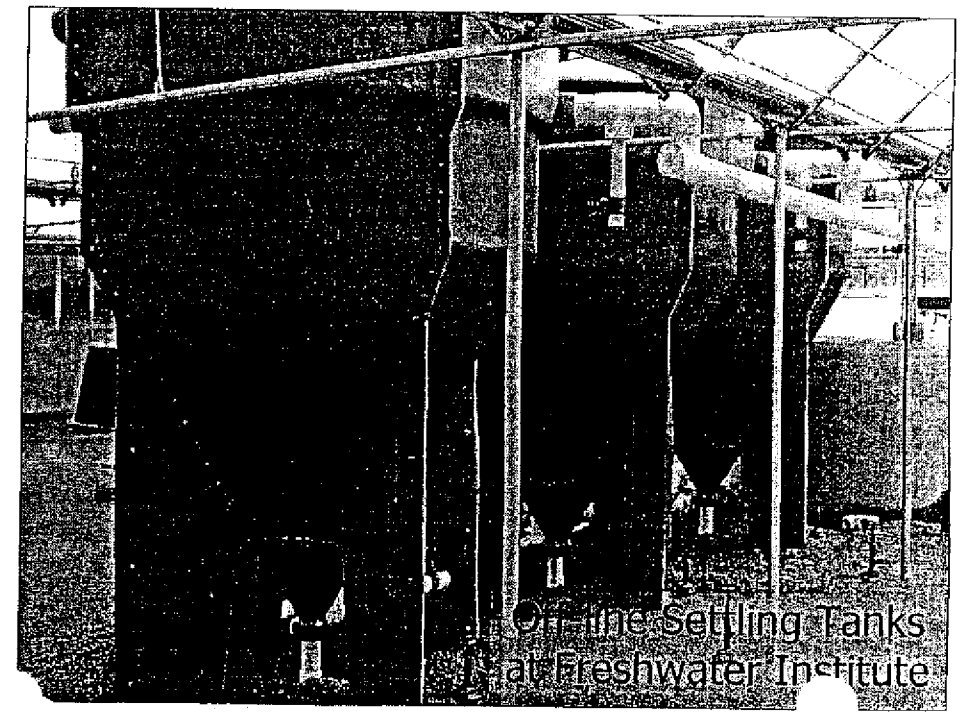
Engineered Storage Products Company

Off-line Settling Basins

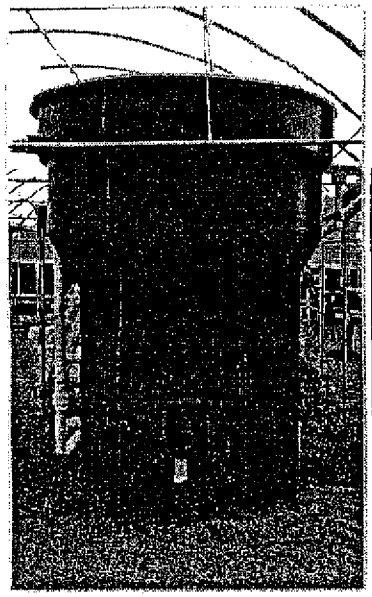
LARGE structures with solids storage capacity







Off-line Settling Tanks at Freshwater Institute



Off-line Settling Tank Performance

	TSS (mg/L)	TAN (mg/L)
In	5,147 ± 1,411	1.7 ± 0.1
Out	151 ± 24	19.5 ± 2.5

- 97% TSS Removal
- 10% TSS in Manure

Off-line Settling Basins

- Idaho DEQ (1998) design criteria for off-line settling basins:
  - ✓ overflow rate of 0.0015 ft^3/sec flow per ft^2 surface area
  - ✓ usually 3.5 ft deep
  - ✓ usually built in pairs
  - ✓ tank MUST capture 85% TSS
  - ✓ TSS effluent CANNOT exceed 100 mg/L in 8 hr composite
  - ✓ settleable solids effluent CANNOT exceed 1.0 ml/L in any sample

Off-line Settling Basins

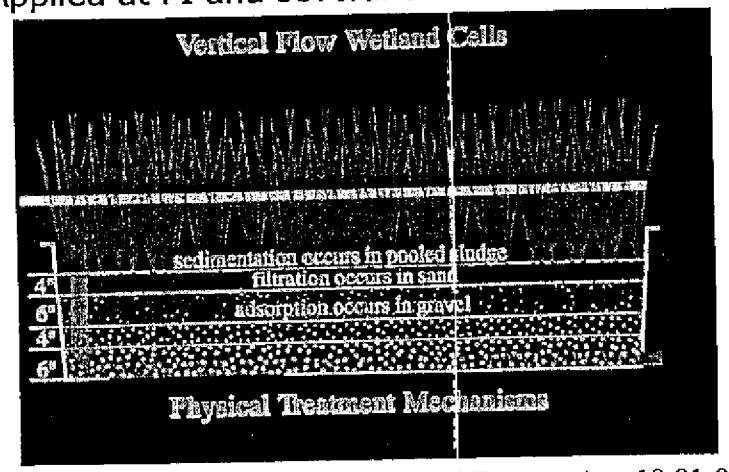
- Bergheim et al. (1993) design criteria for off-line settling basins:
  - ✓ overflow rate of 0.001 ft^3/sec flow per ft^2 surface area
  - ✓ captures 85-90% solids
  - ✓ thicken solids to 5-10% dry matter

Off-line Settling Basin Solids Removal

- OPTION 1: Decant tank, harvest solids with backhoe or front end loader
  - ✓ Let solids dry for several days to 25% to 35% dry weight
- OPTION 2: Sprinkler application to adjacent fields
  - ✓ 0.2% solids dry weight (after mixing solids)
- OPTION 3: Decant tank, then pump out manure
  - ✓ 12% avg. solids dry weight
  - ✓ 20% max. solids dry weight
  - ✓ pumping method influences % solids removed

Reed Drying Beds

- Applied at FI and several other RAS facilities.

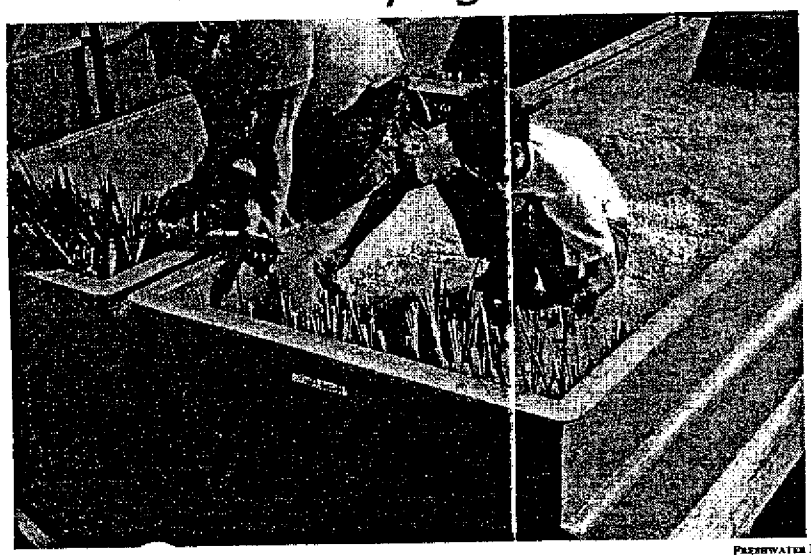


(Summerfelt et al. 1999. *Aquacultural Engineering*, 19:81-92.

Reed Drying Beds

- Reed drying beds are created wetlands:
  - ✓combine solids dewatering and disposal
  - ✓sand drying bed planted with reeds
    - plants facilitate dewatering
  - ✓loading
    - 30-60 kg dry solids per year per m^2 area
    - 7-10 cm sludge at 2% solids every 7-21 days
    - series of beds receive sequential batches
  - ✓store solids for 10 years

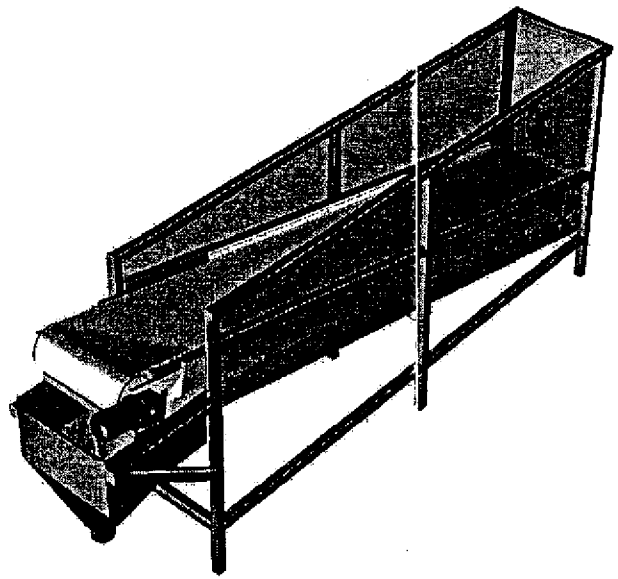
Reed Drying Beds



Reed Drying Beds

- Reed drying bed treatment efficiency
  - ✓96-98% TSS
  - ✓72-91% COD
  - ✓82-93% TKN and P
  - (Summerfelt et al., 1999)

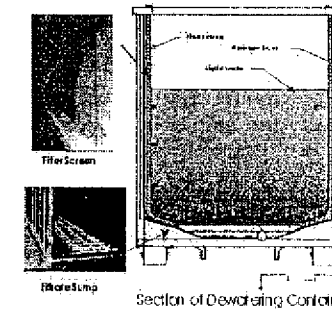
Belt Filters



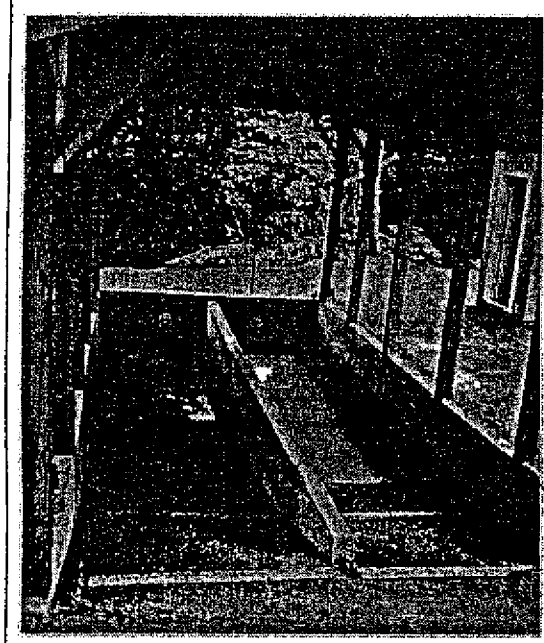
Wedgewire Screen Filters

- Wedgewire screens
  - ✓ Dewatering capacity: < 20,000 gallons
  - ✓ Cycle Time: ½ - 4 days
  - ✓ Percent Solids: 10-30%

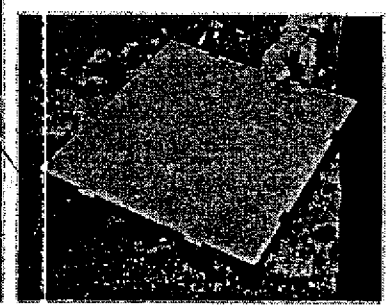
(Green Mountain Technologies)



Section of Dewatering Container



Wedgewire Screen Filter



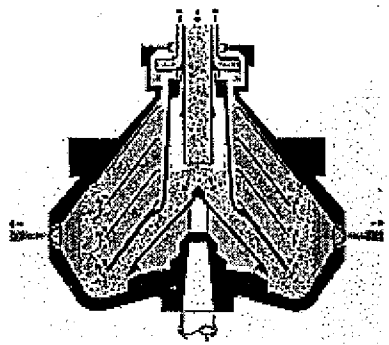
Sweetwater Trout Farm (NC)

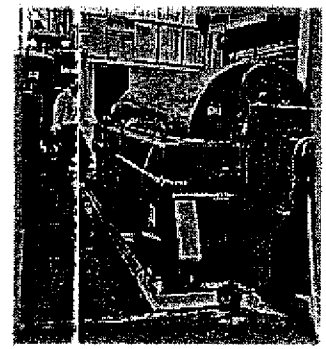
Wedgewire Screen Filter Operation

Sweetwater Trout Farm (NC)

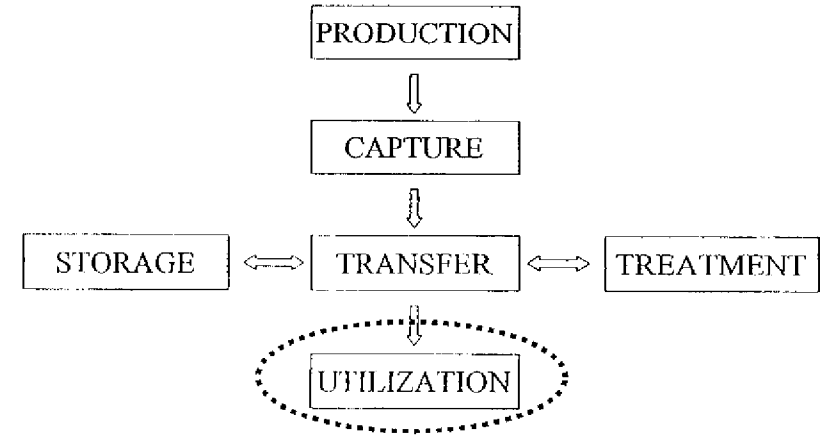
Centrifuge Dewatering

- Alfa Laval Centrifuges:





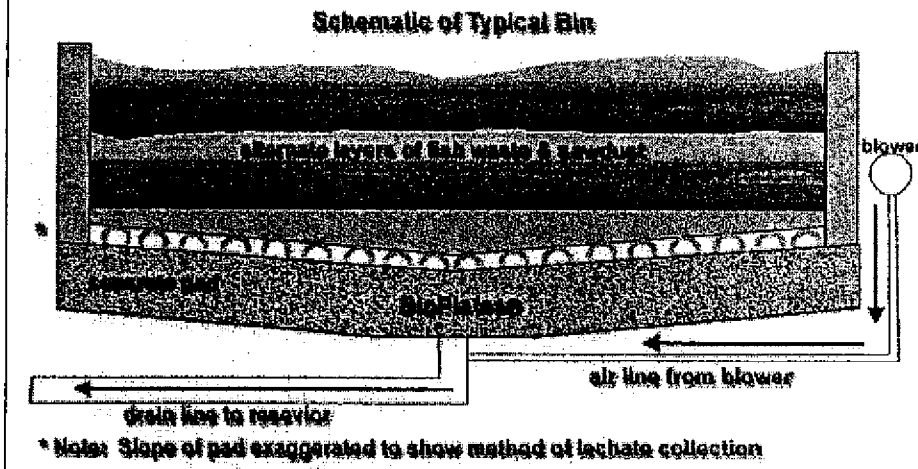
Solids Storage



Utilization/Disposal

- Composting
- Land Application
  - ✓Slurry (<1% solids)
  - ✓Thickened Sludge (>5% solids)
- Contract hauling

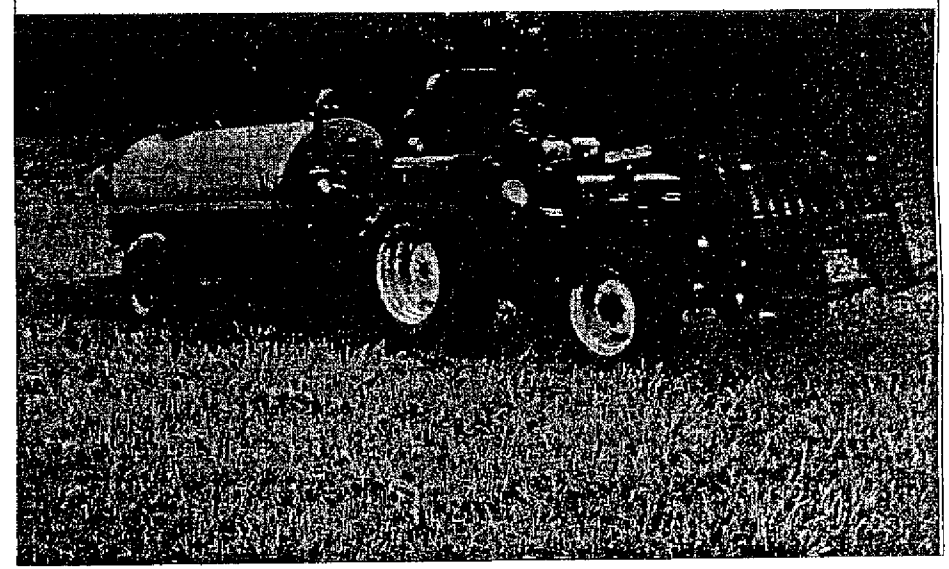
Composting



Land Application

- Liquid/Slurry Application
  - ✓ Solids are easily transferred and distributed when they are >1% solids
  - ✓ Designed as a Slow Rate Land Treatment (crop irrigation)
- Thickened Sludge Application
  - ✓ Designed as a soil amendment or fertilizer (as part of a crop nutrient management plan)
  - ✓ Applied from tanker trucks: surface spreading, incorporation, direct injection

Land Application



Created Wetlands For Effluent Treatment

- To further treat the clarfied or filtered overflow from a fish farm.

Hydroponics for Effluent Treatment

- Further treatment of the clarfied or filtered overflow from a fish farm.
  - ✓Example: lettuce or basil production

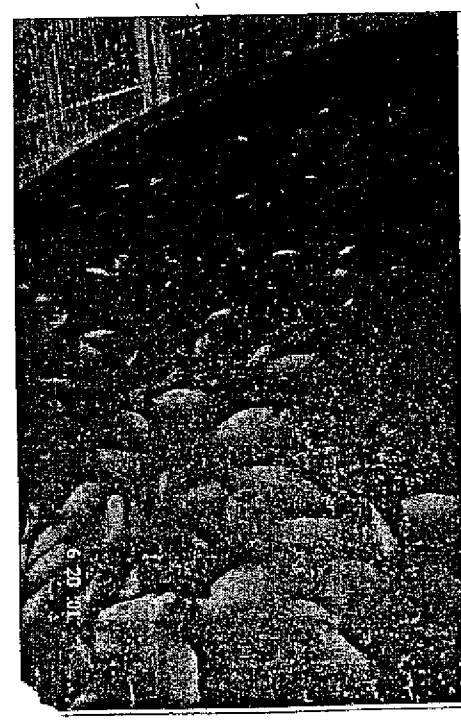
Adler, et al. 2000. *Horticultural Science*, 35(6): 993-999.
Adler, et al., 2000. *Internat. J. Recirc. Aquacult.*, 1: 15-34.

Hydroponics for Effluent Treatment

- Other fruits, vegetables, and herbs can be produced in plant-based treatment

Turf Grass Production

- Further treatment of the clarfied or filtered overflow from a fish farm.

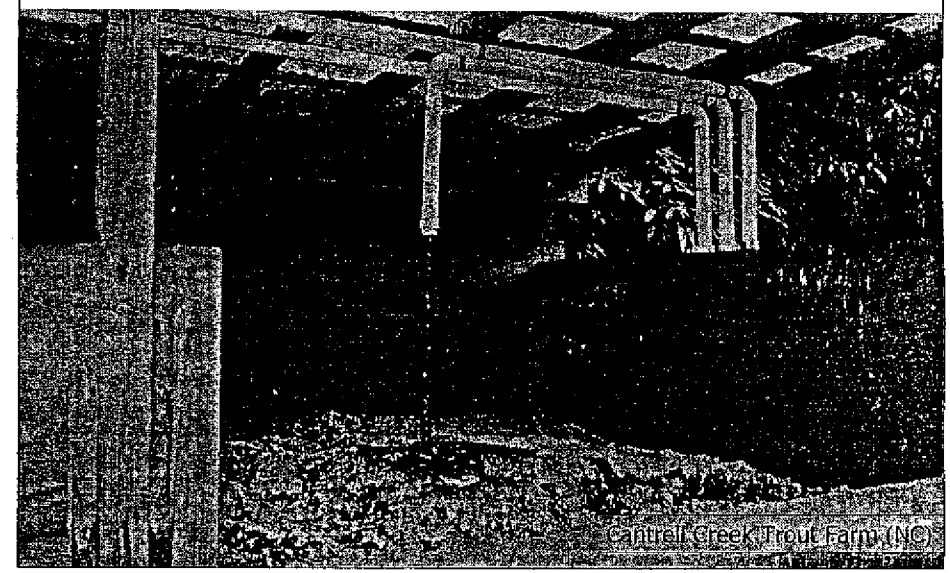


Case Study in Solids Management

Starts with the Fish!
(and feed)

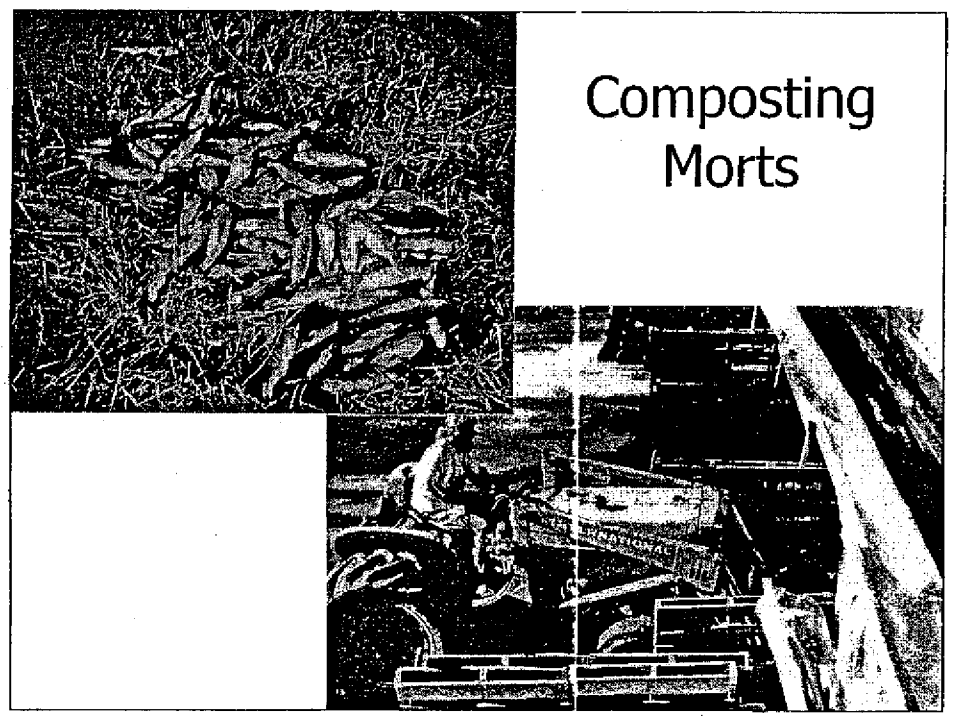
Composting Bin

Composting Fish Manure

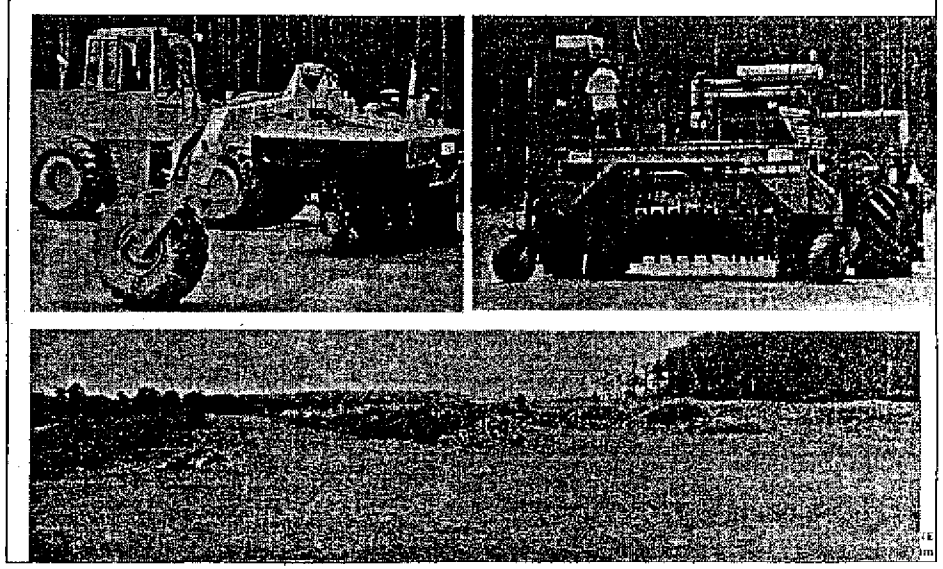


Cantrell Creek Trout Farm (NC)

Composting Morts

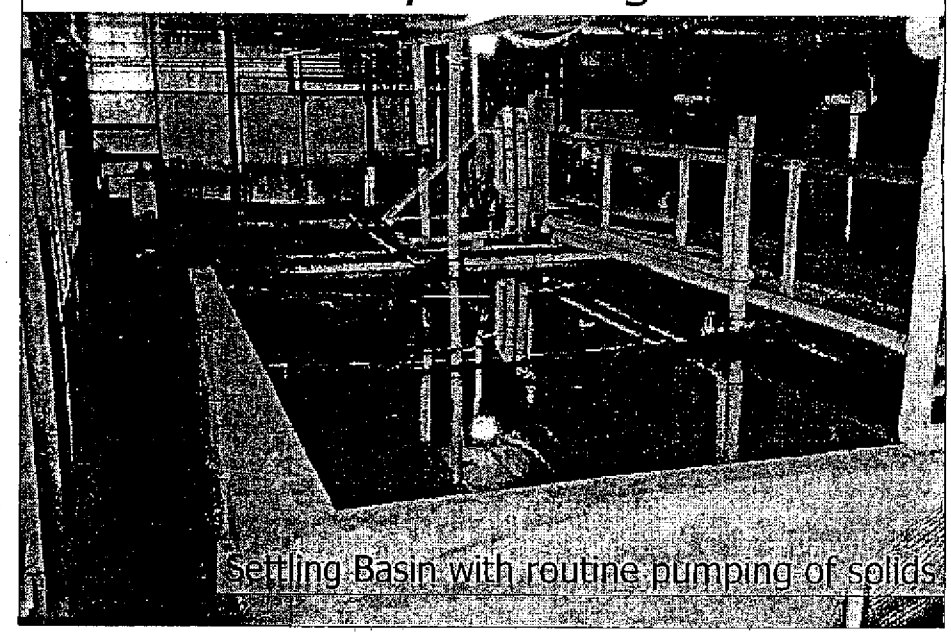


Large-Scale Composting

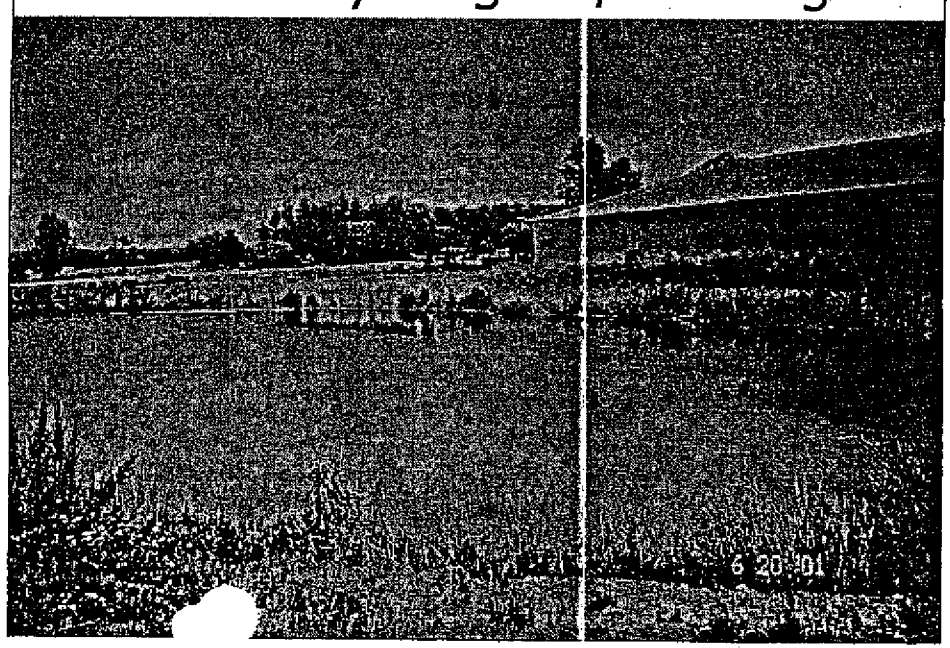


Case Study: Culture Tanks

Case Study: Settling Basin



Case Study: Lagoon/Discharge



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An Assessment of the Environmental Impact Of Alabama Channel Catfish Farming

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Department of Fisheries and Allied Aquacultures
Auburn University, Alabama 36849

Channel catfish farming and other types of aquaculture have grown rapidly in the past few decades, and it finally has become an activity large enough to attract the attention of environmentalists. As a result, the United States Environmental Protection Agency has been coerced by environmental interests to enforce the Clean Water Act in the aquaculture sector. The nature of the regulations and the timing of their implementation are uncertain. However, it is virtually certain that aquaculture pond effluents will be subjected to permitting within the next few years. The Alabama Catfish Producers Association intends to be proactive and become involved in the process through which regulations will be formed. Thus, they provided funding to Auburn University to conduct an environmental assessment of channel catfish farming in Alabama. This study focused on catfish farms in Bibb, Dallas, Greene, Hale, Marengo, Perry, and Tuscaloosa Counties in West Central Alabama where most of the catfish production of the state is realized.

There are about 25,000 acres of ponds with 10.7% of the area for fry and fingerlings and 89.3% for food fish. Most production is from watershed ponds filled by rainfall and runoff, but water levels in many of these ponds are maintained in dry weather with well water. There are some embankment ponds supplied by well water. In 1997, the production of food fish was near 90,000,000 pounds, so the average, annual production per acre was about 3,600 pounds.

Because ponds are filled and maintained primarily with runoff, the water supply is seasonal and water conservation measures must be used. Farmers attempt to store as much runoff in ponds as possible so that water levels can be maintained during summer and fall when there is little runoff. Fish are harvested by seining without drawdown of water levels. However, after about 5 or 6 years, it is necessary to partially drain ponds and renovate fish stocks. After about 15 years, ponds may be completely drained to renovate the embankments and bottoms. Thus, in a 15-year period, about two pond volumes of water are intentionally discharged from ponds, but storm overflow occurs after winter and spring rains.

Although the Alabama industry does not rely heavily on groundwater, as does catfish farming in other southern states, it does use an estimated 23,000,000 gallons of groundwater per day from wells. Seepage from ponds more than replaces the amount of groundwater withdrawn for use in ponds. Thus, Alabama catfish farming does not deplete groundwater supplies on a long-term basis.

Catfish ponds in Alabama serve the dual purpose of fish production and flood control. The large area of ponds retains runoff after heavy rains and releases it gradually. Flooding by larger streams in the area has greatly diminished since catfish farming became a major activity. Overflow from ponds occurs primarily during cool months when fish in ponds are not being fed

at high rates and when pond water quality is generally good. Also, pond overflow occurs at the time that stream flow is high. The timing of overflow from ponds greatly reduces the potential for stream pollution by effluents from ponds.

When ponds are partially drained for harvest, the first 75 to 80% of pond volume released tends to have the same composition as pond water. Only the last 20 to 25% of pond volume released during complete draining of ponds tends to increase in concentration of potential pollutants relative to pond water. Total suspended solids and total phosphorus are the only water quality variables consistently higher in concentration than typical concentration limits for water quality variables in effluent permits for other industries in the southern United States. Total phosphorus in pond effluents is usually associated with suspended solids, and the main sources of total suspended solids are erosion of denuded areas on watersheds, embankments, pond bottoms, and discharge ditches. Concentrations of nitrogen and dissolved phosphorus in effluents are not high, but effluent loads (weights of pollutants in effluents) of these two variables are greater than those for runoff from typical row crops in Alabama. Because of annual draining for harvest, fry and fingerling production ponds have much greater effluent loads of potential pollutants than do food fish production ponds. Watershed ponds that discharge following heavy rains also have greater pollution loads than do embankment ponds that seldom discharge water naturally.

There is little use of medicated feeds in catfish ponds. Sodium chloride is frequently applied to control nitrite toxicity, and copper sulfate is often applied to kill blue-green algae responsible for off-flavor in fish. There is no evidence that sodium chloride applications of 50 to 80 mg/L to ponds will lead to salinization of streams. Copper sulfate may be applied at 0.75 mg/L to ponds 3 or 4 times per year. Copper from copper sulfate quickly precipitates from water and is not present at toxic concentrations in effluents.

Hydrated lime may be applied to ponds 3 or 4 times per year at 50 to 100 kg/ha for algal control and other purposes. This amount of lime will not increase pond water pH enough to cause pH above 8.5 or 9.0 in pond effluent. Pond fertilizers such as triple superphosphate, diammonium phosphate, urea, and 10-34-0 (% N - % P_2O_5 - % K_2O) liquid fertilizer are applied at 5 to 10 pounds N and P_2O_5/acre 2 or 3 times per year in fingerling and fry ponds. Farmers may sometimes apply fertilizer to production ponds which do not develop plankton blooms. The majority of nitrogen and phosphorus in pond effluents originates from feed inputs rather than fertilization.

Seven streams were sampled upstream from any influence of catfish pond effluents and downstream of catfish farm outfalls at monthly intervals for 14 months. The pH was slightly greater downstream of catfish farms than above, but average pH was between 7.1 and 8.2 at all sampling locations. Dissolved oxygen concentrations were essentially the same upstream and downstream of catfish farms, and all average concentrations exceeded 5 mg/L. Specific conductance values tended to be elevated downstream of catfish farms during drier months, but specific conductance never exceeded 600 microSiemens/cm and was within an acceptable range for freshwater aquatic life. The average 5-day biochemical oxygen demand was generally similar between upstream and downstream sites, but there were occasions when the biochemical oxygen demand was greater upstream from catfish farm outfalls than downstream from them. In fall 1997, total ammonia nitrogen tended to be higher below farms than above, but the opposite was true in June 1998. Except for a single sampling date in September 1997, there was little difference in average nitrate-nitrogen and total nitrogen concentrations between upstream and downstream sites. There were no clear trends of difference in either soluble reactive phosphorus

or total phosphorus between sites upstream and downstream of catfish farms. Total suspended solids tended to be greater downstream of catfish farms in March and April 1998, but concentrations were higher above catfish farms than below them in June and July 1998, and similar trends were observed in turbidity values.

The small streams into which Alabama catfish farms discharge drain mostly cropland, pastures, and woods, and they do not have especially high quality water. Upstream of catfish farms, 5-day biochemical oxygen demand values usually are above 5 mg/L and concentrations of total suspended solids are often above 50 mg/L after rains.

There were almost an equal number of cases where water quality variables are higher above catfish farms than below. Furthermore, there were not cases where extremely high concentrations of variables (or very low dissolved oxygen concentrations) were noted downstream of farms. The findings suggest that catfish farm effluents are not having adverse impacts on stream water quality.

A few other observations were made that are of environmental interest. When ponds are drained, dried, and renovated, sediment removed from bottoms is used to repair embankments and not disposed of outside of ponds. Electricity used for pumping water and mechanical aeration was estimated as only 0.41 kilowatt·hours per pound of production. Each ton of fishmeal used in feeds yields about 10 tons of dressed catfish.

Better Management Practices

Evaluation of the environmental status of channel catfish farming in Alabama revealed generally good production practices and absence of widespread, negative environmental impacts. It is not possible to operate channel catfish ponds with current technology and not have effluent. Farmers must discharge water occasionally to renovate fish stocks and repair ponds, and overflow occurs after rainstorms. Water reuse could reduce the amount of discharge when ponds must be drawn down, but this practice would be expensive because farmers would need to purchase and operate pumps to transfer water. On a few farms there is space to construct settling basins, or natural wetlands are available for treating effluents. However, on most farms, an existing pond would have to be used as a settling basin. Renovation of the farm infrastructure to permit the use of existing ponds as settling basins also would be expensive and pumping costs would be incurred.

The catfish industry in Alabama releases little water other than storm overflow, and the major water quality concern is high concentrations of total suspended solids. Because the source of these solids is primarily erosion, it would be possible to greatly reduce total suspended solids concentrations through erosion control techniques.

The use of standard NPDES (National Pollution Discharge Elimination System) permits with the requirement of water quality monitoring to verify compliance would be very difficult and expensive because of the large number of outfalls associated with catfish farming. We feel that the installation of management practices to prevent environmental effects could be an alternative to NPDES permits and an effective means of environmental management for the Alabama catfish industry.

A list of better management practices that could make farm operations more efficient and provide environmental protection will be provided. Some farmers are already using many of these practices, but wide-spread adoption of good management procedures is desirable.

(1) Establish grass cover on denuded areas of pond watersheds to minimize erosion.

(2) Grass cover should be provided on the interior and exterior of pond embankments to minimize erosion.

(3) Divert excess flow of large watershed away from ponds to minimize total suspended solid inputs to ponds.

(4) Use reasonable stocking and feeding rates to reduce nutrient and organic matter inputs and water quality deterioration.

(5) Do not feed more than the fish will eat to reduce feed input.

(6) Do not use fertilizer unless necessary to promote plankton blooms to reduce nutrient inputs.

(7) Use well water conservatively.

(8) Do not install deep-water discharge structures in ponds because surface waters usually are of higher quality than deeper water.

(9) Maintain at least 7.5 to 10 cm of depth below overflow intakes in embankment ponds to conserve rain and runoff in warm months and minimize overflow.

(10) Position mechanical aerators to minimize erosion of pond bottoms and embankments, but use adequate aeration to prevent low dissolved oxygen concentrations.

(11) Do not discharge water during final seining, and when ponds are completely drained, release the final water as slowly as possible to minimize discharge of potential pollutants.

(12) Do not leave ponds empty in winter, and shut valves when ponds are empty to prevent discharge of suspended solids after rains.

(13) Close pond valves when renovating inside earthwork to prevent discharge of suspended solids after rains.

(14) Use sediment removed from pond to repair earthwork rather than disposing of it outside of ponds to reduce erosion potential on farm.

(15) Extend drainpipes beyond the toes of the embankments to prevent erosion of the embankment by discharge.

(16) Construct ditches to minimize erosion and establish grass cover on them.

(17) Use concrete structures or rip-rap to protect areas impacted by rapidly flowing discharge from erosion.

(18) Extend pipes that discharge directly into streams to prevent bank erosion.

(19) Where possible, release pond effluents into natural wetlands to take advantage of natural water treatment.

(20) Store materials such as fertilizers, lime, salt, and other pond amendments so that they are not washed into streams by rainfall.

Inland Shrimp Farming and the Environment

Claude E. Boyd

There is considerable interest in inland farming of marine shrimp in areas where slightly saline water is available and even in some freshwater areas (Jory 1999). There are two ways of obtaining saline waters for inland shrimp ponds. In some areas, there are aquifers containing naturally saline water, and ponds can be filled from wells developed in saline-water aquifers. Where saline water is not available naturally, brine solutions from coastal salt farms or solid salt may be transported to the ponds and mixed with freshwater to provide enough salinity for shrimp production. In some cases, shrimp production has been done in freshwater without adding salt.

There is little historical documentation of inland shrimp farming, but some reliable information is available. In 1989, I visited a site near Mahasarakham in northeast Thailand where salty ground water was being used by a few farmers to produce *Penaeus monodon*. This practice never became established in the area. In the mid 1990s, shrimp farmers in central Thailand began to mix brine solution and irrigation water in inland ponds to culture shrimp. This became a major activity, and a 1997 survey (Musig and Boonnom 1998) reported about 11,500 ha of inland shrimp farms in central Thailand. In the summer of 1998, in response to concerns about salinization of soil and irrigation water, the Thai government banned inland shrimp farming. There has been considerable controversy over the ban, and the Thai government is now attempting to find a way to resolve the controversy and still allow inland shrimp farming.

Inland shrimp farming projects also have been installed in the United States in Arizona (Jory 1999) and in Florida (Scarpa 1998). The project in Arizona relies on ground water from wells that has a salinity of 1 to 2 ppt. The effort in Florida is based on culturing shrimp in

recirculating freshwater (0.4 to 0.5 ppt salinity). In 1999 and 2000, some catfish farmers in west-central Alabama begin to experiment with shrimp culture in ponds filled with ground water from wells that contained 2 to 6 ppt salinity. The effort has been fairly successful and is expected to continue and to expand. During 2000 in Ecuador, several pilot projects where brine solution or salt was used to increase the salinity of freshwater ponds were successful in producing shrimp. There also are areas in Ecuador with saline underground water suitable for using in shrimp culture. Thus, inland shrimp farming is expected to become a viable activity in Ecuador. There are many other areas in the world where inland shrimp farming could be conducted, and this type of shrimp culture could become an important addition to world shrimp supplies.

Because the inland culture of shrimp in the United States is not a large activity, there has been little notice of it by environmental groups. However, it is interesting that some channel catfish farmers in west-central Alabama have been culturing catfish in waters of 2 to 6 ppt salinity for years. This water is highly prized by catfish farmers, because it has considerable therapeutic value to fish, and disease problems are much less than in normal freshwater water used for catfish farming. It is not known how much catfish farming is conducted in saline water, but it is certain that several hundred hectares of ponds are used for this purpose. An environmental impact assessment of channel catfish farming in Alabama (Boyd et al. 2000) did not reveal any negative impacts of catfish culture in saline water. However, it should be noted that all culture in saline water has been conducted in embankment ponds that only overflow after heavy rainfall, ponds are constructed in heavy clay soils where seepage is low, sediment is not removed from ponds, and ponds are not drained more than two times in 15 years for fish harvest because harvest is done by seining (Boyd et al. 2000).

The United States Environmental Protection Agency currently is conducting a rule-making procedure for aquaculture effluents. The initial rule is due in June 2002, and the final rule will be published in June 2004 (Federal Register 2000). Effluents from inland shrimp farms will be considered under the EPA rules. It is assumed that most inland shrimp farms in the USA will reuse water to conserve salinity. However, there will be environmental concerns about inland shrimp farming related to salinization of surface water, ground water, and soils. The issue of inland shrimp farming and the environment deserves careful attention to assure that this type of shrimp culture develops in an environmentally-responsible manner. The following discussion will focus on the situation in Alabama, but the comments are applicable in most other areas.

The saline ground water available for inland shrimp farming in Alabama occurs in the west-central part of the state in Greene, Hale, Marengo, and Tuscaloosa Counties. The aquifers are at depths of 60 to 120 m. Wells yielding 750 to 3,500 L/min usually can be developed. The land available for inland shrimp farming usually is located in the Black Belt Prairie and is gently to moderately rolling, former pastureland. Soils normally have a high content of sticky, expandable clay.

The saline ground water normally has a salinity of 1.5 to 6 ppt. Typical concentration ranges of major constituents and pH are provided in Table 1. Sodium and chloride make up the majority of the concentration of major ions in the water. The saline ground water is much lower in salinity than normal seawater that has an average salinity of 34.5 ppt. However, the proportions of calcium and bicarbonate are much higher in the saline ground water than in seawater, but the opposite is true for magnesium and sulfate (Table 2). A high proportion of calcium and bicarbonate is considered desirable in aquaculture pond waters (Boyd and Tucker 1998), and a low proportion of sulfate also is desirable because sulfate is the source of hydrogen

sulfide in anaerobic pond soils. The significance of a low proportion of magnesium is not known, but pilot studies have shown that shrimp grow well in the saline ground water in Alabama.

The suitability of irrigation water from the standpoint of salt concentration often is expressed in terms of total dissolved solids and sodium adsorption ratio (Boyd 2000). The sodium adsorption ratio (SAR) is calculated as follows:

$$SAR = \frac{(Na)}{1/2\sqrt{(Ca)+(Mg)}}$$

where Na = sodium concentration (meq/L)

Ca = calcium concentration (meq/L)

Mg = magnesium concentration (meq/L)

The SAR for saline ground water in Alabama ranges from 20-40, and total dissolved solids from 1,500 to 6,000 mg/L. The usual influence of SAR and total dissolved solids on plants (Boyd 2000) is summarized in Table 3. Thus, the saline ground water used for inland shrimp farming in Alabama could be expected to harm most plants. It also could cause salinization of surface water or soil if discharged from ponds into natural habitats. Inland shrimp farming in Alabama, and presumably in many other places, should be done in water recirculating systems without discharge of effluents. The exception would be where salinity in pond water is so low that total dissolved solids and SAR of effluent would not lead to soil or water salinization or harm plants (Table 3).

Inland shrimp farming can be conducted without causing adverse environmental effects if certain precautions are followed as follows:

(1) Production should be done only in ponds where discharge can be prevented after rainstorms. The most suitable ponds would be embankment ponds with adequate freeboard to retain rainfall without overflow. Alternatively, ponds could be allowed to discharge but the effluent held in a detention basin without overflow located nearby and on the farm.

(2) Ponds should not seep so that water infiltrates into freshwater aquifers, streams, or non-saline soils. Soils for pond construction should have an adequate particle size distribution to allow for the construction on watertight embankments and bottoms. Proper compaction techniques should be used to further reduce the infiltration potential of bottoms and embankments (McCarty 1998). Anti-seep devices should be installed around pipes extending through embankments. Where soils will not resist infiltration, clay liners or plastic membranes could be used to prevent infiltration (Yoo and Boyd 1994).

(3) Water should be reused and not discharged into natural habitats. Ponds must be drained for harvest, so a reservoir must be provided for holding this water for reuse. The same reservoir can be used to detain overflow from ponds after heavy storms. The reservoir should be large enough to provide 6 or 8 days retention time before water is reused. This will allow for purification of the water by natural processess. Water exchange between production ponds and treatment reservoirs may be done when water quality problems occur in ponds. It is

anticipated that culture ponds will be aerated mechanically. Installation of mechanical aerators in the reservoir would enhance water purification during retention.

(4) It is a common practice to remove sediment from intensive shrimp production ponds between crops. This sediment contains salt, and if disposed of outside of inland shrimp ponds, leaching of spoil piles by rainfall could lead to soil and water salinization (Boyd et al. 1994). Pond bottoms should be dried between crops, and sediment used to reshape the insides of embankments. When sediment must be removed from ponds, it should be stored in a basin where rainwater contacting it can be retained without overflow.

(5) A vegetative barrier should be provided around inland shrimp farms. The continued health of this vegetation would be an indication that salt intrusion is not occurring into the area around the farm. Piezometer tubes should be installed around inland shrimp farms and salinity of ground water measured on a regular schedule to assure that ground water salinization is not occurring. Soil salinity in areas surrounding inland shrimp farms also should be monitored. If the monitoring program suggests that salinization is occurring, practices would need to be improved to prevent it.

(6) Soils in the bottoms of abandoned ponds and surrounding area could be treated with calcium sulfate (gypsum) for reclamation. Gypsum treatment is a common practice for reclaiming saline soils.

Inland shrimp farming has several advantages:

- Allows diversification of land use for food production.
- Shrimp farming can be done outside the coastal zone where possibilities for negative environmental impacts are less.
- Disease problems in shrimp culture can be greatly reduced.
- Inland shrimp farming tends to be more intensive than coastal shrimp farming, so there is more efficient use of land and water resources.
- Logistics often are simpler because transport of supplies and products can be by truck instead of boat as is sometimes the case in traditional shrimp farming.
- The water supply is not shared and there can be better control over water use.

The disadvantage of inland shrimp farming is related almost entirely to the possibility of salinization. However, use of the practices suggested above should allow inland shrimp farms to operate in a responsible manner in freshwater areas with non-saline soils.

Table 1. Typical concentration ranges of water quality variables in salty ground water in west-central Alabama.

Variable	Range
pH	7-8 standard units
Total dissolved solids	1,500-6,000 mg/L
Chloride	500-3,000 mg/L
Sodium	200-1,500 mg/L
Calcium	50-185 mg/L
Magnesium	10-40 mg/L
Potassium	5-15 mg/L
Sulfate	5-20 mg/L
Bicarbonate	85-300 mg/L

Table 2. Comparisons of proportions of individual ions in salty ground water in Alabama and seawater.

	Milliequivalents (% of total)	
Ion	Salty ground water in Alabama	Seawater
Cl	38.5	45
Na	34	38
Ca	12	2
HCO_3	10.5	0.2
Mg	2	9.3
K	2	1
SO_4	1	4.5

Table 3. General standards for total dissolved solids (TDS) and sodium adsorption ratio (SAR) in irrigation water.

Salt tolerance of plants	TDS (mg/L)	SAR
All species, no detrimental effects	500	2-7
Sensitive species	500-1,000	8-17
Adverse effects on many common species	1,000-2,000	18-45
Use on tolerant species on permeable soils only	2,000-5,000	46-100

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Environmental Codes of Practice in Aquaculture

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I am pleased about being asked to write a regular column for the new GAA magazine, and I hope to provide some useful information on aquacultural production in each issue. In this respect, environmental issues are very important to the future of aquaculture, and GAA was formed largely in response to criticisms of negative environment impacts from shrimp farming and other types of aquaculture. Thus, it seems appropriate for the first column to be about aquaculture and the environment.

Aquaculture is important to world food production. The harvest of fish and other aquatic organisms from natural waters apparently has reached its upper limit, but the demand for fishery products is still growing. The difference between catch fisheries production and the demand must be supplied by aquaculture, or there will be a shortage of fisheries products and a sharp rise in their price. Aquaculture has responded to this challenge, and it has increased world production of many species and represents about 20% of world fisheries production. However, the aquaculture industry's image and future may be greatly diminished unless it deals effectively with environmental issues and concerns that have recently arisen.

The aquaculture industry should formulate an environmental agenda with the following objectives:

- Assess production systems to identify the major environmental impacts,
- Develop and implement better environmental management procedures,
- Foster public relations programs to explain the methods of aquaculture, the importance of aquaculture to society, and aquaculture's dedication to the environment,
- Become more active in the political arena—the environmentalists are very involved politically,
- Get involved with environmental management agencies in order to influence the nature of future regulations – the environmentalists certainly are,
- Provide better environmental education for producers.

I have limited knowledge and experience in public relations, political lobbying, managing associations, or extension programs, so I will limit my discussion to technical issues.

The negative impacts of aquaculture, and especially those of shrimp farming, have been outlined many times in the past. The most important concerns are mangrove and other wetland alteration by aquaculture projects, water pollution, wasteful use of fish meal, uncontrolled use of antibiotics, drugs, and other chemicals, excessive water use, salinization of freshwater, changes in land use patterns, introduction of exotic species, and social conflicts. These negative impacts usually result from poor planning or bad management, and they are not routine consequences of aquaculture. Nevertheless, these bad examples have tarnished the image to the entire industry and threaten to cause even more damage. A positive and proactive approach is the logical means of countering the bad publicity and protecting the image of aquaculture.

Better management practices should be adopted to reduce the possible adverse impacts of aquaculture and to demonstrate the industry's commitment to environmental stewardship. The GAA publication, "Codes of Practice for Responsible Shrimp Farming" provides a practical approach to improving environmental management in aquaculture. These codes provide techniques known as best management practices (BMPs) for use in shrimp farming. BMPs are considered the best practical means of reducing environmental impacts to those compatible with water quality or resource management goals. BMPs form the basis for environmental management in many types of agriculture in the United States and other nations. The GAA intends for their general publication on codes of practice to serve as a guideline for others to use in developing country-specific or farm-specific codes of practice for shrimp farming. These more specific codes of practices also should contain greater detail about how to implement BMPs. The adoption of the better practices will be voluntary at first, but a self-evaluation program will be initiated to demonstrate progress in adoption and implementation of better practices.

Several countries already are developing codes of practices and the GAA effort on better practices is serving as a model for these country-level codes of practice. Additional information on good management practices for shrimp aquaculture were presented by C. E. Boyd and Maria Haws at the Symposio 5 Centroamericano de Aquacultura held 18-20 August 1999 in San Pedro Sula Honduras. We started with the GAA model and provided greater details about good management practices specifically for Latin American conditions and shrimp culture methods. A document containing the good management practices was published in the symposium proceedings. This document and the GAA "Codes of Practice for Responsible Shrimp Farming" provide most of the information necessary to make country-specific codes of practice for voluntary adoption by shrimp farmers. By early next year, several countries probably will have adopted codes of practice for shrimp farming. I suspect that other types of aquaculture also will rapidly follow the lead of the shrimp industry and prepare code of practices.

The benefits of adopting better practices include the following:

- Reduce negative environmental impacts,
- Provide a means to interact positively with environmental agencies,
- Improve the efficiency of aquaculture,
- Extend better production methodology to farmers,
- Increase prospects for sustainability,
- Serve as part of future environmental regulations,
- Provide a marketing advantage because some consumers want an environmentally friendly product.
-

The environmental community will probably criticize industry codes of practice because they originated within the aquaculture industry and are voluntary. The criticisms should not deter us, for codes of practice provide the industry a proactive means of dealing with environmental issues that can influence governmental and public perception in a positive way. In the future, these practices could possibly be certified by third party inspectors, become the centerpiece of a certification program, or even take the place of traditional environmental regulations. I believe that the GAA program to encourage preparation and adoption of codes of practice is a very positive and useful approach. Hopefully, it will abate much of the negative attitude that exists in some circles regarding shrimp and fish farming.

Management of Shrimp Ponds to Reduce The Eutrophication Potential Of Effluents

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There is concern about the effects of nutrients in aquaculture pond effluents on natural waters. This concern arises because nitrogen and phosphorus are contained in pond effluents, and these two nutrients can cause eutrophication of natural waters. In eutrophication, nutrient inputs to water bodies increase nutrient concentrations and cause dense phytoplankton blooms. Phytoplankton blooms increase natural productivity of waters, but too much productivity can lead to an excessive demand for dissolved oxygen and cause chronically low dissolved oxygen concentrations. Low dissolved oxygen can result in the loss of ecologically sensitive fauna and lessen biodiversity. Dense phytoplankton blooms also diminish the natural beauty of water bodies, they sometimes cause taste and odor problems in drinking water and off-flavor in aquatic organisms, some species of phytoplankton may be toxic to other forms of aquatic life, and dead or moribund scums of algae may drift to the shore and cause bad odors in the surroundings. Thus, one objective of most water pollution abatement programs is to limit nitrogen and phosphorus concentrations in effluents to minimize the danger of eutrophication in natural waters. Several management practices contained in the Global Aquaculture Alliance "Codes of Practice for Responsible Shrimp Farming' were selected because they will reduce nutrient inputs to natural waters in the vicinity of shrimp farms.

Nutrients in aquaculture pond effluents mainly come from fertilizers and feeds applied to ponds to stimulate the production of the culture species. Organic fertilizers, e.g., animal manures or other agricultural byproducts, are sometimes applied to ponds. These materials contain nitrogen and phosphorus that are released into the water as the organic fertilizer is decomposed by microbes. Chemical fertilizers, e.g. urea, triple superphosphate, diammonium phosphate, mixed fertilizers, etc., dissolve in water to release nitrogen and phosphorus. Feeds also contain nitrogen and phosphorus. Some of the nitrogen and phosphorus in feeds enter the water when unconsumed feed and feces decompose, and more is added when ammonia is excreted by the culture species. Organic nitrogen and phosphorus are both present in the water as a component of living plankton and soluble organic matter. Inorganic nitrogen is dissolved in the water primarily as ammonia nitrogen and nitrate. Inorganic phosphorus in water may be contained on suspended mineral (soil) particles or in soluble phosphate. Phytoplankton and other plants use ammonia nitrogen, nitrate, and soluble inorganic phosphorus for growth. However, nitrogen and phosphorus contained in dead particulate organic matter or soluble organic matter in the water may be transformed by microbial decomposition to ammonia nitrogen, nitrate, or phosphate. Because organic nitrogen and phosphorus can be transformed to soluble inorganic form by microbes, the eutrophication potential of pond effluents increases as the total concentration of

nitrogen and phosphorus increases. In ponds with heavy plankton blooms, most of the nitrogen and phosphorus may be contained in plankton and detritus rather than in soluble form. Effluents from a pond with low concentrations of ammonia nitrogen, nitrate, and phosphate, but with high plankton abundance, may still have as great a pollution potential as an effluent with high concentrations of ammonia, nitrogen, nitrate, and phosphate. This results because the organic matter (plankton, detritus, and soluble organic matter) that enters natural waters via pond effluent will decompose and release ammonia nitrogen, nitrate, and phosphate.

Many shrimp farmers may not think that pond effluents contain much nitrogen and phosphorus because they do not use organic fertilizers, they use chemical fertilizers sparingly and only near the beginning of the culture period, and the feed conversion ratio is good. Some shrimp farmers obtain a feed conversion ratio as low as 1.5. This means that 1.5 kg of shrimp feed results in the production of 1 kg of shrimp. The conclusion may be that only 0.5 kg of waste is generated in the production of 1 kg of shrimp. Even if the feed conversion ratio is as high as 2, the farmer might think that only 1 kg of waste is released in the production of 1 kg of shrimp.

The relationship among feed input, shrimp production, and waste generation will be analyzed more carefully. The feed used in aquaculture normally is a dry pellet. Shrimp feed contains about 90% dry matter and 10% water. Shrimp, on the other hand, contain about 25% dry matter and 75% water. Thus, in the production of 1 kg of shrimp with 1.5 kg of feed (feed conversion ratio of 1.5), 1.35 kg dry matter in feed yields 0.25 kg dry matter in shrimp. From an ecological point of view, 1.35 kg (1.5 kg feed × 0.9) dry nutritive substance has to be used to produce 0.25 kg (1 kg shrimp × 0.25) of dry matter in shrimp. Thus, the dry matter conversion ratio is only 5.4 (1.35 kg dry feed ÷ 0.25 kg dry shrimp). The ratio of shrimp to wastes of 1:0.5 based on the usual method for estimating feed conversion ratio is an apparent ratio, but the true ratio based on dry matter is 1:4.4.

Suppose that a shrimp feed contains 35% crude protein and 1.2% phosphorus. Crude protein is estimated as percentage nitrogen multiplied by 6.25, so this feed has 5.6% N, and 1.5 kg of this feed contains 84 g nitrogen (1,500 g feed × 0.056) and 18 g phosphorus (1,500 g feed × 0.012). The 1 kg of shrimp produced by the feed will contain 0.25 kg dry matter, and shrimp dry matter is about 11% nitrogen and 1.25% phosphorus. It follows that 27.5 g nitrogen (250 g dry shrimp × 0.11) and 3 g phosphorus (250 g dry shrimp × 0.0125) are contained in the shrimp. The differences between the amounts of nitrogen and phosphorus in the feed and in the harvested shrimp represent the amount of nitrogen and phosphorus entering the pond water. In this example, each kilogram of live shrimp would result in 56.5 g nitrogen and 15 g of phosphorus in wastes. On a per ton basis, this would be 56.5 kg nitrogen and 15 kg phosphorus.

In a pond without water exchange, much of the nitrogen and phosphorus will be removed from the water. Nitrogen will be lost to the air by volatilization of ammonia and by microbial denitrification. Some nitrogen will be contained in organic matter deposited in the pond bottom, and phosphorus will be absorbed by sediment. Recent studies suggested that about 50% of the nitrogen and 65% of the phosphorus added in feed could be removed from the water of a pond without water exchange through physical, chemical, and biological processes. Considering that about 25 to 35% of the nitrogen and 15 to 25% of phosphorus added in feed is recovered in shrimp at harvest, only 15 to 25% of the nitrogen and 10 to 20% of the phosphorus applied in feed would be lost in effluent at pond draining. Of course, with water exchange, there would be a greater loss of nitrogen and phosphorus in effluents, because more nitrogen and phosphorus would be flushed out of ponds before being removed by natural purification processes within the

pond. Even in a pond with zero water exchange, the loss of nitrogen and phosphorus at pond draining might be 12.6 to 21 kg nitrogen and 1.8 to 3.6 kg phosphorus where 1 ton of shrimp is produced at a feed conversion ratio of 1.5 (see example above). Thus, for different levels of production, the nitrogen and phosphorus outputs might be as follows:

Production	N (kg/ha)	P (kg/ha)
500	6.3 – 10.5	0.9 – 1.8
1,000	12.6 – 21	1.8 – 3.6
2,000	25.2 – 42	3.6 – 7.2
3,000	37.8 – 63	5.4 – 10.8
4,000	50.4 – 84	7.2 – 14.4

These are rather large amounts of nitrogen and phosphorus, and the effluents from aquaculture can be a threat to cause eutrophication of natural waters into which they are discharged.

Several measures can be taken to avoid or minimize eutrophication as follows:

(1) Minimize water exchange. By retaining water in ponds for a lower time, there is greater opportunity for removal of nitrogen and phosphorus by natural processes.

(2) Use a high quality feed. A feed that is water stable can be eaten more completely by shrimp. Also, a high quality feed results in less feces and metabolic waste.

(3) Use feeds with the lowest nitrogen and phosphorus concentrations that are compatible with good feed quality. This will minimize the amount of nitrogen and phosphorus in wastes.

(4) Feed conservatively. Overfeeding results in wasted feed and increases the amount of waste. It is important for the shrimp to eat all of the feed put into ponds for both economic and environmental reasons.

(5) When draining ponds, try to minimize the velocity of outflowing water so that sediment is not resuspended from pond bottoms. This practice will lower the amount of organic nitrogen and phosphorus in effluents by retaining organic particles within the pond.

(6) Maintain good dissolved oxygen concentrations in ponds by not stocking and feeding too much so that the pond can assimilate most of the wastes. The assimilative capacities of ponds differ, and aerated ponds can assimilate much more waste than unaerated ponds. Good dissolved oxygen concentrations favor oxidation of ammonia to nitrate and nitrate can then be denitrified in the sediment.

(7) Dry pond bottoms and lime acidic bottom soils between harvests to favor organic matter decomposition. This will reduce the accumulation of organic matter in bottom soils.

The reference for the following paper is: Claude E. Boyd and Julio F. Queiroz. 2001. Feasibility of Retention Structures, Settling Basins, and Best Management Practices in Effluent Regulation for Alabama Channel Catfish Farming. Reviews in Fisheries Science 9(2); 43-67.

CATFISH FARMING

A. BMPS TO REDUCE EFFLUENT VOLUME

- New ponds should have watershed area to pond area ratio of 10:1 or less.

- Use terraces to divert excess runoff around ponds as illustrated in Figure 5. Note: sometimes an additional pond may be built to increase storage on the watershed.

- Maintain good vegetative cover on all parts of watersheds, and where feasible, replace short grass with evergreen trees.

- Harvest fish by seining and without partially or completely draining ponds unless it is necessary to renovate fish stocks or repair pond earthwork.

- Maintain at least 20 cm of storage capacity in ponds during summer and fall. The upper 20-cm length of overflow pipe should be painted a bright color to serve as a guide when adding well water to replace water loss.

- Do not flush well or stream water through ponds. This practice does not improve water quality in ponds.

B. BMPS TO MINIMIZE SUSPENDED SOLIDS THROUGH EROSION CONTROL

- Control erosion on watersheds by providing vegetative cover, eliminating gully erosion, and using terraces to route water from areas of high erosion potential.

- Restrict livestock from watersheds of ponds.

- Eliminate steep slopes on farm roads and cover these roads with gravel.

- Provide grass cover on sides of pond dams or embankments and grass or gravel on tops of dams or embankments.

- Do not leave ponds partially or completely empty in winter and spring, and immediately close drains in empty ponds.

- Efficient mechanical aerators should be installed so that water currents caused by these devices do not cause erosion of pond earthwork.

- Sediment should not be disposed of outside of ponds.

- Install structures to prevent drainpipe discharge from impacting and eroding earthwork.

- Construct ditches with adequate hydraulic cross section, and provide grass cover on sides of ditches.

- Provide check dams in ditches to reduce water velocity and allow sedimentation.

- Settling basins are an alternative method for improving the quality of final draining effluent from catfish ponds where space is available.

- Trees or shrubs could be used in critical areas to shelter ponds from excessive wind velocities and reduce wave erosion of embankments.

- Where possible, effluent from catfish ponds should be discharged into natural wetlands.

C. BMPS TO IMPROVE POND WATER AND EFFLUENT QUALITY

- Select high quality feeds that contain adequate, but not excessive, nitrogen and phosphorous.

- Store feed in well-ventilated, dry bins, or if bagged, in a well-ventilated, dry room. The feed should be used by the expiration date suggested by the manufacturer.

- Apply feed uniformly with a mechanical feeder.

- Do not apply more feed than fish will eat.

- Feeding rates should not exceed 30 kg/ha per day in un-aerated ponds. In ponds with 4 kW of aeration per hectare, feeding rates usually can be increased to 100 to 120 kg/ha per day.

- When uneaten feed accumulates in corners of ponds, it should be manually removed.

- Apply fertilizers only when necessary to promote phytoplankton blooms.

- Use chemical fertilizers and avoid use of animal manures.

- Avoid excessive fertilization by using moderate doses and relying on the Secchi disk visibility to determine if fertilization is needed.

- Apply agricultural limestone to ponds with total alkalinity below 20 mg/l.

- Store fertilizers under a roof in a dry place to prevent rain from washing them into surface waters.

- Apply adequate mechanical aeration to maintain dissolved oxygen concentrations above 4 mg/l.

- Do not have deep water intake structures in ponds.

- Install devices to prevent sediment resuspension by water currents entering drains. Such a device is illustrated in Figure 6.

- Restrict livestock from watersheds of ponds.

- Avoid discharge when harvesting fish, but if ponds must be drained completely, hold the final 20% to 25% of pond volume for 2 or 3 days and then discharge it slowly.

D. BMPS FOR USE OF THERAPEUTIC AGENTS AND OTHER CHEMICALS

- Store therapeutants so that they cannot be accidentally spilled to enter the environment.

- Use good water quality management procedures to prevent unnecessary stress to fish.

- Obtain a definite diagnosis for diseases and a recommendation for disease treatment before applying therapeutic agents.

- Follow instructions on labels of therapeutic agents for dose application method, safety precautions, etc.

- Store water quality enhancers under a roof where rainfall will not wash them into surface waters.

- Copper sulfate applications in milligrams per liter should not exceed 1% of total alkalinity also measured in milligrams per liter or a maximum dose of 1.0 mg/l. Pond water should not be released for 72 hours after application of copper sulfate.

- Sodium chloride applications should not exceed 100 mg/l.

- Lime (calcium oxide or hydroxide) applications should not exceed 100 mg/l.

- Agricultural limestone and gypsum (calcium sulfate) applications should not exceed 5,000 kg/ha and 2,000 kg/ha, respectively.

- Calcium hypochlorite or other chlorine compounds should not be applied to catfish ponds.

E. BMPS FOR NEW PONDS OR FARMS

- New ponds should be constructed according to National Resource Conservation Service (NRCS) standards. Riparian vegetation of trees or shrubs should be preserved or established to provide a vegetative buffer zone along streams.

- New ponds should not be located on watersheds that are already impacted by subdivisions, industrial activities, or row-crops.

- Design of new ponds should conform to NCRS standards and be compatible with implementation of BMPs outlined above.

The reference for the following 9 BMP's is: Claude E. Boyd. 1999. Codes of Practice for Responsible Shrimp Farming, Global Aquaculture Alliance, St. Louis, MO, 42 pp.

Mangroves
Code of Practice

Purpose

The Code is designed to foster greater environmental awareness within the shrimp farming industry to assure continued protection of mangrove forests from potentially adverse impacts of coastal aquaculture. Recognizing the multitude of different conditions impacting mangroves in different countries and regional locations, this Code is to be interpreted as a flexible set of criteria to be used to assist any and all interested parties in formulating codes, regulations, and principles for protecting mangrove forests.

The Code helps to achieve several of the "Guiding Principles of Responsible Aquaculture" by encouraging the following:

- The shrimp aquaculture industry will promote responsible and sustainable development and management practices ensuring the preservation of mangroves and the sustainability of shrimp aquaculture.
- Shrimp aquaculture industries will promote alternative development programs aimed at protecting mangroves while benefiting local communities in mangrove areas.
- Producers shall adhere to national and local regulations applicable to mangroves and to shrimp farming.

Management Practices

It shall be the objective of all adherents to this Code to not harm mangrove ecosystems, and whenever possible, to preserve and even enhance the biodiversity of these ecosystems. The following practices will ensure the protection of mangrove ecosystems:

1. New shrimp farms should not be developed within mangrove ecosystems.
2. Realizing that some mangrove must be removed for canals when new shrimp farms are sited behind mangroves, a reforestation commitment of no net loss of mangroves shall be initiated.
3. Farms already in operation will continue ongoing environmental assessments to recognize and mitigate any possible negative impacts on mangrove ecosystems.
4. All non-organic and solid waste materials should be disposed of in an environmentally responsible manner, and waste water and sediments shall be discharged in manners not detrimental to mangroves.
5. The shrimp aquaculture industry pledges to work in concert with governments to develop sound regulations to enhance the conservation of mangroves including regulations regarding restoration of mangrove areas when old farms located in former mangroves are decommissioned.
6. The shrimp aquaculture industry will promote measures to ensure the continued livelihood of local communities that depend upon mangrove resources.

Site Evaluation
Code of Practice

Purpose

The Code is designed to promote site evaluation as a means to ensure that new shrimp-farming projects are harmoniously integrated into local environmental and social settings. Site evaluation can identify limitations that influence the suitability of a site for farm construction and operation, reveal the possibilities of negative environmental and social impacts, and allow estimates of technical and financial requirements for mitigation of unfavorable conditions. Recognizing that enormous variation in environmental and social conditions exists from site to site, this Code presents adaptable guidelines to assist any and all parties interested in making site evaluations for shrimp farms.

The Code helps to achieve several of the "Guiding Principles of Responsible Aquaculture" and promotes the following:

Use of site evaluation to avoid siting farms where significant technical, environmental, and social problems are likely.

Prevention of significant negative environmental and social impacts through use of site evaluation findings in planning mitigation methods. A proper site evaluation will provide most of the information required to produce an environmental impact assessment (EIA).

Management Practices

All adherents to the Code shall thoroughly evaluate potential sites for shrimp farms to assure that local ecological and social conditions are protected and even enhanced. The following practices will ensure that appropriate sites are selected for shrimp farms:

1. Evaluate hydrologic features including tidal patterns, freshwater influences and flood levels, offshore currents, and existing water uses.
2. Determine water quality characteristics of coastal waters in the vicinity of the site.
3. Ascertain the suitability of topography, soil, and ecosystem for siting and construction of ponds.
4. Make sure that previous site use has not resulted in contamination of water or soils.
5. Acquire long-term climatological records to determine the likelihood of drastic events such as flood, droughts, or severe storms that could negatively impact the project.
6. Survey the existing flora and fauna with particular concern for effects of the project on ecologically sensitive areas such as migration routes and nesting grounds or protected areas such as parks and refuges.
7. Document regulatory requirements for the site, and consider alternatives for compliance with regulations.
8. Consider alternatives to mitigate potential negative environmental impacts and to alleviate conditions not conducive to shrimp farm construction and operations.
9. Survey local communities to determine demography, resource use patterns, availability of work force, and compatibility with project goals.
10. Consider alternatives to mitigate potential negative social impacts.
11. Determine if any areas within the site are of significant archeological or historical importance and consider methods for their preservation.

Design and Construction
Code of Practice

Purpose

The Code is intended to promote environmental protection through proper shrimp farm design and good construction methods. Good site selection and incorporation of mitigative features in the farm design are the best ways to avoid problems related to flood levels, storms, erosion, seepage, water intake and discharge points, and encroachment on mangroves and wetlands. Planning of clearing and earth moving activities can prevent or greatly limit ecological damage during farm construction. Recognizing that a site-specific approach to design and construction is necessary, the Code provides basic design and construction criteria for environmentally-responsible shrimp farms.

The Code helps to achieve several of the "Guiding Principles of Responsible Aquaculture" and it promotes:

- Use of design features and good construction methods to overcome site limitations and to prevent or mitigate potential negative environmental and social impacts.
- Adoption of successfully proven and accepted design and construction procedures.

Management Practices

Adherents to the Code shall strive to design and construct shrimp farms in a responsible manner to protect the environment and coastal communities. The following practices can afford this protection:

1. Farms should not be built on ecologically sensitive mangrove areas or other wetlands and in places where it is impractical to correct site-related problems such as highly-acidic, organic, or permeable soils.
2. Comply with all environmental impact assessment (EIA) procedures before initiating construction and abide by EIA restriction during construction.
3. Embankments should be designed to prevent erosion, and where practical, methods for reducing seepage through pond bottoms should be included.
4. Ponds should have separate intake and outlet structures to permit control of filling and draining.
5. Inlet and discharge canals should be separate so that water supply and effluent are not mixed.
6. Storms and flood levels should be considered in earthwork design.
7. Infrastructure and access roads should not necessarily alter natural water flows, cause salinization of adjacent land or water, or impound flood water.
8. Canals should be designed to prevent excessive water velocity and scouring.
9. Water intake point(s) should provide a sufficient volume of high quality water available.
10. Pump intakes should be screened, vegetative buffers provided around pump stations, and containments installed to prevent fuel spills.
11. Where possible, vegetative buffer zones, riparian vegetation, and habitat corridors should be maintained, and vegetative cover provided on exposed earthwork.
12. Sediment traps and basins should be incorporated in the design where suspended solid concentrations are expected to be high in effluents.
13. Outfalls should be designed to prevent erosion and avoid discharge of effluents into stagnant water.
14. Disturb as little area as possible during construction.
15. Erosion should be controlled during construction.
16. Cut and fill construction techniques are preferable, and earthwork should be compacted.
17. Degraded areas such as unused soil piles, barrow pits, and uncontrolled refuse dumps should not be created.

Feeds and Feed Use
Code of Practice

Purpose

The Code is designed to improve the efficiency of supplemental feeds and feed management in shrimp farming and to minimize the waste load in ponds. Feeding is a standard practice in shrimp production, because it permits higher production than can be achieved from natural pond productivity. Recognizing that feed is expensive, it should be used wisely to reduce production costs. However, using good feeds and feeding practices also are important steps towards reducing waste loads in pond effluents. Guidelines presented in this Code can be used by feed manufacturers and shrimp producers to improve feeds and feeding practices.

The Code helps to achieve several of the "Guiding Principles for Responsible Aquaculture" and promotes awareness of two major issues:

- Shrimp feed should be made from high quality ingredients by good manufacturing techniques and stored properly.
- Feed should be used conservatively to ensure efficient conversion to shrimp flesh and minimize waste and expense.

Management Practices

Those supporting the Code shall strive to improve feed quality and feeding with the goal of optimizing the conversion of feed to shrimp and reducing the amount of waste entering ponds. This goal can be achieved through the following practices:

1. Feed ingredients should not contain excessive pesticides, chemical contaminants, microbial toxins, or other adulterating substances.
2. Pellet binders and suitable manufacturing techniques should be used to provide a water-stable pellet.
3. Manufacturing processes should provide adequate vitamin and nutrient concentrations in feed.
4. Feed should be purchased fresh and not stored for more than a few months.
5. Feed should be stored in cool, dry areas to prevent mold and other contamination. Do not use contaminated feed.
6. Feed management practices should be implemented to assure the shrimp consume the maximum amount of supplemental feed and not leave excess amounts decomposing in the pond attributing to poor water quality.
7. Feeding rates should be determined from standard feed curves and adjusted for shrimp biomass, appetite, and pond conditions. Feed trays can be used to monitor feeding and prevent under or overfeeding.
8. The most efficient supplemental feeding can be obtained by distributing the supplemental feed several times through the day and night. Supplemental feed should be widely distributed throughout the pond, either by manual or mechanical dispersement or use of feed trays.
8. Appropriate feed curves commensurate with shrimp biomass and appetite should be utilized on a site specific, species specific basis and with the recommendation of shrimp feed specialists.
9. Medicated feed should be used only if necessary for the control of a specific diagnosis of disease.
10. Cut fish should not be used as shrimp feed.
11. Research to reduce the level of fish and other marine meals in shrimp feed should be encouraged.
12. Pond managers should keep careful records of daily feed application rates so that feed conversion ratio (FCR) can be assessed. Reductions in FCR through careful feeding will improve production efficiency and reduce waste loads.

Shrimp Health Management
Code of Practice

Purpose

The purpose of this Code is to promote shrimp health management as a holistic activity in which the focus is on disease prevention instead of disease treatment. Authorities on shrimp health management recognize that stress reduction through better handling, reasonable stocking densities, good nutrition, and optimal environmental conditions in ponds can prevent most infectious and non-infectious diseases. Treatment should be undertaken only when a specific disease has been diagnosed. Also, effective measures must be taken to minimize the spread of diseases between farm stocks and from farm stocks to natural stocks. This Code provides adaptable guidelines that should provide effective management of shrimp health.

The Code helps to achieve several of the "Guiding Principles for Responsible Aquaculture" and advances three basic premises as follows:

- Many disease problems can be prevented through stress management.
- Disease treatments should be made only after a clear diagnosis of the causative factors.
- Spread of disease should be minimized by reasonable regulation of importations of broodstock and larvae and by isolation and disinfection of affected ponds.

Management Practices

Adherents to the Code shall adopt the principles of good shrimp health management to reduce the incidence of diseases and to protect natural fisheries. The following practices should be used to achieve these goals:

1. Shrimp farming associations should work with governments to formulate and enforce regulations to include quarantine procedures for importations and exportations of broodstock, nauplii, and postlarvae.
2. Healthy postlarvae should be used for stocking ponds. Survival of postlarvae should then be optimized by preparing the pond to ensure adequate availability of natural food, by properly acclimating postlarvae before stocking, and by avoiding stress by using appropriate handling and transportation techniques.
3. Good water quality and bottom soil management should be used. Stocking rates should not be excessive and high quality feed and good feeding practices should be used.
4. Strong chemical treatments that can stress shrimp should not be employed.
5. Shrimp should be routinely monitored for disease, and a definite diagnosis obtained for any observed shrimp health problem.
6. For non-infectious diseases related to pond conditions, carry out the best option for disease treatment or for correcting pond conditions.
7. For mild infectious diseases with potential to spread within a farm, quarantine the pond and carry out the best option for disease treatment.
8. For serious infectious diseases that may spread widely, isolate the pond, net harvest remaining shrimp, and disinfect the pond without discharging any water.
9. Dispose of dead, diseased shrimp in a sanitary manner that will discourage the spread of disease.
10. When disease occurs in a pond, avoid transfer of shrimp, equipment, or water to other ponds.
11. Drug, antibiotic, and other chemical treatments should be done in accordance with recommended practices and comply with all national and international regulations.
12. The shrimp industry should work with governments to develop certification programs for disease diagnosis laboratories and pathologists.
13. Each country or geographical area should develop its own pond dry-out, farm situation, and biosecurity strategy.

Therapeutic Agents and Other Chemicals
Code of Practice

Purpose

The Code is intended to foster greater awareness within the shrimp industry of the proper use of certain potentially toxic or bioaccumulative compounds in shrimp production. Careful control over the use of therapeutants and other chemicals in production will assure that farm-reared shrimp are less likely than wild-caught shrimp to contain residues of pollutants or contaminants. Environmental benefits also will accrue from responsible chemical use. This Code contains flexible criteria that will allow prudent use of certain drugs, antibiotics, and other chemicals in production without endangering food safety or threatening the environment.

The Code helps to achieve several of the "Guiding Principles for Responsible Aquaculture" and promotes three basic objectives:

- The shrimp farming industry in each nation should work with governmental and international agencies to develop lists of approved feed additives, pesticides, drugs, antibiotics, and other chemicals and to specify approved uses for each compound.
- Shrimp farmers who adhere to the Code will rely on good management to prevent water quality and disease problems and chemicals should be used only when necessary.
- Chemical use in ponds should only be done after an accurate diagnosis of the situation and treatments should conform to acceptable protocol.

Management Practices

Adherents to the Code should strive to produce a wholesome product for consumers through responsible use of drugs, antibiotics, and other chemicals. Use of the following practices will assure this goal:

1. Shrimp health management at hatcheries and farms should focus on disease prevention through good nutrition, sound pond management, and overall stress reduction rather than disease treatment.
2. Where countries have approved lists of chemicals and chemical uses, only approved chemicals should be used in ponds and only for the use approved. Where such lists are not available, the shrimp industry and individual producers should work with governments to prepare such lists.
3. Shrimp farmers should follow information on product labels regarding dosage, withdrawal period, proper use, storage, disposal, and other constraints on the use of a chemical including environmental and human safety precautions.
4. When practical, antibiograms should be used to select the best antibiotic for use in a particular case, and the minimum inhibitory concentration (MIC) should be used.
5. When potentially toxic or bioaccumulative chemicals are used in hatcheries and ponds, waters should not be discharged until compounds have naturally decomposed to non-toxic form.
6. Careful records should be maintained regarding use of chemicals in ponds as suggested by the Hazard Analysis and Critical Control Point (HACCP) method.
7. Store therapeutants in a cool place and in a secure manner where they will be inaccessible to unauthorized personnel, children, and animals, and dispose of unused compounds by methods that prevent environmental contamination.
8. The shrimp-farming industry should work with governments to develop regulations for labelling the content and percentage of active ingredients in all chemicals including liming materials and fertilizers.

General Pond Operations
Code of Practice

Purpose

The purpose of the Code is to prevent eutrophication, salinization, reductions in biodiversity, and other environmental perturbations by using responsible pond management practices. Experience demonstrates that it is possible to optimize efficiency of shrimp production and be good stewards of the environment at the same time. This Code contains broad guidelines on pond management that can be used to standardize and improve operations for sustainable shrimp farming.

The Code helps to achieve several of the "Guiding Principles of Responsible Aquaculture" and asserts that:

- Responsible pond operations can protect or even improve environmental quality and enhance sustainability.
- Both profitability and environmental sustainability can be achieved at the same time.

Management Practices

It shall be the objective of adherents to the Code to use pond operation methods that are environmentally responsible while allowing profitable shrimp production. The following practices should be used to promote profitable, yet sustainable shrimp farming:

1. Farms should be encouraged to use hatchery larvae rather than wild-caught larvae.
2. Where wild caught postlarvae are used, a screening method should be used to separate by-catch and return it to the estuary.
3. Native species should be cultured whenever feasible; however, if non-native species are used, all applicable regulations should be obeyed regarding importation and inspection.
4. Only healthy postlarvae should be used.
5. Good water quality should be maintained by using stocking and feeding rates that do not exceed the assimilative capacity of the culture system and by using high quality feeds and good feeding practices.
6. Water exchange should be reduced as much as possible.
7. Fertilizers, liming materials, and all other chemicals should be used in a responsible manner and only as needed.
8. Good shrimp health management should be used.
9. Aerators should be positioned and operated to minimize erosion and creation of sediment mounds in pond bottoms.
10. Freshwater from wells should not be used in ponds to dilute salinity.
11. Effluents, sediment, and other wastes should be disposed responsibly.
12. Bottom soils should be evaluated periodically between crops and necessary treatments applied to remediate deterioration in soil conditions that occur during culture.
13. Water inlets and outlets to ponds should be screened to prevent entrance of competitors and release of culture species.
14. Predator control methods that do not require destruction of ecologically important species should be used.

Effluents and Solid Wastes
Code of Practice

Purpose

The Code is designed to increase the awareness of proper waste management within the shrimp farming industry and enhance protection of coastal land and water resources. Recognizing that a number of production activities produce wastes, shrimp producers and processors should formulate systems of waste management for protecting lands and waters in the vicinity of their activities. This Code provides a set of guidelines that can form the framework for responsible waste management that will benefit all coastal resource users including shrimp farming.

The Code helps to achieve several of the "Guiding Principles of Responsible Aquaculture" and specifically recognizes that:

- The shrimp aquaculture industry should promote responsible methods of effluent and solid waste management to protect environment quality and public health.
- Effluent and solid waste management is a continuous activity, and each member farm should strive to improve waste management procedures and reduce amounts of waste released to the environment.
- In countries where quality and volumes of effluent are not regulated by permits from governmental agencies, adherence to the Code is an alternative way of protecting the environment.

Management Practices

Adherents to the Code should continuously strive to improve waste management. Particular attention should be given to the following practices:

1. Canals and embankments should be maintained to reduce erosion of above water portions.
2. Minimize water exchange to the extent feasible.
3. Use efficient fertilization and feeding practices to promote natural primary productivity while minimizing nutrient inputs.
4. Store and use fuels, feeds, and other products in a responsible manner to avoid accidental spills that could contaminate water. An emergency plan should be made for containing accidental spills.
5. Ponds should be drained in a manner to minimize resuspension of sediment and prevent excessive water velocities in canals and at effluent outfalls.
6. Where feasible, pond effluents should be discharged through a settling basin or mangrove forest.
7. Design outfalls so that no significant impact of effluents on natural waters occurs beyond the mixing zone.
8. Shrimp pond effluents should not be discharged into freshwater areas or onto agricultural land.
9. Sediment from ponds, canals, or settling basins should be put back into areas from which it was eroded, used as earthfill, or disposed in some other environmentally-responsible way.
10. Sanitary facilities for disposal of human wastes should be provided at hatcheries, farms, and processing plants.
11. Garbage and other farm wastes should be burned, put in a land fill, or disposed of by other acceptable methods.
12. Shrimp farms, hatcheries, and processing plants should comply with existing governmental regulations related to effluents and other wastes.
13. Processing plants, and where necessary, shrimp hatcheries should install effluent treatment systems of appropriate type and capacity.
14. Managers should routinely evaluate waste management procedures and continually attempt to improve them.

Community and Employee Relations
Code of Practice

Purpose

The purpose of the Code is to foster good relationships among shrimp farm officials, workers, and local communities. Aquaculture can be a powerful stimulus to improving the standard of living in coastal communities by providing jobs and services, contributing to the tax base, improving the physical and social infrastructure, and creating a larger and more diverse and dynamic economy. Recognizing that public relations and employee welfare are complex issues, this Code is intended to provide some general guidelines for enhancing the prospects for harmonious interactions with workers and the local community. Conditions, expectations, and mores are highly variable from place to place, so considerable flexibility will be necessary in applying these guidelines.

The Code helps to achieve several of the "Guiding Principles for Responsible Aquaculture" and specifically promotes the following:

- Shrimp farms should employ local workers to the extent possible, provide good working conditions, and wages commensurate with local pay scales.
- Shrimp farms should abide by local laws and regulations regarding the rights of local people to use coastal resources.
- Shrimp farms should be supportive of local communities and engage in community activities.

Management Practices

Shrimp farms range in size from small, family operations to large corporate enterprises. Most of the guidelines given below apply primarily to large shrimp farms:

1. Shrimp farm owners should have clear title or right to their property or other current, legal land concession agreements.
2. Shrimp farm management should schedule meetings with local communities to exchange information. This is particularly important in the planning stages for new farms or expansions.
3. Shrimp farm management should attempt to accommodate traditional uses of coastal resources through a cooperative attitude towards established local interests and environmental stewardship.
4. Shrimp farm management should contribute to community efforts to improve local environmental conditions, public health and safety, and education.
5. Local workers should be employed to the extent possible, and all practical means made to prevent conflicts between local people and workers from outside.
6. Workers should be fairly compensated with respect to local wage scales.
7. Healthy and safe living and working conditions should be provided. Procedures should be established for dealing with illness and accidents, and employers must be responsible for making sure that workers are fully aware of these procedures.
8. Shrimp farm management should have clearly-defined and posted security policies.
9. Employees should have a clear understanding of their duties and of company expectations regarding their performance.

BEST MANAGEMENT PRACTICES FOR THE TROUT INDUSTRY

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I. INTRODUCTION

Trout production systems, typically are of the flow-through design, and, more often than not, rearing ponds are arranged in a serial reuse fashion, with water moving from pond to pond. This may repeat itself many times over. A sloping topography is required. The ponds can be arranged linearly or parallel to each other with the flow traveling zig-zag, i.e. back and forth, from pond to pond (Figure 1).

There are major variations in pond design with respect to size, shape, and structurally. Many older facilities may still use earthen ponds, of irregular shape and/or channel-like (raceways). An example of this is shown with Figure 2.

Ponds constructed out of concrete, fiberglass, or other solid material are, in general, either linear and rectangular (raceways) or circular (round). Pond design and structure can significantly affect the capability, and efficiency, of managing waste components, in particular the solids portion (feces and waste food).

II. WASTE MANAGEMENT PRINCIPLES

1. Preventative measures

Prevention is always the best strategy to consider first. For aquaculture there are two main approaches, as well as a long-term strategy.

A. Responsible feed management

In intensive fish production systems feed is the sole source of pollution. Feed waste, as uneaten food, must be avoided as much as possible. Although zero waste may not be practical, such waste should not exceed 3.0-5.0%. Feed losses as high as 20 to 50% have been reported (Cho, et al 1991). All of the waste feed contributes to pollution, and all of it directly affects the economics. The following should be considered in the efforts to reduce feed waste to a minimum.

a) Do not overfeed; administer feed properly.
b) Feed the fish, not the pond.
c) Handle feed with care; prevent fines/remove fines.
d) Know the feed requirements of the fish (%BW/day), through knowledge of the size of the fish, water temperature, projected, i.e. historical, growth rates and, not the least, the fish biomass in the pond (Westers, 1995).

B. Use low-polluting diets

These are diets which are highly digestible, of high nutrient density and with a well balanced protein to energy ratio. Of course, they must be economical as well, but the unit cost can be greater if feed conversions are lower. High energy diets, with up to 30% fat, 40% protein and 13% carbohydrates (nitrogen-free extract) when fed to Atlantic salmon, demonstrated reductions in nitrogen output by 35%, phosphorous by 20% and solids by at least 20% due to a feed intake reduction of 20% versus diets with a fat level of 22% or less (Johnson and Wandsrik, 1991).

C. *Genetic selection and/or genetic engineering*

As new species enter aquaculture, there is a need for domestication, but also a continuing need for improvements in growth rates and feed utilization of traditional species. A "shortcut" in this process is the technology of genetic engineering, the "creation" of genetically modified organisms (GMO). GMO's are extremely controversial. There is much opposition to the use of genetically modified foods. Genetically modified fish (salmon) have been produced, but opposition expressed by anglers, the wildfish lobby, conservationists, and even fish farmers and breeders, because they know that GMO is unacceptable as food, and that includes farmed salmon (Roberts, 2000). However, Roberts also points out that research into this technology should not be choked off outright, but ought to be allowed to proceed with all needed precautions in place. The present administration seems to support that. The Aquaculture News of May 2001, reports that genetically altered salmon will be on the menu of a white house state dinner in honor of the prime minister of France during his USA visit. EPA chief Christine Todd-Whitman voiced concern about the safety of GMO's and indicated that "this administration plans to keep a watchful eye on these products." In other words, let's move ahead with caution.

2. Corrective measures

Feeding fish is synonymous to polluting the water. Solids (feces and waste feed), nitrogen and phosphorus are the main compounds of concern. Solids can be reported as setteable, suspended, dissolved, BOD and COD, nitrogen as inorganic, ammonia, nitrite, nitrate, organic nitrogen, and total nitrogen, phosphorus as inorganic ortho-phosphate and as organic phosphorus in the solids fraction of the waste. Values for each of these sub-components have been reported, showing considerable variation. This is not surprising, because within flow-through systems much variation can be expressed due to management practices, source water quality, monitoring

methods (timing, frequency, etc), system design and operational modes. This problem was pointed out by Cho, et al (1991). They compared the nutritional mass-balance method (biological) with the water chemistry method (limnological), and found significant differences in outcome between these two approaches . Higher than projected TSS values are most likely the result of feed waste. Facility design and management practices can also be responsible for significant variations in concentrations of aquaculture waste components in the effluent. Best management practices are, to a large extent, driven by facility design.

III. System Design Factors

1. Earthen pond systems

It is always difficult to apply corrective action without a good diagnosis of the problem and an intimate knowledge of the characteristics of the production system. All flow-through systems are not equal. The most simple ones are earthen ponds, "down to earth" in construction but complex when it comes to identifying what is happening to the waste and how to manage it. Earthen raceways (ponds) function as settling basins, because of low water velocities. This is a characteristic of all raceways, a truth not always recognized, especially when large flows are used through such rearing vessels. To illustrate, a raceway with these dimensions, length (l) is 30 m (≙ 98') width (w) is 3.0 m (≙ 9.8') and a depth (d) of 0.66 m (≙ 2.2') has a rearing volume of 60 m^3 (2200 ft^3). To create a velocity of 15 cm/s (0.5'), a velocity that would keep solids in suspension, long enough, would require a flow rate of 18,000 lpm (4755 gpm), the water turn-over time would be a short 3.33 minutes, the exchange rate 18 x per hour (R = 18). Normal turn-over rates for most raceways range from 1.0 to 4.0 per hour. The velocity (v) at 4 changes per hour (R = 4.0) would be 3.3 cm/s (0.11 ft/s). Velocity can be determined with: $v = (l \times R)/36$
Where v is velocity in cm/s, 36 is from 3600 s/h divided by 100 because the velocity is expressed as cm/s, the length (l) in m (1.0 m = 100 cm)
For English equivalents: v (as ft/s) = (l (in ft) x R)/3600. In this case the units used for velocity and length are the same.

It is an undisputable fact that raceways function as settling basins. Every fish culturist who has worked with raceways has observed the buildup of solid waste within raceways.

This fact, that raceways function as settling ponds, creates several problems with respect to solid management.

As solids settle and build up, they are resuspended by fish and human activity. This destroys the integrity of the solids (fecal material and wasted feed) and changes relatively large particles (>100 ≙*m*) into many smaller particles. It has been reported that a high proportion (80%) of TSS may end up in size ranges from 5.0 to 20.0 ≙*m* (Boardman, et al, 1998). Such sizes take a long time to settle, they are difficult to remove, and even micro-screens are ineffective because these devises are not very efficient in capturing particles smaller than 80 ≙*m* (Boardman, et al 1998). Also, the finer the particles the easier they leach nutrients (N and P). Their surface area

to volume ratio is very large. For example, it takes 1600 5.0 μm particles to achieve the same volume as one 200 μm particle.

In serial reuse arrangements, such fine solids are passed from pond to pond, degrading the water quality. Although the concentrations are relatively low (< 10.0 mg/l) under normal conditions, they can reach concentrations in excess of 100 mg/l whenever there is activity within the pond, through heavy feeding when fish densities are high and through in-pond activities such as harvesting, sorting, inventorying, cleaning, etc.

In an earthen pond system much of the this waste seems to "disappear." Some of it is converted to new biomass (bacterial, algal, and higher organisms), and these generate their own waste components, such as BOD; COD; CO_2; dead organic matter, and, under anoxic and anaerobic conditions, release phosphorus, and can generate hydrogen sulfide or methane gas. It is therefore difficult, if not impossible, to determine final effluent end products from earthen ponds. The within system dynamics are too complex.

For instance, the BOD, TSS, and TAN concentrations reported (NPDES) for the facility shown as Figure 2, were 5.0, 3.0, and 1.1 mg/l respectively. As for the 1.1 mg/l TAN, this concentration, according to the manager, is the highest on record. Average concentrations have been in the 0.7 to 0.8 mg/l range.

This facility produces about 240,000 pounds of food-sized rainbow trout per year. Daily feed input ranges from 600 to 800 pound per day. The flow rate through the system is about 1200 gpm measured as the discharge flow rate. Assuming the following values per pound of food:

> Solids: 140 g; TAN: 13 g; BOD: 150 g.
> Then the daily totals, based on 800 pounds of feed, are 112 kg TSS (247 lbs); 10.4 kg TAN (23 lbs); and 120 kg BOD (264 lbs).

If these compounds were distributed evenly over a 24-hour period in the flow rate of 1200 gpm, concentrations would be 17.1 mg/l TSS; 1.59 mg/l TAN and 18.3 mg/l BOD. Compared to the measured concentrations, about 82% of TSS, 40% of TAN, and 73% of BOD is unaccounted for.

Trout production in earthen ponds present a difficult challenge for waste management. Settling ponds nor micro-screening can be effective, because routine effluent concentrations are very low and consist, predominantly, of very small particles.

There also is the problem of high TSS concentrations released during pond activities, with concentrations in excess of 100 mg/l. This flow should be diverted to a settling pond. This can be a problem where these flows are needed to supply other ponds in the series.

Over time, accumulated sludge may have to be removed from the pond. This is best accomplished by having the ability to drain the pond down to the level of the sludge and then

pump this material to sludge drying beds, constructed wetlands or land apply. For this, all fish must have been removed and, in most cases, there must be the ability to by-pass the normal flow.

2. Concrete raceways

Concrete raceways for salmonid culture are common with state and federal public fish hatcheries. They are also popular with the large Idaho trout industry and other, relatively large trout production systems throughout the USA and Europe.

Concrete raceways have a distinct advantage over earthen ones. They can accept greater flows of water and are easier to manage.

Nevertheless, even with higher flow-rates, these raceways still function as settling basins. Velocities of 15 cm/s or more are required to make the raceway self-cleaning, but velocities hardly ever exceed 3.0 cm/s. Even at this velocity the flow rate through the 30 m x 3.0 x 0.66 m dimension raceway mentioned earlier, must be as great as 3600 lpm (951 gpm). To accomplish the 15 cm/s velocity requires a flow rate of 18,000 lpm (4755 gpm).

As solids settle and accumulate in raceways, fish activity will, from time to time, resuspend them into the water column, breaking them down into smaller particles which take longer to settle. Eventually some will drift out of the raceway. In general, the TSS concentration in the effluent from raceways vary from 1.0 to 6.0 mg/l (Tables 1 and 2). Such concentrations depend on the amount of feed, the fish size and rearing density and the amount of waste accumulated, i.e., how frequently the pond is cleaned.

Whenever fish stir up solids, in pond TSS concentrations may reach 60 mg/l, but these are of short duration because most will resettle rather quickly (Boardman et al, 1998). Eventually some of these short-duration spikes exit the raceway. Batch sampling seldom "catch" these, and with 24 hour composite sampling, these short-duration spikes do not significantly contribute to the overall concentration.

Things are different with raceway cleaning, harvesting, sorting or any other activity requiring people to walk in the raceway. Shock loading can easily exceed TSS concentrations of 100 mg/l (Boardman, et al 1998). Table 1 shows concentrations during cleaning for seven state of Michigan facilities. These range from 54 up to 145 mg/l TSS, involving cleaning activities lasting 2 to 6 hours.

The 1998 studies by Boardman et al, agree well with studies carried out in 1972. Tables 1 and 2 also show much agreement for TSS concentrations under normal, routine operations, showing average values of 2.88 mg/l (1972) and 3.00 mg/l (1999 and 2000). Mean values for TSS for the three trout farms evaluated by Boardman et al were 3.9, 3.9 and 6.1 mg/l respectively. The ranges for the Michigan facilities are 1.0 to 6.1 mg/l (Table 1), for the Pennsylvania facility 0.6

to 5.7 mg/l (Table 2).
By the way, the negative TSS value of -0.9 mg/l in Table 1 for Baldwin indicates that the raceways trap solids from the source (river) water. In other words, incoming TSS concentrations are greater than the effluent concentrations (river velocities are greater than raceway velocities). This phenomenon has bee reported elswhere.

Table 2 lists the monthly NPDES monitoring values for BOD, TSS, TAN, and TP for 1999 and 2000, from the Big Spring fish culture facility operated by the Pennsylvania Fish and Boat Commission. The facility operates two groups of 40 concrete raceways, each group consisting of 8 parallel raceways, arranged in a 5-pass fashion. The flow from the upper 40 units, can be directed to the lower block of 40 units for another 5 passes. The first column of Table 2 lists the NPDES monitored values for BOD, TSS, TAN, and TP, the second column gives the projected values based on the following generated values per kg feed:

BOD - 0.340 kg (340 g)
TSS - 0.300 kg (300 g)
TAN - 0.030 kg (30 g)
TP - 0.005 kg (5 g)

The third column lists the percent differences between NPDES and the theoretical values. Phosphorus shows a somewhat higher average NPDES concentration then the theoretical value, but the NPDES report for BOD, TSS, and TAN are, on average, 64, 53 and 50% less than the theoretical, feed-based values. This is not that surprising for BOD and TSS but difficult to explain for total ammonia nitrogen in solution. Both effluent BOD and TSS concentrations can be expected to be less than predicted because of the settling characteristics of raceways. As a matter of fact, it appears that at least 50% of the solids end up at the bottom of the raceway and, if it was not for fish activity stirring these up, nearly 100% could be intercepted. It is a matter of knowledge among fish culturists familiar with raceways that these units can be almost self-cleaning if occupied by many large fish constantly stirring up the solids, at the same time destroying the integrity to the point where re-settling would require a very large settling pond. These have been used with raceways. Such large basins are difficult to clean. Not practical.

Studies by Cho et al, 1991, comparing theoretical feed based values for TSS, TAN and TP with effluent water quality monitoring, found the opposite for TSS, i.e. a greater value for the water chemistry analysis than the theoretical feed based value. This difference was 16%. The study was conducted with 4 m x 4 m square fiberglass tanks with rounded corners and a center bottom drain. In other words with a circulating fish rearing unit.

Obviously there are major differences between plug-flow and circulating rearing units. Findings by Cho et al, are opposite to those reported for raceways, i.e. higher TSS values versus lower values when predicted based on feed input. Routine monitoring of raceway effluents for TSS range, most of the time, from about 2.0 to 4.0 mg/l (Tables 1, 2 and Figure 3), these are almost always well below maximum NPDES values. Concentrations for TAN range from 0.05 to 1.00

mg/l (Tables 1, 2 and Figure 4). Similar values for TSS and TAN for three raceway flow-through trout production systems have been reported by Boardman, et al 1998. Again, these values are well below theoretical ones based on feed. Raceways require periodic cleaning to remove the accumulated waste. Removing these solids also help in reducing nutrient loadings. Raceways must be designed to include the capability to divert cleaning flows to sludge collection systems for storage and future processing.

3. Self-cleaning raceways

Westers, 1991 described the use of baffles in concrete raceways to make them self-cleaning. Baffles are thin plates positioned throughout the length of a raceway spaced apart at distances equal to the width of the raceway. They extend to, or above, the water surface and leave a gap between the bottom edge of the baffle and the raceway floor of 6 to 10 cm. As the bulk of the water passes through this narrow gap, the velocity increases. The goal is to create velocities from 15 to 30 cm/s, sufficient to move solids to the next baffle. Settled solids are continuously moved along to the fish retaining screen. Once there, they pass through the screen. As the water passes through the screen, the waste particles are separated and, subsequently, settle very rapidly in the quiescent zone. This zone, the sediment trap, is no longer than the width of the raceway (Figures 5 and 6). Detention time is only a few minutes, yet the bulk of the solids (75 to 85%) settle out most deposited immediately behind the screen. The presence of these screens help in creating a quiescent (non-turbulent) area within the trap, thus optimizing the settling of suspended solids. Because of the trap's limited storage capacity, solids may have to be removed as often as weekly, but, of course, this depends entirely on the feed input. Baffles do work and they work well in concrete raceways, but not in earthen ones. An overall raceway velocity of 3.0 cm/s (0.10') is desirable. This method of waste management has not caught on for these reasons:

a) Baffles interfere with managing the facility in particular where frequent harvesting is practiced from the raceway. This requires removal of these structures, which is viewed as very labor intensive.
b) Baffles provide surface areas for nuisance growth, bacterial and algal.

In countering these objections to baffles, it is very important to understand the function of the baffles and their basic construction and installation requirements.

Baffles are intended to make the raceway "self-cleaning" of fecal matter and waste feed. Baffles do not prevent biological growth on the raceway floor and sides. This growth will also occur on the baffles themselves. Raceways without baffles are routinely cleaned to remove accumulated waste. At the same time the brooms are often used to remove the growth from the bottom and the walls as well..."while we are at it." Fish culturists have been conditioned to keep raceways clean. Baffles will not perform that function, they will only "sweep" out the loose solids, not the attached growth. For most fish culturists this means that baffles really are not self-cleaning because they still have to go in with brooms to remove nuisance growth, not only attached to the floor and sides, but to the baffles as well. Thus, instead of baffles saving labor, they add labor

for cleaning. In addition, baffles interfere with harvesting and handling fish as well. Conclusion, baffles are too labor intensive.

Is it important to remove the algal and bacterial growth? This biological activity uses some of the dissolved nutrients and aid in purifying the water, rather than degrading it. In other words, water quality-wise, such growth is not harmful, rather the opposite. But it looks bad, raceways look dirty, it is a poor reflection on the fish culturist. So what! Fish culturist produce fish. Why waste time on removing such growth. The practice with Michigan's state hatcheries is to operate a full, one year production cycle without ever putting a broom into the raceway. They are only cleaned before the next cycle starts. A pressure washer can quickly clean the baffles.

Baffles do interfere with operations such as harvesting, sorting of fish, etc. Construction and installation of baffles must allow for easy and quick removal and re-installation. Baffles are not much more than heavy curtains hanging in the raceway. They can be constructed of very thin aluminum sheets. The top and bottom should be rolled to provide stiffness and avoid sharp edges as well. A stiff rod can be pushed through the rolled portion to hang the baffles from, as the rod ends rest on the raceway walls. Rods can also be bent 90^0 up out of the baffle and again 90^0 some distance up to permit baffles to be even or just below the surface of the water to expedite feeding. One person can easily and quickly remove and re-install the baffle, but if too bulky, two persons can perform this task very quickly. Weight should be no issue.

The benefits of baffles in managing solids are too great to be ignored. Culturists have to make some adjustments in their raceway cleaning habits. This means no brooms, no fish disturbance, no labor required for cleaning.

Baffles, so managed, result in a net saving of labor while performing an important task in managing solids, the most critical waste component in fish production.

The waste collected in the sediment trap at the end of the raceway can either be pumped out or drained to a solid storage facility for future processing.

4. Circular rearing units

Design and management of aquaculture systems are the critical factor leading to reduced waste output, provided they are affordable.

In contrast to raceways, round tanks can be self-cleaning, they can function as swirl settlers. When provided with two separate discharges, solids can be removed by means of a fraction of the total flow while the bulk of the flow, relatively free of suspended solids, can be reused.

The relatively small discharge through a central bottom drain can move concentrated solids, relatively intact, to a solids interception system consisting of micro-screens and/or settling basin. This flow can be as low as 5% of the total flow rate, while 95% of the flow is discharged from

an elevated drain (Summerfelt, 1998). This water can be reused, either in serial reuse fashion and supplemented with new water to replace the 5% lost, or design can be one of partial recirculation. Double drain circular rearing units can be very effective in managing solids. The more or less instant removal of these solids from the waste stream can contribute to reductions of nutrients as well. This relatively new approach for flow-through trout production appears to be rather promising with respect to efficient use of water and effective waste management.

Figure 7 shows a circular tank equipped with dual drains, an upper side-wall drain and a central bottom drain.

IV. Discussion

Effluent water quality measurements (such as NPDES records) for BOD, TSS, TAN, and TP from most flow-through fish production systems do not present reliable quantitative information about such waste components. In most cases such data does not match up with projected values based on feed input. As was stated earlier, in intensive aquaculture systems, feed can be considered the sole source of all waste.

Figure 8, courtesy of Fish Pro, quantifies the contribution of selected waste parameters per kg feed. Such values are somewhat diet dependent and efforts have been underway to reduce such values. These "low-polluting," nutrient-dense and high-energy diets have indeed been successful in lowering the outputs, as pointed out earlier. To help farmers in their efforts to become more efficient in feed/waste management, it would be helpful to label feed. Such labels could indicate that, under a set of "standard" procedures, that particular diet can be expected to accomplish a feed conversion of 1.0 and generate specific amounts of selected waste components. If, instead of a feed conversion of 1.0, the farm realizes a feed conversion of 1.5, the assumption can be made that a significant portion of the feed remained uneaten. All of the wasted food, the solids, the nitrogen, and the phosphorus would contribute to the pollution of the water.

It has been estimated that a feed conversion increase from 1.0 to 1.5 increases the COD by 186%, the TN by 70%, and the TP by 86% (Bergheim, et al 1991). Feed loss can be, and probably often is, a major contributor to aquaculture waste under farm conditions.

In flow-through trout production systems, where ponds function as settling basins, effluent concentrations of TSS, under routine conditions, under estimate the production of this waste component.

V. In Summary

The best available technology for waste management in trout flow-through production systems depend on design and management flexibility. The following recommendations are presented:

1) Earthen ponds should have the ability to drain down to the level of the accumulated

sludge. This should be pumped to an appropriate sludge storage or processing facility.

2) Concrete raceways must have the flexibility to divert cleaning flows, as well as similar "shock loadings," to appropriate settling basins, or, where equipped with baffles the raceway should have a relatively small sediment trap or quiescent zone from which fish are excluded. Sediment traps should be emptied before slouching of solids, due to buildup, occurs.

3) Round tanks should have a double drain design to allow continuous removal of TSS by means of a relatively small waste stream through a bottom drain. This waste stream can either be treated with micro-screens or settling ponds. Double drains tanks can be arranged in serial reuse or operated as partial reuse system.

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Table 2. NPDES monitoring data from Big Spring, PA state fish hatchery, a concrete raceway flow-through salmonid production system. NPDES values are compared with theoretical values based on feed input (Fd for feed). Differences are expressed in percent (see text).

MONTH/YR	BOD			TSS			TAN			T-PHOSP		
	NPDES	Fd	%	NPDES	Fd	%	NPDES	Fd	%	NPDES	Fd	%
1/99	2.9	9.5	69	2.0	8.4	76	0.38	0.84	55	0.10	0.14	29
2/99	4.0	8.6	53	3.0	7.5	60	0.56	0.75	13	0.13	0.13	0.0
3/99	4.5	7.3	38	1.0	6.4	84	0.59	0.64	8	0.15	0.11	-27
4/99	2.8	5.8	52	3.0	5.1	41	0.36	0.51	29	0.09	0.09	0.0
5/99	2.2	3.2	31	2.0	2.8	29	0.26	0.28	7	0.04	0.05	20
6/99	1.5	4.6	67	2.0	4.1	51	0.22	0.41	46	0.03	0.07	57
7/99	1.6	5.2	69	0.0	4.6	>100	0.23	0.46	50	0.05	0.08	38
8/99	0.6	5.3	89	3.0	4.7	36	0.32	0.47	32	0.07	0.08	12
9/99	2.5	6.7	63	2.0	5.9	66	0.34	0.59	42	0.09	0.10	10
10/99	2.6	7.6	66	3.0	6.7	55	0.37	0.67	45	0.09	0.11	18
11/99	2.9	8.5	66	4.0	7.5	46	0.49	0.75	35	0.06	0.13	54
12/99	4.3	8.6	50	1.0	7.6	87	0.47	0.76	38	0.19	0.13	-31
1/00	2.3	12.5	82	4.0	9.4	57	0.43	0.94	54	0.22	0.16	-37
2/00	4.7	9.6	51	4.0	7.2	44	0.49	0.72	32	0.24	0.12	-100
3/00	4.6	9.1	49	5.0	6.8	26	0.47	0.68	31	0.21	0.11	-91
4/00	1.8	8.5	79	1.0	6.4	84	0.25	0.64	61	0.05	0.11	55
5/00	1.4	5.5	75	2.0	4.1	51	0.31	0.41	24	0.11	0.07	-57
6/00	1.2	5.7	79	2.0	4.3	53	0.12	0.43	72	0.05	0.07	28
7/00	1.5	6.5	77	2.0	4.8	58	0.20	0.48	58	0.06	0.08	25
8/00	3.1	7.4	58	4.0	5.6	29	0.35	0.56	38	0.14	0.09	-56
9/00	3.2	9.0	64	7.0	6.7	-	0.52	0.67	22	0.12	0.11	-9
10/00	5.2	11.9	56	5.0	8.9	44	0.50	0.89	44	0.27	0.15	-93
11/00	5.7	10.9	48	4.0	8.2	51	0.79	0.82	4	0.20	0.14	-38

12/00	3.6	13.7	74	6.0	10.3	42	0.65	1.03	37	0.30	0.17	-76
* -	2.9	8.0	+64	3.0	6.4	+53	0.32	0.64	+50	0.13	0.11	-18

*The plus sign for average values indicate theoretical, feed based, values are greater than NPDES values. The negative sign is the opposite and is only true for TP.

Table 1. Net effluent concentrations in mg/l for five parameters for seven state of Michigan salmonid flow-through production facilities.

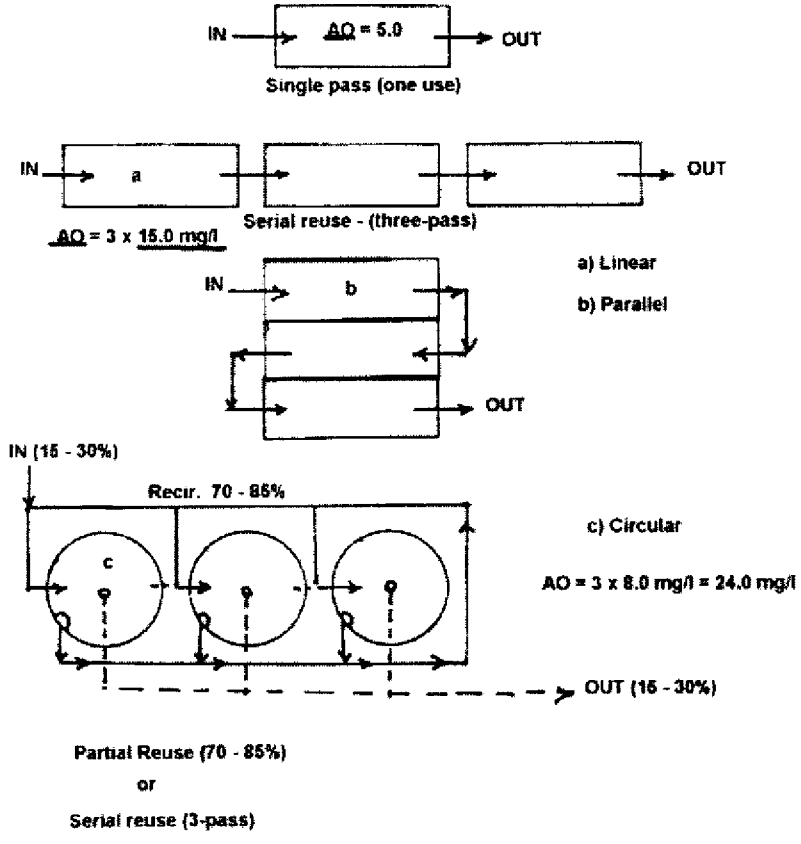
Facility	TSS		BOD		TAN		ORG. N		TOTAL P	
	*N	**CL	N	CL	N	CL	N	CL	N	CL
ODEN	6.1	145	1.9	7.5	0.19	0.28	0.21	9.3	0.12	2.4
MARQUETTE	5.4		-0.3		0.02		0.19		0.04	
THOMPSON	5.0		0.6		0.12		0.13		0.07	
HARRIETTA	1.2	87	1.6	3.5	0.08	0.18	0.02	3.2	0.07	1.00
WOLF LAKE	2.4	59	2.0	9.8	0.07	0.21	0.05	3.8	0.03	0.98
BALDWIN	-0.9		0.5		0.04		0.05		0.01	
STURGEON	1.0	54	-1.7	5.8	0.07	0.14	0.11	5.2	0.03	1.6
AVERAGE	2.88	86	1.20	26.6	0.084	0.20	0.108	5.4	0.053	1.50

*N represents normal (routine) operations

**CL represents raceway cleaning activities of 2 to 6 hour duration

All Samples: 24 hour composite

Figure 1. Flow Through Trout Rearing System



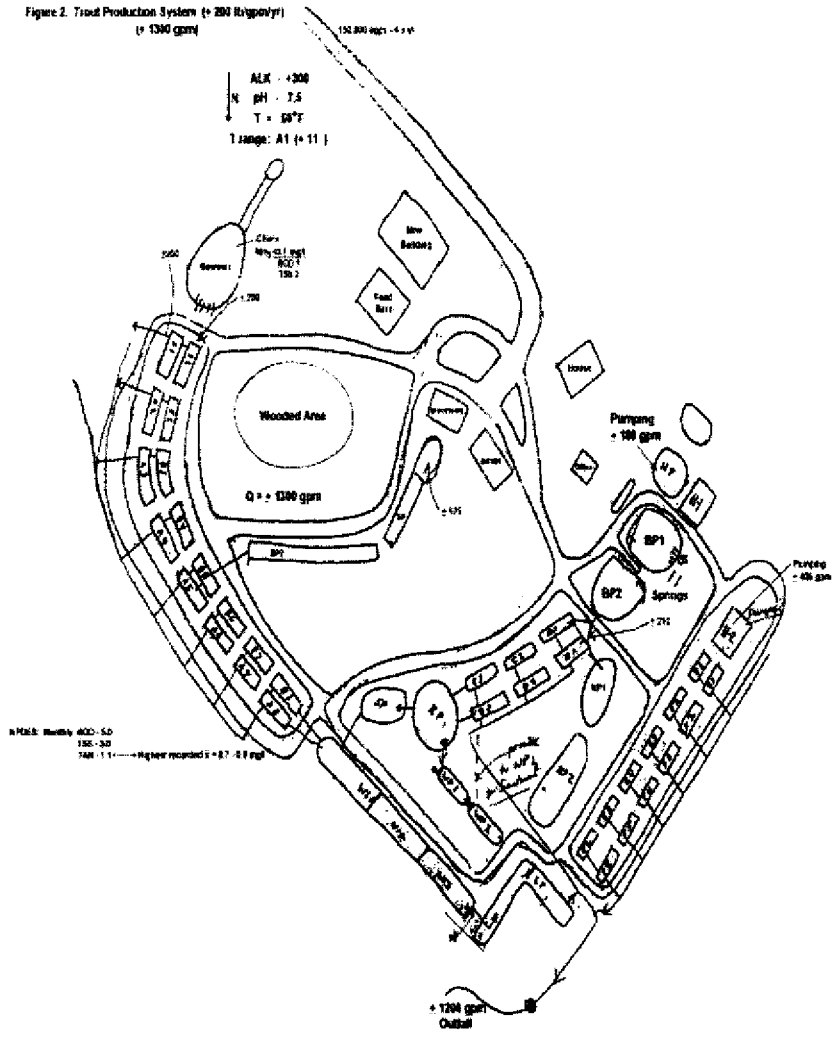


Figure 2. Trout Production System (+ 280 lb/gpm/yr)
(+ 1300 gpm)
ALK - +300
N pH - 7.5
T = 58°F
T range: A1 (+ 11)
Wooded Area
Q = + 1300 gpm
Pumping
+ 100 gpm
BP1
BP2
Springs
+ 1200 gpm
Outfall

Figure 3. Big Spring Historical Effluent Concentrations

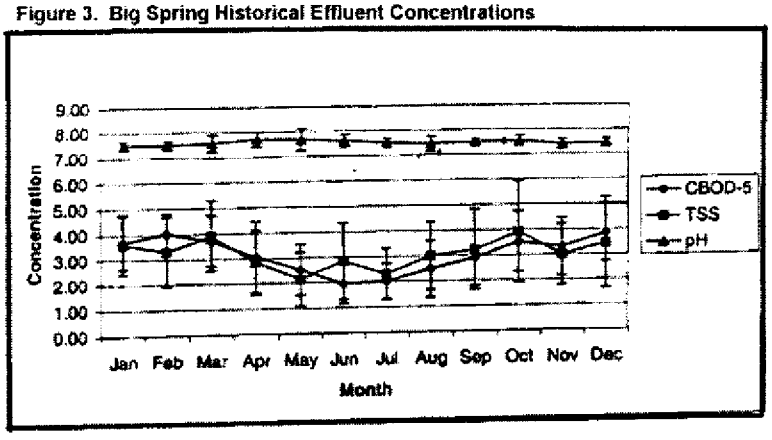


Figure 4. Big Spring FCS Histroical Effluent Concentrations (12 Years of Data)

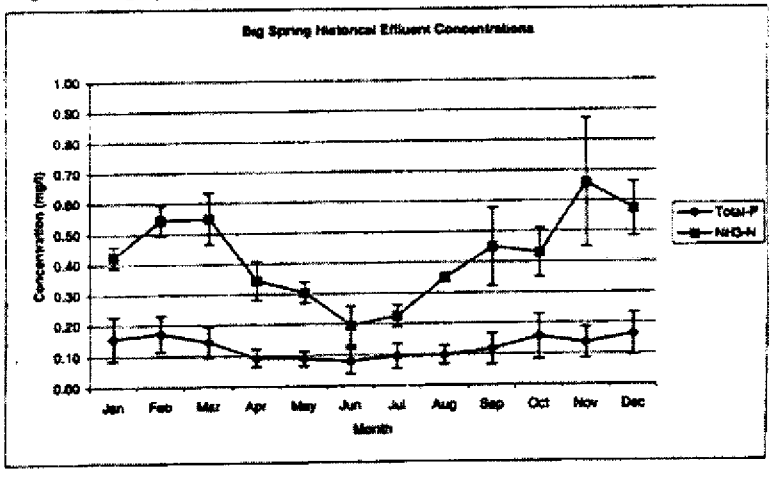


Figure 6. Raceway equipped with baffles.

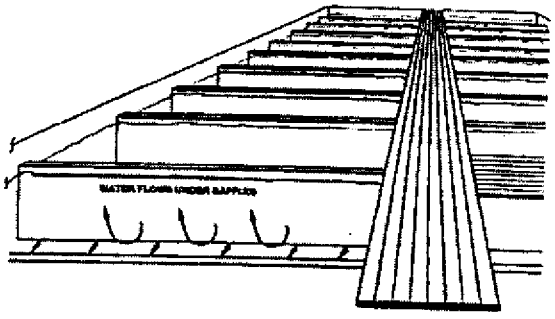


Figure 6. Solids settling characteristics in raceway solids settling section behind fish retaining barrier.

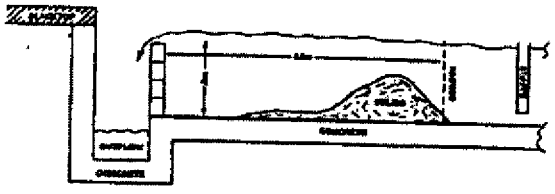
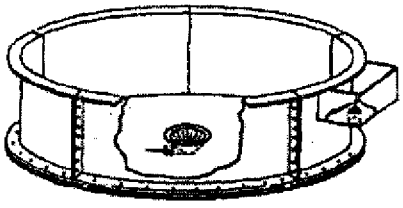


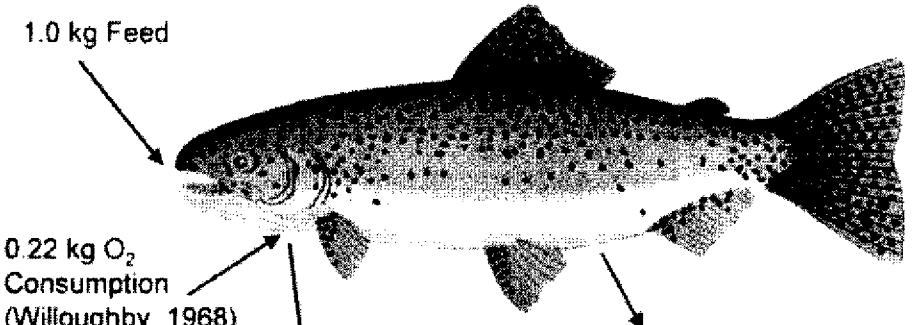
Figure 7 **'Cornell-type' dual-drain tank**



Courtesy of Red Ewald, Inc. (TX)

Figure 8

TROUT METABOLIC REQUIREMENTS AND BY-PRODUCTS



Best Waste Management Practices for the Alligator, Crawfish and Turtle Industries

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Although not necessarily considered "typical" aquacultured crops, alligators, crawfish and turtle hatchlings are all well-established, high-value industries within Louisiana. Each has its own unique nuances in terms of production management and potential environmental impacts, but all can be easily characterized within a best management practice (BMP) framework in terms of their feed, water, and energy inputs as well as their potential for effluent production. Although formal BMP's have not been well-defined for any of these production species, a review of current production practices allows for a rational discussion of potentially beneficial practices. Accordingly, each species will be addressed separately below.

Alligator Production

Alligator farming has grown significantly in Louisiana over the past 2 decades, with farm-gate values approximating $12 million in recent years. This form of aquaculture, as practiced in Louisiana and other southeastern states, involves raising alligator hatchlings to marketable size entirely under indoor confinement. Similar-sized alligators are raised together in groups in insulated "houses," with open floors divided between higher, exposed areas and lower, inundated pools. Houses, and the standing water within them, are maintained at approximately 30 C to promote growth. In this way, alligators can reach marketable lengths of 1.2-1.4 m in 14 to 18 months. Under natural conditions in Louisiana, most alligators would require 3 to 4 years to attain this size.

In additional to a warm, humid growing environment, farm-raised alligators require generous quantities of high protein feed. In recent years, the industry has moved away from unbalanced protein sources such as chicken mortalities, fur-bearer carcasses, and fish wastes and widely adopted dry, artificial rations. Alligators generate high levels of ammonia in their waste, reportedly excrete 4 to 5 times more ammonia per equivalent body weight than humans (Coulson and Hernandez 1983). Within an alligator house, waste excrement accumulates in the stagnant pools of water in the floor, requiring pens to be flushed clean on a daily basis. In this way, however, large volumes of wastewater are typically generated (approximately 6 liters per alligator per day). As a result of daily draining and flushing, large amounts of heat can be lost through effluent discharge at certain times of the year, requiring heating of replacement water prior to re-filling houses.

Alligator Waste Treatment Practices

At this time, alligator farms in Louisiana must treat effluents on site or dispose of them through existing wastewater systems to comply with established water quality standards.

Most Louisiana alligator farms are located in rural areas without access to municipal wastewater treatment. Accordingly, many utilize sludge pits and facultative lagoons for wastewater treatment, often in conjunction with land application or infiltration and these facilities are sized to provide adequate detention times to protect surrounding watersheds.

Alternatives treatment technologies have been evaluated for alligator effluents. Pardue et al. (1994) examined the exclusive use of land application as a means of treatment. The rationale for this evaluation was the potential to simultaneously utilize multiple treatment processes, such as adsorption, absorption, filtration of solids, precipitation, microbial uptake and degradation. The alligator house effluent they sampled resembled municipal domestic wastewater in both high biological oxygen demand and P concentrations, but contained much higher levels of N (approximately twice as much) due to high ammonia excretion rates.

While land application using overland flow consistently removed BOD_5 and N, over a 20-day period the removal of total soluble P became increasingly inefficient, presumably as a result of anaerobic conditions due to inundation and high BOD_5 loading. Based on system performance, the authors determined that a 2000-head alligator farm would require a land application area of approximately 0.16 ha to adequately treat effluents prior to release into the surrounding watershed. One potential problem was cited relating to long term performance: the ability of the system to retain P. Since this element is not converted to gaseous form the only mechanism for permanent removal would be through the harvest of vegetative cover on the land application area. Additionally, avoidance of anaerobic conditions was considered essential to maximize system efficiency and expected performance lifetime.

Delos Reyes et al. (1996) evaluated the effectiveness of water recirculation using floating bead filters to reduce water and energy use in commercial alligator production. The addition of biological and mechanical filtration greatly reduced water and energy requirements, but difficulties were cited in terms of retrofitting current technology to utilize recirculation approaches. In a related study, Langlinais and Soileau (1996) reported an annual operating cost reduction of roughly 65 percent associated with converting conventional flushing management to recirculation, even when taking into account the annualized costs associated with required equipment purchases.

Crawfish Production Practices and Effluent Considerations

Crawfish culture in Louisiana is a $30 million per year industry, based on self-perpetuating populations in shallow ponds and impoundments managed to simulate annual hydrological and vegetative cycles in the species' natural habitat. New-established ponds are initially stocked with adults during spring or early summer while partially flooded. Ponds are subsequently drained to force crawfish to burrow into levees and, to a lesser extent, the pond bottom.

In many parts of south Louisiana it is not uncommon to dig a hole for, say, installation of a fence post or some other purpose, only to find it half-full of water the following day.

When faced with receding surface water, crawfish normally burrow to a depth at which water will seep in from the surrounding soil, which is typically at depths of 30-60 cm in many areas where crawfish are farmed in Louisiana. Burrows are often capped with excavated mud, for protection and/or to conserve moisture and humidity. To survive within its burrow through the summer, a crawfish occasionally dips into the standing water at the bottom of the burrow to moisten its gills and subsequently sits motionless several inches above the water level, relying on the air trapped in the burrow as a source of oxygen. When its gills become too dry, it moistens them again. Metabolic activity drops to a very low level, but many mature females lay their eggs while burrowed, beginning around late August through December.

During summer and fall months, a forage crop of rice or natural vegetation is grown in the pond bottom as crawfish broodstock aestivate in burrows. In mid- to late fall, ponds are flooded to provide habitat for broodstock and newly-hatched juveniles emerging from burrows, providing a population for the new season=s crop. Once a crawfish pond is reflooded in the fall, inundated vegetation serves as the basis of a detrital food chain. Frequent flushing, however, is required to offset the high oxygen demands generated by rapid decomposition of vegetation early in the season.

In the past, many producers utilized baffle levees in an attempt to maximize the benefits derived from flushing their ponds, but this practice still required pumping large volumes to improve water quality, due to dilution with poor quality water throughout the pond. In recent years, a modified fall flushing approach has been adopted by many producers. After initially flooding the established vegetation to a depth of 25 to 30 cm, roughly 10 to 15 cm of water is drained off the field. This process is then repeated one to several times prior to establishing a permanent flood to a depth of 30 to 40 cm. In this way, significant savings in pumping costs are usually realized, as well as significant reductions in water use and effluent generation.

As water temperatures cool with the onset of winter, flushing becomes less of a concern. Warmer temperatures associated with the following spring, however, often require some water exchange to maintain adequate levels of dissolved oxygen. At this time, the activity of crawfish populations generally results in high levels of suspended clay particles, which are discharged whenever water is exchanged. Additionally, regular harvesting activities during the spring tend to disturb pond sediments. As a result, effluent quality during the spring is somewhat poorer than during the fall, although flushing volume is often far lower. Innovative flushing practices, relying on releasing stale water prior to pumping fresh supplies, are also becoming increasingly common during springtime.

Since most of the problems associated with springtime effluents in crawfish production relate to the high levels of suspended clay particles, a number of practical approaches to effluent improvement are being considered by industry and researchers. These include encouraging the growth of natural aquatic vegetation to serve as in-pond buffer zones or strips, surrounding drain outlets. Another approach involves the use of single or multiple porous walls, filled with gravel, around drain outlets. Since crawfish ponds are generally

shallow, this approach does not require complex engineering to construct effective barriers to water flow.

Turtle Hatchling Production

Turtle farming has been practiced for decades in Louisiana, developing around the controlled production of baby turtles, specifically red-ear sliders, for sale as pets. Production of baby red-ear sliders involves holding large numbers of breeding adults at high densities in outdoor ponds. Pond banks are typically hard clay, but may be covered with plastic material to prevent erosion caused by turtles climbing in and out of the water. Under some circumstances, plastic liners can be too slick or smooth, making it difficult for turtles to move about freely. Pond banks are occasionally overlaid with concrete, but this material can cause excessive damage to the turtles' ventral shell surfaces.

Breeding ponds are surrounded with flat areas of sandy soil arranged to provide nesting grounds. Access to laying areas is controlled by removable fencing or bales of hay at the top of the pond bank. Heavy clay soils in nesting areas are usually amended or covered with lighter, sandy soils. River sand is often hauled in to provide an optimum nesting medium. The soft texture of the sand and its tendency to pack together makes it easier for laying females to dig nests. Sandy soils also provide better drainage if excessive rainfall occurs.

Brood pond sizes range widely in Louisiana. Mean and median values reported by Hughes (1999) were 1.1 and 2 hectares, respectively. Pond- and laying-area perimeters are typically enclosed with sheet metal fencing to prevent straying and discourage predators. Turtles are easily frightened, so fences are usually tall and rigid enough to prevent broodstock from seeing outside the pond enclosure. Fences are inspected regularly and maintained to prevent loss of breeding turtles and exclude potential predators.

Broodstock are generally collected from the wild or, occasionally, purchased from other turtle farms. Adult turtles are stocked at 18,500 to 37,000 head per water-hectare. Even when raised in captivity, brood turtles often require 1-3 years to become acclimated to breeding ponds and reproduce reliably. Brood turtles are typically fed floating catfish feed (28 percent protein), although some producers use specially-formulated rations (see broodstock nutrition section below). Daily feeding allowances vary, depending on seasonal temperatures. Feeding rates may reach more than 40 mt per hectare per year, resulting in poor water quality due to high levels of fertility. Nonetheless, this equates to very small amounts of food on a per-turtle basis.

Effluent Considerations – Turtle Brood Ponds

Most practical approaches to reducing effluent impacts from turtle ponds mirror those developed for catfish production. Most turtle producers operate their brood ponds for many years without draining, and many practice water management techniques that allow for capture and storage of rainfall. Few flush their ponds under any circumstances, so that most effluents are associated with rainfall events. When ponds must be drained,

appropriate water retention can be expected to improve the quality of effluents substantially.

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Coulson, R.A. and Hernandez, T. (1983). Alligator Metabolism. Studies on Chemical Reactions In Vivo. Pergammon Press, New York, USA.

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Hughes, D.W. 1999. The contribution of the pet turtle industry to the Louisiana economy. Aquaculture Economics and Management 3(3):205-214.

Langlinais, S.J., and Soileau, R. (1996). Energy conservation and economics in an intensive recirculating alligator production system. Louisiana Department of Natural Resources, Baton Rouge, Project No. PVE-40-95-01.

Pardue, J.H., DeLaune, R.D., Patrick, Jr., W.H. and Nyman, J.A. (1994). Treatment of alligator farm wastewater using land application. Aquaculture Engineering, 13, 129-145.

AQUARIA, INC.
AQUATIC RESEARCH LABORATORY

16 May, 2001

Attendees of the
Aquacultural Waste Management Symposium
July 22-24, 2001

Dear Attendees:

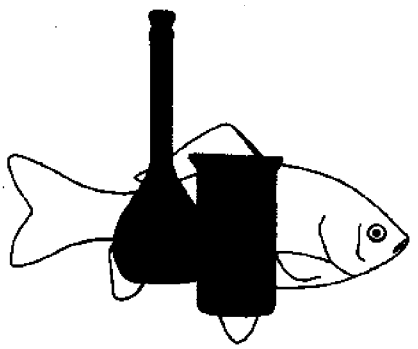
Attached are two articles I have written regarding my research on nitrification and the nitrifying bacteria in culture systems. One is a summary of two papers I have published on the phylogenetics of nitrifying bacteria. The original papers are copyrighted by the American Society for Microbiology but can be freely accessed at their web site (www.asm.org) under Journals and using the search term "Hovanec".

The second article is my presentation given at the World Aquaculture Society meeting held in Orlando Florida this past January.

I hope you find these useful. If you have questions I can be reached at hovanec@marineland.com

Sincerely

Timothy A. Hovanec, Ph.D.
Chief Science Officer



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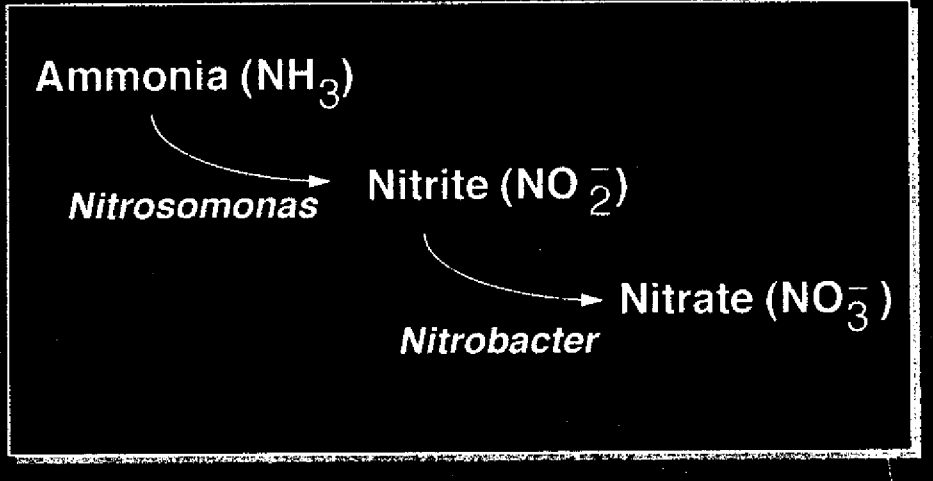
Investigations into the Lack of Efficacy of Starter Bacterial Cultures for Nitrification in Seawater and Freshwater Systems

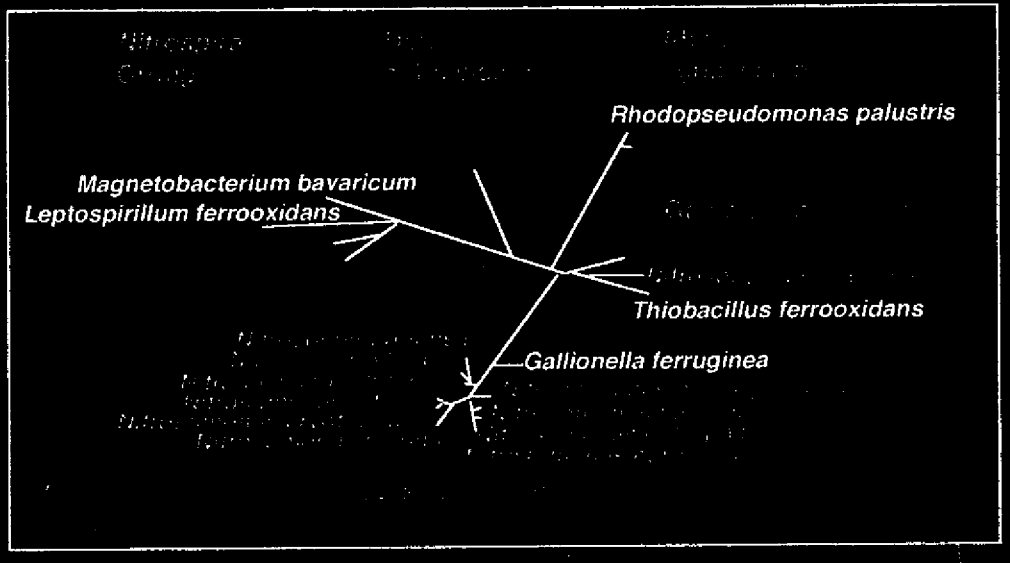
T. A. Hovanec, J. R. Sears-Hartley, L. L. Wilson, Jr., J. Coshland, C. M. Phalen, S. Wirtz, J. Niemans, and P. C. Burrell

Aquatic Research Laboratories, The Aquaria Group, 6100 Condor Dr., Moorpark, CA 93021 (805) 553-4446, hovanec@marineland.com

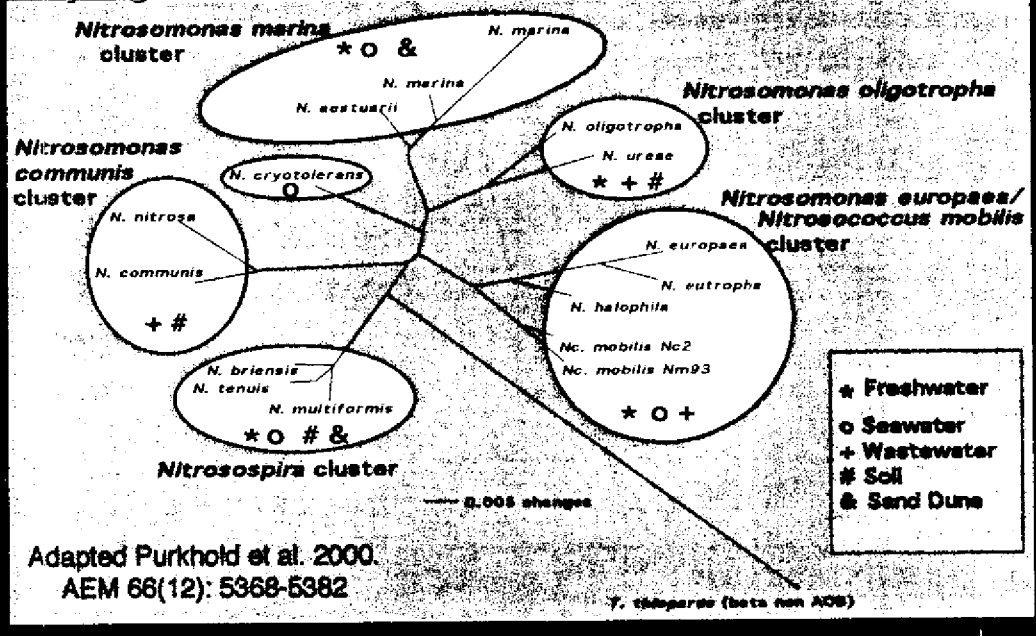
Nitrification - the traditional scheme



Phylogenetic Relationships of AOB & NOB



Phylogenetic Classification of beta-subclass AOB



Nitrospira NOB

Investigator	Environment	Reference
Hovanec et al.	fresh & seawater aquaria	1998. AEM 64:258-264
Burrell et al.	domestic wastewater treatment facility	1998. AEM 64:1878-1883
Juretschko et al.	activated sludge of industrial wastewater	1998. AEM 64:3042-3051
Schramm et al.	lab-scale fluidized bed reactor	1998. AEM 64:3480-3485

AEM - Applied and Environmental Microbiology. published by the American Society for Microbiology

EXPERIMENTAL DESIGN

FRESHWATER		SALTWATER	
with fish (feed)	without fish (dosed NH_4Cl)	with fish (feed)	without fish (dosed NH_4Cl)
3 replicates of each mix (CY, SS, ML) & Control		3 replicates of each mix (CY, SS, ML) & Control	

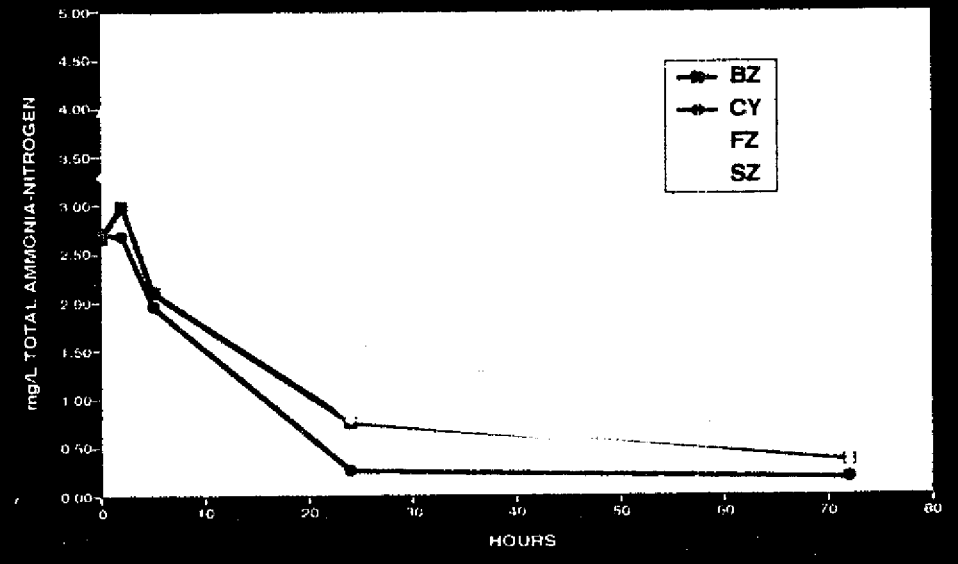
ARE RESULTS DUE TO

- Lack of presence of traditional AOB/ NOB in the available mixtures
- Traditional AOB/NOB cannot survive in current packaging
  - Do mixtures have a short shelf life
- Insufficient numbers of AOB/NOB in the available mixtures

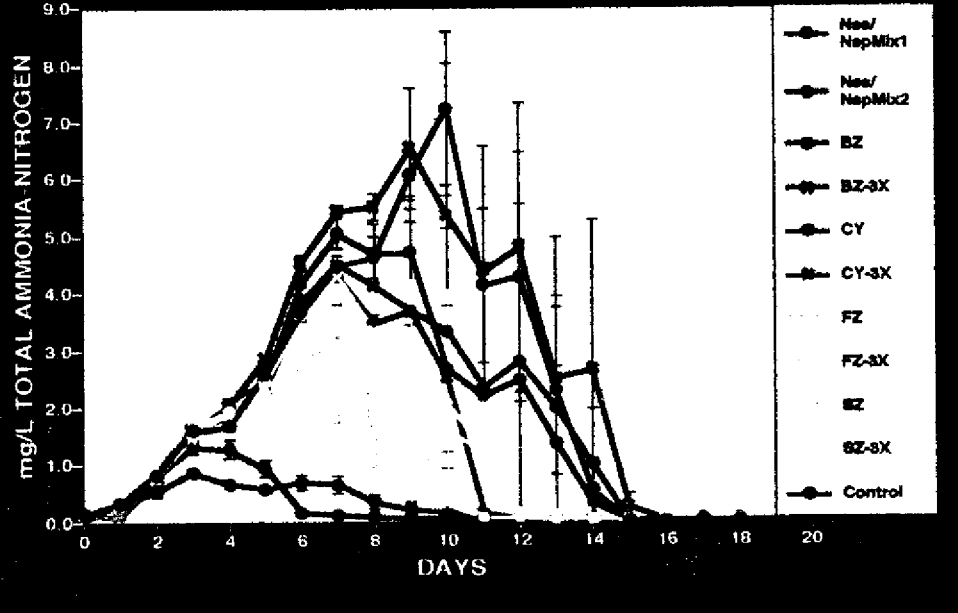
RESULTS OF PCR ON ADDITIVES

Additive	*Nitrosomonas*	*Nitrobacter*
BZ	positive	positive
CY	positive	positive
FZ	positive	positive
SZ	positive	negative

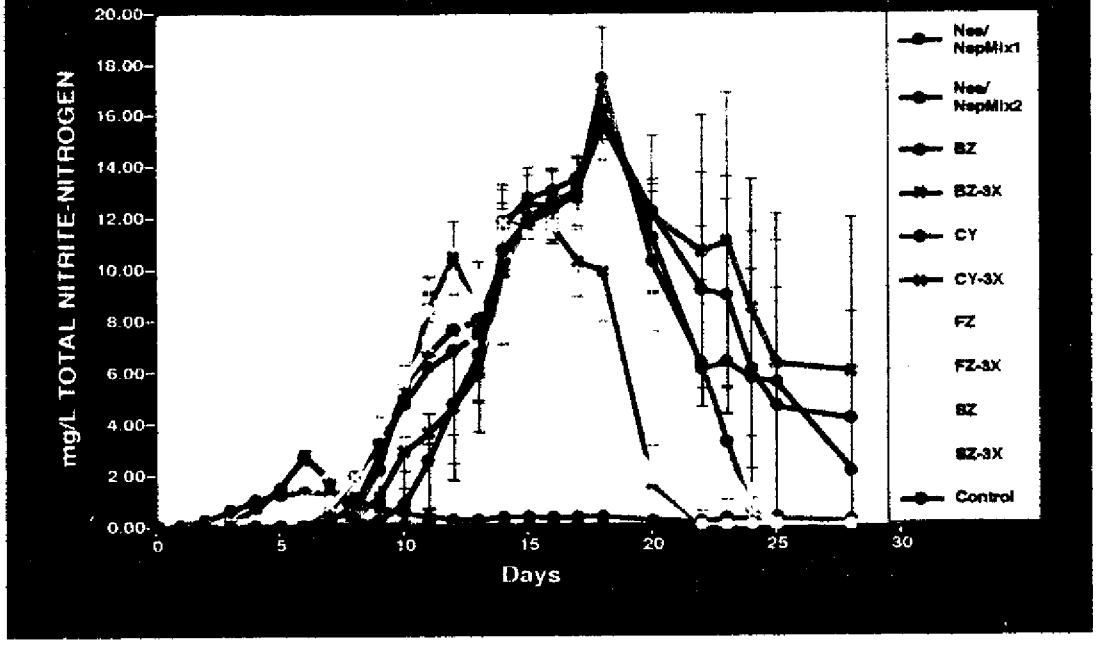
AMMONIA TREND OF COMMERCIAL ADDITIVES IN FLASKS



AMMONIA TRENDS FOR ADDITIVES DOSED 1X and 3X

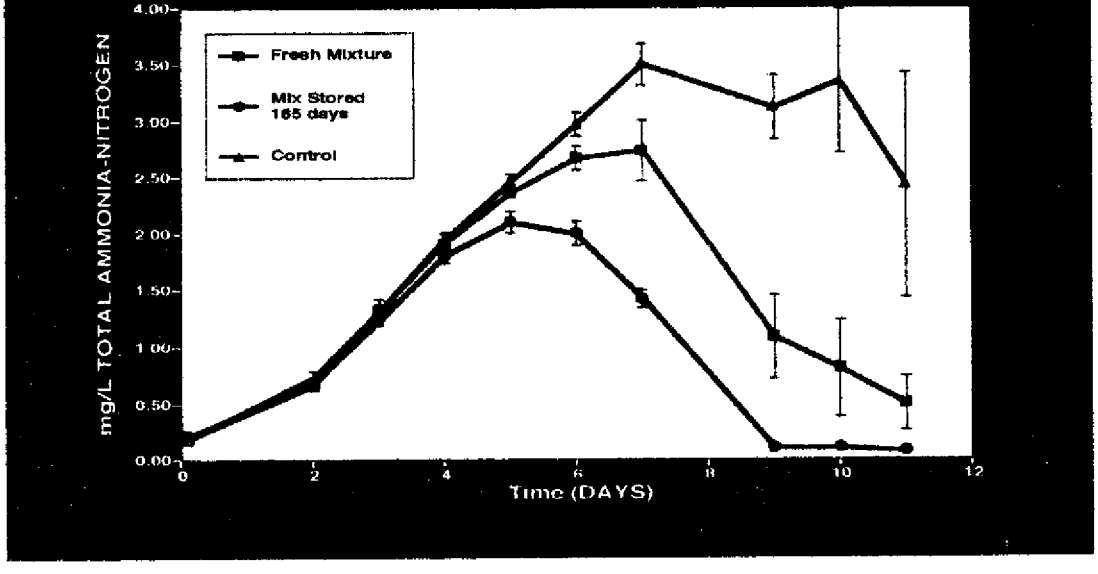


NITRITE TRENDS FOR ADDITIVES DOSED 1X and 3X



STORED VS FRESH

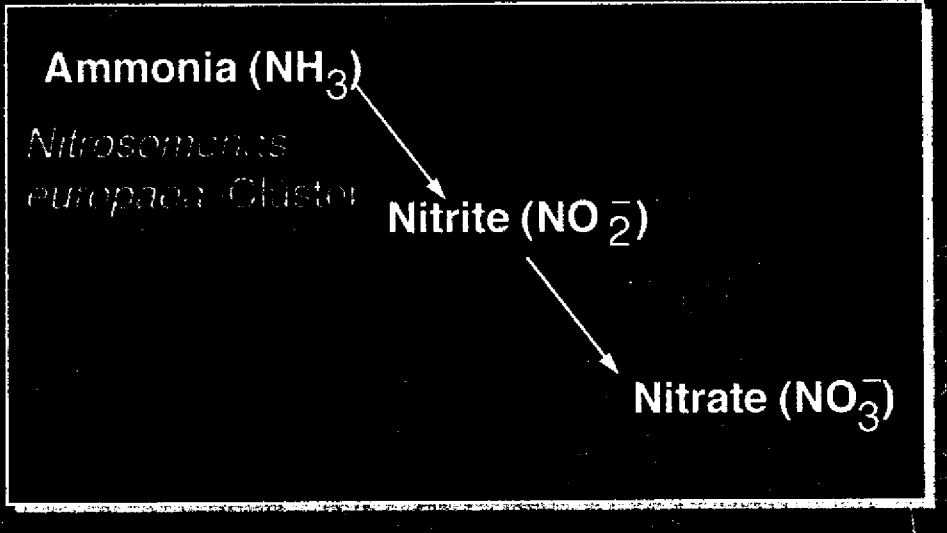
AMMONIA TREND OF STORED EXPERIMENTAL AOB



RESULTS

- Commercial Mixtures of *N. europaea* type AOB & *Nitrobacter winogradskyi* NOB
  - did not significantly accelerate establishment of nitrification in test aquaria
- Experimental Mixtures of *Nitrosomonas/ Nitrosospira* AOB and *Nitrospira* NOB
  - resulted in much lower ammonia & nitrite conc
  - ammonia & nitrite conc. reached zero much faster

Nitrification - current status



Nitrification - current status

Ammonia (NH_3)

~~*Nitrosomonas europaea* Cluster~~

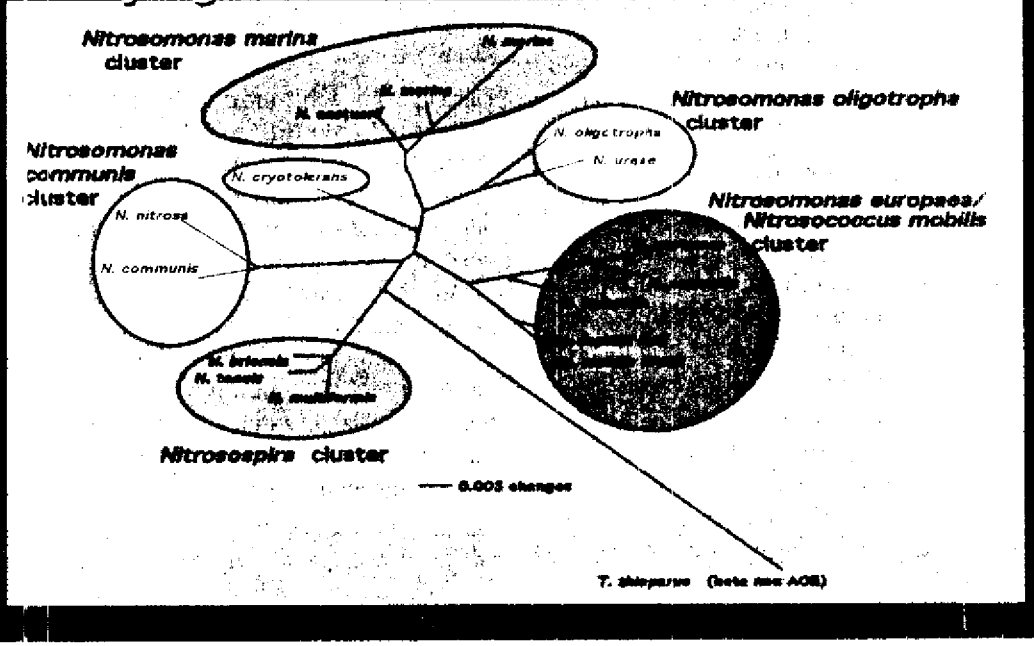
Members of the *Nitrosomonas marina* and *Nitrosospira* Clusters

Nitrite (NO_2^-)

~~*Nitrobacter*~~

Nitrate (NO_3^-)

Phylogenetic Classification of beta AOB



Linking Nitrification and the Nitrifying Bacteria

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For over 3 decades, the bacteria deemed responsible for nitrification, or biological filtration, in closed aquatic systems were thought to be *Nitrosomonas europaea* and *Nitrobacter winogradskyi*. Recent studies using modern methods of molecular biology, including cloning, DNA sequencing, DNA fingerprinting, and fluorescent in situ hybridization (FISH), have demonstrated that this is not the case. The data show that there is significant diversity of ammonia- and nitrite-oxidizing bacteria. The bacteria responsible for ammonia oxidation in freshwater and saltwater systems are newly discovered members of the *Nitrosospira* and *Nitrosomonas* genera. Nitrite-oxidation in freshwater and saltwater systems is performed by bacteria belonging to the phylum *Nitrospira*, which are not closely related to *Nitrobacter winogradskyi*.

INTRODUCTION

Nitrification in closed aquatic systems, whether they are 40 L or 40,000 L in volume, is one of, if not the most critical aspects of water quality management. Nitrification is defined as "the oxidation of ammonia to nitrite, followed by the oxidation of nitrite to nitrate, by bacteria". In closed aquatic systems, nitrification is the most efficient method to remove ammonia from the culture water before the ammonia reaches a toxic concentration. The source of ammonia in the culture water is the aquatic organisms themselves. Ammonia is the principal nitrogenous waste product of teleosts and some invertebrates. Therefore, if not controlled, the ammonia concentration in a closed aquatic system will continually increase. Ammonia is also toxic to aquatic organisms resulting in central nervous system impairment and eventual death. Acute toxicity of ammonia at concentrations of 0.5 to 1.0 mg/L (NH_3-N) have been demonstrated. Chronic low levels of ammonia have also been shown to retard fish growth.

Therefore, a mechanism to rid the culture water of ammonia on a continuous basis is necessary for the survival and development of aquatic species maintained in closed aquatic systems.

For over 100 years, it has been known that certain groups of bacteria oxidize ammonia to nitrite and nitrite to nitrate. These bacteria are classified as belonging to the family *Nitrobacteraceae* but it is realized that the members of this bacterial family are not all phylogenetically related (6).

The most commonly studied ammonia-oxidizing bacterium (AOB) is *Nitrosomonas europaea* while *Nitrobacter winogradskyi* is the most frequently studied nitrite-oxidizing bacterium (NOB). However, other species have been known and isolated from seawater, cooling towers and other aquatic environments (9, 11).

In the field of aquatic filtration, which for the purposes of this review includes wastewater treatment, aquaculture, public aquaria and the ornamental fish hobby, it has been readily accepted that *Nitrosomonas europaea* and *Nitrobacter winogradskyi* are the principal AOB and NOB, respectively, responsible for nitrification. Many scientific papers, books and popular articles have been written about these bacteria and their important role in water filtration.

AQUATIC FILTRATION SYSTEMS

The three major components of a closed aquatic filtration system are: mechanical, chemical and biological. Mechanical filtration is the removal of particulate material from the water by some type of straining. The straining material can be screens, foam pads, sponges or fibrous cartridges (to name but a few). In all cases, they act on particles in the water and not dissolved substances.

Dissolved substances, such as phenols and tannins, which discolor the water, are removed chemically. The most common chemical filtration medium is activated carbon.

While mechanical and chemical filtration are important, almost all aquatic organisms can live in water that is turbid and/or discolored. The same cannot be said for water with a high ammonia concentration.

This is why biological filtration is the most important component of the filtration system. Much work has been done engineering many types of media and kinds of filters on which to grow the nitrifying bacteria. The engineering has also included calculating water flow

rates, water retention times, and various other parameters necessary for building a filtration system. Further, there are many studies on the rates of nitrification and other physiological aspects of the nitrifying bacteria in closed aquatic systems.

There have also been studies on determining the bacteria themselves but these studies were misleading at best because it has been determined that *Nitrosomonas europaea* will generally out-compete other ammonia-oxidizing bacteria when one is attempting to obtain pure cultures of these bacteria.

This was the general state of the accepted knowledge in the field of aquatic filtration in the early 1990's when I started work on my Ph.D. dissertation at the University of California, Santa Barbara.

PHENOTYPIC VERSUS PHYLOGENETIC CLASSIFICATION

To further understand why prior studies were misleading one needs to realize that while phenotypic classification of microorganisms is useful for distinguishing closely related cultivable species, it is unsatisfactory for interrelating distantly related taxa and uncultivable forms (see (12) and references therein). The traditional classification scheme for microorganisms is based on tests of physiological responses and biochemical reactions (7). Thus, in order for a novel organism to be classified, a pure culture of the microorganism in question is needed so the requisite tests can be performed. The accompanying responses, it is assumed, give a clue as to the genetic relationship of the organism to other microorganisms.

While it is clearly not impossible to cultivate novel organisms, and no organism can be considered impossible to culture in the strictest sense, many organisms resist cultivation. The lack of culturability leads to an underestimation of microbial diversity in natural samples. Further, a bias in cultivation can give undue importance to bacteria which, because of their ability to grow in a pure culture versus other members of the assemblage, play a seemingly important role in the environment. However, in the natural setting the cultivable bacteria may be of minor importance, relative to the uncultivated types.

It is estimated that as little as 1 to 3% of the bacteria from a given environment can be cultivated (1). To fully understand the complex world of microorganisms new methods and technologies were needed.

This is where modern molecular biology entered the field of microbial ecology and aquatic biology. The 1990's saw the introduction and rapid advancement of new ways to examine organisms including microbes. These methods included cloning, DNA sequencing, DNA fingerprinting and classifying organism based on their DNA similarity rather than morphological or physiological similarities.

An example of phylogenetic classification for nitrifying bacteria is presented in Fig 1. Ammonia-oxidizing bacteria (always begun with the prefix *Nitroso-*) are confined to the beta subdivision of the *Proteobacteria*, except for one bacterium, while nitrite-oxidizing bacteria (begun with *Nitro-*) are more widespread with no members in the beta subdivision of the *Proteobacteria*.

An initial goal of my research was to develop molecular probes for *Nitrosomonas europaea* and *Nitrobacter winogradskyi*. The probes would then be used to detect and quantify these bacteria from samples

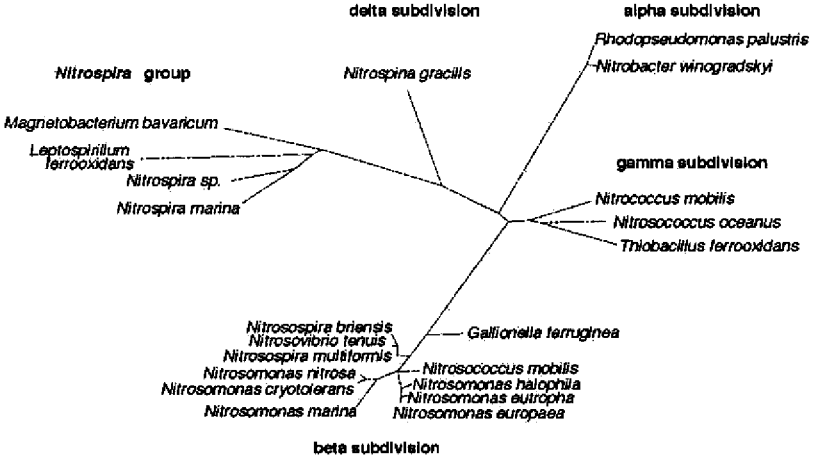

FIG. 1. Phylogenetic relationships of ammonia- and nitrite-oxidizing bacteria.

taken from various filters and locations in functioning test aquaria. Molecular probes are a short sequence of DNA that can be made to match (and so target) a specific bacterium or a group or bacteria.

Once the probes were developed I would then have a novel way to quantify the nitrifying bacteria and could begin the next phase of the project.

The second phase of the project was to use the probes to determine what types of filter media did the AOB and NOB prefer, where do the AOB and NOB actually live on these media, and how close to each other do the AOB and NOB reside. The answers to these questions would allow one to develop better, more efficient biological filtration systems.

A NEGATIVE ANSWER

The results of my first 3 years of work were published in August of 1996 with a paper entitled Comparative analysis of nitrifying bacteria associated with freshwater and marine aquaria co-authored by Professor E.F. DeLong (4).

Briefly, I was able to develop a couple of molecular probes for AOBs and one for NOBs. The AOB probes were targeted towards AOBs such as *Nitrosomonas europaea* and its closest relatives and *Nitrosospira* AOBs. The NOB probe targeted *Nitrobacter winogradskyi* and its closest relatives.

I could get the probes to work on pure cultures of the target AOBs or NOBs but samples from freshwater aquaria always returned negative results. Initially, I thought the problem was with the probes or the technique used to extract the DNA or RNA from the aquarium samples until I tested some samples from saltwater aquaria.

When I examined DNA from saltwater aquaria I got a positive signal for the AOB probes but not the NOB probes. This meant that the extraction techniques were good. I then did an experiment (the results of which were presented at the 96th annual meeting of the American Society for Microbiology held in New Orleans, May 19-23, 1996, Fig. 2) where I set-up six freshwater aquaria and let then go through the establishment of nitrification. When the ammonia added to the tanks could be oxidized to nitrate in less than one day I switched three of the tanks to saltwater and started three new saltwater tanks. I sampled and probed all the tanks at different stages in the test.

The results of this test were that in no case could I get a positive signal for freshwater aquaria from the probes (Fig. 2). However, all the saltwater aquaria, even the ones which had been freshwater, were positive with my AOB probes while all the freshwater aquaria were negative. Both types of aquaria were negative for the *Nitrobacter* probe (Fig. 2).

These results meant that ammonia oxidation was being done by different species of ammonia-oxidizing bacteria in fresh and saltwater aquaria. Otherwise the probes would have detected AOBs in both environments. Furthermore, while the AOBs in saltwater were related to *Nitrosomonas europaea*, the freshwater AOB were most likely novel. Finally, nitrite-oxidation was not being performed by *Nitrobacter winogradskyi* or its close relatives in either environment but by some unknown nitrite-oxidizer.

While these results have upset the conventional wisdom of people associated with the aquarium industry they were not shocking to microbial ecologists. By the time I had published my paper, a few other papers had been recently published regarding nitrifying bacteria.

Hiorns et al. (3) in 1995 used molecular techniques to look for *Nitrosomonas* spp. and *Nitrosospira* spp. and found that samples from soils and activated sludge tested positive for *Nitrosospira* spp. but not *Nitrosomonas* spp.. Further, when they examined lakewater and sediments they could not detect *Nitrosomonas* spp. but did find *Nitrosospira* spp. These results suggested that the importance of *Nitrosomonas* was over-emphasized. Obviously, other AOBs were the primary ammonia-oxidizing bacteria in these environments.

Wagner et al. (10) could not detect *Nitrobacter* cells in samples from river water, a trickle filter or activated sludge and concluded that there were large numbers (or high-level activities) of non-*Nitrobacter* nitrite-oxidizing bacteria in the systems they examined.

Thus, there was plenty of other evidence, besides my work, at this time which pointed towards novel ammonia- and nitrite-oxidizing bacteria being responsible for nitrification in a wide range of aquatic environments.

NITROSPIRA NOT *NITROBACTER*

My work continued and was now aimed at identifying the nitrite oxidizing bacteria in aquatic systems. The results were published in 1998 with a paper entitled *Nitrospira*-like bacteria associated with nitrite oxidation in freshwater aquaria with my co-authors L. T. Taylor, A. Blakis, and Professor E. F. DeLong (5).

When faced with the results that the bacteria initially thought to be present in a sample are not, one has only a few options on how to proceed. One option is to guess which bacteria might be present and design probes for these bacteria. This is no better than trial and error. The second option initially involves more work, but in the long run is really the only way to proceed. For this option, one develops a clone library from the sample. A clone library is a catalog of all the different bacteria in a sample that is built by cloning and sequencing their DNA.

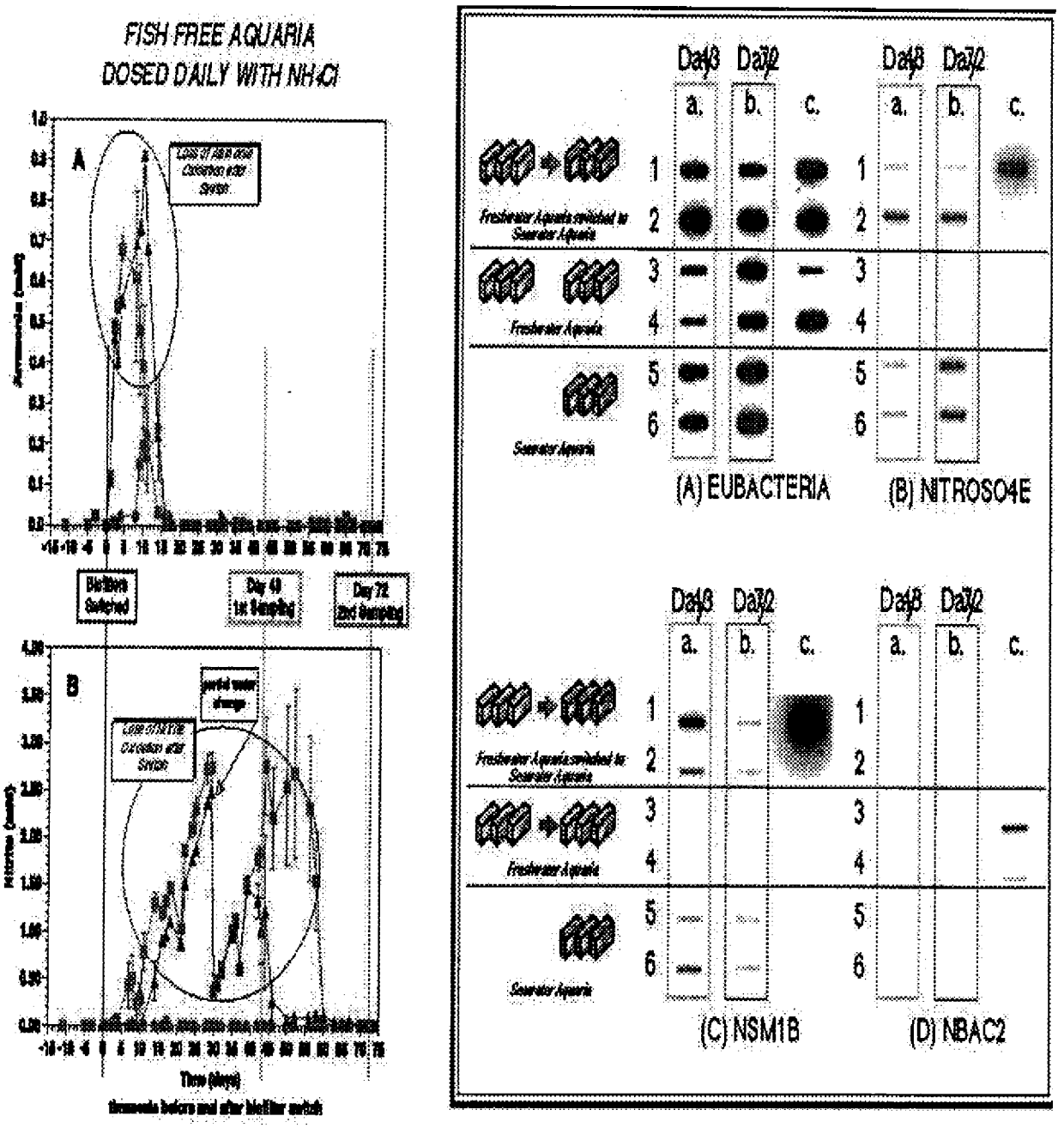

FIG. 2. Results of the slot blotting and molecular probing for ammonia- and nitrite-oxidizing bacteria in freshwater and saltwater aquaria. Presented at the 96th annual meeting of the American Society for Microbiology, New Orleans, LA. The left side of the figure shows the mean (N=3) ammonia (top) and nitrite (bottom) trends for freshwater aquaria, freshwater aquaria switched to seawater, and newly set-up seawater aquaria. The right side shows the results of the slot blotting tests with moeclcular probes for all eubacteria (eubac), two probes for ammonia-oxidizers (NITROSO4E, NSM1B) and nitrite-oxidizers (NBAC2). Only seawater aquaria show a positive signal for ammonia-oxidizers. No *Nitrobacter* spp were detected.

When you have an initial DNA sample from a filter or any other environment, the DNA consists of a mixture of DNA from many different species. There may be the DNA from hundreds or more different bacteria present in the sample. So the first objective is to separate this mixed DNA sample into the individual species DNA by cloning. Next you remove the DNA from the cloning vector, clean it up and sequence it. After you have developed sufficient sequence data, you carefully check the sequence against the sequences of known bacteria using computer programs and a national database. Finally, phylogenetic trees are constructed to

examine the relationship of the bacteria in your sample to known bacteria (see Figure 1).

As is apparent the entire process is very time consuming. Nevertheless, since this is the correct approach it is the one I used to identify the nitrite-oxidizing bacteria in aquaria.

This work resulted in the discovery of new nitrite-oxidizing bacteria belonging to the phylum *Nitrospira*. In the clone library, I was only able to find *Nitrospira* NOBs. Conversely, I never found *Nitrobacter* NOBs. Once I had the correct NOB sequence, I developed unique molecular probes for the *Nitrospira* NOB and probed many freshwater and saltwater aquaria. The results showed that *Nitrospira* was present in relatively large numbers in all the aquarium samples but *Nitrobacter* could not be found in any.

Finally, through a process called denaturing gradient gel electrophoresis (DGGE) I was able to track the appearance of the *Nitrospira* bacteria in newly set-up aquaria and quantify their growing numbers, relative to other bacteria in the sample, as the test aquarium cycled (Fig. 5).

CONFIRMATION BY OTHERS

The responses to the conclusions of my second paper varied depending on the respondent. Most people associated with companies in the aquarium industry did not (and still do not) accept the results and persist in their belief that *Nitrosomonas europaea* and *Nitrobacter winogradskyi* are the nitrifiers in aquaria. Many of these companies sell preserved mixtures of these bacteria as aquarium starter cultures. It is important to note, however, that no one associated with the industry has published any contradictory data, peer-reviewed or not.

Among microbial ecologists and microbiologists who are involved in this line of research the response has been much more positive. Furthermore, my conclusions were strengthened by the publication in May of 1998 of yet another investigation of nitrite-oxidizing bacteria by Burrell et al. (2). These researchers, at the University of Queensland, in Brisbane, Australia, investigated the microbial community of wastewater treatment systems. Through the previously described clone library development and sequencing techniques they found that bacteria belonging to the *Nitrospira* phylum were the putative nitrite-oxidizing bacteria in wastewater systems.

The results of a third study on *Nitrospira* were also published in 1998 (8). These researchers looked at the microbiology of a nitrifying fluidized bed reactor using another molecular method called Fluorescent In Situ Hybridization (FISH). Schramm et al. (8) could not find any ammonia-oxidizing bacteria of the genus *Nitrosomonas* or nitrite-oxidizing bacteria of the genus *Nitrobacter*. Instead they determined that, in their samples, the nitrite-oxidizing bacteria were members of the phylum *Nitrospira* and the ammonia-oxidizing bacteria were members of the genus *Nitrosospira*.

CONCLUSIONS

In the last few years, there have been a number of peer-reviewed papers published in leading scientific journals by a wide array of international researchers on investigations of aquatic nitrifying bacteria from a number of tested environments. There is a commonality amongst these studies: namely, the lack of detection of species of *Nitrobacter* and the finding of *Nitrospira* as the nitrite-oxidizing bacteria. The simplest conclusion from this is that *Nitrobacter* are not, and *Nitrospira* are, the nitrite-oxidizing bacteria in these situations.

In terms of ammonia-oxidizing bacteria, many new species have been discovered and it seems likely that the importance of *Nitrosomonas europaea* has been over-emphasized. Members of the genus *Nitrosospira* and new members of genus *Nitrosomonas* are important ammonia-oxidizing bacteria in environments ranging from ponds, to aquaculture facilities to fish aquaria.

The benefits of my research, and that of others, for the aquatic filtration industry is that we can now develop better biological filtration systems because we have methods to detect and quantify the nitrifiers. Furthermore, the research calls into question the efficacy and use of currently available starter cultures of nitrifiers which are used to accelerate the establishment of nitrification in aquaria. Many of these products are labeled as containing *Nitrosomonas europaea* and *Nitrobacter winogradskyi* while others do not name the bacteria species in their mixtures. The current research of aquatic nitrifying bacteria would strongly suggest that these products are of little to no benefit as they do not contain the correct species of nitrifying bacteria for closed aquatic systems.

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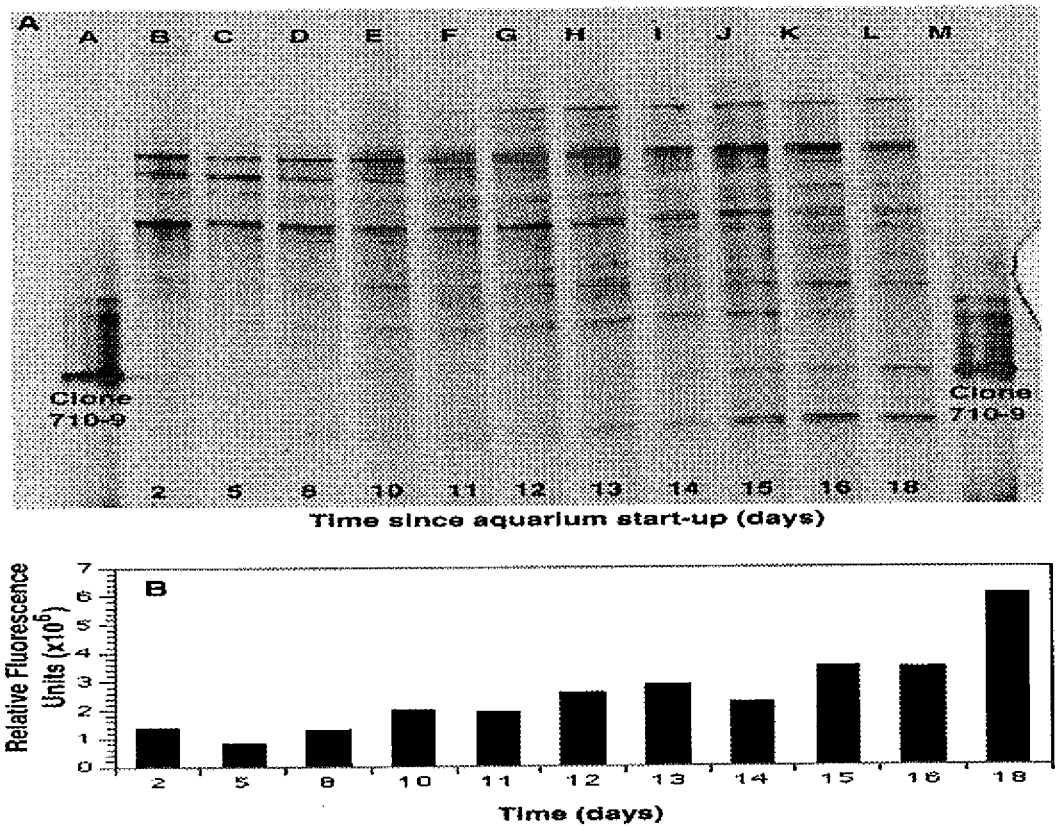

FIG. 3. (A) DGGE of select dates during the first 18 days after the startup of a freshwater aquarium, during which time nitrification became established. Clone 710-9, a *Nitrospira*-like putative NOB, can be seen to appear starting at about day 12 (lane G). (B) Relative intensities of the band for clone 710-9 at each sampling date. From Hovanec et al. 1998 (5).

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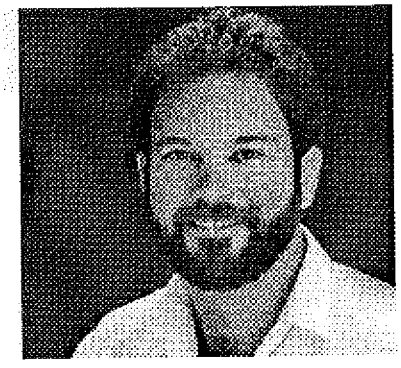

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Comparative Analysis of Nitrifying Bacteria Associated with Freshwater and Marine Aquaria

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Three nucleic acid probes, two for autotrophic ammonia-oxidizing bacteria of the β subdivision of the class *Proteobacteria* and one for α subdivision nitrite-oxidizing bacteria, were developed and used to study nitrifying bacterial phylotypes associated with various freshwater and seawater aquarium biofilters. *Nitrosomonas europaea* and related species were detected in all nitrifying seawater systems and accounted for as much as 20% of the total eubacterial rRNA. In contrast, nitrifying bacteria belonging to the β-proteobacterial subdivision were detected in only two samples from freshwater aquaria showing vigorous nitrification rates. rRNA originating from nitrite-oxidizing α subdivision proteobacteria was not detected in samples from either aquarium environment. The data obtained indicate that chemolithotrophic ammonia oxidation in the freshwater aquaria was not due to β-proteobacterial phylotypes related to members of the genus *Nitrosomonas* and their close relatives, the organisms usually implicated in freshwater nitrification. It is likely that nitrification in natural environments is even more complex than nitrification in these simple systems and is less well characterized with regard to the microorganisms responsible.

The pathways of the nitrogen cycle are highly dependent on microbial activities and transformations. One important pathway in the nitrogen cycle is nitrification, the oxidation of ammonia to nitrite and subsequently to nitrate (17). Traditionally, nitrification has been studied by chemical measurement of ammonia or nitrite disappearance, measurement of the production of nitrite or nitrate, or a combination of these methods (see reference 25 for a review of autotrophic nitrification). Nitrification occurring in a wide range of environments, such as soils (17), ocean water (36), freshwater lakes (11), wastewaters (24), and aquaria (16), is assumed to be due to autotrophic bacteria. While heterotrophic nitrification can occur and may contribute substantially to nitrification in certain environments (17, 29), it is not coupled to energy generation and, therefore, is thought to be a minor component of overall nitrification (4, 25).

A primary concern in fish culture systems ranging from high-density aquaculture operations to the home tropical fish aquarium is the toxic effects of ammonia on fish. To control and maintain safe ammonia levels in fish culture systems, biological filters have been designed to promote the growth of ammonia- and nitrite-oxidizing bacteria. Biological filters use a variety of materials as supports on which the bacteria are cultured. Generally, no special effort is made to distinguish between the types of supports used in different seawater or freshwater culture systems. The general assumption is that species of ammonia- and nitrite-oxidizing bacteria are identical in the two types of environments and that they require only a solid support, good aeration, and an energy source (ammonia or nitrite) to become successfully established.

In freshwater systems, the bacterial genera responsible for the oxidation of ammonia and nitrite are presumed to be predominantly the genera *Nitrosomonas* and *Nitrobacter*, both of which are chemolithoautotrophic members of the class *Proteobacteria* (14, 38). Recent studies in which comparative 16S rRNA analyses of ammonia- and nitrite-oxidizing bacteria were performed have clarified the phylogenetic relationships of these bacteria and have demonstrated that they belong to two separate lineages within the *Proteobacteria* (12, 30). Teske et al. (30) concluded that the nitrifying bacteria may have multiple phylogenetic origins. These authors speculated that nitrifiers have developed independently many times, perhaps from different lineages of photosynthetic bacteria (30). The freshwater autotrophic ammonia-oxidizing bacteria that have been characterized belong exclusively to the β subdivision of the *Proteobacteria* and are typified by *Nitrosomonas europaea* (Fig. 1). These bacteria form a distinct group within the β subdivision and are affiliated with an iron-oxidizing bacterium (*Gallionella ferruginea*) and the photosynthetic bacterium *Rhodocyclus purpureus*, along with methylotrophic bacteria. One ammonia oxidizer, *Nitrosococcus oceanus*, is a marine species that belongs to the γ-proteobacterial lineage.

The most commonly studied autotrophic nitrite-oxidizing bacteria belong to the α subdivision of the *Proteobacteria*, of which *Nitrobacter winogradskyi* is a representative species (Fig. 1). Other chemolithoautotrophic nitrite-oxidizing bacteria that have been characterized are phylogenetically widespread in the class *Proteobacteria*, occurring in the α, δ, and γ subdivisions (Fig. 1). Phylogenetic analysis of the α subdivision of the *Proteobacteria* has shown that *Nitrobacter winogradskyi* is most closely related to *Bradyrhizobium japonicum* and *Rhodopseudomonas palustris* (9, 23, 27, 39).

In this study, we used oligonucleotide probes which target chemolithoautotrophic ammonia-oxidizing and nitrite-oxidizing bacteria to examine nitrifying bacterial populations associated with freshwater and marine aquaria. Various microbial habitats associated with aquarium systems were investigated, including the gravel, water, and biofilter support medium, which is a substratum designed to encourage the growth of nitrifying bacteria. Specific differences between nitrifying bacterial assemblages on freshwater and seawater aquarium biofilters were also investigated.

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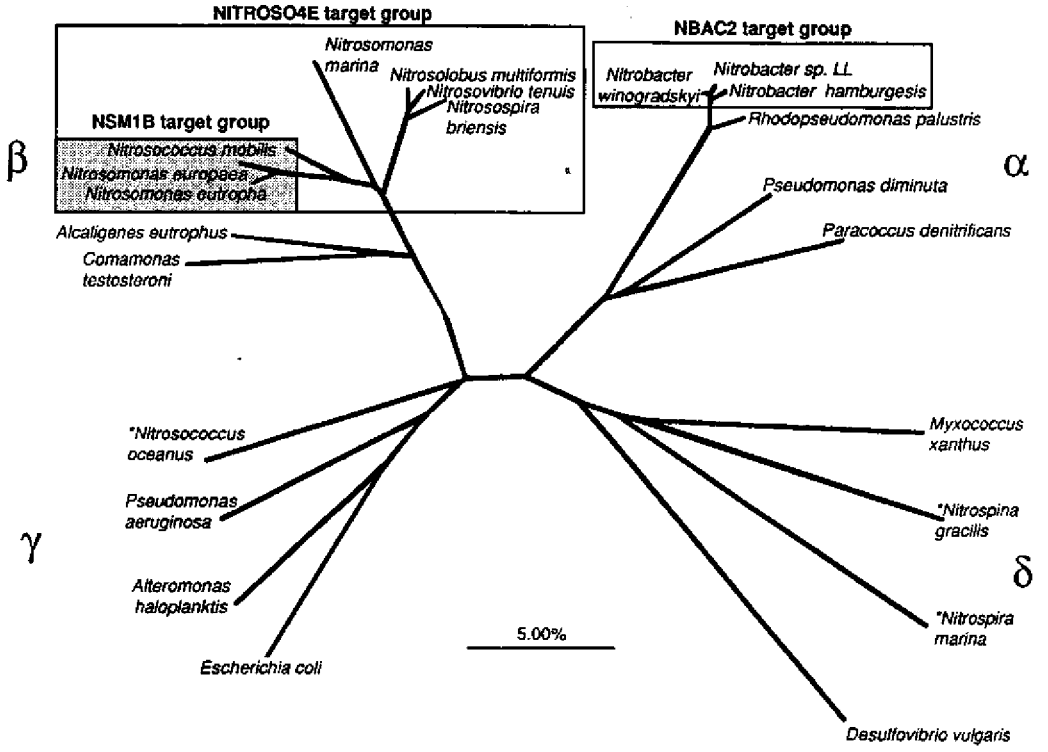

FIG. 1. Phylogenetic relationships of the chemolithoautotrophic ammonia- and nitrite-oxidizing bacteria. Most known ammonia-oxidizing autotrophs belong to the β subdivision of the *Proteobacteria*; the only exception is *Nitrosococcus oceanus*, which is affiliated with the γ subdivision. The nitrite-oxidizing bacteria are more widespread in the *Proteobacteria*, occurring in the α, δ, and γ subdivisions. Nucleic acid probes which correspond to (i) all known β subdivision ammonia oxidizers (probe NITROSO4E), (ii) a clade on a deep branch in the β subdivision (probe NSM1B), and (iii) the nitrite oxidizers belonging to the α subdivision (probe NBAC2) were developed. Nitrifying bacteria which are not targeted by the probes designed in this study are indicated by asterisks. Recent studies indicate that the genus *Nitrospira* may be affiliated with a group outside the δ subdivision of the *Proteobacteria*, in a separate phylogenetic lineage (8).

MATERIALS AND METHODS

Bacterial culture and nucleic acid extraction techniques. Ammonia- and nitrite-oxidizing bacteria were obtained from the American Type Culture Collection or were kindly provided by J. B. Waterbury of Woods Hole Oceanographic Institute, Woods Hole, Mass., and were grown in organic-free media in batch culture by standard methods (Table 1) (2).

Isolation of ribosomal DNA genes of nitrite oxidizers. As expected, the nitrite-oxidizing bacteria grew slowly with low cell yields, and so the PCR was used to generate sufficient ribosomal DNA template to test probe specificities. Prior to the PCR, DNAs from *Nitrobacter winogradskyi* and *Nitrobacter agilis* were extracted. Cells were placed in lysis buffer (40 mM EDTA, 50 mM Tris; pH 8.3) to which lysozyme was added to a final concentration of 1 mg/ml. After incubation at 37°C for 30 min, 50 μl of proteinase K (stock solution concentration, 10 mg/ml) and 50 μl of 20% sodium dodecyl sulfate (SDS) were added to each sample, and then the preparations were incubated at 55°C for 30 min. Cell lysis was monitored by phase-contrast microscopy. In some cases, additional proteinase K and SDS were added and the sample was incubated at 55°C for another 30 min.

After cell lysis, DNA was extracted by sequential extractions with phenol (pH 8.0), phenol-chloroform-isoamyl alcohol (24:24:1), and finally chloroform-isoamyl alcohol (24:1). Each sample nucleic acid was precipitated with 0.3 M sodium acetate and 2 volumes of ethanol and stored at −20°C. The sample was collected by centrifugation, dried, and resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM disodium EDTA). The concentration of DNA was determined by Hoechst type 33258 dye binding and fluorometry (model TKO 100 minifluorometer; Hoefer Pharmacia Biotech Inc., San Francisco, Calif.). Ribosomal DNA was amplified by using primers specific for eubacterial rRNA, as previously described (7).

Isolation of rRNA. Cells of the ammonia-oxidizing and heterotrophic bacteria were harvested by centrifugation for 20 min at 8,000 rpm (model RC5C centrifuge; Sorvall Instruments). Total rRNA was extracted from bacterial cells by cell disruption with glass beads, using a Mini Beadbeater (BioSpec Products, Bartlesville, Okla.). After disruption, a three-step purification procedure (with phenol [Tris buffered, pH 5.1], phenol-chloroform-isoamyl alcohol [24:24:1], and chloroform-isoamyl alcohol [24:1]) was performed (28). The resulting crude nucleic acid was precipitated overnight at −20°C after 0.1 volume of 3 M sodium acetate and 2 volumes of ethanol were added. After precipitation, the nucleic acids were collected by centrifugation and resuspended in 100 μl of TE buffer (pH 8.0). RNA was quantified by measuring A_{260} with a Perkin-Elmer Lambda 3B spectrophotometer by assuming that 1 A_{260} unit corresponds to 40 μg of RNA per ml (28).

Oligonucleotide probe design. 16S rRNA sequences of chemolithoautotrophic ammonia-oxidizing bacteria were aligned in a database by using sequence data obtained from the Ribosomal Database Project (20). Two regions were identified as having potential specificity for the target groups. One 20-nucleotide probe (designated NITROSO4E) targeted all known ammonia-oxidizing members of the β subdivision (Fig. 1), and a second probe (NSM1B) targeted three members of the clade containing *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*.

A third probe (NBAC2) was designed to target the α subdivision nitrite-oxidizing bacteria *Nitrobacter winogradskyi*, *Nitrobacter agilis*, and *Nitrobacter hamburgensis*. The probes were synthesized by Operon Tech, Inc., Alameda, Calif. The nucleotide sequences and positions of the probes are shown in Table 2.

Probe hybridization procedures. To determine the specificity of each probe, probe binding to rRNAs from target and nontarget bacteria was monitored by autoradiography. A temperature series spanning the estimated dissociation temperature of each probe was used to determine the wash temperature empirically.

All probe hybridization experiments were conducted with a slot blot device (Millipore Corp., New Bedford, Mass.). rRNAs from pure stock preparations and samples were denatured with 3 volumes of 2% (vol/vol) glutaraldehyde and then diluted to the final volume (1:100) with dilution water (1 μg of polyriboadenosine per liter, 0.0004% bromophenol blue). The plasmid stock preparations of *Nitrobacter winogradskyi* and *Nitrobacter agilis* were diluted with an equal volume of a mixture containing 1 N NaOH and 3 M NaCl. Samples were applied to nylon filters (Hybond N; Amersham Corp., Arlington Heights, Ill.) fitted into the slot blot device. After air drying, the filters were cross-linked by exposure to 1,200 J of UV irradiation (UV Stratalinker; Stratagene Corp., San Diego, Calif.).

For hybridization experiments, membranes were placed in a heat-sealable bag, 6 or 12 ml (depending on the number of membranes in the bag) of hybridization buffer (0.9 M NaCl, 50 mM $NaPO_4$, 5 mM EDTA, 0.5% SDS, 10× Denhardt's solution, 0.5 mg of polyadenosine per ml) was added, and the bag was sealed and placed in a hybridization oven (model 136500; Boekel Industries, Inc.) for 30 min at 45°C. After 30 min, the bags were removed, and 2×10^7 cpm of ^{32}P-end-

TABLE 1. Sources of the bacteria utilized in the nucleic acid probe validation studies and culture media used to grow them

Species	Proteobacterial subdivision	Strain	Growth medium
Chemolithoautotrophic ammonia-oxidizing bacteria			
Nitrosomonas europaea	Beta	ATCC 19718	ATCC 221
Nitrosococcus mobilis	Beta	NC2 Waterbury[a]	ATCC 928 (25%)[b]
Nitrosolobus multiformis	Beta	ATCC 25196	ATCC 929
Nitrosospira briensis	Beta	C128 Waterbury[a]	ATCC 221
Nitrosovibrio tenuis	Beta	NV12 Waterbury[a]	ATCC 929
Nitrosococcus oceanus	Gamma	ATCC 19707	ATCC 928
Chemolithoautotrophic nitrite-oxidizing bacteria			
Nitrobacter winogradskyi	Alpha	ATCC 25391	ATCC 480
Nitrobacter agilis	Alpha	ATCC 14123	ATCC 96
Nitrococcus mobilis	Gamma	ATCC 25380	ATCC 481
Nitrospira marina	Delta	NB295 Waterbury[a]	ATCC 480
Nitrospina gracilis	Delta	NB211 Waterbury[a]	ATCC 480
Heterotrophic bacteria closely related to ammonia or nitrite oxidizeers			
Alcaligenes eutrophus	Beta	ATCC 17697	Luria-Bertani
Alcaligenes faecalis	Beta	ATCC 15554	Luria-Bertani
Comamonas acidovorans	Beta	ATCC 15668[c]	Luria-Bertani
Comamonas testosteroni	Beta	ATCC 11975[c]	Luria-Bertani
Paracoccus denitrificans	Alpha	ATCC 17741	Luria-Bertani
Rhodopseudomonas palustris	Alpha	ATCC 17001	Luria-Bertani
Pseudomonas diminuta	Alpha	501[c]	Luria-Bertani
Shewanella putrefaciens	Gamma	ATCC 8071	Luria-Bertani
Pseudomonas nautica	Gamma	ATCC 27132	Marine broth
Pseudomonas aeruginosa	Gamma	ATCC 17503	Luria-Bertani

[a] Kindly provided by J. B. Waterbury, Woods Hole Oceanographic Institute.
[b] The medium used was 25% ATCC 928 medium in distilled water.
[c] Received from P. Baumann.

labeled probe was added. Each bag was resealed and returned to the oven. The membranes were incubated overnight in the hybridization oven at 45°C.

After the overnight washing described above, the membranes were removed and washed in a solution containing 1× SET (150 mM NaCl, 1 mM EDTA, 20 mM Tris; pH 7.8) and 1% SDS at room temperature for 30 min on a shaker table. The membranes were then washed in fresh 1× SET–1% SDS at appropriate wash temperatures for 30 min (with shaking every 10 min). After washing, the membranes were allowed to air dry. Autoradiographic signals were quantified by using a gas proportional radioisotope detection system (Ambis, Inc., San Diego, Calif.). Film autoradiographs were also recorded with an intensifier screen for 20 to 24 h at −76°C.

The relative rRNA-specific hybridization signal attributable to each probe was determined by calculating a slope (counts per minute bound per nanogram of RNA) for the serially diluted sample. Values were normalized by using a correction factor determined by dividing the group-specific probe slope derived from known rRNA standards by the slope derived from the eubacterial probe for the same standards (10). Group-specific hybridization signal was calculated by dividing the normalized group-specific probe slope by the eubacterial probe slope of the same sample.

Sampling and extraction of nucleic acids from aquarium samples. A variety of locations in small (water volume, <400 liters) aquaria having two general types of environments (inorganic and organic) were sampled for the presence of chemolithoautotrophic nitrifying bacteria.

The samples consisted of aquarium gravel, aquarium water, and pieces of the aquarium biological filter media. Gravel was collected with a scoop, weighed to the nearest 0.1 g, placed in a polypropylene tube, and immediately covered with low-pH buffer (50 mM sodium acetate, 10 mM disodium EDTA) for rRNA extraction or with cell lysis buffer for DNA extraction. Samples were stored at

TABLE 2. Nucleotide sequences and positions of the three oligonucleotide probes for nitrifying bacteria

Probe	Position (nucleotides)[a]	Base sequence (5′ to 3′)	T_d (°C)[b]/ wash temp (°C)	Targeted group	Nontarget bacteria with exact match to probe sequence
NITROSO4E	639-658	CAC TCT AGC YTT GTA GTT TC	43.2/53.0	β-Proteobacterial ammonia oxidizers	*Nodularia* sp.[c]
NSM1B	479-495	TCT GTC GGT ACC GTC AT	41.2/53.0	*Nitrosomonas europaea*, *Nitrosomonas eutropha*, *Nitrosococcus mobilis*	None[d]
NBAC2	1017-1036.1	GCT CCG AAG AGA AGG TCA CA	49.4/53.0	*Nitrobacter winogradskyi*, *Nitrobacter hamburgensis*, *Nitrobacter agilis*	*Afipia clevelandensis*, *Afipia felis*, *Rhodopseudomonas palustris* strain, *Bradyrhizobium japonicum*[e]

[a] *Escherichia coli* numbering.
[b] T_d, dissociation temperature. Wash temp, experimentally determined wash temperature (see Materials and Methods).
[c] The following two nontarget bacteria have a one-base mismatch with the probe sequence: *Oscillatoria* sp. and *Cylindrospermum* sp.
[d] There are 50 nontarget bacteria or strains of bacteria that have a one-base mismatch with the probe sequence. These bacteria include *Ehrlichia*, *Rhodovulum*, *Rhodobacter*, *Rhodoplanes*, and *Fusobacterium* species, as well as *Anaplasma marginale*, *Thiobacillus thioparus*, *Sebaldella termitidis*, and *Streptobacillus moniliformis*.
[e] The following four nontarget bacteria have a one-base mismatch with the probe sequence: *Photorhizobium thompsonianum*, *Photorhizobium* sp. strain IRBG 230, *Bradyrhizobium* sp., and *Photorhizobium* sp. strain MKAa 2.

−20°C until extraction. Aquarium water was collected in prewashed glass jars and filtered through a Sterivex GV filter by using autoclaved pump tubing and a peristaltic pump. Between 1,000 and 4,000 ml of water was filtered depending on the sample. After filtering, 1.8 ml of cell lysis buffer was added to each unit with a sterile syringe, and the filters were stored at −20°C until processing. Various biological filter media were collected by cutting a piece of material from the filter with alcohol-sterilized scissors and forceps. Each medium sample was placed in a polypropylene tube, covered with 2.0 to 2.5 ml of cell lysis buffer or bead beating solution, and then stored at −20°C until extraction.

The gravel samples were extracted by adding 200 μl of 20% SDS and 3 ml of phenol (Tris buffered to pH 5.1) and shaking the preparations by hand for 5 min; this was followed by incubation in a 60°C water bath for 7 min. After shaking for 3 min, the samples were centrifuged at 1,500 rpm for 10 min (model 1550 centrifuge; Hamilton Bell, Montvale, N.J.). The nucleic acids were aliquoted into three tubes, and the contents of each tube were extracted by using the bead beating protocol described above.

The nucleic acids in the aquarium water samples were extracted by adding 40 μl of lysozyme (from a stock solution containing 25 mg of lysozyme in 500 μl of distilled water) to each thawed sample. The filter was placed on an agitator and shaken at 37°C for 30 min. Then 500 μl of proteinase K (stock solution concentration, 10 mg/ml) was added, and the filter was incubated at 55°C for 1 h with shaking. The solution was drawn out of the Sterivex filter with a syringe into a polypropylene tube. Phenol-chloroform-isoamyl alcohol extraction was performed, and this was followed by a series of chloroform-isoamyl alcohol extractions. The solution was concentrated with a Centricon 100 concentrator (Amicon, Beverly, Mass.), and nucleic acids were precipitated.

Freshwater and seawater aquarium biofilter comparison. Six all-glass aquaria (capacity, 34 liters) were used along with a standard home aquarium filtration system (Penguin model 160B; Marineland Aquarium Products, Moorpark, Calif.). There was no substratum or other material in the aquaria. In the model 160B system the main body of the filter unit hangs on the outside upper back edge of the aquarium. On the upper weir of the filter unit is the dedicated biological filter (BioWheel; referred to below as the biofilter), which sits perpendicular to the water flowing back into the aquarium. The water flow causes the biofilter to continuously rotate such that it functions as a rotating biological contactor, and, therefore, the filter surface alternates between a partially submerged phase and an air-exposed phase.

Initially, the tanks were filled with dechlorinated (activated carbon-treated) tap water; 5 mM ammonia (made with ammonium chloride) was added to each aquarium daily for the first 20 days and then every other day or so. Aquarium water was sampled several times a week and was analyzed by performing a flow injection analysis (FIAstar system; Tecator AB, Höganäs, Sweden) for ammonia (gas diffusion membrane method), nitrite (azo dye method), nitrate (cadmium reduction-azo dye method), and acid-neutralizing capacity (methyl orange to an end point of pH 4.5) as recommended in the manufacturer's application notes. The pH was determined with an electrode and a specific ion meter (Orion Instruments).

After all of the aquaria were exhibiting nitrification, as determined by nitrate production, the water in one group of three aquaria was changed from freshwater to seawater (prepared with artificial sea salts [Marineland Commercial Aquariums, Moorpark, Calif.]). Three additional aquaria were also set up with artificial seawater and filter units with BioWheels which had never been run. Water quality data were collected for the nine aquaria as previously described for another 75 days. At 43 and 72 days after the one freshwater group had been switched to seawater, the biofilm on each biofilter was sampled by cutting out a small piece of the filter. rRNA and ribosomal DNA were extracted as described above. rRNA was analyzed by using oligonucleotide probes as described above.

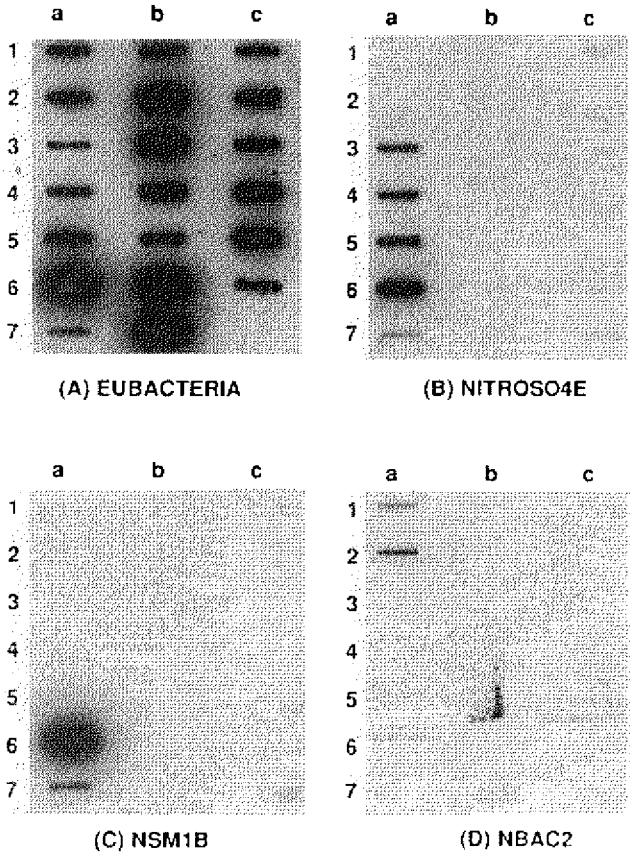

FIG. 2. Autoradiographs demonstrating the specificity of eubacterial probe EUBAC (A), ammonia-oxidizing bacterial probes NITROSO4E (B) and NSM1B (C), and nitrite-oxidizing bacterial probe NBAC2 (D). The rRNA extracts from chemolithoautotrophic nitrifying bacteria and closely related bacteria were blotted in the slots in the following arrangement: slot a-1, *Nitrobacter winogradskyi*; slot a-2, *Nitrobacter agilis*; slot a-3, *Nitrosovibrio tenuis*; slot a-4, *Nitrosospira briensis*; slot a-5, *Nitrosolobus multiformis*; slot a-6, *Nitrosomonas europaea*; slot a-7, *Nitrosococcus mobilis*; slot b-1, *Rhodopseudomonas palustris*; slot b-2, *Pseudomonas diminuta*; slot b-3, *Paracoccus denitrificans*; slot b-4, *Comamonas acidovorans*; slot b-5, *Alcaligenes faecalis*; slot b-6, *Comamonas testosteroni*; slot b-7, *Alcaligenes eutrophus*; slot c-1, *Nitrococcus mobilis*; slot c-2, *Nitrosococcus oceanus*; slot c-3, *Shewanella putrefaciens*; slot c-4, *Pseudomonas nautica*; slot c-5, *Pseudomonas aeruginosa*; slot c-6, *Nitrospina gracilis*; slot c-7, *Nitrospira marina*.

RESULTS

Oligonucleotide probe specificity. The specificities of three of the four ammonia- or nitrite-oxidizing group-specific probes developed in this study are shown in Fig. 2. Database searches and hybridization experiments performed with rRNAs extracted from phylogenetically diverse bacteria indicated that the probes were sufficiently specific to identify various chemolithoautotrophic nitrifying bacteria with the following provisions. There is one nontarget organism for the NITROSO4E probe and there are four nontarget organisms for the NBAC2 probe in which the probe sequence compliments the nontarget sequence exactly (Table 2). In the case of probe NSM1B the sequences of about 50 nontarget organisms out of the database of more than 3,000 sequences have only one mismatch with the target sequence (Table 2).

A range of wash temperatures was tested to determine the optimal conditions for probe specificity. Under appropriate hybridization and wash conditions, the NITROSO4E probe bound the rRNAs of all of the β subdivision ammonia-oxidizing bacteria examined, but not the rRNAs of the closely related heterotrophic bacteria (Fig. 2). The NSM1B probe yielded positive signals with the two targeted β subdivision ammonia-oxidizing bacteria (*Nitrosomonas europaea* and *Nitrosococcus mobilis*) but not with other nitrifying bacteria or closely related heterotrophic bacteria belonging to the same subdivision (Fig. 2). We tested a third probe for the β subdivision oxidizers, NLB1, but this probe cross-reacted with the closely related heterotrophic bacteria at all wash temperatures tested (data not shown). None of the nucleic acid probes designed for the β subdivision ammonia-oxidizing bacteria hybridized to *Nitrosococcus oceanus*, a marine species which is the only known autotrophic ammonia oxidizer not in the β subdivision (Fig. 2).

The results of the specificity test for the nitrite-oxidizing bacterial probe (NBAC2) show that this probe is specific for two known α subdivision nitrite oxidizers (*Nitrobacter winogradskyi* and *Nitrobacter agilis*) and does not cross-hybridize with either the δ or the γ subdivision nitrite-oxidizing bacteria

or closely related α subdivision heterotrophic bacteria, such as *Rhodopseudomonas palustris* (Fig. 2).

The data indicated that three of the four nucleic acid probes tested were sufficiently specific to distinguish autotrophic nitrifying bacteria from closely related heterotrophic bacterial species.

Detection of autotrophic nitrifying bacteria in aquaria. We tested the nitrifying bacterial rRNA probes with nucleic acids extracted from a wide range of samples obtained from actively nitrifying freshwater and seawater aquaria (Table 3). Some of the samples came from biofilters in aquaria which received more than 82 g of fish food or were dosed with 32 mM ammonia each day. Only 2 of the 38 freshwater samples gave a positive result with any of the nitrifier-specific probes (Table 3). These two samples, which exhibited positive signals for the two ammonia oxidizer rRNA probes, were from biofilters which had been dosed with ammonium chloride and were never exposed to the fish waste or organic compounds that are normally associated with a fish tank. The positive signals obtained for these biofilters may have resulted from contamination from seawater biofilters located nearby. These biofilters had been in the culturing system for 76 days before sampling. The NBAC2 probe did not indicate the presence of α subdivision nitrite-oxidizing bacteria in any of the freshwater samples (Table 3). There were large amounts of eubacterial rRNA detected by the eubacterial probe in each sample, and so the lack of signal cannot be attributed to insufficient material on the membranes.

A PCR analysis in which two general eubacterial primers (forward primer 8-27 and reverse primer 1492-1510) (19) were used was performed with some samples to increase sensitivity and to determine whether nitrifying bacterium rRNA genes could be detected in the mixed-community DNA. PCR products were blotted, and hybridization experiments were performed with the nitrifier rRNA probes. No signal was detected in the PCR products, which is consistent with the results of the rRNA hybridization experiments.

Positive results with probes specific for ammonia-oxidizing nitrifiers (NITROSO4E and NSM1B) were obtained for all seawater samples, which were dosed daily with ammonium chloride (Table 3). The lengths of time in the systems for the seawater biofilters tested ranged from 53 to 299 days. As with the freshwater systems, negative results were obtained with the probe for nitrite-oxidizing bacteria (NBAC2). Quantitative oligonucleotide probe hybridization experiments indicated that as much as 20% of the eubacterial rRNA was derived from ammonia-oxidizing bacteria belonging to the β subdivision of the *Proteobacteria* (Table 4). This is consistent with the presumed presence of significant numbers of ammonia-oxidizing bacteria on the biofilters. Furthermore, since the signal of the *Nitrosomonas* species probe (NSM1B) is equivalent to the signal of the more general β-proteobacterial ammonia-oxidizing group-specific probe (NITROSO4E), the nitrifiers on the seawater biofilters appear to be dominated by *Nitrosomonas europaea* and its close relatives rather than *Nitrosospira* types.

Freshwater-seawater biofilter comparison. The mean ammonia, nitrite, and pH data for the three groups of biofilters from aquaria that received different water treatments are presented in Fig. 3. It is clear that established freshwater aquarium biofilters experienced a complete loss of nitrification when the water in the aquaria was switched to seawater. This caused an increase in the ammonia concentrations in the aquaria (Fig. 3). After the switch to seawater, it took the previously freshwater biofilters nearly as long to reestablish ammonia oxidation as it took the newly set up seawater biofilters. However, the maximum ammonia concentration reached during the establishment period was less in the switched biofilters than in the newly set up seawater biofilters (Fig. 3). There was a small, temporary increase in the ammonia concentration in the freshwater aquaria from day 9 to day 17 (after the switch), which coincided with a drop in the pH to less than 7.00. The pH rose (and ammonia disappeared) after the addition of $NaHCO_3$.

Nitrite oxidation was established faster in the newly set up seawater biofilters than in the biofilters switched from freshwater, with complete oxidation occurring by day 50 and by day 60 (after the switch), respectively (Fig. 3). Furthermore, the nitrite concentration reached a much higher value and remained higher for a longer period of time in the switched biofilters than in the newly set up seawater biofilters. The nitrite concentration in the continuously freshwater biofilters was low for the duration of the measuring period (Fig. 3). A partial water change was performed on day 29 (after the switch) in all aquaria, and this change is reflected by the sudden drop in the nitrite concentrations in the seawater and freshwater-to-seawater groups. The nitrite concentration steadily increased again after day 29 in both groups until it finally decreased before the end of the measuring period because of establishment of nitrite oxidation.

The pH trends for the three groups of biofilters were similar except for a period of 8 days early in the test (days 9 to 17) when the pH in the freshwater biofilter group fell to less than 7.00. This pH change was compensated for by the addition of $NaHCO_3$.

Oligonucleotide probe hybridization experiments revealed positive signals with both ammonia-oxidizing bacterial probes for all seawater filters regardless of age (newly set up filters and filters switched from freshwater) (Fig. 4). Freshwater biofilters consistently yielded negative results with all of the nitrifier-specific probes (Fig. 4). The results indicated that *Nitrosomonas europaea* or its close relatives were well represented on the seawater biofilters. The results obtained with the probe for nitrite-oxidizing bacteria were negative for all samples (Fig. 4). Thus, both *Nitrobacter winogradskyi* and *Nitrobacter agilis* were either absent or present only at concentrations below our limits of detection, even though the nitrate concentrations steadily increased during the test.

DISCUSSION

Definitive studies correlating nitrification rates with nitrifying microorganisms in natural samples are difficult. Until recently there were few available methods for identifying and quantifying specific bacteria or groups of bacteria in environmental samples without cultivation, an approach known to sometimes lead to biased representation (1, 32). Cultivation of nitrifying bacteria is especially challenging because of the slow growth rates of these bacteria and the frequent occurrence of culture contamination by heterotrophic bacteria (22, 31). Ward (35) utilized immunofluorescence techniques to enumerate nitrifying bacteria, but this technique also required cultivation of the target group to raise antibodies. More recently, PCR primers have been developed and used to detect *Nitrosomonas* spp., *Nitrosospira* spp., and *Nitrobacter* spp. in diverse environments (6, 13, 21, 22, 31). Wagner et al. (33) developed fluorescent in situ hybridization probes specific for certain β-subdivision proteobacterial ammonia oxidizers. These authors found that up to 20% of the total bacteria in activated sludge samples from an animal waste-processing facility could be ammonia oxidizers.

In this study, oligonucleotide probes were used successfully to detect ammonia-oxidizing chemolithoautotrophic bacteria in environmental samples (i.e., seawater aquarium biofilters). Furthermore, the data obtained indicated that the bacteria responsible for ammonia oxidation in freshwater aquaria are

TABLE 3. Results of probing rRNAs extracted from biofilms attached to various aquarium biofiltration media or aquarium water with domain- and group-specific oligonucleotide probes[a]

Sample	Aquarium environment[b]	Biofilm substrate[c]	Daily amt of ammonia[d]	Ammonia source[e]	Signal detected by the following oligonucleotide probes[f]:			
					Bacterial	NITROSO4E	NSM1B	NBAC2
1301	Freshwater	Bulk water	4 g	Fish	+	−	−	−
1302	Freshwater	Gravel	4 g	Fish	+	−	−	−
1303	Freshwater	Gravel	4 g	Fish	+	−	−	−
1304	Freshwater	Filter fiber	4 g	Fish	+	−	−	−
1306	Freshwater	Bulk water	4 g	Fish	+	−	−	−
1307	Freshwater	Gravel	4 g	Fish	+	−	−	−
1309	Freshwater	Polypropylene	4 g	Fish	+	−	−	−
1312	Freshwater	Bulk water	32.1 mM	NH_4Cl	+	−	−	−
1315	Freshwater	Bulk water	32.1 mM	NH_4Cl	+	−	−	−
1316	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
7501	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
7502	Freshwater	Bulk water	82.84 g	Fish	+	−	−	−
7503	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
7504	Freshwater	Polyfiber	82.84 g	Fish	+	−	−	−
710r	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
711r	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	−	−
CAQBW	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	+	+	−
CAQBW	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	+	+	−
E8T32B	Freshwater	Sponge	1.4 g	Fish	+	−	−	−
E8T33B	Freshwater	Polypropylene	1.4 g	Fish	+	−	−	−
E8T34B	Freshwater	Filter fiber	1.4 g	Fish	+	−	−	−
Flwrte5	Freshwater	Gravel	10 mM	NH_4Cl	+	−	−	−
Flwrte8	Freshwater	Gravel	10 mM	NH_4Cl	+	−	−	−
Flwrte9	Freshwater	Gravel	10 mM	NH_4Cl	+	−	−	−
FWSW4	Freshwater	Polypropylene	5 mM	NH_4Cl	+	−	−	−
FWSW6	Freshwater	Polypropylene	5 mM	NH_4Cl	+	−	−	−
MejBW-A	Freshwater	Polypropylene	82.8 g	Fish	+	−	−	−
MejBW-B	Freshwater	Polypropylene	82.8 g	Fish	+	−	−	−
T408	Freshwater	Detritus	3.5 g	Fish	+	−	−	−
T408	Freshwater	Gravel	3.5 g	Fish	+	−	−	−
T825	Freshwater	Gravel	0.8 g	Fish	+	−	−	−
T825	Freshwater	Gravel	0.8 g	Fish	+	−	−	−
WDF1025	Freshwater	Sponge	2.0 g	Fish	+	−	−	−
WDF1026	Freshwater	Polypropylene	2.0 g	Fish	+	−	−	−
WDF1036	Freshwater	Polypropylene	3.2 g	Fish	+	−	−	−
WDF1036	Freshwater	Gravel	3.2 g	Fish	+	−	−	−
WDF1039	Freshwater	Polypropylene	3.2 g	Fish	+	−	−	−
WDF1039	Freshwater	Gravel	3.2 g	Fish	+	−	−	−
714r	Seawater	Polyfiber	714 mM	NH_4Cl	+	+	+	−
715r	Seawater	Polyfiber	714 mM	NH_4Cl	+	+	+	−
FWSW2	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
FWSW3	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
FWSW8	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
FWSW9	Seawater	Polypropylene	5 mM	NH_4Cl	+	+	+	−
SW117	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW123	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW129	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW134	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW148	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW152	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW159	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−
SW202	Seawater	Polyfiber	2.5 mol	NH_4Cl	+	+	+	−

[a] A continued negligible concentration of ammonia in the systems which had daily inputs of fish food or ammonium chloride was considered evidence that nitrification occurred.

[b] The type of aquarium water.

[c] The medium from which the bacterial cells were extracted.

[d] The values in grams are the amounts of fish food put into the aquaria each day; the molar and millimolar values indicate the amounts of ammonia added to the aquaria or systems in which the biofilters were located each day.

[e] Fish means that the aquarium had a fish population and ammonia was generated by the fish; NH_4Cl means that there were no fish in the tank and the ammonia was from ammonium chloride added daily.

[f] +, signal detected; −, no signal detected.

different from the bacteria responsible for ammonia oxidation in seawater aquaria. In seawater aquaria, *Nitrosomonas europaea* and related phylotypes appear to be present at high levels and are presumably the active ammonia-oxidizing bacteria, which is consistent with the results of previous studies. However, previously characterized β subdivision ammonia-oxidizing bacteria were detected in vigorously nitrifying freshwater aquaria in only 2 of 38 samples.

TABLE 4. Levels of hybridization (normalized to the eubacterial probe) of the two probes specific for β-subdivision ammonia-oxidizing bacteria to rRNAs extracted from the biofilms of two seawater aquarium filters[a]

Sample	Age of biofilter (days)	Daily ammonia dose (mol)	% Hybridization to:	
			NITROSO4E probe	NSM1B probe
SW202	53	2.5	20.4	23.8
SW148	98	2.5	18.5	17.4

[a] The biofilters were part of a larger group of 35 filters dosed daily with 2.5 mol of ammonia. The NITROSO4E probe targets all β ammonia oxidizers, while the NSM1B probe targets a subgroup of these bacteria (Fig. 1).

There are three possible explanations for our observations: (i) there were few nitrifiers relative to other bacteria in the samples examined, and so the method used was not sensitive enough; (ii) heterotrophic bacteria were responsible for the oxidation of ammonia and nitrite in the environments studied; or (iii) the responsible species of autotrophic ammonia-oxidizing bacteria belong to another phylogenetic group which the probes did not detect. These possibilities are discussed below.

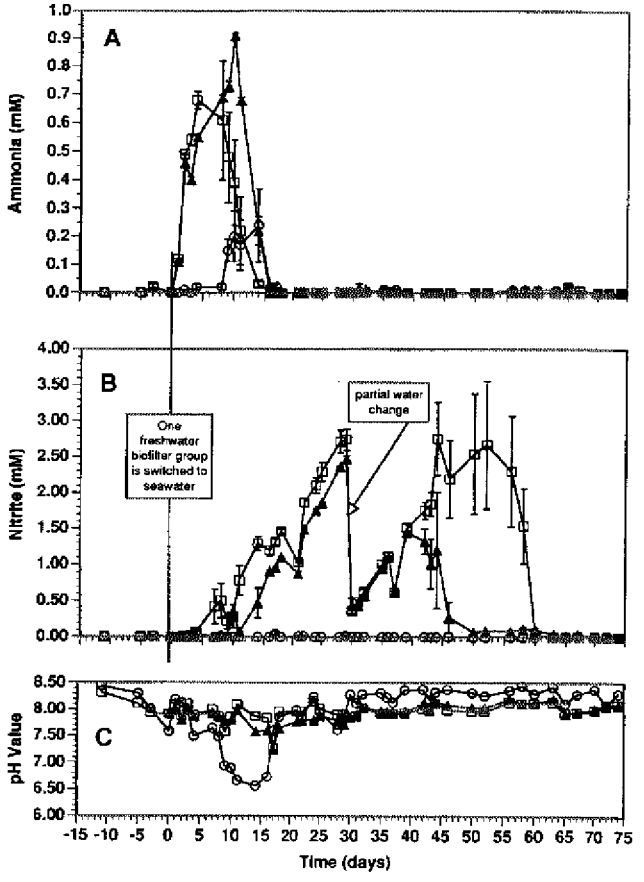

FIG. 3. Mean values ($n = 3$) for ammonia concentration (A), nitrite concentration (B), and pH (C) for the biofilters from the following three aquarium environments: freshwater changed to seawater (□), freshwater (○), and seawater (▲). Bars indicate standard errors. Each aquarium received 5 mM ammonia (as ammonium chloride) each day for the first 20 days and then nearly every other day; none of the aquaria contained fish. Establishment of nitrification is shown by the sudden decrease in the ammonia concentration for the seawater group and the group in which freshwater was changed to seawater near day 15 (after the switch). This was followed by a rapid decrease in the nitrite concentration between days 45 and 60. A partial water change was performed on all aquaria on day 29, and this resulted in the large temporary decrease in nitrite concentration evident at this time.

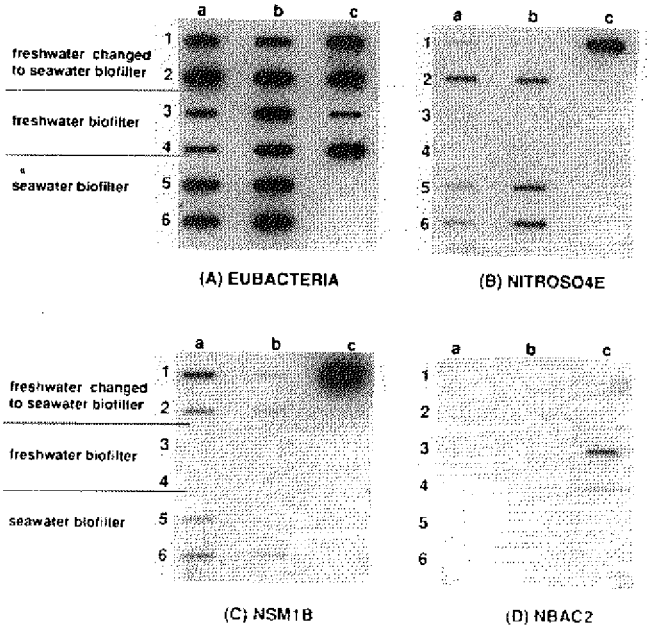

FIG. 4. Slot blot analysis of rRNAs extracted from the biofilters of two freshwater aquaria changed to seawater (rows 1 and 2), two continuously freshwater aquaria (rows 3 and 4), and two seawater aquaria (rows 5 and 6) and hybridized with the eubacterial probe (A), the NITROSO4E probe (B), the NSM1B probe (C), and the NBAC2 probe (D). Water chemistry was tested three times a week for these filter units (see Fig. 3), and the data confirmed that active nitrification occurred. Lanes a, rRNA samples taken 43 days after the switch from fresh water to seawater; lanes b, rRNA samples taken 72 days after the switch; lanes c, rRNAs extracted from control strains (slot c-1, *Nitrosomonas europaea*; slot c-2, *Comamonas testosteroni*; slot c-3, *Nitrobacter winogradskyi*; slot c-4, *Rhodopseudomonas palustris*).

The minimum detection limit for radiolabelled nucleic acid probes is between approximately 0.1 and 1.0% of the total rRNA (1). While absolute bacterial cell numbers cannot be inferred from the results of hybridization experiments, this method does provide a reasonable indication of the relative biomass or metabolic activity of the targeted group. The biofilter experiments demonstrated that our method was sufficiently sensitive to detect nitrifiers in this environment, since all seawater samples produced a strong positive signal. It is reasonable to assume that in the aquaria, whose sole energy input was ammonia, the bacteria responsible for nitrification were active and constituted a large fraction of the total bacterial assemblage. The positive results obtained with the autotrophic ammonia-oxidizing bacterial probes for seawater biofilters exposed to the same environmental conditions as parallel freshwater filters indicate that the extraction and hybridization procedures were sufficiently sensitive to detect ammonia oxidizers belonging to the β-proteobacterial subdivision on filters.

Heterotrophic nitrification has been shown to be potentially greater than autotrophic nitrification in certain environments (26). There are several species of heterotrophic bacteria which use ammonia as a substrate and produce either nitrite, nitrate, or a less common nitrogen cycle intermediate, such as hydroxylamine (18). Tate (29) found insufficient numbers of *Nitrosomonas* and *Nitrobacter* cells to account for the nitrate production in histosols. Instead, using inhibitors, he determined that an *Arthrobacter* population was responsible for a major

portion of the nitrification occurring in these soils. Castignetti and Hollocher (5) identified six heterotrophic bacteria, including *Pseudomonas denitrificans*, *Pseudomonas aeruginosa*, and two strains of *Pseudomonas fluorescens*, that exhibited nitrification activity. While there is no direct evidence that heterotrophic nitrification was the dominant process in the present study, this possibility cannot be totally discounted. However, such heterotrophic nitrification seems unlikely since the aquaria received only inorganic ammonia (ammonium chloride) as an energy source. Carbon dioxide was the sole carbon source available as there were no significant inputs of organic carbon to support heterotrophic bacterial growth beyond trace contamination. It is doubtful that heterotrophic bacterial growth was significant in this lithotrophic environment.

The possibility that the autotrophic nitrifying bacteria in the freshwater aquaria studied belong to subdivisions other than the β subdivision of the *Proteobacteria* seems to be the most likely explanation for our observations. The probes were sensitive enough to detect ammonia-oxidizing bacteria in seawater systems. The freshwater systems had similar rates of nitrification, but ammonia oxidizers were not detected. Furthermore, *Nitrosomonas* spp. were detected on biofilters only after a shift from freshwater to seawater. This suggests that there were changes in the population of nitrifying bacteria, as indicated by the appearance of *Nitrosomonas* spp. and their relatives, as well as the transient decrease in nitrification immediately following the shift from freshwater to seawater. In total, our data suggest that microorganisms other than the usually implicated nitrifiers (members of the β subdivision of the *Proteobacteria*, such as *Nitrosomonas* spp. and their relatives) are the major agents responsible for nitrification in the freshwater aquarium environments examined.

To date, only one ammonia-oxidizing bacterium which does not belong to the β subdivision of the *Proteobacteria* has been cultured. The emphasis on *Nitrosomonas* types, especially *Nitrosomonas europaea*, as the major ammonia oxidizers in environments may be partially a result of culture bias. It is possible that *Nitrosomonas europaea* grows better in enrichment cultures and pure cultures than other, more ecologically significant nitrifiers which flourish and outcompete *Nitrosomonas* spp. in mixed populations. Belser and Schmidt (3) observed selectivity among the different genera of ammonia oxidizers, with *Nitrosomonas* spp. generally dominant over *Nitrosospira* and *Nitrosolobus* spp., possibly because of a faster growth rate. Furthermore, these authors found that while a medium could support the growth of either *Nitrosomonas* species or *Nitrosospira* species, these bacteria generally never grew together in the same enrichment culture. This may explain the data of Hiorns et al. (13), who suggested that *Nitrosomonas* spp. were prevalent only in enrichment cultures that were not obtained from environmental samples.

In the case of nitrite-oxidizing bacteria, the data suggest the possibility that the responsible bacteria were not *Nitrobacter* species, since we were unable to detect *Nitrobacter* cells in any sample examined. Nitrite-oxidizing α subdivision proteobacteria were also not detected by Wagner et al. (34), who examined river water, a nitrifying trickle filter biofilm, and activated sludge samples by using fluorescent probes specific for various *Nitrobacter* species. These authors concluded that the most probable reason for their results was that there were large numbers (or high-level activities) of non-*Nitrobacter* nitrite-oxidizing bacteria present in the systems which they examined. The chance that our results were due to the relatively low level of sensitivity of quantitative rRNA hybridization experiments does exist. However, DNAs from aquarium samples amplified by PCR with general eubacterial primers and subsequent hybridization experiments with the amplified DNAs also yielded negative results with the *Nitrobacter* probe. The fact that there are several nitrite oxidizers in other subdivisions of the *Proteobacteria* could readily explain our results. Three known autotrophic nitrite-oxidizing bacteria are in the δ subdivision, and one such organism is in the γ subdivision, although a recent study has suggested that the phylogenetic placement of the genus *Nitrospira* may need to be reconsidered (8). The δ subdivision nitrite-oxidizing bacteria were isolated from marine environments, but the salinity of the water from which the sample of *Nitrospina gracilis* was isolated was low (12.870 ppt) (37). Ehrich et al. (8) recently isolated a new obligately chemolithoautotrophic nitrite-oxidizing species, *Nitrospira moscoviensis*, which was cultured in freshwater media. Since few strains of the other nitrite-oxidizing bacteria have been cultivated, it may be premature to design probes based on only a few isolates.

There have been no definitive studies of the microbiology of aquarium biofilters. Johnson and Sieburth (15) used scanning electron microscopy to investigate nitrifying bacteria obtained from the biological filters and waters of three aquaculture operations (one freshwater and two seawater). These authors were unable to detect bacteria with *Nitrosomonas*-like cytomorphological features in actively nitrifying freshwater salmon culture systems. In addition, they could not find *Nitrobacter winogradskyi* in any of the natural systems which they sampled, but these bacteria were found in subsequent enrichment cultures. These results are consistent with our results obtained with freshwater aquaria, in which no "classical" *Nitrosomonas* species could be detected.

To a certain extent, models of nitrification are dependent on the known biochemical properties and pathways of the classical nitrifiers. The data from our study indicate that the bacterial species responsible for nitrification in simple freshwater systems remain unknown. It is likely that nitrification and the associated nitrifying bacterium diversity in natural systems are even more complex. Therefore, models which assume, in a general fashion, that *Nitrosomonas* spp. are the major nitrifiers may have to be revised as novel species of nitrifying bacteria are identified, isolated, and characterized and the biochemical properties of these species are determined. Molecular phylogenetic methods, along with classical isolation and culture techniques, all of which are aimed at determining the responsible organisms and their physiological properties, should provide a more complete understanding of biogeochemical processes mediated by nitrifying bacteria.

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Nitrospira-Like Bacteria Associated with Nitrite Oxidation in Freshwater Aquaria

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Oxidation of nitrite to nitrate in aquaria is typically attributed to bacteria belonging to the genus *Nitrobacter* which are members of the α subdivision of the class *Proteobacteria*. In order to identify bacteria responsible for nitrite oxidation in aquaria, clone libraries of rRNA genes were developed from biofilms of several freshwater aquaria. Analysis of the rDNA libraries, along with results from denaturing gradient gel electrophoresis (DGGE) on frequently sampled biofilms, indicated the presence of putative nitrite-oxidizing bacteria closely related to other members of the genus *Nitrospira*. Nucleic acid hybridization experiments with rRNA from biofilms of freshwater aquaria demonstrated that *Nitrospira*-like rRNA comprised nearly 5% of the rRNA extracted from the biofilms during the establishment of nitrification. Nitrite-oxidizing bacteria belonging to the α subdivision of the class *Proteobacteria* (e.g., *Nitrobacter* spp.) were not detected in these samples. Aquaria which received a commercial preparation containing *Nitrobacter* species did not show evidence of *Nitrobacter* growth and development but did develop substantial populations of *Nitrospira*-like species. Time series analysis of rDNA phylotypes on aquaria biofilms by DGGE, combined with nitrite and nitrate analysis, showed a correspondence between the appearance of *Nitrospira*-like bacterial ribosomal DNA and the initiation of nitrite oxidation. In total, the data suggest that *Nitrobacter winogradskyi* and close relatives were not the dominant nitrite-oxidizing bacteria in freshwater aquaria. Instead, nitrite oxidation in freshwater aquaria appeared to be mediated by bacteria closely related to *Nitrospira moscoviensis* and *Nitrospira marina*.

The oxidation of nitrite to nitrate by chemolithoautotrophic nitrite-oxidizing bacteria (NOB) in fish culture systems, ranging from home aquaria to commercial aquaculture systems, is an important process. The accumulation of high concentrations of nitrite, which is toxic to fish and other aquatic organisms, is prevented by active nitrite removal by nitrifying microorganisms. Nitrite is formed in aquarium systems from the oxidation of ammonia, the principal nitrogenous waste of teleosts, by autotrophic ammonia-oxidizing bacteria (AOB). Thus, closed aquatic filtration systems usually provide a solid substratum, which is termed a biological filter or biofilter, to promote the growth of AOB and NOB. A variety of materials can form the substratum of a biofilter, ranging from gravel to specially engineered molded plastics. Biofilters can be submerged in the flow path of the filtration system or can be located such that the water trickles or percolates through a medium situated in the atmosphere outside the aquarium, before flowing back into the tank.

Traditionally, the bacteria responsible for the oxidation of ammonia and nitrite in aquaria were considered to be *Nitrosomonas europaea* and *Nitrobacter winogradskyi* or their close relatives, respectively (17, 18). However, there is some indication that both *N. europaea* and *N. winogradskyi* may not be predominant components of actively nitrifying freshwater aquaria (9). In seawater aquaria, however, *N. europaea* and close relatives do appear to comprise a significant proportion of the total eubacterial community, but *N. winogradskyi* was below detection limits (9).

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Chemolithoautotrophic NOB are phylogenetically diverse, occurring in several subdivisions of the class *Proteobacteria* (Fig. 1). The most well-studied members of this group of organisms (i.e., *N. winogradskyi* and close relatives) belong to the α subdivision of the class *Proteobacteria* (16). *Nitrospina gracilis* and *Nitrococcus mobilis*, which were first isolated by Watson and Waterbury (16), were determined to be members of the δ and γ subdivisions of the class *Proteobacteria*, respectively (14). Another NOB, *Nitrospira marina*, is phylogenetically affiliated with non-NOB such as *Leptospirillum ferrooxidans* (7, 14, 16). Based on phylogenetic analysis of 16S rRNA sequences, Erlich et al. (7) proposed a new phylum within the domain *Bacteria* for these organisms (Fig. 1). A newly discovered NOB from a freshwater environment (a corroded iron pipe in a heating system), *Nitrospira moscoviensis*, was recently found to be phylogenetically related to *N. marina* (7).

Whether in pure culture or on biofilters, NOB are slowly growing organisms with doubling times from 12 to 32 h (3, 5, 7). Therefore, in newly set up aquaria, ammonia and nitrite can reach concentrations toxic to fish before a sufficient biomass of AOB and NOB becomes established. To reduce the length of time for establishment of NOB on biofilters, commercial preparations of these organisms, in various forms of preservation, are available to seed the aquarium environment. These preparations range from essentially pure cultures of *Nitrobacter* species to mixed cultures of autotrophic AOB and NOB organisms and to products which combine autotrophic nitrifying bacteria with various species of heterotrophic bacteria. Past studies have generally shown these mixes to be ineffectual but have not elucidated specific reasons for their poor performance (4, 15).

In this study, we observed that *Nitrospira*-like species rather than *Nitrobacter* species appeared responsible for oxidation of nitrite to nitrate in freshwater aquaria. A combination of

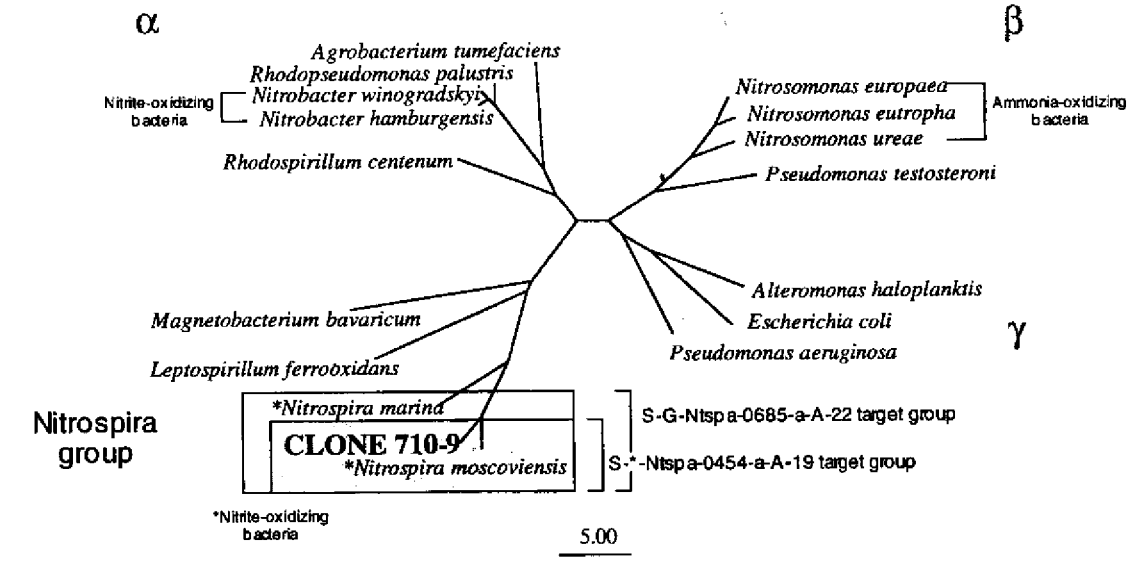

FIG. 1. Phylogenetic relationships of autotrophic NOB in the α subdivision of the class *Proteobacteria* and the *Nitrospira* group. Clone 710-9, an rDNA clone originating from aquaria with active NOB populations, is most similar to NOB of the *Nitrospira* group. The specificities of two oligonucleotide probes designed for *Nitrospira* spp. are indicated by the boxed sections.

methods was used to investigate concurrently the appearance of NOB on biofilters and the oxidation of nitrite to nitrate. Oligonucleotide probes, which target *Nitrospira* and close relatives, were developed and used to quantify this group at different times during the establishment of nitrification. Denaturing gradient gel electrophoresis (DGGE) was used to monitor the appearance of *Nitrospira*-like bacteria during the onset of nitrite oxidation. The effectiveness of a commercial mix of AOB and NOB was also evaluated.

MATERIALS AND METHODS

Nucleic acid sampling and extraction. For rRNA extractions from aquarium gravel, individual gravel samples (10 g) were placed in a polypropylene tube and covered with 2.5 ml of low-pH buffer (50 mM sodium acetate, 10 mM disodium EDTA) and processed as previously described (9). For DNA extraction, gravel samples were resuspended in cell lysis buffer (40 mM EDTA, 50 mM Tris-HCl, 0.75 M sucrose) and processed as described previously (9). Samples were stored at −20°C until extraction.

DNA was quantified by Hoechst type 33258 dye binding and fluorometry (DynaQuant 200; Hoefer Pharmacia Biotech, Inc., San Francisco, Calif.). rRNA was quantified by measuring A_{260} (Lambda 3B; Perkin Elmer), assuming that 1 A_{260} U corresponds to 40 μg of RNA per ml.

Clone libraries of PCR-amplified rRNA genes. Clone libraries were derived from nucleic acid extracts of aquarium samples. Bacterial rRNA gene fragments were amplified with the primers S-D-Bact-0011-a-S-17 (8f; GTT TGA TCC TGG CTC AG) and 1492r (eubacterial; GGT TAC CTT GTT ACG ACT T) or S-*-Univ-0519-a-A-18 (519r; GWA TTA CCG CGG CKG CTG). PCR conditions, cycle parameters, and reaction components were as previously described (6). PCR products were evaluated by agarose gel electrophoresis. PCR fragments were cloned with a TA cloning kit (Invitrogen, Carlsbad, Calif.), as previously described (6).

DGGE analysis and profiling. For DGGE analysis, ribosomal DNA (rDNA) fragments were amplified with the forward primer 358f (eubacterial; CCT ACG GGA GGC AGC AG) with a 40-bp GC clamp on the 5′ end as described by Murray et al. (11) and the reverse primer S-*-Univ-0519-a-A-18 (519r; GWA TTA CCG CGG CKG CTG). PCR was performed on a Stratagene Robocycler Gradient 96 (La Jolla, Calif.) with the manufacturer's reagents. PCR conditions included a hot start (80°C) and a touchdown procedure (11). Initial denaturation at 94°C for 3 min was followed by a denaturation at 94°C for 1 min, a touchdown annealing from 65 to 55°C for 1 min and 29 s (the annealing time during the touchdown increased by 1.4 s per cycle), and primer extension at 72°C for 56 s (the extension time was increased 1.4 s per cycle). The final temperature series of the above thermal cycle was repeated for 20 total cycles, followed by a final extension at 72°C for 5 min. Amplicons were examined by agarose gel electrophoresis.

DGGE was performed with a Bio-Rad D-GENE System (Bio-Rad Laboratories, Hercules, Calif.). All gels were 8.5% acrylamide–bis with Bio-Rad reagents (D-GENE Electrophoresis Reagent kit). Gel gradients were poured with Bio-Rad reagents (D-GENE Electrophoresis Reagent kit) with a denaturing gradient of 20 to 60% (where 100% denaturant is a mixture of 40% deionized formamide and 7 M urea) and the Bio-Rad gradient delivery system (model 475; Bio-Rad). All gels were run at 200 V for 6 h. The gels were visualized in one of two ways. For visualization and recovery of discrete DNA bands, the gels were first stained for 10 min in 250 ml of 1× Tris-acetate-EDTA (TAE) buffer, in which 100 μl of ethidium bromide (1 mg/ml) was added, and then were washed for 10 min in 1× TAE buffer. For documentation purposes, some gels were stained in Vistra Green (diluted 1:10,000) (Molecular Dynamics, Sunnyvale, Calif.) for 20 min, followed by a 20-min wash in 1× TAE buffer, and then were scanned with a FluorImager SI (Molecular Dynamics).

Individual bands were excised from the DGGE gels with alcohol-sterilized scalpels. Extraction of DNA from the gel followed the methods of Ferris et al.

TABLE 1. The nucleotide sequences and positions of oligonucleotide probes for NOB

Probe[a]	Position (nucleotides)[b]	Base sequence (5′ to 3′)	T_d (°C)[c]	Wash temp (°C)	Targeted group	Nontarget bacteria with exact match to probe sequence
S-G-Ntspa-0685-a-A-22	664–685	CAC CGG GAA TTC CGC GCT CCT C	63.0	60.0	*N. moscoviensis*, *N. marina*, and 710-9 clone	None
S-*-Ntspa-0454-a-A-19	435–454	TCC ATC TTC CCT CCC GAA AA	58.5	56.0	*N. moscoviensis* 710-9 clone	None

[a] Probe names designated by the standard proposed by Alm et al. (1).
[b] *E. coli* numbering.
[c] T_d, temperature at which 50% of the bound probe is released from the homologous hybrid.

TABLE 2. Similarity ranking for clone 710-9 isolated from freshwater aquaria and members of the *Nitrospira* group

rDNA source	% Similarity to rDNA of:					
	710-9 sequence	*N. moscoviensis*	*N. marina*	*Leptospirillum* sp.	*L. ferrooxidans*	*M. bavaricum*
710-9 sequence[a]						
N. moscoviensis	96.1					
N. marina	87.4	87.6				
Leptospirillum sp.	79.9	80.3	80.2			
L. ferrooxidans	78.1	78.4	77.9	91.0		
Magnetobacterium bavaricum	78.2	77.9	79.7	78.3	77.0	

[a] Positions 24 to 1284 of 710-9 (*E. coli* numbering).

(8). The excised band was placed in a sterile 2-ml screw-cap tube with 500 μl of sterile deionized water. The tubes were half filled with glass beads (catalog no. 11079-101; BioSpec Products, Inc., Bartlesville, Okla.) and placed in a mechanical bead beater (Mini-beadbeater-8; BioSpec Products) for 3 min at the highest setting. The processed DNA remained in the tubes at 4°C overnight. After overnight storage, the tubes were centrifuged at 3,200 × *g* for 8 min at 4°C to concentrate the gel fragments. The supernatant was transferred to a clean Eppendorf tube.

To check the extraction efficiency, the supernatant was reamplified with the DGGE primers and reanalyzed by DGGE. An extraction was considered acceptable if it yielded a single band in DGGE analysis which comigrated with the original DGGE band in the mixed population sample.

Oligonucleotide probe development and hybridization procedures. Two oligonucleotide probes were designed which specifically hybridize with *N. marina*, *N. moscoviensis*, and the *Nitrospira*-like rRNA gene sequence isolated in this study from biofilters. One probe (S-G-Ntspa-0685-a-A-22) targets the biofilter-derived *Nitrospira*-like bacterium and both *N. marina* and *N. moscoviensis*. The second probe (S-*-Ntspa-0454-a-A-19) targets the biofilter-derived *Nitrospira*-like bacterium and its closest cultivated relative, *N. moscoviensis* (Fig. 1). Probe matches were initially screened by BLAST (2) and CHECK_PROBE (10). The probes were synthesized by Operon Tech, Inc. (Alameda, Calif.). The nucleotide sequences and positions of the probes are shown in Table 1.

Since no pure rRNA of the biofilter-derived *Nitrospira*-like bacterium is yet available, in vitro-transcribed 16S rRNA was used as a template for temperature of dissociation (T_d) determinations and as a control in hybridization experiments. In vitro-transcribed 16S rRNA was synthesized as described by Polz and Cavanaugh (12).

The T_ds of the oligonucleotide probes were determined by measuring the amounts of probe eluted over a series of increasing wash temperatures (13). For these tests, 200 ng of template was immobilized on a nylon membrane (Hybond-N; Amersham) and hybridized overnight at 45°C with ^{32}P-labelled probe. After hybridization, the membrane was washed at room temperature in 1× SET (150 mM NaCl, 1 mM EDTA, 20 mM Tris; pH 7.8)–1% sodium dodecyl sulfate (SDS) for 30 min on a shaker table. Individual filter strips were then placed in a 0.5-ml Eppendorf tube containing 500 μl of 1× SET–1% SDS preheated to the initial test temperature. The Eppendorf tubes were placed in a thermal cycler (Perkin-Elmer) and incubated for 30 min. The membrane was transferred to a new Eppendorf tube containing 1× SET–1% SDS, and the temperature was increased and maintained at the elevated temperature for 30 min. After each wash, the wash buffer was transferred to a scintillation vial containing 3 ml of scintillation cocktail (Liquiscint; National Diagnostics, Atlanta, Ga.) and was mixed, and radioactivity was quantified by liquid scintillation counting. Each profile was performed in duplicate.

rRNA from aquaria was slot blotted and quantified with nucleic acid probes developed in this and an earlier study (9) under conditions previously described (9). The methods for determining the relative amounts of rRNA-specific hybridization signal from each probe were the same as those previously described (9).

Sequencing. Sequencing of SSU rDNA excised from DGGE gels or clones was performed directly with Sequenase 2.0 (U.S. Biochemicals, Cleveland, Ohio).

Experimental aquarium systems. Three sets of experiments in aquaria were run to (i) study the establishment of nitrifying bacteria and (ii) determine the effect of a bacterial additive. New aquaria, filter systems, and gravel were used for each test. Samples of aquarium water for the three tests were analyzed for ammonia (gas diffusion membrane method), nitrite (azo dye method), and nitrate (cadmium reduction-azo dye method) by flow injection analysis as previously described (9).

(i) Bacterial additive test. Six all-glass aquaria were established with an airlift undergravel filtration system (model KF720; Neptune Products, Moorpark, Calif.) in a temperature-controlled laboratory (mean air temperature, 26.0 ± 1.5°C). The aquaria were covered with glass lids but were not illuminated other than by room ceiling lights which were on a 14- and 10-h light and dark cycle, respectively. A 6.8-kg amount of natural aquarium gravel (Kaytee Products, Irwindale, Calif.) was placed on top of the filtration plate. A 30-liter volume of city tap water, passed through activated carbon, was added to each aquarium. Filtered air was supplied to each aquarium from a common air source. Six fish (*Danio aequipinnatus*) were placed in each aquarium and fed 0.5 g of fish feed (Aquarian; Kal Kan Foods, Vernon, Calif.) daily over two feedings. Three of the aquaria (the treatment group) were each given doses of 8 ml of bacterial additive (Cycle; Rolf C. Hagen Inc., Mansfield, Mass.) on the first day and once every 7 days afterwards for an additional 3 weeks. The other three aquaria were the control group and did not receive an additive.

Two samples of 10 g of gravel were collected from each aquarium on a weekly basis, and nucleic acids were extracted and analyzed as described above.

(ii) Time of NOB appearance. Three all-glass aquaria were established as described above. A 34-liter sample of city tap water, which was passed through activated carbon, was added to each aquarium, which contained 4.53 kg of gravel. Initially, 0.71 mmol of filter-sterilized (0.2-μm-pore-size filter) ammonium chloride was added to each tank, followed by an additional dosing of 5.0 mmol of NH_4Cl on the fourth day. On days 10, 15, 18, 23, and 30, further ammonia additions of 8.9 mmol were made to each aquarium. During the test, a total of 50.4 mmol of ammonia was added to each aquarium. Water samples were collected daily.

Two 10-g samples of gravel were collected from each aquarium daily for 33 days. To one sample, 2 ml of lysis buffer was added and the sample was frozen (−20°C) until rDNA was extracted by previously described methods. rDNA was subjected to DGGE after undergoing PCR with the primers and conditions described above. The other sample was preserved with 2 ml of bead beating buffer.

(iii) Time series. Three aquaria were set up as previously described with 4.53 kg of gravel and were filled with 30 liters of city water which had been passed through activated carbon. The test was run for 138 days, during which the aquaria were individually dosed with 8.9 mmol of filter-sterilized (0.2 μm) ammonia (as ammonium chloride) on the first and second days of the test. From days 12 to 78 of the test, further additions of 8.9 mmol of ammonia were done on average every 3 days. A total of 246 mmol of ammonia was added to each tank during the test. The water was sampled three times a week for chemical analysis. The aquaria were run for 80 days with freshwater, at which time the water was switched to seawater (32 ppt) by draining and refilling with water mixed with artificial sea salts (Marineland Commercial Aquariums, Moorpark, Calif.). After the switch, the testing continued for an additional 57 days.

Nucleotide sequence accession no. The nucleotide sequence reported in this paper for clone 710-9 has been deposited in the GenBank database under accession no. AF035813.

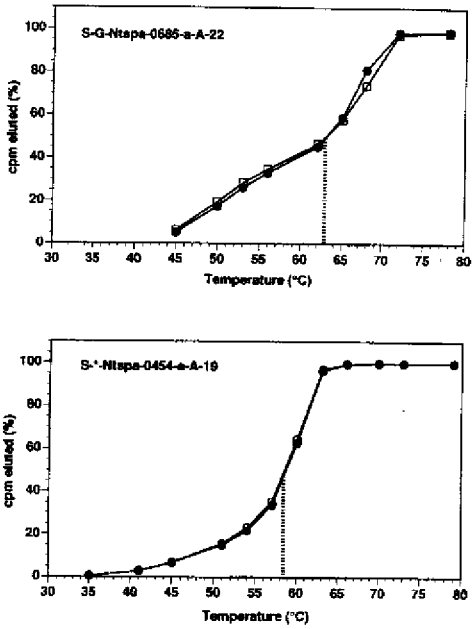

FIG. 2. Results of the T_d experiments for the probes S-G-Ntspa-0685-a-A-22 and S-*-Ntspa-0454-a-A-19, with the 50% T_d indicated by the vertical line. □, rRNA of *N. marina*; ● and ○, transcribed RNA of clone 710-9.

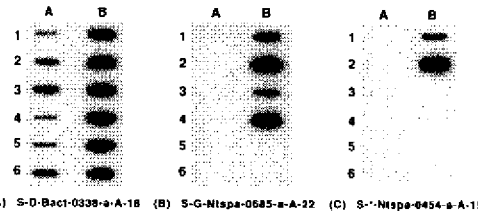

FIG. 3. Specificities of the oligonucleotide probes targeting NOB of the *Nitrospira* group and the 710-9 clone identified in this study. Probe order was eubacterial probe S-D-Bact-0338-a-A-18 (A), *Nitrospira*-like NOB probe S-G-Ntspa-0685-a-A-22 (B), and *Nitrospira*-like NOB probe S-*-Ntspa-0454-a-A-19 (C), with rRNA, transcribed RNA (trRNA), or PCR-amplified rDNA, in the following arrangement: slot A-1, *Comamonas testosteroni*; slot A-2, *Alcaligenes eutrophus*; slot A-3, *Alcaligenes faecalis*; slot A-4, *Comamonas acidovorans*; slot A-5, *N. winogradskyi* (rDNA); slot A-6, *Nitrobacter agilis* (rDNA); slot B-1, clone 710-9 (rDNA); slot B-2, clone 710-9 (trRNA); slot B-3, *N. marina* (rDNA); slot B-4, *N. marina* (trRNA); slot B-5, *N. gracilis*; slot B-6, *Shewanella putrefaciens*. See text for description of methods.

RESULTS

Isolation of putative NOB. Two approaches were taken to identify NOB in aquarium samples. The first approach was to develop clone libraries from gravel samples from an aquarium at several times during the establishment of nitrification. Samples were taken 17 and 31 days after the aquarium establishment and ammonia additions started. A third library was constructed from DNA extracted from the material of a commercial biofilter constructed of thermoplastic material (model CBW-1; Aquaria, Inc.). This filter had been set up for 109 days in a system with daily dosing of ammonium chloride.

The second approach used to monitor and identify nitrifying microorganisms was DGGE. The DNA extracted from aquarium gravel samples taken during the establishment of nitrification was subjected to DGGE to produce a pattern of discrete bands. The banding patterns were compared to each other and to band patterns produced by a mix of known nitrifiers. Unique bands were excised from the gels and sequenced.

The sequences from the clone libraries and DGGE were compared to bacterial sequences found in public databases (BLAST [2] and RDP [10]). Some clones, which showed a close similarity to those of known nitrite-oxidizing organisms, were more completely sequenced.

Identification of putative *Nitrospira*-like NOB. Five samples were screened for NOB by either clone library development or DGGE. A total of 96 clones or excised bands were partially sequenced. Of these, 11 were highly similar to members of the *Nitrospira* group but none were similar to *Nitrobacter* spp. The partial sequences were most highly similar to those of *N. ma-*

TABLE 3. Results of probing rRNA extracted from aquarium biofilms with nucleic acid probes for NOB

Sample label	Aquarium environment[a]	Biofilm substrate[b]	Daily ammonia amt[c]	Ammonia source[d]	Signal with the following oligonucleotide probes[e,f]:			
					S-D-Bact-0338-a-A-18	NOB		
						S-*-Nbac-1017-a-A-20	S-G-Ntspa-0685-a-A-22	S-*-Ntspa-0454-a-A-19
710r	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	+	+
711r	Freshwater	Polyfiber	32.1 mM	NH_4Cl	+	−	+	+
T825	Freshwater	Polypp	0.8 g	Fish	+	−	+	+
T825	Freshwater	Gravel	0.8 g	Fish	+	−	+	+
WDF1036	Freshwater	Polypp	3.2 g	Fish	+	−	+	+
WDF1036	Freshwater	Gravel	3.2 g	Fish	+	−	+	+
WDF1026	Freshwater	Polypp	2.0 g	Fish	+	−	+	+
WDF1039	Freshwater	Gravel	3.2 g	Fish	+	−	+	+
WDF1038	Freshwater	Sponge	2.0 g	Fish	+	−	+	+
WDF1035	Freshwater	Polypp	2.0 g	Fish	+	−	+	+
FLRT6	Freshwater	Gravel	2.0 g	Fish	+	−	+	+
EXP8B	Freshwater	Polypp	1.4 g	Fish	+	−	+	+
FWSW4	Freshwater	Polypp	5 mM	NH_4Cl	+	−	+	+
FWSW6	Freshwater	Polypp	5 mM	NH_4Cl	+	−	+	+
BC2-8	Freshwater	Gravel	5 mM	NH_4Cl	+	−	+	+
BC2-10	Freshwater	Gravel	5 mM	NH_4Cl	+	−	+	+
BC2-12	Freshwater	Gravel	5 mM	NH_4Cl	+	−	+	+
BC2-13	Freshwater	Gravel	5 mM	NH_4Cl	+	−	+	+
BC2-16	Freshwater	Gravel	5 mM	NH_4Cl	+	−	+	+
BC2-4	Freshwater	Gravel	2.0 g	Fish	+	−	+	+
BC2-16a	Freshwater	Gravel	2.0 g	Fish	+	−	+	+
714r	Seawater	Polyfiber	714 mM	NH_4Cl	+	−	+	−
715r	Seawater	Polyfiber	714 mM	NH_4Cl	+	−	+	+
FWSW2	Seawater	Polypp	5 mM	NH_4Cl	+	−	+	−
FWSW3	Seawater	Polypp	5 mM	NH_4Cl	+	−	+	−
FWSW8	Seawater	Polypp	5 mM	NH_4Cl	+	−	+	−
FWSW9	Seawater	Polypp	5 mM	NH_4Cl	+	−	+	−

[a] Type of aquarium water.
[b] Media from which the bacterial cells were extracted, Polypp, polypropylene.
[c] Fish, the aquarium had a fish population and ammonia was generated by the fish; NH_4Cl, the tank had no fish and the ammonia was from daily dosing with ammonium chloride.
[d] Values in grams are the amounts of fish feed put into the aquarium each day; molar or millimolar values are the concentrations of ammonia added to the aquarium or system in which the biofilter was located each day.
[e] +, signal detected by probe; −, no signal detected.
[f] S-*-Nbac-1017-a-A-20 was originally called NBAC2 (9) and targeted *N. winogradskyi* and *N. agilis*. Probe S-G-Ntspa-0685-a-A-22 targeted *N. marina*, *N. moscoviensis*, and clone 710-9. Probe S-*-Ntspa-0454-a-A-19 targeted *N. moscoviensis* and clone 710-9.

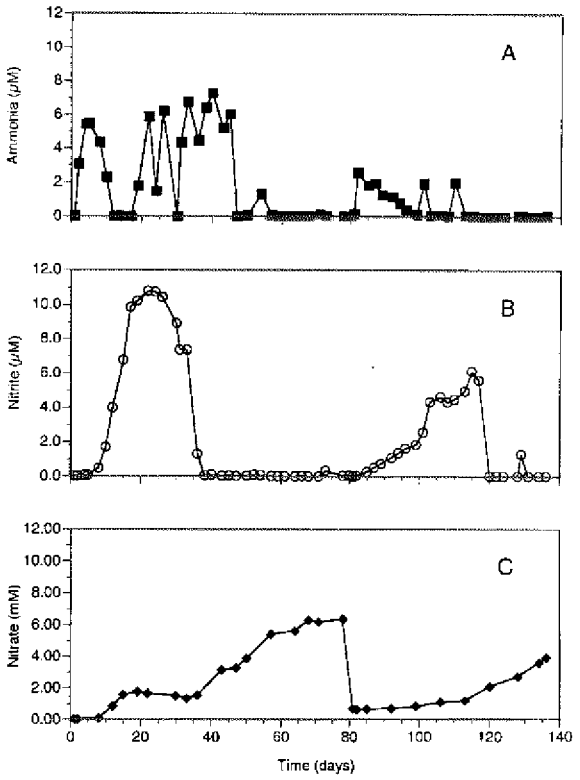

FIG. 4. Ammonia (A), nitrite (B), and nitrate (C) chemistry for an aquarium from startup through 138 days. The saw-toothed pattern for ammonia is the result of the increasing frequency of dosing with ammonium chloride as nitrification was being established. The water was switched from freshwater to seawater on day 80.

rina and *N. moscoviensis* (data not shown). The 16S rDNA of a representative clone which contained the *Nitrospira*-like rDNA was fully sequenced, and a phylogenetic tree was inferred. Phylogenetic analysis indicated a high similarity between this cloned rDNA (710-9) and members of the *Nitrospira* group, *N. moscoviensis* and *N. marina* (Fig. 1). The rDNA contained in clone 710-9 was 96.1% similar to that of *N. moscoviensis* and 87.4% similar to that of *N. marina* (Table 2).

Oligonucleotide probe specificity. Oligonucleotide probe sequences, positions (*Escherichia coli* numbering), T_ds, wash temperatures and target groups for the probes are indicated in Table 1. For probe S-*-Ntspa-0454-a-A-19, the T_d was 58.5°C, while the T_d was 63.0°C for the S-G-Ntspa-0685-a-A-22 probe (Fig. 2).

Slot blot experiments confirmed that the probe S-G-Ntspa-0685-a-A-22 was specific to the known NOB of the *Nitrospira* group, as well as to the clone 710-9 (Fig. 3). As predicted, probe S-*-Ntspa-0454-a-A-19 hybridized to clone 710-9, but not *N. marina*. Furthermore, experiments demonstrated that neither probe hybridized with NOB which are members of the α or δ subdivisions of the class *Proteobacteria* (Fig. 3).

Detection of NOB in aquaria. Table 3 summarizes the results from the probing of several aquarium biofilms with the NOB probes. Probe S-G-Ntspa-0685-a-A-22 yielded a positive signal with all freshwater and saltwater aquaria tested. The probe S-*-Ntspa-0454-a-A-19 detected *Nitrospira*-like bacteria in all freshwater aquaria, but not in all the saltwater aquaria (Table 3). There were no cases of positive detection by a probe which targets α proteobacterial *Nitrobacter* species (Table 3).

Time series. The ammonia, nitrite, and nitrate values for a representative test aquarium dosed with ammonium chloride for 138 days are shown in Fig. 4. The data show the expected pattern for the establishment of nitrification in aquaria. Initially, the concentration of ammonia increased and then decreased to undetectable levels by day 12 (the saw-toothed pattern of the ammonia values is the result of the increasing frequency of ammonia additions). By day 12, the amount of nitrite increased, reaching its maximum value on day 22. By day 38, the amount of nitrite was essentially 0 and that of nitrate was steadily increasing (Fig. 4). The change from freshwater to seawater at day 80 resulted in an immediate increase in the amounts of ammonia and, subsequently, nitrite. It took nearly 20 days for ammonia oxidation to become reestablished. Reestablishment of nitrite oxidation took approximately 40 days.

A DGGE profile for selected days over the first 101 days for this aquarium shows that the *Nitrospira*-like rDNA sequence appeared faintly on day 15, corresponding to the onset of nitrite oxidation (Fig. 5). By day 22, the band corresponding to the *Nitrospira*-like rDNA sequence increased in relative intensity and remained intense over the next two sampling dates. After the switch to seawater, the relative intensity of the *Nitrospira*-like band diminished. The general band pattern also changed qualitatively between freshwater and seawater sampling dates. The banding pattern for day 87 (7 days after the switch) appeared to more closely resemble the pattern for day 57 (freshwater) than the pattern for day 101 (seawater) (Fig. 5).

Time of *Nitrospira*-like bacterial appearance. The daily concentrations of ammonia, nitrite, and nitrate over the first 33 days after setup of a new aquarium are presented in Fig. 6. The trends were as expected, with ammonia peaking about day 12. Nitrite values increased starting at day 12, peaked at day 21, and decreased to below detection limits by day 26. Nitrate values steadily increased from about day 15 onwards. DGGE showed that the band corresponding to clone 710-9, the putative NOB, first appeared on day 12, with the relative intensity of the 710-9 band increasing daily based on relative fluorescence units of rDNA amplicons (Fig. 7).

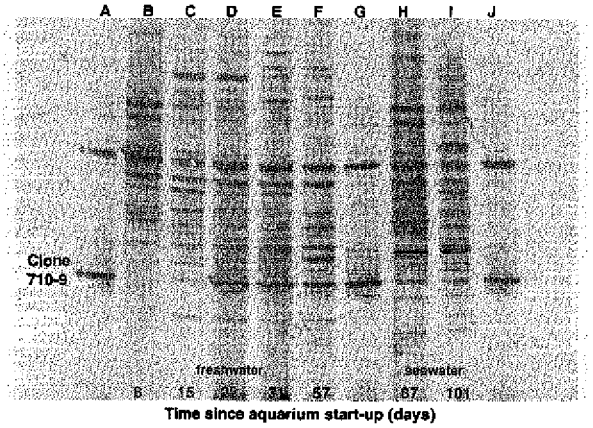

FIG. 5. DGGE time series profile from a biofilm of a freshwater aquarium during the establishment of nitrification. The aquarium water was switched to seawater on day 80. Lanes A, G, and J contain two clones, including clone 710-9, a putative NOB showing close similarity to the *Nitrospira* group. The band corresponding to this organism first appears with significant intensity on day 22. Lanes B, C, D, E, and F are sampling dates before the switch to seawater. Lanes H and I are sampling dates after the switch to seawater. The water chemistry for various forms of nitrogen in this aquarium is indicated in Fig. 4.

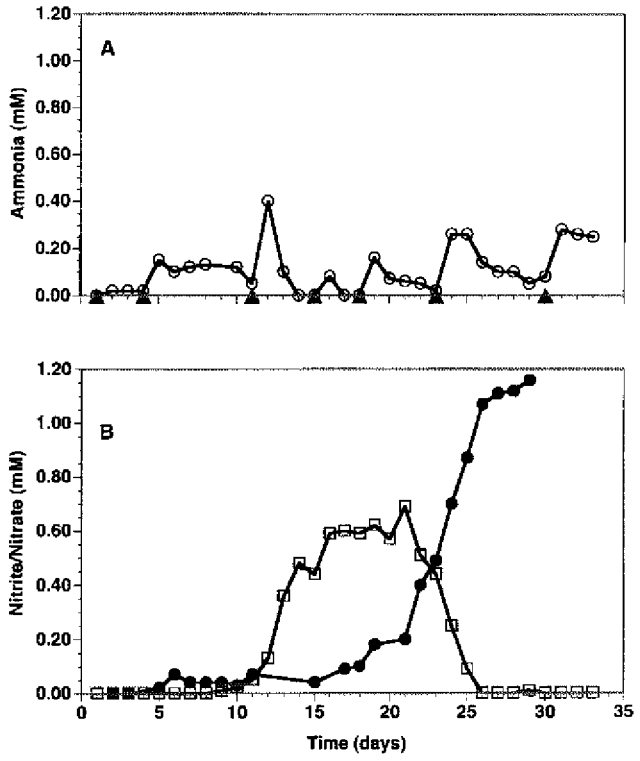

FIG. 6. Inorganic nitrogen values for a newly established freshwater aquarium dosed with ammonia chloride over 33 days. (A) Ammonia (○) values along with dates of ammonia additions (▲); (B) nitrite (□) and nitrate (●) values for the same aquarium. A DGGE profile of the nitrifying assemblage associated with this aquarium is presented in Fig. 7.

Commercial additive. The addition of a commercial bacterial mixture which contained *Nitrobacter* sp., but not *Nitrospira* sp., did not result in the detection of *Nitrobacter* species by oligonucleotide probe hybridization experiments (Fig. 8). However, a band which comigrated with a control derived from pure *Nitrobacter* DNA could be detected in the original commercial mixture by DGGE analysis (data not shown). *Nitrospira*-like rRNA was readily detected in the aquarium. *Nitrospira* group-specific probes indicated that the tank which received the additive had a significantly greater percentage of the *Nitrospira* species rRNA (Fig. 8). By day 16, approximately 5% of the eubacterial rRNA hybridized with the general *Nitrospira* group-specific probe, compared to only 0.33% of the eubacterial rRNA in the tank which did not receive an additive (Fig. 8). By day 50, the values were 3.39 and 1.52% for the additive and nonadditive aquaria, respectively (Fig. 8).

Nitrite concentrations in the two aquaria decreased as the relative percentages of *Nitrospira*-like rRNA increased. By day 22, the nitrite value had reached a maximum in the tank which received the additive. Nitrite concentrations reached maxima in the nonadditive aquarium on about day 32. By day 38, the nitrite levels in both aquaria were essentially below our limits of detection, and nitrate levels were equivalent in the treated and nontreated aquaria (Fig. 8).

DISCUSSION

Our results from DGGE analysis, rRNA probing, and sequencing generally indicate that *Nitrospira*-like bacteria are the most likely candidates responsible for nitrite oxidation in freshwater aquaria. The combined use of molecular phylogenetic techniques and monitoring of water chemistry suggested a correspondence between changes in the biofilm microbial community which coincided with the onset of ammonia and nitrite oxidation. The commencement of nitrite oxidation coincided with the appearance of the putative nitrite-oxidizing *Nitrospira*-like bacterium. The results lend support to the conclusion of an earlier study, which suggested that α subdivision proteobacterial NOB (*Nitrobacter* types) were not major components of nitrite oxidation bacterial populations in freshwater or marine aquaria (9).

Results regarding the beneficial effects of the addition of a bacterial additive containing *Nitrobacter* species were equivocal. While nitrite levels in treated aquaria decreased earlier than those in nontreated aquaria, there was no evidence that *Nitrobacter* species were actively growing in these aquaria. It is possible that the levels of *Nitrobacter* species were below the limits of detection of our techniques. However, since *Nitrospira*-like bacteria were readily detected and that their establishment coincided with nitrite oxidation we postulate that *Nitrospira*-like organisms, and not *Nitrobacter* species, are the major nitrite oxidizers in the freshwater aquarium environment. It is possible that the addition of bacterial mixtures supplies vitamins and other nutrients which generally stimulate the growth of the nitrifying assemblages, fostering their growth and development and indirectly stimulating nitrite oxidation.

In the present study, we identified *Nitrospira*-like putative NOB by amplification of rDNA with general bacterial PCR primers and DGGE analyses. We chose to use universal and domain primers rather than group-specific primers, since previous analysis suggested that nitrite oxidizers other than

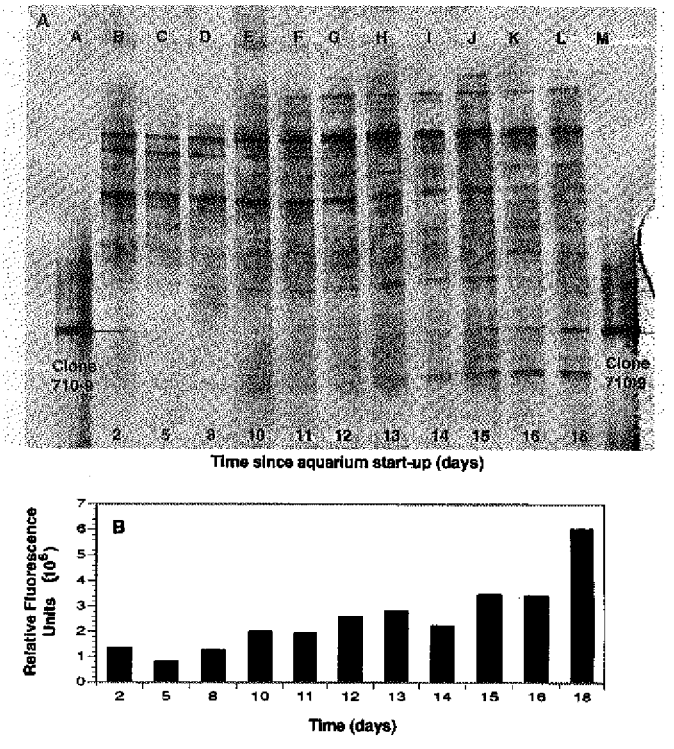

FIG. 7. (A) DGGE of select dates during the first 18 days after the startup of a freshwater aquarium, during which time nitrification became established. Clone 710-9, a *Nitrospira*-like putative NOB, can be seen to appear starting at about day 12 (lane G). (B) Relative intensities of the band for clone 710-9 at each sampling date. Associated water chemistry data for this aquarium are presented in Fig. 6.

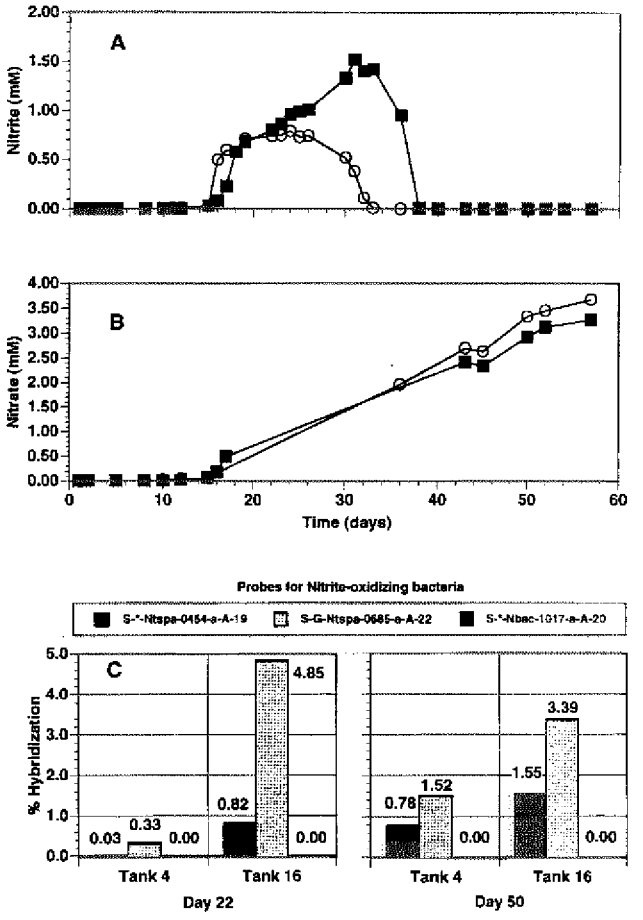

FIG. 8. Water chemistry data and nucleic acid probe hybridization results for a freshwater aquarium during the first 57 days after startup. Nitrite (A) and nitrate (B) are for two tanks, i.e., tank 4 (■), which did not receive a commercial bacterial mixture, and tank 16 (○), which received weekly additions of a commercial bacterial mixture for the first 4 weeks. Problems with nitrate analysis equipment resulted in no data for days 18 through 40. (C) Percent hybridization (relative to that of a eubacterial probe, S-D-Bact-0338-a-A-18) to probes specific for NOB. Probes S-G-Ntspa-0685-a-A-22 and S-*-Ntspa-0454-a-A-19 target *Nitrospira* spp., while probe S-*-Nbac-1017-a-A-20 is for NOB of the α subdivision of the class *Proteobacteria*.

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When comparing biofilters, researchers in the past have been generally limited to assessing mainly water chemistry changes, such as ammonia disappearance and nitrate appearance. The use of molecular probes for the relevant nitrifying bacteria in different systems should provide a more detailed understanding of the interaction between the biology and chemistry of the systems. This in turn provides information relevant to better filter design and may allow the effects of various conditions to be assessed with respect to their effects on the biology as well as the chemistry of the system.

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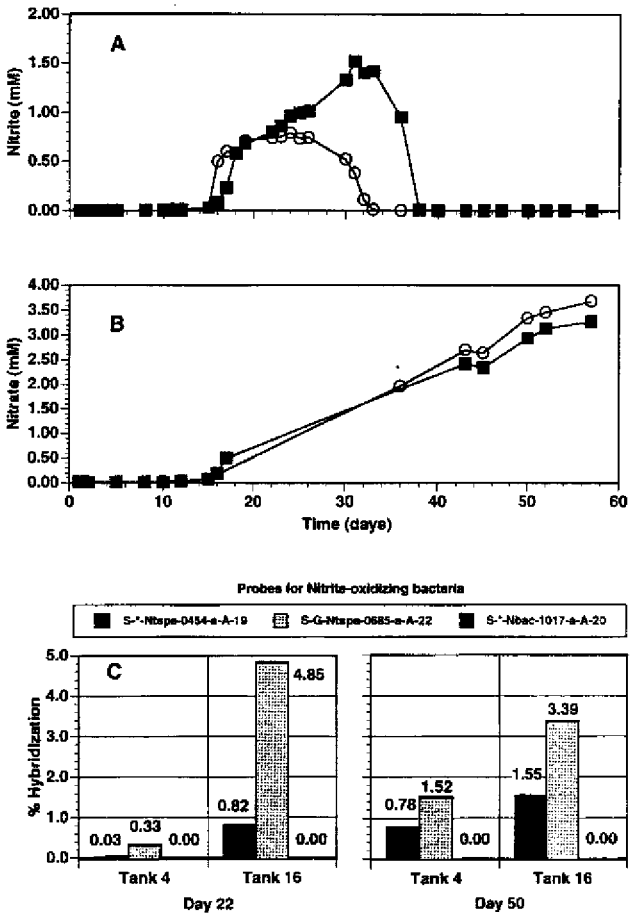

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Microbiology of a Nitrite-Oxidizing Bioreactor

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The microbiology of the biomass from a nitrite-oxidizing sequencing batch reactor (NOSBR) fed with an inorganic salts solution and nitrite as the sole energy source that had been operating for 6 months was investigated by microscopy, by culture-dependent methods, and by molecular biological methods, and the seed sludge that was used to inoculate the NOSBR was investigated by molecular biological methods. The NOSBR sludge comprised a complex and diverse microbial community containing gram-negative and gram-positive rods, cocci, and filaments. By culture-dependent methods (i.e., micromanipulation and sample dilution and spread plate inoculation), 16 heterotrophs (6 gram positive and 10 gram negative) were identified in the NOSBR sludge (RC), but no autotrophs were isolated. 16S ribosomal DNA clone libraries of the two microbial communities revealed that the seed sludge (GC) comprised a complex microbial community dominated by *Proteobacteria* (29% beta subclass; 18% gamma subclass) and high G+C gram-positive bacteria (10%). Three clones (4%) were closely related to the autotrophic nitrite-oxidizer *Nitrospira moscoviensis*. The NOSBR sludge was overwhelmingly dominated by bacteria closely related to *N. moscoviensis* (89%). Two clone sequences were similar to those of the genus *Nitrobacter*. Near-complete insert sequences of eight RC and one GC *N. moscoviensis* clone were determined and phylogenetically analyzed. This is the first report of the presence of bacteria from the *Nitrospira* phylum in wastewater treatment systems, and it is hypothesized that these bacteria are the unknown nitrite oxidizers in these processes.

Nitrification is the initial step in the removal of nitrogenous compounds from wastewaters. It involves the two-step conversion of ammonia to nitrite (ammonia oxidation) and nitrite to nitrate (nitrite oxidation) (10). Denitrification of the nitrate to nitrogenous gas removes the nitrogen from solution (18). If nitrogen removal fails, the nitrogenous compounds passing into waterways may cause a series of environmental and medical problems (2).

There are a range of autotrophic (11) and heterotrophic bacteria (8) capable of nitrification. Unlike heterotrophic bacteria, autotrophs are dependent on this reaction to generate energy for cell maintenance and growth. In wastewater treatment systems, autotrophs constitute only a small percentage of the mixed liquor microbial community, but they are responsible for the bulk of nitrification (17, 18).

In wastewater treatment systems, the genera *Nitrosomonas* (an ammonia oxidizer) and *Nitrobacter* (a nitrite oxidizer) are the two groups of autotrophs presumed to be responsible for nitrification (11). Although ammonia oxidizers have been intensively studied by the use of molecular methods (26, 27), the nitrite oxidizers have not been similarly studied. In one study of activated sludge flocs (15), clusters of *Nitrosomonas* and *Nitrobacter* spp. were adjacent to each other as revealed by fluorescent in situ hybridization (FISH) probing. However, in other studies, *Nitrobacter* could not be detected, and it was speculated that other bacteria were likely responsible for nitrite oxidation (12, 27).

To investigate the identity of the nitrite oxidizers in wastewater treatment plants, a nitrite-oxidizing sequencing batch reactor (NOSBR) was operated. After 6 months of operation of the NOSBR, the developed sludge (RC) was investigated by microscopy, by culture-dependent methods, and by molecular biological methods. In addition, the sludge used to inoculate the NOSBR (GC) was investigated by molecular biological methods and compared with the NOSBR biomass.

MATERIALS AND METHODS

Mixed liquor from the Merrimac Wastewater Treatment Plant at the Gold Coast, Queensland, Australia, was used as inoculum for the NOSBR. The Merrimac plant is a full-scale biological nutrient removal (BNR) plant operating for nitrogen and phosphorus removal. Mixed liquor from the aerobic stage was collected and brought to the laboratory on ice. A volume of 1 liter was used to initiate the NOSBR, while further aliquots were stored at −20°C.

Operation of NOSBR. The NOSBR was operated according to methods previously reported (6). Briefly, the reactor was a chemostat with an operating volume of 1 liter, and the reactor feed comprised the following (per liter): 400 mg of KNO_2, 3.75 g of $MgSO_4 \cdot 7H_2O$, 250 mg of $CaCl_2 \cdot 2H_2O$, 10 g of KH_2PO_4, 10 g of KH_2PO_4, 200 mg of $FeSO_4 \cdot 7H_2O$, and 20 g of $NaHCO_3$ (pH 7.2). There were four stages to each cycle and a hydraulic retention time of 12 h. The stages were (i) feed, 500 ml of fresh medium for 30 min (0 to 0.5 h); (ii) aerobic reaction, 4.5 h (0.5 to 5 h); (iii) settle, 40 min (5 to 5.7 h); and (iv) decant, 500 ml of supernatant for 20 min (5.7 to 6 h). The total time per cycle was 6 h.

After the NOSBR was operated for a period of approximately 6 months, a 10-ml grab sample of mixed liquor was removed from the reactor during the middle of the aerobic reaction stage and used immediately for analyses.

Microscopy. Approximately 50 μl of the NOSBR mixed liquor was Gram stained, and micrographs were taken with a Nikon Microphot FXA microscope.

Culture-dependent methods. The NOSBR sludge (400 μl) was washed twice with phosphate-buffered saline (PBS; 135 mM NaCl, 2.5 mM KCl, 10 mM Na_2HPO_4, 1.75 mM K_2HPO_4 [pH 7.5]), resuspended in 400 μl of PBS, and serially diluted to 10^{-7}. A volume of 50 μl of each dilution was then spread inoculated onto two types of agar media. These were Nutrient Agar (NA; Oxoid, England) and autotrophic nitrite agarose (ANA; composed of the reactor feed [see above] solidified with 10 g of agarose per liter). In addition, a range of the diluted sludge samples was briefly sonicated, and individual cells were isolated by micromanipulation (21) and inoculated onto ANA. Plates were then incubated at 28°C until growth occurred. A range of colonies with different morphologies grew on the NA and ANA inoculated with sludge samples by spread inoculation and on ANA inoculated with micromanipulated cells. These colonies were subcultured to ensure purity. The 16S ribosomal DNA (rDNA) sequence was partially determined and analyzed for a range of these isolates by previously published methods (3, 4).

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Molecular biological methods. The total community DNAs from the NOSBR sludge (RC) and from the sludge used as inoculum for the NOSBR (GC) were isolated, and the 16S rDNAs were PCR amplified and cloned.

DNA extraction. The biomass (500 μl) was centrifuged at 12,000 × *g* for 5 min. The supernatant was discarded, and the pellet was resuspended in 500 μl of saline-EDTA (150 mM NaCl, 100 mM EDTA [pH 8.0]). A volume of 100 μl of freshly prepared 100-mg/ml lysozyme was added to the mixture and incubated at 37°C for 1.5 h. The mixture was then subjected to four cycles of freezing and thawing at −20 and 65°C, respectively. Following this, 100 μl of 25% (wt/vol) sodium dodecyl sulfate and 50 μl of 2% (wt/vol) proteinase K were added to the mixture and the mixture was incubated at 60°C for 1.5 h. The DNA was recovered from the tube by phenol-chloroform extraction (19). The nucleic acids from the 0.5-ml aqueous phase were precipitated by adding 0.12 ml of sterile 3 M sodium acetate and 1 ml of ice-cold 100% ethanol and incubating for 1 h at −70°C. The DNA pellet was recovered by centrifuging the solution at 12,000 × *g* for 20 min at 4°C. The pellet was washed by adding 500 μl of 70% ice-cold ethanol and was recovered by centrifuging at 12,000 × *g* for 10 min at 4°C. The pellet was then air dried, and the nucleic acids were dissolved in 100 μl of sterile milliQ-purified (mQ) water. Residual RNA was removed from the nucleic acid solution by adding 3 μl of 10-mg/ml RNase and incubating at 37°C for 1 h. The DNA was visualized by agarose gel electrophoresis (19).

Amplification of the 16S rRNA genes (16S rDNA). Amplification of the near-complete 16S rRNA genes from the extracted DNA was done by employing the bacterial conserved primers 27f and 1492r (14) in a PCR. The components of the PCR were 10 to 100 ng of DNA, 1 U of *Tth* Plus DNA polymerase (Biotech, Perth, Australia), 10 μl of 10× reaction buffer (Biotech), 200 μM (each) dATP, dCTP, dGTP, and dTTP (deoxynucleoside triphosphates [dNTPs]), and 1 μl of 200-ng/μl (each) primer in a final volume of 100 μl made up with sterile mQ water. The reaction mixture was then overlaid with 80 μl of mineral oil and placed in a thermal cycler (Perkin-Elmer DNA Thermal Cycler 480). A cycling program of 30 cycles of 94°C for 60 s, 48°C for 60 s, and 72°C for 120 s with a final extension of 1 cycle of 48°C for 60 s and 72°C for 300 s was used. The amplicons were visualized by agarose gel electrophoresis and were purified by the Wizard PCR Cleanup Kit (Promega, Sydney, Australia) according to the manufacturer's instructions.

Cloning of the 16S DNAs. Amplicons were used immediately in a ligation reaction mixture comprising 1 μl of T4 DNA ligase (1 U/μl), 10× buffer, 1 μl of pGEM-T vector (50 ng), 2 μl of amplicons (75 ng), and 5 μl of sterile mQ water. All components except the amplicons were from the TA Cloning Kit (Invitrogen, Calif.). Ligation occurred at 15°C for 16 h.

Ultracompetent Epicurian Coli XL2-Blue MRF' cells (Stratagene, Sydney, Australia) were thawed on ice in preparation for the transformation step. A volume of 100 μl of thawed cells was gently placed in a chilled 50-ml Falcon tube, and 1.7 μl of β-mercaptoethanol was added. The mixture was incubated on ice for 10 min with regular gentle swirling. Then, 2 μl of the ligation mixture was added to the cells, and the cells were incubated on ice for 30 min. A heat shock step was done by immersing the Falcon tube in a 42°C water bath for exactly 30 s. Cells were then returned to ice for 2 min. A volume of 900 μl of warm (42°C) sterile SOB (20 g of Bacto Tryptone, 5 g of yeast extract, 0.5 g of NaCl per liter) was added to each tube of transformed cells. These were then shaken at 37°C for 1 h.

A volume of 25 μl of transformed cells was spread inoculated onto Luria-Bertani (LB) agar plates containing ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and isopropyl-β-D-thiogalactopyranoside (LB Ampicillin/X-gal/IPTG), (19) which were incubated at 37°C for 12 to 16 h and then at 4°C for 1 h. Positive clones (those containing 16S rDNA PCR inserts) appeared white and negative clones (no inserts) were blue. Positive clones were picked and patched onto LB Ampicillin/X-gal/IPTG agar plates to ensure that the first screening was correct. Positive clones were picked, homogenized into 300 μl of sterile 50% glycerol, and stored at −20°C until required. These clones constituted the clone libraries.

Amplification of clone inserts. Stored clones from the library were patched onto LB Ampicillin plates from glycerol stocks and grown overnight at 37°C. A sterile tip from a P200 micropipettor was used to obtain a barely visible amount of overnight growth, which was placed into a microcentrifuge tube containing 63 μl of sterile mQ water and 10 μl of 10× reaction buffer, and the mixture was covered with mineral oil. The tube was placed into the thermal cycler and incubated at 96°C for 10 min. Then, 200 μM (each) dNTP, 1 μl of *Tth* Plus DNA polymerase, and 1 μl of each of the plasmid primers (SP6 and T7) (200 ng/μl; Invitrogen) were added to each tube. PCR cycling and observation of amplicons were performed as described above.

Restriction enzyme analysis (REA) of clone inserts. For the NOSBR (RC) library, the amplicons from the SP6-T7 PCR from individual clones were subjected to *Hae*III (Sigma, Sydney, Australia) digestion. *Hae*III is a restriction enzyme that recognizes and cuts the tetranucleotide sequence 5' GG-CC 3', i.e., it is a "4-bp cutter." The digestion mixture consisted of 0.5 μl of *Hae*III enzyme (10 U/μl), 2 μl of NEB Buffer 2 (Sigma), 7 μl of sterile mQ water, and 10 μl of amplicon. The reaction was carried out at 37°C for 3 h. The restriction-digested fragments were visualized by electrophoresis in a 3% Tris-acetate-EDTA agarose gel (19) for 55 min at 80 V.

Clones containing inserts that produced identical restriction patterns were grouped into operational taxonomic units (OTUs), and representatives of each OTU were selected for insert sequencing and analysis.

Partial and near-complete sequencing of clone inserts. Amplicons from the SP6-T7 PCR from individual clones were purified with the Wizard PCR Cleanup Kit and sequenced with the ABI dideoxy sequencing kit (ABI, Melbourne, Australia) according to the manufacturers' instructions and with primer 530f (14). For some clones, near-complete insert sequence data were obtained. In this case, PCR of the inserts with the 27f and 1492r primers was employed. A range of bacterial conserved primers (27f, 357f, 530f, 927f, 1114f [4]) were used to determine the sequences. PCR and sequencing were performed as described above.

Analysis of sequence data. The partial 16S rDNA sequences were compared with those on publicly accessible databases by using the program Basic Local Alignment Search Tool (BLAST [1]). The sequences were also manually aligned, considering secondary structural constraints, with sequences from members of the domain *Bacteria*. Phylogenetic analysis of aligned data sets was carried out by using the Phylogeny Inference Package (PHYLIP version 3.5) according to previously published methods (4).

RESULTS

Microscopy. Over the 6-month period of exposure to a very simple medium that favored the growth of autotrophic nitrite oxidizers, a diverse microbial community in terms of morphology (cocci, rods, and filaments) and Gram stain reaction developed.

Culture-dependent methods. Results for partial 16S rDNA sequences and identities for 16 pure cultures of bacteria obtained from the NOSBR are shown in Table 1. A range of bacteria were able to grow on the ANA medium, although in some cases, the growth took up to 14 days. In addition, prolific growth of a range of bacteria was observed on the NA. Clearly, the NOSBR contained heterotrophs in addition to autotrophic nitrite oxidizers. A range of other bacteria in addition to the 16 reported were isolated. However, none were closely related to known autotrophic nitrite-oxidizing bacteria.

Molecular biological methods. Inserts from a total of 102 clones from the RC library were examined by REA, and they were found to fall into 13 different OTUs (Fig. 1). A total of 90 clones (88%) were grouped into OTU 1, while the remaining 12 OTUs were each composed of individual clones (each of these OTUs was 1% of the total number of clones). Each individual OTU clone insert and six representatives from OTU 1 (RC7, RC11, RC16, RC25, RC73, and RC99) were partially sequenced. Results from BLAST comparisons are given in Fig. 1. According to the BLAST results, the vast bulk of clone inserts in the RC clone library originated from bacteria whose closest relative is *Nitrospira moscoviensis*. It is recognized that BLAST analysis is a fairly crude way to align sequences from clones with those of specific bacterial genera or species, and a selection of the *N. moscoviensis*-like clones was analyzed in much more detail. Also according to BLAST analysis, two other clone inserts, RC44 (OTU 3) and RC57 (OTU 4) (Fig. 1), most closely matched sequences from the genera *Nitrobacter* and *Bradyrhizobium*, respectively. These genera along with the genera *Afipia* and *Rhodopseudomonas* are very closely related according to rRNA comparisons (16, 23). Phylogenetic analysis and direct pairwise comparisons of clones RC44 and RC57 with their closest relatives did not clearly align them with any one of these genera. However, the closest matches from BLAST are given in Fig. 1.

Inserts from a total of 77 clones from the GC library were partially sequenced with the 530f primer and analyzed. The groups to which these clone inserts were affiliated are shown in Table 2. The majority of the clone sequences grouped with the proteobacterial phylum (56%), while 4% (3 clones; GC3, GC86, and GC109) grouped with the phylum *Nitrospira*. The sequences of GC3 and GC86 were 99% similar, while the se-

TABLE 1. Results for isolates obtained from the NOSBR by either sample dilution and spread plate inoculation or micromanipulation of individual cells to ANA

Isolate[a]	Information from BLAST comparison			Gram stain and cell morphology
	Closest match	No. of nucleotides compared	% Similarity with closest match	
1-NA-S	*Acinetobacter* sp.	422	90	Single, gram-negative rods
2-NA-S	*Bacillus firmus*	422	98	Large, long, gram-positive rods; chains
3-NA-S	*Pseudomonas mendocina*	500	96	Single, paired, gram-negative rods
4-NA-S	*Pseudomonas alcaligenes*	380	97	Long, thin, gram-negative rods
5-NA-S	*Acinetobacter* sp.	400	96	Short rods; gram negative
6-NA-S	*Acinetobacter* sp.	425	100	Short rods; gram negative
7-NA-S	Bacterial sp.	375	99	ND[b]
8-ANA-S	*Rhodococcus* sp.	420	97	Gram-positive filaments
9-ANA-S	*Rhodococcus rhodochrous*	434	98	Short, fat rods; gram positive
10-ANA-S	*Rhodococcus* sp.	218	92	Gram-negative rods
11-ANA-S	*Mycobacterium fallax*	280	94	Long, thin, gram-negative rods
12-ANA-S	*Staphylococcus epidermidis*	381	95	Gram-positive tetrads
13-ANA-S	*Paracoccus aminovorans*	300	96	Medium-length, gram-negative rods
14-ANA-M	Unidentified actinomycete	328	97	ND
15-ANA-M	*Stenotrophomonas* sp.	315	96	ND
16-ANA-M	*Comamonas testosteroni*	365	97	ND

[a] NA, Nutrient Agar; ANA, autotrophic nitrite agarose; S, spread plate inoculation; M, micromanipulation.
[b] ND, not described.

quence similarities between clone GC109 and clones GC3 and GC86 were 91.4 and 92.6%, respectively.

Analysis of *Nitrospira* clones. Near-complete insert sequences were determined for eight RC clones (seven from OTU 1 [RC7, RC11, RC14, RC19, RC25, RC73, and RC99] and the one from OTU 2 [RC90]) (Fig. 1), one of the three GC *Nitrospira* clones (GC86), and four clones (SBR1015, SBR1024, SBR2016, and SBR2046) phylogenetically grouped in the *Nitrospira* phylum and from a clone library prepared by Bond et al. (5). The data were phylogenetically analyzed as shown in Fig. 2. A similarity matrix of the 13 clone insert sequences and all those from *N. moscoviensis* and *Nitrospira marina* is shown in Table 3.

DISCUSSION

Our goal was to discover the possible nitrite-oxidizing microorganisms in wastewater treatment systems, since Wagner et al. (27) had unequivocally shown that *Nitrobacter* was not found in sludges by FISH probing. Until then, wastewater treatment personnel had presumed that *Nitrobacter* was the dominant nitrite oxidizer because it was commonly isolated from sludges. Additional support for this notion came from Mobarry et al. (15) who used FISH to observe clusters of *Nitrobacter*, closely juxtaposed with clusters of *Nitrosomonas*, in activated sludge and biofilm samples. However, by quantitative methods of rRNA extraction and slot blot hybridization, it was concluded that the contribution of *Nitrobacter* to nitrification was minor (15).

Nitrobacter (α subclass of the class *Proteobacteria*) can grow heterotrophically, while the remaining known nitrite oxidizers, *Nitrospina* (δ subclass), *Nitrococcus* (γ subclass), and *Nitrospira* (*Nitrospira* phylum), are unable to grow heterotrophically (9). To preclude the selective advantage that *Nitrobacter* may have gained from heterotrophic growth, we employed strategies that selected chemoautotrophic nitrite oxidizers. We attempted to significantly narrow the microbial community from a complex mixture with multiple functions (carbon, nitrogen, and phosphorus removal from domestic wastewater) to a single function (autotrophic nitrite oxidation) of reduced diversity. Our NOSBR has excellent nitrification capacities (7), and we investigated its microbial community structure.

Microscopy and culture-dependent methods. The community is composed of complex morphological types and still retains the floccular nature of activated sludge. We were able to isolate a range of heterotrophs on both ANA and NA by classical sample dilution and spread plate inoculation and by micromanipulation. The occurrence in the NOSBR of hetero-

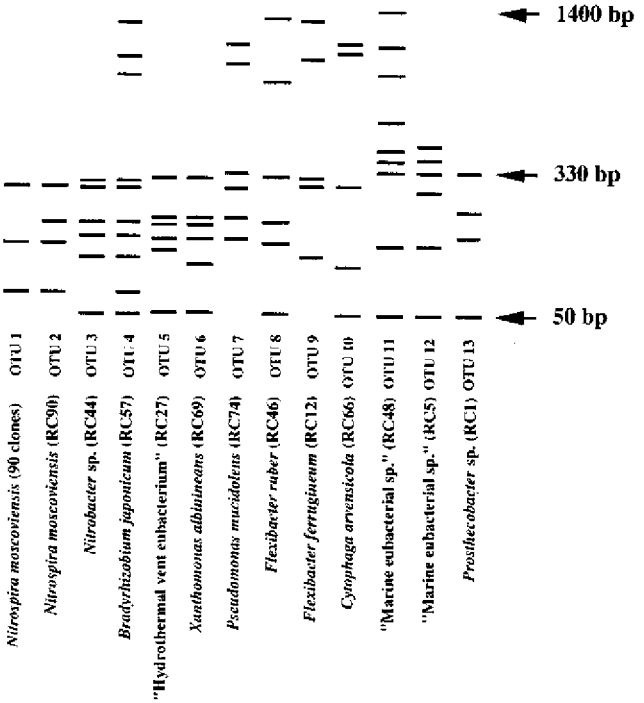

FIG. 1. Diagrammatic representation of the banding profiles of the 13 OTUs in the RC clone library and their closest matches by BLAST comparisons with partial 16S rDNA sequencing of inserts.

TABLE 2. Phyla from the domain *Bacteria* represented in the seed sludge (GC) clone library determined by BLAST comparisons of partial clone insert sequences

Phylum	% of clone library
Proteobacteria	
Alpha subclass	5
Beta subclass	29
Gamma subclass	18
Delta subclass	4
High G+C gram positive	10
Low G+C gram positive	7
Flexibacter/Cytophaga/Bacteroides	5
Nitrospira	4
Planctomycetales	9
Unaffiliated	9

trophic nitrification and of other reactions such as aerobic denitrification cannot be ruled out, but the paucity of organic carbon in the reactor would slow such reactions. Organic carbon can theoretically come from the extracellular polymers that the bacteria in the flocs are producing and from dead microbial cells. The possibility of heterotrophic nitrification and aerobic denitrification is currently being further investigated. We were unsuccessful in isolating an autotrophic nitrite oxidizer. However, the isolation procedures used are perhaps not likely to favor this since we employed only growth on solid media and other groups have employed liquid media for the isolation of nitrite oxidizers (9).

Molecular biological methods. Previous studies generating clone libraries with nonselected activated sludges (e.g., that of Bond et al. [5]) indicated that the diversity of the community would be too great to simplify by REA. Consequently, for the seed sludge library (GC), partial insert sequencing was immediately done rather than REA for grouping. As with other sludges, the diversity of the Merrimac sludge was significant. As well, the proteobacterial phylum dominated the library, comprising 56% of clones, with the majority of these being of the beta subclass of the class *Proteobacteria* (29% of all *Bacteria*). The next largest group was the high G+C gram-positive bacteria (10%). These findings are similar to those from other researchers where bacteria of the beta subclass and/or high G+C gram-positive bacteria are dominant in BNR systems (5, 13, 24, 25). We did not recover any *Nitrobacter* clones in the GC library but did identify three clones (4% of the library) most closely related to *N. moscoviensis*.

We employed REA for the grouping of clones from the NOSBR sludge library (RC) because we hypothesized that the microbial diversity should be reduced in this clone library. Culture-dependent methods had not supported such a hypothesis, but it is well recognized that these methods are heavily biased and the results obtained with them are unrepresentative of the true microbial composition (13). However, because of a range of biases in the methods, clone libraries are not considered adequate for generating quantitative information about the diversity of the microbial community from which the library was prepared (5). Nevertheless, REA proved extremely useful in grouping clones in the RC library because, when 102 clones were examined, one grouping comprised 90 (88% of the total) clones. Of the remaining 12 clones, 11 contained inserts originating from different bacteria with five from the *Flexibacter/Cytophaga/Bacteroides* phylum and five others from the proteobacterial phylum. Two *Nitrobacter*-like clones could not be unequivocally aligned with any genus, but more sequence data from these clones could clarify their affiliation. None of the clone inserts came from gram-positive bacteria, but 6 of the 16 isolates reported were gram positive. In addition, gram-positive bacteria were microscopically observed in the sludge. Cell lysis methods may not have been rigorous enough to lyse the gram-positive bacteria or the primers may have preferentially bound to the non-gram-positive templates in the PCR. Bond et al. (5) also found very few gram positives in two clone libraries from sludges, but Wagner et al. (25) hypothesize that gram

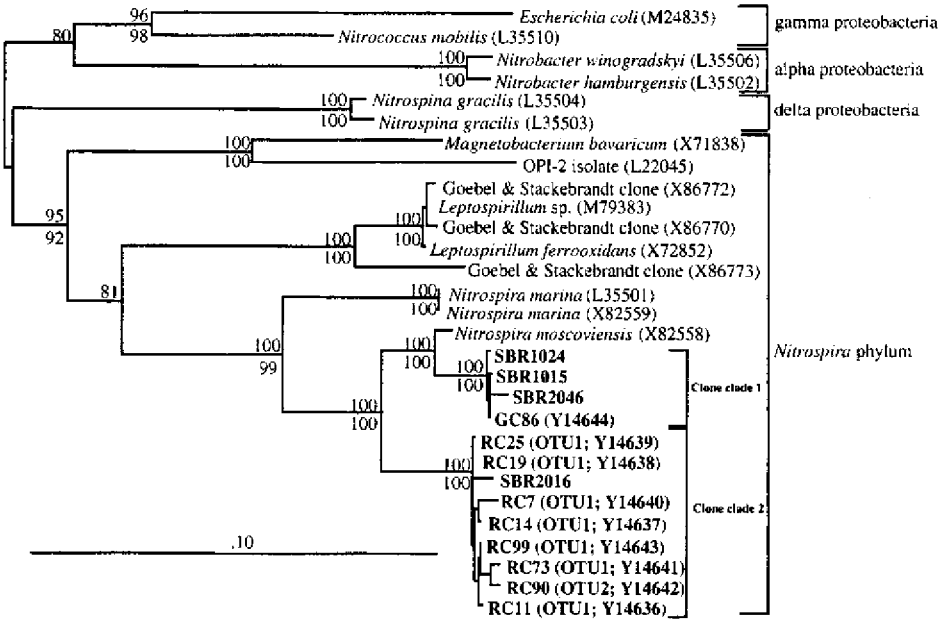

FIG. 2. Evolutionary distance tree of the *Nitrospira* phylum and other known nitrite oxidizers in the domain *Bacteria* based on a comparative analysis of 1,030 nucleotides. Most bootstrap values greater than 92% from 100 resamplings for distance (numbers above branches) and parsimonious (numbers below branches) analyses are presented at the nodes. The outgroup, *Bacteroides fragilis*, is not shown in the tree. The bar represents 0.1 estimated change per nucleotide.

TABLE 3. Similarity matrix showing the percent similarities among 16S rDNA sequences of *N. moscoviensis*, *N. marina*, and 13 near-complete sequences of clone inserts obtained from biomass from a full-scale BNR activated sludge plant or an NOSBR and clones for which the partial sequences had been previously reported[a]

Strain no.	Species or clone (accession no.)	% Sequence similarity with species of strain no.:														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*N. moscoviensis* (X82558)															
2	SBR1024	96.3														
3	SBR1015	96.1	**99.6**													
4	GC86 (Y14644)	96.1	**99.6**	**99.4**												
5	SBR2046	95.8	**99.3**	**99.4**	**99.2**											
6	RC25 (Y14639)	93.4	93.4	93.6	93.6	93.1										
7	RC19 (Y14638)	93.2	93.1	93.0	93.2	92.7	**98.8**									
8	SBR2016	93.0	92.7	92.8	92.6	92.4	**99.1**	**98.7**								
9	RC7 (Y14640)	92.9	93.1	93.2	92.9	92.8	**98.7**	**98.7**	**98.5**							
10	RC14 (Y14637)	92.8	93.0	93.1	93.1	92.7	**98.7**	**98.9**	**98.5**	**99.3**						
11	RC99 (Y14643)	92.7	92.9	93.0	93.0	92.6	**98.5**	**98.7**	**98.4**	**99.2**	**99.6**					
12	RC11 (Y14636)	92.6	92.8	93.0	92.9	92.5	**98.5**	**98.7**	**98.4**	**99.0**	**99.5**	**99.7**				
13	RC73 (Y14641)	92.2	92.5	92.6	92.6	92.1	**98.0**	**98.2**	**97.9**	**98.7**	**99.1**	**99.4**	**99.4**			
14	RC90 (Y14642)	92.1	92.1	92.3	92.2	91.8	**98.1**	**98.6**	**98.0**	**98.1**	**98.6**	**98.8**	**98.8**	**99.0**		
15	*N. marina* (X82559)	88.7	88.2	88.3	88.3	87.8	88.1	87.6	87.2	87.2	87.1	87.1	87.1	86.5	86.6	
16	*N. marina* (X35501)	88.0	88.0	88.2	88.1	87.7	87.9	87.5	87.2	87.2	87.1	87.1	87.1	86.5	86.6	99.9

[a] GC86 was obtained from a BNR activated sludge plant. RC clones were obtained from an NOSBR. Partial sequences for SBR clones are reported in reference 5. Values in boldface indicate the two environmental *Nitrospira* clone clades.

positives could be responsible for phosphorus removal in BNR systems because increases in this population, as determined by FISH probing, were correlated with initiation of phosphorus removal in activated sludge systems.

The RC clone library was predominantly composed of clones (89% from OTU 1 and OTU 2) with inserts originating from bacteria whose closest relatives are in the *Nitrospira* phylum and are most similar to *Nitrospira moscoviensis*. Direct pairwise sequence comparisons between sequences in the two *Nitrospira* clone clades (see Fig. 2) showed that clone clade 1 (SBR1015, SBR1024, SBR2046, and GC86) had an average 16S rDNA similarity value of 99.4% (Table 3), while for clone clade 2 (RC7, RC11, RC14, RC19, RC25, RC73, RC90, RC99, and SBR2016) this value was 98.7% (Table 3). The average sequence similarity between the two clone clades was 92.8% (Table 3), while those between *N. moscoviensis* and clone clades 1 and 2 (Fig. 2) were 96.1 and 92.8%, respectively. The highest comparative value between an RC clone sequence and *N. moscoviensis* was 93.4% for RC25 (Table 3). From the sequence data analysis, the two clone clades would likely represent two separate species. This conclusion is drawn from discussions by Stackebrandt and Goebel (22), who note that organisms with rRNA sequence similarity values of less than 97.5% most likely represent different species.

Conclusions. From the data presented in this paper, we suggest that the unknown nitrite-oxidizing bacteria in activated sludges belong in the *Nitrospira* phylum. In the meantime, both Wagner et al. (27) and Schramm et al. (20) have discovered clones originating from *Nitrospira* in industrial activated sludge and biofilm processes, respectively. Both the seed sludge from the Merrimac plant and the highly selected autotrophic nitrifying bioreactor biomass contain these organisms. Clones with inserts originating from *Nitrobacter* were not recovered in the GC library, but two RC clones (RC44 and RC57) were closely related to this bacterium and its relatives. In the meantime, we have prepared *Nitrospira*-specific primers and in preliminary studies involving a PCR test (data not shown) have positively correlated the presence of *Nitrospira* with excellent nitrification in full-scale activated sludge plants. In addition, in processes where nitrification is poor, these bacteria are absent. These PCR experiments will be complemented with FISH studies to quantify the numbers of *Nitrospira* in nitrifying systems. However, we will use our PCR test as a screening in advance of FISH probing of sludges to show the presence of nitrospiras. Data from studies on nitrification kinetics from the enhanced nitrite-oxidizing culture in the NOSBR (7) will be combined with numbers of nitrospiras from FISH probing and used in mathematical modelling.

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Waste Management in Closed Recirculating Aquaculture Systems

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Aquaculture, as with any form of animal agriculture, produces waste products. But unlike with terrestrial animals, the wastes are in a dilute liquid form. These wastes are generated in proportion to the scale of production, specifically the amount and quality of the feed provided to the fish, and to the efficiency of the methods which are used for growing the fish. The management of aquaculture wastes has become a topic of intense regulatory scrutiny, as the Environmental Protection Agency develops new waste management regulations for the entire industry. The resulting legislation will have an increasing impact on the economics of raising fish in the future.

In the United States, "traditional" forms of aquaculture have included catfish farming in ponds, trout farming in flow-through raceways, and salmon farming in cages. Most of the finfish cultured here are still produced by these methods. Each of these techniques results in a direct environmental impact, mostly in the form of the release of organic nutrients as solid wastes (fish feces), and dissolved wastes (nitrogen and phosphorus). And each uses either public or private waters to provide for the dilution and discharge of the wastes. However, increasing competition for water use, and the responsibility of government agencies to predict and regulate environmental impact, is resulting in more restrictive limits being set on water use and wastewater effluents.

All methods of aquaculture will have to adjust their management techniques to meet these regulations. The traditional aquaculture systems, due to the inherent qualifications of their design, will have significant difficulties doing so. These methods rely heavily on dilution to provide acceptable water quality for fish production. The subsequent dilution of the waste products makes its concentration and removal proportionately difficult and expensive. And while new methodologies are available which can provide for a more concentrated waste effluent, the costs of renovation and retrofitting of these aquaculture systems may be prohibitive.

Alternative methods for culturing fish have now been developed using recirculation technologies to recycle the culture water, thus reducing the volume of water required to produce a specific biomass of fish. Using these alternative methods of aquaculture have reduced the demand for the tremendous volumes of high quality water previously necessary for the production of commercial quantities of fish, and have resulted in increased options for managing and treating the levels of wastes produced.

In order to compare their water resource requirements, it is important to define the terms used in describing recirculating aquaculture systems. For instance, recirculation can refer to the reuse of water in flow-through systems, in which no water control technologies are employed except water exchange. A number designating percent recirculation in these instances denotes the amount of water that is reused after a single pass via pumping, thereby increasing the volume of water available per pass. Semi-closed systems employ various water treatment technologies, but may still require the exchange of one or more tank volumes daily. A "closed" recirculating system describes a system which exchanges less than 10% of the total system volume daily.

For the purposes of comparison of flow-through, semi-closed and closed systems, and the impact on waste management requirements, consider the following. A single trout raceway with a volume of 20,000 gallons of water and receiving 500 gallons per minute will use 720,000

gallons of water each day to maintain water quality. A semi-closed recirculating system of the same volume and capacity may be provided with appropriate tank design, biofiltration, clarification and aeration/oxygenation technologies to reduce the necessary influent volumes to a level replacing the tank water only once per day (14 gpm). A closed aquaculture system of the same volume, which recirculates 95% of its water, will use a total volume of only 1000 gallons per day, and require a influent flow rate of only 0.7 gpm.

These comparative effluent volumes are extremely important, as they relate to the waste management considerations. For the flow-through system, all of the wastes generated by the fish are eliminated from the culture tank in a very dilute form. Since these suspended solids must be concentrated from the total effluent volume of water, the methods used will require significant engineering adaptation to capture the waste solids, and will therefore be extremely expensive.

The recirculating technologies used by the semi-closed and closed systems result in the accumulation of the solid wastes in a concentrated form. The techniques which are then available to remove these solids are simplified and much less expansive. However, for dissolved wastes the treatment methods available to semi-closed and closed aquaculture systems are less comparable, considering the volumes of dilute effluent discharged. Closed systems produce a concentrated solid waste as well as a concentrated dissolved waste, both of which can be successfully and economically treated. Semi-closed systems produce a significant volume of dilute effluents that may be more difficult to treat.

The recirculating technologies, which are used in closed systems, when coupled with good management practices, also reduce the actual levels of waste generated. As with all aquaculture systems, it is essential to use good quality feeds, eliminate excessive fines in the feed, and optimize the nutritional aspects of the feed for each species cultured. Beyond this, the use of controlled-environment systems allow for more effective feed management strategies which control the number of feedings per day, eliminate uneaten feed and under-feeding, and maintain optimal water quality and feed conversion, which will result in more efficient growth, and a reduction in wastes.

However, the concentrated wastes in the effluents from closed systems represent as much danger to the environment as the dilute wastes from flow-though systems, if released. It is the concentration of these wastes through the operation of the internal water treatment systems of closed systems that provide for a number of alternative waste management strategies.

The reduction of the volume of water replaced each day from a system, and the subsequent concentration of the solids within the effluents, defines the potential for waste management in aquaculture systems. The more "closed" a system is, the easier will be the management of the wastes. However, there is a significant difference in the sophistication, and the level and cost of technologies required to maintain water quality in recirculating systems using more or less water. The dilution required by semi-closed systems significantly reduces the water quality control requirements of the recirculation technologies. Even within closed systems, the design and management requirements of a 90% recirculating system are greatly reduced from those of a 95-100% closed design. The increased costs of a recirculating system design which recycles a higher percentage of its water daily, must be balanced against the advantage of reduces water use, increased energy conservation, and the ability to deliver a more concentrated effluent stream.

Discharge to Municipal Sewer Systems

Closed recirculating systems, which are constructed in urban or suburban locations, should be eligible to release their wastes directly to the municipal sewer. The level and type of waste produced is seldom a restrictive consideration, as long as the municipality has the capacity to receive the volumes produced. The costs involved with this method of waste management must be considered, and will vary significantly from place to place.

Larger scale systems may consider the construction of their own self-contained waste treatment facility. The use of an on-site Sequencing Batch Reactor (SBR) can provide economical treatment of aquaculture wastes through both aerobic and anaerobic processes. This results in the control of the solid wastes (BOD and COD) and dissolved wastes (nitrates and phosphates) required for the direct elimination of the entire water volume into adjacent waterways.

Land Application

Rural aquaculture systems can discharge their wastes onto agricultural lands at an irrigation rate specified by the USDA, EPA and/or State Department of Natural Resources. With irrigation capabilities, systems adjacent to agricultural sites should have no problem discharging their wastes during temperate months. However, this is a seasonal approach, since the waste cannot be applied during winter months. It may be necessary to provide for the accumulation of the effluents during the off-season, for application to farmlands in the spring. Again the concentration of the waste will greatly affect the volume of the holding facilities involved.

In some cases, it may be possible to continually apply aquaculture wastes to such ground cover as is available on sod or tree farms. It will be necessary to work with the local agriculture extension agency to determine the potential for land application of fish culture wastes.

Discharge to Waterways

There are a number of options available to prepare the aquaculture effluents to be released to ponds, streams, lakes, rivers or other bodies of water. Release of effluents to surface waters requires an NPDES (National Pollution Discharge Elimination System) permit. This permit will require the reduction of the BOD, nitrogen and phosphorus within the effluents to specified levels. A combination of techniques including further solids concentration (dewatering), aerobic stabilization, hydroponics, and the use of submerged or constructed wetlands may be necessary to produce effluents of sufficient quality that they can be discharged to the environment.

The following example demonstrates the preparation of the wastewater effluents from a closed, recirculating aquaculture system for discharge.

Solids Concentration

A closed system employs various techniques within its water treatment design to concentrate the solid wastes of the fish. A self-cleaning tank employing the proper flow design must deliver the entire tank volume for clarification at a rate of more than once every hour. The clarifier must remove the solids from the water flow continuously, and then eliminate this collected waste from the system in a concentrated stream. For example, a microscreen clarifier with a 60 micron sieve will deliver an effluent stream with 100% of the waste suspended solids produced by the fish daily, within 5-10% of the system water volume.

The concentration of the effluent stream can be further increased using various methods, including inclined plate separators, swirl separators or settling basins. These methods are more or less effective depending on the effectiveness of the system in concentrating the wastes in a minimum effluent volume.

Once the solids are separated from the effluent stream, the supernatant must also be considered for treatment. The levels of nitrate and phosphate will determine whether this portion of the waste stream must be further treated before release. Treatment options available include anaerobic digestion and hydroponic removal.

Aerobic Stabilization

For most waste treatment applications, after concentration of the waste solids, the resultant slurry of concentrated wastes must be further treated. To prepare the wastes for land application, if significant odors are a concern, the slurry must be vigorously aerated as it is accumulated. This "stabilizes" the waste and results in the reduction of any odors during surface applications. This process is also necessary when considering application to a constructed wetland or reed bed treatment system.

The concentrated wastewater from the aquaculture systems can be accumulated within large reservoirs, and aerated via compressed air pumps. One reservoir is filled, then continually aerated for 30-40 days, while a second reservoir begins the accumulation process. Once fully stabilized the first reservoir can be applied to the reed bed, and is available again to accumulate the continually developing aquaculture wastes.

Reed Beds

Perhaps the most promising alternative method of aquaculture waste management is the use of constructed wetlands. The "reed bed" has been used as an economical approach to the handling, dewatering and disposal of municipal wastewaters for many years, and seems ideally suited to the management of aquaculture wastes. The reed bed uses conventional sludge drying beds planted with a common reed. Liquid sludge is applied periodically for up to 10 years, with the end product, dried solids finally disposed of as agricultural fertilizer or landfill.

The reed system is composed of common reeds (*Phragmites comminus*) planted in conventional sludge drying beds. The root system grows through the dried and stored sludge, and through an upper sand layer. The plants supply oxygen to the root systems, which harbor a rich bacterial environment, which feeds on the organic matter and promotes vigorous plant growth. The root system keeps channels open to the sand and lower gravel layers of the reed bed allowing gravity drainage of the beds.

The sludge is further dewatered through evaporation and the transpiration of water through the reed leaf systems. The waste solids layer becomes completely dry between applications, resulting in a crusty surface devoid of odors, and allowing for years of accumulation within the concrete confines of the reed bed.

The concentrated and aerobically stabilized wastes are applied about every 30 days. This "dosing" of the beds provides the necessary time for the drying of the sludge between applications. There are no odors involved because the sludge has been thoroughly stabilized through the aeration process during accumulation.

Most of these processes can be mechanized, and reed beds can significantly reduce the man-hours and costs required to dewater and treat sludge. Actual operation includes application of the liquid sludge and harvesting of the dormant plant material after the growing season. The plants are cut off approximately 20 cm above the sludge surface, gathered, and then removed. This plant material is removed so that new shoots can emerge from the root system in the next season. The beds will continue to operate throughout the winter, with oxygen supplied to the lower layers of the bed through the cut stems of the reeds.

Sludge can be accumulated in the beds until it reaches a depth of approximately 1 m. This may take 8 to 10 years. The sludge can then be removed using mechanical means such as a backhoe, and applied as landfill, or as fertilizer.

Hydroponics

Besides resulting in the accumulation of concentrated solid wastes, aquaculture in closed systems also produces concentrated dissolved wastes, specifically nitrates from the biofiltration process. Phosphates are also accumulated in sufficient levels to support the production of vegetation. The hydroponic recovery of these nutrients derived from aquaculture processes, through the integrated culture of aquatic or terrestrial vegetables, is called "aquaponics".

The use of aquaponics as a waste management alternative involves the removal of the dissolved nutrients from the aquaculture effluents, following the removal of the solids. Some closed systems exclusively use hydroponic techniques as the biofiltration component of the design, with the plant roots directly removing ammonia from the culture water. However, the balance of fish culture to hydroponic vegetable production in this case is strongly skewed towards the production of vegetables. A very limited level of fish production results in sufficient dissolved nutrients for acres of aquaponic vegetable production.

There are much more appropriate plants for the treatment of aquaculture wastes than those terrestrial vegetables grown hydroponically as secondary crops, such as lettuce, tomatoes or cucumbers. Wastewater treatment using aquatic plants such as water hyacinth and watercress demonstrates significant potential towards polishing water for subsequent reuse or discharge. Water hyacinth especially is extremely efficient at absorbing dissolved nutrients and transforming it into plant biomass. Solids must first be efficiently removed from the wastewater, or the roots of the aquatic plants will be quickly fouled. But after sufficient contact with the roots of the plants, the wastewater stream can be transformed into an extremely clear effluent, sufficiently treated for discharge.

Of course, plants like water hyacinth simply transform the wastes from a dissolved form into plant biomass. The resultant quantities of plant material must be continually harvested and disposed of, usually as compost, and subsequently land applied.

It is tempting to further reuse the effluents flows derived after hydroponic treatment within the aquaculture systems. The water appears pristine and free of any pollutants (assuming organic hydroponic methods were employed). However, if the reuse of hydroponically treated effluents precludes the addition of adequate makeup water to the aquaculture systems, there may be deficiencies in various micronutrients necessary both to the fish, and to the bacteria within the biofilters. Under extremely high levels of recirculation, approaching 100%, many species of fish will demonstrate various problems, presumably due to the presence of a concentration of dissolved metabolites, or to the lack of some trace elements. In closed aquaculture systems, the

addition of a relatively small percentage of make-up water may be the least expensive treatment process available.

SUMMARY

Recirculating aquaculture systems are often considered too expensive or too impractical to serve the aquaculture industry at an influential level. Despite the many advantages afforded them, including water conservation, complete environmental control, the production of a quality-controlled product, with increased marketing advantages of the product, the increased cost and complexity of such systems have delayed their incorporation into the aquacultural mainstream.

However, as commercial aquaculture facilities are required to more effectively treat their effluents before discharge, the advantages afforded to closed, recirculating aquaculture systems for effective waste management alternatives, are becoming increasingly apparent and important. Before long it may become obligatory to apply recirculating technologies to all land-based methods of fish farming. And as the true cost of raising fish using traditional methods increases accordingly, the cost of using recirculating technologies will be considered much more reasonable. This process will transform what has often been considered an impractical method of aquaculture, into the most environmentally, and even economically, acceptable culture method available.

COMPOSTING FUNDAMENTALS

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INTRODUCTION

Composting of organic waste has been used for centuries. Its use in biosolid stabilization increased in the 1970's and 80's as alternatives to land fill, ocean dumping and incineration. As municipalities face disposal problems for their organic materials, so do food processors and farmers. In addition to stabilizing organic materials, compost has some additional benefits. Reynk, et al., 1992 suggest some potential benefits are as follows:

- Enhanced soil fertility and tilth
- Destruction of undesirable microorganisms
- Reduce or eliminate unpleasant odors
- Environmental risk reduction

This presentation will be limited to discussing the fundamentals of composting.

WHY COMPOST WORKS

Composting is a biological process of decomposing organic materials into a humus like product. The process will occur naturally, but can be "speeded up" and controlled if proper ingredients are blended together. The controlled composting process is usually considered an aerobic process, which requires oxygen. The decomposition process is a "slow cooking" process and not a rotting process. Microorganisms are considered the "work horses" of the composting process.

HOW COMPOST WORKS

In order to generate a healthy compost process, some key inputs elements are as follows: a proper nutrient mix; moisture; oxygen; temperature; pH control; particle size; porosity; and time.

A proper nutrient mix is often referred to as the recipe; this is a blending of carbonaceous and nitrogenous materials together to form a desired carbon:nitrogen ratio (C:N). The ratio may vary from 20 to 35:1. Lower C:N ratios will produce rapid activity at the beginning, however, more odors will be given off in the process. A C:N ratio of 20:1 should be considered the minimum in formulating compost mix recipes. To assist in "recipe making" Brodie, 1994, at the University of Maryland, developed a computer spreadsheet. The spreadsheet allows the user to select the organic material(s) to be composted, then the program will indicate least cost recipe(s) based on the carbon sources available. Many scenarios can be evaluated, and a compost mix selection made in a very short period of time. In addition to the C:N ratio, Rubin and Sheldon (1993) suggest a C:P ratio of 100:1 to 150:1. A proper recipe is very important to successful composting. The end product is no better than the feedstocks used to make the recipe.

Moisture in the range of 40-60 percent is acceptable for composting. There are times when the moisture will be at the extremes of the range. In research at Maryland, 50 percent moisture has worked well in our composting efforts. Without a scale and convective or microwave oven, how can the moisture be estimated in a compost mix? One field method is the hand squeeze test. In the hand squeeze test, a hand full of the compost mix is obtained and squeezed into a ball by forming a fist. One or two drops of water may be squeezed from the ball. As the fist is released, the ball should expand but remain intact. The hand will be moist by not too wet. The squeeze test, as described, will approximate 50 percent moisture in the compost mix. Moisture levels greater than 60 percent may also cause a supernate (liquid) to leach from the compost mix and cause anaerobic (odor-causing) and other undesirable situations. Moisture is a key ingredient. If the moisture is too low or too high, the composting process will not function properly.

Oxygen is required to maintain the composting process in an aerobic state. It is desirable to maintain aerobic conditions for odor control and multiplication of thermophilic bacteria associated with this process. As the oxygen is depleted, one of the indicators may be the lowering of temperature in the compost mix. However, measuring the oxygen content of the compost mix is a more reliable way to determine oxygen depletion. In compost mixes having very high BOD_5 loads, oxygen requirements will be great. It may not be possible to supply the oxygen requirements by just turning. To overcome this situation some systems may be aerated with a fan and piping system or a combination of mechanical and aerated systems.

Temperature is generated in a compost mix by the metabolism of the microorganisms (bacteria, fungi and actinomycete). If the recipe, including proper moisture and oxygen, has been blended together correctly, the microbes will begin the metabolism process. The bacteria associated with process are mesophilic (moderate heat loving) and thermophilic (high heat loving) species. Mesophilic bacteria operate at temperatures less than 110° F.; thermophilic bacteria operate at temperatures ranging from 110 to 150° F. Good composting temperatures range from 135°-140° F.

Composting temperatures of 150° F. for organics from animal, poultry or seafood origin are desirable to assure the destruction of pathogenic bacterial and viral organisms.

PH is another item that may be critical at times, particular if it exceeds 8. If a compost mix has a pH of 8 or greater, ammonia (NH_3) volatilization may become a problem as it will cause odors. The desirable pH range is between 5.5 and 7.5. In some processes, depending upon the material, the pH will decrease over time to approximately 7; in others the pH will increase. You have to be on the guard for shifts in pH. If the pH is out of the desirable range, appropriate chemical action to alter the pH may be desirable. If the pH is too high, blending ferrous sulfate into the compost mix has been found to be an effective pH control agent (Carr and Brodie, 1992).

Particle size for the carbonaceous materials should range in size from 1/4 to 1 inch (Rubin and Shelton, 1993). For good bio-oxidation, the smaller particles are desirable because the surface-to-volume ratio is greater than in larger particles. Bio-oxidation occurs on the particle surface thus better decomposition. Sawdust is an excellent material to use in a recipe.

Porosity as defined by Rynk, et al. 1992 is as follows: "a measure of the pore space of a material or pile of materials. Porosity is equal to the volume of the pores divided by the total volume. In composting, the term porosity is sometimes used loosely, referring to the volume of the pores occupied by air only (without including the pore space occupied by water)." Particles greater than 1-inch in size used as bulking agents can assist in creating pore space and are not usually part of the C:N ratio determination.

Time for compost to mature is dependent on the mix recipe, moisture, feedstocks used, particle size, turning frequency, temperature and end use of the product. A compost requiring only pathogen reduction and can be utilized as a green compost will require much less time to process than a mature compost. Check with your state regulators concerning their requirements you have to follow as a compost site operator.

COMPOSTING TECHNIQUES

Four composting techniques will be discussed. These techniques will "speed up" the composting process over natural composting. The techniques are: static pile; aerated static pile; windrow and in-channel.

Static pile is where the compost mix is piled and not disturbed for a long period of time. It may be turned, but not frequently. To assist in natural aeration, the initial compost mix should have a porosity of approximately 30 percent or use a bulk density of approximately 900 lb./yd^3.

Aerated static piles can be active or passive in mode of operation. The active piles normally draw air through the compost mix by using pipes or plenums placed in the

compost mix and fans attached to the duct system. Air discharge from the fan system can be filtered through a biofilter for odor control. Another aerated pile system is passive in operation. The passive system uses a series of perforated 4 or 5 inch plastic pipes underneath the compost pile. The pipe ends are left open and a natural convective process provides oxygen to the compost mix. A porosity of approximately 30 percent or use a bulk density of approximately 900 lb./yd^3 is also desirable for the aerated pile system.

Windrow composting can be accomplished outside or in a large, covered structure. Windrows are normally turned with some type of turning equipment. The equipment can be as simple as a front-end loader or self-propelled equipment that straddles the windrow and turns it in one pass or equipment that requires 2 passes. However, good mixing may not be as effective with the front-end loader as the turning device. A porosity of approximately 30 percent is desirable or use a bulk density of approximately 900 lb./yd^3.

In-channel techniques primarily use a turning device that runs down a rail of some type. It is possible to have parallel bays with common walls so the turning device can be moved from bay to bay. This type system is expensive, but may be a better system for long term composting. The in-channel system may also be used in conjunction with an aerated system. Fans and air ducts are placed through out the system and will speed up the composting process by continuously providing oxygen in the compost mix. This may be of great benefit if the compost mix is highly volatile. Air from the fans can be discharged into a biofilter for odor control. A 30 percent porosity or use a bulk density of approximately 900 lb./yd^3 will also assist in this process.

QUALITY CONTROL

Thought must be given to the compost product use before developing the initial compost mix. The end product will be no better than the feed stock used to make the initial mix. Therefore, it is very important to have a reasonably current nutrient analysis of each feedstock used in "recipe making". Tables 1 and 2 illustrate nutrient parameters associated with crab compost ingredients and the final compost, respectively. (Brodie, et al., 1994).

Table 1. Analysis of Compost Ingredients

Product Parameter	Crab Waste	Pine Sawdust	5 wk-Old Compost
% Carbon	29.8	46.6	47
% Nitrogen	5.7	<0.06	0.6

C:N	5.2	>750	71
Bulk density lb/yd^3 (wb)	540	675	610

Table 2. Crab Scrap-Sawdust Compost Nutrient Analysis

Chemical Parameter		Compost - 1 [a]	Compost - 2 [b]
N	%	0.59	0.72
NH_4 - N		0.02	0.03
$P_2 O_4$	%	0.99	1.5
$K_2 O$	%	0.20	0.25
Ca	%	5.7	6.1
Mg	%	0.28	0.51
S	%	0.12	0.14
MN	ppm	86.2	104.1
Zn	ppm	49.7	189.4
Cu	ppm	13.7	19.5

[a] Compost-1: 35 days old; sawdust mix with crab once at a total volume ratio of 1 part crab to 1 part sawdust.

[b] Compost -2: 60 days old; compost-1 mixed with crab two times at a mix volume ratio of 2 parts crab to 5 parts compost-1 resulting in a total volume ratio of 1.8 parts crab to 1 part sawdust.

A decision has to be made concerning end use and compost quality. If the compost is going to be used as a field fertility source, the refinement or quality of feedstocks does not have to be as great as that used in home landscaping.

To assist in determining if compost is cured, respiration rates of the compost can be determined by laboratory procedures. A field determination can be made by collecting a compost sample, saturating it with water (but not soaking, dripping wet), place in a sealed plastic bag and store in a warm place (70 - 85 F) for one week. After one week open the bag, if there are no bad odors, the compost has stabilized.

Quality compost will have a C:N ratio of about 15:1. The time to achieve quality compost will depend on the technique used to compost. It may take one year or more to achieve a quality compost using static piles, whereas, a quality compost may be achieved in 2-3 months using mechanical systems.

SUMMARY

A brief overview of composting fundamentals has been presented in this paper. The final compost will be no better than the initial mix of feedstocks and the practices utilized during the process. Current nutrient analyses of the feedstocks are necessary in formulating the initial mix recipe. Refinement of feedstock quality of a compost mixture will be determined by its end use.

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A COMMERCIAL COMPOSTING FACILITY SERVING THE FARM AND FOOD PROCESSING INDUSTRIES OF THE DELMARVA PENINSULA

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The purpose of this paper is to describe the operation and evolution of a rural compost facility by New Earth Services, Inc.. A 30 acre site is operated in rural Dorchester County Maryland near Hurlock. The facility is located adjacent to the county landfill; county officials hoped to divert organics from the landfill. The primary feedstock which caused the creation of the site was the waste shell generated by the county's crab meat packing industry. Dorchester County picks and packs one million pounds of crabmeat every year; this results in several million pounds of "chum" needing disposal. Throughout the years various solutions were tried, but to no avail. Returning waste shell to the Bay created a loading problem at the point of discharge, creating a green "paint" along the bottom. Landfilling was awkward and created odors difficult to control. In the summer of 1992, New Earth began composting the tons of chum, turning it into an odor free humus, a composted garden product, "Chesapeake Blue" now bagged and sold in six states.

With the success of composting as a solution to the crab chum problem, New Earth turned its attention to other food processing by-products. Traditional disposal methods for these by-products had been land application. But with increasing demands on the land mass and the development of farmland it has become increasingly difficult to access this solution except for the most benign waste (i.e. vegetable cuttings). New Earth's next feedstock was clam processing waste. This is the solid portion of the processing water that is belt pressed into cake. The clam residue has a high pH due to addition of lime as a floc. The clam residue was another difficult to handle waste due to its clay like consistency, high pH, and ready to rot clam protein. The task was made easier when our next feedstock was introduced. All of the Grey Poupon mustard in the country is made in Cambridge, Maryland. The waste from this product amounts to several tons a day, just about the same as the clam. The mustard bran however has entirely different physical characteristics. The bran has a low pH, is very viscous, and has a rather pleasant odor, reminiscent of a day at the ballpark. This then became a

perfect match to blend with the clams, helping us overcome all of the problems associated with them. Ordinarily, we compost feedstocks separately, unless there is good reason to mix.

In addition to these two seafood based by-products the facility handles some of the waste from the poultry processing industry. Waste solids from poultry processing water is known as DAF, derived from the practice of floating the solids from the waste water. This material is sludge like and may be thick or thin, it makes an excellent source of moisture into the compost recipe. We also receive feathers, which the industry normally processes into chicken feed, but due to the high volume and occasional breakdown of rendering equipment, the feathers sometimes find their way to our site in sporadic bursts. They are a good source of nitrogen into the recipe.

Some years ago, New Earth began working with poultry litter. This was well before pfiesteria precipitated a hard look at manure disposal practices. Although initial work that included time and motion studies showed that it cost almost $30 /ton to compost litter, New Earth continued improving that equation until composting litter became economically viable.

Although some of our feedstocks present unique handling challenges, the economics of a composting facility is probably the most difficult aspect of a successful operation. All organic material can be composted, but at what cost? There are a lot of good sources of ingredients that will make good composted products, but the collection of a tipping fee is necessary. The compost process takes time, up to a year in a lot of instances; equipment and labor are expensive; we often need to pay for components of the recipe that may be in short supply (in our case woodchips); and the process results in a loss of approximately half the mass. The last example is illustrated by the realization that you can only sell half of what you take in, although your expenses are based on the latter. This means front end dollars are twice as important as back end.

Our facility regularly turns away good compostables from food processors that have good alternatives. A vegetable processor has an easier time sending material to a hog farm or to land application than does a clam shucker or crab packer. We believe that more difficult access to land application is a trend that will continue and works in favor of the compost alternative.

This is not to diminish the importance of receiving good value for the finished product. There are many obstacles to this however. The main problem is consumer education; an uneducated consumer is less likely to buy your product at a price that you may ask. Other obstacles include competition from municipally, or otherwise, subsidized compost operations. This can include a county grass and leaf compost facility that practices recycling of these materials via composting, but often lacks the appreciation of value in the finished product. The product is often given away or sold at a price unrelated to the cost of production. Similarly, biosolids compost facilities can command a tipping fee beyond that of a food residue facility. These products too find their way into the market at lower prices.

It is probable that a small facility must always be able to compete on quality. Our operation starts with good science. Analyses are taken on all potential incoming materials. A recipe is then formed that will indicate to us what our expenses will be, this then determines the fee. The beginning is also a good place to stop problems before they occur. This is the time to have an understanding with the generator that the material needs to come in fresh and devoid of contaminants. Pieces of glass and/or plastic can drastically diminish the value of your compost.

After the recipe is determined, the physical handling routine is established. Will the material go directly to mixing or will it be drained onto a bed of woodchips for incorporation later? Typically we spend a couple of months saturating woodchips with our food processing liquids; we then mix wet woodchips with drier nitrogenous materials. This prevents leachate that might occur were you to saturate the poultry litter or crab chum. Once the mix is right the compost process begins. We take care to monitor its process, primarily by way of temperature recordings. Temperatures are a guide to when to turn the material and are an indicator of when the process is nearing completion. It is required by the state that we document a pathogen kill that entails reaching certain temperatures over a certain time with a number of turnings. This ensures a safe product in the marketplace.

When the compost is finished, we usually prefer to give it time to mature; this is as simple as letting it sit for another couple of months. There are some advantages to that, although some applications do not require it. We seek to establish our composts as superior to others by quality control, consistency, and creating brand names. Chesapeake Blue was our first compost and was named for the Chesapeake Blue Crab. Chesapeake Green later was able to capitalized on that and was named because it turns grass green. Other names such as Chesapeake Clam Bake and Chesapeake Chicken are used informally to test market reaction.

Most of our markets do require it and most are higher end. These include the bagged goods and bulk homeowner market, landscapers, horticultural growers, golf courses, and state DOTs. In addition to the type of compost produced, we have the ability to blend finished products when the market calls for it. As an example, the state of Maryland composted biosolids that over time developed quite a loyalty as a turf dressing. The product was high in iron (for green) and high in lime (providing the pH preferred by turf). When the state closed the facility, New Earth was able to blend composts that resulted in this analysis, thus capturing market share. Another instance is to blend composts as a horticultural media. University tests reveal performance of blended composts as superior to any one compost. As a final example of blending products to achieve performance, the company is currently blending a compost based organic fertilizer to be crop and soil specific.