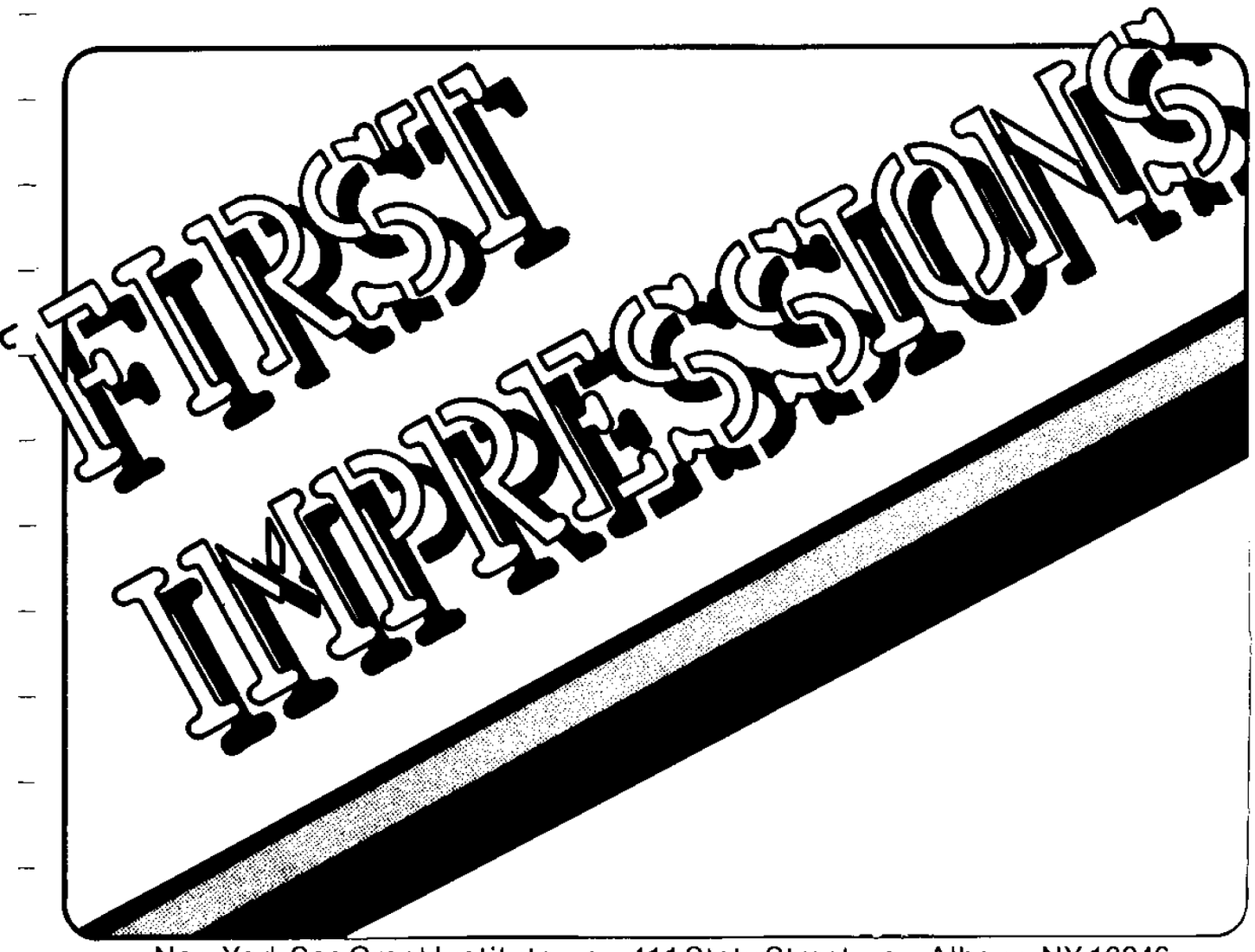


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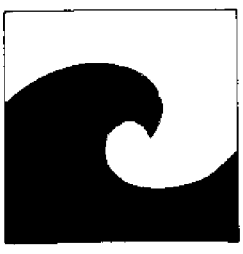

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Animal Health Management
for Aquaculture:
Shellfish Series

by Ralph Elston

# Animal Health Management for Aquaculture

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## Bacteriologic Sampling in Bivalve Mollusc Hatcheries

Algal food stocks, water source(s) and the brood stock, spawn or purchased seed are the three primary sources of unwanted bacterial contamination.

Locations to take bacteriologic samples:

- Sea water source
- Any intermediate seawater holding tanks
- Processed seawater as it is utilized
- Algal stocks
- Expanded algal cultures
- Larval and spat tanks

Sample water column and surfaces

## Collection and Plating of Bacteriologic Samples

Aquatic culture systems can be easily sampled for bacteria both quantitatively and qualitatively.

Materials

- loop, flame, glass spreader
- tubes containing 5 ml sterile saline
- sterile swabs
- Marine agar and TCBS agar plates
- breed loop for quantitative samples (as described below)
- standard loop for qualitative samples when desired.

Container surface samples: Collect sample from a defined square area (1 to 2 cm) with a cotton swab. This must be an area which has been removed from underwater but not dried. Either drain tanks down slightly or use standard sheets of fiberglass, glass or similar material which can be easily removed from tanks for surface sampling. Rinse swab thoroughly in 5.0 ml sterile seawater or 2.0% saline. (When sampling areas with very dense bacterial populations - a greater initial dilution or successive dilutions will be necessary). Using a breed loop (0.01 ml) plate the sample on marine agar and TCBS agar plates. Spread the bacteria with a glass rod. Incubate the plates at room temperature and examine in 24 to 72 hours.

Water samples: Sample container water with a breed loop and plate as above. Make a 1 to 500 dilution in sterile saline and plate as above. Again, further dilutions may be required, depending on your conditions.

With careful technique and proper dilutions you should obtain clearly separated, countable colonies. Many bacteria which spread or swarm (and are uncountable) on typical agar media (such as Difco Marine Agar) will form discrete, countable colonies on TCBS.

Note: Dispose of used bacteriologic plates carefully (by sterilization if this is practicle) to avoid contamination of your system.

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## Materials and Preparation of Media for Routine Bacteriologic Sampling in the Shellfish Hatchery

Workplace: clean dry area, without excessive drafts

Equipment:
- refrigerator
- autoclave (sterilizer) or pressure cooker
- gas burner or range top
- flame - such as 'BernzOmatic' propane torch kit
- 'clorox' diluted 1 to 20 with tap water
- standard bacteriologic loop
- bacteriologic breed loop
- plastic disposable petri dishes (100 x 15 mm)
- 'Marine agar' bacteriologic medium
- 'TCBS' bacteriologic medium
- 25 ml pipettes
- scale or weighing device (capable of weighing 1 gram units)
- pyrex glass tubes with screw caps (at least 10 ml capacity)
- sterile cotton swabs

The bacteriologic media can be obtained from a variety of scientific supply distributors or directly from the manufacturer:

DIFCO Laboratories
P.O. Box 1058A
Detroit, Michigan 48232

Two other scientific supply companies which can provide the other materials needed are:

Fisher Scientific Company
15 Jet View Drive
P.O. Box 8740
Rochester, New York 14624
(716) 464-8900
(also other regional offices)

Scientific Products
2 Townline Circle
Rochester, New York 14623
(716) 475-1470
(also other regional offices)

Many of these materials may also be available from local hospital supply companies.

Procedures for preparing media:

For Marine Agar follow label directions except that tap water can be substituted for distilled water to rehydrate the powdered medium. If the tap water is chlorinated, the chlorine should be neutralized or the water allowed to sit at room temperature for 24 hours to allow the chlorine to escape into the atmosphere. Instead of using an autoclave (sterilizing machine) the medium can be boiled in a pressure cooker at 15 lbs for 10 minutes. As an alternative, if a pressure cooker or autoclave are not available, carefully boil the medium for 10 minutes on a range top. This will usually accomplish sterilization. Media containing agar boils over easily so once the boiling point is attained the material must be watched carefully. After sterilization is complete transfer 15 to 20 ml of medium to each 100 x 15 mm petri dish using 'sterile technique.' Allow the medium to remain at room temperature for up to 12 hours to remove excessive moisture Package in sealed plastic bags and refrigerate until use.

Use similar technique for TCBS agar, except noting label directions for boiling procedure. Also, seawater diluted to about 10 parts per thousand should be used instead of distilled water. Sea water can be diluted with tap water.

Wipe down work surface areas with the 1/20 'clorox' solution.

See other leaflets for sampling procedures.

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## Presumptive Identification of Pathogenic Bacteria and Efficacy of Antibiotics for their Control

*Vibrio* spp. are the bacteria most commonly associated with diseases of shellfish. Members of other genera, such as *Pseudomonas* may be potential pathogens. It should also be noted that these and similar rapidly growing bacteria can be very abundant as part of the normal marine bacterial fauna. The way in which they are managed in mariculture systems will determine whether or not they interfere with the production of animals in an intensive culture system. *Vibrios* are characterized by being gram negative usually motile rods with polar flagella, oxidase positive, non-aerogenic, and facultatively fermentive. They usually grow on TCBS agar, are sensitive to the pteridine compound O/129 and the antibiotic novabiocin and are insensitive to penicillin. A very tentative identification for practical purposes in aquaculture systems can be made by growing the suspect bacterium on TCBS agar and observing its sensitivity to O/129, novabiocin and penicillin. The usefulness of such a preliminary observation will depend partly on the prior experience of the particular facility and the numbers and location of the isolated bacteria. However, even routine presumptive identification requires further tests, which are included below. Antibiotic sensitivity can easily be tested with the use of sensitivity discs. Although antibiotics are not usually recommended for long-term or prophylactic use they can be helpful in some aquaculture systems.

### General Materials

Loop and flame
24 hour cultures of suspect bacterium for specific biochemical tests or culture samples for preliminary plating.

### Plating on Culture Plates

#### Materials

Marine agar, TCBS agar or other suitable medium in petri dishes.

#### Procedure

Using standard aseptic technique streak the suspect bacterium or sample from culture system.

#### Reactions

Observe the growth of the bacterium (which may become visible from 12 to 72 hours). Note whether colonies are uniform or there is a mixture. Note the shape, texture mode of growth, size and color. Note also effects on the medium employed. All of these characteristics will aid in future identification of the bacteria.

Proceed with the following tests with suspect bacteria.

## Gram Stain

### Materials

1. Crystal Violet Solution (Stain)
   Crystal Violet 1 gm
   Distilled Water 100 mls.

2. Grams Iodine Solution (Mordant)
   Iodine 2 gm
   IN NaOH 10 mls.
   Distilled Water 90 mls.

3. Decolorizing Solution
   Acetone 30 mls.
   95% Ethylalcohol 70 mls.

4. Safranin (Counterstain)
   Safranin O 2 gm
   Distilled Water 100 mls
   Glass slides, distilled $H_2O$

### Procedure

1. Clean a glass slide with 70% alcohol, wipe dry and clean. Mark a circle, with a wax pencil, in the center of the slide, and place a drop of sterile distilled water in the center of the circle.

2. With a sterile bacteriologic loop, employing sterile techniques, scrape the surface of a 24 hour agar slant culture and transfer the bacterial contents of the loop into the sterile drop on the slide and mix.

3. Fix the bacteria to the slide by quickly passing the slide through the flame of the Bunsen burner several times.

4. When the bacterial slide is dry and cool, flood the surface of circled area of the slide with crystal violet solution for 1 min.

5. Rinse the stained areas of the slide with tap water to remove excess dye.

6. Flood the circled area and smear with the Iodine solution and leave on for 1 minute.

7. Rinse slides with tap water.

8. Flood slides with decolorizer until decolorizer flows colorless from the slide. Do not apply excessive decolorizer to the preparation.

9. Rinse with tap water.

10. Counterstain with safranin for 10 sec.

11. Rinse with tap water and allow to dry.

Reactions

Gram positive: blue organisms

Gram negative: red organisms

Microscopic Examination of Bacteria - Hanging Drop method to determine motility.

Materials
24 hour broth culture of suspect bacterium
Depression slide
cover slip
petrolatum

Procedure
Ring a cover slip with petrolatum. Place a drop of the broth culture on the center of the cover slip. Lower the depression slide onto the cover slip and invert the entire unit.

Reactions
Observe under the microscope for motility.

Oxidase Test

Materials
Oxidase differentiation discs, or fluid oxidase reagent, distilled $H_2O$.

Procedure
Wet an oxidase disc with d $H_2O$. Rub the bacteriologic loop on the disc to determine that the loop does not cause a positive reaction. From an agar culture transfer a loop-full of bacteria to the disc. Alternatively, transfer a loop-full of bacteria to a piece of filter paper wet with oxidase reagent.

Reactions
Observe for color change on disc:
Positive reaction: purple color around culture
Negative reaction: no color change

Gas Production

Materials
Purple base broth culture tubes reconstituted with 1.5% NaCl and 1% glucose. Culture tubes contain gas tubes.

Procedure
Inoculate broth.

Reactions
Observe for accumulation of gas within the gas tube. A color change to yellow indicates the production of acid from glucose.

O/F Test

Materials
Two tubes of OF media- (reconstituted with 1.5% NaCl and 1% carbohydrate - usually glucose) or MOF media (with 1% carbohydrate).
Sterile mineral oil.

Procedure
Inoculate both tubes. Overlay one with the mineral oil.

Reactions
Observe at 24 hours: Both tubes yellow indicates fermentive metabolism. Open tube yellow, closed tube with no change or alkaline indicates oxidative metabolism only.

Antibiotic discs (including O/129)

Materials
Antibiotic discs.
O/129 discs - "home made"
(0.5% solution of O/129 phosphate[1] in d $H_2O$ - filter sterilized solution pipetted onto filter paper discs, about 0.02 ml/disc)

Procedure
Make a uniformly streaked plate of test bacterium using sterile technique place test discs at least 1.5 cm apart on the agar plate.

Reactions
Observe plates at 24 hours for circular zones of inhibition around discs. Record radius of zone from edge of disc to edge of bacterial growth. An approximate dosage level can be estimated for therapeutic use of antibiotics by taking into account the concentration of antibiotic in the disc and the area of the zone of inhibition. Several doses based on this rough calculation can then be tried experimentally to determine an effective dose.

[1]Available from Calbiochem, San Diego, CA 92112.
See other leaflets for suppliers of general materials for microbiology.

# Animal Health Management for Aquaculture

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## Water Treatment and Maintenance of Small Hatchery Systems

### Water Treatment:

Sea water quality and the degree of water treatment required are dependent on local conditions such as: use of open seawater or estuarine water, presence of commercial and residential effluents and time of year. The further the seawater source is removed from the ocean (for example, in long shallow bags) the more it tends to be influenced by seasonal fluctuations and man's activities.

The first objective of water treatment is to remove marine organisms and organic debris which contribute to excessive organic loading in the system. A second objective is to reduce the microbiological components in seawater. In many cases, the use of untreated seawater or estuarine water is satisfactory for hatchery operation. In other cases, water may only have to be treated in the warmest months of the year when organic loading is the heaviest. The removal of large particles and organic debris will significantly reduce bacteria which enter the system. Bacteria which commonly interfere with intensive culture are those which are attached to particulate material in the water column and which subsequently become attached to culture system surfaces.

Some water treatment systems available are:

Sandfilters. For removing larger particles, usually 50 um or larger. Contact swimming pool supply distributors for information on these filters or a primary distributor such as: Baker Industries (Hydro-rate filters)
P.O. Box 845
Hillsboro, Oregon 97423
(503) 642-3508

Bagfilters. Various mesh sizes available. Usually used in the 5 um to 25 um range. Routinely available down to 1 um. Effective and inexpensive, can be used in series with various size bags or other treatment modes. These can be cleaned by hosing or in the washing machine. Frequent cleaning required when particulate load is high. A series of bag filters is an inexpensive method of obtaining particulate free seawater.
Two manufacturers are:

GAF Corporation
140 West 51st Street
New York, N.Y. 10020

American Felt and Filter Co.
Filter Products Department
Newburgh, New York 12550
Toll free (800) 431-8708
or
(914) 561-3560

Chlorination. An effective method widely in use in freshwater systems. Some large-scale marine bivalve hatcheries report that this is an effective mode of water sterilization. Equipment for continuous monitoring and injection of chlorine gas and neutralizing sodium sulfite can be obtained from companies producing waste water equipment. One such company (with nationwide distributors and technical support) is:

Wallace-Tiernan
25 Main St.
Belleville 9, New Jersey 07109

Batch treatment of standing water can be accomplished by sterilization with commercial bleaches such as 'Clorox' and subsequent neutralization of the unreacted Clorox with sodium thiosulfate. Usually a treatment of 10 ppm/sodium hypochlorite for one hour is effective for sterilization. Some operations use 50 mg/L and greater for up to 24 hours but this is excessive unless the treated water exhibits an unusually high oxygen demand. Depending on local conditions and tests for sterility you may find it necessary to treat above the 10 ppm level. It is also advisable to neutralize the chlorine with excessive sodium thiosulfate since no practicle hatchery method exists to measure residual chlorine at the very low levels at which it can be toxic. Sodium thiosulfate is relatively very non-toxic. In excess, however, it can depress oxygen levels and raise pH.

An example of the use of sodium hypochlorite at 10 ppm and its excess neutralization is as follows:

> To treat 100 liters seawater at 10 ppm sodium hypoclorite add Clorox (5.25% sodium hypoclorite) at the rate of 0.19 ml / liter. That is, add 19.0 ml to 100 liters of water. To neutralize this in excess add sodium thiosulfate at the rate of 50 mg/L. For 100 liters, add 5 grams sodium thiosulfate.

Ultraviolet irradiation. An effective method of sterilization requiring good water clarity to be effective. Usually requires prefiltering and maintenance. Large scale systems are expensive and not cost-effective, especially when compared to alternatives. In practical usage, the maintenance requirement is often not adhered to for units which require dismantling of the quartz tube system. Modified systems with teflon tubes and accessible ultra-violet units are available from the second manufacturer listed below. Ultraviolet may have more practicality for sterilizing small volumes of water, such as for algal cultures. Two manufacturers of ultraviolet equipment are:

Aquafine Corp.
1869 Victory Place
Burbank, Calif. 91504
(213) 842-6158

Ultraviolet Technology, Inc.
7835 Wilkerson Court
San Diego, CA 92111
(714) 560-0060

Ozonation. an effective method of water sterilization but not useful for treating influent waters for cultured animals because of the difficulty in controlling excess oxidation products. Greater usefulness where effluent treatment is required

Centrifugation. The 'Wells-Glancy' method developed in the Long Island region in the 1920's. Used for removing particulates and large algal cells. Generally becoming obsolete with the advent of other systems, such as bag filtration.

Maintenance of the System

The most important feature of maintenance of the entire hatchery water system is very simple: keep it clean. Design water delivery and holding containers so as to avoid pockets where debris can collect. Also, size pipes and install valves so that the system can be cleaned with sponge 'pigs', chlorination or other methods should this become necessary. Avoid the accumulation of debris in animal tanks by: 1) not overstocking or overfeeding the system. 2) providing adequate water exchange. 3) regular cleaning of animal holding tanks.

When bacteriologic contamination becomes a problem, or is a potential problem, holding tanks can be easily and inexpensively cleaned between use by mopping the walls (with a broom) with 'Clorox' diluted about 50 times with tap water. Then rinse the tank thoroughly. If bacterial contamination becomes a serious and persistent problem the entire system, including water lines and pumps can be flushed and rinsed with the diluted 'Clorox' solution. This 'Clorox' solution is much more concentrated than the recommended water treatment dose to facilitate rapid cleaning of a system.

# Animal Health Management for Aquaculture

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## Treatment Strategies for Shellfish Aquaculture

### Systems Containing Diseased Animals

Identify cause(s) and sources of disease

Remove and isolate diseased individuals or groups known to be infected. Sterilize tanks holding these animals.

Modify management techniques.

- Water treatment
- Selection and treatment of incoming animals
- Feeding and handling considerations
- Maintenance of a clean system

Treat system and animals with antibiotics or appropriate antimicrobials.[1]

Disassemble system, sterilize and start over.

[1]Antibiotics are not usually recommended for routine usage since it is possible to promote the development of resistant strains of bacteria. Experience has demonstrated in commercial scale hatcheries, however, that antibiotics can be a very useful and sometimes essential tool. Usually, they are best used during 'crisis' periods or phases during which seawater or bay water contains high bacterial counts.

See Shellfish Series-3 for determining the proper type and dose of antibiotic.