View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by College of William & Mary: W&M Publish

W&M ScholarWorks

Reports

2002

Standing policy for Virginia Institute of Marine Science non-native oyster research in Virginia

Standish K. Allen
Virginia Institute of Marine Science

Eugene M. Burreson
Virginia Institute of Marine Science

Follow this and additional works at: https://scholarworks.wm.edu/reports

Part of the Aquaculture and Fisheries Commons

Recommended Citation

Allen, S. K., & Burreson, E. M. (2002) Standing policy for Virginia Institute of Marine Science non-native oyster research in Virginia. Virginia Institute of Marine Science, College of William and Mary. https://doi.org/10.25773/bvkb-jw20

This Report is brought to you for free and open access by W&M ScholarWorks. It has been accepted for inclusion in Reports by an authorized administrator of W&M ScholarWorks. For more information, please contact scholarworks@wm.edu.

Standing Policy For Virginia Institute of Marine Science Non-Native Oyster Research In Virginia

Standish K. Allen, Jr.
Director
Aquaculture Genetics and Breeding Technology Center

Eugene M. Burreson
Director
Research and Advisory Services

Virginia Institute of Marine Science
College of William and Mary

Approved by VMRC

January 22, 2002

Printed Feb. 2002

Table Of Contents

Introduction 1

The NOR Policy in operation 1

Levels of biosecurity for non-native resources 2

Biosecurity at VIMS for non-native oyster research 3

1) Level-1 quarantine 3
  Current system 3
  Upgrades (2002) 4

2) Hatchery, Gloucester Point 4

Transition from level-1 to level-2 quarantine 5

3) Nursery, Gloucester Point 5

4) Extended nursery/ adult flow-through holding system) 5
  Ambient water temperature >12° C 5
  Ambient water temperature ≤12° C 6

5) Brood stock conditioning 6

Standard operating procedure for field trials 6

F_1 or greater progeny for the field 6

Disease-certified 7

Use of non-reproductive oysters 7

Contingency for reversion (monitoring) 8

Other contingencies 9
  Mode of deployment 9
  Unusual weather-related precautions 9

Summary 9

Appendices 10

Standing Policy For Virginia Institute of Marine Science Non-Native Oyster Research In Virginia

Introduction

In 1995, VIMS began research on non-native oyster species *Crassostrea gigas* and *C. ariakensis* under the State-mandated *Rational Plan for Testing Application of Non-Indigenous Oyster Species.* The goal of the *Rational Plan* was to provide a science-based foundation for public policy decisions concerning the introduction of non-native species. Central to this research was *in situ* testing of non-natives in Chesapeake Bay. These tests are nearing an end, but the era of non-native research is just beginning. With the advent of the Virginia Institute of Marine Science (VIMS) Aquaculture Genetics and Breeding Technology Center (ABC) in 1997, a broader canvas for non-native research was stretched. The scope of non-native work at ABC includes genetic technologies to test the feasibility of non-natives for aquaculture directly, and also to use non-natives as a resource for germ plasm to accelerate selective breeding.

Germ plasm is essentially any form of hereditary material (eggs, sperm, chromosomes, chromosome segments, genes, or DNA sequences) that might be useful for genetic evaluation or breeding. There is an immense resource of shellfish germ plasm available throughout the country and around the world that could provide interesting genes or gene combinations for breeding efforts. ABC is taking some major steps to accommodate research on non-natives and their germ plasm. Some are listed below:

- VIMS is in final planning stages for a land-based, non-native holding facility, located at the Kauffman estate in Topping. The Kauffman Aquaculture Center will be a unique facility for holding brood stock of non-natives in isolation from Bay waters.
- Down- and upweller capabilities are being upgraded at Gloucester Point to accommodate early nursery stage of non-natives, also in isolation from the Bay.
- Capabilities to cryopreserve gametes, especially sperm, are under development from selected non-native (as well as native) stocks for future conservation or use.
- ABC's molecular genetics group has been isolating unique DNA sequences and will eventually be "fishing out" genes of import, native and non-native. These sequences will be archived for future reference.

It follows that with the collection and preservation of germ plasm resources, we will need to evaluate them through various types of lab, land-based, or field tests. The "Non-native Oyster Research Policy" (NOR Policy) will govern future activities.

The NOR Policy in operation

VIMS, and in particular the Aquaculture Genetics and Breeding Technology Center, will have recurring requests to deploy non-native oysters in the field in Virginia. Some proposed field tests will be funded

from outside sources and some as part of general funded (State-mandated) research. All need biosecurity measures. It seems redundant however to continually restate what are essentially standard measures of precaution and monitoring for these projects. The NOR Policy serves as a standard operating procedure that will apply to all cases of non-native research activity in the future. Activities with non-natives by other organizations would be subject to separate VMRC review, although the NOR Policy protocols establishes guidelines for others.

VIMS will notify VMRC at least 90 days in advance of deployment with a Project Plan containing details of the project goals and objectives, scope, location(s), duration, monitoring, and unusual circumstances, all as defined below. Accompanying this description will be a statement of assurance that the project will conform to NOR Policy.

- **Goals & Objective(s)** – A statement of the purpose (goal) for the research and the information that defines the end-point of the project (objective). For example, the deployment of triploids to track reversion has the overall **goal** of risk assessment in triploids with the **objective** of determining the relative rates of reversion in various environments.
- **Scope** – Statement about numbers of test groups and the number of oysters per test group, replications, and the size (age) of oysters to be used.
- **Location(s)** – Whereabouts of the test sites and principal party(ies) charged with monitoring stocks and overall site conditions.
- **Duration** – Amount of time required to realize objectives of the project.
- **Monitoring** – The method for monitoring the population for reversion with indication of termination criteria.
- **Unusual Circumstances** – Any situation that is at odds with the NOR Policy but might be warranted for the particular objective under investigation. Unusual circumstances need to be accompanied by a justification. For example, a request to put chemically induced triploid or tetraploid, non-native seed (but not yet certified) in natural waters outside of the spawning season would be at odds with the NOR Policy. The justification might be that growth needs to be accelerated in natural waters to get oysters to a critical size.

Levels of biosecurity for non-native resources

Effective biosecurity for non-native oysters is best organized by combining information on the purpose for holding them, where they are handled, and the risks they pose. The first broad category of biosecurity includes measures at research facilities at VIMS. Here, we deal with all life history stages of oysters and biosecurity measures are applied at five levels of control. *These procedures are not technically under VMRC purview, but we include them as general operating principals of the NOR Policy.*

1. **Level-1 quarantine** – newly acquired (imports from other parts of the country or world), fertile brood stock are held in relatively small numbers in our brood stock room in static tanks with controlled temperature regimes, are fed cultured algae, and require complete changes of water at regular intervals.

2. **Hatchery** – gametes, larvae, and non-reproductive spat (1-2 mm post-set animals) are handled on the hatchery floor in large numbers in static tanks of various sizes, are fed cultured algae, and require complete changes of water at regular intervals
3. **Nursery** – post-set (> 2 mm), non-reproductive juvenile animals are held in flow-through upwelling systems that provide the animals with natural algal food with continuous production of effluent water.
4. **Extended nursery and adult flow-through** – potentially reproductive juveniles and adult animals are held prior to deployment or as potential brood stock in outdoor flow-through upwellers and raceways where they feed on natural food with continuous production of effluent water.
5. **Disease-certified brood stock for conditioning** – F_1 or greater adult oysters are held in relatively small numbers in our brood stock room, as above.

The second broad category of control is field deployment of non-natives, which are subject to VMRC approval The NOR Policy describes standard operating principles for VIMS experiments.

Each of these categories clearly presents different risks of introduction. Adult brood stock animals held in the brood stock room are unable to escape but may release gametes and/or may harbor commensal, parasitic, or disease organisms that could escape confinement in waste water without special treatment. Subsequent hatchery-reared generations of brood stock that have been certified to be free of other organisms present only the threat of releasing gametes. Gametes, larvae, and < 2 mm spat handled on the hatchery floor are very small and could, therefore, escape directly unless wastewater is treated so as to retain or kill escapees before they are released. Larger juvenile animals held in flow-through systems can be effectively barred from escape using simple mesh screens. Large animals held in flow-through raceways are not able to escape, but are capable of releasing gametes, and all effluent from these animals must be treated as if it contained live gametes or larvae during the period where reproduction is possible.

Biosecurity at VIMS for non-native oyster research

1. Level-1 quarantine

Current system – Level-1 quarantine is intended to isolate imported species or strains from outside the Chesapeake Bay area and control against the potential release of exotic diseases and associated fauna that could become pests to the Bay. The level-1 biosecurity precautions at the Gloucester Point hatchery are designed to hold adult animals in a secure, climate controlled environment called the "brood stock room." The brood stock room currently doubles as our conditioning room for ripening adult oysters and our level-1 quarantine area. Water quality and operating conditions are monitored on a daily basis. Access to the room is limited and footbaths with chlorine bleach are placed at all door entrances.

Ten 250-L tanks are located in the brood stock room. Each is sufficient for holding 50-100 adult animals (depending on size). When new imports or strains are brought in for quarantine, animals are cleaned in a chlorine bleach solution and the shipping material is destroyed. If there is more than one species (or stock) in the quarantine room, each is kept separate and precautionary measures are taken to

guard against the transmission of gametes, pathogens or pests between different stocks. Quarantined tanks are cordoned off from others by use of curtains. The oyster pathology lab at VIMS (under Burreson's direction) examines any mortalities in a population for evidence of pathology.

Water in the tanks is changed about every two days. Before discharge, water is sterilized to eliminate any potential progeny, pathogens, and pests. Sterilization consists of a stepwise procedure of water treatment (also see Appendix I: Brood Stock Room).

i. First water is chemically treated with chlorine bleach to obtain a working dose of 200 ppt for at least 24 hours.

ii . The water is then dechlorinated with sodium thiosulfate.

iii. Dechlorinated water is heated through a heat exchanger (to a minimum of 80° C for 1-2 hours) and cooled to ambient temperature for a day, then reheated to 80° C and cooled to ambient temperature before discharge.

*iv.*Finally, chlorinated, dechlorinated and heat-treated water is discharged into a dry well that perks into the ground adjacent to the hatchery.

Upgrades (2002) – The Kauffman Aquaculture Center (KAC) will be the newest addition to the infrastructure of ABC. Funded entirely by private funds, this facility will be built at the Kauffman estate in Topping, Virginia for the purposes of holding non-native and other sensitive brood stocks in isolation from Chesapeake Bay waters. KAC will be a first-of-its-kind, world-class facility closely complimenting the work and facilities of the VIMS and ABC. Biosecurity for the facility has been engineered by a team of aquatic specialists. Storing the majority of non-native brood stock at KAC will take the pressure off of our facility at Gloucester Point. By the beginning of 2003, the site at Gloucester Point will be primarily for rearing spat to a size they can be transferred to KAC.

2. Hatchery, Gloucester Point

The Gloucester Point hatchery is set-up to handle spawning and culturing of small batches of many discreet families or strains of oysters. We have biosecurity measures to safely spawn and culture non-native animals, safeguarding against release of larvae (also see Appendix II: Hatchery Floor). Before non-native work is started, all other work (with native species) is finished and areas are cleaned and rinsed.

All non-native work is done in a designated area only. In this area, hatchery drains are opened and the drainage is diverted to holding tanks outside the hatchery. These tanks collect all water from the hatchery floor. After spawning or larval work is completed, the area is treated with a chlorine bleach solution (12%) and rinsed thoroughly. The quarantine drains are then closed and the water in the holding tanks is chlorinated for at least 24 hours, followed by dechlorination with sodium thiosulfate.

We use sets of larval screens built specifically for each species so that larvae cannot be transmitted from culture to culture. All screens are rinsed, soaked in chlorine bleach solution, and rinsed again before storage.

Transition from level-1 to level-2 quarantine

We have defined level-1 quarantine as that level of safety required to ensure that newly imported brood stock (whether non-native or derived from outside the region, e.g., *C. virginica* from Louisiana and *C. gigas* from the West Coast) do not bring exotic pathogens or diseases, epifauna, or other pests to the Chesapeake Bay. Imported brood stock are therefore maintained in level-1 quarantine as outlined above.

Level-1 quarantine is labor intensive. Our goal is to get newly imported brood stocks to level-2 quarantine where we are guarding only against the release of gametes from diploid non-natives, and past the concern of pathogens and pests. To do this, International Council for the Exploration of the Seas (ICES) guidelines recommend spawning imported brood stocks to produce an F_1 generation. All brood stock in level-2 quarantine are therefore at least F_1 generations. To qualify for level-2 quarantine the F_1 progeny (and the adult brood stock, if possible) are screened for obvious pathology caused by protists or bacteria. A sample of 50 - 100 spat (5 - 10 mm minimum size) are sent to the oyster pathology lab at VIMS and if free of pathogens, upgraded to level-2 quarantine.

3. Nursery, Gloucester Point

The biosecurity system for nursery of non-native oysters coming out of the hatchery is based on providing physical barriers to loss of individuals. Spat (disease certified, if needed) are placed directly into our upweller system receiving raw water from the York River. The water passes through the upweller system once and then drains directly into the VIMS boat basin. Screens are placed on the outlets of the upwellers to prevent escape of the spat themselves. At this period of their life cycle – 1 to 10 mm – oysters are reproductively inactive and incapable of spawning. When spat reach about 10-mm average size, they are transferred to either our extended nursery system or our adult flow-through holding system.

The upweller nursery is composed of three systems: one for native use, one for early nursery of non-natives, and one for extended nursery/ adult-flow through holding system of non-natives. Biosecurity measures are listed below (also see Appendix III: Upwellers):

- Upwellers for native and non-native spat (<10-mm) use incoming raw water from York River. Non-native silos are separated from native ones by a physical barrier. Non-native silos have covers screening at the outflow of the silo, and screening at the outflow of the upweller unit itself to prevent escape of spat. Filtration of discharge water is unnecessary because of redundancy in the screen sets and specific work procedures during handling (Appendix III: Upwellers).
- Extended nursery/ adult flow-through holding system for non-native groups of average size >10-mm will be accomplished in the two part description below:

4. Extended nursery/ adult flow-through holding system

Ambient water temperature >12° C – Originally, the adult flow-through holding system was built for holding a small number of non-native adults temporarily, or for small scale experiments. (Again, all non-natives in this system are certified disease and pest free.) Presently, this system comprises six shallow

raceways that are fed by a continuous flow of seawater. The seawater drains into a holding pond and the effluent percolates through the bottom sediments. The percolation rate limits the flow rate to each raceway to only about 5 gpm and, consequently, the number of adults to only 2000-5000 animals.

Because of the advent of KAC, described above, we will soon be able to restrict the number of animals held at Gloucester Point to those being used in the hatchery or for research projects.

Ambient water temperature ≤ 12° C – We employ a winter strategy for the adult flow-through system/ extended nursery system. We bypass the active filtration system for flow-through quarantine when temperatures are <12° C. There are several reasons for this. The practical reason is that operation of the system is labor intensive, and any reduced operations would be helpful. The other reasons are that, biologically, it is unnecessary to filter the discharge in the colder months of the year. At temperatures of <12° C, oysters will be sexually immature. Even if there was residual gonad, temperatures <12° C would not induce spawning. And finally, even if there were ripe animals and they were able to spawn sufficiently to create embryos – both highly unlikely – the larvae could not survive to settlement in the wild.

5) Brood stock conditioning

F_1 and greater non-native brood stock are normally kept in the adult flow-through system or will be kept at KAC. When these brood stock are needed for spawning, we bring them into the brood stock room to intentionally induce them to attain sexual maturity – a process called conditioning. Quarantine procedures for these brood stock are the same as for Level-1 quarantine, except we do not run the chlorinated, dechlorinated water through the heat exchanger.

Specifically, water in the tanks is changed about every two days. Before discharge, water is sterilized to eliminate gametes. Sterilization consists of a stepwise procedure of water treatment.

i . First water is chemically treated with chlorine bleach to obtain a working dose of 100 ppt for at least 24 hours.

ii. The water is then dechlorinated with sodium thiosulfate.

iii. Finally, chlorinated, dechlorinated water is discharged into a dry well that perks into the ground adjacent to the hatchery.

Standard operating procedure for field trials

F_1 or greater progeny for the field

East Coast stocks of both *C. gigas*, *C. ariakensis* and *C. rhizophora* are available at VIMS. *C. gigas* has been bred through four generations, beginning at Rutgers University and continuing here in Virginia by ABC. *C. ariakensis* has been through 3 generations since the original importation. *C. rhizophora* are F_1s. These we call "domesticated stocks" because they are now under our genetic control. Other stocks are under domestication including two stocks of *C. ariakensis* from China obtained in 1999. The first step in preparing oysters for field testing then is the "domestication" process, creating F_1's from imported parents.

Disease-certified

All stocks used for testing in the field will be free of exotic diseases. To ensure this, only F_1 (or greater) progeny will be used and then, only after certification of the population for absence of pathology. We consider two generations of such testing adequate for all subsequent use of the stock. For example, we imported some *C. ariakensis* from Southern China in May 1999. Parents were spawned to produce an F_1 generation that has subsequently been certified as pathogen free. We have spawned the F_1 to produce an F_2, and that generation has been certified as well. However, we feel that it is unnecessary to continue the certification past F_2, since absence of exotic diseases through two generations is ample evidence to classify a brood stock "disease certified."

Use of non-reproductive oysters

Only triploids will be deployed in the field under the NOR Policy. Exceptions will be noted as "Unusual Circumstances" under the Project Plan. Triploids are obtained from either of two methods: chemical or natural.

Chemical triploids are made in the hatchery at the time of fertilization of the egg. The developing egg is forced to retain an extra polar body (containing the third set of chromosomes) by administration of some agent, typically a chemical one. The treatment for triploidy is applied to millions of eggs at the same time, but there is variability among those eggs and not all the treated embryos become triploid. Oysters resulting from chemical treatments are mostly, but not all, triploid. Therefore, in order to use 100% triploids from chemical triploids for field tests, each individual must be certified before deployment. Typically, it is only possible to deploy in the order of thousands of chemical triploids because of the burden of certification.

Natural triploids are produced by mating tetraploid male oysters with female diploid ones. It is expected that all progeny from such matings are triploid. In general this is true, but there are a few ways that populations of <100% triploids might result. First, if sperm from a diploid accidentally got into the spawning process, a few percent of the offspring could be diploid. Second, if a tetraploid male used for spawning is imperfect, that is, produces some haploid gametes along with diploid ones, some diploids could result. Third, if there is mixing of larvae among batches accidentally, a foul up that we take extraordinary precautions to avoid, some diploids might contaminate the natural triploids. Recognizing the potential pitfalls of 100% triploid production, we have instituted several key checkpoints for each "natural triploid" spawn.

- Sperm from the tetraploid male will be tested for DNA content by flow cytometry (FCM). Only sperm with no evidence of haploid cells will be used for spawning.
- Larvae will be tested by FCM after 2 and 4 days in culture to see if there are any diploids present. If so, larvae will be discarded and the spawn repeated.
- Larvae will be tested again by FCM at eyed stage to ensure that no accidental mixing has occurred during the larval cycle.

- Before deployment, 200 spat will be sampled randomly from the natural triploid group and tested for ploidy by FCM. The group will be certified "ready for deployment" if every single spat sampled is triploid.

Contingency for reversion (monitoring)

Over the last several years it has become clear that triploids of *Crassostrea* species manifest chromosome set instability. That is, triploids tend to lose chromosomes over time. The result of this loss is that the oyster, instead of having only triploid cells, will have some percent of its cells triploid and the rest diploid. An oyster with part triploid and part diploid cells is called a **mosaic**. The rate of chromosome loss (called **reversion**) seems to vary according to where they are grown and whether the triploids are "chemical" or "natural," but the process is generally very slow. Typically, the rate of reversion in chemical triploids is 2-3 times higher than that in natural triploids. Once an oyster begins to lose chromosomes, the process continues over time so that the percentage of triploid cells goes down and the percentage of diploid cells goes up. After three or four years, mosaic *C. gigas* and *C. ariakensis* can have anywhere from 10 to 70% diploid cells, with the mean about 40%. The problem with reversion is not loss of chromosomes *per se* but the possibility that mosaics might regain their reproductive capacity.

The eventual restoration of reproductive capacity in mosaics **is not a given**. For example, we have followed the reproductive potential of mosaics very closely in both *C. gigas* and *C. ariakensis*. After as many as four years and with the percentage of diploid cells as high as 50%, we have found no reproductive capability in mosaics. In other words, mosaics seem to occur with regularity but restoration of reproductive capacity in mosaics seems to either not occur or to occur very late in life – i.e., later than we have sampled so far.

Despite the lack of reproductive potential, mosaics are a concern. We will institute routine methods of sampling for mosaics during field trials. This sampling may vary with the nature of the experiment. Several examples are given below:

- *Example 1:* Direct sampling – If field deployed oysters are to be sampled (non-lethally) during the experiment anyway, then we already have a built in control.
- *Example 2:* Sentinel population – Other experiments might be deployed but not sampled. Instead, a cohort group coming from the same spawn would be deployed next to it and sampled non-lethally over time as an indicator of what was happening in the experimental group. The cohort group should consist of at least 50 individuals that are all sampled individually and repeatedly at least 3 times a year.
- *Example 3:* Subsampling – enough oysters may be deployed to allow periodic sampling from the experimental population, as was the case for the non-native work on *C. gigas* and *C. ariakensis* from 1996 to 1999.

Typically, mosaics will **not** be removed from field experiments, at least until we have a better handle on their biology. Said another way, mosaics are far more valuable in the field where we can study them, than

removed. We deem this of little risk because mosaics apparently lack or fail to regain reproductive capability, at least until 3 or 4 years old.

What results of the sampling would result in termination of the experiment? We are constantly adding to our database on the biology of reversion. But we have enough data to flag what appears to be high rates of reversion within a population. A high rate of reversion might be attributable to the genetics of the experimental group or to the environment where they are deployed. In addition to tracking the percentage of triploid oysters reverting to mosaics, we can also monitor the rate at which individual oysters lose triploid cells (in examples 1 and 2 above). That is, how fast are diploid cells replacing triploid ones. A "red flag" would cause us to monitor the experimental group very carefully, especially during the spawning season, when mosaics will be monitored for reproductive capacity.

The strongest and quickest indication of reproductive capacity is to sample (lethally) a mosaic for evidence of haploid sperm production. In fact there is no other way to unequivocally demonstrate reproductive capability other than doing test crosses, and even test crosses are subject to variables that could confound the results. Therefore, we will take the presence of haploid cells in the gonad of mosaics to indicate a problem with the deployment. The experiment will be terminated, or restructured with additional approval from VMRC.

Other contingencies

Mode of deployment – Only sites where we feel we have high levels of security from poaching and vandalism will be used. These generally consist of sites adjacent to docks or domiciles of cooperating Virginia citizens, public groups, or industry. Under the NOR Policy, oysters will be deployed in containers always, and secondarily secured in place as a precaution against usual weather related turbulence.

Unusual weather-related precautions – Each project will have written "hurricane" plans on file and these plans will be distributed and explained to all participants, both VIMS employees and cooperators. In the event of storms, oysters will be secured or relocated as appropriate to conditions. Hurricane plans for biosecurity at VIMS facilities for non-native research are described in Appendix IV.

Summary

The NOR Policy (and accompanying Appendices) lays out a standard operating procedure for the experimental use of non-native species at VIMS and in Virginia waters. The principal features of this Policy relate to due diligence on introduction of foreign diseases and pests, biosecurity relating to reproductive potential, and monitoring for biological status of test animals. Most of the standard operating procedures have been part of VIMS protocols from the beginning of our work with non-natives, but bears repeating for the record. Standard operating procedures for testing non-native oysters in the field has been evolving over the last few years of tests, and through comments by the *ad hoc* Panel of the Chesapeake Bay Program's Living Resources Sub-Committee.

Appendices

(standard operating procedures)

for

Standing Policy For Virginia Institute of Marine Science Non-Native Oyster Research In Virginia

Mark D. Camara

Standish K. Allen, Jr.

Ann Arseniu

Aquaculture Genetics and Breeding Technology Center

Appendix I: Brood Stock Room

Overview

There are two distinct levels of quarantine procedures for the brood stock room corresponding to the risks posed by either newly acquired animals or by subsequent hatchery-raised generations. Level-1 procedures are meant to prevent not only the introduction of non-native oysters themselves, but also the introduction of other commensal and /or parasitic organisms and diseases. These procedures use both bleach treatment and heat pasteurization to ensure that they kill viruses as well prokaryotic and eukaryotic organisms. Level-2 procedures are meant to prevent the escape of gametes and/or larvae from unintentional spawning activity of certified disease- and commensal-free animals in holding tanks and relies on bleach treatment only.

Level-1 procedures

1. Go to the tanks on the loading dock in front of the hatchery and verify that the drain valves on the tank to be used are closed.
2. In the brood stock room, remove the animals from their tank(s) and put them into unoccupied tank(s) of seawater.
3. Add bleach to the water of empty tank(s) at the rate of 10 ml/ liter of water. These tanks hold about 200 liters to the bottom of the stopper, so the corresponding volume is 2000 ml of bleach. Aerate the tank to mix well.
4. Maintain bleach treatment for at least 24 hours.
5. Using a submersible pump, pump chlorinated water from the brood stock tank into the loading dock tank.
6. Add 1 ml of sodium thiosulphate stock solution (80 g/ L) per ml of chlorine used to chlorinate the tanks on the previous day. Aerate and verify neutrality using chlorine test strips.
7. When the loading dock tank is full, pump the water using the dock seawater system up through the heat exchanger until the water temperature reaches 80° C. The water must remain at 80° C or above for one hour. Open the heat exchanger all the way to accomplish this.
8. Let water cool to ambient temperature.
9. Repeat heating/cooling cycle: 80° C for one hour; cool to ambient temperature.
10. Water can then be drained into dry well.

Level-2 procedures

1. Go outside to the tanks at the back of the hatchery. Verify that the drain valve on the tank you are going to drain the brood stock tank(s) into is closed and that the fill valve is open. Also check that the fill valve to the other tank is closed so that all drained water will go into the appropriate tank.
2. In the brood stock room, remove the animals from their tank(s) and put them into unoccupied tank(s) of seawater.
3. Add bleach to the water of empty tank(s) at the rate of 5 ml/ liter of water. These tanks hold about 200 liters to the bottom of the stopper, so the corresponding volume is 1000 ml of bleach. Aerate the tank to mix well.
4. Maintain bleach treatment for at least 24 hours.
5. Add 1 ml of sodium thiosulphate stock solution (80 g/ L) per ml of chlorine used to chlorinate the tanks on the previous day. For standard chlorination, one liter of sodium thiosulphate will be added. Aerate and verify neutrality using chlorine test strips. Verify that the fill valve on the tank(s) is (are) closed.
6. Drain the dechlorinated tank(s) into drains that run into the tanks behind the hatchery by opening the valve(s) at the base(s) of the tank(s).
7. Rinse tanks with freshwater with the drain valves open so that the rinse water goes into the quarantine system with the drained water.

Appendix II: Hatchery Floor

Overview

Spawning, setting, and routine larviculture of non-natives in the hatchery building requires special care as the small gametes and larvae are difficult to control. The basic precautions in this area are similar to those used to prevent the escape of gametes and larvae in the brood stock room in that they rely on chlorination. The hatchery floor is sloped toward both sides from its middle, and both sides are equipped with quarantine drains that lead to a holding tank behind the hatchery where the water is treated. Due to the need for a constant outflow of water and the impracticality of treating this much water, only the loading dock (front) side of the hatchery is used for working with non-natives and the normal drains on the back wall are typically open and flowing. For this reason, no non-native animals, gametes, rinse water or equipment used to handle them are taken into the "native" side of the hatchery. Sieves and other equipment are also color-coded by species. Currently *C. virginica* equipment is marked with blue labels, *C. gigas* equipment with yellow labels, and *C. ariakensis* with red labels.

When several kinds (species and ploidy-levels) of non-natives are to be handled on the same day, there is a chance for cross-contamination of cultures as well as for escape. This is most dangerous if potentially reproductive material (e.g. diploid larvae or gametes) contaminate putatively non-reproductive cultures (e.g. triploids) or if non-natives contaminate native cultures. Since contamination is most likely to move from material left on work surfaces or equipment into cultures handled at a later time, it is important to work with the least "dangerous" materials first (natives first, 100% "natural" triploid non-natives next, high percentage chemical non-native triploids next, diploid non-natives last). In this way, the accidents are minimized.

Hatchery Floor Procedures

1. Notify hatchery manager and assistant manager of any quarantine work before proceeding. This is necessary for:
   - Spawns
   - Draining larval tanks
   - Water changes in downweller systems

2. Do quarantine work in designated areas only. **No non-native animal should ever be carried outside of the marked area.**

3. Clean and rinse all work surfaces, hatchery floors, and water troughs with fresh water.

4. Close non-quarantine hatchery drains and open quarantine drains on the loading dock side of the hatchery. (Quarantine drains are ALWAYS CLOSED with wing-nut plugs when NOT doing quarantine work.)

5. Use only the appropriately labeled sieves for quarantine work and fill sink with bleach water to soak them between uses.
6. When finished, soak all sieves and other equipment in bleach, spray down floor and troughs into quarantine drains with fresh water hoses.
7. Empty bleach buckets with quarantine drains open.
8. Re-spray floor and troughs with 12% bleach solution.
9. Close quarantine drains and open hatchery drains.

Appendix III: Upwellers

Overview

Since the animals kept in downwelling and upwelling nursery tanks are too small to be reproductive, the only quarantine measures required are those necessary to prevent the animals themselves from escaping. Animals at this stage are still quite small and may be held in substantial numbers, so attention to handling is paramount. Quarantine procedures at this stage consist of both the proper choice of mesh size on silos to prevent animals passing through their bottoms as well as redundant screening of effluent water, with additional screening added while animals are being handled. This is achieved by placing screens of the proper sizes: on the bottoms of the silos themselves, over the outflow tubes of the silos, over the standpipe drains in the upwelling tanks, and on the system drain outflow.

Upweller procedures

Post-set animals to downwellers and transferring to larger-mesh upweller silos

1. Sieve the animals very thoroughly on a graded series of hatchery screens either under quarantine in the hatchery or outside the hatchery where any spills will put animals on dry ground.
2. Choose a silo with a smaller mesh size than the one on which the animals caught in the sieving process, and carefully transfer the animals to the silo.
3. For upwellers, fit the outflow tube of the silo with a PVC t-fitting and insert screens of the same mesh size as the bottom of the silo to prevent any floating animals from entering the outflow.
4. Check the standpipe of the upwelling tank into to be sure that it is protected by a screen of the same or smaller mesh size.
5. Place a bag made of the same mesh size over the fire-hose outflow from the upwelling tank into the drain system.
6. Transfer the silo(s) to the tank and ensure that no animals are caught in the surface film.
7. Remove the bag from the fire-hose drain, but leave all others in place.

Drain-downs of upwelling tank

1. Check that the mesh screen on the tank standpipe and the system outflow is as fine as the finest silo in the tank.
2. Put an equally fine mesh bag over the fire-hose tank drain where it enters the upweller drain system.
3. Pull the standpipe to drain the tank.

4. With the screen still over the tank drain, rinse the silos and the tank.
5. Make transfers (if any) according to the procedures above for moving animals among silos.
6. For upwellers, check that all the t-fittings and their screens are properly sized and in place.
7. Replace the standpipe and check that it's screen is in place and properly sized.
8. Refill the tank.
9. Check that everything is secure and that no animals are floating in the silos.
10. Remove the screen from the fire-hose drain.

Appendix IV: Hurricane Plan

Overview

Catastrophic storms obviously provide an opportunity for the escape of non-native animals if the hatchery facilities are damaged and/or inundated. The only possible measures to prevent this are the removal of quarantine animals from all tanks, raceways, and field enclosures and storing them in a secure area.

Brood Stock Room

1. Collect extra water in temporary tanks for water exchanges if SPO cannot be accomplished.
2. For Level-2 stocks, where possible, bag up animals and move to secure cold storage.
3. Chlorinate water to working level of 200 ppt (Appendix I).

Hatchery

1. Drop all larval cultures and capture larvae on appropriate sieves.
2. Ball larvae into wet paper towels and put into secure cold storage.
3. Treat drain water as per SOP (Appendix II).

Upwellers

1. Remove and bag all animals.
2. Store in secure cold storage.
3. Drain tanks according to SOP (Appendix III).

Raceways

1. Bag up all animals.
2. Store in secure cold storage.
3. Shut off water to drainage pond and allow to continue to perk.

Field Experiments

1. Written plan will be developed on a case by case basis, depending on locations.