LICENSING AND REGULATION OF VETERINARY BIOLOGICS FOR FISH IN THE UNITED STATES

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ABSTRACT

The U.S. Department of Agriculture (USDA) regulates biologics for fish, including vaccines, bacterins, and diagnostic test kits, produced in, imported into, or exported from the United States. The regulatory process is designed to ensure that biologics under USDA jurisdiction are not contaminated, worthless, dangerous, or harmful. The Animal and Plant Health Inspection Service (APHIS), an agency within the USDA, licenses and inspects biologics production facilities, and licenses and tests veterinary biological products. Veterinary biological products should be pure, safe, potent, and efficacious. A biologics-producing firm located in the United States may sell its products provided the firm possesses a valid U.S. Veterinary Biological Product License for each product produced for sale, as well as a valid U.S. Veterinary Biologics Establishment License. A permittee (i.e., the legal representative in the United States of a biologics-producing firm located outside the United States) may import biologics into the United States provided the permittee possesses a valid U.S. Veterinary Biological Product Permit. Biologics available in the United States for fish are manufactured by Alpharma NW Inc., Aqua Health Ltd., and DiagXotics. Monovalent and multi-faction bacterins (i.e., antigenic suspensions of inactivated bacterial organisms) are available for the vaccination of fish to aid in the prevention of furunculosis caused by Aeromonas salmonicida, enteric septicemia of catfish caused by Edwardsiella ictaluri, columnaris disease caused by Flavobacterium columnare, vibriosis caused by Vibrio anguillarum and V. ordalii, cold water vibriosis caused by V. salmoninarum, and enteric redmouth diseases caused by Yersinia ruckeri. Qualitative and quantitative test kits to diagnose the presence of the bacterial kidney disease antigen Renibacterium salmoninarum in fish are also available in the United States. A bacterin recommended as an aid in the prevention of winter ulcers caused by V. viscosa is produced in the United States for export only.

INTRODUCTION

The U.S. Department of Agriculture (USDA) regulates biologics for fish produced in, imported into, transported through, or exported from the United States. Veterinary biologics include viruses, serums, toxins, and analogous products (e.g., vaccines, bacterins, allergens, antibodies, antitoxins, toxoids, immunosubstituents, cytokines, etc.) which act through an immune mechanism to prevent, diagnose, manage, or cure disease of animals.

DISCUSSION

Biologics currently available for fish in the United States include bacterins and diagnostic test kits. Bacterins (i.e., an antigenic suspension of inactivated bacterial organisms) are used for the vaccination of fish as an aid in the prevention of furunculosis caused by Aeromonas salmonicida, enteric septicemia of catfish caused by Edwardsiella ictaluri, columnaris disease caused by Flavobacterium columnare, vibriosis caused by Vibrio anguillarum and Vibrio ordalii, cold water vibriosis caused by Vibrio salmoninarum,
and enteric redmouth diseases caused by *Yersinia ruckeri*. Qualitative and quantitative test kits to diagnose the presence of the bacterial kidney disease antigen *Renibacterium salmoninarum* in fish are also available in the United States. A bacterin recommended as an aid in the prevention of winter ulcers caused by *Vibrio viscous* is produced in the United States for export only. Depending on the specific biological product being considered, bacterins may contain a single fraction or multiple fractions, and may be recommended for administration to fish by immersion, by injection, or by ingestion.

The regulatory process is designed to ensure that all biologics under USDA jurisdiction are pure, safe, potent, and efficacious, and not worthless, contaminated, dangerous, or harmful. The Animal and Plant Health Inspection Service (APHIS), an agency within the USDA, licenses and inspects biologics production facilities, and licenses and tests biologics produced in licensed biologics-manufacturing facilities.

The Center for Veterinary Biologics (CVB) is the veterinary biologics regulatory program within APHIS and is composed of three units with defined functions. The Licensing and Policy Development (CVB-LPD) unit establishes licensing standards and policy; reviews prelicense documentation; reviews test methods, outlines of production and labels; and issues, suspends, or revokes licenses and permits. The Inspection and Compliance (CVB-IC) unit inspects production facilities, methods, and records; releases serials (lots or batches) of biologics for distribution in the marketplace; performs post-release product surveillance; and investigates suspected law violations and consumer complaints. The Laboratory (CVB-L) unit develops test methods, standards, and reagents; performs prelicense, surveillance, and field problem testing; and trains personnel from other laboratories.

The authorities and procedures for the regulation of biologics are defined in a variety of published documents, including the Virus-Scrum-Toxin Act of 1913 (amended in 1985), Title 9 of the Code of Federal Regulations, Veterinary Biologics Memorandums, Veterinary Biologics Notices, Veterinary Biologics General Licensing Considerations, and Supplemental Assay Methods. Further sources of information and guidance include: the semi-annual Veterinary Biological Products publication listing the licensees, permittees, and veterinary biologics produced, the CVB internet home page, and APHIS-sponsored public meetings.

Veterinary biologics eligible for distribution and sale in the United States may be manufactured in facilities located either in the United States or abroad. In order to sell a veterinary biologic in the United States, a biologics manufacturer located in the United States must possess two types of Federal licenses: a U.S. Veterinary Biologics Establishment License for the production facility, and a separate U.S. Veterinary Biological Product License for each biological product. In order to import from abroad and sell a veterinary biological product in the United States, a foreign veterinary biologics manufacturer’s legal representative (permittee) in the United States must possess a U.S. Veterinary Biological Product Permit for the biologic(s) to be imported. With only minor differences, the licensing process for domestically produced veterinary biologics is the same as the permitting process for veterinary biologics imported from abroad.

The applicant for an establishment license or a product permit should submit the following documents to the CVB:

2. Application for U.S. Veterinary Biological Product Permit (APHIS Form 2005): a one-page document completed by the permittee of a foreign biologics-manufacturing establishment indicating general information regarding the permittee, the foreign biologics producer, and the biological product(s).
3. Articles of Incorporation: a legal document indicating the business operating status of the manufacturing establishment.
4. Water Quality Statement: a document required for domestic veterinary biologics manufacturers only indicating the manufacturing establishment’s status regarding applicable
U.S. effluent water quality control standards.


6. Qualifications of Veterinary Biologics Personnel (APHIS Form 2007): a one-page document indicating specific information regarding the educational and work background of employees involved in biologics production.

7. Facilities Documents: blueprints, plot plans, and legends describing the biologics production facilities.

In support of the product license or permit applications, the applicant should prepare in an acceptable manner and submit to Licensing and Policy Development the following items (some variation may exist depending on the particular veterinary biological product being considered):

1. Outline of Production and Related Special Outlines: documents describing the protocol for manufacturing and testing of a particular biologic.

2. Master Seed Purity and Identity Test Report: the Veterinary Biologics Production and Test Report form (APHIS Form 2008) indicating the test results for the organism selected and permanently stored at a specified passage level from which all additional passages are derived.

3. Master Cell Stock Purity, Stability, and Non-tumorigenic Quality Test Report: the Veterinary Biologics Production and Test Report form (APHIS Form 2008) indicating the test results for the cells within a specific passage level range used to grow seed organisms for biologics production.

4. Backpassage Test Report: results of reversion to virulence studies for conventional modified live or live recombinant-derived vaccines indicating the Master Seed's genetic stability and reversion to virulence potential following administration to the host animal.

5. Efficacy Report: study results indicating the effectiveness of the veterinary biological product to perform as indicated on the product label. Vaccines recommended as an aid in the prevention of a specific fish disease are typically evaluated for efficacy by vaccination-challenge studies. The vaccine (produced with the lowest antigen level and at the highest passage level from Master Seed approved in the filed Outline of Production) should be administered according to label directions (e.g., injection, immersion, or oral) to the youngest age or smallest size fish for which the product shall be recommended. After an appropriate post-vaccination observation period, the vaccinated fish and other non-vaccinated control fish are challenged with a virulent strain of microorganism for which protection is recommended, and all post-challenge findings are accurately recorded. The precise challenge method and the criteria for determining protection vary with the immunizing agent. For products with two or more fractions, data should be submitted to evaluate any in vivo or in vitro interference of the various fractions.

6. Serial Purity, Safety, and Potency Test Report: the Veterinary Biologics Production and Test Report form (APHIS Form 2008) indicating all required test results for each of at least three consecutively produced prelicense serials (batches or lots) of finished product:

   a. Purity test results indicate if extraneous viable bacteria and fungi are present in the finished product. The permittee of imported veterinary biologies is charged a monetary fee if APHIS conducts additional testing of the finished biological product for exotic viruses.

   b. Laboratory safety test results indicate if there are any adverse reactions attributable to the vaccination of susceptible fish with the biological product during the pre-challenge observation period.

   c. Potency test results indicate the relative strength of the biological product, and are designed to correlate with the approved host animal vaccination-challenge efficacy study. Potency tests for killed viral or killed
bacterial products typically utilize laboratory animal or host animal evaluations or else quantitative in vitro methods. The potency of live virus and bacterial vaccines is typically measured by means of bacterial counts or virus titrations. The bacterial count of a live bacterial vaccine must be sufficiently greater than that shown to be protective in the immunogenicity (efficacy) test to ensure that at any time prior to the expiration date the count will be at least twice that used in the immunogenicity test. The virus titer of a live viral vaccine at release should be at least 1.2 logarithms greater than that shown to be protective in the immunogenicity test to ensure that at any time prior to the expiration date the titer will be at least 0.7 logarithms greater than that used in the immunogenicity test.

d. Other Outline of Production finished product test results indicate specific required information, e.g., microorganism identity, residual-free formaldehyde, viricidal activity, etc.

7. Field Safety Report: study results indicating the level of unsuspected adverse product-related reactions that may not have been observed during product development. Two or more prelicense serials are tested at three or more distinct geographic locations on a large number of appropriately sized fish that do not belong to the manufacturer. The manufacturer receives authorization to conduct field safety studies only after submission of acceptable efficacy data and satisfactory testing results of three consecutively produced prelicense serials. The field study is approved only if the test conditions are adequate to prevent the spread of disease, and the firm has obtained permission from the proper animal health authorities for each state where the tests will take place. Before beginning the field safety test, the firm should submit to Licensing and Policy Development for review a detailed protocol indicating the proposed observation and recording methods.


9. Label: the insert, container label, and carton label indicating the true product name, the name and address of the producer (and also the importer for imported products), the establishment license or permittee number, the recommended storage temperature, the full instructions for use, the withdrawal time if the biologic is administered to food animals, the expiration date, the serial identification number, the recoverable quantity and number of doses, the presence of any antibiotic used as a preservative, the indication to use the entire contents of a multi-dose container when the container is first opened, the recommendation to burn the container and unused contents of all live organism products, and any special restrictions. The label may not contain any information which is false or misleading. All label claims must be supported by data submitted and filed by Licensing and Policy Development as acceptable.

Before issuing an establishment license or permit for general distribution and sale, APHIS will conduct an on-site inspection of the biologics production facilities and equipment to determine that these are acceptable for producing, testing, and distributing veterinary biologics using good manufacturing procedures and good laboratory techniques. The permittee for a foreign biologics manufacturer is charged a monetary fee to pay for the on-site inspection of a foreign biologics production facility. Biologics manufacturers should use good sanitary measures in compliance with Federal regulations and the Outline of Production. At the prelicense inspection, APHIS reviews all aspects of the manufacturing process, including accurate record keeping and product sampling. Following submission by the firm to APHIS of satisfactory results for all required prelicense serial release tests, APHIS will conduct confirmatory prelicense testing of representative samples of three consecutively produced prelicense serials at the CVB-L. APHIS will issue the appropriate establishment and product licenses or permit only after all prelicensing requirements have been satisfied.
There are several types of U.S. Veterinary Biological Product Licenses and U.S. Veterinary Biological Product Permits. A regular biologics product license authorizes the distribution of a veterinary biological product manufactured in the United States, with or without restrictions (e.g., for use by or under the supervision of a veterinarian only, intra-state distribution limited to authorized recipients or approved laboratories, use only on premises having a history of the disease, for export only, etc.). A conditional product license is issued in an expedited procedure to make a biologic needed (e.g., in an emergency or limited market situation) available following the demonstration of product purity and safety (even though product efficacy and potency studies remain in progress). A permit for general distribution and sale allows for the importation into the United States and distribution (with or without restrictions) of a specified biologic or biologics. Permits may also be issued to allow the importation of biologics into the United States for research and evaluation purposes or for transit shipment only.

EPILOGUE


Qualified personnel at APHIS' CVB are available to assist biologics manufacturers and permittees in the application process. For further information regarding the regulation of veterinary biologics, contact: U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services, Center for Veterinary Biologics, Licensing and Policy Development, 4700 River Road, Unit 148, Riverdale, Maryland 20737-1231; telephone: (301) 734-8245, fax (301) 743-8910. Information regarding the CVB is available from the Internet web site <http://www.aphis.usda.gov/vs/cvb>.
EFFECTS OF REARING CONDITIONS ON BLIND SIDE HYPERMELANOSIS IN JAPANESE FLOUNDER

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ABSTRACT

The effects of light direction, intensity, and bottom substrate on the hypermelanosis in Japanese flounder were examined. Hatchery-produced juvenile or young flounder were kept in aquaria with transparent tops and bottoms to illuminate the fish with upward and downward light. When the bottom of the aquarium was a transparent plastic plate, hypermelanosis occurred in all the fish tested regardless of light direction or intensity (downward/upward illumination: 1300/100, 1300/60, and 150/7 lux). However, only 14% of the fish showed hypermelanosis in the aquarium with a sandy bottom and no upward light. Percentage occurrence of fish with hypermelanosis decreased drastically when the bottom was covered with glass sand, even if the fish were exposed to a high intensity of upward light (1400 lux). Similar trends were observed in the enlargement of the blind side pigmented area. None of the fish showed visible expansion of the pigmented area in the aquarium with sand or glass sand on the bottom; however, the pigmented area was enlarged in half of the fish in the aquarium with a transparent plastic plate bottom. From these results, it is considered that, not light, but the presence of sand on the aquarium bottom is the primary cause of blind side hypermelanosis in Japanese flounder.

INTRODUCTION

The Japanese flounder Paralichthys olivaceus is one of the most important marine culture fish species in Japan. The wild fish of this species generally has a white blind side, while almost all the cultured flounder show a dark pigmented area on their blind side (amibocoloration or hypermelanosis on the blind side). This color anomaly is a serious problem in flounder culture, because it usually decreases the market price of the fish.

Norman (1934) divided hypermelanosis of flatfishes into three types by its characteristics: staining, spotting, and true amibocoloration. The staining type is the most common in cultured Japanese flounder (Yamamoto and Oda 1991). Some factors such as illumination on the blind side, food, and stocking density are considered to affect this type of hypermelanosis in Japanese flounder (Seikai 1991, Suzuki 1994, Takahashi 1994). Among these factors, illumination to the blind side seems to be the most plausible, because there are many studies not only on Japanese flounder (Seikai 1991) but also on other flatfish species which showed a relationship between light and hypermelanosis (Cunningham 1891, 1893, 1895, Osborn 1940, 1941, Stickney and White 1975). However, because most of the studies were conducted in aquarium in which the bottom was covered with sand to prevent upward light, the sandy substrate may have affected their results. In this study, rearing experiments were conducted to determine if light direction and intensity or bottom substrate are the most important factors for hypermelanosis in cultured Japanese flounder.

MATERIALS AND METHODS

Fish of about 1g body weight were obtained from commercial hatcheries, and were kept in a tank with a recirculating seawater system until the start of experiments.

Experiments were carried out in aquaria equipped with a closed recirculating system (Fig.
1). The aquaria with transparent acrylic bottoms were placed on transparent acrylic plates. The insides of aquarium walls were made of matted black vinyl chloride plates, and the top was covered with a transparent acrylic plate. Downward and upward illumination was provided with fluorescent lights installed above and below the aquaria. These lights were turned on for 12 h/day. Fish were fed with a commercial pelleted diet twice a day for 5 days/wk during the experimental period. Temperature was maintained at 23°C.

Experiment 1 was designed to examine the effect of light intensity and direction on the hypermelanosis of the flounder in transparent plastic bottom aquaria with or without sand. Experimental conditions are shown in Table 1. The bottoms of two aquaria were covered with a 1-cm layer of coarse sand (particle size 0.5 - 1.0 mm). Fifteen fish of about 6 g body weight without visible pigmentation on the blind side were reared for 16 wk. At the end of the experiment, all surviving fish were anesthetized, and photographs of their blind sides were taken individually to examine the pigmentation.

In experiments 2 and 3, three aquaria with upward light and different bottom conditions were prepared as follows (Tables 2 and 3): (1) a transparent plastic plate and strong upward light, (2) a white opaque plate and weak upward light, (3) similar light conditions as the first, but the bottom was covered with a 1-cm layer of transparent glass sand (particle size 0.5 - 1.0 mm).

### Table 1. Effects of downward and upward light intensity on the blind side hypermelanosis of Japanese flounder reared with or without sand on the bottom of the aquarium, experiment 1.

<table>
<thead>
<tr>
<th>Bottom material of aquarium</th>
<th>Light intensity (lux)</th>
<th>Percentage of hypermelanosis fish (%)</th>
<th>Survival rate (%)</th>
<th>Specific growth rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Downward</td>
<td>Upward</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transparent plate</td>
<td>150</td>
<td>7</td>
<td>100</td>
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<td>1300</td>
<td>60</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>1300</td>
<td>1100</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>Sand (1 cm thickness)</td>
<td>200</td>
<td>0</td>
<td>7</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>1900</td>
<td>0</td>
<td>14</td>
<td>50</td>
</tr>
<tr>
<td>Bottom material of aquarium</td>
<td>Light intensity (lux)</td>
<td>Percentage of hypermelanosis fish (%)</td>
<td>Survival rate (%)</td>
<td>Specific growth rate (%)</td>
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<tr>
<td></td>
<td>Downward</td>
<td>Upward</td>
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<td></td>
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<tr>
<td>Transparent plate</td>
<td>1800</td>
<td>1000</td>
<td>88</td>
<td>68</td>
</tr>
<tr>
<td>White opaque plate</td>
<td>1800</td>
<td>10</td>
<td>100</td>
<td>72</td>
</tr>
<tr>
<td>Glass sand</td>
<td>1900</td>
<td>1400</td>
<td>27</td>
<td>88</td>
</tr>
</tbody>
</table>

Table 2. Effects of upward light and the presence of sandy substrate on the bottom of the aquarium on the blind side hypermelanosis of Japanese flounder, experiment 2.

<table>
<thead>
<tr>
<th>Bottom material of aquarium</th>
<th>Light intensity (lux)</th>
<th>Percentage of hypermelanosis fish (%)</th>
<th>Survival rate (%)</th>
<th>Specific growth rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Downward</td>
<td>Upward</td>
<td></td>
<td></td>
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<tr>
<td>Transparent plate</td>
<td>1800</td>
<td>1000</td>
<td>88</td>
<td>85</td>
</tr>
<tr>
<td>White opaque plate</td>
<td>1800</td>
<td>10</td>
<td>100</td>
<td>60</td>
</tr>
<tr>
<td>Glass sand</td>
<td>1900</td>
<td>1400</td>
<td>18</td>
<td>85</td>
</tr>
</tbody>
</table>

Table 3. Effects of upward light and the presence of sandy substrate on the bottom of the aquarium on the blind side hypermelanosis of Japanese flounder, experiment 3.

Twenty-five fish of about 4 g body weight were reared for 12 wk in experiment 2 and 20 fish of about 1 g were reared for 16 wk in experiment 3. None of the fish used in the experiments had visible pigmentation at the start. Fish with hypermelanosis were described in experiment 1.

Experiment 4 was carried out to examine the effect of bottom conditions on the enlargement of the dark pigmented area on the blind side with three aquaria prepared as follows: (1) bottom of transparent plate with strong upward light, (2) bottom covered with a 1-cm layer of transparent glass sand and strong upward light, (3) bottom covered with a 1-cm layer of coarse sand to prevent upward light (Table 4). Fish of about 25 g body weight, all of which had partial dark pigmentation on their blind side, were reared for 4 wk. Photographs of the blind side of individual fish were taken at the start and at the end of the experiment and the changes in pigmented area were compared.

RESULTS AND DISCUSSION

The results of experiment 1 are shown in Table 1. In the aquarium without sand on the bottom, all fish showed hypermelanosis on the blind side at the end of the experiment, regardless of light intensity. However, only 7 and 14% of the fish showed hypermelanosis in the aquaria with sand on the bottom (Fig. 2). Specific growth rate of the fish was lower in the aquaria with a sand bottom than those without sand.

The results of experiments 2 and 3 were similar to each other (Tables 2, 3). Namely, 100% of fish had hypermelanosis in the aquarium with the white opaque plate bottom, and 88% in the aquarium with the transparent plate bottom. In contrast to these, less than 30% of fish showed
dark pigmentation on the blind side with glass sand on the bottom in spite of the strong upward light. Specific growth rate was almost the same among treatments in both experiments.

Sand on the bottom of the aquarium was also effective for preventing enlargement of the pigmented area on the blind side (Table 4). No fish showed enlargement of their pigmented area in the aquaria with sand or glass sand on the bottom, while half of the fish enlarged their pigmented area in the aquarium with a transparent plate bottom. None of the fish died during the experimental period under any conditions, and the specific growth rate varied with treatment.

Previous papers concerning the pigmentation on the blind side in flatfish species suggested that light is the primary factor for such an abnormal coloration. However, from the results of this study, it is better to consider that, not light, but the presence of sandy substrate on the bottom of the culture tank has an important role in this phenomenon. In this study, sandy substrate on the bottom prevented the occurrence of hypermelanosis as well as its enlargement. As there is no other information that supports our results, more research is needed to determine how much the occurrence of hypermelanosis depends on sandy substrate or light. Furthermore, sand on the bottom of culture tanks is not considered to be practical, because it will easily cause deterioration of the culture environment by producing anaerobic areas. Therefore, alternative substrates will be required.

Figure 2. Photographs of the blind side of Japanese flounder at the end of experiment 1: a - Fish showing typical hypermelanosis, reared in an aquarium with a transparent acrylic plate on the bottom. b - Fish without visible pigmentation on the blind side, reared in aquarium with coarse sand on bottom.

<table>
<thead>
<tr>
<th>Bottom material of aquarium</th>
<th>Light intensity (lux)</th>
<th>Percentage of fish with hypermelanosis area enlargement (%)</th>
<th>Survival rate (%)</th>
<th>Specific growth rate (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Downward</td>
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<td></td>
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<tr>
<td>Transparent plate</td>
<td>1300</td>
<td>400</td>
<td>43</td>
<td>100</td>
</tr>
<tr>
<td>Glass sand</td>
<td>1500</td>
<td>300</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Sand</td>
<td>1300</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 4. Effects of bottom substrate, light intensity (lux), and direction on the enlargement of blind side hypermelanosis of Japanese flounder, experiment 4.
for practical usage.

Most of the pigmentation on the blind side of the flounder was shown at a margin of the trunk, caudal peduncle, and base of pectoral fin in this study (Fig. 2), and its location was the same as generally seen in cultured Japanese flounder (Seikai 1991, Yamamoto and Oda 1991).

ACKNOWLEDGMENT

We wish to thank Dr. Harry V. Daniels, North Carolina State University, for critical review of the manuscript.

LITERATURE CITED


MICROBIOLOGY OF EARLY LARVAL STAGES OF SUMMER FLOUNDER PARALICHTHYS DENTATUS GROWTH IN A RECIRCULATING WATER SYSTEM

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ABSTRACT

Flounder in early larval stages of growth can suffer high mortality in aquacultural facilities because of diseases and nutritional problems. Recent studies suggest that bacteria associated with the live feed and hatchery environments that colonize flounder can have beneficial or detrimental effects on fish health. A local commercial facility that grows summer flounder in a recirculating water system has been the subject of microbiological studies for their first four production runs. The culture of summer flounder is in its infancy and the microbiology of these fish is not well characterized. Samples of fish, tank water, and feed collected at times of change in feeding regime, metamorphosis and epistatic high mortality and disease events were analyzed for different bacteria. Growth media targeting total heterotrophs, total vibrios and Vibrio anguillarum were used to enumerate and isolate bacteria. Isolates were identified to species and/or genus. Differences and similarities in microbial community diversity and abundance at different life stages and feeding regimes were noted. The results provide an initial database for determining the role of bacteria in the onset of disease and the health of early stages of summer flounder growth.

INTRODUCTION

Aquaculture is becoming widespread and growing rapidly in northern New England, USA, and throughout the world. Among many uncertainties, one of the biggest is the incidence of disease in the fish being cultured. Diseases can cause significant fish mortalities, especially in early life stages, and such events are obviously catastrophic to any industry.

Bacterial pathogens that cause diseases in fish often enter the host with ingested food or feces and colonize the intestinal tract (Romalde et al. 1996). The bacterial diversity is enormous in fish tissue and hatchery environments (Muroga et al. 1987, Nicolas et al. 1989, Sorgeiloos 1994), making it difficult to identify pathogens or monitor for predicting the onset of disease. Prophylactic and direct treatment of diseases often involves use of antibiotics and vaccination (Cahill 1990, Joosten et al. 1995). There are many disadvantages to using antibiotics, including the potential for evolution of drug resistant strains (Kapetanaki et al. 1995), harmful effects on fish eggs (Munro et al. 1995), negative effects of seawater (Barnes et al. 1993), and complex governmental regulations. In recirculating aquaculture systems (RAS), the use of antibiotics is even more limited because of the need for establishing stable microbial communities on biofilters needed for removing wastes. An alternative approach to disease management is the use of probiotic bacteria. This approach employs use of the beneficial or benign natural microflora associated with healthy fish to establish and maintain a
microflora that can suppress potential pathogens and promote fish growth. Inhibition can be accomplished by production of toxins (Fouz et al. 1995), siderophore production (Pybus et al. 1994) or by competitive exclusion of pathogens (Smith and Davey 1993).

Great Bay AquaFarms (GBA) is a land-based RAS facility located in Portsmouth, New Hampshire, that is unique in North America for the combination of system and the cultivation of summer flounder, Paralichthys dentatus. Different aspects of the culture and diseases of two other biologically similar flatfish species—Japanese flounder and turbot—have been studied. However, little is known about the microbiology of summer flounder, especially in an RAS. An early study identified Vibrio anguillarum as a common pathogen associated with kidney tissue in dead fish, both feral and cultured, from the coasts of New Hampshire and Maine (Strout et al. 1978). More recent work in New Hampshire has focused largely on the incidence and ecology of human pathogenic Vibrio sp. in the Great Bay estuary (Jones et al. 1991, O’Neill et al. 1992, Jones et al. 1997), the source of water for GBA culture tanks. The purpose of this study was to determine the effects of intestinal microflora and the culture environmental conditions on the health and survival of larval summer flounder.

METHODS

Great Bay AquaFarms, Inc. is a commercial hatchery dedicated to the culture of summer flounder. Young larvae are grown from fertilized eggs, provided by brood fish on site, in recirculating culture tanks until the juveniles reach a size of 5–10 g (5–8 cm), at which time they are transferred to on-growing operations. The focus of this study was the fourth production run since the start of GBA in 1996, which began on 22 March 1997. The conditions in the rearing tanks were subject to many changes during the 100-day study, including feeding regime, tank disinfection and cleaning, and movement of fish between tanks. Larvae were fed algae and rotifiers in eight larval rearing tanks for the first 20 days, then Artemia nauplii followed by enriched Artemia for the next ~20 days prior to metamorphosis. After 35–40 days, metamorphosed fish were transferred to 12 weaning tanks and fed artificial feed weaning diets.

Samples for microbiological analyses were taken from different tanks on a weekly basis. The justification for not sampling specific tanks in a consistent fashion was that fish reared in specific larval tanks were mixed into different weaning tanks, and some weaned fish were remixed between weaning tanks. These factors made it difficult to conduct analyses under controlled experimental conditions, so sampling was eventually focused on tanks with clearly distinguishable healthy and unhealthy fish. Sampling for sick and healthy fish involved paired fish samples from the same tank on any given sample date. Tank water temperature remained relatively constant, ranging from 16.4 to 19.9°C. Salinity ranged from 18 to 32 ppt.

Accurate estimates of fish densities in all rearing tanks were not available, so percent survival could not be calculated. The densities in tanks ranged from 100,000 to 200,000 fish in tanks not affected by disease, and substantially lower in tanks where disease had been present. Assessment of the degree of mortality of fish was based on quantifying dead fish on a daily basis in each tank. Sick fish were identified by altered pigmentation and feeding behavior.

Fish, feed, and water samples were collected using sterile containers and transported on ice to the Jackson Estuarine Laboratory for analysis. Samples were processed within 2 h of collection. The fish were anesthetized, measured, surface sterilized, and ground with a mortar and pestle. Tissue, water and feed samples were diluted in sterile buffered peptone water and aliquots from a range of dilutions were collected onto membrane filters and placed on different agar media. Total heterotrophs were cultured from 2216E medium, total vibrios were cultured from thiosulfate–citrate–bile salts–sucrose (TCBS) medium, and V. anguillarum was cultured from VAM agar (Alsina et al. 1994), all incubated at room temperature (18–22°C). Focus was placed on vibrios because they have been shown to be important in other aquacultural settings both as agents of disease and as beneficial 'probiotic' organisms, and are the dominant bacteria in the intestines of larval and juvenile marine fish (Murogo et al. 1987). In addition, the source water from the Great Bay estuary is known
to have abundant vibrios (Jones et al. 1997), particularly during the time of the production run under study.

Dominant and unique colonies on all plates were identified using an identification scheme similar to Muruga et al. (1987). Colony morphology and color plus carbohydrate utilization reactions were noted. Isolated colonies were re-streaked onto TSA medium and the colony morphology and color plus pigment production were noted for re-grown isolates. Cell morphology, motility, oxidase reaction, and gram reaction were determined. Gas production, growth, and acid production with single carbon sources were determined along with amino acid decarboxylase reactions. Growth at different salinities and temperatures were also used to identify bacterial isolates.

RESULTS

Figure 1 illustrates the dynamics of mortality in all of the larval-rearing and weaning tanks. The first spike in fish mortality occurred within a week after feeding on Artemia that began on day 21. The salinity in the tanks dropped from ~25 ppt to 18 ppt between days 27 and 29, when 9.7 cm of rain fell in 48 h, dropping the salinity of the source estuarine water in the process. A consistent, medium level of mortality persisted in some of the tanks during the first 3 wk of weaning diet, followed by a slight drop in mortality rate. The persistent mortality in tanks after day 60 was nearly all associated with delayed mortality in tanks that had shown good survival early in the weaning period.

Total vibrio concentrations increased dramatically in the rearing tanks after day 20 when Artemia feeding began (Fig. 2). The highest concentration of vibrios occurred during the time when the first heavy mortality occurred. Total heterotroph concentrations increased only after day 30, following the spike in vibrio numbers. A small
peak in concentrations of putative (yellow colonies on VAM agar) *V. anguillarum* coincided with the total vibrio peak (data not shown).

Relatively low concentrations of all bacteria were apparent in the tank water from day 45 to day 69, followed immediately by a second large peak in total vibrio concentrations (Fig. 2). This second vibrio peak occurred at the beginning of June, when estuarine temperatures began to increase above 15°C and microbial communities dramatically change, typically characterized by significant increases in the diversity and population sizes of *Vibrio* sp. (O’Neill et al. 1992). The salinity in the culture tanks also increased from the low of 18 ppt on day 29 to 32 ppt on day 70. The second peak in total vibrios also corresponded with the onset of another incidence of elevated mortality, nearly all of which occurred in tanks that had relatively healthy fish early in the weaning phase. Thus, the microbial dynamics in the fish tanks had some relationship to the occurrence of disease/mortality in the fish. Peaks in total heterotrophs, total vibrios, and *V. anguillarum* also occurred on day 90 post-hatch.

Figure 2. Bacterial concentrations in larval and weaning tanks. cfu = colony-forming units.

Figure 3. Bacterial concentrations in "healthy" fish. cfu = colony forming units; DW = dry weight.
The delay in onset of increases in total heterotroph populations relative to total vibrios was not seen in the healthy fish. Simultaneous peaks in concentrations for both total heterotrophs and total vibrios occurred on days 33, 77, and 95 (Fig. 3), a three-peak pattern similar to the microbial dynamics in the rearing tanks. A comparison of total vibrios in healthy and unhealthy fish taken from the same tanks on five sample dates from day 41 through day 83 showed unhealthy fish had higher concentrations of total vibrios than healthy fish on four of the five sample dates, with overall average concentrations in unhealthy fish >10 times higher than in unhealthy fish (Fig. 4). In contrast, total heterotroph concentrations were higher in healthy fish in the first four of the five samples (Fig. 5). Concentrations of total heterotrophs in unhealthy fish were much higher in the fifth sample and the overall averages for unhealthy and healthy fish were similar. The TCBS medium for recovery of total vibrios recovered higher numbers of bacteria than the 2216E medium.

Predominant bacterial isolates from live feed and tank water were identified to species and/or genus (Table 1). The results are biased toward isolation of vibrios because of the isolation media used. The feed had a more predominant presence of *Vibrio* sp. although vibrios occurred in both the feed and the water. Numerous bacteria were present in the rotifers and the *Artemia*. The tank water contained many species, with major changes in composition accompanying changes in the feed and tank environment. *Moraxella* sp. was the most consistently prevalent organism. Otherwise, there were few similarities between isolates from the tank water and the feeds.

**DISCUSSION**

Bacterial numbers and species composition varied widely during the early stages of sum-
<table>
<thead>
<tr>
<th>Age:</th>
<th>1-20 days</th>
<th>21-33 days</th>
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<tbody>
<tr>
<td>Feed:</td>
<td>Rotifers/algae</td>
<td>Artemia</td>
</tr>
<tr>
<td><strong>Live feed</strong></td>
<td>Moraxella sp.</td>
<td>Moraxella sp.</td>
</tr>
<tr>
<td></td>
<td>Vibrio sp.</td>
<td>Vibrio sp.</td>
</tr>
<tr>
<td></td>
<td>Vibrio sp. III</td>
<td>Vibrio sp. III</td>
</tr>
<tr>
<td></td>
<td>V. alginolyticus</td>
<td>V. damsela</td>
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<tr>
<td></td>
<td>Enterobacteriaceae</td>
<td>Enterobacteriaceae</td>
</tr>
<tr>
<td></td>
<td>Flavobacterium sp.</td>
<td>V. parahaemolyticus</td>
</tr>
<tr>
<td></td>
<td>Aeromonas sp.</td>
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<thead>
<tr>
<th>Tank water</th>
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</thead>
<tbody>
<tr>
<td>Acinetobacter sp.</td>
<td>Argobacterium sp.</td>
<td></td>
</tr>
<tr>
<td>Aeromonas sp.</td>
<td>Enterobacteriaceae</td>
<td></td>
</tr>
<tr>
<td>Moraxella sp.</td>
<td>Moraxella sp.</td>
<td></td>
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<tr>
<td>Pseudomonas sp. III/IV</td>
<td>Pseudomonas sp. III</td>
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</tr>
<tr>
<td>V. fluvialis</td>
<td>Vibrio sp.</td>
<td></td>
</tr>
<tr>
<td>V. anguillarum</td>
<td>V. alginolyticus</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V. parahaemolyticus</td>
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Table 1. Bacterial species in live feed and tank water for summer flounder at Great Bay Aquafarms.

Summer flounder growth at GBA. Factors that could have affected the abundance and succession of bacterial species include the microflora of the live feed, nutritional differences in feeds, fish growth and changes in physiology, seasonal changes in source water, the transfer of fish between tanks, and environmental conditions in the culture system. Others have reported similar microbial community dynamics and species composition in a variety of cultured finfish (Campbell and Buswell 1983, Muroga et al. 1987, Nicolas et al. 1989, Sorgeloos 1994). The similarity in species diversity and abundance of different bacteria with other studies suggests that there are no unique microbiological characteristics of summer flounder or northern New England culture conditions.

There were peaks in both total heterotrophs and total vibrios that corresponded roughly with elevated mortality episodes. These peaks were observed in both the tank water and the fish tissue. Munro et al. (1995) clearly demonstrated that *V. anguillarum* is a pathogen of larval turbot, and Nicolas et al. (1989) reported a domination of the rotifer microflora by *Vibrio* sp. associated with high mortality of larval turbot. In this study, vibrios were associated with unhealthy fish, but also with healthy fish and their tank water, even in high numbers at certain times during the early growth stages of the summer flounder. However, fish considered healthy were present in tanks that also contained unhealthy fish, making cross contamination highly probable. The simple presence of vibrios is apparently not a clear indication of disease potential in summer flounder. However, the general trend of higher numbers of total vibrios in unhealthy compared to healthy fish suggests that total vibrio counts may provide a better reflection of disease than total heterotroph counts. The earlier occurrence of a peak in total vibrios compared to total heterotrophs in tank water just prior to the first episode of high mortality suggests that monitoring total vibrio concentrations in tank water may be useful in predicting disease.

The microflora of the feed and culture environment was dominated by *Vibrio* sp. The use of traditional culture methods provides results that are strongly influenced by the composition of the isolation media and the isolation conditions used. Because heterotrophs other than vibrios were also present, the lower numbers of bacteria recovered on TCBS medium compared to TCBS suggests that a better medium for recovery of total heterotrophs is needed. Others have reported that *Vibrio* sp. are the dominant bacteria in the intestines of larval and juvenile marine fish (Muroga et al. 1987). The use of TCBS and VAM agars in this study anticipated
this, biasing the results in order to provide isolates that may be useful in future studies on probiotic bacteria and pathogens. The phylogeny of the fish pathogens and general microflora would be better determined using molecular methods (Amann et al. 1995), although this was clearly not the purpose of this study.

There were a few apparent differences between the fish and tank water microflora, as well as in the abundance of bacteria in healthy and unhealthy fish. These preliminary observations suggest that detection of the selection of bacterial species in fish both during colonization of the fish intestine from live feed and during disease episodes may be difficult using the methods in this study. More detailed data on abundance of bacterial species and speciation of isolates from healthy and unhealthy fish during the targeted production run and other runs at GBA are currently being analyzed, and the results will hopefully provide clearer results for a future publication. Further work and refinement of methods are needed to more clearly identify probiotic and pathogenic bacteria. This study also suggests that further work should be done to better understand the relationship between the microflora of the live feed and the eventual colonization of larval and juvenile fish.

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