Phenol-water and trichloroacetic acid extracts of Vibrio anguillarum were both antigenic and immunogenic when injected into coho salmon (Oncorhynchus kisutch). Cross-protection against virulent Vibrio anguillarum was observed with whole-cell bacterins and phenol-water extracts prepared from two serologically distinct groups. Gel diffusion studies indicated that intact somatic antigens were serotype specific. Breakdown products or impurities in serotype specific phenol-water extracts displayed antigenic cross-reactivity. Generation times for Vibrio anguillarum LS 1-74 were shorter than for MAN 1669. Both isolates reached higher levels of growth at 18 than at 30 C. Spheroplasting and lysis was more characteristic of Vibrio anguillarum MAN 1669. An artificial, waterborn challenge was developed for Vibrio anguillarum LS 1-74. Similar challenges with members of the serologically unrelated group (Vibrio anguillarum
MAN 1669 and MSC 2-75) were unsuccessful. Studies with motile and non-motile strains of *Vibrio anguillarum* indicated that the flagellar antigen is not required for immunogenicity.

An economical, efficacious vaccine delivery system for immunizing fish has been developed which employs a high-pressure liquid spray apparatus operated at 6.3 to 7.0 kilograms per square centimeter (90 to 100 pounds per square inch). Bacterin consisting of formalin-killed *Vibrio anguillarum* culture plus 0.15 percent bentonite was both antigenic and immunogenic when sprayed on various size coho salmon. The technique, referred to as spray vaccination, was found to confer higher levels of immunity against virulent *Vibrio anguillarum* than oral vaccination. Immune serum raised by the technique was protective in passively immunized coho. Bacterins containing bentonite were found to be most effective when adjusted to pH 3.0. Spray vaccinated fish possessed detectable agglutinating antibody after 112 days and were protected against challenge after 125 days. *Aeromonas salmonicida* and Bacterial Kidney Disease bacterins induced increased humoral antibody production in spray vaccinated fish.
Development of a New Vaccine Delivery System for Immunizing Fish and Investigation of the Protective Antigens in Vibrio anguillarum

by

Rowan Wayne Gould

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# TABLE OF CONTENTS

## INTRODUCTION

1

## LITERATURE REVIEW

3

- Immunization of Fishes
  - Injection of Vaccine Preparations
  - Oral Vaccination
  - Immunization by Vacuum Infiltration
  - Immunization by Hyperosmotic Immersion
  - Immunization with Attenuated Strains

3

- *Vibrio anguillarum*, the Causative Agent of Vibrosis in Salmonids

22

## MATERIALS AND METHODS

26

- Culture Media
- Bacterial Strains
  - Description of Cultures
  - Preservation of Cultures
- Growth Curves for *Vibrio anguillarum*
  - Comparison of *Vibrio anguillarum* LS 1-74 and MAN 1669 at 18 and 30 C
  - Tryptone Glucose Yeast Broth Growth Curves for *Vibrio anguillarum* MSC 1-73
  - Mean Generation Times for Various Isolates of *Vibrio anguillarum*
- Bacterial Antigen Production
  - Mass Culture Technique
  - Injectable Antigens
  - Oral Antigens
  - Spray Antigens
- Production of Lyophilized Sonicate of *Vibrio anguillarum*
  - Sonicated Antigen for Gel Diffusion
- Extraction Techniques
  - Cell Wall Production
  - Trichloroacetic Acid Extractions
  - Phenol-water Extractions
- Toxicity of Extraction Products for Mice
- Serological Techniques
  - Bacterial Identification by Slide Agglutination
  - Harvesting Fish Serum
RESULTS

Electron Micrographs of *Vibrio anguillarum* LS 1-74 and MSC 2-75

Growth Studies

Comparison of *Vibrio anguillarum* LS 1-74 and MAN 1669 growth curves at 18 and 30 C

Tryptone Glucose Yeast Broth Growth Curves for *Vibrio anguillarum* MSC 1-73

Toxicity of Extraction Products in Mice

Determination of the LD$_{50}$ for Injected *Vibrio anguillarum* MSC 2-75 Injected into Coho Salmon

Determination of the Requirement for *Vibrio anguillarum* Flagella in Bacterin Preparations

Immunodiffusion Studies on the Whole-Cell Antigens and Phenol-water Extracts of *Vibrio anguillarum* LS 1-74 and MSC 2-75

Protection of Coho Salmon by an Injected Phenol-water Extract of *Vibrio anguillarum* LS 1-74

Cross-Protection of Various Bacterins and Extraction Products Produced from Selected Isolates of *Vibrio anguillarum*

Agglutinating Antibody Titers and Degree of Protection Elicited by Injection of Graded Amounts of *Vibrio anguillarum* LS 1-74 Lyophilized Sonicate and Boivin Extract into Coho Salmon
Efficacy of the Spray Vaccination Technique for Administration of *Vibrio anguillarum* LS 1-74 Bacterin 88
Effect of Different *Vibrio anguillarum* LS 1-74 Bacterin Preparations for Spray Vaccination 94
Longevity of Antibody Response in Coho Salmon Spray Vaccinated with *Vibrio anguillarum* LS 1-74 Bacteria Containing 0.15 percent Bentonite at pH 9.0 95
Effect of pH on *Vibrio anguillarum* LS 1-74 Spray Vaccination Preparations Containing 0.15 percent Bentonite 97
Passive Immunization of Coho Salmon against Vibriosis by Injection of Immune Serum from Coho Salmon Immunized by Spray Vaccination 99
Comparison of Agglutinating Antibody and Protection against *Vibrio anguillarum* LS 1-74 in Groups of Coho Salmon Vaccinated Separately and in Combination by the Spray and Oral Method 101
Polyvalent Bacterins Delivered by Spray Vaccination 103
Spray Vaccination of Coho Salmon with Preparations Containing Heat-Killed Bacterial Kidney Disease Bacteria 105
Spray Vaccination with Different Preparations of Formalin-killed *Aeromonas salmonicida* SS-70 Immunization of Production Coho Salmon against *Aeromonas Salmonicida* by the Spray Vaccination Method 110

DISCUSSION 116

SUMMARY AND CONCLUSIONS 130

BIBLIOGRAPHY 133
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Electron micrograph of a <em>Vibrio anguillarum</em> LS 1-74 cell in longitudinal section showing polar insertion of flagellum and a small area which may be a part of the polar plate.</td>
<td>55</td>
</tr>
<tr>
<td>1B</td>
<td>Electron micrograph of a <em>Vibrio anguillarum</em> LS 1-74 cell in longitudinal section showing a portion of the polar plate.</td>
<td>55</td>
</tr>
<tr>
<td>1C</td>
<td>Electron micrograph of a <em>Vibrio anguillarum</em> LS 1-74 cell in both longitudinal and cross-section showing the polar plate.</td>
<td>55</td>
</tr>
<tr>
<td>2A</td>
<td>Electron micrograph of two <em>Vibrio anguillarum</em> MSC 2-75 cells in longitudinal sections.</td>
<td>57</td>
</tr>
<tr>
<td>2B</td>
<td>Longitudinal and cross-section of <em>Vibrio anguillarum</em> MSC 2-75 cells.</td>
<td>57</td>
</tr>
<tr>
<td>3</td>
<td>Comparison of growth of two strains of <em>Vibrio anguillarum</em>, LS 1-74 and MAN 1669, as measured by viable count at 18 C.</td>
<td>58</td>
</tr>
<tr>
<td>4</td>
<td>Comparison of growth of two strains of <em>Vibrio anguillarum</em>, LS 1-74 and MAN 1669, as measured by viable count at 30 C.</td>
<td>59</td>
</tr>
<tr>
<td>5</td>
<td>Viable cell count versus optical density at 18 and 30 C for <em>Vibrio anguillarum</em> LS 1-74.</td>
<td>61</td>
</tr>
<tr>
<td>6</td>
<td>Viable cell count versus optical density at 18 and 30 C for <em>Vibrio anguillarum</em> MAN 1669.</td>
<td>62</td>
</tr>
<tr>
<td>7</td>
<td>Growth curve of <em>Vibrio anguillarum</em> MSC 1-73 as measured by viable count in Tryptone Glucose Yeast broth at 18 C.</td>
<td>65</td>
</tr>
<tr>
<td>8A</td>
<td>Shadow-cast electron micrograph of non-motile <em>Vibrio anguillarum</em> LS 1-71 FF.</td>
<td>70</td>
</tr>
</tbody>
</table>
8B  Shadow-cast electron micrograph of motile *Vibrio anguillarum* LS 1-74.

9  Immunodiffusion comparison of sonicated cellular antigens of the motile *Vibrio anguillarum* LS 1-74 and the non-motile *Vibrio anguillarum* LS 1-71 FF against rabbit antiserum for *Vibrio anguillarum* LS 1-74.

10A  Immunodiffusion comparison of sonicated cellular antigens and trichloroacetic acid extracts of *Vibrio anguillarum* LS 1-74 (LS) and *Vibrio anguillarum* MSC 2-75 (MSC) against rabbit antiserum for *Vibrio anguillarum* LS 1-74.

10B  Immunodiffusion comparison of sonicated cellular antigens and trichloroacetic acid extracts of *Vibrio anguillarum* LS 1-74 and *Vibrio anguillarum* MSC 2-75 against rabbit antiserum for *Vibrio anguillarum* MSC 2-75.

11  Immunodiffusion comparison of sonicated *Vibrio anguillarum* LS 1-74 (LS) and *Vibrio anguillarum* MSC 2-75 (MSC) antigens as well as different trichloroacetic acid extracts of *Vibrio anguillarum* LS 1-74 and *Vibrio anguillarum* MSC 2-75 against side-by-side wells of corresponding rabbit anti-*Vibrio anguillarum* serum.

12  Cumulative loss in two groups of 1975-brood coho salmon containing approximately 60,000 fish each at Siletz River Salmon Hatchery.
### LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Isolates of <em>Vibrio anguillarum</em>, <em>Aeromonas salmonicida</em>, and the agent of Bacterial Kidney Disease used in this study.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>27</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Dry weight determinations for each of two <em>Vibrio anguillarum</em> LS 1-74 whole-cell bacterins and two <em>Vibrio anguillarum</em> LS 1-71 FF bacterins.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td>----------------------------------------------------------------------------------------------------------------</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Mean generation times (G) for <em>Vibrio anguillarum</em> isolates grown in Brain Heart Infusion or Tryptone Glucose Yeast broth.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt; values in mice to cell wall extracts of <em>Aeromonas salmonicida</em> SS-70, <em>Vibrio anguillarum</em> LS 1-74 and <em>Vibrio anguillarum</em> MSC 2-75 administered intraperitoneally.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.</td>
<td>----------------------------------------------------------------------------------------------------------------</td>
<td>66</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Mortalities and mean day-to-death for groups of coho salmon injected with different dilutions of virulent <em>Vibrio anguillarum</em> MSC 2-75.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Comparison of protection against virulent <em>Vibrio anguillarum</em> LS 1-74 conferred by oral and injected bacterins derived from motile <em>Vibrio anguillarum</em> LS 1-74 and non-motile <em>Vibrio anguillarum</em> LS 1-71 FF.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>77</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Determination of protection against virulent <em>Vibrio anguillarum</em> LS 1-74 and agglutinating antibody titers conferred in coho salmon injected with partially purified phenol-water extracts of <em>Vibrio anguillarum</em> LS 1-74 cell walls.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>80</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Efficacy of injected bacterins prepared from various isolates of <em>Vibrio anguillarum</em> against challenge by virulent <em>Vibrio anguillarum</em> LS 1-74.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>82</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table</th>
<th>Efficacy of injected bacterins prepared from various isolates of <em>Vibrio anguillarum</em> MSC 2-75.</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.</td>
<td>-------------------------------------------------------------------------------------------------------------</td>
<td>83</td>
</tr>
</tbody>
</table>
10. Determination of protection against virulent Vibrio anguillarum LS 1-74 conferred in coho salmon by injected chemical extracts of Aeromonas salmonicida SS-70, Vibrio anguillarum LS 1-74 and Vibrio anguillarum MSC 2-75.

11. Determination of protection against virulent Vibrio anguillarum MSC 2-75 conferred in coho salmon by injected chemical extracts of Aeromonas salmonicida SS-70, Vibrio anguillarum LS 1-74 and Vibrio anguillarum MSC 2-75.

12. Agglutinating antibody titers in coho salmon induced by injecting graded amounts of Vibrio anguillarum LS 1-74 lyophilized sonicate.

13. Level of agglutinating antibody titer and protection conferred against virulent Vibrio anguillarum LS 1-74 in coho salmon injected with trichloroacetic acid extract of Vibrio anguillarum LS 1-74.

14. Protection against virulent Vibrio anguillarum LS 1-74 and production of agglutinating antibody titers in coho salmon sprayed with homotypic bacterins in conjunction with fluorescent dye.

15. Comparison of agglutinating antibody and protection against virulent Vibrio anguillarum LS 1-74 in coho salmon sprayed with various concentrations of Vibrio anguillarum LS 1-74 bacterin in conjunction with fluorescent dye.

16. Effect of different Vibrio anguillarum LS 1-74 bacterin preparations for spray vaccination.

17. Longevity of antibody response in coho salmon sprayed with Vibrio anguillarum LS 1-74 bacterin (pH 9.0) containing 0.15 percent bentonite.

18. Agglutinating antibody produced in coho salmon sprayed with Vibrio anguillarum LS 1-74 bacterins containing 0.15 percent bentonite adjusted to different pH levels.
19. Passive immunization of coho salmon against vibriosis by injection of immune serum from coho salmon immunized by spray vaccination.

20. Comparison of agglutinating antibody and protection against *Vibrio anguillarum* LS 1-74 in groups of coho salmon orally vaccinated, spray vaccinated, and vaccinated by both methods in combination.

21. Geometric mean agglutinating antibody titers against constituent antigens of various bacterin combinations included in monovalent or polyvalent spray vaccination preparations.

22. *Aeromonas salmonicida* SS-70 challenge of coho salmon spray vaccinated with different bacterin preparations.

23. Anti-Baceterial Kidney Disease bacteria agglutinating antibody induced in coho salmon by spray vaccination with bacterins containing bentonite.

24. Agglutinating antibody production in coho salmon and rainbow trout spray vaccinated with different preparations of *Aeromonas salmonicida* SS-70 bacterin.
DEVELOPMENT OF A NEW VACCINE DELIVERY SYSTEM FOR IMMUNIZING FISH AND INVESTIGATION OF THE PROTECTIVE ANTIGENS IN VIBRIO ANGUILLARUM

INTRODUCTION

Disease has had a major influence on the success or failure of many fish cultural operations, both public and private. In the past, disease problems have been handled primarily by chemical or antibiotic treatments. In some cases effective management of stocks has proven beneficial. The emergence of drug resistance in bacterial fish pathogens, as well as concern that indiscriminate use of antibiotics might transfer drug resistance factors to human pathogens has necessitated the search for other disease prevention techniques. Vaccination is a logical alternative. The method is effective in higher animals and, in addition, is prophylactic rather than therapeutic. The problems faced in fish vaccine development have consisted primarily of developing efficacious preparations administered to large populations in an economic manner.

In the last few years the advent of a growing salmonid mariculture industry has increased the need for various means of controlling fish vibriosis. This study examined the protective antigens of the etiological agent, Vibrio anguillarum. Efforts were also directed toward evaluating a new technique for delivering fish vaccines. The objective was to provide information leading to the best
methods for preparation and administration of the vaccines against this disease. A consequence of these efforts was extended application of the delivery system to other troublesome fish pathogens.

The techniques used to study bacterial antigens consisted of extraction and immunological examinations, followed by in vivo immunization and challenge against the specific pathogen. Information was obtained concerning: (1) the cross-protective nature of different serotypes of *V. anguillarum*, (2) the requirement for flagellar antigens in vaccine preparations, and (3) the specific components which play a role in immunogenicity. Similar techniques were used to determine the effects and potency of different compounds, conditions, and fish pathogens related to a new fish vaccine delivery system, spray vaccination.
LITERATURE REVIEW

Immunization of Fishes

The immune response in fishes has been of interest to investigators since the turn of the century. Recent reviews by Corbel (1975), Finn (1970), and Ridgeway et al. (1966) have concluded that immune mechanisms in fishes are not dissimilar to those found in other vertebrates. Components of non-specific, cell-mediated and humoral antibody systems have all been demonstrated. A major difference between the immune response of fishes and those of higher vertebrates is dependence on environmental temperature. Avtalion et al. (1973) provided an extensive review on the subject.

Much of the work on the immune response in fishes is related to protective immunization against disease (Corbel, 1975; Anderson, 1974; Dorson, 1972a; Klontz and Anderson, 1970; Finn, 1970; Snieszko, 1970; Muroga and Equsa, 1969b; Ridgeway et al., 1966). The recipient species, target disease, and mode of vaccine application are varied in the literature. Generally, the commercially important bacterial pathogens and food fishes have received the most emphasis. The delivery systems employed include parenteral injection, feeding, vacuum infiltration, and immersion.
Injection of Vaccine Preparations

It has long been known that fish produce agglutinins in response to bacterial antigens. Babes and Riegler (1903) noticed that blood from crucians \( \textit{Carassus carassius} \) and perch \( \textit{Perca fluviatilis} \) agglutinated \textit{Proteus piscidus versicolor} just 24 h after infection with the homologous organism. This phenomenon was noticed even when the blood was diluted 1:50. They reported that the ability increased if the fish survived for longer periods. Bergman (1911) and Aaser (1925) were both able to detect agglutinating activity against \textit{Vibrio anguilla} in northern pike \( \textit{Esox lucius} \).

The immunity of fishes to subsequent reinfection after either having received injections or survived epizootics was the subject of many early investigations. Schaperclaus (1965) reported that in 1929, his injections of polyvalent strains of \textit{Aeromonas hydrophila} proved to be effective in protecting carp \( \textit{Cyprinus carpio} \) from infection by bacterial dropsey. Nybelin (1935) demonstrated agglutinating antibody in eels \( \textit{Anguilla anguilla} \) injected with pathogenic \textit{Vibrio} at 18 C. He found no response to injection at 8 C. Both Pliszka (1939a, b) and Snieszko (1954) demonstrated that carp produce agglutinating antibody when heat-killed \textit{A. hydrophila} is injected intraperitoneally. Both workers demonstrated the presence of different serotypes of \textit{A. hydrophila} based on cross-agglutination studies. Titers as high as 1:10,240 were obtained at 22 C after 24 d. These titers were
higher than those reported by Sniezko et al. (1938) when killed *A. hydrophila* was injected into rabbits. Pliszka (1939b) found that antibody production is very limited at 9-11 C. When fish are injected and held at low temperature for 6 wk and then placed at 20 C, high titers are subsequently observed in 3 wk. Pliszka also demonstrated that both heat-killed and phenol-killed bacterins are equally effective. Smith (1940) injected heat-killed *Aeromonas salmonicida* into carp, brown trout (*Salmo trutta*) and rainbow trout (*Salmo gairdneri*) at 10 C. He demonstrated increased agglutinating antibody in all three species. Smith pointed out that carp appear to be immune to trout furunculosis.

Parenteral immunization of rainbow trout was examined in depth by Post (1963). He injected fish intramuscularly or intraperitoneally with either saline or Freunds adjuvant suspensions of killed *A. hydrophila*. Post noticed no major difference in agglutinating antibody response when fish were injected by either route. Detectable antibody was observed with two injections of saline suspension or one injection of Freunds suspension. Immunized fish were resistant to 1 LD$_{90}$ (90% lethal dose) challenge of virulent bacteria if a $\geq 1:4$ titer was detectable in the blood.

Krantz et al. (1963, 1964a) found that brook trout (*Salvalinus fontinalis*) and brown trout held at 11 C produce agglutinating antibody against mineral oil suspensions of formalin-killed *A. salmonicida* at
a slower rate than rabbits receiving the same dose. Peak titers were reached after 1 mo in rabbits and in trout after 3 mo. Titers (1:1,280) achieved with the adjuvant preparations were stable for at least 24 mo. Titers (1:160) in response to saline suspensions were only present for 3 mo. Krantz also demonstrated protection against injected virulent *A. salmonicida* in fish receiving bacterin plus adjuvant. No protection was observed in fish receiving bacterin alone.

Japanese scientists have contributed considerable information on injectable preparations for immunizing fish against disease. Saito et al. (1964) and Hayashi et al. (1964b) both examined injectable bacterins for the control of vibriosis. Both investigators reported a high level of immunity. Hayashi et al. (1964b) demonstrated a correlation between level of agglutinating antibody and level of protection. They also noted that increased challenge levels resulted in decreased protection. Saito et al. (1964) injected both heat-killed and phenol-killed *Vibrio piscium* bacterins. Maximum titers (1:800) were noted in rainbow trout after 2 mo. Antibody levels dropped to 1:200 after 3 mo.

Spence et al. (1965) demonstrated the protective nature of immune serum components by passively immunizing juvenile coho salmon with rainbow trout anti-*A. salmonicida* serum. Specific serum components other than agglutinating antibody obtained in response to
an injected bacterin were studied by Ridgeway et al. (1966). They reported 12 of 20 rainbow trout injected with heat-killed "redmouth" bacteria had saline agglutinins detectable at titers of 1:4 and 1:8. All 20 had antibodies detectable with a Coombs-type anti-rainbow trout globulin.

Just recently, Anderson and Nelson (1974) reported investigations on injected "redmouth" bacterins. They inoculated rainbow trout subcutaneously with 0.1 ml of 0.85% saline suspension containing 1 mg lyophilized vaccine. Challenge by injection indicated that protection was evident in 1 wk. Fish were still protected after 3 mo. This observation was confirmed by "natural" exposure to dead and moribund fish.

In studies to determine the class of serum protein resulting from increased antibody production, Summerfelt (1966) injected golden shiners (Notemigonus crysoleucas) with formalin-killed *A. hydrophila*. Antibody titers of 1:1,280 were reached in 59 d. Weekly injections of $1 \times 10^7$ killed bacteria were administered. Injected challenge with different levels of virulent *A. hydrophila* demonstrated increasing protection with time.

Mikryakov et al. (1967) used anti-*A. hydrophila* immune carp serum to examine its effect on *in vitro* cultures of *A. hydrophila*. In immune serum, there was a pronounced suppression of the homologous bacteria. Bacterial growth approached that of the controls as
the serum dilution approached 1:320. The immune serum had little effect on *Pseudomonas fluorescens*.

Goncharov and Mikryakov (1968) found that infectious dropsey in carp was prevented by injection of killed *A. hydrophila* and *Pseudomonas fluorescens*. Natural challenge resulted in 18% loss in the vaccinated groups and 80-84% in the controls. Goncharov (1971) found that a booster vaccination of killed *A. hydrophila* produced increased titers in carp. Vladimirov (1969) injected killed *A. hydrophila* into trout and carp in both a saline and Freund's adjuvant suspension. He noted markedly increased titers in both preparations, higher with Freund's adjuvant. He assumed, but did not confirm, that the animals were protected. Further work on injected *A. hydrophila* bacterins was carried out by Schaperclaus (1970). He recommended the simultaneous intraperitoneal injections of chloramphenicol and a killed bacterin containing mixed serotypes at the onset of disease. Schaperclaus pointed out that bacterial constituents or growth products may be converted to effective vaccines by extraction or inactivation.

*Vibrio anguillarum*, the causative agent of fish vibriosis has received considerable investigation as an injected vaccine. Novotny (1973) reported injected bacterins to be of considerable benefit to salmonid aquaculture. Tanji (1967), in studies with both *Vibrio* and *Aeromonas liquefaciens*, found that injected formalin-killed bacterins
produced increased agglutinating antibody in both rainbow trout and eels. Repeated attempts to demonstrate precipitating antibody failed. Muroga and Equsa (1969a) and Muroga (1975) found that eels are capable of producing titers of 1:800 to 1:1,600 when injected with merzonin-killed _V. anguillarum_ at 15 C to 27 C. No measurable titer was produced at 11 C. Adjuvant did not appear to be a major benefit. Fletcher and White (1973) found, using passive haemagglutination, that injected adjuvant preparations of heat-killed _V. anguillarum_ produced high, lasting titer in plaice (_Pleuronectes platessa_ L.). Only small amounts of antibody could be detected in cutaneous mucous and intestinal washings. Bacterin alone in saline did not produce an equivalent response and antibody was not detectable after 1 yr. Harrell et al. (1975) demonstrated by passive immunization and antibody absorption that components in anti- _V. anguillarum_ serum are important in protection against vibriosis. They also were able to passively protect rainbow trout for up to 2 mo with both rabbit and fish immune serum. A comparison of bacterin production techniques indicated that heat-killed preparations elicited higher agglutinating antibody than formalin-killed preparations. Abe (1972) found that injected trichloroacetic acid (TCA) extracts of _V. anguillarum_ were immunogenic in salmonids against challenge by virulent _V. anguillarum_. Rohovec et al. (1975) demonstrated that injected formalin-killed whole-cell _V. anguillarum_ protected coho salmon from vibriosis when
challenged naturally at a saltwater impoundment endemic for the disease. Antipa (1976) reported considerable success in protecting chinook salmon by intraperitoneal injection of heat-killed *V. anguillarum*. Heat-killing was more effective than formalin-killing.

Because of its economic importance, *Aeromonas salmonicida* has been the subject of many injected bacterin studies. Anderson and Klontz (1970, 1973) injected small amounts of alum-precipitated, sonicated antigen into albino rainbow trout. Precipitating and agglutinating antibody was detected in 5 wk. Maximum titers were reached in 8 wk. A second inoculation resulted in decreased lag time and more rapid rise in antibody titer. Large increases in production of antibody were not observed. Paterson and Fryer (1974a) indicated that 100% of the coho salmon (*Oncorhynchus kisutch*) injected with formalin-killed *A. salmonicida* in Freund's adjuvant showed a response after 6 wk. Fish as small as 1.2 g produced antibodies 4 wk after injection. These fish were protected from injected virulent *A. salmonicida*. Paterson and Fryer (1974b) also examined the effect of dose and temperature on the antibody response of coho. They found that injection of *A. salmonicida* TCA extract produced responses at 6.7 C, 12.2 C, and 17.8 C in 4, 2 and 1 wk, respectively. As little as 1 μg was antigenic when injected with or without adjuvant. The injected extract was not toxic for fish, but killed mice at an LD<sub>50</sub> of 535 μg. Hara et al. (1976) demonstrated that injected saline or Freund's
adjuvant suspensions of formalin-killed \textit{A. salmonicida} are immunogenic in cultured Amago (\textit{Oncorhynchus rhodurus}) and Yamame (\textit{Oncorhynchus masou}). Agglutinating antibody responses were similar to those noted by Krantz et al. (1964a).

Parenteral injection has been studied for fish diseases such as kidney disease and columnaris. Evelyn (1971) inoculated immature sockeye salmon (\textit{Oncorhynchus nerka}) with heat-killed, adjuvant-suspended cells of the causative agent of bacterial kidney disease. Agglutinating antibody was produced and remained detectable for 16 mo. An anamnestic response was evident when, after 13 mo, a second injection was given. Titers of 1:2,560 and 1:10,240 were observed.

Fujihara and Nakatani (1971) were able to elicit titers of 1:5,120 in rainbow trout vaccinated with Freund's adjuvant suspensions of killed \textit{Flexibacter columnaris}. Schachte and Mora (1973) inoculated channel catfish (\textit{Ictalurus punctatus}) with killed \textit{Flexibacter columnaris}. The subcutaneous as well as the intramuscular routes were used. Both methods produced high agglutination titers. Sanders et al. (1976) demonstrated the presence of type-specific antigens within \textit{F. columnaris}. They suggested that a polyvalent vaccine might be required.

Various studies have demonstrated in trout the development of antibody to injected viruses (Wolf and Quimby, 1969; Jorgensen, 1971; Dorson, 1972b). Amend and Smith (1974) injected rainbow trout
with IHN virus (Infectious Hematopoietic Necrosis) and demonstrated that the raised antiserum passively protected other fish from virulent IHN challenge. Agniel (1975) demonstrated passive transfer of immunity to IPN virus (Infectious Pancreatic Necrosis) in trout. Unprotected controls suffered a 58% mortality when challenged with the virulent virus. Passively protected groups suffered 21% to 5% loss, depending on the dose of immune serum.

**Oral Vaccination**

Immunization by injection has long been known to be effective in controlling disease. The method becomes impractical, however, when vaccinating large numbers of fish. For this reason, oral immunization has been studied extensively because of the efficiency of the technique and its lack of traumatic effect on immunized animals.

Duff (1942) was the first investigator to study the effects of oral immunization. He added growth from one Roux flask of chloroform-killed *A. salmonicida* to every 1.3 to 2.3 kg of feed. Cutthroat trout (*Salmo clarki*) received bacterin following various schedules. A waterborn challenge with virulent *A. salmonicida* resulted in loss of 75% in control lots and 24% in experimental lots. Agglutinating antibody was higher in the immunized fish. Background titers were observed in the controls. Protection appeared to be related to number and duration of feedings.
Snieszko and Friddle (1949) fed heat-killed *A. salmonicida* bacterins to brook trout. They mixed 0.5 ml of centrifuged saline suspensions of heat-killed bacterin to each kg of food. Vaccine diet was administered for 8 d. The fish were challenged by placing virulent *A. salmonicida* in the water. Feeding continued every 2 d thereafter. The original challenge failed to induce an epizootic in 10 d. A massive dose of virulent *A. salmonicida* was administered intraperitoneally. All fish in control and experimental lots were killed.

Interest in the oral bacterins declined for about 10 yr with the advent of chemotherapeutics. Much of the next reported work originated in Japan. Endo (1961, 1962a, 1962b, 1962c) and Endo et al. (1962a) fed polyvalent preparations containing several killed pathogens including the causative agent of vibriosis. He administered two feedings per day to rainbow trout for 10 d. Natural loss to fresh-water vibriosis (*Vibrio piscium*) subsided within 1 wk. He also noted that 3 to 4 d were required before the fish would readily accept diet containing bacterin. Two liters of polyvalent preparation was added to every 87.3 kg of multi-constituent diet. There was increase in agglutinating antibody in fish receiving bacterin. A characterization of the serum protein of the immunized fish indicated an increase in a component which migrated as a β-globulin. Endo (1962c) indicated that one or two feedings are not sufficient to induce protection. He suggested that well-mixed diets containing 1% bacterin should be
administered immediately to fry. He also suggested that adult rainbow trout required oral vaccination before the hot months and eels required vaccination before the dormancy period. Endo et al. (1962b) demonstrated some benefit from feeding preparations containing inactivated *Saprolegnia*.

Hoshina (1962) orally vaccinated eels with heat-killed *A. hydrophila*. He found neither agglutinating antibody production nor protection. Hayashi et al. (1964b) fed formalin-killed polyvalent preparations of various isolates of *Vibrio* sp. He found that rainbow trout produced 1:64 titers in 3 wk and 1:128 titers in 4 wk. Intramuscular injection of virulent organisms resulted in 100% loss in the controls and 70% loss in the experimental fish. Saito (1964) fed heat-killed *V. piscium* to rainbow trout in one "continuous feeding ball" so that each fish received 1 mg of bacterin. No protection was observed with injected challenges of various dilutions of virulent bacteria.

Post (1963) fed heat-killed *A. hydrophila* to rainbow trout which were held at 16 C. Circulating antibodies were observed in the vaccine-fed groups after 316 d. Challenge experiments after 183 d with 1 LD$_{90}$ of virulent *A. hydrophila* resulted in 70% mortality in orally vaccinated groups and 90% mortality in control groups.

The equivocal results reported by previous investigators led Krantz et al. (1964b) to include oral *A. salmonicida* bacterins in
experiments on the trout immune response. They fed a preparation similar to that used by Duff (1942). Krantz and his coworkers found neither protection nor production of antibody titer.

Ross and Klontz (1965) prepared phenol-killed redmouth bacteria for incorporation into the diet of rainbow trout. Fifteen milliliters of wet-packed cells were included in every 4.5 kg of feed. The preparation proved to be protective for longer than a year. After 70 d of feeding, exposure to 1 LD$_{90}$ of injected virulent redmouth bacteria resulted in 10% mortality in experimental lots and 80% mortality in control lots.

Spence et al. (1965) fed yearling coho salmon for 98 d with formalin-killed _A. salmonicida_ in OMP diet (Oregon Moist Pellet). When the fish were naturally challenged at a hatchery endemic for virulent _A. salmonicida_, there was no protection or circulating antibody formed. A second type of oral _A. salmonicida_ vaccine was examined by Klontz in 1965 (Klontz and Anderson 1970). An alum-precipitated supernatant solution obtained from sonicated _A. salmonicida_ (FSA) was found to protect brook trout when fed at 60 µg per fish per day. Anti- _A. salmonicida_ antibody was detected in the blood after 37 d. After 90 d, a natural-exposure challenge against the virulent homotypic organism resulted in 58% loss in control fish and no loss in vaccine-fed fish. Subsequent field trials of the FSA oral vaccine at Issaquah Hatchery (Washington Department
of Fisheries) and Siletz Hatchery (Oregon Fish Commission) resulted in substantial protection against natural furunculosis epizootics (Klontz and Anderson, 1970; Overholser, 1968). Further field trials resulted in either inadequate challenges, inconclusive data, or lack of protection. The successful vaccinations employed laboratory preparations while the unsuccessful vaccinations used commercial preparations. That observation has yet to be tested to further understand the inconsistencies of FSA vaccine. Hara et al. (1976) was partially successful with fed modified FSA in Yamame (O. masou) and Amago (O. rhodurus). Slightly increased survival was observed in the vaccinated fish. Results tended to vary from culture station to culture station.

The FSA extraction technique was tried on redmouth bacteria by Klontz in 1968 (Snieszko, 1970). Immunization with 100 mg per fish of alum precipitated antigen given over an 80 d period resulted in only a 1% loss in vaccinated groups and very heavy loss in controls. Later, however, the immunized fish succumbed to a second epizootic of the disease, indicating either short duration of immunity or a second invading serotype.

Fujihara (1969) and Fujihara and Nakatani (1971) examined antibody production and immune responses of rainbow trout and coho salmon to Flexibacter columnaris. They orally vaccinated three month old coho with heat-killed bacterial sonicates. Dried OMP was
rehydrated with saline suspensions of bacterin at a level of $4 \times 10^7$ cells per gram-wet-weight of food. Vaccinated fish developed agglutinating antibody titers of 1:168 after 3 mo feeding. Control fish had titers of 1:17. Ten weeks after natural exposure to the pathogen, 48% of the control fish and 8% of the vaccinated fish died of columnaris disease.

Muroga and Egusa (1969b) reviewed the various results of oral immunization of fishes. Experiments by Muroga (1975) and Muroga and Egusa (1969b) indicated that feeding eels merizonin-killed *V. anguillarum* for 4 mo resulted in no significant protection or antibody production. Eels fed viable cells produced circulating antibody and suffered less loss to vibriosis.

Anderson and Ross (1972) compared different oral preparations of redmouth bacterin in rainbow trout. Fish were challenged by subcutaneous injection of various dilutions of the virulent organism. Three percent chloroform-killed unwashed bacterin proved to be vastly superior to several washed preparations. Subsequently, Anderson and Nelson (1974) fed the chloroform-killed redmouth bacterin to 150 rainbow trout. No specific agglutinating antibody was found in the orally vaccinated fish but protection was observed when fish were challenged by injection of graded amounts of virulent redmouth bacteria. The $LD_{50}$'s for control fish were $8.5 \times 10^5$ bacteria per ml versus $9.0 \times 10^9$ (estimate) bacteria per ml for the vaccinated
Schaperclaus (1972) reported his findings on oral immunization of carp using polyserotype vaccines of phenol or chloramphenicol-killed *A. hydrophila*. In addition, he studied formalin-killed vaccines prepared from cultures which had been allowed to stand for up to 3 wk at 37°C. Incubation period was based on a certain level of hemolytic activity in the medium. With both phenol and chloramphenicol-killed oral vaccines, agglutinating antibody levels in carp were elevated. Laboratory and field challenges confirmed low levels of immunity in carp immunized with all three preparations. The formalized, 3 wk culture appeared slightly more effective.

Probably the most successful use of oral bacterins has been reported in salmonids against fish vibriosis. Fryer et al. (1972) found significant protection in chinook salmon from orally administered, formalin-killed, lyophilized sonicates of *V. anguillarum*. As little as 200 μg per fish per d for 20 d was sufficient for immunization. Natural challenges resulted in a 95% loss in control lots versus 37% in experimental lots. Booster feedings did not appear to provide significantly increased protection. Rohovec et al. (1975) reported that formalin-killed whole-cell *V. anguillarum* bacterin is just as effective as the lyophilized sonicate. Increasing feeding times from 3 wk to 6 wk did not significantly increase protection. As little as 0.5 mg per g of diet for 15 d was effective. Of great significance
When studying the immune response in salmonids is Rohovec's finding that oral vibrio bacterin is effective in fish held at temperatures from 4 C to 21 C.

Gunnels et al. (1976) attempted to immunize fall chinook salmon by both feeding and injecting heat and formalin-killed *V. anguillarum*. No protection was conferred by either vaccination method. In addition, no agglutinating antibody was observed in the orally immunized groups. In the injected groups, after 21 d, the mean agglutinating antibody titer was not quite 1:4. Passive immunization with anti-*V. anguillarum* serum produced for an earlier study was protective.

Fletcher and White (1973) demonstrated by passive hemagglutination that plaice (*P. platessa*) orally immunized against *V. anguillarum* contain higher titers in the mucous of the intestinal tract than in cutaneous mucous or the blood.

**Immunization by Vacuum Infiltration**

Recently, several attempts have been made to develop vaccine delivery systems which allow fast, efficacious immunization of fish with minimal handling. One such method is called vacuum infiltration. Fender (1974) and Amend (1976) reported that fish immersed in vaccine solutions are placed in a vacuum chamber and subjected to one-fifth normal atmospheric pressure for about 15 sec. The vacuum seal is then released and pressure is returned to normal as quickly
as possible. The procedure is repeated three times within 2 min. The exact route of antigen access is unknown, but it was reported that protection was equal to that of parenteral injection.

**Immunization by Hyperosmotic Immersion**

Another recent technique receiving extensive study is referred to as hyperosmotic immersion (Amend and Fender, 1976; Ament, 1975). Fish are allowed to stand in 8% NaCl solution for about 2 min. They are then placed in vaccine solution for about 2 min. The technique is thought to induce uptake of protective antigen primarily through the lateral line system and secondarily through the gills. Presently, hyperosmotic immersion is being employed for commercial immunization against redmouth disease.

**Immunization with Attenuated Strains**

The use of attenuated strains for immunization against viral and bacterial diseases is common in human medicine. This method is not common in fish vaccine preparations. Braaten and Hodgins (1976) exposed steelhead (*Salmo gairdneri*) to living low-virulence *V. anguillarum*. Subsequently, the fish were challenged both by waterborn exposure and by injection of virulent homotypic organisms. Mortality in the fish exposed to low-virulence bacteria was significantly lower than in the controls. Braaten and Hodgins (1976)
indicated that the protection conferred by the low-virulence exposure was no more effective than immunization with killed preparations.

The use of attenuated strains for immunization may have more applicability with viral diseases of fish. Zwillenberg et al. (1968) and Jorgensen (1971) reported experimentation with attenuated Egtved virus. Attenuation was accomplished by continued passage in cell culture. Repeated intraperitoneal injection by Jorgensen (1971) resulted in low titers against Egtved virus in rainbow trout. Zwillenberg et al. (1968) reported that virus passed in tench (Tinca vulgaris) cell lines conferred protection to rainbow trout against infection by the virulent virus.

Dorson et al. (1975) reported that IPN was attenuated by the tenth passage in RTG 2 cells at 14 C. Incorporation into the food of \(10^7\) plaque forming units of twice passed IPN resulted in 70% loss in rainbow trout. The same dose of virus passed ten times was non-infective. Protection in animals to virulent IPN challenge was not examined.

Fryer et al. (1976) developed an attenuated strain of IHN virus. The attenuated strain was administered in the water supply of susceptible sockeye salmon (Oncorhynchus nerka). After 25 d and 110 d the fish were protected from waterborn challenge by virulent virus.
Vibrio anguillarum the Causative Agent of Vibriosis in Salmonids

Vibriosis in fishes is caused by the bacterium, V. anguillarum. Various aspects of the disease and etiology of the organism have been reviewed by Anderson and Conroy (1970) and Fryer et al. (1972).

Vibrio anguillarum has been described by Fryer (1972) as a gram-negative, slightly curved, small, nonspore-forming rod (2-3 μm by 1 μm) and motile by means of a single polar flagellum. The organism is a facultative anaerobe, catalase negative and cytochrome-oxidase positive. Vibrio anguillarum is an anaerogenic fermenter of glucose and certain other carbohydrates. Like other vibrios, the organism is sensitive to the vibriostatic agent 0/129 (2, 4-diamino-6, 7-di-isopropyl pteridiene) and novobiocin. Muroga and Egusa (1967) have shown that the organism grows optimally at salt concentrations of 1.5% to 3.5%.

Rohovec (1974) described the pathology and signs of vibriosis in salmonids. They include erythema at the base of the fins, around the mouth, and around the vent. If the disease runs a chronic course, red, necrotic lesions can be found in the abdominal and body musculature. Petechiae may also be present. There may be hemorrhaging in the eye and a bloody discharge from the vent. Internally, the signs are similar to those seen in most septicemic diseases. Hemorrhaging and bloody fluid may be found throughout the body cavity. In the
acute form of the disease, the external symptoms may be absent and only slight observable internal pathology noted, i.e. petechia and reddening.

Vibrio anguillarum was first isolated by Canestrini (1893) from eels with "Red Pest" disease. He designated the bacteria Bacterium anguillarum. Vibrio anguillarum first got its name from Bergman (1909) when he isolated the organism from eels being cultured in Swedish waters.

Numerous strains of V. anguillarum were examined by Nybelin (1935b) to determine if they all exhibited similar biochemical properties. He found two biochemical types:

Type A - Acid but no gas from sucrose and mannitol, indol positive.

Type B - Will not attack sucrose and mannitol, indol negative.

Subsequently, Smith (1961) introduced a third biochemical type:

Type C - Acid but no gas from sucrose and mannitol, indol negative.

Pacha et al. (1958) described four serological groups in several northwest isolates of V. anguillarum. All four had a common somatic antigen, except for an isolate from herring (later described as a Pseudomonad [R. E. Pacha, personal communication]). The members of the several serological groups differed in the presence or absence of eight additional somatic antigens.
Pacha and Kiehn (1969) studied 13 isolates of *V. anguillarum* to determine if serological differences existed. Examination of the thermostable antigens in each isolate indicated the presence of three serologic groups. The first group consisted of *V. anguillarum* isolates from the Pacific Northwest in the USA; the second group consisted of European isolates; and the third group consisted of isolates from herring in the Pacific Northwest of the USA. An examination of the DNA homologies and base compositions confirmed their observations (Kiehn and Pacha, 1969). In terms of the biochemical system of Nybelin (1935b) the isolates from the first group, Pacific Northwest isolates, tended to fall into group A. Anderson and Ordal (1972), on the basis of DNA homologies and base compositions, found Kiehn and Pacha's groups 1 and 3 genotypically indistinguishable.

The distribution of vibriosis outbreaks throughout the world has been reviewed by Anderson and Conroy (1970). Isolations have been reported in numerous species of fish. The outbreaks are especially devastating in commercially cultured species (Fryer et al. 1972). Epizootics in salmonids caused by *V. anguillarum* have been a problem in Europe, Japan, and the United States. Smith (1959, 1961) described an epizootic in brown trout from Scotland, and identified the causative agent as *V. anguillarum*. Ghittino (1972) reported an outbreak of vibriosis in rainbow trout in Italy. McCarthy et al. (1974) isolated
V. anguillarum from rainbow trout in England. Hoshina (1956, 1957) first described vibriosis in rainbow trout from Japan. Later, other reports of vibriosis in salmonids from Japan appeared in the literature (Hayashi et al., 1964a, b; Tanji, 1966; Egusa, 1969). Salmonid culture in the marine environment is relatively new in the United States. Even so, vibriosis has proven to be a major obstacle to aquaculture enterprises (Novotny, 1973). Rucker et al. (1953) first isolated V. anguillarum in diseased salmonids from the Pacific Northwest. Since then, several investigators have attributed salt-water epizootics and one fresh-water epizootic in salmonids to vibriosis (Rucker, 1959; Ross et al., 1968; Cisar and Fryer, 1969; Evelyn, 1971).
MATERIALS AND METHODS

Culture Media

The culture media used for this study included Brain Heart Infusion (BHI) broth (Difco) BHI agar (Difco), Tryptone Glucose Yeast (TGY) broth, and Cytophaga Seawater Agar. Most routine culturing of either V. anguillarum or A. salmonicida was on BHI agar or in BHI broth. Tryptone Glucose Yeast broth was used for mass culture of bacteria. Its formulation consists of 1.0% tryptone (Difco), 0.5% yeast extract (Difco), 0.25% glucose (Difco), and 0.25% sodium chloride (Mallinckrodt) in distilled water. Cytophaga Seawater Agar deeps were used as a maintenance medium for V. anguillarum cultures. The formulation (Pacha and Ordal, 1967) consists of 0.02% beef extract (Difco), 0.05% tryptone (Difco), 0.05% yeast extract (Difco), 0.02% sodium acetate (Baker), 2.5% Rila salts (Rila Products, Teaneck, N. J.), and 0.4% agar (Difco) in distilled water.

Bacterial Strains

Description of Cultures

The bacterial strains employed in this study are described in Table 1. The various cultures of V. anguillarum were selected based on work by Dr. J. S. Rohovec (personal communication) who found
Table 1. Isolates of *Vibrio anguillarum*, *Aeromonas salmonicida*, and the agent of Bacterial Kidney Disease used in this study.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Origin and Species from Which Isolated</th>
<th>Date of Isolation</th>
<th>Person Responsible for Isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Vibrio anguillarum</em> LS 1-74</td>
<td>Lint Slough, Waldport, Oregon - Coho Salmon</td>
<td>1974</td>
<td>J. Rohovec</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> MSC 1-73</td>
<td>Marine Science Center, Yaquina Bay, Newport, Oregon - Chum Salmon</td>
<td>1973</td>
<td>D. Ransom</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> MAN 775</td>
<td>Manchester Bay, Washington - Coho Salmon</td>
<td>1974</td>
<td>B. Friedman</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> MSC 2-75</td>
<td>Marine Science Center, Yaquina Bay, Newport, Oregon - Coho Salmon</td>
<td>1975</td>
<td>D. Ransom</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> MAN 1669</td>
<td>Manchester Bay, Washington - Coho Salmon</td>
<td>1974</td>
<td>B. Friedman</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> ATCC</td>
<td>ATCC 19624, Denmark - Cod</td>
<td>1956</td>
<td>Bagge and Bagge</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> LS 1-71 FF</td>
<td>Lint Slough, Waldport, Oregon - repeated passage on artificial media - Chinook Salmon</td>
<td>1971</td>
<td>J. Rohovec</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em> SS-70</td>
<td>South Santiam Salmon Hatchery (ODFW), Oregon - Chinook Salmon</td>
<td>1970</td>
<td>W. Paterson</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em> Sil-67</td>
<td>Siletz Salmon Hatchery (ODFW), Oregon - Coho Salmon</td>
<td>1967</td>
<td>W. Paterson</td>
</tr>
<tr>
<td>Bacterial Kidney Disease Lea 1-74</td>
<td>Leaburg Salmon Hatchery (ODFW), Oregon - Chinook Salmon</td>
<td>1974</td>
<td>J. Sanders</td>
</tr>
</tbody>
</table>
that the LS 1-74 and MAN 775 isolates appeared to be physiologically and serologically different from the MSC 2-75 and MAN 1669 isolates. The MSC 1-73 isolate is most nearly related to LS 1-74. The ATCC culture was included as a standard reference. *Vibrio anguillarum* LS 1-71 FF was carried repeatedly on artificial media and is non-motile.

*Aeromonas salmonicida* SS-70 was chosen because of its pathogenicity in salmonids. *Aeromonas salmonicida* Sil-67 was chosen because of its lack of a clumping phenomena, characteristic of *A. salmonicida* SS-70. This characteristic made the Sil-67 strain ideal as antigen for agglutinating antibody titer determination. Kidney disease bacteria for the spray immunization studies were generously provided by Mr. J. E. Sanders, Fish Pathologist, Oregon Department of Fish and Wildlife.

**Preservation of Cultures**

To maintain antigenic uniformity, each isolate of *V. anguillarum* was maintained in the lyophilized state. In addition, lyophilized cultures of several other isolates from other locations throughout the world are maintained by this laboratory. A modification of the method employed by Floodgate and Hayes (1961) was used to freeze-dry the stock cultures. The method is as follows:

1. An isolate of *V. anguillarum* is incubated in BHI broth at 18°C
until cells are just into stationary phase.

2. A 25 ml sample of the bacterial culture is centrifuged at 1,500 x g for 10 min and the supernatant solution aseptically removed.

3. The remaining pellet is resuspended in 3 ml of sterile "mist desiccans" (1 part nutrient broth/3 parts horse serum/7.5% [W/V] glucose).

4. The suspension is transferred in 0.25 ml aliquots to sterile lyophilization vials. After dipping in liquid nitrogen to fast-freeze the bacteria, the vials are placed on the lyophilizer (Virtis Research Equipment, Inc.). When freeze-drying is complete (approximately 2 h) the vials are sealed and checked for vacuum. The cultures are stored at -26 C.

5. Freeze-dried cultures are reconstituted in BHI when required. For routine laboratory use, reconstituted cultures were maintained in Cytophaga Seawater Agar deeps at 4 C.

Both strains of _A. salmonicida_ were provided by Dr. W. D. Paterson in lyophilized 20% skim-milk suspensions. Cells were reconstituted in BHI broth. Inoculum for experimental cultures was obtained from isolated colonies on a BHI agar plate.
Growth Curves for Vibrio anguillarum

Comparison of Vibrio anguillarum LS 1-74 and MAN 1669 at 18 and 30 C

The two different northwest serotypes of V. anguillarum were first recognized because they displayed different growth characteristics. One type seemed to prefer water temperatures near 18 C and had a rapid generation time. The second type was isolated from an epizootic occurring at a colder temperature (around 10 C) and had a slower generation time in artificial media. To determine the growth characteristics of these two types of V. anguillarum and to provide graphs for approximate enumeration of cultures, growth studies were initiated.

The two strains used for this study were V. anguillarum LS 1-74 and MAN 1669. The growth curves for both isolates were determined at 18 C and 30 C. The lower temperature was chosen to approximate the normal environmental condition and the higher temperature was chosen because 30 C is utilized for mass culture of V. anguillarum bacteria.

Fifty milliliter aliquots of sterile BHI broth were dispensed into optically matched 300 ml Bellco side-arm flasks. Duplicate samples for each bacterial strain were equilibrated to both temperatures. Inoculum consisted of 0.1 ml of 48 h BHI culture adjusted to the
density of a no. 1 McFarland nephelometer tube (Kolmer et al., 1951).

After inoculation, one ml samples were drawn at selected intervals—every 4 h in lag phase, every 2 h in log phase, and every 8 h in stationary and death phase. Each sample was enumerated in triplicate in pour plates using standard dilution technique. Dilution blanks consisted of 9 ml aliquots of 0.01 molar phosphate buffered saline (PBS) at pH 7.0 (Buffer no. 35A, Williams and Chase, 1968). Plating media consisted of 15 ml aliquots of liquid BHI agar maintained at 42 C combined with 1 ml samples of the appropriate PBS dilution.

After each sample was taken for plate count enumeration, the optical density in the flask was read on a Bausch and Lomb, Spectronic 20 at a wavelength of 525 nm. An uninoculated BHI broth culture was used as a standard reference.

Tryptone Glucose Yeast Broth Growth Curves for Vibrio anguillarum MSC 1-73

A second growth curve experiment with V. anguillarum MSC 1-73 was run in TGY at 18 C. The experimental procedure was similar to the previous experiment. The inoculum was 0.1 ml of 0.01 molar PBS washed BHI broth culture. The inoculating media was adjusted to the turbidity of a no. 5 McFarland nephelometer tube.
Mean Generation Times for Various Isolates of Vibrio anguillarum

Mean generation times were calculated by the method of Stanier et al. (1970). The formula used is as follows:

\[
G = \frac{T}{\log_2 N_2 - \log_2 N_1}
\]

\(G\) = Generation time  
\(T\) = Time interval \((T_2 - T_1)\)  
\(T_2\) = Final time  
\(T_1\) = Initial time  
\(N_2\) = Final concentration  
\(N_1\) = Original concentration

Bacterial Antigen Preparation

Mass Culture Technique

Mass culture for extraction of cell wall components and production of large quantities of bacterin were prepared in a Fermacell Fermentor Model CF-50 (New Brunswick Scientific Co., Inc.). Tubes containing 10 ml of TGY broth were inoculated from cultures reconstituted directly from lyophilization tubes. The tubes were incubated at 18 C for 24 h. This cell suspension was used to inoculate two, 500 ml quantities of sterile TGY broth. These cultures were also
incubated at 18°C for 24 h, but with agitation. The growth in each flask was examined for purity and identity by streaking for isolation and performing a slide agglutination with specific antisera. These 500 ml cultures served as inoculum for 30 l of TGY broth which had been sterilized in the fermentor. Five milliliters of Antifoam Y-30 (Dow Corning) was added to the medium before sterilization. After inoculation, this culture was allowed to incubate at a sparging rate of 5.7 l of air per min, agitation speed of 200 rpm, and temperature of 18°C. After 12 h, 500 ml of sterile 20% glucose solution and 25 ml of 10 N sodium hydroxide were added to the medium. For extraction purposes, the incubation period was 24 h. For vaccine production, the incubation period was 96 h. Again, after the prescribed incubation time, the culture was examined for purity and identity as before. When necessary, cultures were harvested with a continuous-flow Sharples Super Centrifuge Type IP.

**Injectable Antigens**

Injectable whole-cell bacterins for the *Vibrio anguillarum* study were provided by Dr. J. S. Rohovec. Eight ounce bottle slants of BHI agar were inoculated with 1.0 ml of a 12 h broth culture of the desired organism. After incubation, the bacteria were removed from the agar surface with 0.3% formalin-saline solution. The cells remained in solution for 1 h and were washed three times by
centrifugation (0 C, 3,000 x g, 10 min) in 0.01 molar PBS. Cells were resuspended to the turbidity of a McFarland no. 3 nephelometer tube.

Injectable whole-cell bacterins for the study of motile and non-motile *V. anguillarum* were prepared by inoculating the desired organism into BHI broth from a Cytophaga Seawater Agar carrying slant. The culture was incubated at 18 C for 24 h. Cells were killed by adding formalin to a concentration of 0.5% and allowed to incubate overnight. Cells were washed three times by centrifugation and re-suspension in sterile 0.01 molar PBS (0 C, 3,000 x g, 10 min) and finally resuspended to 10 X the turbidity of a McFarland no. 3 nephelometer tube.

**Oral Antigens**

Formalin-killed whole-cell antigens were used to orally immunize fish in the examination of the immunogenicity of motile and non-motile *V. anguillarum*. The same material was used in the comparison of oral immunization and spray immunization. Oral vaccines for the flagella study were as follows: 10 ml of BHI broth was inoculated with LS 1-74 and 10 ml with LS 1-71 FF. Cultures were incubated for 24 h at 18 C. Each culture was used to inoculate two, 1,500 ml quantities of sterile BHI broth. These cultures were grown at 18 C for 24 h. Cultures were checked for purity and identity by streaking
for isolation and slide agglutination. LS 1-71 FF antiserum was not available so agglutinations were performed on all cultures with anti-LS 1-74 rabbit serum. After incubation, all flasks received formalin to equal a 0.5% concentration and were allowed to stand at room temperature for 8 h. The contents of each flask were harvested by centrifugation at 3,000 x g.

Dry weights were determined for each sample, two LS 1-74 bacterins and two LS 1-71 FF bacterins (Table 2). These values were used to calculate the exact amount of each preparation which was incorporated into the food. Final concentration was equal in all cases. For each sample, three aliquots from different areas of the bacterin pellet were dried to constant weight in vacuo at 60 C.

Table 2. Percent dry weight determinations (w/w) for each of two Vibrio anguillarum LS 1-74 whole-cell bacterins and two Vibrio anguillarum LS 1-71 FF bacterins.

<table>
<thead>
<tr>
<th>Sample</th>
<th>First Replicate</th>
<th>Second Replicate</th>
<th>Third Replicate</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS 1-74 no. 1</td>
<td>16.5%</td>
<td>16.4%</td>
<td>17.7%</td>
<td>16.9%</td>
</tr>
<tr>
<td>LS 1-74 no. 2</td>
<td>18.3%</td>
<td>16.7%</td>
<td>17.1%</td>
<td>17.4%</td>
</tr>
<tr>
<td>LS 1-71 FF no. 1</td>
<td>18.7%</td>
<td>19.5%</td>
<td>21.1%</td>
<td>19.8%</td>
</tr>
<tr>
<td>LS 1-71 FF no. 2</td>
<td>19.4%</td>
<td>19.3%</td>
<td>18.4%</td>
<td>19.0%</td>
</tr>
</tbody>
</table>

The two replicates for each isolate were incorporated into Oregon Moist Pellet (OMP) diet at 2 mg per g of food. The required
amount of bacterin was added as follows:

a. A known weight of frozen OMP mash was placed in a lyophilizer chamber. The material was dried under vacuum for 8 h. Water loss was calculated. The dry mash was powdered further in a covered Waring blender cup at high speed.

b. The bacterin required to constitute 2 mg per g of food was added to the amount of water lost in the drying process.

c. The dry powder and water containing bacterin were thoroughly mixed. The resulting product was pelleted (0.32 cm) in an Alemite hand compressor.

Oral bacterins employed in the comparison of the spray and oral immunization techniques were produced by the method of Rohovec et al. (1975). The mass culture procedure is similar to that previously described. The major difference lies in the incubation temperature; 30 C vs. 18 C. After incubation of the 30 l culture, 100 ml of formalin solution was added. The culture was allowed to stand for 1 h. Sterility was checked and the cells harvested by centrifugation. Wet-packed whole cells were stored at -26 C until incorporation into OMP diet at 5 mg per g of food.

Spray Antigens

The original spray preparations consisted of fluorescent marking dye (Scientific Marking Material, Seattle, Washington) mixed with a
known amount of wet-packed, whole-cell bacterin. After drying under vacuum the preparations were powdered in a Waring blender or in a large mortar and pestle.

Later the spray preparations consisted of BHI broth cultures (TGY for mass cultures) of a desired isolate, grown at 18°C for 96 h. After incubation, the bacteria were killed by overnight exposure to 0.5% formalin. Bacterins were checked for sterility and purity with BHI agar streak plates and with slide agglutinations with specific antisera. This liquid bacterin was sprayed alone, or with other components to investigate any beneficial effects. The compound found to be most beneficial, bentonite, was usually added at 0.15% (W/V).

**Production of Lyophilized Sonicate of Vibrio anguillarum**

Lyophilized sonicate of *Vibrio anguillarum* was utilized to determine the level of injected antigen required to provide protection against virulent homotypic organisms. One liter of BHI broth was inoculated with *V. anguillarum* LS 1-74. The culture was incubated at 18°C for 24 h. Cells were harvested by centrifugation at 3,000 x g and washed three times with 0.01 molar PBS. The cells were resuspended in 50 ml of deionized water and disrupted on a Branson W185 Sonifer Cell Disruptor (Heat Systems, Inc.). The culture was lyophilized after freezing in dry ice-acetone.
The protein nitrogen in the final product was determined using the Nessler reaction following Kjeldahl digestion (Williams and Chase, 1968). Samples of 4 mg and 1 mg of lyophilized sonicate were tested. The reference was 100 μg nitrogen as ammonium sulfate. Bovine serum albumin (1 mg/ml) was included to check the efficiency of Kjeldahl digestion. Lyophilized sonicate was determined to contain 100 mg nitrogen per 1.13 mg dry weight of lyophilized preparation.

**Sonicated Antigen for Gel Diffusion**

Gel diffusion studies requiring soluble bacterial antigen were prepared prior to each experiment. An isolate was grown up in BHI broth for 24 h at 18°C. The bacteria were harvested by centrifugation at 3,000 x g and washed three times in 0.01 molar PBS. Heavy suspensions (1×10^11 cells per ml) were sonicated using the Branson W185 Sonifer Cell Disruptor. Sonication was continued in 15 sec intervals until an opalescent solution was obtained.

**Extraction Techniques**

**Cell Wall Production**

Cell walls were extracted from 700 g wet weight of mass cultured *V. anguillarum* LS 1-74. Packed cells were washed once in 3 l of 0.01 molar PBS and resuspended in 5 l of cold, deionized distilled
water. The suspension was sonicated in ice-bath cooled, 60 ml aliquots with a Branson W185 Sonifier Cell Disruptor. Lysis required full power output in three, 30 sec exposures. To remove unlysed cells the combined sonicates were resuspended to 15 l and centrifuged twice for 20 min at 3,000 x g. Cell walls were harvested by centrifugation at 12,000 x g for 1 h. The supernatant solution was discarded. Pelleted material was washed once in deionized water and again centrifuged for 1 h at 12,000 x g. The cell walls were divided into two aliquots and stored at -26 C.

**Trichloroacetic Acid Extractions**

Trichloroacetic acid (TCA) extractions were made of cell walls of *V. anguillarum* LS 1-74. A modification to the technique described in Williams and Chase (1968) was used. Acetone dried cell walls were suspended in five times their weight of distilled water. An equal amount of either 0.25N, 0.5N or 1.0N TCA was added and the mixture stirred in the cold for 4 h. The extracted suspension was warmed to room temperature and centrifuged at 12,000 x g for 30 min. The solution was adjusted to pH 6.5 with 1 N NaOH and cooled to 0 C. Two volumes of cold 95% ethanol were added slowly with stirring. The material which had precipitated overnight at 0 C was collected and dialyzed for 2 d against running tap water and 2 d against twice changed distilled water. The final product was dissolved in deionized distilled
water, centrifuged at 3,000 x g and the pellet discarded. The final supernatant solution was diluted to 1 mg per ml of deionized distilled water and 1:5,000 merthiolate added as preservative.

Trichloroacetic acid extracts of *A. salmonicida* were provided by Mr. L. R. Udey in the lyophilized form. Solutions were made up of 2 mg per ml dried extract in deionized distilled water containing 1:5,000 merthiolate as a preservative.

**Phenol-water Extractions**

Phenol-water extractions on cell walls of *V. anguillarum* were carried out by the method described in Williams and Chase (1968). Five grams (dry weight) of cell walls were suspended in 88 ml of distilled water maintained at 65 to 68 C. An equal volume of 90% phenol, preheated to 65 C was added with stirring and the solution maintained at that temperature for 15 min. The mixture was cooled to 10 C and centrifuged at 3,000 x g for 30 min. The upper aqueous layer was carefully removed without disturbing the material at the phenol-water interface. The aqueous solution was centrifuged at 3,000 x g and the small remaining pellet discarded. The supernatant solution was dialyzed for 3 d against daily changes of deionized distilled water. The dialyzed solution was recentrifuged at 3,000 x g and the pellet discarded. The remaining solution was lyophilized.

The crude lyophilized extract was resuspended in 3% by weight and centrifuged in a Beckman model L preparative ultracentrifuge (Beckman type 30 rotor) at 105,500 x g for 2 h. The mucous material
at the bottom of the pellet was cut out of the cellulose nitrate tube and the clear, gel-like material resuspended in distilled water. The centrifugation was later repeated at the same speed for 5 h. No mucoid material was noted. The pellet was resuspended to 2 mg per ml of deionized distilled water. Merthiolate was added at 1:5,000 as a preservative.

Extractions for the cross-protection study were made on *V. anguillarum* LS 1-74 and MSC 2-75 according to the procedure outlined in Nowotny (1969). Mass cultured cells were acetone dried using the procedure of Williams and Chase (1968). Cells were washed twice with 4 l volumes of 0.01 molar PBS and dried by washing three times with acetone (about 500 ml per 100 g of wet cells or 15 g of dry cells) at 5 C. Ten grams of dried cells were mixed in 300 ml of distilled water, while immersing the container in a 70 C water bath. When a homogenous suspension was obtained, 300 ml of phenol was added and the temperature brought to 70 C. The stirred solution was maintained at that temperature for 10 min. The material was quickly cooled and centrifuged at 3,000 x g for 30 min. The upper aqueous layer was carefully removed by suction. A second aliquot of 300 ml of deionized, distilled water was added and the extraction procedure repeated. A third aliquot of 300 ml was added and the extraction procedure repeated a third time. The pooled aqueous phases were placed in large, washed dialysis bags (Brewer et al., 1974) and dialyzed
against twice daily changes of distilled water for 3 d.

After dialysis, the solution was concentrated to 100 ml at 30 °C, using a Brinkman vacuum distillation unit (Scientific Products). Three-hundred milliliters of a solution of cold absolute methanol (5 C) containing 20% magnesium chloride was added slowly with stirring. The precipitate which was formed during 60 min incubation was centrifuged from the solution at 3,000 x g for 30 min. The precipitate was dissolved in 100 ml of distilled water and cooled to 5 C. To this solution, 200 ml of methanol without magnesium chloride were slowly added with stirring and the solution incubated for 60 min. The precipitate was again collected by centrifugation. This procedure was repeated once more. The final reprecipitated material was resuspended in 100 ml of distilled water and reduced under vacuum to 50 ml, rediluted to 100 ml and reconcentrated to 50 ml. This solution was lyophilized.

Toxicity of Extraction Products for Mice

Each extraction product was tested for toxicity in mice. The two products obtained after extraction and purification from V. anguillarum cell walls were injected into five Webster white mice weighing approximately 19 g each. Each mouse received 1 mg of extracted product in a 0.5 ml intraperitoneal injection.

The phenol-water extractions for cross-protection studies and
the *A. salmonicida* TCA extracts were checked for toxicity in mice by determination of the LD$_{50}$. Webster white mice weighing 16 to 18 g were placed in groups of five in each of six mouse cages. The animals received either 2.0, 1.0, 0.5, 0.25, or 0.125 mg of the desired extraction product in 0.5 ml intraperitoneal injections. Five mice received sterile 0.01 molar PBS as a negative control. Dead animals were collected for 3 d and examined for the characteristic gross pathology associated with endotoxin injection. LD$_{50}$'s were calculated using the method described in Meynell and Meynell (1965).

**Serological Techniques**

**Bacterial Identification by Slide Agglutination**

Rabbit antiserum against all of the bacterial antigens employed in this study were obtained from Dr. J. S. Rohovec. They were prepared by injecting live, Freunds adjuvant suspensions of the desired bacteria subcutaneously into each of four sites near the axillary lymph nodes of a New Zealand white rabbit. All serum titers were greater than 1:512. The rapid slide agglutination technique employed in this study is described in Kabat and Meyer (1961).

**Harvesting Fish Serum**

Blood from fish was harvested by severing the caudal artery and bleeding into a small, disposable capillary tube. The blood in
the tube was allowed to clot at room temperature, then placed at 5 C overnight. After any attached clot was carefully separated from the tube, the samples were centrifuged at 500 x g for 15 min. The serum was either collected for storage or used immediately for antibody titer determinations. Stored samples were frozen and maintained at -26 C.

Determination of Agglutinating Antibody Titers

The antigen for titer determinations was grown in BHI broth until slightly turbid. The cells were washed by centrifugation at 3,000 x g for 20 min and resuspended in 0.01 molar PBS. This process was repeated three times. After the final centrifugation, the cells were resuspended to correspond to an optical density of 0.85 at 525 nm on a Baush and Lomb Spectronic 20. Serum samples containing 0.025 ml were diluted in 0.025 ml quantities of 0.01 molar PBS in a disposable microtiter plate (round bottom) using a microtiter diluter and 0.025 ml calibrated dropper pipette (Cooke Engineering, Alexandria, Virginia). An equal volume of antigen was added to each dilution of serum (1:4 to 1:8192). Plates were incubated for 1 h at room temperature and then at 5 C overnight. Titers were read as the last dilution that displayed agglutination when viewed at 20 x magnification on a dissecting microscope.
Immunodiffusion Techniques

Double-diffusion plates (Ouchterlony) were prepared using 1% agarose in immunodiffusion buffer, pH 7.0. The buffer consisted of 3.58 g of NaH₂PO₄, 10.95 g of Na₂HPO₄·7H₂O, 1.86 g ethylenediamine tetraacetate (EDTA), 0.65 g of sodium azide per 1 of deionized, distilled water (Williams and Chase, 1968). Wells were cut in the desired pattern and excess media withdrawn by low vacuum. After the antigen and antisera were added to the wells, the plate was allowed to incubate for approximately 48 h in a humid chamber. Bands were photographed in the dark on a light box using high contrast copy film (Kodak, HC 135-36) with 20 mm and 36 mm combined extension tubes on a standard 55 mm lens.

Electron Microscopy

The presence or absence of flagella in V. anguillarum LS 1-71 FF was determined by shadow-casting electron microscopy. Vibrio anguillarum LS 1-74 was included in the examination as a positive control. Both cultures were grown at 18°C for 18 h. Cells were washed three times with 0.01 molar PBS. The final suspension was adjusted to an optical density of 0.85 at 525 nm on a Spectronic 20. A drop of cells was placed on a polyvinyl formvar coated 300 mesh copper screen. The excess liquid was blotted off with a tissue. The
suspension was air dried. Grids were shadow cast with platinum-palladium (4:1) at an angle of 20° at 5 cm in a Varian Model VE-10 vacuum evaporator. The vacuum was $1 \times 10^{-5}$ Torr. Samples were observed on a Phillips EM-300 transmission electron microscope operated at an acceleration potential of 60 KV. Images were recorded on Kodak glass electron image plates and developed with Kodak HRP developer.

Thin-section electron micrographs were made of both _V.anguillarum_ LS 1-74 and _V.anguillarum_ MSC 2-75 which had been grown in BHI broth overnight at 15 C, fixed in 0.5% glutaraldehyde, washed three times by centrifugation and resuspended in 0.01 molar PBS (500 x g for 10 min). Cells were stained with 1% osmium-tetroxide and dehydrated with a graded acetone series. The second to last 70% acetone wash contained uranyl acetate. Cells were embedded in an araldite-epon mixture, sectioned, and post-stained with alkaline lead citrate. Samples were observed as previously described.

**Experimental Salmonids**

**Species and Origin**

Experimental animals consisted primarily of 1974-brood coho salmon (_O. kisutch_) obtained from the Oregon Department of Fish and Wildlife's Research Division, Corvallis, Oregon; Alsea Salmon
Hatchery; or Cascade Salmon Hatchery. A small lot of 1975-brood coho from Alsea Salmon Hatchery were used to determine the effects of spray vaccination on small fish. Coho from Alsea Salmon Hatchery (1974-brood) were used for the study of motile and non-motile _V. anguillarum_. A group of rainbow trout (_S. gairdneri_) from Wizard Falls Trout Hatchery were used to determine the amount of agglutinating antibody against furunculosis elicited by spray vaccination.

**Holding Facilities**

Both stock and experimental fish were maintained at the Oregon State University Fish Disease Laboratory, Corvallis, Oregon. Water is drawn from a well at a constant ambient temperature of 12.2 C. All stock fish, prior to use in experimentation, were held at ambient temperature in 0.91 or 1.52 m circular fiberglass, self-cleaning tanks. Aerated water was delivered at approximately 10 l per min. Experimental 68 l, self-cleaning fiberglass tanks were supplied with either heated or chilled water capable of maintaining temperatures between 3.9 C to 23.3 C. Water was delivered to each tank at approximately 1.5 l per min.

**Marking Techniques**

Very often it was necessary to mark fish for easy identification and separation. Three techniques were used in this study;
cold-branding, fluorescent dyes, and fin-clips. Most of the experiments used fish which were marked with a brass-tipped brand, cooled in dry-ice and acetone. The brand was placed on different areas of the fish in various configurations. Fluorescent dyes were sprayed on fish with a standard commercial sandblasting gun operated at pressures of 6.3 to 7.0 kg per cm$^2$ (90 to 100 p.s.i.). The various colors of dye were read with a UVL 56 hand held ultraviolet lamp (Ultraviolet Products, Inc.). Fin clips were made by removing either the left or right pelvic fin with scissors. After marking, fish were treated by a 10 sec dip in 1:10,000 malachite green to prevent fungus growth (Wood, 1968).

**Immunization Techniques**

**Intraperitoneal Injection**

Intraperitoneal injections with a 26 gauge, 1 cm needle were made just dorsal of the insertion of the left pelvic fin. Fish were anesthetized in benzocaine solution (0.5 ml per 1 of a 10% benzocaine in 95% ethanol solution). Each fish received 0.1 ml of the immunizing material. After immunization, the fish were held at 12.2°C for a specified length of time for immunity to develop.

**Oral Immunization**

Oral immunization with the motile and non-motile *V. anguillarum*
bacterins was carried out at 12.2°C. Each tank of 25 coho (mean wt approx 9 g each) received 14 daily rations of 12.5 g of oral vaccine which was equivalent to 1 mg vaccine per fish per day. The diet was accepted readily the first day, but was rejected the next day (commonly observed in bacterin feeding experiments). On the third day, approximately one-fourth of the diet was consumed, on the fourth day approximately three-fourths. After that, no hesitation in feeding was noted. Average vaccination dose per fish totaled 12 mg in a 14 d period. After receiving vaccine, the fish were rested for 7 d before challenge with virulent *V. anguillarum* LS 1-74.

Orally immunized fish for the comparison of the oral and spray immunization technique were fed production diets containing 5 mg vaccine per g of OMP (Rohovec et al., 1975). The fish received the diet for 45 d.

**Spray Vaccination**

The delivery system consists of a commercial sandblasting gun operated at 6.3 to 7.0 g per cm² with bacterin in the venturi-feeding reservoir. Approximately 450 to 900 g of fish (number depending on size) were placed in a standard dipnet (30 by 46 by 25 cm) and sprayed for 5 to 10 sec. The fish were moved in the net in such a manner that each fish received a direct application of vaccine on some surface of the body. The tip of the spray apparatus was positioned 20 to 25 cm
from the fish. After immunization, the fish were held for immunity to develop.

Challenge Procedures

Waterborn Challenge by *Vibrio anguillarum* LS 1-74

The waterborn challenge against *V. anguillarum* LS 1-74 proceeded as follows:

1. The various groups of marked treated and untreated experimental animals were held at or equilibrated to 12.2 C. Coho salmon were used in sizes ranging from 9 g to 50 g. Each treatment should contain lots allowing duplicate or triplicate challenges.

2. Challenge tanks were prefilled with 12.2 C water. This laboratory utilizes three, stainless steel, 93 l, raceway challenge tanks with a common water supply entering each tank at approximately 6 l per min.

3. Fish were transferred from their holding tank to the challenge tanks. At this point, the water temperature was increased to 18 C for 1 h. During this period, the viable count of an overnight BHI broth culture of *V. anguillarum* LS 1-74 was estimated by its optical density at 525 nm on a Spectronic 20. An inoculum size for the 93 l tank was determined to achieve a
final concentration of $1 \times 10^6$ bacteria per ml. A standard plate count on the original culture was used to determine exact inoculum size.

4. After the 1 h temperature-change period, the incoming 18°C water was shut off. The appropriate inoculum was introduced into the challenge tank. The fish were agitated to distribute the inoculum and increase the level of stress. After 15 min, the 18°C water was turned back on.

5. Dead fish were collected daily and examined for cause of death.

Waterborn Challenge of *Aeromonas salmonicida* SS-70

The waterborn challenge against virulent *A. salmonicida* was essentially the same as that described for *V. anguillarum* LS 1-74 with the following exceptions:

1. The water level was dropped in the challenge tank to 30 l, prior to introduction of inoculum.

2. Inoculum was added to give a final concentration of approximately $1 \times 10^7$ bacteria per ml during the 15 min while the water was off.

Determination of LD$_{50}$ for Injected *Vibrio anguillarum* MSC 2-75

Artificially cultured *V. anguillarum* MSC 2-75 does not display
sufficient invasiveness for fresh-water, waterborn challenge of coho salmon. For this reason, an injectable LD$_{50}$ for MSC 2-75 was determined. Ten Alsea coho (mean wt approx 30 g each) were injected intraperitoneally with 0.1 ml of several 0.01 molar PBS dilutions of a log phase BHI broth culture. The fish were held for 10 d at 18 C. Dead fish were collected daily and examined for cause of death. The LD$_{50}$ was calculated by the method outlined in Meynell and Meynell (1965).

Injected Challenge by Vibrio anguillarum MSC 2-75

To challenge coho salmon against _V. anguillarum_ MSC 2-75, fish were injected with 0.1 ml of 0.01 m PBS diluted culture containing 1 LD$_{90}$ of bacteria ($1 \times 10^4$). Experimental animals were maintained for 14 d after injection. Dead fish were collected daily and examined for cause of death.

Examination of Experimental Salmonids

Fish collected daily from challenges were examined for gross pathological symptoms and cultured from the kidney onto BHI agar. After incubation at room temperature, plates were examined for colonies displaying the growth and gram-stain characteristics of the challenge organism. Final identification of the cultured bacteria was confirmed by rapid slide agglutination with specific rabbit antiserum.
RESULTS

Electron Micrographs of *Vibrio anguillarum* 
**LS 1-74 and MSC 2-75**

Because of the different growth and physical characteristics of the serologically distinct *V. anguillarum* LS 1-74 and MSC 2-75, electron micrographs were prepared to compare the ultrastructure of the two isolates. Both cell types appeared typical of gram-negative bacteria (Figures 1 and 2). The cytoplasm of each contained ribosomes and various granular inclusions. For LS 1-74, the polar insertion of a single flagellum can be seen in Figure 1A. Although not pictured, MSC 2-75 possesses a single polar flagellum. The cell walls of both isolates consist of a typical cytoplasmic membrane, an almost imperceptible solid membrane and a pronounced cell membrane. The LS 1-74 isolate differs from MSC 2-75 in the amount of periplasmic space and the presence of "polar plates."

**Growth Studies**

Comparison of *Vibrio anguillarum* LS 1-74 and 
**MAN 1669 growth curves at 18 and 30 C**

Bacterial growth curves of *V. anguillarum* LS 1-74 and MAN 1669 at 18 C and 30 C are presented in Figures 3 and 4. At both temperatures LS 1-74 achieved a higher viable count in a shorter
Figure 1A  Electron micrograph of a *Vibrio anguillarum* LS 1-74 cell in longitudinal section showing polar insertion of flagellum and a small area which may be a part of the polar plate. Note the large periplasmic area and lack of readily discernible peptidoglycan layer (X60,000)

Figure 1B  Electron micrograph of a *Vibrio anguillarum* LS 1-74 cell in longitudinal section showing a portion of the polar plate (X60,000)

Figure 1C  Electron micrograph of a *Vibrio anguillarum* LS 1-74 cell in both longitudinal and cross-section showing the polar plate. Note the location of the plate in the longitudinal section and three patch distribution in the cross-section. This electron micrograph suggests a three fingered cup originating at the site of flagellar insertion (X60,000)
Figure 2A  Electron micrograph of two *Vibrio anguillarum* MSC 2-75 cells in longitudinal sections (X60,000)

Figure 2B  Longitudinal and cross-section of *Vibrio anguillarum* MSC 2-75 cells. Note the amorphous appearance of the cell wall and the apparent lack of a readily discernible peptidoglycan layer (X80,000)
Figure 3. Comparison of growth of two strains of *Vibrio anguillarum*, LS 1-74 and MAN 1669, as measured by viable count at 18°C.
Figure 4. Comparison of growth of two strains of *Vibrio anguillarum*, LS 1-74 and MAN 1669, as measured by viable count at 30 C.
time than MAN 1669. Both isolates demonstrated higher counts at 18°C than at 30°C. MAN 1669 went into death phase much sooner than LS 1-74 at both temperatures. To quickly enumerate growing cells in BHI broth cultures, Figures 5 and 6 compare viable counts at 18°C and 30°C, respectively, to optical density at 525 nm during logarithmic growth of LS 1-74 and MAN 1669. Generation times for both isolates are presented in Table 3.

**Tryptone Glucose Yeast Broth Growth Curves for Vibrio anguillarum MSC 1-73**

Vibrio anguillarum MSC 1-73 is serologically similar to *V. anguillarum* LS 1-74 (Dr. J. S. Rohovec, personal communication). This strain was used to determine growth characteristics of cells grown in the mass culture media, TGY broth. Figure 7 relates viable-count to optical density at 525 nm during logarithmic growth. The generation time is shown in Table 3.

**Toxicity of Extraction Products in Mice**

One of the common biological properties of phenol-water or TCA extracts from gram-negative bacteria is toxicity for mice. Toxicity of the preparations used in the cross-protection study were determined (Table 4). The TCA extract of *A. salmonicida* SS-70 was the most toxic with an LD$_{50}$ of 177 μg. The phenol-water
Figure 5. Viable cell count versus optical density at 18 and 30 C for Vibrio anguillarum LS 1-74.
Figure 6. Viable cell count versus optical density at 18 and 30°C for *Vibrio anguillarum* MAN 1669.
Table 3. Mean generation times\textsuperscript{a} (G) for \textit{Vibrio anguillarum} isolates grown in Brain Heart Infusion or Tryptone Glucose Yeast broth.

<table>
<thead>
<tr>
<th>Media (Broth)</th>
<th>Isolate</th>
<th>Temperature (°C)</th>
<th>G (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHI</td>
<td>LS 1-74</td>
<td>18</td>
<td>1.07</td>
</tr>
<tr>
<td>BHI</td>
<td>LS 1-74</td>
<td>30</td>
<td>0.75</td>
</tr>
<tr>
<td>BHI</td>
<td>MAN 1669</td>
<td>18</td>
<td>1.60</td>
</tr>
<tr>
<td>BHI</td>
<td>MAN 1669</td>
<td>30</td>
<td>1.20</td>
</tr>
<tr>
<td>TGY</td>
<td>MSC 1-73</td>
<td>18</td>
<td>2.14</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Time required for doubling of the cell population in the culture.
extracts of *Vibrio anguillarum* LS 1-74 and MSC 2-75 were of similar toxicities, 750 μg and 928 μg. Mice were examined during the experiment for signs related to endotoxicity. All injected animals had closed eyes and reduced activity after 3 h. After 10 h all had ruffled hair and displayed reduction in activity. Mice succumbing to injection of endotoxin displayed diarrhea. Necropsy revealed hemorrhaging in the lungs and, in some cases, the peritoneal cavity.

The two products obtained after extraction and purification of *V. anguillarum* cell walls were found to be non-toxic in mice. The animals demonstrated the typical outward signs of endotoxicity up to 10 h, but did not succumb after a 3 d waiting period.

**Determination of the LD\textsubscript{50} for *Vibrio anguillarum* MSC 2-75 Injected into Coho Salmon**

The mortality and mean day-to-death of coho salmon (mean wt approx 30 g each) injected with different dilutions of *V. anguillarum* MSC 2-75 are presented in Table 5. The LD\textsubscript{50} was calculated to be 2.76 x 10\textsuperscript{3} bacteria per fish (95% conf. int. between 1.16 x 10\textsuperscript{3} and 6.61 x 10\textsuperscript{3}). Based on this data, an injected dose of 1 x 10\textsuperscript{4} bacteria per fish should induce 80% mortality.

**Determination of the Requirement for *Vibrio anguillarum* Flagella in Bacterin Preparations**

*Vibrio anguillarum* LS 1-71 FF was non-motile, and shadow-cast
Figure 7. Growth curve of *Vibrio anguillarum* MSC 1-73 as measured by viable cell count in Tryptone Glucose Yeast broth at 18 C. Inset compares optical density of log phase culture to viable count.
Table 4. LD\textsubscript{50} values in mice to cell wall extracts of *Aeromonas salmonicida* SS-70, *Vibrio anguillarum* LS 1-74 and *Vibrio anguillarum* MSC 2-75 administered intraperitoneally.

<table>
<thead>
<tr>
<th>Extraction Method</th>
<th>Species</th>
<th>LD\textsubscript{50} ((\mu g))^a</th>
<th>95% Confidence Interval for LD\textsubscript{50} ((\mu g))^b</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td><em>A. salmonicida</em> SS-70</td>
<td>177</td>
<td>36 to 831</td>
</tr>
<tr>
<td>Phenol-water</td>
<td><em>V. anguillarum</em> LS 1-74</td>
<td>750</td>
<td>209 to 2685</td>
</tr>
<tr>
<td>Phenol-water</td>
<td><em>V. anguillarum</em> MSC 2-75</td>
<td>928</td>
<td>307 to 2805</td>
</tr>
</tbody>
</table>

^a Each group contained five mice.

^b If another value is obtained outside of the defined limits, that observation may be rejected with 95% confidence.
electron microscopy showed that LS 1-71 FF had no flagella (Figure 8). Figure 9 compares sonicated LS 1-74 and LS 1-71 FF antigens against antiserum for \textit{V. anguillarum} LS 1-74 in gel diffusion plates. The extra precipitin band present with motile LS 1-74 presumably indicates flagellar antigen which is not present with non-motile LS 1-71 FF. This isolate was used to determine the requirement for flagellar antigen in bacterin preparations. Duplicate preparations of both oral and injected LS 1-74 and LS 1-71 FF bacterins were administered to groups of 25 coho salmon (mean wt. approx. 30 g each). Each group of fish was marked by cold-brand 7 d prior to immunization. During the course of immunization, the water supply to one replicate of each treatment group was accidentally left off, killing all the fish in the oral treatment and 14 fish in the injected LS 1-71 FF treatment. The fish were challenged 21 d after initial immunization. Final concentration in the challenge tank was \(8.3 \times 10^5\) bacteria per ml.

The results of the challenge experiments with oral and injected bacterins produced from the motile and non-motile \textit{V. anguillarum} are presented in Table 6. Both oral bacterins and injected bacterins were effective to some degree. The injected preparations confer more protection than the oral preparations.
Table 5. Mortalities and mean day-to-death for groups of coho salmon injected with different dilutions of virulent Vibrio anguillarum MSC 2-75.

<table>
<thead>
<tr>
<th>Number of Bacteria Injected</th>
<th>Number of Fish Injected</th>
<th>Number of Deaths</th>
<th>Mean Day to Death</th>
<th>% Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1.7 \times 10^8$</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>$1.7 \times 10^7$</td>
<td>10</td>
<td>10</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>$1.7 \times 10^6$</td>
<td>10</td>
<td>10</td>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>$1.7 \times 10^5$</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>$1.7 \times 10^4$</td>
<td>10</td>
<td>9</td>
<td>6</td>
<td>90</td>
</tr>
<tr>
<td>$1.7 \times 10^3$</td>
<td>10</td>
<td>4</td>
<td>7</td>
<td>40</td>
</tr>
<tr>
<td>$1.7 \times 10^2$</td>
<td>10</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>-none-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.01 molar PBS</td>
<td>10</td>
<td>0</td>
<td>-</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 8A  Shadow-cast electron micrograph of non-motile *Vibrio anguillarum* LS 1-71 FF (X15,000)

Figure 8B  Shadow-cast electron micrograph of motile *Vibrio anguillarum* LS 1-74 (X15,000)
Immunodiffusion comparison of sonicated cellular antigens of the motile *Vibrio anguillarum* LS 1-74 and the non-motile *Vibrio anguillarum* LS 1-71 FF against rabbit antiserum for *Vibrio anguillarum* LS 1-74. Note the predominant band present in the motile strain as compared to the non-motile strain (→).
Table 6. Comparison of protection against virulent *Vibrio anguillarum* LS 1-74<sup>a</sup> conferred by oral and injected bacterins derived from motile *Vibrio anguillarum* LS 1-74 and non-motile *Vibrio anguillarum* LS 1-71 FF.

<table>
<thead>
<tr>
<th>Challenge no.</th>
<th>Bacterin Prepared from:</th>
<th>Vaccination Method</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LS 1-74</td>
<td>Injected</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral</td>
<td>25</td>
<td>15</td>
<td>13</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>LS 1-71 FF</td>
<td>Injected</td>
<td>25</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oral</td>
<td>25</td>
<td>14</td>
<td>12</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Unvaccinated Control</td>
<td></td>
<td>25</td>
<td>22</td>
<td>21</td>
<td>88</td>
</tr>
<tr>
<td>2</td>
<td>LS 1-74</td>
<td>Injected</td>
<td>25</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>LS 1-71 FF</td>
<td>Injected</td>
<td>11&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Unvaccinated Control</td>
<td></td>
<td>30</td>
<td>15</td>
<td>15</td>
<td>50</td>
</tr>
</tbody>
</table>

<sup>a</sup>The waterbom challenge level in both challenge tanks was 8.3 x 10⁵ bacteria per ml. A resting period of 21 d followed initial immunization.

<sup>b</sup>The loading in the two challenge tanks was different, so mortality in the controls cannot be compared.

<sup>c</sup>Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

<sup>d</sup>Both oral groups and 14 fish from the LS 1-71 FF injected group were lost due to mechanical failure.
Immunodiffusion Studies on the Whole-cell Antigens and Phenol-water Extracts of _Vibrio anguillarum_ LS 1-74 and MSC 2-75

The serological cross-reaction of antigens produced by either sonication of whole cells or extraction with TCA was examined by gel diffusion. Figures 10 and 11 show diffusion bands of sonicates and extracts of both isolates against rabbit anti- _V. anguillarum_ serum prepared against both LS 1-74 and MSC 2-75. Both strains possess serologically similar antigens, but TCA extracts of both strains produce different banding patterns against the two antisera. Cross-reactive bands are present with the faster migrating antigens (Figure 11).

Protection of Coho Salmon by an Injected Phenol-water Extract of _Vibrio anguillarum_ LS 1-74

A partially purified lipopolysaccharide fraction was injected into coho salmon to determine if it was protective. The material (as previously described) was prepared from cell walls isolated and partially purified by differential centrifugation. Each of 25 coho salmon (mean wt approx 22 g each) received 0.1 ml intraperitoneal injections of partially purified phenol-water extract of LS 1-74 (1 mg per ml). An uninjected control group (30 fish) and a 0.01 molar PBS injected control group (30 fish) were included in the experiment. The fish were maintained in 68 l tanks for 37 d. At that time
Figure 10A  Immunodiffusion comparison of sonicated cellular antigens and trichloroacetic acid extracts of *Vibrio anguillarum* LS 1-74 (LS) and *Vibrio anguillarum MSC 2-75* (MSC) against rabbit antiserum for *Vibrio anguillarum* LS 1-74. Note the presence and absence (→) of a slow migrating band in the whole-cell sonicates. Also note that these bands are similar in the TCA extracts (←). Many cross-reacting antigens are observed in both isolates.

Figure 10B  Immunodiffusion comparison of sonicated cellular antigens and trichloroacetic acid extracts of *Vibrio anguillarum* LS 1-74 (LS) and *Vibrio anguillarum MSC 2-75* (MSC) against rabbit antiserum for *Vibrio anguillarum* MSC 2-75. See explanation of symbols above.
Figure 11. Immunodiffusion comparison of sonicated *Vibrio anguillarum* LS 1-74 (LS) and *Vibrio anguillarum* MSC 2-75 (MSC) antigens, as well as different trichloroacetic acid extracts of *Vibrio anguillarum* LS 1-74 and *Vibrio anguillarum* MSC 2-75 against side-by-side wells of corresponding rabbit anti-*Vibrio anguillarum* serum. Note (→) the cross-reacting bands in 0.25 N trichloroacetic acid extracts of both isolates.
anti-*V. anguillarum* titers were determined and the fish challenged with virulent waterborn *LS 1-74* at $1.7 \times 10^5$ bacteria per ml. The injected extract is both antigenic and protective (Table 7). Also indicated is that the injection process had little detrimental effect when compared to "less stressed" uninjected controls.

**Cross-protection of Various Bacterins and Extraction Products Produced from Selected Isolates of *Vibrio anguillarum***

Various isolates of *V. anguillarum* were injected into coho salmon to determine if they were cross-protective when used as an immunizing bacterin. The challenge organisms, *LS 1-74* and *MSC 2-75*, were chosen because of their different antigenic composition.

One-hundred coho salmon (mean wt approx 16 g each) were marked by cold-branding and then intraperitoneally immunized with 0.1 ml of one of the following strains of formalin-killed injectable bacterin: *V. anguillarum* *LS 1-74*, *MSC 2-75*, *MAN 775*, *MAN 1669*, and ATCC. All bacterins were injected into 100 coho salmon. In addition, 100 fish were marked and injected with 0.1 ml of 0.01 molar PBS to serve as an unimmunized control. After injection four, 0.91 m holding tanks each received 25 fish immunized with each isolate as well as 25 controls. The fish were maintained at 12.2 C for 21 d. At that time the fish from two of the holding tanks were placed in separate challenge tanks and exposed to waterborn,
Table 7. Determination of protection against virulent *Vibrio anguillarum* LS 1-74\(^a\) and agglutinating antibody titers conferred in coho salmon injected with partially purified phenol-water extracts of *Vibrio anguillarum* LS 1-74 cell walls.

| Treatment                  | Number of Fish Bled | Geometric Mean Agglutinating Antibody Titer\(^1\) | Number of Fish Challenged | Number of Deaths | Deaths Caused by Vibriosis | % Mortality Caused by Vibriosis
|---------------------------|---------------------|-----------------------------------------------|------------------------|------------------|---------------------------|-------------------------------
| Phenol-water Extract      | 6                   | 203                                           | 22                     | 0                | 0                         | 0\(^c\)                        |
| Injected PBS Control      | 5                   | 0                                             | 20                     | 10               | 9                         | 47                            |
| Uninjected Control        | 5                   | 0                                             | 25                     | 13               | 13                        | 52                            |

\(^a\) The waterborne challenge level was 1.7 x 10\(^5\) bacteria per ml. A resting period of 37 d followed initial immunizations.

\(^b\) Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific death.

\(^c\) Difference from the combined controls is significant at the 95% confidence level. \(X^2 = 16.5\).
The bacterial concentration in each challenge tank was $1.4 \times 10^6$ per ml. An attempt to challenge the fish in one of the two remaining holding tanks with waterborn MSC 2-75 was unsuccessful. After 48 d, the fish from the remaining holding tank were challenged with 1 LD$_{90}$ of injected MSC 2-75 ($9.65 \times 10^3$ bacteria per fish).

All groups of fish immunized with the various bacterins and challenged with virulent _V. anguillarum_ LS 1-74 displayed some degree of immunity as compared to unimmunized controls (Table 8). The homotypic and MAN 775 bacterins were equally effective and elicited the highest degree of protection. The MSC 2-75, MAN 1669, and ATCC bacterins were also effective, but less so than LS 1-74 and MAN 775. When the challenge strain was MSC 2-75, the results were similar, all bacterins elicited strong protection (Table 9). In this case, there was no clear-cut distinction between the level of protection in the various groups.

Coho salmon (mean wt approx 16 g each) were injected with phenol-water extracts of either _V. anguillarum_ LS 1-74 or MSC 2-75 to determine if cross-protective immunity developed against challenge by both isolates. Seventy coho (two groups of 20 and two groups of 15) were injected intraperitoneally with 0.1 ml of LS 1-74 extract (10 mg/ml in distilled water). Forty-four coho (four groups of 11) were similarly injected with the same concentration of MSC 2-75 extract.
Table 8. Efficacy of injected bacterins prepared from various isolates of *Vibrio anguillarum* against challenge by virulent *Vibrio anguillarum* LS 1-74, a

<table>
<thead>
<tr>
<th>Strain of <em>V. anguillarum</em> Used for Bacterin Preparation</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis b</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS 1-74</td>
<td>25</td>
<td>1</td>
<td>1</td>
<td>4c</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAN 775</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAN 1669</td>
<td>25</td>
<td>4</td>
<td>4</td>
<td>16c,d</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7</td>
<td>5</td>
<td>22</td>
</tr>
<tr>
<td>MSC 2-75</td>
<td>25</td>
<td>7</td>
<td>7</td>
<td>28d</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7</td>
<td>7</td>
<td>28</td>
</tr>
<tr>
<td>ATCC</td>
<td>25</td>
<td>6</td>
<td>5</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Injected PBS</td>
<td>25</td>
<td>19</td>
<td>19</td>
<td>76</td>
</tr>
<tr>
<td>Control</td>
<td>25</td>
<td>16</td>
<td>16</td>
<td>64</td>
</tr>
</tbody>
</table>

a The waterbom challenge level was $1.4 \times 10^6$ bacteria per ml. A resting period of 21 d followed immunization.

b Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

c The difference between the means of the MAN 1669 and LS 1-74 mortalities, 18% and 2% respectively, are significant at the 95% confidence level. $X^2 = 7.49$.

d The difference between the means of the MAN 1669 and MSC 2-75 mortalities, 18% and 28% respectively, are not significant at the 95% confidence level. $X^2 = 0.25$. 
Table 9. Efficacy of injected bacterins prepared from various isolates of *Vibrio anguillarum* against challenge by virulent *Vibrio anguillarum* MSC 2-75, a

<table>
<thead>
<tr>
<th>Strain of <em>V. anguillarum</em> used for Bacterin Preparation</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Number of Deaths Caused by <em>Vibriosis</em> b</th>
<th>% Mortality Caused by <em>Vibriosis</em> c</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS 1-74</td>
<td>25</td>
<td>3</td>
<td>2</td>
<td>8 d</td>
</tr>
<tr>
<td>MAN 775</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MAN 1669</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MSC 2-75</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ATCC</td>
<td>25</td>
<td>3</td>
<td>2</td>
<td>8 d</td>
</tr>
<tr>
<td>Injected PBS Control</td>
<td>26</td>
<td>22</td>
<td>22</td>
<td>85</td>
</tr>
</tbody>
</table>

a The injected challenge level was $9.65 \times 10^3$ bacteria per fish. A resting period of 48 d followed immunization.

b Challenge monitoring period was 14 d.

c Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

d The difference between mortality in LS 1-74 and ATCC (8%) and the other groups receiving bacterin (0%) is not significant at the 95% confidence level. $X^2 = 2.08$. 
Seventy coho (two groups of 20 and two groups of 15) were injected with 0.01 molar PBS as a control. Since endotoxins are known to enhance non-specific immunity, 100 coho (four groups of 25) were injected with 1 mg of _A. salmonicida_ TCA extract. All fish were marked by cold branding.

After injection, one group of fish immunized with the extract of each isolate (LS 1-74, MSC 2-75 and _A. salmonicida_) was placed in each of four 0.91 m circular holding tanks. A control group was also placed in each tank. The fish were maintained at 12.2 C for 21 d. At that time, the fish in two of the holding tanks were moved to separate challenge tanks and exposed to waterborn, virulent LS 1-74. The bacterial concentration in each challenge tank was $2.4 \times 10^6$ per ml. The fish in the other two holding tanks were placed in separate challenge tanks containing 18 C water. Each animal was injected intraperitoneally with 0.1 ml of 0.01 molar PBS containing $1.2 \times 10^5$ virulent MSC 2-75 per ml.

Phenol-water extracts of _V. anguillarum_ LS 1-74 and MSC 2-75 both elicited protection against challenge by the virulent homotypic organisms (Table 10, 11). Trichloroacetic acid extract of _A. salmonicida_ (very toxic for mice) failed to induce a non-specific immunity (Table 10, 11).
Table 10. Determination of protection against virulent *Vibrio anguillarum* LS 1-74\(^a\) conferred in coho salmon by injected chemical extracts of *Aeromonas salmonicida* SS-70, *Vibrio anguillarum* LS 1-74 and *Vibrio anguillarum* MSC 2-75.

<table>
<thead>
<tr>
<th>Extracted Strain of Bacteria</th>
<th>Extraction Method</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. salmonicida</em> SS-70</td>
<td>TCA</td>
<td>25</td>
<td>18</td>
<td>18</td>
<td>72(^c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>18</td>
<td>17</td>
<td>71</td>
</tr>
<tr>
<td><em>V. anguillarum</em> LS 1-74</td>
<td>Phenol-water</td>
<td>20</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>V. anguillarum</em> MSC 2-75</td>
<td>Phenol-water</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Injected PBS Control</td>
<td>---</td>
<td>20</td>
<td>12</td>
<td>11</td>
<td>59(^c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>10</td>
<td>10</td>
<td>67</td>
</tr>
</tbody>
</table>

\(^a\) The waterborne challenge level was 2.4 x 10\(^6\) bacteria per ml. A resting period of 21 d followed immunization.

\(^b\) Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific death.

\(^c\) The difference between the means of the control and *A. salmonicida* SS-70 mortality, 63% and 71% respectively, is not significant at the 95% confidence level. \(X^2 = 0.85\).
Table 11. Determination of protection against virulent *Vibrio anguillarum* MSC 2-75<sup>a</sup> conferred in coho salmon by injected chemical extracts of *Aeromonas salmonicida* SS-70, *Vibrio anguillarum* LS 1-74 and *Vibrio anguillarum* MSC 2-75.

<table>
<thead>
<tr>
<th>Extracted Strain of Bacteria</th>
<th>Extraction Method</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. salmonicida</em> SS-70</td>
<td>TCA</td>
<td>25</td>
<td>15</td>
<td>13</td>
<td>57&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>16</td>
<td>15</td>
<td>63</td>
</tr>
<tr>
<td><em>V. anguillarum</em> LS 1-74</td>
<td>Phenol-water</td>
<td>20</td>
<td>2</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>1</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td><em>V. anguillarum</em> MSC 2-75</td>
<td>Phenol-water</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Injected PBS Control</td>
<td>---</td>
<td>20</td>
<td>13</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15</td>
<td>12</td>
<td>12</td>
<td>80</td>
</tr>
</tbody>
</table>

<sup>a</sup> The injected challenge level was \(1.2 \times 10^5\) bacteria per ml. A resting period of 22 d followed immunization.

<sup>b</sup> Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

<sup>c</sup> The difference between the means of the *A. salmonicida* endotoxin group and the controls, 60% and 71% respectively, are not significant at the 95% confidence level. \(X_2^2 = 1.04\).
Agglutinating Antibody Titers and Degree of Protection Elicited by Injection of Graded Amounts of 
Vibrio anguillarum LS 1-74 Lyophilized Sonicate and Boivin Extract into Coho Salmon

The amount of antigen required to protect a fish was examined to better understand the amount of purification necessary to compare the immunogenic characteristics of various *V. anguillarum* cell-wall components. Fifty coho salmon (mean wt approx 23 g each) were injected intraperitoneally with 0.1 ml PBS suspension of *V. anguillarum* LS 1-74 lyophilized sonicate (1 mg nitrogen per ml). The procedure was repeated for ten-fold dilutions of the suspension to 10 ng nitrogen per ml. Boivin extract of *V. anguillarum* cell walls was also injected into groups of 30 coho salmon (mean wt approx 23 g each) at various dilutions. The levels injected were 100 μg, 10 μg, 1 μg, 500 ng, 50 ng, and 5 ng. Injected and uninjected controls were included. All fish were marked by cold-brand and held in 68 l tanks at 12.2 C. Agglutinating antibody titers on five randomly selected fish receiving lyophilized sonicate were determined after 31 d. Waterborn LS 1-74 challenge of fish receiving lyophilized sonicate failed to produce death in the unimmunized controls and were thus unsuccessful. The groups receiving Boivin extract were challenged after 37 d with $1.7 \times 10^5$ virulent LS 1-74 bacteria per ml. Agglutinating antibody titers were determined on six individuals randomly
selected from each group prior to challenge.

Coho salmon, injected with ten-fold dilutions of lyophilized sonicate of *V. anguillarum* LS 1-74, produced measurable agglutinating antibody responses in all fish received doses equal to, or greater than 0.1 µg nitrogen (Table 12). When fish were injected with Boivin (TCA) extract, all fish receiving doses of 5 ng (dry weight) or greater produced agglutinating antibody and were protected from challenge by virulent *V. anguillarum* LS 1-74 (Table 13).

**Efficacy of the Spray Vaccination Technique for Administration of Vibrio anguillarum LS 1-74 Bacterin**

The results of the previously outlined experiment indicated that coho salmon can be immunized with small amounts of injected *V. anguillarum* LS 1-74 antigen. A common technique for marking fish is spraying with fluorescent dye and reading the mark with an ultraviolet light. The dye can still be detected after 3 yr on adult chinook salmon (*Oncorhynchus tshawytscha*) returning from sea (R. L. Garrison, ODFW, personal communication). For this reason vaccine was included with dye (100 mg dry wt per g) as previously described and sprayed on 50 coho (mean wt approx 15 g each). Fifty control fish were sprayed with dye only. After 38 d at 12.2 C in 68 l holding tanks, a five fish sample was removed from each group to determine agglutinating antibody levels. The remainder
Table 12. Agglutinating antibody titers \(^{-1}\) in coho salmon induced by injecting graded amounts of *Vibrio anguillarum* LS 1-74 lyophilized sonicate.

<table>
<thead>
<tr>
<th>Level of Lyophilized Sonicate Injected (μg nitrogen)a</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer(^{-1})b</th>
<th>% Showing Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>6</td>
<td>162</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>40</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>51</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>45</td>
<td>100</td>
</tr>
<tr>
<td>0.01</td>
<td>6</td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td>0.001</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>PBS Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a Level of diluted lyophilized sonicate was standardized to nitrogen content as determined by the Nessler reaction.

b A resting period of 31 d elapsed from injection to sampling.
Table 13. Level of agglutinating antibody titer\(^{-1}\) and protection conferred against virulent *Vibrio anguillarum* LS 1-74\(^a\) in coho salmon injected with trichloroacetic acid extract of *Vibrio anguillarum* LS 1-74.

<table>
<thead>
<tr>
<th>Amount of TCA Extract Injected (μg)(^b)</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer(^{-1})</th>
<th>Percent Showing Response</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>6</td>
<td>114</td>
<td>100</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>152</td>
<td>100</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>362</td>
<td>100</td>
<td>22</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>.5</td>
<td>6</td>
<td>228</td>
<td>100</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>.05</td>
<td>6</td>
<td>128</td>
<td>100</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>.005</td>
<td>6</td>
<td>36</td>
<td>100</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td>10</td>
<td>9</td>
<td>47</td>
</tr>
<tr>
<td>Uninjected Control</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>25</td>
<td>13</td>
<td>13</td>
<td>52</td>
</tr>
</tbody>
</table>

\(^a\) The waterbom challenge level was \(1.7 \times 10^5\) bacteria per ml. A resting period of 37 d followed immunization.

\(^b\) The level of TCA extract was standardized by dry weight.

\(^c\) Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.
Table 14. Protection against virulent *Vibrio anguillarum* LS 1-74 and production of agglutinating antibody titers in coho salmon sprayed with homotypic bacterins in conjunction with fluorescent dye. 

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer of Single Fish</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yellow dye and bacterin</td>
<td>5</td>
<td>196</td>
<td>45</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Blue dye control</td>
<td>5</td>
<td>0</td>
<td>45</td>
<td>39</td>
<td>38</td>
<td>86</td>
</tr>
</tbody>
</table>

*a* The waterborn challenge level was $1.8 \times 10^6$ bacteria per ml. A rest period of 28 d followed immunization.

*b* Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

*c* The preparation contained 100 mg dry wt of bacterin per g of dye.
of the fish were challenged by virulent waterborn *V. anguillarum* at 1.8 x 10^6 bacteria per ml. Levels of agglutinating antibody and protection against virulent *V. anguillarum* LS 1-74 are shown in Table 14. Only fish receiving bacterin produced agglutination antibody and displayed immunity.

Whole-cell bacterin was mixed with fluorescent dye at various levels as previously described. Each of these preparations was sprayed on 50 coho salmon (mean wt approx 15 g each). Tenfold dilutions of bacterin ranged from 100 mg to one mg dry wt per g of dye. Fish were marked by cold-brand and maintained at 12.2 C in 68 l tanks for 24 d. At that time five fish samples were removed from each lot for agglutinating antibody determination and the remainder challenged by virulent *V. anguillarum* LS 1-74. The final concentration in the challenge tank was 2.0 x 10^6 bacteria per ml. The fish produced an agglutinating antibody response at all levels tested except 0.001 mg bacterin per g dye (Table 15). The antibody response was minimal at and below a spray level of 1 mg bacterin per g of dye. A noticeable degree of protection against challenge by the virulent homotypic organism was produced at spray levels equal to or above 1 mg bacterin per g of dye (Table 15).
Table 15. Comparison of agglutinating antibody and protection against virulent *Vibrio anguillarum* LS 1-74 in coho salmon sprayed with various concentrations of *Vibrio anguillarum* LS 1-74 bacterin in conjunction with fluorescent dye.

<table>
<thead>
<tr>
<th>Concentration (mg bacterin/g dye)</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer of Single Fish Samples</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>5</td>
<td>5</td>
<td>23</td>
<td>15</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>5</td>
<td>21</td>
<td>22</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>21</td>
<td>22</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>32</td>
<td>29</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5</td>
<td>147</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>5</td>
<td>147</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No Bacterin</td>
<td>5</td>
<td>2</td>
<td>25</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a The waterborn challenge level was $2.0 \times 10^6$ bacteria per ml. A rest period of 24 d followed immunization.

b Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

c The differences between the mean control mortality and mean 0.001 mg/g and 0.1 mg/g mortality are significant at the 95% confidence level. $X^2 = 5.39$ and $5.61$, respectively. The difference between the mean control mortality and 0.01 mg/g level is not significant at the 95% confidence level. $X^2 = 0.29$. 

---

$X^2$ = 5.39 and 5.61, respectively. The difference between the mean control mortality and 0.01 mg/g level is not significant at the 95% confidence level. $X^2 = 0.29$. 

---
Several different bacterin preparations were tested, both for their ability to elicit protection and for their ability to elicit high agglutinating antibody titers. The parameters which were examined were liquid bacterins, abrasives, and absorption carriers.

Formalin-killed bacterin grown for 96 h at 30°C was sprayed on 50 coho salmon (mean wt approx 15 g each). Also included was a group of fish sprayed with the original fluorescent dye-vaccine preparation (100 mg vaccine per g dye) slurried in water (50:50 by wt). Another group was sprayed with a dry bacterin-alumina combination (10 mg of bacterin per g of alumina). The bacterin was combined with the alumina in the same manner as fluorescent dye.

In a separate experiment, 40 coho salmon (mean wt approx 23 g each) were sprayed with 0.5% formalin-killed, 96 hr incubated LS 1-74. A second flask containing the same bacterin plus 0.15% bentonite was used to spray 30 more coho. Control fish were sprayed with uninoculated BHI broth. All fish were marked by cold-brand and held in 68 l tanks at 12.2°C.

Five fish from the 96 hr incubation group, bentonite group and BHI broth control group were examined for agglutinating antibody after 30 d. All other fish were challenged as previously described.

The results of challenge experiments and/or agglutinating
antibody determinations are presented in Table 16. This data indicates that liquid suspensions of bacterin-dye preparations are effective spray vaccines. The addition of alumina as a scarifier resulted in no significant improvement over the spray-dye combination (see Table 15). Spray vaccinating fish with formalin-killed cultures grown at both 18 and 30°C caused the production of agglutinating antibody. The 30°C culture was protective against challenge by virulent *V. anguillarum* LS 1-74. The addition of bentonite to the 18°C, formalin-killed spray vaccination culture caused a ten-fold increase in antibody titer.

**Longevity of Antibody Response in Coho Salmon Spray Vaccinated with *Vibrio anguillarum* LS 1-74**

| Bacterin Containing 0.15 Percent Bentonite at pH 9.0 |

The duration of time agglutinating antibody remained in coho salmon sprayed with formalin-killed *V. anguillarum* was examined. Groups of 40+ fish (mean wt approx 23 g each) were sprayed with bacterin suspensions containing 0.15% bentonite at pH 9.0. A second solution containing only formalin and sterile BHI broth was sprayed on another group. Antibody titers were read in five fish samples at 30 d, 40 d, 54 d, 62 d, 81 d, and 112 d. At 123 d the nine remaining fish in the control group and eight remaining fish in the vaccinated group were challenged in separate 68 l tanks. The challenge was
### Table 16. Effect of different *Vibrio anguillarum* LS 1-74 bacterin preparations for spray vaccination.

<table>
<thead>
<tr>
<th>Spray Preparation</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer of Single Fish Samples</th>
<th>Number of Fish Challenged&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Number of Deaths</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3% formalin-killed 96 h culture, 30 C incubation</td>
<td>5</td>
<td>48</td>
<td>23</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Liquid suspension of 100 mg bacterin per g of fluorescent dye</td>
<td>5</td>
<td>256</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 mg bacterin per g of alumina</td>
<td>5</td>
<td>147</td>
<td>21</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>0.5% formalin-killed 96 h culture, 18 C incubation</td>
<td>5</td>
<td>32</td>
<td>--&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>0.5% formalin-killed 96 h culture, 18 C incubation + 0.15% bentonite</td>
<td>5</td>
<td>388</td>
<td>--&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Fluorescent dye spray control</td>
<td>5</td>
<td>2</td>
<td>25</td>
<td>18</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td>BHI Broth + 0.5% formalin</td>
<td>5</td>
<td>0</td>
<td>--&lt;sup&gt;c&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup>The waterborne challenge level was 2.0 x 10<sup>6</sup> bacteria per ml. A rest period of 24 d followed immunization.

<sup>b</sup>Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

<sup>c</sup>These groups were not challenged.
performed in a manner different from the standard procedure. Fish were placed in the tank at 12.2 °C and incoming water temperature changed to 18 °C. After 1 h, the water level was reduced to 20 l and incoming water turned off. Ten milliliters of virulent *V. anguillarum* were introduced into each tank from a common culture which had a plate count of $1.86 \times 10^9$ bacteria per ml. Total challenge in the tank was $9.5 \times 10^5$ bacteria per ml. Incoming water at 18 °C was restored after 15 min exposure. The tanks were again allowed to fill to 68 l.

Table 17 shows that for the 112 d period, agglutinating antibody could be detected in only spray vaccinated fish. The levels dropped sharply at 81 d. When challenged at 123 d, seven of the nine control fish died of vibriosis (78%). None of the vaccinated fish died.

**Effect of pH on *Vibrio anguillarum* LS 1-74 Spray Vaccination Preparations Containing 0.15 Percent Bentonite**

The binding ability of the bacterin to bentonite, if it is critical to spray immunization, may be affected by the pH of the solution. To examine this possibility, standard bacterin as described previously, containing 0.15% bentonite was split into five batches. Each was adjusted to a different pH; 3.0, 5.0, 7.0, 9.0, 11.0 with hydrochloric acid or sodium hydroxide. Six groups of 10 coho salmon (mean wt approx 23 g each) were each sprayed with bacterin at one of the pH levels, the last group was unimmunized. After 25 and 45 d, samples
Table 17. Longevity of antibody response in coho salmon sprayed with *Vibrio anguillarum* LS 1-74 bacterin (pH 9.0) containing 0.15 percent bentonite.

<table>
<thead>
<tr>
<th>Time After Vaccination (Days)</th>
<th>Group</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer$^{-1}$ of Single Fish Samples</th>
<th>% Showing Antibody Response</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>Vaccine</td>
<td>5</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>40</td>
<td>Vaccine</td>
<td>5</td>
<td>194</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>54</td>
<td>Vaccine</td>
<td>5</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>62</td>
<td>Vaccine</td>
<td>5</td>
<td>128</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>81</td>
<td>Vaccine</td>
<td>5</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>112</td>
<td>Vaccine</td>
<td>5</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
of five fish were examined for agglutinating antibody against *V. anguillarum* LS 1-74.

Bacterins adjusted to pH 3.0 produced higher agglutinating antibody levels after both 25 and 45 d (Table 18). Not much difference was noted in titers elicited by bacterin at pH's 5.0, 7.0 and 9.0. The least effective preparation was at pH 11.0.

**Passive Immunization of Coho Salmon against Vibriosis by Injection of Immune Serum from Coho Salmon Immunized by Spray Vaccination**

The protective nature of components in serum collected from spray vaccinated fish was demonstrated by passive immunization. One-hundred coho salmon were sprayed with *V. anguillarum* LS 1-74 bacterin containing 0.15% bentonite. After 25 d, 50 fish were bled and the serum collected. The serum was sterilized by passage through a 0.22 μm Millipore filter and the level of agglutinating antibody determined. The next day, 40 fish were injected with immune serum and 44 fish injected with normal rainbow trout serum. The anti-*V. anguillarum* agglutinating antibody in the normal serum had previously been determined to be zero. Four hours after injection of both types of serum, 40 immunized and 38 passively immunized fish were challenged. The fish receiving the normal serum were challenged in the same manner. Immunized and passively immunized fish received a challenge level of $3.3 \times 10^6$ bacteria per ml. The controls
Table 18. Agglutinating antibody produced in coho salmon sprayed with *Vibrio anguillarum* LS 1-74 bacterins containing 0.15 percent bentonite adjusted to different pH levels.

<table>
<thead>
<tr>
<th>pH</th>
<th>Rest Period after Immunization</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer&lt;sup&gt;-1&lt;/sup&gt; of Single Fish Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>25 45</td>
<td>5 6</td>
<td>512 128</td>
</tr>
<tr>
<td>5</td>
<td>25 45</td>
<td>5 6</td>
<td>256 40</td>
</tr>
<tr>
<td>7</td>
<td>25 45</td>
<td>5&lt;sup&gt;a&lt;/sup&gt; 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>256 32</td>
</tr>
<tr>
<td>9</td>
<td>25 45</td>
<td>5 7</td>
<td>256 60</td>
</tr>
<tr>
<td>11</td>
<td>25 45</td>
<td>5 5</td>
<td>169 18</td>
</tr>
<tr>
<td>Unvaccinated</td>
<td>25 45</td>
<td>5 5</td>
<td>0 0</td>
</tr>
</tbody>
</table>

<sup>a</sup>Remainder of fish succumbed to excess nitrogen in the water supply.
received $2.4 \times 10^6$ bacteria per ml. Six fish each from the passively immunized and control group were bled just before challenge and the anti-*V. anguillarum* agglutinating antibody titer determined.

Fish receiving immune serum were protected while fish receiving normal serum were not (Table 19). Immune serum injected intra-peritoneally 4 h prior to sampling resulted in a measurable agglutinating antibody titer in circulating blood.

**Comparison of Agglutinating Antibody and Protection against *Vibrio anguillarum* LS 1-74 in Groups of Coho Salmon Vaccinated Separately and in Combination by the Spray and Oral Method**

A comparison of orally protected and spray protected coho salmon (mean wt approx 30 g each) was made at Sandy River Hatchery (ODFW). Members of the Fish Culture Division, Oregon Department of Fish and Wildlife had fed oral bacterin (5 mg per g of OMP) for 41 d when two lots of orally immunized and two lots of unimmunized fish, 80 fish per lot, were removed from raceways and placed separately in 0.95 m circular tanks. One lot of each treatment was sprayed with formalin-killed *V. anguillarum* LS 1-74 containing 0.15% bentonite and red fluorescent dye. The remaining lots were sprayed with BHI broth containing formalin, bentonite, and green fluorescent dye. The fish had originally been marked with pectoral fin clips. The orally immunized fish continued to receive oral vaccine for an additional 4 d, 45 total. Thirty-four days after spraying, the fish
Table 19. Passive immunization of coho salmon against vibriosis by injection of immune serum from coho salmon immunized by spray vaccination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Fish Bled</th>
<th>Mean Agglutinating Antibody Titer $^{b}$</th>
<th>Number of Fish Challenged $^{a}$</th>
<th>Number of Deaths</th>
<th>Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray vaccinated against Vibrio anguillarum LS 1-74</td>
<td>50</td>
<td>$512^{b}$</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Intraperitoneal injection of 0.1 ml immune serum from spray vaccinated fish</td>
<td>6</td>
<td>$16^{c}$</td>
<td>17</td>
<td>2</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Intraperitoneal injection of 0.1 ml normal Rb trout serum</td>
<td>6</td>
<td>0</td>
<td>38</td>
<td>37</td>
<td>37</td>
<td>97</td>
</tr>
</tbody>
</table>

$^{a}$ The waterborne challenge level for immunized and passively immunized fish was $3.3 \times 10^{6}$ bacteria per ml; for the unimmunized fish, $2.4 \times 10^{6}$ bacteria per ml. A resting period of 26 days followed spray vaccination.

$^{b}$ Twenty-five days elapsed from spray vaccination to sampling. Pooled samples were utilized.

$^{c}$ Geometric mean agglutinating antibody titer of single fish samples was determined four hours post-injection of immune serum, at time of challenge.

$^{d}$ Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.
were transported to the Oregon State University Fish Disease Laboratory for challenge. The fish were held in 0.91 m circular tanks at 12.2 C. Two days after arrival, the fish were split into three 15 fish groups and challenged. The level in the challenge tank was \(6.1 \times 10^5\) bacteria per ml. Ten fish were bled from each group for agglutinating antibody titer determination. Results presented in Table 20 indicate that in coho salmon, both oral and spray vaccination conferred immunity against vibriosis. The spray technique, however, resulted in a higher level of immunity than the oral method. When the two methods are used in combination, indications of a depressed anti-\(V\). \textit{anguillarum} LS 1-74 antibody level were observed. The degree of immunity in both cases is high.

**Polyvalent Bacterins Delivered by Spray Vaccination**

The use of polyvalent vaccines would markedly reduce the time required for administration of protective antigens, lessen the stress on the fish, and significantly reduce the cost of vaccine administration. This experiment was designed not only to examine the effect of sprayed polyvalent vaccines, but was also a pilot designed to test pumice, or any scarifier, for benefit in spray preparations. The vaccine consisted of 0.3\% formalin-killed \(A\). \textit{salmonicida} SS-70, \textit{V}. \textit{anguillarum} LS 1-74, and \textit{V}. \textit{anguillarum} MSC 2-75. Spray preparations of each bacterial type consisted of 500 ml of deionized
Table 20. Comparison of agglutinating antibody and protection against *Vibrio anguillarum* LS 1-74 in groups of coho salmon orally vaccinated, spray vaccinated, and vaccinated by both methods in combination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Antagglutinating Antibody Titer$^{-1}$ of Single Fish Samples</th>
<th>Number of Fish Challenged$^a$</th>
<th>Number of Deaths Caused by Vibriosis</th>
<th>% Mortality Caused by Vibriosis$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed 45 d with standard OMP-sprayed with sham vaccine, 0.5% formalin in BHI + 0.15% bentonite</td>
<td>10</td>
<td>0</td>
<td>20</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Fed 45 d with OMP containing 5 mg bacterin per g of diet-sprayed with sham vaccine, 0.5% formalin in BHI + 0.15% bentonite</td>
<td>10</td>
<td>0</td>
<td>23</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Fed 45 d with standard OMP-spray vaccinated with bacterin + 0.15% bentonite</td>
<td>10</td>
<td>147$^c$</td>
<td>20</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fed 45 d with OMP containing 5 mg bacterin per g of diet-spray vaccinated with bacterin + 0.15% bentonite</td>
<td>10</td>
<td>78$^c$</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ The waterbom challenge level was $6.1 \times 10^5$ bacteria per ml. A resting period of 36 d followed spray vaccination; 32 d followed oral immunization.

$^b$ Percent equals deaths caused by vibriosis/Number of fish challenged minus non-specific deaths.

$^c$ The difference between the means of log$_2$ transformed single fish titers from both groups were significant at the 90% confidence level, but not at the 95% confidence level. $t = 1.68$, d.f. = 18.
water, 50 g of ground pumice (grade FFF), and 1 g (wet wt) whole-cell bacterin (prepared according to Rohovec et al., 1975). Fifty coho salmon (mean wt approx 18 g each) were sprayed with the selected polyvalent preparation. A second group of 50 coho were sprayed with BHI broth containing formalin and pumice. The fish were kept in 68 l tanks for 29 d at 12.2 C. After that time, five single fish samples were tested for agglutinating antibody against each of the three bacterial antigens. The remainder of the fish were challenged as previously described by virulent A. salmonicida. The concentration during challenge was $5.3 \times 10^7$ bacteria per ml.

Background titers against A. salmonicida were present in all groups sampled (Table 21). The only increased anti-A. salmonicida SS-70 titer was induced by spray vaccinating with preparations containing all three antigens. Titers against the two Vibrio species were induced in all cases where the vibrio antigen was present. The combination of three antigens provided increased titer for all constituents compared to other preparations containing only two antigens. Protection against challenge by virulent A. salmonicida SS-70 was either minimal or non-existent in all groups (Table 22).

**Spray Vaccination of Coho Salmon with Preparations Containing Heat-Killed Bacterial Kidney Disease Bacteria**

Bacterial Kidney Disease (BKD) is one of the most troublesome
Table 21. Geometric mean agglutinating antibody titers\(^{-1}\) against constituent antigens of various bacterin combinations included in monovalent or polyvalent spray vaccination preparations.\(^{a}\)

<table>
<thead>
<tr>
<th>Constituents of Bacterin</th>
<th>Aeromonas salmonicida Sil-67 Antigen</th>
<th>Vibrio anguillarum LS 1-74 Antigen</th>
<th>Vibrio anguillarum MSC 2-75 Antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas salmonicida SS-70</td>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aeromonas salmonicida SS-70 Vibrio anguillarum LS 1-74</td>
<td>64</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>Aeromonas salmonicida SS-70 Vibrio anguillarum LS 1-74 Vibrio anguillarum MSC 2-75</td>
<td>147</td>
<td>28</td>
<td>169</td>
</tr>
<tr>
<td>Vibrio anguillarum LS 1-74 Vibrio anguillarum MSC 2-75</td>
<td>64</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>None</td>
<td>55</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{a}\)Five fish samples after 29 d rest period following immunization.
Table 22. *Aeromonas salmonicida* SS-70 challenge of coho salmon spray vaccinated with different bacterin preparations. 

<table>
<thead>
<tr>
<th>Constituents of Preparation</th>
<th>Number of Fish Challenged</th>
<th>Number of Deaths</th>
<th>Deaths Caused by Furunculosis</th>
<th>% Mortality Caused by Furunculosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aeromonas salmonicida</em> SS-70</td>
<td>33</td>
<td>27</td>
<td>25</td>
<td>81</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em> SS-70* Vibrio anguillarum LS 1-74</td>
<td>34</td>
<td>22</td>
<td>20</td>
<td>63</td>
</tr>
<tr>
<td><em>Aeromonas salmonicida</em> SS-70* Vibrio anguillarum LS 1-74* Vibrio anguillarum MSC 2-75</td>
<td>31</td>
<td>23</td>
<td>22</td>
<td>73</td>
</tr>
<tr>
<td><em>Vibrio anguillarum</em> LS 1-74* Vibrio anguillarum MSC 2-75</td>
<td>26</td>
<td>21</td>
<td>21</td>
<td>81</td>
</tr>
<tr>
<td>None</td>
<td>27</td>
<td>24</td>
<td>24</td>
<td>89</td>
</tr>
</tbody>
</table>

\( ^{a} \text{The waterborn challenge level was } 5.3 \times 10^{7} \text{ bacteria per ml. A rest period of 29 d followed immunization.} \)

\( ^{b} \text{Percent equals death caused by furunculosis/Number of fish challenged minus non-specific deaths.} \)
fish pathogens encountered by Pacific Northwest fish culturists. An experiment was carried out to see if spray vaccination with low levels of heat-killed kidney disease antigen plus bentonite might increase agglutinating antibody. Coho salmon (mean wt approx 57 g each) not known to be infected with BKD were separated into three lots, 30 fish per lot. One group was sprayed with 0.01 molar PBS + 0.5% bentonite, another group was sprayed with 0.01 molar PBS + 0.5% bentonite + 0.3% BKD antigen. The last group was the same as the second except that the preparation was sonicated. After 23 d the agglutinating antibody against BKD was determined. The antigen for titer determination was heat-killed BKD Lea 1-74. Cells were washed three times in isotonic ethanol (1.29% in deionized distilled water) and finally resuspended in isotonic sodium chloride (0.9% in deionized distilled water) to an optical density of 1.0 on a Spectonic 20 (525 nm). This procedure was necessary to eliminate a clumping tendency characteristic of BKD. The diluent for the standard microtiter procedure was isotonic sodium chloride. Five fish samples of each test group were titered individually. Spray preparations containing whole-cell, heat-killed BKD bacteria increase agglutinating antibody levels in coho salmon (Table 23). Sonicates of heat-killed BKD bacteria were not effective in this experiment.
Table 23. Anti-Bacterial Kidney Disease bacteria agglutinating antibody induced in coho salmon by spray vaccination with bacterins containing bentonite. a, b

<table>
<thead>
<tr>
<th>Spray Preparation</th>
<th>Number of Fish Bled</th>
<th>Geometric Mean Agglutinating Antibody Titer$^{-1}$ for Single Fish Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-killed Bacterial Kidney Disease bacterin + 0.5% bentonite</td>
<td>5</td>
<td>168</td>
</tr>
<tr>
<td>Sonicated Heat-killed Kidney Disease bacterin + 0.5% bentonite</td>
<td>5</td>
<td>64</td>
</tr>
<tr>
<td>PBS Control + 0.5% bentonite</td>
<td>5</td>
<td>70</td>
</tr>
</tbody>
</table>

a Bacterins were prepared from Bacterial Kidney Disease bacteria, strain Lea 1-74.

b The time from spray vaccination to sampling was 23 d.
Spray Vaccination with Different Preparations of Formalin-killed *Aeromonas salmonicida* SS-70

The following groups were immunized to determine if agglutinating antibody production could be stimulated by spraying various preparations of *A. salmonicida* SS-70 formalin-killed bacterin:

75 Rainbow Trout (mean wt approx 3 g each) - No treatment

75 Rainbow Trout (mean wt approx 3 g each) - BHI broth + formalin only

75 Rainbow Trout (mean wt approx 3 g each) - Formalin-killed *A. salmonicida* 96 h, BHI culture + bentonite

30 Coho Salmon (mean wt approx 23 g each) - *A. salmonicida* culture bacterin

30 Coho Salmon (mean wt approx 23 g each) - BHI broth + formalin only

Fish were maintained in 68 l tanks at 12.2 C.

Two pooled groups consisting of 15 fish each were titered against *A. salmonicida* Sil-67 from the treated control and immunized rainbow trout lots. To determine the mechanical and handling stress of spray vaccination, dead fish in the untreated and treated controls were monitored during the 21 d post-vaccination period. No mortality was experienced in either group.

Fourteen fish were randomly sampled out of each coho group
after 23 d. Agglutinating antibody against _A. salmonicida_ Sil-67 was separately determined for each fish. Formalin-killed, 96 h, 18 C incubation cultures of _A. salmonicida_ containing 0.15% bentonite caused a substantial increase in rainbow trout agglutinating antibody titer when administered by the spray vaccination method (Table 24); however, spray vaccination of coho salmon with the above preparation without bentonite also caused increased agglutinating antibody titer.

**Immunization of Production Coho Salmon against _Aeromonas salmonicida_ by the Spray Vaccination Method**

Siletz River Salmon Hatchery (ODFW) is known to have an annual predictable epizootic of furunculosis each year. Usually the disease can be diagnosed during the month of May, when the water temperatures begin to rise into the high 50's or low 60's (°F). For this reason, this site has been the location of several immunization studies. Siletz Hatchery does not maintain its own stock of coho salmon, but imports its fish from different locations throughout the state. This year the coho stock was spawned and reared at Alsea Salmon Hatchery (ODFW) to approximately 0.9 g each.

On April 20, 1976 two groups of coho salmon (0.87 g) consisting of approximately 60,000 each were sprayed with either the formalin-killed _A. salmonicida_ SS-70 + 0.15% bentonite (as previously described) or with a sham vaccine containing sterile BHI broth, 0.5%
Table 24. Agglutinating antibody production in coho salmon and rainbow trout spray vaccinated with different preparations of *Aeromonas salmonicida* SS-70 bacterin.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Species</th>
<th>Number of</th>
<th>Days After Immunization</th>
<th>Geometric Mean Agglutinating Antibody Titer$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5% formalin-killed 96 h culture, 18 C incubation + 0.15% bentonite</td>
<td>Rainbow Trout</td>
<td>15</td>
<td>21</td>
<td>512$^a$</td>
</tr>
<tr>
<td>BHI broth + 0.5% formalin</td>
<td>Rainbow Trout</td>
<td>15</td>
<td>21</td>
<td>32$^a$</td>
</tr>
<tr>
<td>0.5% formalin-killed 96 h culture, 18 C incubation</td>
<td>Coho Salmon</td>
<td>14</td>
<td>23</td>
<td>172</td>
</tr>
<tr>
<td>BHI broth + 0.5% formalin</td>
<td>Coho Salmon</td>
<td>14</td>
<td>23</td>
<td>55</td>
</tr>
</tbody>
</table>

$^a$Pooled samples
formalin, and 0.15% bentonite. A group of 75 coho (0.64 g) had been sprayed earlier at the lab with the same suspensions to make sure there were no deleterious effects. The time required for four workers to immunize 60,000 fish was 45 min. The amount of bacterin preparation required was about 6 l. The fish were immunized in dip nets containing approximately 450 g of fish each. The coho used for this experiment were in the last stages of recovery from an epizootic of cold-water disease (*Cytophaga psychrophilia*) which had been treated with oxytetracycline (TM 50). The fish were removed from the medicated diet the day of spraying. Loss during immunization was negligible, 15 fish per lot.

The fish were held in separate raceways until moved to Siletz Hatchery on May 3, 1976. The water temperature at Alsea Salmon Hatchery was 44 F and at Siletz River Hatchery, 54 F. It was found at that time that only 59,760 immunized fish were transported versus 63,664 in the sham treatment group. The fish were approximately 1.3 g each.

After arrival at Siletz River Salmon Hatchery, the lots were monitored frequently for the onset of furunculosis or any other disease. On June 9, 1976 a sample of 20 fish from each treatment group were bled and the serum pooled to determine agglutinating antibody titer against *A. salmonicida*. The titers against *Aeromonas salmonicida* Sil-67 were between 1:256 and 1:512 for the vaccinated fish and 1:64
for the unvaccinated fish. Figure 12 traces the cumulative mortality in each group after the fish were moved to Siletz River Salmon Hatchery (13 d from vaccination to transfer). After 12 d, an epizootic of costia was diagnosed in both groups. The vaccinated fish were more affected. Treatment consisted of one exposure to 1:6,000 formalin for 1 h. Furunculosis was first diagnosed at day 50. No significant furunculosis loss occurred in either group after 60 d.
Figure 12. Cumulative loss in two groups of 1975-brood coho salmon containing approximately 60,000 fish each at Siletz River Salmon Hatchery.  
1. Non-immunized.  2. Spray immunized with *Aeromonas salmonicida* bacterin.
DISCUSSION

This study was directed toward two separate but related aspects of fish immunization, protective antigens and delivery systems. *Vibrio anguillarum* was chosen as the experimental model because of its devastating impact on maricultured salmonids. Because more than one serotype of *V. anguillarum* was responsible for separate outbreaks of vibriosis in the northwest, as well as the recent interest in large scale immunization, there was a need for more study of the cellular antigens responsible for protection. Such information would aid in formulation of both the composition and production protocol of vaccines.

All *V. anguillarum* isolates in this study readily formed spheroplasts and were prone to lysis. A possible explanation for the delicate nature of these bacteria lies in the relative small amounts of peptidoglycan in the rigid layer of the gram-negative cell wall. Decreased peptidoglycan and subsequent difficulty in observing a solid membrane in electron micrographs has been reported in the marine pseudomonad B-16 (Forsberg et al., 1972). Electron micrographs of both LS 1-74 and MSC 2-75 (Figures 1 and 2) reveal no readily observable solid membrane as compared to the tri layered cell walls of certain other gram-negative bacteria, i.e. *Escherichia coli* and *Spirillum serpens* (Murray et al., 1965) or *Aeromonas salmonicida*. Instability in *V. anguillarum* is more pronounced in cultures grown at 30 versus
Survival in lyophilization tubes is correspondingly increased at the lower temperature. The decreased stability of cells at 30°C with increased generation time is conducive to bacterin production, especially if lysis products or material sloughed-off in stress conditions are immunogenic and retainable from the culture.

The observations by Rohovec (personal communication) that different isolates of *V. anguillarum* fall into two separate serological groups are further substantiated by the growth characteristics of the serologically distinct *V. anguillarum* LS 1-74 and *V. anguillarum* MAN 1669. The latter organism, termed "slow grower" by many, does in fact have longer generation times at both 18 and 30°C. In addition, MAN 1669 and the related MSC 2-75 are more prone to spheroplast formation and lysis than LS 1-74.

Electron micrographs reveal slight differences in cell wall structure of LS 1-74 and MSC 2-75. The quantity and electron density of the periplasmic space in LS 1-74 appears greater than MSC 2-75. Even though steps were taken to treat both samples in an identical manner, it is possible that the difference may be a result of fixation, staining, or thickness of section. The "polar plate" or "polar membrane" observed in the micrographs of LS 1-74 has been reported in conjunction with lophotrichous flagella of *Spirillum serpens* (Murray and Birch-Anderson, 1963) and *Ectothiorhodospira mobilis* (Remsen et al., 1968). The structure has also been observed in
polarly flagellated **Campylobacter (Vibrio) fetus** (Keeler et al., 1966) and **Vibrio metchnikovii** (Vaituzis and Doetsch, 1969). The only report of a polar plate in peritrichous bacteria was by Munn and Orpin (1975) in Eadies ovals from sheep rumen. The polar plate was not observed in MSC 2-75. This obvious difference should be studied further to preclude the chance that proper sections were not examined.

Vaituzis and Doetsch (1969) suggested that contraction of the polar plate may act to tilt the basal plates of the flagella, thus causing the characteristic flagellar movement. It has also been suggested that the polar plate serves as a site for electron transport and energy production (Keeler et al., 1966; Murray and Birch-Anderson, 1963). If the structure is indeed found in **Vibrio anguillarum** LS 1-74 and not MSC 2-75, it may act as a rigid base for more efficient movement. Flagellar action may then conform to the currently accepted DePamphilus model (DePamphilus and Adler, 1971). If the structure is indeed found in LS 1-74 and not MSC 2-75, it may act as a rigid base for more efficient movement. Flagellar action may then conform to the currently accepted DePamphilus model (DePamphilus and Adler, 1971). **Vibrio anguillarum** LS 1-74 is more effective in waterborn challenge of coho salmon than MSC 2-75. The increased efficiency of movement or rigidity imparted by a polar plate may partially explain the increased invasiveness. If this is true, the polar plate could be considered a virulence factor. More "virulent" and "avirulent" culture should be examined by electron microscopy to confirm the correlation.

Experiments to determine the efficacy of bacterin preparations
against *V. anguillarum* require that immunized animals (coho salmon) be challenged by pathogenic bacteria. Previously, these challenges were effected by transporting experimental animals to areas supporting epidemic vibriosis. The disease contraction period was seasonal, occurring during the warm-water months which limited challenge experimentation to a small period of time. Other problems with natural challenge include inability to control the level of exposure, secondary invasion by unwanted species or serotypes, and fluctuation in environmental parameters. These and other problems, coupled with the increased technical requirements, required the development of an artificial, waterborn, fresh-water challenge. Thus far, as was previously pointed out, the serotypes similar to LS 1-74 are the only isolates displaying enough invasiveness to produce a successful challenge in coho salmon. Two parameters which have not been examined that may eliminate this problem are continuous low-level waterborn challenge for a long period of time and examination of different species susceptibilities.

Experiments with motile and non-motile *V. anguillarum* indicate that flagella are not required in efficacious vaccines. This observation does not preclude the possibility that flagella are immunogenic. Only by extraction and exhaustive purification can the protective nature of flagella be adequately examined. If flagella are immunogenic, the fact that many gram-negative flagella are
serologically cross-reactive may make their presence beneficial in vaccine preparations.

Injected whole-cell and cell-wall extracts of serologically distinct groups of \textit{V. anguillarum} are cross-protective under the challenge conditions employed. The data in Table 8 indicates that cross-protection may not be complete. Immunodiffusion studies demonstrate that both serotypes possess identical antigens. Even though cross-protection exists, the data are not sufficient to dismiss the possible need for polyserotype vaccines. Several aspects of immunity must be examined before such an action can be taken. With a standard dose and delivery system, the first consideration, level of immunity, can be estimated by determining the amount of protection conferred against several challenge levels of each serologically dissimilar disease-causing organism. Since level of immunity is also related to physiological state and availability of immune mechanisms, these criteria must be standardized as much as possible by using a homogeneous population sample. The second consideration, duration of immunity, could best be determined by repeating the challenges on the just described sample population at specified intervals. If possible, the challenges should approximate as closely as possible the natural situation. A third aspect of immunity which must be examined is different disease susceptibilities between species--of great importance depending on the application of the product. Only after testing
on this basis can a good approximation of practical, conferred immunity be made. This information, as well as an understanding of the environmental conditions faced by the immunized animal, can help determine a specific type of vaccine required.

As was previously mentioned, phenol-water extracts of both _V. anguillarum_ serotypes are cross-protective. In addition, a phenol-water extract of purified LS 1-74 cell walls conferred immunity and stimulated the production of agglutinating antibody. This latter preparation was twice ultracentrifuged to remove contaminating nucleic acid. Other extracts have been used to protect fish from vibriosis. Abe (1972) used TCA extracted endotoxin to protect chinook salmon. The difference in final product between the TCA and phenol-water extraction methods lies in the amount of covalently bound protein attached to the somatic lipopolysaccharide. The phenol-water method gives a much less contaminated LPS fraction although up to 2% bound amino acids are invariably still present (Nowotny, 1969). Other investigators have had success with immunogenic cell-wall extraction products. Lipopolysaccharide (LPS) was used by Verwey et al. (1965) and Watanabe and Verwey (1965) to protect mice against infection by Inaba and Ogawa strains of *Vibrio cholera*. Verwey et al. (1965) demonstrated that high temperatures or phenol-water extraction inactivated the immunogenic portion of the Inaba strain but not the Ogawa strain.
Even though the extracted LPS fraction is highly antigenic and immunogenic against *V. anguillarum* (Table 7), the exact nature of the protective antigen is still in question. Antipa (1976) has demonstrated efficacy of injected *V. anguillarum* bacterins, inactivated by boiling at 100°C for 1 hr. This data, as well as that of Verwey et al. (1965), along with the results presented in this work, suggest the protective entity lies in the LPS fraction. Luderitz et al. (1966) has stated that (a) the LPS and polysaccharide are only weakly or non-antigenic, (b) the antigenic determinant lies in the polysaccharide moiety, (c) immunogenicity requires the presence of protein, and (d) the LPS and polysaccharides make excellent antigens when coupled with a carrier protein. With phenol-water or TCA extracts, Kabat (1971) has described the gel diffusion pattern as a large major lipo-polysaccharide band fusing into a second band of degraded polysaccharide. Further banding is probably due to small amounts of degraded polysaccharide or impurities. Figure 10 and 11 show that the primary endotoxin bands of two serotypes of *V. anguillarum* are not cross-reactive with heterologous antiserum. Cross-reaction occurs in the faster diffusing secondary bands. Results shown in Tables 10 and 11 demonstrate cross-protection with phenol-water extracts. If the immunogenic portion is degraded polysaccharide composed of strain specific side chains and/or group specific basal sugars (causing cross-protection), protection would be contrary to the observations of
Luderitz et al. (1966). If the polysaccharide were immunogenic when attached to non-specific carrier compounds from either the bacterial cell or the immunized animal, denatured protein or not, the efficacy of various immunogenic agents from *V. anguillarum* could be explained. Vaccination preparations of highly purified polysaccharide extractions from purified LPS, both attached and not attached to unrelated carrier protein could be tested for immunogenicity. It is also possible that enough contaminating protein is available to stimulate protection. Even though heat denaturation does not seem to affect the efficacy of *V. anguillarum* protective preparations, enough reannealing may occur upon cooling to restore biological activity. The results presented in Table 13 indicate that an extremely small amount of TCA extract (5 ng) will produce both agglutinating antibody activity and protection against challenge. If protein is required, detection of that amount in test preparations is extremely difficult.

Since small amounts of antigen are required to protect coho against vibriosis, the possibilities for delivery techniques increase. Several vaccines for fish have been delivered by intraperitoneal injection, by incorporation in food or water, by vacuum infiltration, and, more recently, by placing fish in hyperosmotic solutions followed by vaccine baths. Each of these techniques has its inherent advantages and disadvantages. Peritoneal injection of large antigenic mass (with and without adjuvent) has proven to be the most effective. The method
suffers when administered to large populations because it is cumbersome and costly. Incorporation of vaccine into the diet is probably the most desirable of the above delivery systems. However, the method has only been shown to be effective in a limited number of cases. The degree and duration of immunity conferred by the oral route is generally less than by injection. Vacuum infiltration and hyperosmotic immersion are newer delivery systems and lend themselves to vaccination of small fish. Both techniques would be time consuming and costly when applied to larger fish. Unless disease causing agents are constantly present to reinforce immunity, or if duration of immunity is short, revaccination of larger fish might be required.

Vaccination of larger fish prior to release into areas endemic for vibriosis could be necessary for increased survival. With this in mind, the observation that fluorescent marking techniques are useful for long periods of time suggested a means of bacterin delivery. Since small amounts of protective antigen are required, it was thought that by combining bacterin with dye, enough antigenic mass would be delivered to elicit an immune response. The data indicates that this does occur (Table 14). The original use of dye as a carrier was very expensive and inefficient, especially when bacterin was incorporated at 100 mg per g. Even at 1 mg per g (Table 15) based on observed consumption of dye-bacterin, it would take 500 g (wet wt) of bacterin
plus 10 kg of dye to vaccinate between 30,000 and 50,000 fish (approx 45 g each). The cost of preparation, material, and delivery would be prohibitive (not to mention messy). These considerations led to examination of liquid and culture media preparations. The success of these vaccines (Table 16), especially with the addition of the adsorbant-cation exchanger bentonite (Table 16 and 24) solved these problems. One group of 18,000 chinook salmon (approx 6 g each) spray vaccinated with *Vibrio* bacterin containing 0.15% bentonite required 45 min and 6 l of bacterin at a total cost of approximately 0.15 cents per fish. Spraying 60,000 coho (approx 0.9 g each) in the *A. salmonicida* production experiment required the same time and amount of bacterin at approximately 0.05 cents per fish.

Culture media preparation of spray vaccination bacterins were incubated for 96 h. The long incubation period was employed because lysis products or excreted cellular material might lead to better protection, possibly by increased availability of the protective moiety for bentonite attachment. Abrasives, both alumina and pumice, did not seem to add to the efficacy of the spray vaccination preparations.

Pumice was added to the polyvalent bacterin preparations in an attempt to increase titers. The low level of bacterin (whole-cell) and the lack of effect by pumice probably led to the low titers observed in Table 21, especially against *A. salmonicida*. Higher titers were observed in rainbow trout spray vaccinated with *A. salmonicida*.
bacterins plus bentonite (Table 24). In the polyvalent vaccine experiment, it is interesting to note that increasing the number of bacterins in a preparation resulted in increased antibody titer for all constituents. There appears to be a synergistic effect. It is also noteworthy that when \textit{V. anguillarum} LS 1-74 bacterin is fed for 45 d and then sprayed, the mean agglutinating antibody response is lower than if the bacterin was sprayed with no oral immunization. Further studies on this phenomenon as well as the polyvalent synergism should be carried out.

The mechanism of immunity stimulated by spray vaccination is probably similar to that stimulated by injection. Unlike oral immunization, detectable agglutinating antibody is produced. Passive protection has been reported in salmonids with injected immune serum against \textit{A. salmonicida} (Spence et al., 1965) and \textit{V. anguillarum} (Harrell et al., 1975; Gunnels et al., 1976). In this work, the antibody titer (1:16) reported for passively immunized fish is the titer at the time of challenge and is not necessarily the titer present after challenge was complete. Distribution of the immune serum from the intraperitoneal injection site may not have been completed. Thus, the level of agglutinating antibody sufficient for protection is not adequately estimated in the passive immunization experiment. In other tests, protection was present when lowest titers of 1:24 to 1:48 were recorded. It is entirely probable that lower agglutinating
antibody titers (undetectable) may be sufficient for conferred immu-
nity. Fish of known size and blood volume could be injected with
graded amounts of immune serum and then challenged to get an esti-
mate of antibody required for protection.

Bentonite, the substance found to increase the efficacy of spray
preparations, is a naturally occurring, very fine grained material
composed of the clay mineral, montmorillonite. Chemically, it is
a hydrous aluminum silicate consisting of one octahedral aluminum
sheet between two tetrahedral silicon sheets. These three sheets,
or unit cell, are stacked in a parallel array. The distance between
the unit cells depends mostly on the amount of water adsorbed to the
hydroxyl ions radiating from the surface of the silica sheets (inter-
lamellar adsorption). These ions also act as a cation-exchange site.
The cation-exchange capacity for bentonite is 70 to 100 meq/100 g of
clay (Clem and Doehler, 1963). The interlamellar surfaces and swelling
characteristic of montmorillonite explain the high cation-exchange
capacity compared to most other clays.

Materials other than small cations will adsorb to the external
or interlamellar surfaces of bentonite. MacEwan (1961) reported
various organic materials including protein which adsorb (via dipole
moments, electrostatic forces, or van der Waal's forces) to the
bentonite particle. Recent evidence indicates that organic solutes
which do not have cation-exchangable groups will not migrate to the
inner surfaces of the bentonite but are restricted to external surfaces (Cartensen and Su, 1971).

Because of the many possible adsorption mechanisms and adsorption sites on bentonite, the exact reason for increased bacterin efficiency at low pH is unknown, especially since the exact nature of the protective antigen or its required form of presentation is unknown. If protein were required, either singly or attached, the lower pH would lead to increased numbers of positive charges which in turn would lead to stronger attachment to the bentonite.

Several aspects of spray vaccination must be further studied to ascertain the effectiveness of the technique. Experiments with spray vaccinated, sham-sprayed and unvaccinated fish exposed to homologous versus heterologous challenge, i.e. vaccinate against _V. anguillarum_ and challenge subgroups and controls separately with either _V. anguillarum_ or _Flexibacter columnaris_, would yield useful information on the effect of spraying on general resistance of the fish. Another aspect to be established is some standardization of spray dose, both in the bacterin itself and the amount applied to fish. Recyling and collection of spray preparations would give a good rough estimate of the amount of bacterin delivered.

The experiment at Siletz River Salmon Hatchery was unsuccessful as of July 9, 1976. Only a low level of challenge had been observed. The difference between the level of loss in the vaccinated and
unvaccinated fish was minimal. Loss was slightly higher in the vaccinated fish. The fact that fish receiving bacterin suffered higher losses due to *Costia* and slightly higher loss due to furunculosis (even though both control and vaccinated groups were sprayed) makes experiments designed to check the effect of bacterin on general predisposition to disease very important.

Spray vaccination lends itself to mechanization. Spray devices can be attached to pre-existing fish movement apparatus, possibly with a reservoir and recycling system. The use of bentonite as the vaccine carrier should be studied further. The amount of bentonite added can be varied. Phase-microscopy examination of spray preparations reveal bentonite particles completely surrounded by bacterial cells and cellular debris. At the same time, much bacterial material is still free in the media. Increasing bentonite levels may lead to increased response. An advantage to bentonite is its natural derivation and should have little deleterious effect on the fish and the environment. Charcoal, another substance used as an adsorbant, as well as beidellite might also be examined as a carrier.
SUMMARY AND CONCLUSIONS

1. From growth studies on *Vibrio anguillarum*, at 18 and 30 C, the isolate termed LS 1-74 had a shorter generation time than the isolate termed MAN 1669. Both isolates reached higher levels of growth at 18 versus 30 C. At 30 C, the MAN 1669 isolate went into death phase in a very short period of time. Both organisms were susceptible to spheroplasting and lysis, the MAN 1669 more-so than the LS 1-74.

2. A reproducible waterborn challenge against *V. anguillarum* LS 1-74 was developed. Similar challenges with a second serotype of *V. anguillarum* MSC 2-75 failed to produce death. Intraperitoneal injection of MSC 2-75 into coho salmon (mean wt approx 30 g each) resulted in an LD$_{50}$ of $2.6 \times 10^3$ bacteria per fish.

3. Injection of whole-cell bacterins and phenol-water extracts prepared from isolates of two *V. anguillarum* serotypes conferred cross-protection against challenges by *V. anguillarum* LS 1-74 and MSC 2-75. The challenge levels resulted in mortalities ranging from 59% to 85% in the controls. Gel diffusion studies indicate that the different antigens present in LS 1-74 and MSC 2-75 include the intact somatic O fraction. Breakdown products may be cross-reactive.
4. Vaccination with non-flagellated, non-motile *V. anguillarum* resulted in protection against challenge by virulent *V. anguillarum*. This result indicates that flagella are not a necessary protective antigen.

5. Injected partially-purified, phenol-water extracts of *V. anguillarum* cell-wall preparations are antigenic in coho salmon and are protective against challenge by virulent *V. anguillarum* LS 1-74. Boiven extracts are antigenic and confer protection in injected doses as low as 5 ng.

6. Fish can be immunized against vibriosis by being sprayed via a sandblasting apparatus operated at 6.3 to 7.0 Kg/cm$^2$ with bacterins containing 0.15% bentonite. The addition of scarifiers such as alumina and pumice do not increase antigenicity or potency.

7. Bacterins consisting of formalin-killed cultures of *V. anguillarum* grown at either 18 or 30 C are protective in coho salmon when applied by spray vaccination. The addition of 0.15% bentonite increased the production of agglutinating antibody ten-fold.

8. Coho salmon sprayed with *V. anguillarum* bacterin containing bentonite possessed a 1:24 agglutinating titer after 112 d. The fish were protected from challenge by virulent *V. anguillarum* after 125 d.
9. At both 25 and 45 d, fish sprayed with bacterins containing bentonite adjusted to pH 3 showed higher agglutinating antibody levels than fish sprayed at pH 5, 7, 9, and 11.

10. Coho salmon were passively immunized against challenge by virulent *V. anguillarum* by injection of 0.1 ml of 1:512 immune sera raised in the spray-vaccinated homotypic species.

11. Studies with oral and spray vaccination preparations against *V. anguillarum* indicate that spray vaccination results in a higher level of protection than oral immunization. The level of agglutinating antibody produced may be depressed if both methods are employed on the same population.

12. Studies on polyvalent-spray vaccines indicate that with *Aeromonas salmonicida* and *V. anguillarum* LS 1-74 and MSC 2-75, inclusion of all three in the sprayed preparation results in a synergistic agglutinating antibody response.

13. Spray vaccination preparations containing heat-killed Bacterial Kidney Disease bacterin plus 0.5% bentonite produced higher coho salmon anti-BKD antibody than was observed in the unvaccinated controls.

14. Spray vaccination preparations of 0.5% formalin-killed, 96 h, 18 C cultures of *A. salmonicida* SS-70 resulted in agglutinating antibody titers of 1:512 compared to 1:32 in the unvaccinated controls.
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