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Title: Microbial Flora of Pink Shrimp, Pandalus borealis

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Eight samples of Pandalus borealis shrimp were taken in the estuaries of southeastern Alaska during a period extending from October, 1969 to April, 1970. A total of 759 bacterial isolates from these samples were grouped into categories using a dichotomous identification scheme. The groups of microorganisms present in greatest numbers varied with the season. The Acinetobacter phenons III and IV group (36 to 70% of total isolates) predominated in the October samples. In November, the Pseudomonas-Aeromonas-Vibrio group (45%) and an unclassified group (45%, which would formerly have been classified as Achromobacter) were present in the largest proportions. The Flavobacterium-Cytophaga group (50 and 42%) predominated in February and April, while an unclassified group (37%) of polar-flagellated, oxidase-negative organisms predominated in March.

Two comparisons were made between the microbial flora of Smooth pink shrimp (Pandalus jordani) and Pink shrimp (Pandalus borealis). In the first comparison the microbial compositions were quite similar, but in the second comparison, the Pseudomonas-Aeromonas-Vibrio group (35%) predominated in the Smooth pink shrimp sample, in contrast to the Pink shrimp sample where it comprised only 10% of the total isolates. Other differences were noted in the second comparison.

When male-or-transitional sex shrimp were compared with female shrimp sampled in parallel, striking differences in microbial composition were noted. In the first comparison, the female sample had a larger percentage of Flavobacterium-Cytophaga isolates (female, 75%; male-or-transitional, 13%). Also, Acinetobacter phenon II isolates which comprised 80% of the male-or-transitional sample were totally absent in the female sample. In the second comparison, it was the male-or-transitional sample that had the higher proportion of Flavobacterium-Cytophaga isolates (69% vs 7%). In this comparison, the other major difference in microbial flora between the sexes was the large proportion of unidentified polar-flagellated, oxidase-negative isolates in the female sample (43%).

The uncertainty of the taxonomic position of certain groups of isolates is noted. Variables which might have been responsible for the observed fluctuations in the microbial composition of the samples are discussed.

Microbial Flora of Pink Shrimp, *Pandalus borealis*

by

David Isaac Wieler

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MICROBIAL FLORA OF PINK SHRIMP, *PANDALUS BOREALIS*

INTRODUCTION

The shelf life and safety of foods subject to microbial degradation is directly influenced by the types of microorganisms present and the physical conditions to which the foods are subjected. Interactions among the organisms present additional variables that must be considered in order to predict the shelf life and safety of a food product. Large saprophytic microbial populations provide some protection against proliferation of public health significant bacteria by competing for nutrients and otherwise modifying the microenvironment. The spoilage caused by the saprophyte population can serve as a safety factor by making the food organoleptically unacceptable before large numbers of public health significant microorganisms can arise.

Practical Value

One would expect that the partial destruction of the saprophyte population by a process such as heat or radiation pasteurization would reduce the safety factor and present a lesser competitive obstacle to bacteria of public health significance that might survive the treatment or gain access to the food after treatment. Before adopting such a process, one would have to weigh the beneficial effects of longer shelf life against the possible hazards of unchecked proliferation of public health significant bacteria. The

quantities and types of saprophytes that survive the pasteurization would affect both the shelf life and the safety of the food product.

Purpose

The purpose of this study is to enumerate the natural aerobic saprophytic flora of the Pink shrimp, Pandalus borealis. The methodology developed in achieving this goal, and the data obtained, will be used in further studies designed to evaluate the safety of food processes involving the partial destruction of the saprophytic flora of Pink shrimp. Knowledge of the types and abundance of the natural flora is a necessary first step in understanding changes in the microbial flora that take place during processing and storage.

HISTORICAL REVIEW

Information on the microbial flora of shrimp is primarily limited to shrimp of the Gulf of Mexico (Campbell and Williams, 1951) (Carroll, Reese and Ward, 1968). Harrison and Lee (1969) investigated the microbial composition of Smooth pink shrimp (P. jordani) sampled at different sites along the processing line in two Oregon plants. They found that the initial microbial flora, in order of predominance, was Acinetobacter-Moraxella, Flavobacterium, Pseudomonas, Gram-positive cocci, and Bacillus species. Gram-positive cocci, the majority of which were coagulase-negative, increased proportionately at each stage of processing.

The outgrowth of public health significant microorganisms is influenced by competition and antagonism with the saprophytic microbial flora. Oberhofer and Frazier (1961) investigated the influence of sixty-six cultures of food microorganisms on the outgrowth of four strains of Staphylococcus aureus, two of which were enterotoxigenic and two were not. They found that the degree of inhibition of S. aureus varied considerably with the competing culture. Some cultures were even found to be stimulatory. Peterson, Black and Gunderson (1961) found that the greater the naturally occuring saprophytic population in precooked frozen foods, the greater the protection against staphylo-

coccal growth on defrosting. Oblinger and Kraft (1970) reported inhibition of Salmonella, Staphylococcus, and Streptococcus by Pseudomonas strains. It is apparent that changes in composition of the saprophytic flora can influence the likelihood of outgrowth of public health significant microorganisms.

Investigating the relationships between groups of microorganisms associated with food spoilage has been facilitated by the development of simplified identification schemes. The dichotomous scheme presented by Shewan, Hobbs and Hodgkiss (1960), was a breakthrough that permitted identification of marine Gram-negative bacteria without a great investment in time and labor. The authors developed the scheme to facilitate identification of large numbers of bacteria isolated in fish iced-storage studies. Motile isolates were broadly grouped on the basis of oxidase reaction and type of flagellation. Non-motile isolates were broadly grouped on the basis of pigmentation and cellular morphology. Other identification criteria consisted of colonial appearance, antibiotic and 0/129 sensitivity tests, presence of diffusible fluorescent pigments, 2-ketogloconic acid formation, and reaction on Hugh and Leifson's media.

Corlett, Lee and Sinnhuber (1965) adapted the scheme of Shewan et al. to solid media which was inoculated by replica plating (Lederberg and Lederberg, 1952). The results were coded and analyzed by computer. The differential characteristics

consisted of pigmentation, cellular morphology and Gram reaction, and growth response on SS agar, Staph 110 agar, potato dextrose agar, and a variety of antibiotic media. Development of the multipoint replicator (Seman, 1967), which permitted rapid multiple inoculations of liquid media, made it practical to use the criteria of motility and type of flagellation proposed by Shewan et al., rather than the more difficult to interpret antibiotic sensitivity tests of Corlett et al.

The non-motile non-pigmented group designated as Achromobacter-Alkaligenes in the Shewan et al. scheme was reclassified by Shaw and Shewan (1968) as Moraxella-like organisms. Thornley (1967) classified this group as Acinetobacter and found five phenons within this group. Baumann, Doudoroff and Stanier (1968) proposed that the members of this group possessing cytochrome oxidase c be classified as Moraxella, and the oxidase-negative members be classified as Acinetobacter. In their proposed definition of the genus Moraxella, the authors include sensitivity to penicillin G at a concentration of one unit per milliliter. Lee and Harrison (1968) found that those isolates resistant to one unit of penicillin were equally resistant to three units, but the latter concentration more clearly differentiated the sensitive species. Harrison and Lee (1969) reported difficulties in applying the criteria of Baumann

et al. because of the presence of oxidase-positive but penicillin-resistant organisms and the presence of penicillin-sensitive but oxidase-negative organisms.

MATERIALS AND METHODS

Identification Scheme

The identification scheme is diagrammed in Figure 1. The dichotomous identification scheme proposed by Shewan, Hobbs and Hodgkiss (1960) was adapted for identifying the Gram-negative flora. The group formerly classified as Achromobacter-Alkaligenes by Shewan et al. was subdivided according to Thornley (1967). These non-motile coccoid rods, cocci, or short rods, often in pairs, not producing pigment on nutrient agar milk were divided into Acinetobacter phenon II (oxidase-negative, penicillin-resistant) and Acinetobacter phenons III and IV[1] (oxidase-positive or oxidase-negative, penicillin-sensitive). Penicillin sensitivity at a level of three units per milliliter was selected for differentiation in this study.

A more complete identification scheme could have been developed for the Pink shrimp microbial flora, but it was decided to keep the number of individual tests to be performed to a minimum in order to have time to screen a large number of isolates. Also tests requiring long incubation periods or requiring intensive labor were rejected. The exception to this was the motility examination and subse-

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[1] The polar-flagellated, oxidase-positive organisms in the Shewan et al. scheme may include some of the organisms classified by Thornley as belonging to Acinetobacter phenon V.

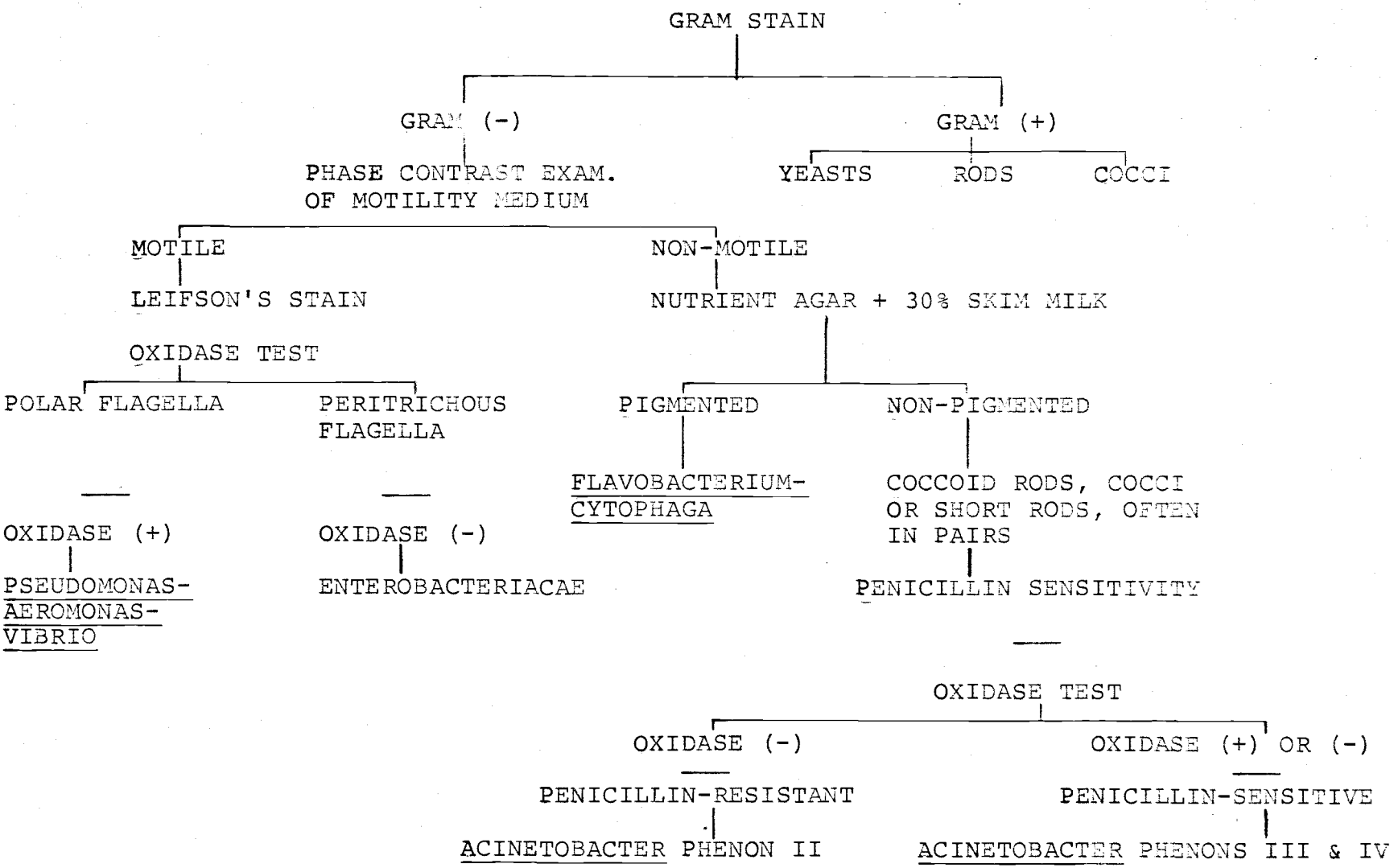

Figure 1. The identification scheme for microorganisms isolated from shrimp.

quent Leifson's flagella stain of motile isolates. It was felt that the unequivocal dichotomy afforded by this procedure would compensate for the time required to perform it.

The Gram-positive flora were simply divided into rods and cocci.

Media

Eklund's TPY medium (Miyauchi et al., 1963) with 0.2% dextrose was selected for use as the primary isolation medium because of its higher recovery than Standard Methods Agar and a variety of other media. In addition, the author has had previous experience with this media (Eklund, Poysky and Wieler, 1967) and is familiar with the colonial appearance of some of the more common marine microbial flora grown on it. The formula for Eklund's TPY is as follows: 0.5% Difco yeast extract, 1.5% BBL Trypticase, 0.5% NaCl, 0.5% Colab soy peptone powder, 1.5% Difco agar, pH adjusted to 7.1. TPY medium was compared twice with Lee's TPN medium (Corlett, Lee and Sinnhuber, 1965) for recovery at 20°C. The formula of TPN is: 0.5% Difco peptone, 0.5% Difco tryptone, 0.25% Difco yeast extract, 0.5% NaCl, 0.1% dextrose, 2.0% Difco agar, pH adjusted to 7.1. In the first comparison, TPY counts were slightly greater than TPN when three-day-old Smooth pink shrimp (P. jordani) caught in April 1969 were used (2.3×10^6 vs. 1.5×10^6 organisms per gram). When this comparison was repeated in April of 1970 using Pink shrimp (P. borealis), the TPY recovered a lesser number

of organisms than the TPN (7.1×10^4 vs. 1.7×10^5 organisms per gram). The comparison of TPY with TPN showed that these two media were selective for the same groups of organisms and that the isolates recovered were present in about the same proportions.

Oxoid nutrient agar with 30% Difco skim milk (Shewan, Hobbs and Hodgkiss, 1960) was used for identification of the Flavobacterium-Cytophaga group. Three units per milliliter Squibb potassium penicillin G was added to tempered TPY agar to prepare the plates for penicillin sensitivity testing. TPN agar was used for detection of diffusible fluorescent pigments produced by certain pseudomonads.

In the scheme of Shewan et al. (1960), Lab Lemco nutrient agar was used as the primary isolation and cultivation medium, and nutrient broth was used as the motility medium. When TPY agar was used as the primary isolation medium and nutrient broth was used as the motility medium in the preliminary experiments of this study, growth in the nutrient broth was poor and sometimes observation of motility was unrepeatable. It was hypothesized that a motility medium more like the primary isolation medium would result in a greater number of isolates showing motility and that the results would be more reproducible. To test this assumption, 20 isolates were replicated into 5 sets of media and observed for motility after 24 hours incubation at 20°C. The results are shown in Table I. TPY broth and TPY broth

TABLE I. COMPARISON OF VARIOUS MOTILITY MEDIA

	MEDIA[1]	% MOTILE	% GOOD GROWTH[2] (motile & non-motile)
TPY broth	A	65	65
Brain-heart infusion broth	B	45	50
Nutrient broth	C	25	25
Heart infusion broth	D	40	35
TPY broth with dextrose	E	65	55

[1] All media contained 0.1% KH_2PO_4

[2] Good growth is defined as a visibly turbid suspension throughout the tube.

without dextrose proved to be satisfactory media for the demonstration of motility when used in conjunction with a TPY agar primary isolation. Following the advice of Leifson (1960), the TPY without the fermentable carbohydrate was selected for routine use.

Shrimp

The shrimp used in these experiments were caught with commercial gear, either by beam-trawl or pot, in the inside waters of southeastern Alaska in the vicinity of Wrangell (Eastern Passage) and 50 miles south in Behm Canal (Shrimp Bay). The beam-trawl-caught shrimp came from a depth of 25 to 30 fathoms and were almost exclusively P. borealis. The pots were set in depths from 55 to 70 fathoms and contained primarily Spot shrimp (P. platyceros) and Side-stripe shrimp (P. dispar). The smaller and less valuable Pink shrimp were an incidental catch in the pots. On several occasions, pots contained both P. borealis and P. jordani. On one occasion, one string of pots (10 to 12) contained only P. borealis, while 30 meters away another string contained only P. jordani. It was necessary to examine each shrimp taken for sampling to avoid mixing species.[2]

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[2] P. borealis has spines on the third and fourth abdominal segments, whereas P. jordani is smooth (Butler, 1964).

Because the shrimp came from depths predominately inhabited by the female sex, the randomly selected samples reflected the sexual composition of the catch. Table II shows the sexual stage and other characteristics of the samples.

Testing Procedures

Sample Preparation

Sterile forceps were used to transfer the shrimp caught in the pots or trawl to sterile jars. The jars were held in ice until they could be air-taxied back to the laboratory. In most cases, the shrimp were less than four hours out of the water and still alive when received at the lab. Immediately following the receipt of samples, species of shrimp was verified, and following the procedures of Lewis and Angelotti (1964), 50 g of P. borealis was aseptically weighed into a tared beaker. The weighed sample was transferred to a sterile one-quart Oster blender using part of the 450 ml of diluent (buffered phosphate with 0.1% peptone added) to rinse into the blender jar any shrimp adhering to the weighing beaker. The remainder of the 450 ml of diluent was added to the blender jar and the contents were blended for one minute at low speed. It was found that better homogenization occurred if the blender was stopped for 5 seconds after 10 seconds of blending, to allow the antennae and

TABLE II. SPECIES, METHOD OF CATCH, SEX AND LOCATION OF SAMPLES

Sample	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Date	Sept.		Oct.					Nov.	Feb.			Mar.			Apr.
	8	8	8	15	22	29	29	14	24	24	24	3	3	3	8
	1969								1970						
Species:															
P. borealis		X	X	X	X	X		X	X	X	X	X	X	X	X
P. jordani	X						X								
Method of catch:															
pot	X	X	X	X	X	X	X	X							
beam-trawl									X	X	X	X	X	X	X
Sex characteristics:															
Majority with eggs						X	X	X	X	X		X	X		
Male-or-transitional											X			X	
Female only	X	X	X	X	X					X			X		X
Sampling site:															
Shrimp Bay	X	X	X	X	X	X	X	X							
Eastern Passage									X	X	X	X	X	X	X

pieces of shell to settle to the bottom where they could be cut up by the blades.

Log dilutions were prepared, and 0.1 ml of each dilution to be used was spotted on a pre-dried TPY agar plate. Bent glass rods were used to spread the inoculum.

Replication

After four days incubation at 20°C,[3] the plates were counted and colonies were picked with sterile toothpicks onto fresh TPY master plates, 20 colonies per plate. Tiny, slow-growing colonies were picked onto different master plates than the larger, fast-growing colonies. Large-colony plates were incubated for two to three days and small-colony plates were incubated for three to four days. When the colonies on the master plates had attained sufficient size, a multipoint replicator (Seman, 1967) was used to inoculate the identification media in the following order: TPN, TPY-PEN, nutrient agar-milk, TPY, TPY motility medium, and TPY. The multipoint replicator was reinoculated between the first TPY plate and the TPY motility medium to assure an adequate inoculum level throughout the identification media. By using TPY as the last plate in the replication series, it was possible to verify the successful transfer of all the isolates to the preceding media.

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[3] The uniform incubation temperature of 20°C was selected as a compromise between rapid outgrowth, and recovery of obligate psychrophiles.

Motility Examination and Flagella Staining

After 18 to 24 hours at 20°C, a small drop of liquid from the surface of the TPY motility tube was removed by sterile capillary tube and examined as a hanging drop preparation under phase contrast. If the preparation showed no growth, the tube was reincubated an additional 24 hours. If motility was observed, the contents of the tube (4 to 5 ml) was added to 0.3 ml formalin and 1.0 ml distilled water and centrifuged at 1000 RCF for 10 minutes. The supernatant was then decanted and the tube lip washed while the tube was still inverted. Two ml of water was added, and the contents were mixed by blowing bubbles before repeating the above operation. To make a barely turbid suspension, 0.5 to 2.0 ml of distilled water was added, mixed by bubbling, and one drop was taken for a Leifson's stain (Leifson, 1960). Two smears were placed on a slide and four slides were stained at a time. Preparations were observed by light microscope at 1000x and the flagella were recorded as polar or peritrichous.

Morphological and Biochemical Observations

Characteristics of the isolates on the plate media were observed after three to four days incubation. Table III shows the tests used to identify isolates. A long wave (3660 Å) ultraviolet light source was used to detect the presence of diffusible fluorescent pigments produced by

TABLE III. TESTS USED TO IDENTIFY ISOLATES.

Medium	Observation
TPY agar	Colonial appearance and pigmentation Gram stain Cellular morphology Oxidase test (Barry and Bernsohn, 1969)
TPN agar	Diffusible fluorescent pigments with U.V. light Colonial appearance and pigmentation
TPY motility medium	Motility, flagella stain (Leifson, 1960), cellular morphology
Nutrient agar + 30% skim milk	Pigment production by Flavobacterium-Cytophaga group, casein proteolysis
TPY agar with 3 units per milliliter penicillin G	Penicillin sensitivity

isolates on the TPN plates. When the isolates replicated on TPY-PEN did not grow or were less than half the size of the colony on the corresponding TPY plate, the isolate was considered to be sensitive to penicillin. The presence of yellow or orange pigmentation of the colonies on the nutrient agar-milk plates was recorded. Gram stains were made from the TPY plates and at the same time, the test for the presence of cytochrome oxidase c was performed using test papers prepared according to Barry and Bernsohn (1969). Cellular morphology of the isolates was observed from the Gram stains and was recorded as rod, cocci, or cocco-bacillary form. Secondary characteristics that were observed and recorded but were not used in the dichotomous identification scheme included pigmentation on TPY and TPN plates, proteolysis of casein on the nutrient agar-milk plates, and size and shape of the cells in the TPY motility medium.

RESULTS AND DISCUSSION

The results of eight samplings of P. borealis shrimp are shown in Table IV. A total of 759 isolates from the eight samples were classified. Total aerobic counts of the samples ranged from 2.9×10^3 to 6.5×10^6. The number of isolates examined and the total aerobic counts of the samples are shown in Table V. Figure 2 is a graphic depiction of these results in which Gram-positive rods and Gram-positive cocci are grouped together, the peritrichous oxidase-positive isolates are included in the unidentified Gram-negative group, and the Enterobacteriacae and yeasts and molds categories are not shown. Four of the eight samples shown were taken in the month of October. Rough weather and ice precluded sampling during the months of December and January.

Samples analyzed but not shown in Table IV or Figure 2 include P. jordani samples, female-only samples, male-or-transitional samples, and samples assayed before the identification procedures were standardized. Funds were unavailable after April for further sampling.

Acinetobacter Phenons III and IV

As shown in Figure 2 and Table IV, Acinetobacter phenons III and IV were the predominant microbial flora in the October samples. In sample E (10-22-69), this group reached a peak of 70% and had declined to 47% by the next

TABLE IV. MICROBIAL FLORA OF EIGHT SAMPLES OF *P. BOREALIS*

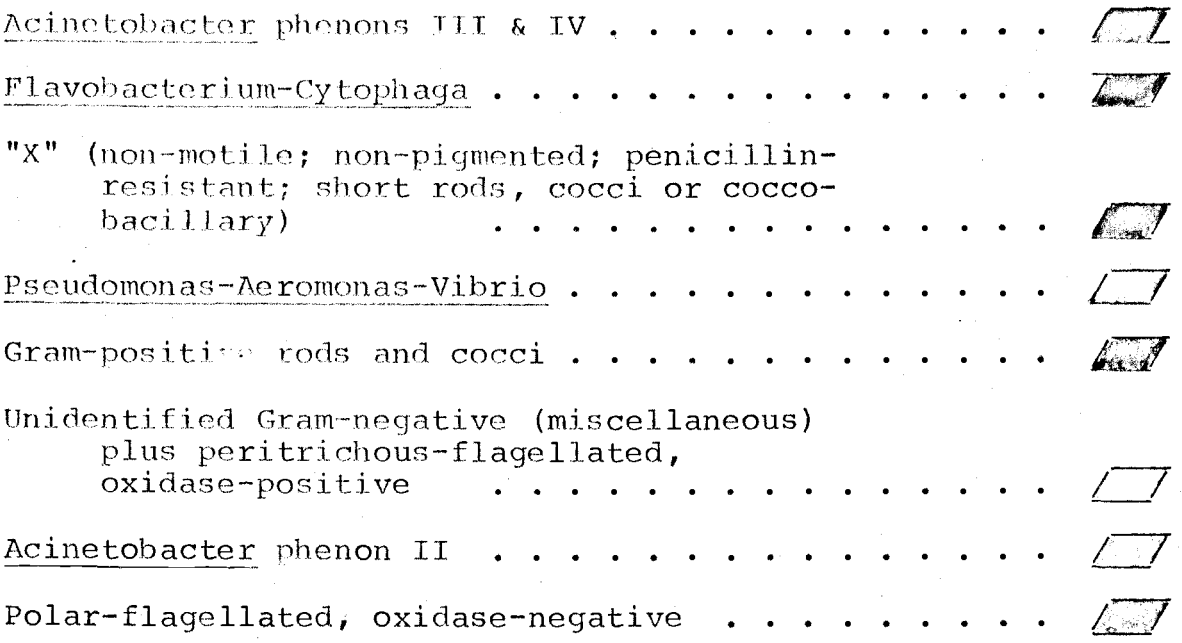
Sample	C	D	E	F	H	I	L	O
Pseudomonas-Aeromonas-Vibrio	10.4	10.0	3.9	9.5	44.8	0.0	10.5	0.0
Enterobacteriacae	0.0	0.0	1.3	2.2	0.0	0.0	0.0	0.0
Flavobacterium-Cytophaga	26.7	10.0	5.2	14.7	0.0	50.0	10.5	41.7
Acinetobacter phenon II	0.9	1.8	0.0	1.4	0.9	10.0	21.0	0.0
Acinetobacter phenons III & IV	36.2	50.9	69.7	47.3	9.5	3.3	10.5	28.7
Gram-positive rods	6.8	1.8	1.3	5.1	0.0	10.0	1.8	0.8
Gram-positive cocci	1.7	1.8	1.3	0.0	0.0	0.0	0.0	0.0
Unidentified Gram-negative	3.4	1.8	3.9	5.1	0.0	3.3	0.0	0.0
"X" (non-motile; non-pigmented; penicillin-resistant; short rods, cocci or cocco-bacillary)	12.9	20.9	11.8	14.0	44.8	13.3	7.0	28.7
Polar-flagellated, oxidase-negative	0.0	0.9	1.3	0.0	0.0	10.0	36.8	0.0
Peritrichous-flagellated, oxidase-positive	0.9	0.0	0.0	0.0	0.0	0.0	1.8	0.0
Yeasts and Molds	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0
TOTAL %	99.9	99.9	99.7	100.0	99.9	99.9	99.9	99.9

TABLE V. NUMBER OF ISOLATES TESTED[1]

Sample	C	D	E	F	H	I	L	O
Number of Isolates	116	110	76	136	116	30	60	115
Total Aerobic Count	2.9×10^6	6.5×10^6	4.4×10^6	9.2×10^4	1.0×10^6	3.4×10^4	2.9×10^3	7.1×10^4

[1] A total of 759 isolates were tested from eight samples. The number of isolates tested per sample was determined by the total number of isolated colonies obtainable from the initial isolation plates.

Figure Legend



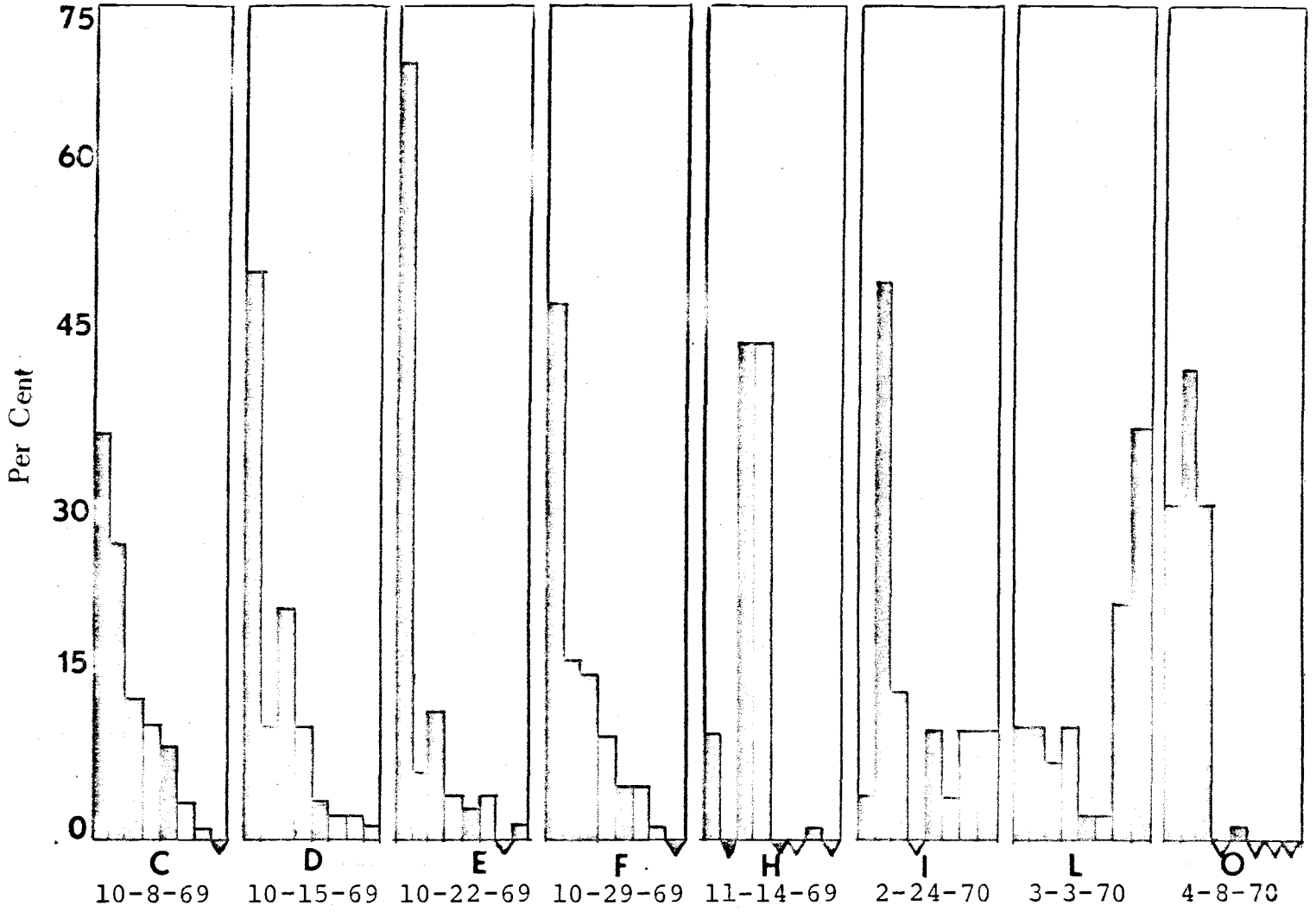


Figure 2. Summary of microbial flora of eight samples of P. borealis.

week. In November (sample H, 11-14-69), this group was down to 10% of the total. No samples were available during the months of December and January. In February, sample I (2-24-70), the Acinetobacter phenons III and IV group made up only 3% of the total. The relative numbers of this group increased in sample L (3-3-70) and in sample O (4-8-70), this group made up 29% of the total.

These data suggest that the proportion of members of the Acinetobacter phenons III and IV group decreases during the cold months of the year.

Flavobacterium-Cytophaga

At 27% of the total isolates, the Flavobacterium-Cytophaga group was the second most common group in sample C (10-8-69). The relative numbers of this group declined in the next two samples, but increased to 15% in sample F (10-29-69). None were found in sample H (11-14-69). In sample I (2-24-70), this group was predominant at 50% of the total. In sample L (3-3-70), this group was only 10%, but in sample O (4-8-70), it was up to 42%. These limited data do not suggest a seasonal pattern for this group.

On several occasions, attempts were made to enumerate members of the order Myxobacteriales (Breed, Murray and Smith, 1957), which includes the genus Cytophaga, directly by spread plating 0.5 ml quantities of shrimp homogenate dilutions on CPM agar plates following the procedures of Carlson and Pacha (1968). Less than 0.5% of the isolates

recovered by this method were identified as myxobacteria.

Pseudomonas-Aeromonas-Vibrio

This group which made up from 4 to 10% of the isolates in the October samples, increased to 45% in sample H (11-14-69). In sample I (2-24-70) and in sample O (4-8-70), no members of this group were found. Ten per cent of the isolates of sample L (3-3-70) were members of this group.

Acinetobacter Phenon II

Except in sample I (2-24-70) and M (3-3-70), only low levels of Acinetobacter phenon II were encountered. In sample I, 10% of the isolates were members of this group. In sample M, 21% of the isolates were members of this group.

Gram-Positive Rods

Gram-positive rods which consisted of 7% of the isolates of sample C (10-8-69), were present in small amounts in the next two samples, and went up to 5% in sample F (10-29-70). Totally absent in sample H (11-14-70), they reappeared in Sample I (2-24-70) where they comprised 10% of the microbial flora. They were present in samples L (3-3-70) and O (4-8-70) in small numbers. Spores were not recovered in any of the isolates.

Gram-Positive Cocci

Gram-positive cocci were found in small numbers in the first three October samples. Thereafter they were not encountered.

Yeasts

Yeasts, although occasionally encountered, were not present in significant numbers. Occasional platings of shrimp homogenates on acidified malt agar plates did not result in recovery of additional numbers of this group.

Unidentified Gram-Negative

At times, a significant proportion of the unidentified Gram-negative isolates was comprised of an apparently homogenous group of non-motile, non-pigmented, penicillin-resistant, cytochrome oxidase-positive organisms having coccobacillary or short rod cellular morphology and producing whitish colonies on TPY. This group of organisms does not fit into the taxonomic schemes proposed by Baumann et al. (1968) nor by Thornley (1967). In the scheme of Baumann et al., in which the oxidase-positive, penicillin-sensitive species are placed in the genus Moraxella and the oxidase-negative, penicillin-resistant organisms are placed in the genus Acinetobacter, the oxidase-positive organisms that are resistant to penicillin cannot be classified.

In Thornley's scheme, the oxidase-positive, penicillin-resistant species are placed in phenons I and V. These phenons, however, have the additional characteristic of motility, whereas the group of isolates found on P. borealis shrimp are non-motile. In the scheme of Shewan et al. (1960), Acinetobacter phenons III and IV, Acinetobacter phenon II, and "X" are simply grouped together under "Achromobacter-Alkaligenes." It was decided to report these organisms as a sub-group of the unidentified Gram-negative isolates. This sub-group is denoted as "X" in the pertinent figures and tables.[4]

The "X" sub-group was present in all of the samples tested. In the C, D, E and F samples (10-8-69, 10-15-69, 10-22-69, and 10-29-69, respectively), this group was either the second or the third most common group of isolates. In sample H (11-14-69), "X" isolates were tied with the Pseudomonas-Aeromonas-Vibrio isolates for first place at 45%. In sample I (2-24-70), 13% of the isolates belonged to this group. From a low of 7% in sample M (3-3-70), this group rose to 29% in sample O (4-8-70).

Another group of organisms that did not fit into the scheme was the polar-flagellated cytochrome oxidase-negative group. This group was present in large numbers in

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[4] Dr. Robert Levin, from the University of Massachusetts is currently investigating this group of organisms in hopes of clarifying their taxonomic position.

only two samples. In sample I (2-24-70), this group comprised 10% of the isolates examined. In sample L (3-3-70), this group comprised 37% of the isolates tested. None of the sample O (4-8-70) isolates were members of this group.

Comparison of Male-or-Transitional with Female Samples

Pandalus borealis shrimp undergo a sex change from male to female. The sex reversal occurs at an age of about two years in British Columbia waters (Butler, 1964) and at about two and one-half years off Denmark (Smidt, 1968). Smidt reported that the smaller shrimp (male-or-transitional) predominate at shallower depths than the larger shrimp (females). Shrimp fishermen in southeastern Alaska are of the opinion that the female P. borealis shrimp are found at greater depths than the males except when the females come in closer to shore to release their eggs.

Because the male-or-transitional and female P. borealis shrimp are found at different depths, the possibility exists that the microflora might also be different. To investigate this possibility, female-only and male-or-transional-only samples were collected and assayed in parallel with the usual randomly selected mixed-sex sample. The samples were taken on February 24, 1970 (I, J, K) and March 4, 1970 (L, M, N), at a time of the year when the females have eggs. Sex determination was made on the basis of size, and the

presence or absence of eggs. The shrimp in the mixed-sex samples were about 90% females. The results are shown in Table VI.

The most striking difference between the male-or-transitional samples and the female samples in the I, J, K set is in the much larger percentage of Flavobacterium-Cytophaga and the absence of Acinetobacter phenon II, in the female samples, whereas the male-or-transitional samples show a larger percentage of Acinetobacter phenon II and a lower percentage of Flavobacterium-Cytophaga. In the L, M, N samples, taken only eight days after the I, J, K samples, the situation with respect to the Flavobacterium-Cytophaga group had reversed, with the majority of the male-or-transitional isolates belonging to this group and only a small proportion of the females. Another difference between the male-or-transitional samples and the female samples was the larger proportion of the unidentified polar-flagellated, oxidase-negative group in the female samples. This group was practically absent in the male-or-transitional samples.

The results of these two comparisons suggest that there is a definite difference between the male-or-transitional shrimp flora and the female shrimp flora. The mixed-sex samples which consisted primarily of females (about 90%) reflected their composition by more closely resembling the female shrimp isolates than the male-or-transitional isolates.

TABLE VI. COMPARISON OF MALE-OR-TRANSITIONAL, FEMALE, AND NATURALLY PROPORTIONED MIXED-SEX SAMPLES OF *P. BOREALIS* SHRIMP: MICROBIAL FLORA.

Sample	I mixed	J female	K male	L mixed	M female	N male
Pseudomonas-Aeromonas-Vibrio	0.0	4.2	1.1	10.5	3.4	0.0
Flavobacterium-Cytophaga	50.0	75.0	13.3	10.5	6.7	68.8
Acinetobacter phenon II	10.0	0.0	80.0	21.0	3.4	0.0
Acinetobacter phenons III & IV	3.3	0.0	2.2	10.5	19.0	11.2
Gram-positive rods	10.0	4.2	2.2	1.8	13.8	16.2
Unidentified Gram-negative (miscellaneous)	3.3	4.2	0.0	1.8	1.7	1.2
Other unidentified Gram-negative: "X" (non-motile; non-pigmented; penicillin-resistant; short rods, cocci or cocco-bacillary)	13.3	12.4	0.0	7.0	8.6	0.0
polar-flagellated; oxidase-negative	10.0	0.0	1.1	36.8	43.1	2.5
Total %	99.9	100.0	99.9	99.9	99.7	99.9
Total Plate Count at 20°C	3.4×10^4	2.4×10^4	9.5×10^4	2.9×10^3	3.3×10^3	6.7×10^4

Variables Discussed

The microbial composition in the samples fluctuated widely over the period observed. The variables that might have been responsible for these fluctuations, shown in Table II, include the depth, location, time of the year and method by which the shrimp were caught. Another variable noted was the presence of eggs on the shrimp taken from late October through early March.

Because the microbial floral of the eggless samples C, D, and E shrimp closely resembles the microbial flora of the sample F (10-29-69) shrimp, the majority of which carried eggs, it can be assumed that the presence of eggs does not greatly affect the relative proportions of the isolates.

The location, depth, and method of catch are related because the pot-caught shrimp came from a depth of 55 to 70 fathoms in Shrimp Bay, whereas the beam-trawl-caught shrimp came from a depth of 25 to 30 fathoms in the Eastern Passage near Wrangell. Figures 3 and 4 show these locations.

Total aerobic counts of beam-trawl-caught samples (Table V) were considerably lower than total aerobic counts of pot-caught samples. It is possible that this difference may be due to the time of the year in which the samples were taken. However, the similarity between the microbial flora

Figure 3. General area of shrimp sampling.

Figure Legend

Location	Samples Taken	Date
Shrimp Bay	A-H	Sept.-Nov., 1969
Eastern Passage	I-O	Feb. -Apr., 1970

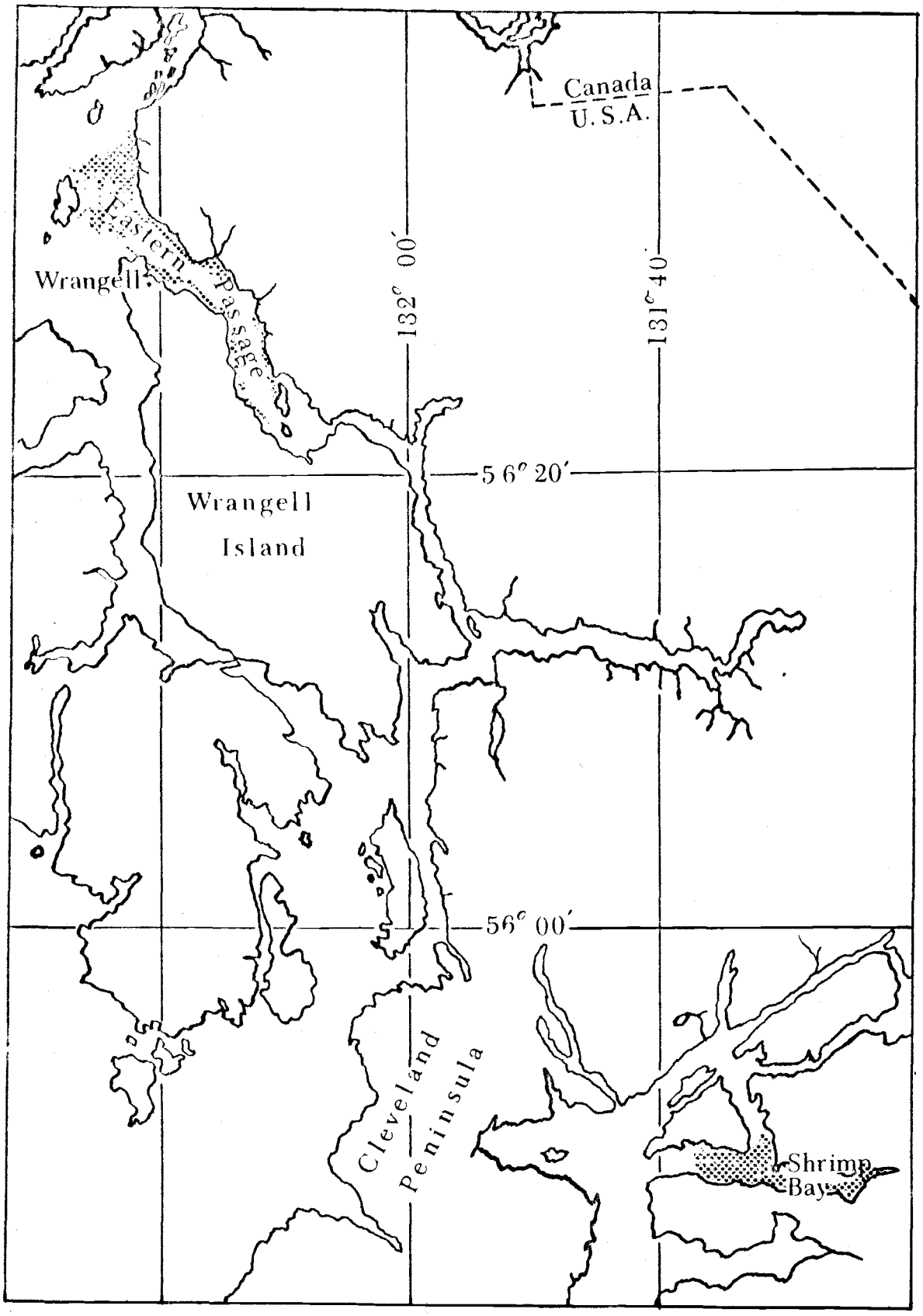

Figure 4. The shrimp grounds.

of sample C taken in Shrimp Bay in early October, 1969, with the microbial flora of sample O, taken in Eastern Passage in early April, 1970, suggests a possible re-establishment of the October pattern. If this is true, the possible variables of location, method of catch, and depth of catch can be discounted.

Sample H, which was obtained in the same manner as the October samples C, D, E and F (pot-caught, 55 to 70 fathoms, Shrimp Bay) manifested a difference in microbial composition showing that seasonal variation alone could have accounted for these fluctuations in microbial flora.

Further support to the seasonal variation hypothesis is lent by the fact that samples I, L and M, which were beam-trawl-caught, (25 to 30 fathoms, Eastern Passage) also showed differences in microbial composition. The evidence suggests that the time of year is an important factor in determining the microbial flora of P. borealis.

Comparison with P. Jordani Microbial Flora

On two occasions, sufficient quantities of P. jordani were collected in parallel with P. borealis for a comparison of microbial flora to be made. Unfortunately, the first comparison made was with the very first sample set, before TPY motility medium was substituted for nutrient broth. It is for this reason that the results of the first two sample

sets were not included with the rest of the results. The effect of mis-matching the primary isolation media and the motility medium resulted in a large number of unidentified Gram-negative isolates in the first sample set. Many of these unidentified organisms resembled pseudomonads in colonial morphology but did not show motility. When some of these isolates were retested using TPY motility medium, 40% of the test group previously observed to be non-motile were actively motile.

Although identification of the first samples was incomplete, a comparison can still be made because the same procedures were applied to both P. jordani and P. borealis samples. Table VII shows the results of two parallel assays.

In the first comparison (9-8-69), the P. borealis isolates were similar to the P. jordani isolates. The biggest differences, which were none too great, were the larger percentage of the Pseudomonas-Aeromonas-Vibrio group and the smaller percentage of the Acinetobacter phenons III and IV group in the P. borealis sample. In the second comparison (10-29-69), the P. jordani sample showed larger percentages of the Pseudomonas-Aeromonas-Vibrio group and Enterobacteriacae, with corresponding lesser percentages of the Flavobacterium-Cytophaga group and Acinetobacter phenons III and IV. Further testing is necessary to establish the differences of microbial flora on these two shrimp species.

TABLE VII. COMPARISON OF MICROBIAL FLORA OF *P. BOREALIS* WITH *P. JORDANI*

	A (*borealis*)	B (*jordani*)	F (*borealis*)	G (*jordani*)
Pseudomonas-Aeromonas-Vibrio	25.4	14.9	9.5	34.7
Enterobacteriacae	0.0	3.4	2.2	12.7
Flavobacterium-Cytophaga	4.2	3.4	14.7	6.8
Acinetobacter phenon II	2.8	0.0	1.4	4.2
Acinetobacter phenons III & IV	28.1	35.6	47.3	29.7
Gram-positive rods	5.6	8.0	5.1	0.0
Gram-positive cocci	2.8	2.3	0.0	0.0
Unidentified Gram-negative	23.9	18.3	5.1	0.8
"X" (non-motile; non-pigmented; penicillin-resistant; short rods, cocci or cocco-bacillary)	4.2	10.3	14.0	11.0
Polar-flagellated, oxidase-negative	2.8	3.4	0.0	0.0
Yeasts and Molds	0.0	0.0	0.7	0.0
Total %	99.8	99.6	100.0	99.9

Results of Sample A

As previously mentioned, the results of sample A (9-8-69), the first sample set, were not included with the results of the later assays because the motility test initially used gave too many false-negative results. If one assumes that the large proportion of unidentified Gram-negative isolates were actually composed of motile organisms incorrectly classified as non-motile, the incorrectly identified isolates could belong to the Pseudomonas-Aeromonas-Vibrio group, the Enterobacteriacae group, or possibly the polar-flagellated, oxidase-negative group (a sub-group of the unidentified Gram-negative group). A fourth possibility is that the unidentified isolates belong to none of the aforementioned groups.

Although the possibility that a large proportion of the unidentified isolates rightfully belong in the Enterobacteriacae group should not be ignored, the insignificant levels of this group in the later samplings suggest against this possibility. Judging from the composition of the following C, D and F samples, the unidentified Gram-negative isolates of sample A are more likely members of the Pseudomonas-Aeromonas-Vibrio group than members of the polar-flagellated, oxidase-negative sub-group. And the slender-rod cellular morphology and the proteolysis of casein on the nutrient agar-milk plates of many of these unidentified isolates is highly suggestive of the Pseudo-

monas-Aeromonas-Vibrio group.

If the sample A results were to be included with the results of the other eight mixed-sex P. borealis samples, assuming that most of the unidentified isolates were actually members of the Pseudomonas-Aeromonas-Vibrio group, the overall picture of the microbial flora changes would not be seriously affected. The Pseudomonas-Aeromonas-Vibrio group would be seen to decline from the September high to a low in mid-October. If there is a cyclical pattern to the relative abundance of this group, these data do not clearly show it. Likewise, the increase in the Flavobacterium-Cytophaga group from the September low would not define a pattern. The hypothesis previously brought forth that the Acinetobacter III and IV group decreases rapidly during the cold months would not be challenged by the inclusion of the sample A results. The low proportion of the sub-group of unidentified Gram-negative isolates denoted as "X" in the A sample, neither adds nor detracts from the total picture. Enterobacteriacae, Acinetobacter phenon II, Gram-positive cocci, yeasts and molds, and the polar-flagellated, oxidase-negative unidentified Gram-negative isolates would remain unchanged.

CONCLUSIONS

The microbial flora of P. borealis shrimp fluctuated seasonally. The data collected in the course of this study do not clearly demonstrate cyclical fluctuations in the microbial composition, although if sampling and identification had proceeded over a longer period of time, cyclical fluctuations might have been observed. A distinct difference was observed between the female P. borealis microbial flora and the male-or-transitional P. borealis microbial flora. However, the mere presence of eggs on the shrimp does not appear to be an important influence on the shrimp microbial composition.

A group of polar-flagellated, oxidase-negative isolates did not fit the identification scheme adapted from that of Shewan, Hobbs and Hodgkiss (1960). Another group, classified as the Achromobacter-Alkaligenes group in the broad grouping of Shewan et al. and denoted as "X" in this study, did not fit either the scheme of Baumann et al. (1968) or Thornley (1967). Other groups in large proportions (greater than or equal to 10% of the total isolates) at various times in the course of this study include Acinetobacter phenon III and IV, Flavobacterium-Cytophaga, Pseudomonas-Aeromonas-Vibrio, Acinetobacter phenon II, and Gram-positive rods.

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