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The Occurrence and Distribution of Bacterial Types on Flatfish

BY J. LISTON

Department of Scientific and Industrial Research, Torry Research Station, Aberdeen

SUMMARY: An investigation of the bacterial flora of the representative flatfish, skate and lemon sole, was carried out by direct counts of special groups of bacteria and by the analysis of over 1700 strains of bacteria isolated from the fish. Luminous and agar-digesting bacteria occurred seasonally on fish. Luminous strains occurred mainly in the gut contents. A group of sea-water-loving *Pseudomonas* spp. which seem to require sea water for growth on initial isolation was present on the flatfish in variable numbers throughout the year. A more or less distinct intestinal flora was present in North Sea flatfish in which a homogeneous group of micro-organisms provisionally labelled Gut Group vibrios predominates; this group includes luminous bacteria. The bacterial populations of skin and gills were similar in both the fish studied and were composed principally of Gram-negative rods of the *Pseudomonas* and *Achromobacter* genera. The composition of the bacterial flora of the two flatfish, as calculated from an analysis of strains isolated from sea-water agar plates, was: Lemon sole: *Pseudomonas* 60 %, *Achromobacter* 14 %, *Alcaligenes* 8 %, *Flavobacterium* 5 %, corynebacteria 1 %, cocci 1 %, Gut Group vibrios 9 %, miscellaneous 2 %. Skate: *Pseudomonas* 53 %, *Achromobacter* 13 %, *Alcaligenes* 6 %, *Flavobacterium* 9 %, corynebacteria 2 %, cocci 3 %, Gut Group vibrios 12 %, miscellaneous 2 %.

Representatives of most of the known bacterial genera have been isolated from marine sources (ZoBell, 1946). As is to be expected, in view of the low nutrient content of unpolluted sea water, the largest populations of bacteria in the oceans are found associated with solid surfaces such as the sea bed and the bodies of marine animals and plants where the concentration of organic nutrients is relatively high (Russel, 1893; Harrison, Perry & Smith, 1926; Waksman, 1934; Wood, 1953). In particular, large numbers of bacteria occur on the exposed surfaces and in the gut of fish (Lumley, Piqué & Reay, 1929).

It was early recognized that the spoilage of fish as a foodstuff is due almost entirely to the activities of bacteria (Anderson, 1907), and in particular to bacteria of the species normally occurring on fresh fish (Schönberg, 1930). Consequently, several investigations into the bacteriology of commercially important species of fish have been carried out in the last thirty years (see Shewan, 1949; Tarr, 1954). The results of most of these investigations indicate that the bacterial flora of living or freshly caught fish is primarily made up of Gram-negative bacteria, mainly belonging to the genera *Pseudomonas*, *Achromobacter* and *Flavobacterium*. However, some authors (Wood, 1940; Dyer, 1947) have reported that micrococci constituted a large proportion of the types present on the fish they sampled.

There is available therefore a good deal of information, albeit sometimes conflicting, about the nature of the bacterial flora of some types of fish. Little or nothing, however, has been reported concerning the bacterial populations

growing on flatfish, though a few authors have described one or two bacterial strains isolated from individual flatfish (Gibbons, 1934; Thjøtta & Sømme, 1943). The results described in this paper were obtained from an investigation of the bacterial flora of two types of flatfish, skate (*Raja* spp.) and lemon sole (*Pleuronectes microcephalus*). The investigation was both qualitative and quantitative; much of the quantitative data obtained has been published in an earlier paper (Liston, 1956).

METHODS

From October 1952 until December 1954 viable counts were carried out several times a month, using the pour plate method, on samples aseptically excised from the skin, gills and gut contents of freshly caught fish transported to the laboratory in closed sterile tins. The fish were often still alive on arrival at the laboratory and had to be killed before sampling; so the bacteria derived from them can be considered to be representative of the flora of living fish. Counts were carried out in duplicate by using the sea-water agar medium (SWA) and the tap-water agar medium (HHA) described in the earlier paper (Liston, 1956); plates were incubated at 0°, 20° and 37°. From March to December during 1954, plates incorporating *c.* 3 units of penicillin/ml. were additionally included for the counts of skin and gill samples.

Qualitative counts were carried out directly by enumerating the number of luminous and agar-digesting colonies on the normal count plates and, less directly, by comparing the number of colonies appearing on the plates containing penicillin with the number appearing on corresponding plates without penicillin. The luminous colonies were counted in a dark room. The enumeration of the agar-digesting colonies was facilitated by flooding the plates with iodine solution which stains the agar medium except in the area surrounding agar-digesting colonies which are thus readily recognized.

During the first year of the investigation representative colonies were picked directly from the count plates into tap-water or sea-water broth, depending on whether they were derived from HHA or SWA media. In selecting the colonies an attempt was made to ensure that the preponderant types were fairly represented numerically among the isolates, according to their occurrence on the plates. However, in addition, colonies of unusual appearance (e.g. agar-digesting colonies) which did not represent any significantly large part of the flora were also selected so that some estimate of the range of bacterial types occurring on fish could be obtained. The media into which the colonies were picked were incubated at 20° for 3 days in the case of those derived from count plates incubated at 20° and 37° and at 0° for *c.* 14 days when derived from 0° plates. The cultures were then plated out on sea water or nutrient agar to determine their purity (20° and 0° incubation temperatures).

Pure cultures obtained from these plates were examined for morphology and their growth characteristics on solid and in liquid media determined. They were tested (at 20°) for ability to liquefy gelatin, reduce nitrate, alter litmus milk and produce acid from glucose, lactose, sucrose, mannitol, maltose and starch. Most of the cultures were also tested for sensitivity to penicillin,

chloramphenicol, streptomycin, oxytetracycline, and the vibriostatic compound 0/129 (2:4-diamino 6:7-di-*iso*propylpteridine) in accordance with the method outlined by Shewan, Hodgkiss & Liston (1954) for the rapid differentiation of non-pathogenic asporogenous rods.

RESULTS

Penicillin-resistant bacteria constituted a variable proportion of the bacterial flora of skate and lemon sole skin and gills during 1954. This is apparent from Figs. 1 and 2 where the penicillin-resistant count is shown as a percentage of

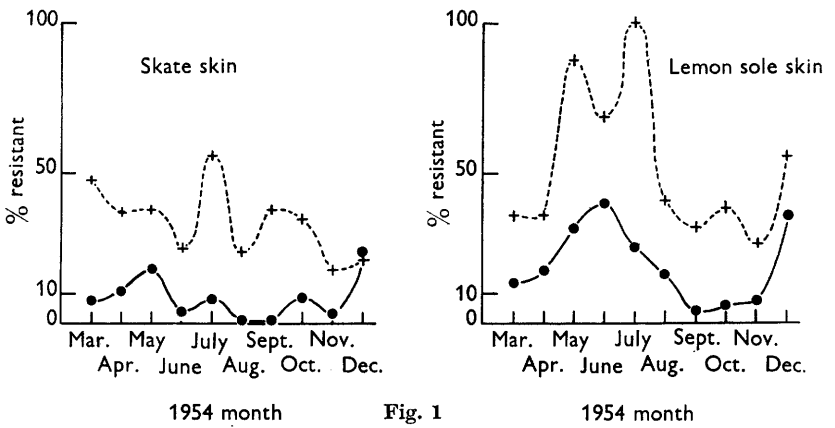


Fig. 1

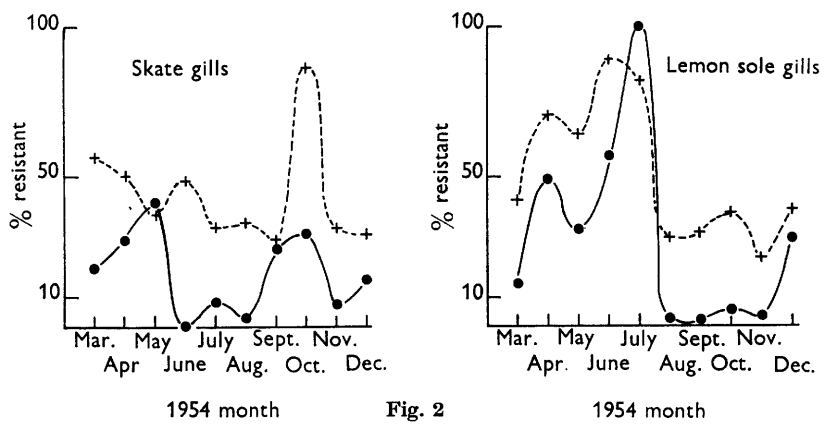
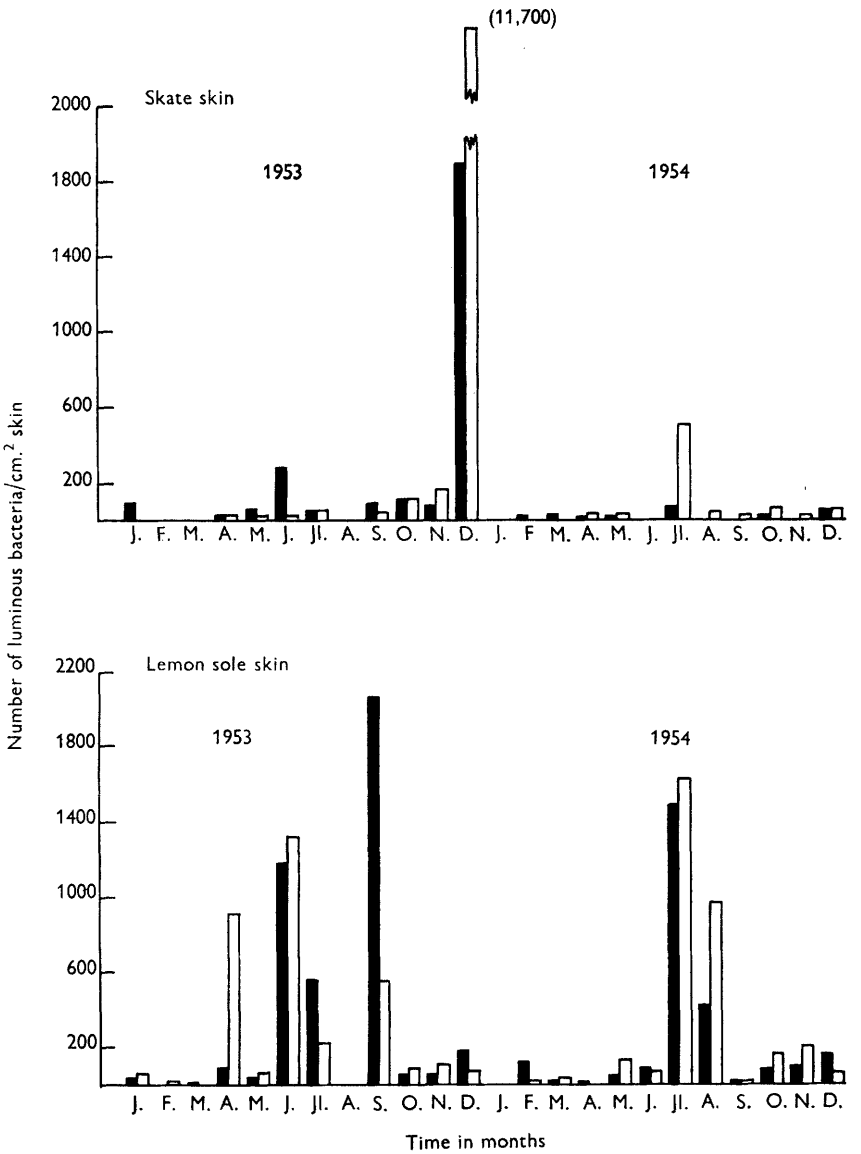


Fig. 2

Figs. 1, 2. Percentage of the bacterial flora resistant to 3 units penicillin/ml., during 1954. Fig. 1. Skin flora. Fig. 2. Gills flora. Broken line, counted on SWA medium. Unbroken line, counted on HHA medium.

the corresponding total viable count at 20°. In the case of lemon sole the penicillin-resistant bacteria, on both skin and gills, constituted a relatively large proportion of the organisms present in summer and a relatively small proportion of those occurring in winter. No distinct pattern of occurrence of

penicillin-resistant organisms is apparent for skate skin, but it is noteworthy that they never constituted more than 50 % of the bacterial flora at any time. On the gills of skate there appears to have been a fluctuation in the repre-

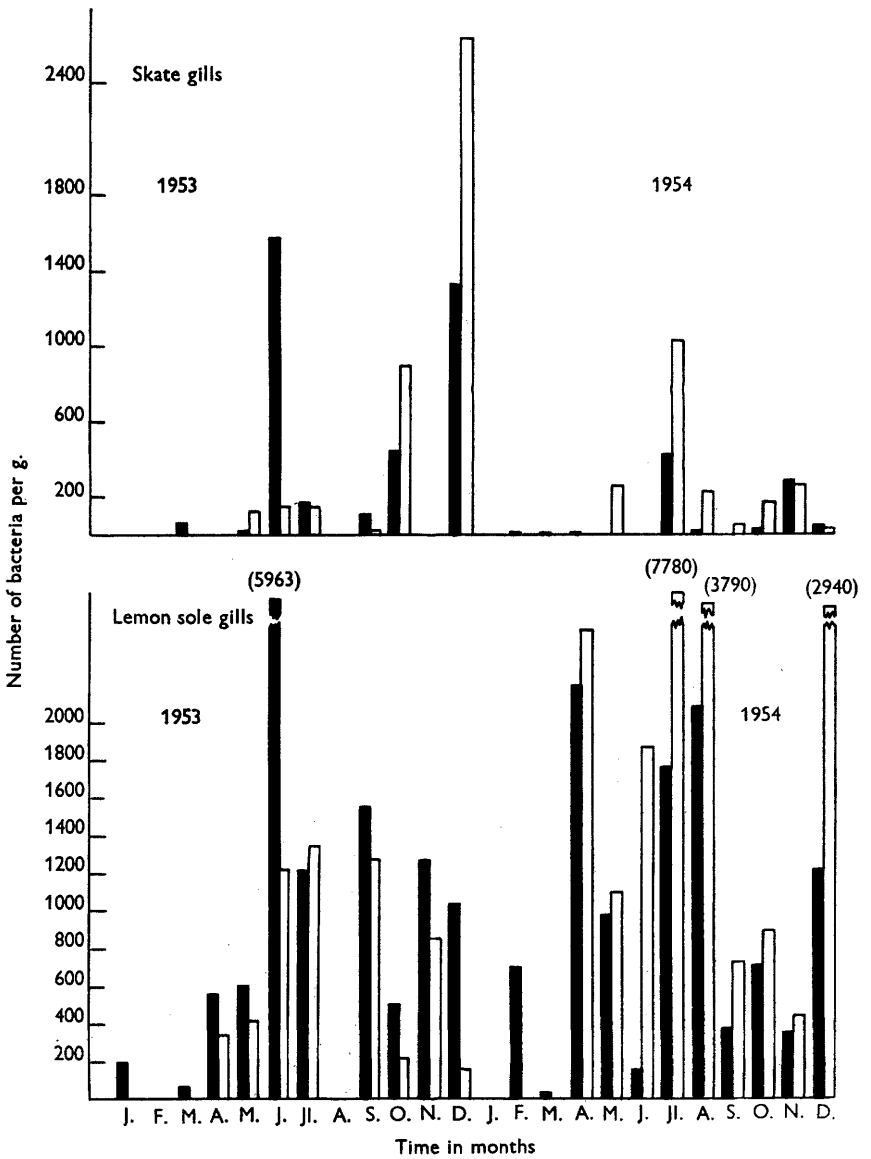


Figs. 3 and 4. For legend see Figs. 5 and 6.

sentation of penicillin-resistant bacteria from a relatively high proportion in spring, through a period of low incidence in summer to a high proportion again in the autumn. While the curves derived from results obtained using SWA and HHA media are similar in shape they are not co-incident and the values

obtained using SWA medium are in general higher than those obtained using HHA medium.

The incidence of luminous bacteria among the organisms appearing on 20° count plates is shown in Figs. 3–9. It has been necessary, because of the large numbers of luminous bacteria often present in gut samples, to express the

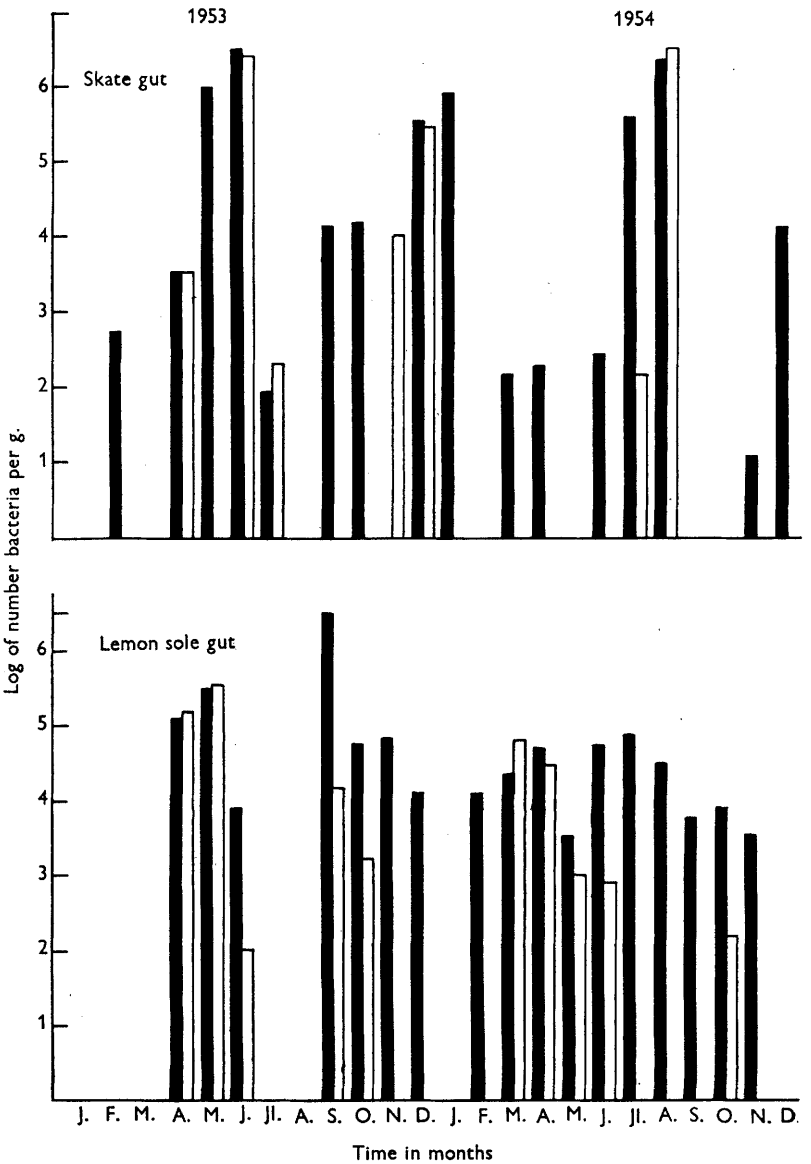


Figs. 5 and 6

Figs. 3–6. Occurrence of luminous bacteria on flatfish during the period January 1953 (J) to December 1954 (D). Fig. 3. On Skate skin. Fig. 4. On Lemon sole skin. Fig. 5. On Skate gills. Fig. 6. On Lemon sole gills. ■, Counts in HHA medium; □, counts in SWA medium.

results of gut counts as log. count/g. From these block diagrams it can be seen that luminous bacteria were more common on the gills than on the skin, in both fish, and on lemon sole than on skate, comparing fish with fish. Very large numbers of luminous bacteria may occur in the gut contents of both fish.

Agar-digesting bacteria were encountered only in very small numbers and never exceeded 1 % of the total bacterial flora appearing at 20° or 0°. They



Figs. 7, 8. Occurrence of luminous bacteria in flatfish gut during the period January 1953 (J) to December 1954 (D). Fig. 7. Skate gut. Fig. 8. Lemon sole gut. ■, counts in HHA; □, counts in SWA.

appeared most frequently on the skin and gills of both fish in the summer and towards the end of the year, but were virtually absent in spring and autumn.

More than 1500 colonies were picked off count plates incubated at 0° and 20°; 212 strains were also isolated from 37° counts. However, the counts at 37° rarely exceeded 1 % of the counts at 20° and therefore the 37° isolates were not investigated in great detail as they were not considered to represent a significant portion of the total bacterial population of the fish examined. Sufficient data have been collected about the organisms isolated at 37° to determine the overall distribution of these bacterial types between the two fish (Table 1). In both skate and lemon sole cocci and Bacillus types constitute the bulk of the flora growing at 37°.

Table 1. *Distribution of organisms isolated at 37° from skate and lemon sole*

	Cocci (%)	Bacilli (%)	Gram-negative rods* (%)	Coryne-bacteria† (%)	Yeasts and moulds (%)
Skate	51	26	13	6	4
Lemon sole	56	24	16	4	0

* Mainly pseudomonads and Enterobacteriaceae.
† *Corynebacterium sensu lato* as defined by Jensen (1952).

This distribution between the two fish, according to genera of the bacteria growing at 0° and 20°, as determined by analysis of the characteristics of bacteria isolated from SWA and HHA media is represented in Table 2. The most important features of this distribution are the overwhelming preponderance of Gram-negative rods in the flora of both fish, the apparent reversal of the relative importance of the groups Pseudomonas and Achromobacter among the organisms derived from HHA and SWA media, respectively, and the similarity between the groups present on the two fish.

Table 2. *Distribution of organisms isolated at 0° and 20° from skate and lemon sole*

	Skate: isolated from		Lemon sole: isolated from	
	SWA medium	HHA medium	SWA medium	HHA medium
	Percentage			
Pseudomonas	52·5	21·7	60·3	19·4
Achromobacter	13·4	24·4	14·1	25·0
Alcaligenes	5·4	14·4	8·2	18·1
Flavobacteria	9·4	3·3	5·0	5·2
Corynebacteria	1·5	4·2	0·9	4·3
Cocci	3·4	1·9	1·4	4·3
G. G. vibrios	12·5	26·9	8·7	22·0
Miscellaneous	1·9	3·2	1·4	1·7

Again in Table 3 the generic distribution of these 0° and 20° isolates is presented according to their original sites of growth on the two fish. From

Table 3 it is apparent that most of the so-called Gut Group vibrios (G.G. vibrios) were derived from gut-samples and were not present in significant numbers in samples from other parts of the fish. The luminous organisms belong to this group of vibrios and their occurrence in very large numbers in the gut has been noted already (Figs. 7, 8). The apparent incidence of G. G. vibrios in gut samples deduced from the analysis of isolates, high as it is, is in fact lower than the actual incidence. Direct observations of count plates during the course of the investigation revealed that these organisms frequently

Table 3. *Distribution by genera of organisms isolated at* 0° *and* 20° *according to sites of isolation from the fish*

Key to genera: Ps. = *Pseudomonas*; A = *Achromobacter*; Al. = *Alcaligenes*; C. = corynebacteria; F. = flavobacteria; G.G.V. = Gut Group vibrios; M. = miscellaneous.

	Medium													
	Sea-water agar							Tap-water agar						
	Genera													
	Ps.	A.	Al.	C.	F.	G.G.V.	M.	Ps.	A.	Al.	C.	F.	G.G.V.	M.
	Percentage													
Skate														
Skin	63·0	8·6	6·4	5·4	10·2	—	6·4	22·3	38·3	17·5	2·1	4·3	4·2	11·3
Gut	26·7	12·2	2·4	1·6	4·9	48·0	4·2	10·9	3·0	3·0	3·0	1·8	74·0	4·3
Gills	59·5	13·7	6·9	4·0	11·3	—	4·6	24·7	30·6	21·8	2·9	5·3	7·6	7·1
Lemon sole														
Skin	57·0	16·7	9·5	—	9·5	—	7·3	20·2	30·9	22·3	1·6	9·1	5·3	10·6
Gut	34·6	7·7	1·9	5·8	9·6	34·6	5·8	6·1	7·6	7·6	13·6	1·5	59·1	4·5
Gills	62·2	14·5	10·0	—	11·1	1·1	1·1	31·8	31·8	16·5	1·2	3·5	9·4	5·8

constituted nearly 100 % of the bacteria in the gut. The discrepancy between the two results is due to the method of selection of colonies from plates which, as described above, was partly directed towards obtaining examples of the total range of types occurring on fish. Colonies of organisms other than the G.G. vibrios were thus purposely selected from gut sample plates.

The constitution of the bacterial populations of skin and gills is similar not only for each kind of fish but also from fish to fish. Furthermore, the effect of medium of isolation upon the apparent relative proportions of Achromobacter and Pseudomonas types, noted in the data listed in Table 2, can be seen from Table 3 to be exerted without reference to the origin of the strain—the picture in the case of the gut flora is complicated by the factors of selection already noted.

DISCUSSION

The results obtained in this investigation are in agreement with the hypothesis that the bacterial flora of fish, like the flora of the sea (ZoBell, 1946), is composed principally of Gram-negative rods. No evidence of a preponderance of cocci was noted at any time, though if the organisms isolated at 37° are alone considered, cocci do appear to predominate. This finding is, however, of little

importance since the 37° count is negligible as compared with the 20° count. Nevertheless, the findings of other workers who have reported significantly high proportions of cocci in the bacterial flora of fish cannot be ignored (Stewart, 1932; Wood, 1940; Dyer, 1947). In most of their experiments incubation temperatures of *c.* 20° were used so that the selective effect of temperature cannot be responsible for the results. Recent investigations concerning the bacterial populations of cod, caught in the same area as the flatfish which were used in this investigation, has revealed that Gram-negative rods again predominate (Mr D. Georgala; personal communication). Consequently, the effect of species of fish on the associated bacterial flora may also be discounted as a cause of the high occurrence of cocci reported by these authors. The most likely reason for their findings seems to be the effect of environment and this is supported by the observations of Wood (1953). Wood noted that organisms isolated in warm Australian waters usually grew well at 37°, while the common finding in the case of bacteria isolated from the cold Northern seas is that they rarely grow at temperatures above 30°.

The relative proportions of the various types of bacteria occurring on flatfish do not remain constant throughout the year. Insufficient information was obtained to determine whether the fluctuations in numbers of penicillin-resistant bacteria occurring on fish is a regular seasonal phenomenon. However, such seems to be the case for the luminous and agar-digesting bacteria, which appear in their greatest number at approximately the same times each year. It is possible that these seasonal variations represent annual fluctuations in the bacterial populations of the sea in which the fish live. Beijerinck (1915) reported an accumulation of luminous bacteria in the North Sea in August and September. Allowing for limited variations of timing from year to year, this period approximately coincides with one of the periods of high incidence of luminous bacteria on flatfish.

The overwhelming preponderance of organisms of the Pseudomonas and Achromobacter groups in the bacterial flora of flatfish was recognized from a preliminary investigation of strains isolated during the first year of the investigations, as was also the apparent difference in the proportion of the two groups among the bacteria appearing on SWA and HHA media. In general also it was found that, counts on SWA medium were higher than the corresponding counts on HHA medium (Liston, 1956). Since *Pseudomonas* spp. are generally insensitive to penicillin while *Achromobacter* species are sensitive (Shewan *et al.* 1954) it was decided to include plates containing penicillin in the count pattern to obtain some information about the distribution of penicillin-resistant (mainly *Pseudomonas* spp.) and sensitive (mainly *Achromobacter* spp.) organisms occurring on count plates. It appears from the results obtained by this method that the additional colonies which grew on SWA plates were those of *Pseudomonas* spp. which, initially unable to develop on HHA medium, are responsible for the increased proportion of Pseudomonas types in the flora derived from such plates. These *Pseudomonas* spp. are obviously sea-water-exacting or at least sea-water-loving so far as primary growth on defined media is concerned; they thus belong to the group of true marine bacteria as

defined by ZoBell (1946). In this respect, however, it should be noted that all strains isolated initially on SWA medium eventually proved able to grow on tap-water media, though some required to be carried through several sub-cultures on sea-water-based media before they would do so. That none of the Achromobacter types was inhibited by sea-water agar was proved by the ready growth of all strains on this medium and the isolation of all the types from both media.

Some investigators have reported a distribution of the Gram-negative rods occurring on fish, in which the Achromobacter types represent an even greater proportion than appears in Table 2 (HHA columns; see Shewan, 1949). This is undoubtedly due to a great extent to the changing criteria of classification. Reference to the published descriptions of organisms isolated by earlier workers from fish (e.g. Stewart, 1932) shows that many strains which they classified as Achromobacter would now be classified as Pseudomonas in accordance with the definitive description of this group published in the 6th edition of *Bergey's Manual* (1948). This is particularly true of the proteolytic non-pigmented *Pseudomonas* spp. which are extremely common on fish and which, because of their failure to produce pigment, were usually classified as Achromobacter in the past. The antibiotic sensitivity test of Shewan *et al.* (1954) has proved very useful as a method for quickly identifying these organisms.

The third largest group, after the Pseudomonas and Achromobacter groups, was that of the Gut Group vibrios, so called because they were consistently isolated from gut sample count plates and only occasionally from other sources; a brief description of this group has been published already (Liston, 1954). It should be noted that the isolation of smaller numbers of this type of organism from SWA plates than from HHA plates is due, not to any inhibitory effect of sea water, but to the overall nutritional inadequacy of SWA medium. The organisms seem to be nutritionally exacting and will grow well in a sea-water based medium containing 1·0 % peptone and 0·5 % Lab Lemco. Prominent among the organisms in this group, but constituting only a minority of it, is the luminous group.

Alcaligenes types from fish seem to have been classified simply as *Achromobacter* spp. by earlier workers. However the types isolated in this investigation constituted a distinct and very homogeneous group.

The corynebacteria occurring on flatfish have been identified as *Corynebacterium* spp. *sensu lato* as defined by Jensen (1952). It is only within recent years that corynebacteria have been reported to occur on fish (Wood, 1940; Shewan & Hodgkiss, 1952) but, according to their published descriptions, some strains isolated from fish by earlier workers and described by them as *Flavobacterium* spp. would now be classified as *Corynebacterium* spp. *sensu lato*.

The relatively small number of cocci isolated at 0° and 20° were mainly of the Micrococcus-Sarcina type and were, in general, similar to cocci obtained from fish by earlier workers.

The generic distribution of bacteria in the populations of skin and gills was similar in both fish but there were small differences which probably reflect the

varying conditions of the different growth loci on the two fish. Between the bacterial flora of the gut and those of skin and gills, however, there was a very marked difference. It has been repeatedly reported that the gut contents in fasting fish (of many different species) are sterile (see Margolis, 1953) and this finding has been confirmed in the present investigation. Consequently, the bacterial flora of the intestine of fish should depend on the bacteria present in the food ingested by the animal. However, the constant occurrence of Gut Group vibrios as the predominating organism in the gut contents of skate and lemon sole, which consume a fairly mixed diet of small fish, crustaceans and worms of various kinds, implies that the bacterial population of the fish gut does not arise from simple mechanical transfer of organisms from the food. Obviously the conditions in flatfish gut are such as to exert a selective effect on the bacteria ingested and on the evidence of this investigation only the Gut Group vibrios are able to survive in large numbers. It is interesting to speculate on whether these organisms are in this respect analogous to the coliform population of the mammalian gut, though of course the phenomenon of long fasts of several months duration which is normal in the case of fish, does not occur in most mammals.

That a more or less typical commensal bacterial flora occurs on the skin and gills of flatfish is suggested by the repeated isolation of the same type of organism from these areas on fish caught at different times of the year. However, the diverse nature of these bacterial populations of skin and gills is probably at least partly due to the ease of access of these sites of growth from the surrounding water and the generally favourable conditions of growth provided by the slime secreted continuously by fish.

As two lists have been obtained for both of the fish examined, purporting to describe the constitution of their bacterial flora and corresponding to the two media used for isolating individual micro-organisms, it is necessary to decide which list most accurately represents the true state of affairs on the living fish. Since SWA medium has been shown to allow the development of the greatest number of colonies from fish samples and since (excepting the Gut Group vibrios) all isolated strains, whether initially derived from SWA or HHA media, grow readily on SWA medium, the lists obtained from an analysis of organisms isolated on this medium are probably the more accurate. However, the special case of the Gut Group vibrios must be borne in mind, and it is probable that in any future investigation an even better estimate of the nature of the bacterial flora of fish would be obtained by using a sea-water based medium containing larger amounts of peptone and Lemco than the SWA medium used in this investigation.

I wish to thank Mr D. Georgala for supplying me with information on the bacterial flora of North Sea cod. The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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