Direct Bacterial Count as a Rapid Freshness Test for Fish Fillets

PETER LERKE AND LIONEL FARBER

Seafood Research Laboratory, The George Williams Hooper Foundation, University of California Medical Center, San Francisco, California 94122

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Comparison of various indices of deterioration of refrigerated fish fillets showed that the direct bacterial count can be used to predict the storage life of the foodstuff. For direct counts, a thin film made from fillet surface material was spread on a microscope slide, stained, and read. Initial counts were found to correlate well with keeping quality; a period of freshness of 24 or 48 hr at 5 C could be reliably predicted. Preliminary data indicated that hypoxanthine estimation could probably also be used for the prediction of shelf life but that the relative complexity of the procedure detracted from its usefulness.

Several methods are now used to measure spoilage in fish flesh, but until recently none were available for the estimation of shelf life or keeping quality. The flesh of fish, once caught, remains apparently fresh until the onset of the usual signs of spoilage, primarily the development of unpleasant and offensive odors; by means of various chemical procedures, the extent of spoilage can readily be measured. Such tests are useful in assessing, for example, the effectiveness of a preservative treatment but are relatively useless in estimating the quality of fresh fish. Throughout the "fresh" period between catching and the onset of detectable spoilage, fish muscle seems uniformly fresh, judging from appearance, odor, and chemical spoilage tests. But the few initial contaminating bacteria multiply rapidly during this time without producing detectable concentrations of spoilage products. The result is that toward the end of the period of freshness the bacterial population approaches a level at which spoilage products can be smelled or detected chemically by means of spoilage tests [volatile reducing substances (VRS), volatile amines, etc.]. But by this time, chemical methods can only quantitate the results already sensed olfactorily.

The ability to assess the stage of freshness of any given lot of fillets, in short, to be able to estimate how much storage life remains, is important in many aspects of the unfrozen fish industry, and several attempts have been made to achieve this end. Thus, Shewan and Liston (8) used the ability of bacteria to reduce tetrazolium salts to measure the freshness of iced fish. More recently, Spinelli et al. (9) measured freshness by a long and highly specialized procedure that enabled them to estimate the extent of nucleotide breakdown in fish muscle cells. This method is the only true freshness test in present use.

In seeking a simpler and faster test we reasoned, as have many before us, that since bacteria are the main cause of fish muscle spoilage, the most logical approach would be to estimate bacterial numbers. A viable count was ruled out, because it requires from 24 to 48 hr and therefore would hardly be useful to predict the shelf life of fresh fish which already may have expired when the test results become known. The alternative was the direct microscopic count. Although it is usually frowned on for well-known reasons, Tarr (10) used it to assess the bacteriological quality of fish fillets and concluded that the method had merit but was not sensitive enough when bacterial levels were below $10^6$ cells per g. In the present paper, we report on the successful use of the direct count to measure freshness of rock cod and sole fillets and compare this method with hypoxanthine estimation.

MATERIALS AND METHODS

Unfrozen fillets of English and petrale sole (Parophrys vetulus and Eopsetta jordani) and of Pacific Coast rockfish (Sebastodes spp.) were obtained from local commercial processors. After initial tests, the fillets were stored at either 5 or 10 C.

We made direct counts by lightly scraping half of the surface of each of two fillets with the intact narrow edge of a clean, 1 by 3 inch (2.54 by 7.62 cm) microscope slide, mixing the material collected on a spot of the fillet itself (using the slide), then picking up a small amount of the mixture and spreading it in a thin film on another slide as for a blood smear. After the film was air-dried, heat-fixed, and Gram-stained, we examined it under an oil immersion lens (field diam-
eter, 190 μm) and counted all of the gram-negative bacteria present in either 20 or 50 fields, depending on the level of bacteria as discussed below. Then we calculated the average number of cells per field.

We dispersed the Gram stain in later counts because micrococci were the only gram-positive organisms found and then only on very fresh fillets. They are easily distinguishable from the only other bacteria with which they possibly can be confused, the Moraxella-like organisms. Therefore, we substituted a simple 30-sec staining with safranin for the complete Gram stain, and this had the added advantage of producing a less cluttered-looking field.

Preliminary experiments showed that the counts of interest to us ranged from 0 to about 5 cells per field, as samples with higher counts had such a short remaining shelf life that the prediction was pointless. From samples consisting of 200 readings each, taken at various levels within that range, we calculated 95% confidence intervals, some of which are shown in Table 1. On the basis of these findings, we decided to count 20 fields for population means of 2 or more and 50 fields for populations means of less than 2.

Hypoxanthine determinations were made according to Dugal (4). Ultraviolet absorption was measured with a Bausch & Lomb 505 recording spectrophotometer. Only a plateau or a maximum at around 290 nm was considered as definitely indicating the presence of uric acid, which was derived from the hypoxanthine in the sample. All tests were duplicated, and hypoxanthine values were read from a calibration chart made by using standard hypoxanthine solutions.

To find the relationship between the two freshness tests and shelf life, fillets stored at either 5 or 10 °C were periodically examined for signs of spoilage. We estimated organoleptically the quality of the fish, and from the fillet halves not used in the freshness tests we aseptically prepared a press juice, which was used as sample material for the objective tests of spoilage.

To obtain the viable counts, we surface-plated various dilutions of the juice on Penassay Agar (Difco) and incubated them at 20 °C for 48 hr.

VRS were determined by the method of Farber and Ferro (5), as modified by Farber and Lerke (6), and were expressed as microequivalents of reduction per 5 ml of press juice.

Trimethylamine nitrogen (TMN) was estimated according to Conway (2) and expressed as milligrams of nitrogen per 100 ml of press juice.

## RESULTS

### Direct count as a freshness test

Figure 1 shows the results of a representative experiment and contrasts the growth of the bacterial population with the chemical indices of spoilage of fresh fillets of English sole stored at 5 C. During the interval between points A and B (about 95 hr), no spoilage products were detected by the chemical methods used. This observation supported our organoleptic judgment that the fish was fresh (perfectly acceptable). This interval is that period of freshness previously referred to. Obviously, freshness was being exhausted as point B was approached, for within a few hours beyond B all indices of spoilage turned sharply upward. Organoleptically the fish then became borderline or unacceptable. However, all through the period of freshness, while the spoilage indices remained low, the bacterial population was rapidly increasing, with the viable count roughly paralleling the direct count.

The relationship between the bacterial level, as

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**TABLE 1. 95% Confidence limits as a function of cell density and number of fields counted**

<table>
<thead>
<tr>
<th>Avg no. of cells per field</th>
<th>No. of fields counted</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02</td>
<td>20</td>
<td>0–0.082</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0–0.058</td>
</tr>
<tr>
<td>0.16</td>
<td>20</td>
<td>0.08–0.24</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.11–0.21</td>
</tr>
<tr>
<td>2.9</td>
<td>20</td>
<td>2.4–3.4</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2.6–3.2</td>
</tr>
</tbody>
</table>

**FIG. 1. Relationship between certain chemical indices of spoilage (VRS and TMN) and indices of freshness in refrigerated fillets of sole. Units: VRS, microequivalents of reduction per 5 ml of press juice; TMN, milligrams of nitrogen per 100 ml of press juice; viable count, logs of the number of cells per ml of press juice; and direct count, logs of the number of cells per field.**

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measured by the direct count, and the shelf life of the fillets is shown in Table 2. The data summarized therein were gathered over several years from experiments similar to those detailed in Fig. 1 through 3, except that in all cases covered in Table 2 the holding temperature was 5°C.

Based on correlations with organoleptic judgments, either a VRS level of 10 microequivalents or more or a TMN level of 1 mg or more were chosen as marking the onset of spoilage. In all cases, the TMN was first to reach the critical level. Although it appears from Fig. 1 through 3 that this level should have been closer to 2 mg, we wanted to use the earliest chemically detectable level of spoilage. The VRS values sometimes fluctuated below 10 microequivalents, but once above this level the rise became sustained. Thus, 10 microequivalents of reduction unmistakably denoted the onset of spoilage. On the other hand, trimethylamine, as determined by the Conway method, first remained undetectable, then appeared abruptly and rose steadily. Thus, trimethylamine is a more sensitive and reliable indicator of the onset of early spoilage in sole and rock cod, and a correspondingly lower threshold value can be selected. These values represent a degree of spoilage that is at the very bottom of the spoilage scale, and the fillets remain "commercially" acceptable up to 24 hr beyond this point.

Under these conditions, an initial direct count of 0.1 cell per field or less (corresponding to log -1.0 or less) assures a shelf life of at least 48 hr at 5°C (Table 2). When the direct count is between 0.2 and 1.0 cell per field, only 24 hr of freshness can reliably be expected. Finally, when the direct count is 1.1 or more per field, even 24 hr of freshness becomes unlikely.

For the past 8 years, we have used this method to estimate the shelf life of nonfrozen fish purchased weekly by the University of California Hospitals. A projection of 24 or 48 hr was needed. In most instances, the fish received was to all outward appearances fresh, and decisions to accept or reject it were made strictly on the basis of the data in Table 2. In almost all cases, our estimates were correct: when a 48-hr shelf life was predicted, the fish always remained fresh that long, but when the fish was rejected because of a high count, in about 4% of the cases it was still acceptable at the end of the storage period.

**Hypoxanthine determination as a freshness test.** Most of the work on the direct count was necessitated by the need for a quick and simple method of freshness estimation; other, lengthier methods were consequently not investigated. More recently, however, because of the widespread interest in hypoxanthine determination as a freshness test, its usefulness was compared to that of the direct count in a series of experiments similar to those reported in Fig. 1. An additional purpose was to once more verify previously obtained data. Typical results, shown in Fig. 2 and 3, indeed confirm those obtained in the previous experiments and also indicate that the hypoxanthine curve is similar to that of the direct count inasmuch as the greatest increase takes place early in the period of freshness, thereby making possible the prediction of shelf life. For example, it appears from the data in Fig. 2 that for fillets of sole a level of 2 μmoles or less of hypoxanthine per g of flesh would assure a 2-day keeping time

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**Table 2. Relationship between the initial direct count and the keeping quality of unfrozen fish fillets stored at 5°C**

<table>
<thead>
<tr>
<th>Group</th>
<th>Avg no. of bacteria per field on day 0</th>
<th>No. of samples spoiled/no. tested at 24 hr</th>
<th>No. of samples spoiled/no. tested at 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.1 or less</td>
<td>0/18 (0%)</td>
<td>0/16 (0%)</td>
</tr>
<tr>
<td>2</td>
<td>0.2-1.0</td>
<td>0/11 (0%)</td>
<td>7/10 (70%)</td>
</tr>
<tr>
<td>3</td>
<td>1.1-5.0</td>
<td>13/22 (59%)</td>
<td>17/19 (90%)</td>
</tr>
</tbody>
</table>

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**Fig. 2. Relationship between certain chemical indices of spoilage (VRS and TMN) and indices of freshness in refrigerated fillets of sole. Units: VRS, micro-equivalents of reduction per 5 ml of press juice; TMN, milligrams of nitrogen per 100 ml of press juice; hypoxanthine, micromoles per gram of flesh; viable count, logs of the number of cells per ml of press juice; and direct count, logs of the number of cells per field.**
The direct bacterial count appears to be a good indicator of freshness: it increases markedly during the period of freshness, whereas the chemical indices of spoilage do not. This demonstrates the uselessness of the latter for the prediction of shelf life. From Fig. 1 it is apparent that the viable count, too, would be of limited use for that purpose because by the time its results became known the period of freshness would be to a great degree expended. Furthermore, unlike the direct count, the viable count sometimes lags and may even show an initial drop (Fig. 2 and 3). The direct count, however, gives almost immediate results, permitting the user to take full advantage of its ability to predict the amount of storage life remaining. The drawback of having to rely on very small numbers remains, but sensitivity is increased through the use of surface slime as the sampling material; this prevents the dilution that takes place in the preparation of muscle homogenates (10).

The random errors associated with the direct count have been estimated (Table 1). Within each confidence interval, however, there remains a large percentage variation around the mean. In spite of this, the method appears to work well, as we have shown experimentally as well as in practical applications. This may be explained by the observation (Fig. 1 through 3) that, during the time the chemical indices of spoilage remain unchanged, the increase in the direct count is 3 or 4 log units. Against this kind of change the possible variation within the confidence interval is of little significance. Furthermore, although the distinction between an initial count of, say, 0.1 (Table 2, group 1) and 0.2 (group 2) cells per field appears rather fine, it distinguishes between samples showing no spoilage and samples showing some spoilage after storage for 48 hr. The number of samples spoiled is immaterial; the method is not designed to predict shelf life beyond the point at which 100% remain fresh. Thus, although a sample in the lower end of group 2 (0.2 or 0.3 cell per field, for example) could probably, but not certainly, last 2 days, the method will allow us to predict only 1 day of freshness. On day 2, some of the samples would be fresh and some spoiled. The ratio of fresh samples to spoiled ones would vary depending on the value of the initial direct count within the group and would reflect the error involved in the method. We are not concerned with these mixed groups, and in this manner the error is automatically eliminated. The initial ranges shown in Table 2 were arbitrarily selected to give 100% reliability in predicting freshness under defined conditions, at the price of condemning some samples that might last through the storage period. Thus, the method is reliable in one direction only: that is, with a given initial count either 1 or 2 days of freshness can be guaranteed; on the other hand, predictions of spoilage are not reliable, as seen in Table 2, where even in group 3 not all samples were spoiled after 48 hr.

Aside from the quantitative aspects of the method, just what is the relationship between the bacterial cells we count under the microscope and the spoilage process? First, a certain proportion of the bacterial cells are nonviable and therefore do not cause spoilage. Further, it is universally agreed that bacterial activity is responsible for
the spoilage of fish muscle at ordinary refrigerator temperatures. Figure 2 shows that the bacterial population increases during storage and, having reached a rather high level, apparently causes the appearance of detectable amounts of spoilage products. The data do not, of course, prove cause and effect; however, we know that sterile fish muscle or juice therefrom, inoculated with a mixture of bacteria from fish (fresh or spoiled), will spoil, whereas its noninoculated counterpart will remain fresh (7).

On the other hand, we have shown elsewhere that not all bacteria present on fish are able to spoil it (at least, not in the sharp, offensive way that fish spoils after being stored for 3 to 4 days at around 5 C) and that active spoilers make up a rather small percentage of the total population (1). Thus, the percentage of morphologically indistinguishable active spoilers included in the direct count could affect the results. For example, the shelf life of a sample having a high count but few spoilers would be judged unreliable on the basis of total count, but could, on storage, last 48 hr or more. Any such error would be reflected in the mixed results column of Table 2 and does not affect the usefulness of the method as it is set up. The reverse, an unusually large percentage of spoilers in a low total count, could also happen. The probability of such occurrences is small, however, for we have shown (1) that the percentage of spoilers among the flora of fish fillets is fairly constant. Nevertheless, variation in that percentage is for the time being a potential source of error.

Admittedly, the importance of these considerations is debatable. We have chosen to look at fish spoilage as the result of the activities of certain bacteria that we call spoilers. When grown on fish muscle or juice, these bacteria are quickly able to produce offensive odors as well as several chemical substances commonly associated with spoilage. However, other organisms, the so-called nonspoilers, multiply as well as the spoilers but give rise after a long period of storage either to no odors at all or to faint musky ones. It can be argued that fish flesh bearing a high load of any kind of bacterium, "spoiler" or "nonspoiler," is unfit for human consumption. Neither view detracts from the usefulness of the direct count.

A few other considerations may affect the performance of the direct count. We developed this test using only fillets of sole and of rock cod and a storage temperature of 5 C. For any other variables, whether fish or holding temperature, new ranges will have to be determined.

Variations in handling of the fish before it is tested can also affect results. For instance, lack of refrigeration may have started the bacteria on their logarithmic phase of growth while overall numbers are still low. Under these conditions, the fish could spoil sooner than predicted. In our experience, however, no such anomalies were ever encountered.

Finally, gram-positive cocci are often present on perfectly fresh fillets but are rapidly overgrown during storage. Again, the relationship is unidirectional: the presence of cocci indicates freshness, but their absence does not preclude it. They should not be included in the direct count.

We have shown that the appearance of hypoxanthine in refrigerated fillets follows a course similar to that of the direct count, thus confirming the suggestion (9) that the concentration of hypoxanthine in fish flesh can be used to predict shelf life. Insufficient data were presented here to form a basis for such a use of the hypoxanthine test; in addition, it is not clear that further work is justified with regard to the application discussed in this report, since the ease and rapidity of the direct count, as contrasted with the sophisticated biochemical techniques and equipment as well as the time required for hypoxanthine determination, place the latter at a considerable disadvantage. The two methods do have somewhat different ranges of applicability, and hypoxanthine might, for example, be an excellent way to assess the quality of frozen fish where the direct count is of little use. On the other hand, it has been shown (3) that nucleotide degradation occurs at different rates in different fish and that in salmon, for example, the final level of hypoxanthine is very low. In this instance, the direct count might prove to be the better method of predicting freshness.

**LITERATURE CITED**