Analysis of Total Aerobic Viable Counts in Raw Fish by High-Throughput Optical Oxygen Respirometry

A. HEMPEL,† N. BORCHERT,‖ H. WALSH,† K. ROY CHOUDHURY,‖ J. P. KERRY,‖ and D. B. PAPKOVSKY†§

1School of Food and Nutritional Sciences, 2School of Statistics, and 3Biochemistry Department, University College Cork, Cork, Ireland

ABSTRACT

A simple, miniaturized, and automated screening assay for the determination of total aerobic viable counts in fish samples is presented here. Fish tissue homogenates were prepared in peptone buffered water medium, according to standard method, and aliquots were dispensed into wells of a 96-well plate with the phosphorescent, oxygen-sensing probe GreenLight. Sample wells were covered with mineral oil (barrier for ambient oxygen), and the plate was monitored on a standard fluorescent reader at 30°C. The samples produced characteristic profiles, with a sharp increase in fluorescence above the baseline level at a certain threshold time, which could be correlated with initial microbial load. Five different fish species were analyzed: salmon, cooL, mackerel, and whiting. Using a conventional agar plating method, the relationship between the threshold time and total aerobic viable counts load (in CFU per gram) was established, calibration curve generated, and the test was validated with 169 unknown fish samples. It showed a dynamic range of 10^2 to 10^7 CFU/g, accuracy of ± 1 log(CFU/g), assay time of 2 to 12 h (depending on the level of contamination), ruggedness with respect to the key assay parameters, simplicity (three pipetting steps, no serial dilutions), real-time data output, high sample throughput, and automation. With this test, quality of fish samples, CFU-per-gram levels, and their respective time profiles were determined.

Fish in general is a highly perishable product because of high water activity (1), relatively high pH, and the presence of autolytic enzymes (19). Commercially, this high rate of perishability often makes the transport and marketing of fish a challenge (16). Fresh fish by its nature has low microbial loads, both internally and externally. Muscle tissues are usually sterile in healthy fish, whereas large populations of bacteria are present on the external surfaces, gills, and intestines. There may be as much as 10^2 to 10^6 bacteria per cm^2 on skin surfaces (6, 20). As soon as fish is caught and processed, a series of bacteriological, chemical, physical, and histological changes occur in the muscle tissue (10). Significant microbial spoilage and chemical changes in fish produce sensory changes to a degree that the product becomes unacceptable to the consumer. Autolytic, chemical, and microbiological processes generate undesirable sensory changes in fish, which include discoloration, changes in texture, odor, and flavor, as well as slime and gas formation. Microbial growth is the main reason for the development of off flavors and odors rendering fish products unacceptable or spoiled (7, 20). The high degree of perishability has limited fish consumption in a fresh state to areas close to capture.

To preserve the freshness of fish products, especially during prolonged transportation and storage, and to extend their shelf life, various packaging and holding temperature techniques are used, including freezing, cooling, refrigeration (3), vacuum, and modified atmosphere packaging (8, 22). Simultaneously, prolonged storage and transportation requires efficient control measures to ensure high quality and safety of fish products (including whole fish and pieces). In particular, microbial load has to be controlled carefully and maintained below acceptable threshold levels.

The conventional microbiological method called total aerobic viable counts (TVC) provides quantification of viable microorganisms in a sample. Traditionally, TVC in fish and other samples has been done by agar plating, which takes 24 to 48 h to generate results (9). This macromethod involves multiple dilutions of each sample and manual or semiautomated readout (counting of grown colonies). When dealing with such a perishable product as fish, a micromethod more rapid, simple, and automated, making possible determination of TVC in large number of samples, is highly desirable.

A number of alternative tests and systems suitable for TVC determination in fish have been described, which utilize different chemistries, detection principles, and instrumenta-

* Author for correspondence. Tel/Fax: +353-21-490-1698; E-mail: d.papkovsky@ucc.ie.
† These authors contributed equally to this work.
In particular, optical microrespirometry uses fluorescence-based oxygen sensing probes, standard 96-well plates, and fluorescent reader detection to monitor growth of aerobic cells and microorganisms via respiration. Its initial food safety application has been described for raw meat (12), where TVC are determined directly in crude homogenates in peptone-buffered water (PBW) medium. This screening assay has been successfully validated and certified by the AOAC International. It has now been adopted by a number of meat producing companies and food safety laboratories.

In this study, we applied an optical oxygen microrespirometry assay methodology (18), utilizing the commercial GreenLight probe to develop a similar TVC test for fresh fish samples. In such a test, the probe produces a large increase in fluorescence on the depletion of dissolved oxygen by growing microorganisms, which occurs when a certain level of respiration is reached (the threshold). For different samples, fluorescent profiles are expected to be shaped similarly, but shifted with respect to each other according to respective initial TVC loads: samples with higher TVC values ascend earlier, with low TVC loads, later. The samples are partially sealed with mineral oil to reduce back diffusion of atmospheric oxygen. With five different species of fish, we investigated matrix effects on assay performance, performed optimization of assay parameters (dilutions, volumes, and timing), generated calibrations for each species, and combined calibration. The assay was validated with a panel of real (unknown) samples and benchmarked against a conventional agar-plating TVC test.

MATERIALS AND METHODS

Materials. Samples of salmon, cod, whiting, plaice, and mackerel fillets were purchased from local retailers in Cork, Ireland. Refrigerated storage room was set at 4, 14, or 24°C (room temperature) for fish storage trials. Sterile PBW was prepared fresh with components from Sigma-Aldrich Corp. (St. Louis, MO) and MilliQ water (Millipore, Billerica, MD). The stomacher machine and sterile stomacher bags were from Seward, Ltd. (London, UK). Sterile 96-well, flat-bottom microplates with clear polystyrene lids were from SARSTEDT AG and Co. (Nümbrecht, Germany). The GreenLight oxygen probe and mineral oil were from Luxcel Biosciences (Cork, Ireland). Plate agar was obtained from Merck (Darmstadt, Germany).

Respirometric assay. The fluorescent reader Safire (Tecan Group, Ltd., Switzerland) was set up as follows: measurement mode, fluorescence; excitation filter, 380 nm; emission filter, 650 nm; and gain, 60. The temperature of the microplate compartment was set at 30°C. Measurement of the microplate was carried out in kinetic mode, with each test well measured every 10 min, over 2 to 12 h.

The assay was performed as follows. The GreenLight probe was reconstituted in 10 ml of sterile PBW to produce stock solution. Fish samples (stored at 4, 14, or 24°C), were taken by cutting 10-g squares from the edges of each fillet containing skin on the outside, placed in a stomacher bag with 90 ml of PBW, and homogenized for 1 min. After this, 100-μl aliquots of the homogenate were transferred to the wells of a 96-well plate. Subsequently, 100-μl aliquots of probe stock and 100 μl of mineral oil (seal from ambient oxygen) were dispensed into each well. One fillet per type of fish represented a fish sample (one 10-g replicate) and was used per testing day, analyzed in triplicate by the respirometric method, and in duplicate by conventional TVC. Negative controls (PBW with probe) and blanks (PBW without probe) were also included. The plate was then placed in the fluorescent reader and monitored at 30°C, using the above settings to determine threshold time (TT) for each sample. TT was taken as the time to reach the threshold of fluorescence intensity set to 400 fluorescence units. At this threshold, a sample containing quickly proliferating microorganisms starts to deplete rapidly the dissolved oxygen. Initially (at relatively low cell numbers), the sample remains oxygenated, and the probe fluorescent signal remains flat and low, being quenched by dissolved oxygen. When the threshold is reached, the sample undergoes rapid deoxygenation (seen as a steep increase of probe fluorescence), and then levels off when dissolved oxygen is depleted (unquenched probe).

The procedure of this fish TVC assay includes six simple steps, as presented in the flow chart shown in Scheme 1. Compared with the previously described assay for raw meat (12), the procedure has been streamlined to three 100-μl pipettings, requiring just one micropipette. The homogenization step and medium used are the same as those used in conventional agar-plating TVC methods.

During the initial setup of the assay, positive controls (medium spiked with Escherichia coli) and blanks (medium without probe) were also included to ensure sufficient sensitivity and proper operation of the instrument, whereas at later stages, these controls are not necessary. Plate preparation time was kept to a minimum (<20 min). Where required, the same homogenates were also used in an agar plating TVC test (see below). The respirometric assay can also be run on other fluorescent readers, for example VICTOR3 (PerkinElmer, Waltham, MA) and Omega (BMG LABTECH GmbH, Offenburg, Germany) readers, which are spectrally compatible with the probe and allow temperature control and measurements in kinetic mode in 96-well plates.

To determine possible matrix effects, measurements were conducted at several different dilutions of fish homogenates: the
standard 1:10 dilution, 1:20, 1:40, and 1:80 dilutions. Spiking with E. coli was also used to assess matrix effects on microbial growth and calibration. In this case, frozen cod fillet with low TVC (tested by agar plating) was thawed for 3 h at room temperature, homogenized in PBW, and then spiked with E. coli stock to produce concentrations between $5 \times 10^5$ and $5 \times 10^7$ CFU/g, and measured as above.

To validate the new TVC assay, a panel of fresh fish samples with unknown levels of microbial contamination (salmon, cod, mackerel, whiting; $n = 169$) was obtained from local retailers on different days, with several samples taken each day. Each sample was tested by the new respirometric test, and respective values (expressed as CFU per gram) were determined by applying the combined calibration. In parallel, the samples were analyzed by a conventional TVC test (International Organization for Standardization [ISO] Method 4833:2003), and the results were compared with, and plotted against, each other.

To test the ruggedness of the respirometric assay, two different errors were introduced, pipetting volume and probe concentration (Table 1). As standard protocol involves three consecutive additions of 100-μl volumes (probe, sample, and oil), an error in each was introduced by applying a lower (70 μl) and a higher (120 μl) pipetting volume. An error in probe concentration was introduced by using a lower (50%) and a higher (150%) probe dilution, as compared with the standard conditions. The effects of these two errors were tested at two different contamination levels, $10^4$ to $10^5$ CFU/g (low) and $10^6$ CFU/g (high), with negative controls (media only, <$10^3$ CFU/g) included in each test. Samples were taken from cod fillets, which were stored at 24°C for 2 days (high CFU per gram) and at 4°C (low CFU per gram), and analyzed in five repeats ($n = 5$).

In the storage trials, fish samples were kept at 4, 14, and 24°C, and analyzed periodically by the respirometric and conventional TVC tests: daily for 4 and 14°C tests, and hourly for 24°C (due to fast deterioration).

**Conventional TVC test.** A conventional TVC test on agar plates was performed according to ISO Method 4833:2003, using PBW medium, incubation at 30°C, and counting the colonies of bacteria after 48 h (9).

**Statistical analysis.** The possibility of the calibration relation between TVC (in CFU per gram) and TT (in hours) being modulated by other factors such as species of fish or trial effects was investigated by fitting a general linear model of the form:

$$
\text{th}_{ij} = \mu + b \log(\text{TVC}_{ijk}) + \gamma_i + \beta_j + \gamma_i \log(\text{TVC}_{ijk}) + \delta_i \log(\text{TVC}_{ijk}) + j_{ij} + \varphi_{ij} \log(\text{TVC}_{ijk}) + e_{ijk}
$$

where $\text{th}_{ijk}$ stands for the threshold recorded on the $ith$ species on the $jth$ trial for the $ith$ species, and similarly for TVC$_{ijk}$. Here, $\mu$ stands for overall mean of TT values; $b$ (TVC) for the overall slope of the regression of TT on log(TVC); $\gamma_i$ (species) and $\beta_j$ (trial) for the main effect on the mean of the $ith$ species and $jth$ trial, respectively; $\gamma_i$ (TVC: species) and $\delta_i$ (TVC: trial) for the effect on the slope of the $ith$ species and $jth$ trial, respectively; $\varphi_{ij}$ (species:trial) for the interaction (combined) effect on the mean of the $ith$ species and $jth$ trial; $e_{ijk}$ (error) for measurement error. For estimation and hypothesis testing, measurement errors were assumed to have a Gaussian distribution with identical variance and to be mutually independent. The significance of effects in equation 1 was measured with $F$ tests computed by a three-factor analysis of variance (ANOVA) (26). The acronyms in parentheses were used to represent each effect in the ANOVA table. Based on the significant terms identified by the ANOVA procedure, a reduced model of the form

$$
\text{th}_{ijk} = \mu + b \log(\text{TVC}_{ijk}) + e_{ijk}
$$

was fit to the data by least squares. The fitted calibration model was examined for adequacy by examining the residuals (estimated errors) for outliers and constancy of variability. Outliers identified by this process were removed for estimating the final calibration model. The assumption of Gaussianity of measurement error was checked with a quantile–quantile plot (25). The quality of fit was quantified by the $R^2$ statistic.

The respirometric TVC assay (TVC$_R$) was computed with the relation

$$
\text{TVC}_R = \frac{\text{th} - \mu}{b}
$$

where $\text{th}$ is the observed TT. The quantities $\mu$ and $b$ are obtained from the final calibration model in equation 2. For validation, we compared TVC$_R$ values against standard TVC values by using agar plating across a range of validation samples, $i = 1, \ldots, 169$, by linear regression:

$$
\text{TVC}_{Ri} = c + m \text{TVC}_i + e
$$

For a perfect validation, one would expect $c$ to equal 0 and $m$ to equal 1 (the line $y = x$), but the actual values are likely to be different due to sampling variability. However, one can prove adequacy of the validation by checking if 95% of the data values are within ±1.96 standard deviation (SD) of the ideal line, where SD due to sampling variability is estimated from the residual error of the fitted regression model in equation 4 (26).

Analysis of ruggedness testing was performed with a two-factor ANOVA (26), where the factors were (i) the level of sample contamination (high and low CFU per gram) and (ii) either the assay volume (70, 100, and 120 μl) or the probe concentration (50, 100, and 150 ml). Significances of effect were measured by standard ANOVA $F$ tests (26). Statistical analysis was done with the R package (http://www.cran.r-project.org).

### RESULTS AND DISCUSSION

**Assay setup.** The probe was added to crude homogenates of fish tissue in growth-promoting medium (PBW) during plate preparation, and then changes in fluorescence were monitored at 30°C. Prominent probe fluorescence changes due to microbial growth allowed unambiguous identification of positive samples and quantification of their TVC loads, based on measured TT. The TT were determined for all the samples on the plate and converted to CFU per gram by using predetermined calibration. Theoretical sensitivity of the respirometric assay is 1 CFU
per well (14); however, sample volume (0.1 ml) and dilution during the homogenization (1:10) should be factored in for food samples. Statistical variability at low cell numbers (1 to 10), possible matrix effects, and data scattering reduces further the sensitivity, down to $10^3$ to $10^4$ CFU/g (limit of detection). Samples producing flat profiles with low fluorescent signal were defined as negative (below the limit of detection). Up to 96 samples could be analyzed on a plate in one run.

**Analysis of fish matrix effects and optimization of assay conditions.** To assess matrix effects in the respirometric assay, fish samples with relatively low levels of contamination (a range of $10^5$ to $10^4$ CFU/g, verified by conventional TVC) were initially measured at different dilutions of the homogenates: 1:10, 1:20, 1:40, and 1:80. Representative respiration profiles for one such salmon sample are shown in Figure 1. As with pure microbial cultures (14) and raw meat homogenates (12), the samples produced characteristic sigmoidal profiles. In contrast, negative samples gave flat profiles, as their oxygen concentrations did not change.

From these profiles, a good linearity between measured TT values and sample dilution was seen (Fig. 1, processed data). The threshold is the point at which the fluorescence signal shows a sharp increase from the basal level. Corresponding TT was compared with the results of conventional TVC CFU per gram, which showed that sample matrix had no significant effect on assay performance, and that at different sample dilutions the microorganisms proliferated at about the same rate (exponential growth).

To mimic the responses at different initial microbial load, homogenates of cod sample with low level of microbial contamination (<$10^2$ CFU/g, 1:20 homogenate dilution) was spiked with increasing concentrations of E. coli and measured. Figure 2 shows that spiked homogenates produced consistent profiles in the assay and gave a linear relationship between TT and E. coli concentration (in CFU per milliliter). In this matrix, doubling time of E. coli, the limit of detection, and maximal monitoring time were determined: 25.6 min, 50 CFU/g, and 10 to 12 h, respectively.

From these experiments, 1:20 dilutions of fish homogenates were selected as standard for further work, as this method provided convenience with pipetting (standard 100-μl aliquots throughout) and no undesirable matrix effects.

**Establishment of calibration.** To establish the relationships between the TVC (CFU per gram) and TT (hours), and to generate calibrations that can be used for the analysis of samples with unknown microbial load, we analyzed panels of samples of different fish types (fresh salmon, cod, whiting, plaice, and mackerel). The selection of fish was made to encompass the spectrum of different types of tissue, i.e., white and red tissue, fresh and seawater fish, flat and thick-bodied fish, low-fat fish, and oily fish. Each sample homogenate underwent parallel analysis by the conventional agar plating TVC method and by the respirometric assay. Accounting for potentially slower growth rates of microorganisms present in fish samples, plate monitoring time was extended to 12 to 16 h.

From the ANOVA analysis (results not shown), it was apparent that the only significant source of variation in the calibration relation is TVC level. More specifically, the calibration relation is not significantly different across species or trial, or any combination of factors. This justifies a simple regression model of equation 2, in which other factors are not included.

Figure 3 shows the combined calibration for the four fish species (salmon, cod, whiting, and mackerel) after

![Figure 1](http://example.com/figure1.png)

*FIGURE 1. Typical profiles (a) of salmon homogenate measured at different dilutions: 1:10, 1:20, 1:40, and 1:80 (from left to right) and negative control (flat line), and the relationship (b) between TT (at 400 fluorescent units) and sample dilution. Doubling time (DT) calculated from the slope is shown on the graph.*

![Figure 2](http://example.com/figure2.png)

*FIGURE 2. Respiration profiles (a) of cod homogenate samples (~$10^2$ CFU/g) spiked with different concentrations of Escherichia coli (red line represents the threshold), and the resulting relationship (b) between TT and E. coli concentration.*
The results of assay ruggedness test with respect to exclusion of the top two outliers. The majority of points lie within the ±1.39 SD band, as expected. However, the $R^2$ value is moderate, indicating substantial variability in the data. The relationship obtained from the fitting and the analytical equation for conversion of measured TT values into CFU per gram are given in Figure 3. It is worth noting that inclusion of the two outliers significantly changes the calibration relation (it becomes $TT = -2.40 \text{ CFU/g} + 18.69$). By individual treatment of these fish types, the $R^2$ and the parameters of equation are as follows: for salmon, $TT = -2.94 \text{ CFU/g} + 21.35$ ($R^2 = 0.72$) and for whiting, $TT = -2.36 \text{ CFU/g} + 18.73$ ($R^2 = 0.70$)—a higher $R^2$ obtained than that obtained for cod, where $TT = -2.41 \text{ CFU/g} + 19.01$ ($R^2 = 0.53$) and for mackerel, $TT = -1.49 \text{ CFU/g} + 13.00$ ($R^2 = 0.39$).

Again, fish is a difficult product to work with. It is less known the chemical and physical changes fish develop in muscle tissue postmortem and how this can influence a respirometric method. In an earlier work performed on different types of raw meat (beef, pork, lamb, and poultry) by the same method (12), a combined $R^2 = 0.86$ was obtained, as compared with a fish $R^2$ of 0.56.

**Problematic samples.** At the same time, certain fish samples, particularly fresh plaice, were seen to produce high scattering of results of the respirometric assay and worse correlation with the conventional TVC test (Fig. 4). We explain this by plaice being a flat fish with a low ratio of muscle tissue volume to skin surface, resulting in a sampling of surface bacteria less predictable than that for the other fish species tested. Likewise, the scattering of results from frozen fish samples was significantly greater than it was for fresh fish, although the calibration equation was similar. This suggests that freezing affects the bacteria in fish tissue and normal microbial growth duringassyaying. Reasons could include freeze damage to microorganisms by the crushing and spearing action of ice crystals, as well as lethality resulting from cell dehydration effects. The rate of freezing, storage temperature, and temperature fluctuations during storage influence the extent of sublethal injury and death of microorganisms. Thawing is more injurious to microorganisms than freezing is, and the effects vary according to species. Even simple thawing of a frozen microbial population without intervening storage causes slight to moderate reduction in the number of live organisms (20). Due to the large variance of results, plaice and frozen fish samples were excluded from further testing in the respirometric TVC assay.

**Assessment of ruggedness of respirometric TVC assay.** The results of assay ruggedness test with respect to pipetting volume are summarized in Table 2. With this data, ruggedness was tested against two factors, (i) the level of sample contamination (high and low CFU per gram), and (ii) the pipetting volume (70, 100, and 120 µl). To examine the relative contributions of these factors to measurement variation, we modeled log(response) as a function of them, yielding the ANOVA results presented in Table 3.

As expected, Table 3 shows that the main source of variability is sample microbial load (in CFU per gram), whereas variability due to pipetting volume and residual error appear to be negligible by comparison (relative means square of 0.1 and 0.02%, respectively). Further analysis showed a marginally significant measurements trend ($P = 0.04$) due to change in assay volume. We note that the significance occurs due to the very small value of residual error (because of replication).

The probe concentration ruggedness test produced similar results (not shown). The main source of variability was again sample microbial load (CFU per gram), whereas variability due to probe concentration and residual error appeared to be negligible by comparison (relative means square of 0.2 and 0.01%, respectively). Further analysis showed a significant measurements trend ($P = 0.003$) due to change in probe concentration. We note that the
significance occurs due to the very small value of residual error (because of replication).

**Assay validation.** Figure 5 shows the correlation between the two methods. The diagonal line in the figure demonstrates the ideal correlation between the two methods (predicted line ~ observed line). Although the validation trend line produced by linear regression fit of all the data points did not match this ideal line, one can see that 93.5% (158 of 169) of data points lie within ¡ 1.96 SD of the ideal line. This is close to the expected 95%. One can see that respirometric assay provides the accuracy of TVC determination in fish samples of approximately ¡ 1 log(CFU/g).

For a simple, fast, high-throughput screening test, this analytical performance is considered good (though not as good for raw meat samples).

In addition, storage trials were carried out to ascertain the natural spoilage rates of fish at different temperatures. It is known that bacteria grow faster at high temperatures. The Q_{10} rule implies that for every 10°C increase in temperature, the growth doubles, i.e., Q_{10} = 2 (5, 23). Of course, this can vary, depending on bacteria and sample used. Representative data for salmon are shown in Fig. 6.

After linearization of the three curves, slopes were observed, which increased from 4 to 24°C, with a Q_{10} factor of 2.5: particularly, 4°C ~ 0.31, 14°C ~ 0.78, and 24°C ~ 1.97.

In conclusion, a simple, rapid, and robust screening test for TVC in raw fish sample was developed, which relies on fluorescence-based microrespirometry in standard 96-well plates. Assay conditions including pipetting volumes, sample dilution, and matrix effects were optimized to streamline the procedure and produce reliable results. The test was applied to five different fish types—fresh cod, salmon, whiting, mackerel, and plaice, as well as frozen fish.

**TABLE 2. Experimental data for pipetting volume ruggedness test**

<table>
<thead>
<tr>
<th>Pipetting vol (μl)</th>
<th>Log sample (high CFU)</th>
<th>Log sample (low CFU)</th>
<th>Log sample (negative control)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Replicate no. 70</td>
<td>100</td>
<td>120</td>
</tr>
<tr>
<td>Log sample (high CFU)</td>
<td>1 6.79 6.96 6.98</td>
<td>3.75 3.88 3.99</td>
<td>&lt;3 &lt;3 &lt;3</td>
</tr>
<tr>
<td></td>
<td>2 6.79 6.96 6.99</td>
<td>3.66 3.77 3.93</td>
<td>&lt;3 &lt;3 &lt;3</td>
</tr>
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</tr>
<tr>
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<td>3.11 3.38 3.66</td>
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</tr>
<tr>
<td>Avg</td>
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</tr>
<tr>
<td>SD</td>
<td>0.00 0.01 0.01</td>
<td>0.25 0.19 0.15</td>
<td>NA NA NA</td>
</tr>
</tbody>
</table>

**TABLE 3. Two-factor ANOVA for sample volume ruggedness experiment**

<table>
<thead>
<tr>
<th>Factor</th>
<th>df&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F statistic</th>
<th>P value</th>
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<tr>
<td>Microbial load</td>
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<td>78.83</td>
<td>78.83</td>
<td>3,976</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(CFU/g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Assay volume</td>
<td>2</td>
<td>0.29</td>
<td>0.147</td>
<td>7.4</td>
<td>0.002</td>
</tr>
<tr>
<td>Error</td>
<td>26</td>
<td>0.52</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> df, degrees of freedom.

<sup>b</sup> NA, not applicable, based on data.

**FIGURE 5.** Assay validation with unknown fish samples from different retailers. Solid line was produced with linear regression fit of the respirometric and standard TVC test values. Dotted line shows the ideal case: y = x. From regression, SD = 0.97 CFU/g. Dashed lines indicate sampling variability range (ideal is ±1.96 SD).

**FIGURE 6.** Time profiles of microbial load [log(CFU per gram)] for salmon fillets stored at different temperatures: 24°C (○), 14°C (■), and 4°C (▲).
(all used as crude homogenates in PBW)—for which individual calibrations and combined calibration were generated. The test showed good correlation with a conventional TVC test (ISO 4833:2003), analytical performance, and ruggedness, with respect to variation of key assay parameters (probe concentration and pipetting volume). Although linear regression fit was not perfect ($R^2 = 0.56$), a vast majority of data points lay within $\pm 1.39$ SD. At the same time, plaice and frozen fish yielded lower correlations when using the conventional TVC method, which can be explained by the generic structure of these fish samples, resulting in a sampling less predictable and a higher scattering of data. The respirometric test was then validated with a panel of unknown fish samples ($n = 169$), where it correlated well with the conventional TVC test. Although the correlation trend line produced by linear regression does not match the ideal line, 93.5% of points lie within $\pm 1.96$ SD, i.e., close to the anticipated 95%.

Overall, the respirometric fish TVC test provides general simplicity (homogenization and pipetting) and miniaturization, a dynamic range ($10^4$ to $10^7$ CFU/g), accuracy of $\pm 1 \log$(CFU/g), high speed, and automation. Highly contaminated samples can be identified quickly (in 2 to 12 h, depending on the level of contamination) (Fig. 3); positive samples can be seen as the measurement progresses (real-time data output). Its ability to assess highly perishable products such as fish in less than 12 h shows good industry application usage for testing samples far more quickly and reliably, and making safety and quality assessments in large numbers of samples. The test offers simple setup (conventional microplates and fluorescent reader), significant savings on labor, laboratory space, and waste requirements, and it overcomes many drawbacks of conventional TVC testing.

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