SISTEMA DE MICRIOBOLOGÍA RÁPIDA

BACTRAC

CONTENIDOS:

Principios de Medida

La detección de microorganismos por impedancia eléctrica del sistema **BacTrac** se fundamenta en la medida de su actividad metabólica. De este modo, mucho antes de que una colonia pueda ser visible en un medio de cultivo sólido, es posible detectar la actividad de los microorganismos.

El sistema BacTrac permite realizar ensayos tanto **cualitativos** (presencia/ausencia) como **cuantitativos** (recuentos)

Existen dos métodos diferentes de detectar microorganismos por Impedancia: Directa o Indirecta.

IMPEDANCIA DIRECTA:

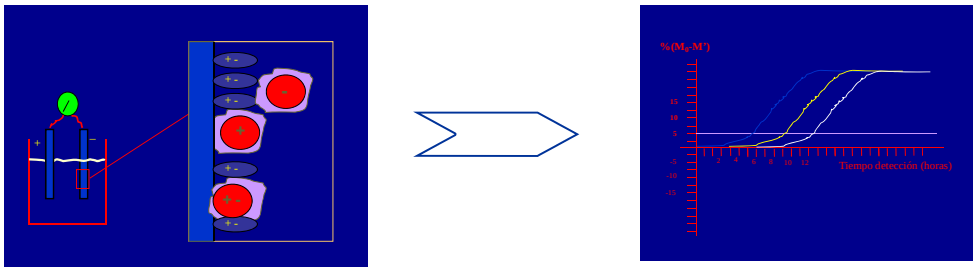
La detección de microorganismos por Impedancia Directa se basa en la capacidad de los microorganismos de metabolizar las moléculas del medio de cultivo, para crecer. Los nutrientes del medio de cultivo (glúcidos, proteínas...) son moléculas eléctricamente neutras o están débilmente ionizadas. Estas moléculas son metabolizadas por los microorganismos en crecimiento, y transformadas en moléculas más pequeñas, con polaridad y/o carga eléctrica, como por ejemplo ácido láctico, acético, cítrico, aminoácidos...

El efecto final de esta actividad metabólica es un incremento de la conductividad eléctrica del medio de cultivo, medible mediante dos electrodos sumergidos en el medio de cultivo.

De este modo se puede establecer una correlación entre cambios en la impedancia eléctrica y la concentración inicial de microorganismos en la muestra: cambios rápidos de la conductividad del medio de cultivo son debidos a elevadas poblaciones microbianas, y lentos, a una menor carga microbiana inicial.

De este modo se puede establecer una correlación entre cambios en la impedancia eléctrica y la concentración inicial de microorganismos en la muestra: cambios rápidos de la conductividad del medio de cultivo son debidos a elevadas poblaciones microbianas, y lentos, a una menor carga microbiana inicial.

Medios de cultivo deshidratados



Del mismo modo la no variación de la impedancia del medio de cultivo a lo largo del tiempo es una indicación de ausencia de microorganismos viables.

IMPEDANCIA INDIRECTA

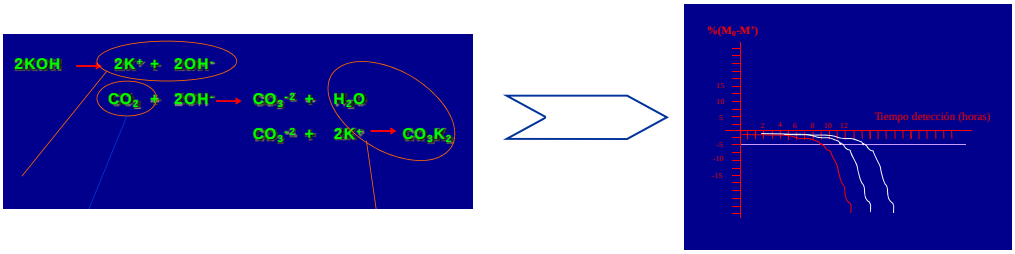
BACTRAC
Microbiología
Rápida

Algunos microorganismos, como los mohos y levaduras, de crecimiento lento, pueden ser detectados más rápidamente registrando la producción de CO_2 debida a su metabolismo.

Para registrar la producción de CO_2 la muestra se inocula en un vial junto con el medio de cultivo. Este vial se introduce a su vez en una celdilla que contiene una solución de KOH (potasa).

El CO_2 generado por los microorganismos se irá disolviendo en la potasa, modificando su impedancia.

La reacción química entre el CO_2 y el KOH se basa en la siguiente reacción química:



Ventajas Sistema BacTrac

La detección de contaminación microbiana por impedancia elimina la necesidad de dilución o separación de los microorganismos, así como su siembra en placa, reduciendo considerablemente el tiempo de preparación de la muestra.

Los caldos líquidos utilizados en el análisis por impedancia responden mejor a la demanda de proliferación de los microorganismos que los medios semisólidos de agar. La tasa de proliferación se acelera y el tiempo de análisis se reduce. Una mayor reducción del tiempo se logra gracias a la sensibilidad de la detección eléctrica.

La detección de la señal eléctrica debida a contaminación microbiana es determinada a cargas microbianas muy bajas, en comparación con el método de placa, donde se requiere una proliferación del orden de 10^8 - 10^9 CFU/ml para poder detectar una colonia visible. La detección por impedancia es del orden de 1000 veces más sensible que por placa.

BACTRAC
Microbiología
Rápida

En general, el tiempo total de análisis por impedancia no **excede de 24 horas**. **En la mayor parte de los casos los resultados se obtienen sólo en unas pocas horas**, con la ventaja de que los resultados se obtienen más rápido cuanto mayor es la contaminación de la muestra.

Las ventajas del análisis por impedancia se pueden resumir de la siguiente forma:

- **Automatización de un proceso basado en la técnica más contrastada: Microbiología Clásica**
- **Detección de Patógenos y recuentos de parámetros de calidad microbiológica.**
- **Reducción del tiempo de detección Simplificación en la preparación de la muestra para determinaciones cuantitativas.**
- **Reducción de materiales y tiempo de trabajo.**
- **Informatización de la documentación.**
- **Certificación y Validación en Normas Oficiales DIN y AFNOR**

Validaciones Oficiales

Francia

AFNOR, NF V08-105
Use of impedance technology in the analysis of **food and animal feeds**

AFNOR NF V08-106
Impedance detection of **E.coli** in seafood

AFNOR Validation
Impedance method for the detection of **Enterobacteriaceae** in dairy products, according to **ISO 16140**

Alemania

DIN Standards and $35 LMBG official method for foods

DIN 10115 General Impedance Standard, **§35 LMBG: L00.00-53**

DIN 10120 Salmonella Standard, **§35 LMBG: L00.00-67**

United Kingdom

The processed Animal Protein Order 1989, Salmonella in animal feeding stuffs

Austria

ÖNORM-DIN 10115
General Impedance Standard, part of the official Codex for testing of foods

BACTRAC
Microbiología Rápida

Celdillas de Medida

Parámetros Microbiológicos

MICROORGANISMOS DETECTABLES

- **Aerobios mesófilos y termófilos**
- **Enterobacterias**
- **Coliformes**
- **E.coli**
- **Samonella**
- **Listeria**
- **Pseudomonas**
- **Bacterias Lácticas**
- **Staphylococcus aureus**
- **Bacillus cereus**
- **Clostidia sp. y Clostridium perfringes.**
- **Mohos y levaduras**

BACTRAC
Microbiología
Rápida

APLICACIONES

- **Detección de patógenos**
- **Recuentos**
- **Test de esterilidad**
- **Evaluación de procesos de esterilización y test de inhibidores.**
- **Estudios de inactivación**
- **Test de vitalidad**

Journal of Food Protection, Vol. 57, No. 5, Pages 369-376
Copyright©, International Association of Milk, Food and Environmental Sanitarians

Rapid Detection of Salmonellae by Means of a New Impedance-Splitting Method

P. PLESS[1]*, K. FUTSCHIK[2] and E. SCHOPF[1]

[1]*Institute of Meat Hygiene and Technology, University of Veterinary Medicine, Linke Bahngasse 11, A-1030 Vienna, Austria and*
[2]*Institute of Fundamentals and Theory of Electrotechnics, Bioelectricity and Magnetism Division, University of Technology Gußhausstraße 27, A-1040 Vienna, Austria*

(Received December 7, 1992/Accepted January 10, 1994)

ABSTRACT

An Impedance-Splitting method is proposed for the rapid detection of salmonellae in foods. The measuring system, BacTrac™ 4100, permits the registration of changes, caused by bacterial metabolism, not only of the impedance of the culture medium but also of changes in the ionic layers at the measuring electrodes, which has advantages in case of high salt concentrations. These changes are expressed as percentage decreases of the initial values, M-value and E-value, respectively. Food samples were pre-enriched 14 to 16 h at 37°C in peptone water by addition of mannitol, which facilitated the detection of salmonellae on selective culture media. Following this, 0.1 ml of the preenrichment culture was transferred to 9.9 ml of Impedance-Splitting Salmonellae (ISS) medium which consisted of magnesium chloride (hydrated), malachite green oxalate, novobiocin, phosphate buffer, mannitol, peptone and yeast extract. Despite the high magnesium chloride concentration in this medium, salmonellae produced changes of the E-value up to 100%, while the changes in M-values were limited to a few percent. The impedance changes were automatically recorded during incubation in the measuring system for up to 22 h at 40°C, and the time required to exceed a threshold value of 15% (E reaction time) was evaluated. Comparative testing of the ISS method with standard cultural analysis of 250 unknown food samples showed high sensitivity and selectivity in detecting salmonellae. From all of the 122 *Salmonella*-positive samples, the largest number (119) was obtained by the ISS method, as compared to that obtained by conventional testing with the selenite-cystine (106), Rappaport Vassiliadis soya (95), Rappaport Vassiliadis (92) and tetrathionate brilliant green medium (64). Six samples were false positive by *Enterobacter cloaceae*. One strain each of *Salmonella enteritidis* PT8 and *Salmonella panama* were not recorded. The ISS method is very suitable as a screening test, all the more since a negative investigation result will be obtained within 38 h. In view of the practicability, this method is superior to the enzyme-immunological and molecular-biological procedures.

Key Words: Rapid methods, impedance, salmonellae, foods

Increased incidences of food infections caused by salmonellae make it essential to maintain stricter controls in food manufacturing processes. The conventional cultural methods of detection are labor intensive, and a result cannot be expected within less than 3 to 5 days. Increasing numbers of tests lead to greater expense of work and materials and a huge increase in analytical cost. In addition, increased storage costs for food manufacturers can be expected and/or shelf-life problems may arise with easily perishable foodstuffs due to this preclearance storage of foods under test.

A series of rapid investigation methods have been developed in recent years in the fields of gene hybridization (*6,7*) and the enzyme immunoassay technique (*3,5*) saving from 1 to 2 days in time.

Measurements of impedance and/or conductance have gained recently in significance for the rapid detection of salmonellae due to its high degree of automatization. As a precondition of this procedure, selective enrichment on culture media are necessary.

A medium for the measurement of conductance (selenite cystine trimethylamine-N-oxide/dulcitol medium, SCT/D medium) on the basis of selenite-cystine (SC) broth, by addition of dulcitol as a carbon source and trimethylamine-N-oxide (TMAO) to increase conductance changes (*4*), has been developed for the Malthus Growth Analyzer. The replacement of dulcitol by mannitol (selenite cystine trimethylamine-N-oxide/mannitol medium, SCT/M medium) as later carried out by Gibson (*9*) and Ogden and Cann (*11*), to detect also dulcitol-negative salmonellae strains, has led to a shorter detection time; however, the sensitivity of the medium was decreased by mannitol. The application of a lysine conductance medium [lactalbumin hydrolysat, L-lysin, glucose, sodium biselenite, sodium chloride (NaCl)] by Ogden (*10*), and some modified versions (*12-14*) resulted in higher conductance changes and the detection of nondulcitol fermenting salmonellae, too, although the sensitivity and selectivity were not as good as compared to the SCT/D medium. Another impedance detection medium was described (*2*), where lysine iron cystine neutral red medium was modified for employment in the Bactometer M123 system.

With the new BacTrac™ 4100 measuring system (Bacteria Tracer, SY-Lab, 3002 Purkersdorf - Vienna, Austria), an apparatus based on the measurement of impedance has been marketed. The first of its kind to operate according to

the Impedance-Splitting method described by Futschik et al. (*8*) (later termed the IS method). In comparison with other available impedance (conductance) measuring systems, which determine the changes of the sum impedance (medium and electrode impedance), the IS method allows for the simultaneous and separate determination of changes of the impedance of the culture medium and of the electrode impedance, respectively (see materials and methods). In various culture media, the recording of this additional parameter leads to a considerable rise in the sensitivity of the measuring system and thus also to a reduction of the investigation time.

The aim of this study was to develop a salmonellae selective medium for the IS method. The following electrode impedance procedure holds great promise as a rapid and reliable procedure for the detection of foodborne salmonellae.

MATERIALS AND METHODS

Principle of the IS method

Microbial metabolic processes producing electrically measurable changes in the growth medium were utilized to detect bacteria which metabolize high-molecular weight nutrients into smaller charged ionic components, thereby increasing the electrical conductivity of the medium. The electrical impedance of the growth medium will subsequently decrease. The M-value measures the change in impedance of the growth medium with time and is expressed as percentage decrease of the initial value.

In addition to the M-value, the IS method also detects the changes of ionic layers in the vicinity of the electrodes. For the purpose of measurement, these ionic layers represent, additional impedances, so-called "electrode impedances". Changes in the molecular composition of the growth medium also cause changes of these ionic layers and therefore changes of the electrode impedance. The mechanisms of the latter are much more complex than those of the medium impedance. Principally, an increase in mobile ions causes a decrease of the electrode impedance (similar as for medium impedance). In media of high salt content, where additional ions only cause a negligible decrease in medium impedance, the electrode impedance can change considerably if new types of ions are electrically adsorbed to the electrode surface. As a third cause, dipole molecules or uncharged substances, which thus do not contribute to conductivity, can cause high changes of the electrode impedance by chemical adsorption. By the IS method, these changes of the electrode impedance are measured and expressed as a percentage decrease of the initial value (E-value). Thus, two separate parameters are available for the characterization of growth. Which parameter is used (the M-value, the E-value, or both) depends on the case of application.

Two characteristic quantities are drawn upon for the evaluation. The duration from the beginning of the investigation up to the moment when the M-value reaches an arbitrarily selectable threshold value is defined as the M-reaction time (T_M). Similarly, the concept of the E-reaction time is described as T_E. The level of the selected threshold value (e.g., 5% threshold for the M-value, 20% threshold for the E-value) is dependent upon the case of application. By means of appropriate calibration, the number of colony-forming per ml (CFU/ml) can be calculated.

Test strains

Organisms used in this work (Table 1) were stock cultures made available by the Austrian Federal Research Institute of Bacteriology and Serology at Graz. Additionally, a series of bacterial cultures from our own laboratory was included in the investigation.

TABLE 1. *Organisms used in this work.*

Strain	No. of strains	Origin of strain[a]
Salmonella typhimurium	1	ATCC 14028
S. typhimurium	1	SZ; 1,4,5,12: i: 1,2
S. typhimurium	2	LS
S. enteridis	1	SZ; 1,9,12: g,m:-
S. enteridis	5	LS
S. saint-paul	1	SZ; 1,4,5,12: e,h: 1,2
S. saint-paul	2	LS
S. heidelberg	1	SZ; 1,4,5,12: r: 1,2
S. heidelberg	2	LS
S. agona	1	SZ; 4,12: f,g,s:-
S. agona	1	LS
S. virchow	3	SZ; 6,7: r: 1,2
S. virchow	1	LS
S. infantis	1	SZ; 6,7: r: 1,5
S. braenderup	1	SZ; 6,7: e,h: e,u,z, 5
S. newport	1	SZ; 6,8: e,h: 1,2
S. blockley	1	SZ; 6,8: k: 1,5
S. bovis morbificans	1	SZ; 6,8: r: 1,5
S. kentucky	1	SZ: 8,20: i, z: 6
S. berta	1	SZ; 9,12: f,g,t:
S. dublin	1	SZ; 1,9,12: g,m:-
S. panama	1	SZ; 1,9,12: l,v: 1,6
S. london	1	SZ; 3,10: l,v: 1,5
S. meleagridis	1	SZ; 3,10: e,h: l,w
S. senftenberg	1	SZ; 1,3,19: g,s,t:-
S. senftenberg	1	LS
S. livingstone	1	LS
S. thompson	1	LS
S. münchen	1	LS
S. hadar	1	LS
S. schwarzengrund	2	LS
S. isangi	1	LS
Enterobacter cloaceae	1	ATCC 23355
E. cloaceae	1	DSM 30054
E. cloaceae	3	LS
E. aerogenes	1	LS
Klebsiella pneumonieae	1	ATCC 13883
K. pneumonieae	2	LS
K. aerogenes	1	LS
Citrobacter freundii	1	ATCC 8090
C. freundii	2	LS
Escherichia coli	1	ATCC 25922
E. coli	4	LS
Proteus vulgaris	1	ATCC 13315
P. vulgaris	2	LS
Pseudomonas aeruginosa	1	ATCC
Pseudomonas sp.	2	LS

[a] ATCC, DSM: international type strains; SZ: strains from the Graz central station; LS: laboratory strains (salmonellae strains type specified at the salmonellae station).

Various substances used for media formulations

The following substances were used for the selective media formulations suitable for the IS method: Elective substances were

yeast extract (Oxoid L 21), peptone (Oxoid L 34), mannitol (Merck M-4125), L-lysine monohydrochloride (Sigma N-1628), L-cystine (Merck 2838), NaCl (Labchemie 706380), KH_2PO_4 (Merck 4873), trimethylamine-N-oxide (TMAO) (Aldrich 17686-9), dulcitol (Merck 3589), buffered peptone water (BPW) (Oxoid CM 509). Selective substances were magnesium chloride hexahydrate (Merck 5833), malachite green oxalate (Merck 1398), novobiocin sodium (Sigma N-1628), potassium tetrathionate (Merck 5169), sodium biselenite (Merck 6340), brilliant green (Merck 1310) and ox bile (Merck 3756).

Cultural procedures for the determination of salmonellae in foodstuffs

According to international standards (*1*) a nonselective preenrichment stage in BPW for 16 h is required. As discussed later, a modified preenrichment medium (adding mannitol, omitting phosphate buffer) of the following composition (g/L) is used: NaCl (8.5), peptone (1.0), mannitol (5.0) demineralized water.

To compare the results obtained with the IS method with those from conventional cultural procedures, the same preenrichment culture was used for both procedures. After overnight preenrichment, 0.1 ml was inoculated into 10 ml selective enrichment broth and incubated for 24 h at 42°C. This procedure was not only applied for the Rappaport Vassiliadis medium (RV medium, Merck 10236) and the Rappaport Vassiliadis soya peptone medium (RVS medium, Oxoid CM 866) but also for the tetrathionate brilliant green bile broth (TBG broth, Merck 5172) and the selenite-cystine broth (SC broth, Merck 7709) because we found that when testing samples with high numbers of competing microorganisms, better results were obtained using 0.1 ml preenrichment culture.

The selective plating media used for control were modified brilliant green agar (BGA, Oxoid CM 329) and xylose Lysine desoxycholate agar (XLD agar, Oxoid CM 469 with novobiocin sodium addition of 0.04 g/L). Suspect colonies were confirmed serologically (polyvalent I-test serum, ORMT 10/11, Bering, Germany).

Salmonellae determination by the IS method

Preenrichment was obtained by homogenizing the sample or rinsing it in the previously described modified preenrichment medium. After incubation for 14 to 16 h at 37°C, 0.1 ml was transferred to 10 ml of the various selective media being investigated and was then incubated for 22 h in the measuring system BacTrac 4100 at a temperature of 40°C, the impedance course being simultaneously recorded. Then the impedance tubes were subcultured directly on modified BGA and XLD agars; these were incubated at 37°C and suspect colonies were confirmed serologically.

Sample material

Excluding the pure cultures listed, 250 samples comprising whole poultry carcasses, cut-up poultry, eggs and minced meat, obtained from the Vienna market (about 20 retail shops) and from slaughterhouses were analyzed.

RESULTS

Investigation of the various selective and nutrient substances for their suitability in the IS assay yielded the following results.

Formulation of a selective tetrathionate, brilliant green and ox bile medium

In order to obtain preliminary information on the applicability of the TBG broth, it was used in its original composition. At an incubation temperature of 37°C, however, no significant changes of impedance were found during bacterial growth. Omission of calcium carbonate and increasing the nutrients by the addition of mannitol (5 g/L), lysine (5 g/L), and yeast extract (1 g/L) produced changes of up to 8% in the M-value and up to 50% in the E-value (Fig. 1). This medium was, however, found to be unsuitable for some kinds of salmonellae (serological variants of *S. panama, Salmonella senftenberg, Salmonella virchow*), which showed no growth, while *Enterobacter aerogenes* and *E. cloaceae* did grow and developed curves similar to those of salmonellae. The M and E reaction times (threshold values 5%) of various strains with an initial cell concentration of 10^5 CFU/ml were reached after 4 to 20 h of incubation.

Lowering the tetrathionate concentration from 20 to 10 g/L or less made it possible to detect further strains of salmonellae with increased impedance changes and reduced reaction times but considerably decreased the selectivity of the medium. Raising the tetrathionate concentration and incubation temperature from 37 to 42°C restricted the

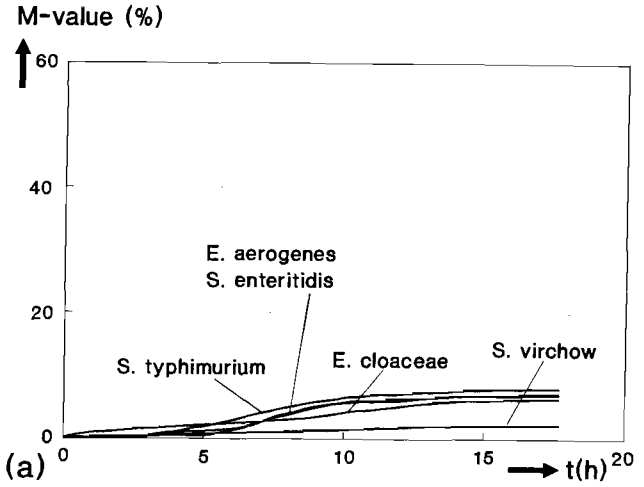


Figure 1a. *Recorded course of (a) M-values and (b) E-values during the growth of* E. cloaceae, E. aerogenes, S. enteritidis, S. typhimurium *and* S. virchow, *in TBG medium.*

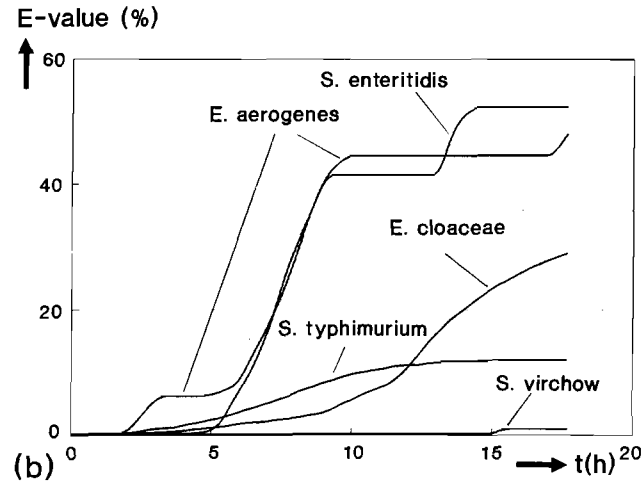


Figure 1b.

growth of competing microorganisms, but no new strains of salmonellae were detected.

SCT/D and SCT/M medium (4,9)

Next, the performance of the SCT/D medium (*4,9*) was tested. With measurement lasting up to 30 h, at an incubation temperature of 37°C and inoculation of the test strains of 10^3 CFU/ml, the M and E reaction times (threshold values 5%) varied between 9 h (*Salmonella agona*) and 27 h (*Salmonella saint-paul*). Some problems arose with the strains *Salmonella typhimurium* and *S. panama*, which produced no discriminating impedance changes (Fig. 2 and 3).

Replacing dulcitol (SCT/D) with mannitol (SCT/M) markedly increased impedance changes in M- and E-values (Fig. 3). However, a strain of *S. panama* was not detected. Investigations on the selectivity of the SCT/M medium showed that *Citrobacter freundii* precipitated changes in M and E-values of 15%, which could lead to false-positive results.

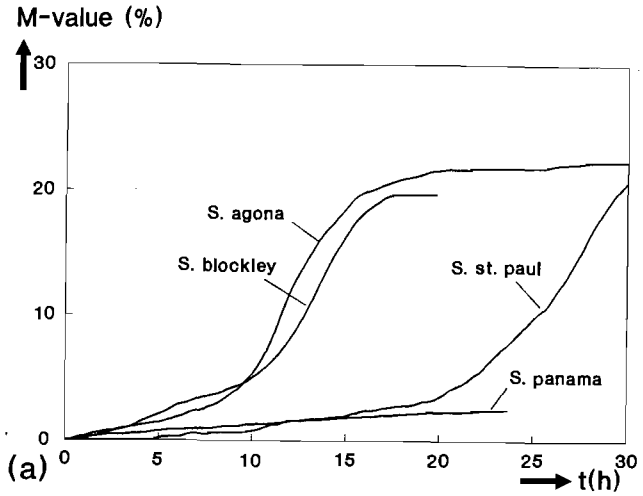


Figure 2a. *Recorded course of (a) M-values and (b) E-values during incubation of* S. agona, S. panama, S. saint-paul *and* S. blockley *in SCT/D medium.*

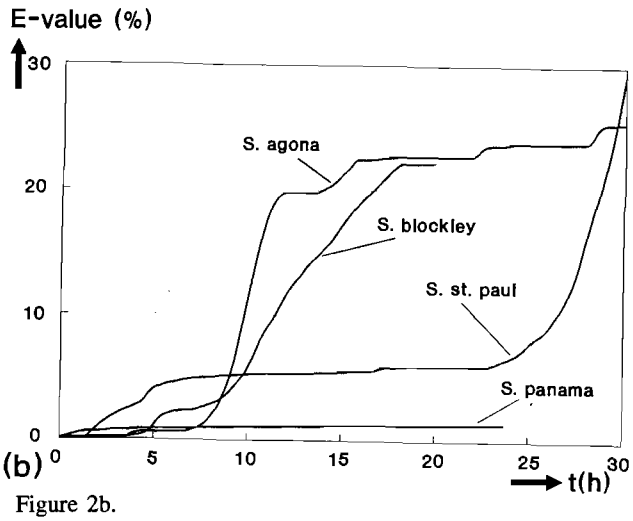


Figure 2b.

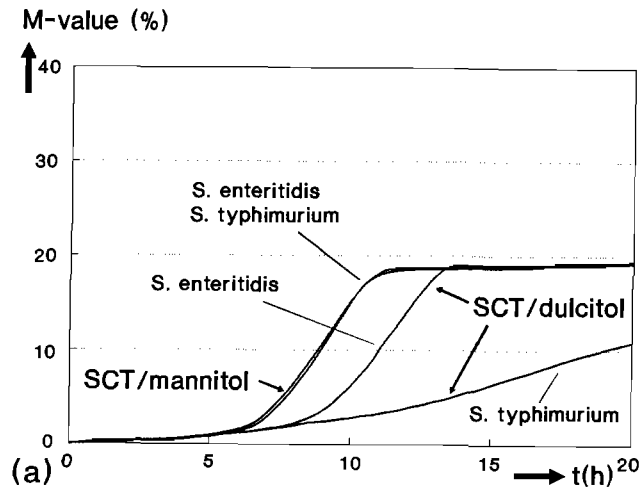


Figure 3a. *Recorded course of (a) M-values and (b) E-values during incubation of* S. enteritidis *and* S. typhimurium *in SCT/D and SCT/M media.*

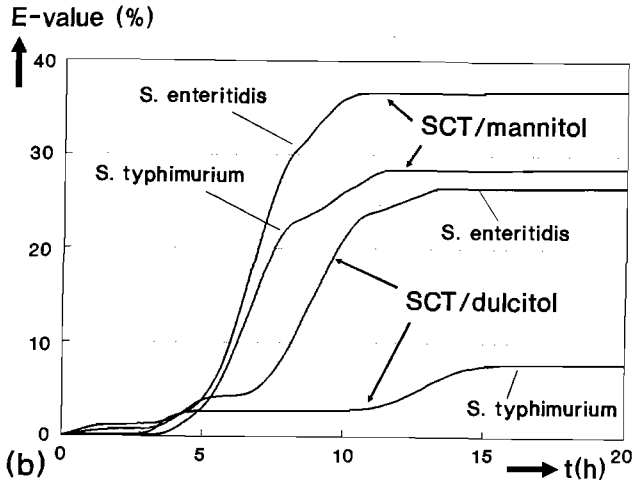


Figure 3b.

Magnesium chloride and malachite green as selective agents in ISS medium

The first applications of this selective substances for the ISS medium were conducted similar to the Rappaport Vassiliadis Salmonella (RVS) medium (*15,16*), in concentrations of up to 28 g/L magnesium chloride (hydrated), 0.04 g/L malachite green, and the novobiocin quantity, recommended by the manufacturer, 0.04 g/L. Peptone (5 g/L) was used as a nitrogen source, mannitol (5 g/L) as carbon source, and yeast extract (3 g/L) as a universal growth-promoting substance. The pH value was adjusted to 7.0. Use of phosphate buffers and NaCl prevented a marked decrease in medium pH due to the metabolism of mannitol, which gave rise to nonspecific impedance courses.

Using an incubation temperature of 37°C, changes in the E-value of up to 100% occurred as a result of the growth of salmonellae but only a few percentage points in the M-value. Testing of the medium with naturally contaminated food samples showed poor selectivity, however, making correction of the amount of selective substances necessary.

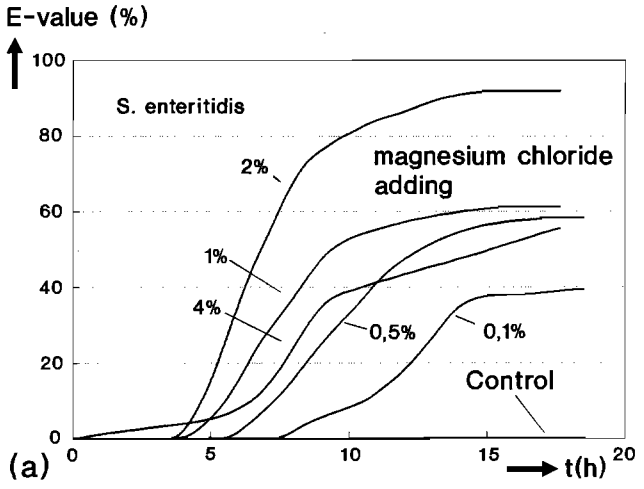


Figure 4a. *Effect of $MgCl_2$ concentration on the growth of (a)* S. enteritidis, *(b)* C. freundii *and (c)* E. cloaceae.

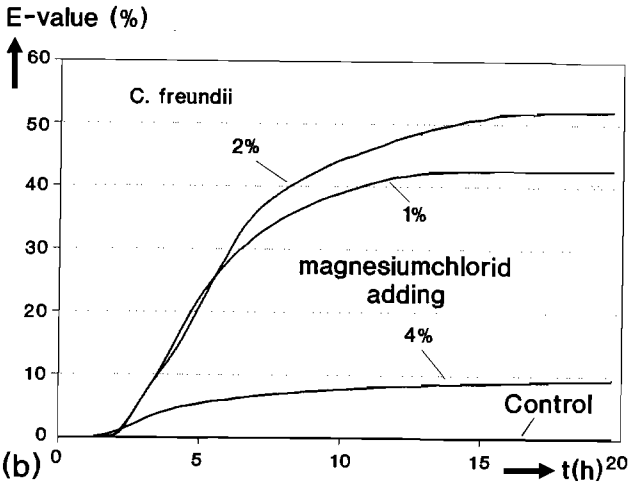


Figure 4b.

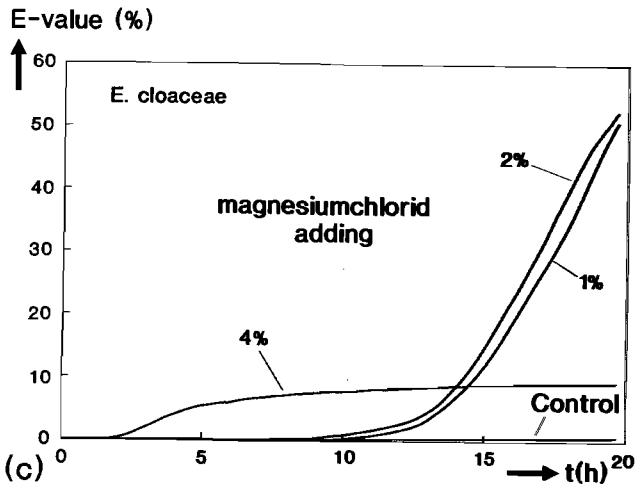


Figure 4c.

Variations in the amounts of selective agents without concurrent changes in the nutrient level of the medium demonstrated that when magnesium chloride was omitted, both salmonellae and competing organisms were inhibited by the action of malachite green oxalate, and consequently, no impedance changes occurred either (Fig. 4).

It was interesting that the addition of 1 g/L magnesium chloride led to reduction of the effect of malachite green oxalate and to sharp rises in the E-values (about 50%) both with salmonellae and competing organisms. A further increase in the magnesium chloride concentration produced inhibition of the competing microorganisms but not of the salmonellae (e.g., *Escherichia coli* was inhibited at a concentration of 20 g/L). In order to inhibit the growth of *C. freundii* (Fig. 4b) and *E. cloaceae* (Fig. 4c); the concentration of magnesium chloride had to be increased to 40 g/L. Additionally, with the *E. cloaceae*, the incubation temperature also had to be raised to 40°C. These provisions to inhibit the growth of competing microorganisms had negligible effects on the detection of salmonellae; only a transient increase of 0.6 h was found (Fig. 4a).

In Figures 4 to 6 only changes in the E-values are illustrated. Due to the high salt content of this medium, the ionic increase caused by metabolism is small compared with the ionic concentration already present in the medium, so that changes in M-values are very small and therefore not easily evaluated. Despite the high salt concentration, the E-value showed significant changes during the growth of salmonellae, and thus, allows for the effective use of inorganic salts in media formulation.

After the selective substance concentrations had been adjusted to optimum values, mannitol, peptone and yeast extract of the ISS medium were replaced by other growth-promoting substances. No better results were obtained with either lysine, dulcitol, sorbitol, tryptone, TMAO or combinations of these. When growth-promoting substances were included separately, it was shown that a combination of mannitol, peptone and yeast extract brought about maximum changes of the E-value in the range between 40 and 100% (Fig. 5). Detection of one strain of *S. panama* was found to be as difficult with this medium as with other media.

The optimum composition for 1 L of ISS medium is magnesium chloride (hydrated) (40 g/L), phosphate buffer (1 g/L), malachite green oxalate (0.04 g/L), mannitol

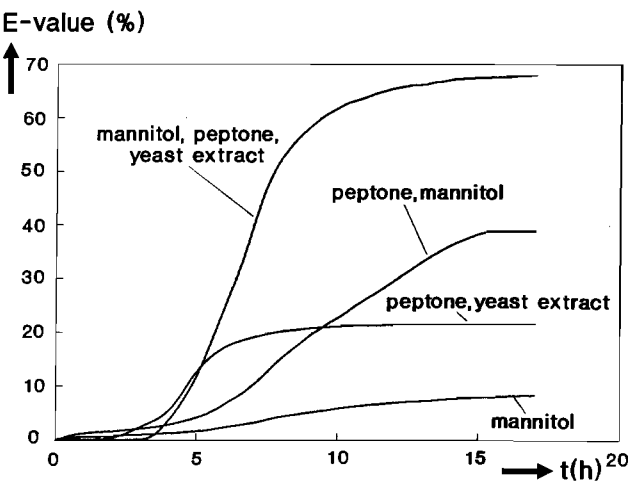


Figure 5. *Effect of various growth-promoting substances on the growth of* S. enteritidis.

(5 g/L), novobiocin sodium (0.04 g/L), peptone (5 g/L), NaCl (2 g/L) and yeast extract (3 g/L).

Reproducibility

The reproducibility of T_E of a number of salmonellae strains was investigated using five replicate determinations. With a threshold value of 15%, they were within 0.5 h (Fig. 6) and were achieved within 3 to 8 h at initial inoculation levels of 10^3 to 10^4 CFU/ml.

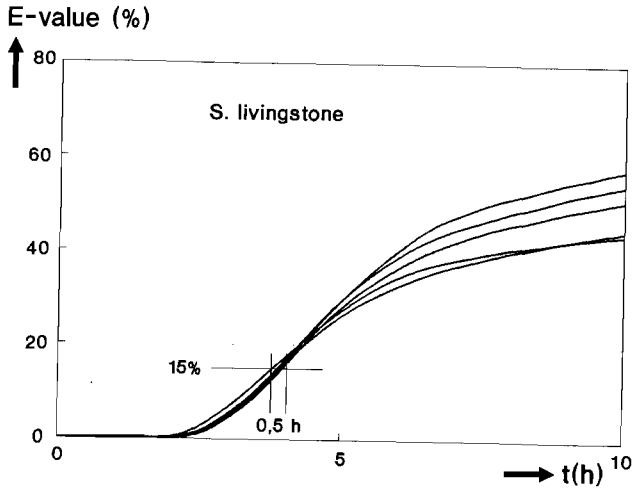


Figure 6. *Course of E-values (five-fold repetition) taken during the growth of* S. livingstone.

Effect of preenrichment on T_E

Preenrichment in BPW is specified in standard cultural procedures (*1*). The addition of mannitol and TMAO to the preenrichment medium is recommended by Easter and Gibson (*4*) because it provides for the more rapid detection of foodborne salmonellae. In this study it was investigated to what extent the addition of mannitol alone to BPW would influence the results, since, as already discussed (*4*), the use of TMAO is costly. Comparative studies (data not shown) indicated that the addition of mannitol to BPW exerted little effect on T_E but dramatically decreased the incidence of competing microorganisms on plating media.

Proposed procedure

In order to carry out an initial practical test of the developed ISS medium, rinsed samples of slaughtered poultry carcasses, containing coliforms or enterobacteriaceae in a population of about 10^5 CFU/ml rinsing fluid, were inoculated with salmonellae strains in populations of 10^0 to 10^4 CFU/300 ml of BPW preenrichment broth. These artificially contaminated carcass rinse samples produced, after preenrichment for 14 h and subsequent incubation in the measuring system at 40°C for 22 h, the E reaction times shown in Table 2.

The following suggested method is derived from the above investigations. It is henceforth described as the ISS method: a) preenrichment of the samples for 14 to 16 h at 37°C in BPW preenrichment broth; b) inoculate 0.1 ml preenrichment culture into 9.9 ml ISS medium; c) incubate samples in the measuring system BacTrac™ 4100 at 40°C for 22 h; d) positive sample if E > 15%; and e) if necessary, plating positive samples on BGA or XLD; serologically confirm suspect isolates.

TABLE 2. *E reaction times determined from rinsing fluid samples contaminated with various salmonellae strains.*

Salmonella strain	T_E for 10^4 CFU/300 ml (h)	T_E for 10^0 CFU/300 ml (h)
Salmonella enteritidis	6.0	14.0
S. blockley	5.5	12.0
S. dublin	7.0	20.0
S. typhimurium	8.0	20.0
S. heidelberg	5.5	12.0
S. livingstone	7.0	16.5

Comparison of the ISS method with cultural procedures

A comparison of the ISS method and standard cultural methods using 250 test samples showed that the highest percentage (48%) of salmonellae-positive findings could be obtained using the ISS method. In five samples assessed as positive by the ISS method, no salmonellae could be cultured from the ISS medium. Generally, the plating media (BGA, XLD) were overgrown with *E. cloaceae*. In one sample, salmonellae were isolated culturally from the ISS medium but could not be identified by the ISS method. This was a strain of *S. enteritidis*, PT8, which however, could also not be detected by conventional enrichment procedures (RV and RVS media) at an incubation temperature of 42°C. Testing standard cultural procedures the highest (42%) and the lowest (26%) salmonellae-positive samples were obtained with the SC broth and the TBG, respectively. The results for the 250 samples investigated are given in Table 3.

TABLE 3. *Results of analysis of 250 unknown samples for salmonellae using different methods and media.*

Methods/selective enrichment	No. of positive samples (%)	No. of false (+)	No. of false (–)
All methods combined	122 (48,8)	–	–
ISS method	119 (47,6)	6	9
ISS medium[a]	114 (45,6)	–	8
TBG broth	64 (25,6)	–	58
RV medium	92 (36,8)	–	22
RVS medium	95 (38,0)	–	25
SC medium	106 (42,4)	–	16

[a] Results of plating enrichment culture from the ISS method onto BGA and XLD.

DISCUSSION

Taking into account that the duration of the detection procedure for salmonellae should be shortened, methods based on gene hybridization, enzyme immunoassay and impedance techniques have recently been developed. In addition to reducing the investigation time to no more than

2 or 3 days, some methods have yielded a reduction in work and cost outlays (*2-4,13*). The ISS method which provides for the more rapid identification of negative samples remains a screening test whereas positive samples need to be confirmed culturally.

As with conventional procedures, rapid diagnostic tests require the preenrichment and selective enrichment of food samples. In some immunological and gene probe techniques, a postenrichment step in nonselective broth (e.g., GN broth) is necessary to increase numbers of salmonellae to the threshold level of sensitivity of individual test kits. The efficiency of these methods is essentially dependent on the growth of salmonellae to a detectable microorganism concentration (approximately 10^5 to 10^6 CFU/ml).

The impedance method does not require a postenrichment step because threshold levels of salmonellae are attained in the selective enrichment broths within ~20 h, with a concomitant impediometric detection of the target organism. The decisive factor for the applicability of impedance methods as a rapid procedure for the detection of salmonellae is the use of a salmonellae-selective medium, which allows growth of the microorganisms with simultaneous changes in impedance. In this study, a suitable medium was developed for the new IS method.

The use of selective broth media such as tetrathionate, brilliant green and ox bile in the impedance assay met with limited success because of the low medium selectivity (Fig. 1).

The addition of further selective substances, such as magnesium chloride, malachite green, selenite and cystine, led to considerably better results. Selenite and cystine had already been used by Easter and Gibson (*4*) in the SCT/D conductance medium for the Malthus system. In the present study, however, dulcitol-negative salmonellae strains were encountered, and, in addition, false-positive reactions were obtained with *C. freundii*. There were problems with the SCT/D medium with *S. panama* and *S. typhimurium*, which did not show the characteristic increase in M and E-values (Fig. 2 and 3). Of 50 poultry samples examined from one poultry slaughterhouse, 20 were contaminated with the *S. typhimurium* strain; these samples were not detected when this medium was used.

A modification of the SCT/D medium (exchanging dulcitol for mannitol SCT/M medium) resulted in shortening of T_E (e.g., *S. typhimurium*, Fig. 3). Testing this medium for its selectivity towards competing microorganisms showed that not only *C. freundii* but also *E. cloaceae* and *Klebsiella oxytoca* were not inhibited in their growth and thus gave false-positive results.

Because of the deficiencies of the above media, the selective substances of the RV medium (*15,16*) were tested for the ISS method. When including these substances, consideration was also given to the fact that there would be considerably fewer problems with regard to both laboratory work with these substances and their disposal, as compared to the toxic selenite media.

Magnesium chloride and malachite green as a selective basis for a medium to be used in the ISS method produced best results when combined with mannitol, peptone and yeast extract. Very good selectivity of the medium could be obtained with magnesium chloride concentrations of 40 g/L. With high salt content, however, the increased concentration of ionic species due to bacterial metabolism was slight compared to the nominal ionic content of the sterile broth medium, so that only slight and practically not detectable changes of media impedance (or conductance) were available. The growth of salmonellae could only be observed with this high salt concentration by using the E-values (Fig. 4a). Competing microorganisms, such as *C. freundii* or *E. cloaceae*, were interfering in the detection of salmonellae (Fig. 4). A threshold E-value of 15% was, therefore, chosen for the determination of T_E, in order to exclude false-positive results caused by *E. cloacea* and *Pseudomonas aeruginosa* which can produce slight changes in the E-values of about 5 to 10% (data not shown). With the growth of salmonellae, on the other hand, changes in the E- value are extremely marked (up to 100%), and the rises are very steep, so that there is only a slight increase of T_E despite the utilization of the 15% threshold value.

A modification of the standardized salmonellae preenrichment by adding mannitol to the peptone water had no particular effect on the recorded course of the curves. By adding mannitol, however, a selection advantage for salmonellae was found, expressed as a lower occurrence of competing organisms upon subculturing into selective broth media, or as increased occurrence of pure cultures.

A matter of particular concern in formulating the media was to obtain results permitting firm statements as regard to foodstuffs containing large and problematical quantities of competitive flora (*C. freundii, E. cloaceae, E. aerogenes, K. oxytoca, E. coli*), as is the case with raw products (e.g., slaughtered poultry, minced meat).

Information on the reliability of the ISS method and the developed ISS medium was provided by the examination of 250 food samples. Very good agreement was found between the ISS method and cultural detection from the ISS medium. In five cases the salmonellae-positive results by the ISS method could not be confirmed culturally from the ISS medium. Subculturing the ISS medium onto plating media revealed large populations of *E. cloaceae*.

One strain of *S. enteritidis* PT8 yielded a false-negative result by the ISS method and also could not be recovered from the ISS medium when plated on BGA/XLD agars. This strain, suggestively, was heat sensitive and failed to multiply sufficiently in the ISS medium incubated for 22 h at 42°C.

When the ISS method is employed for screening test, the small number of erroneous results encountered in the present study are quite acceptable. It must be noted that the number of positive findings of salmonellae recognized and confirmed by the ISS method is up to 12% higher than those found with the SC medium and up to 86% higher than with the TBG broth. In this connection, it may be permissible to point out that the ISS medium could also fundamentally be employed as a selective enrichment medium for the cultural detection of salmonellae at an incubation temperature of 40°C. This recommendation derives from the fact that when 250 food samples were examined with the help of this medium, significantly higher salmonellae-positive results were obtained as compared with those from the SC broth and the RV medium.

The employment of only one single selective medium for the detection of salmonellae is a general problem. Ogden (*10*) has already advised the use of two selective media in order to raise the safety level. It would be possible to use the SCT/D medium in addition to the developed ISS medium.

In summary, it is established that on the basis of the investigation results achieved, the ISS method is very suitable as a screening test, all the more since a negative investigation result will be shown within 38 h. In view of the practicability, this method is superior to the enzyme-immunological and gene probe procedures.

ACKNOWLEDGMENTS

The authors express their thanks to Professor E. Brandl (University of Veterinary Medicine, Institute of Milk Hygiene and Technology, Vienna) and to Professor H. Pfützner, (University of Technology, Institute of Fundamentals and Theory of Electrotechnics, Vienna) for reading the manuscript and for helpful discussions, as well as to the SY-LAB company for making available the BacTrac™ 4100 measuring system.

REFERENCES

1. Anonymous. 1990. General guidance on methods for the detection of salmonellae. Intl. Org. Standardization, Geneve, Switzerland, ISO 6579.
2. Bullock, R. D. and D. Frodsham. 1988. Rapid impedance detection of salmonellas in confectionery using modified LICNR broth. J. Appl. Bacteriol. 66:385-391.
3. D'Aoust, J. Y. and A. M. Sewell. 1986. Detection of salmonellae by the Enzyme Immunoassay (EIA) Technique. J. Food Sci. 51:484-507.
4. Easter, M. C. and D. M. Gibson. 1985. Rapid and automated detection of salmonellae by electrical measurement. J. Hyg. Camb. 94:245-262.
5. Emsweiler-Rose, B., W. D. Gehle, R. W. Johnston, A. Okrend, A. MoRan and B. Bennett. 1984. An enzyme immunoassay technique for the detection of salmonellae in meat and poultry products. J. Food Sci. 49:1010-1020.
6. Fitts, R. 1985. Development of DNA-DNA-hybridization test for the presence of salmonellae in foods. Food Technol. 39:95.
7. Flowers, R. S., M. A. Mozola, M. S. Curiale, D. A. Gabis and J. H. Silliker. 1987. Comparative study of a DNA hybridization method and the conventional culture procedure for detection of salmonellae in foods. J. Food Sci. 52:781-785.
8. Futschik, K., H. Pfützner, A. Doblander and H. Asperger. 1988. Automatical registration of microorganism growth by a new impedance method. Abstr. Int. Meet. Chem. Eng. & Biotechnol, Achema 88:3p.
9. Gibson, D. M. 1987. Some modification to the media for rapid automated detection of salmonellas by conductance measurement. J. Appl. Bacteriol. 63:299-304.
10. Odgen, I. D. 1988. A conductance medium to distinguish between salmonellae and Citrobacter spp. Int. J. Food Microbiol. 7:287-297.
11. Ogden, I. D. and D. C. Cann. 1987. A modified conductance medium for the detection of salmonellae spp. J. Appl. Bacteriol. 63:459-464.
12. Ogden, I. D. 1990. Salmonellae detection by a modified lysine conductance medium. Lett. Appl. Microbiol. 11:69-72.
13. Smith, P. J., A. Boardman and P. C. Shutt. 1989. Detection of salmonellas in animal feeds by electrical conductance. J. Appl. Bacteriol. 67:575-588.
14. Smith, P. J., F. J. Bolton, V. E. Gayner and A. Eccles. 1990. Improvements to a lysine medium for the detection of salmonellas by electrical conductance. Lett. Appl. Bacteriol. 11:84-86.
15. Vassiliadis, P. 1983. The Rappaport-Vassiliadis (RV) enrichment broth for the isolation of salmonellas: An overview. J. Appl. Bacteriol. 54:69-76.
16. Vassiliadis, P., V. Kalapothaki, C. Mavrommati and D. Trichopolus. 1984. A comparison of the original Rappaport medium (R-medium) and the Rappaport-Vassiliadis medium (RV-medium) in the isolation of salmonellae from meat products. J. Hyg. Camb. 93:51-58.

ELSEVIER

Journal of Microbiological Methods 44 (2001) 205–208

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

Rapid detection of *Bacillus stearothermophilus* using impedance-splitting

S.H. Flint [a,*], J.D. Brooks [b]

[a] *New Zealand Dairy Research Institute, Private Bag 11029, Palmerston North, New Zealand*
[b] *Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand*

Received 17 August 2000; received in revised form 21 December 2000; accepted 23 January 2001

Abstract

An impedance splitting method was used to detect *Bacillus stearothermophilus* in suspension and attached to stainless steel surfaces. The effects of bacterial metabolism on the impedance of the culture medium and the ionic layers of the measuring electrodes were recorded using the BacTrac™ 4000 microorganism growth analyser. Impedance changes were measured at 55°C. Seven of the eight media produced changes in the electrode impedance (*E*-value) and all media produced negligible changes in the impedance of the culture medium (*M*-value). Good correlations were obtained between cell numbers and the *E*-value measured over 18 h ($r > 0.9$) for the two strains of *B. stearothermophilus* tested in trypticase soy broth. The *E*-value correlations were used to estimate the numbers of both vegetative and spore forms of *B. stearothermophilus* as either planktonic or adhered cells. For the detection of *B. stearothermophilus* using impedance, only methods where the *E*-value impedance is recorded, can be used. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: *Bacillus stearothermophilus*; Impedance; Rapid methods

1. Introduction

Bacillus species are among the main spoilage organisms in food due to their versatile metabolism and heat-resistant spores (Deák and Temár, 1988) *Bacillus stearothermophilus* is a thermophilic *Bacillus* causing flat sour spoilage in low-acid foods (Ito, 1981). The spores of this organism are ubiquitous in nature; the organism is difficult to characterise in its growth, germination and sporulation requirements and is difficult to destroy with heat and chemicals (Ito, 1981). *B. stearothermophilus* represents about 33% of the thermophilic isolates in foods (Deák and Temár, 1988) and 64.7% of the isolates in some milk powders (Chopra and Mathur, 1984).

The growth of *B. stearothermophilus* on the surface of a milk powder manufacturing plant has been recorded (Stadhouders et al., 1982) and it is likely that the contamination of milk powders with thermophilic bacteria is due to the detachment of microorganisms from biofilms that have developed on stainless steel surfaces.

Detection of *B. stearothermophilus* normally relies on cultivation in agar or broth media producing results after 24–48 h of incubation at 55°C. Rapid methods for the detection of these bacteria would be

* Corresponding author. Tel.: +64-6-350-4649; fax: +64-6-356-1476.
E-mail address: steve.flint@nzdri.org.nz (S.H. Flint).

0167-7012/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0167-7012(01)00223-8

useful in making decisions on product quality. Impedance is a rapid and efficient method for quantifying bacteria and has been applied to the detection of biofilms (Flint et al., 1997). Once a medium has been selected or designed for the optimum detection of specific microorganisms (Brooks, 1986) this method can be used to rapidly determine the effects of disinfectants in controlling biofilms (Dhaliwal et al., 1992; Holah et al., 1990; Mosteller and Bishop, 1993; Johnston and Jones, 1995).

In this study, *B. stearothermophilus* was quantified using impedance. Different media were compared to select the optimum for detection and the impedance changes in the medium and on the electrode surface were compared. Calibration curves were prepared using serial 10-fold dilutions of isolates and these curves were used for the estimation of planktonic and attached cell densities.

2. Materials and methods

2.1. Cultures

B. stearothermophilus ATCC 7953 (reference strain) and B10 (from a milk powder manufacturing plant) were used to develop initial calibration data in this trial. Other strains (TP7, TP10, 5b) from dairy sources were also used to develop calibration curves for spores and vegetative cells and to test the growth of cells on stainless steel surfaces.

2.2. Culture media

The cultures used in this trial were grown in trypticase soy broth (TSB) (BBL, Becton Dickinson, Cockeysville, MD, USA). The impedance responses obtained in the following media were compared: TSB, TSB + 1% yeast extract, TSB + 1% NaCl (BDH, Palmerston North, New Zealand), Col-M (Flint et al., 1997), Brain heart infusion (BHI) (BBL), M17 (Difco, Fort Richard, Auckland, New Zealand), *Bacillus* Growth Medium (BGM) [11] and 10% reconstituted skim milk (RSM).

2.3. Preparation of spores

Spore suspensions were prepared by inoculating sporulation agar (Husmark and Rönner, 1992) and incubating at 55°C for 7 days. The colonies were washed from the plates using sterile deionised water, and the suspensions were treated (100°C for 10 min) to destroy any vegetative cells and then stored at −20°C.

2.4. Impedance measurement

Impedance was measured using the BacTrac™ 4000 microorganism growth analyser (SyLab, Purkersdorf-Vienna, Austria). This enabled electrical changes in the growth medium resulting from microbial metabolic processes to be measured (*M*-value). In addition, changes in the ionic layers on the electrode surfaces were measured (*E*-value).

2.5. Impedance calibration

Serial 10-fold dilutions of either strain ATCC 7953 or strain B10 were prepared in sterile deionised water. For each dilution, 0.1 ml was inoculated into duplicate tubes of the BacTrac™ 4000 containing 10 ml of TSB and incubated at 55°C for 18 h and the change in impedance was recorded. Agar plates of standard methods agar (BBL) were inoculated with 0.1 ml of each dilution, inverted, dried at 55°C for 10 min, and then placed in a plastic bag and incubated at 55°C for 18 h. The number of colonies was recorded and a calibration of colony counts against impedance detection time was prepared using the BacTrac™ software.

2.6. Determination of numbers of planktonic and adhered vegetative cells and spores

The total number of viable planktonic cells was determined by inoculating duplicate impedance tubes containing TSB with 0.1 ml inocula of fresh 12-h cultures of *B. stearothermophilus*. Estimates of the number of viable spores were made by inoculating duplicate tubes containing TSB with 0.1 ml inocula of a spore suspension heat treated (100°C for 10 min) before inoculation. Adhered cells were determined by inoculating tubes containing TSB with 1 cm^2 coupons of stainless steel that had been exposed to suspensions of *B. stearothermophilus* or had been colonised with a biofilm of *B. stearothermophilus* in a continuous flow reactor (Flint, 1998).

3. Results and discussion

Impedance splitting refers to the measurement of two impedance values—the *E*-value, which is often referred to as a capacitance change at the electrode surface, and the *M*-value, which is the change in conductivity in the growth medium. In all impedance tests on *B. stearothermophilus*, changes in the *E*-value but not the *M*-value could be related to the numbers of *B. stearothermophilus*. This may be explained by dipole molecules or uncharged substances, which do not contribute to conductivity, causing large changes in the electrode impedance by chemical adsorption (Pless et al., 1994). Estimates of cell numbers of *B. stearothermophilus* will be possible only with equipment that enables impedance splitting.

In seven of the eight media tested, *B. stearothermophilus* produced changes in the *E*-value but not the *M*-value. The *E*-value (capacitance) is most strongly affected by the presence of small ions and pH (Firstenberg-Eden and Eden, 1984). Therefore, media that allow bacterial growth to affect these two factors are likely to be the most sensitive. The time taken for an impedance change with different media varied (Table 1). The TSB medium with yeast extract produced the fastest change in impedance. However, a bimodal impedance change, which was difficult to relate accurately to cell numbers, occurred. This bimodal phenomenon may be analogous to the diauxie effect where two different components of the medium are metabolised. Media producing satisfactory changes in impedance were TSB + 1% NaCl, BGM, Col-M, RSM, TSB and BHI. No changes in impedance occurred in M17 medium. As Col-M and RSM produced a drift rather than a sharp *E*-value response, these media were regarded as being unsuitable for the development of calibration curves. The *E*-value response in BHI was considerably longer than for the other media; therefore, BHI was also regarded as being unsuitable for the development of calibration curves. TSB + 1% NaCl produced a sharp, rapid and reproducible change in the *E*-value. However, concern that different isolates may vary in their sensitivity to salt made this medium less desirable. TSB and BGM produced sharp and reproducible (although less rapid than for TSB + 1% NaCl) changes in the *E*-value. TSB was selected to develop calibration curves further.

Table 1
Detection times ($h \pm$ standard deviation) based on the time taken for an impedance change at a 5% threshold for *B. stearothermophilus* in eight different media

Media type	Detection times (h) for inocula of 1×10^6 cells of *B. stearothermophilus* ATCC 7953
TSB	3.85 ± 0.28
BHI	6.36 ± 0.69
M17	no detection
BGM	3.37 ± 0.27
RSM	5.36 ± 1.42
TSB + 1% yeast extract	0.25 ± 0.04
TSB + 1% NaCl	2.37 ± 0.12
Col-M	3.44 ± 0.08

Table 2
Calibrations for vegetative cells and spores of *B. stearothermophilus* based on correlations of impedance detection times with agar plate counts at 55°C

Strain	Vegetative cells	Spores
TP7	$-0.3949t + 7.1362$, $r = -0.9767$	$-0.4556t + 9.5292$, $r = -0.9993$
TP10	$-0.3501t + 6.5877$, $r = -0.9751$	$-0.3337t + 10.7692$, $r = -0.9998$
5b	$-0.4396t + 7.1970$, $r = -0.9907$	$-0.5127t + 10.7247$, $r = -0.9463$

Calibration curves for planktonic, vegetative cells of *B. stearothemophilus* strains B10 and ATCC 7953 produced reproducible correlations using a 5% threshold for changes in *E*-value readings. Regression equations for triplicate runs with each strain are given below.

Regression equations for strain B10:

$-0.6693t + 7.8033 \quad r = -0.9902$
$-0.7246t + 7.2390 \quad r = -0.9566$
$-0.6603t + 6.8054 \quad r = -0.9632$

Regression equations for strain ATCC 7953

$-0.7319t + 7.9708 \quad r = -0.9843$
$-0.7018t + 8.7862 \quad r = -0.9378$
$-0.7877t + 7.8629 \quad r = -0.9785$

Calibrations were also done for strains TP7, TP10 and 5b, with different calibration curves for the vegetative and spore forms of *B. stearothermophilus* (Table 2). This reflects the requirement for germination of spores prior to vegetative growth.

The BacTrac™ was used to estimate the numbers of bacteria (B10) on stainless steel coupons or growing in stainless steel tubing. These results showed good reproducibility on replicate tests, with an estimate of $1.0 \pm 0.6 \times 10^4/\text{cm}^2$ being a typical example. This method enables estimates of low numbers of cells ($< 10^4/\text{cm}^2$) and cells on the inner surfaces of tubes, which are difficult to estimate by any other means.

Impedance splitting enabled rapid (< 8 h for estimates of 100 vegetative cells or more) and reproducible estimates of *B. stearothermophilus* based on changes in the *E*-value. This method has potential to estimate the levels of this thermophilic *Bacillus* species in foods. Furthermore, the ability to detect viable cells on stainless steel coupons will enable investigations into the attachment and biofilm development of *B. stearothermophilus*. Although the calibration curves for different strains do vary, single strains colonise the stainless steel surfaces in a dairy manufacturing plant (Flint, 1999, unpublished data). This method has potential for monitoring thermophile growth in dairy manufacturing plant, with results obtained much sooner than the 48 h for standard agar plating methods (Frank et al., 1985).

4. Conclusion

Impedance splitting in trypicase soy broth is a reliable method to enumerate *B. stearothermophilus*.

References

Brooks, J.D., 1986. Impedance microbiology. Food Technol. Aust. 38, 338–340.

Chopra, A.K., Mathur, D.K., 1984. Isolation, screening and characterisation of thermophilic *Bacillus* species isolated from dairy products. J. Appl. Bacteriol. 57, 263–271.

Deák, T., Temár, É., 1988. Simplified identification of aerobic spore-formers in the investigation of foods. Int. J. Food Microbiol. 6, 115–125.

Dhaliwal, D.S., Cordier, J.L., Coz, L.J., 1992. Impedimetric evaluation of the efficiency of disinfectants against biofilms. Lett. Appl. Microbiol. 15, 221–271.

Firstenberg-Eden, R., Eden, G., 1984. What is impedance microbiology. Impedance Microbiology. Research Studies Press, Hertfordshire, pp. 7–22.

Flint, S.H., 1998. Formation and control of biofilms of thermo-resistant streptococci on stainless steel. PhD Thesis, Massey University, Palmerston North, New Zealand.

Flint, S.H., Brooks, J.D., Bremer, P.J., 1997. Use of the Malthus conductance growth analyser to monitor biofilms of thermophilic streptococococci on stainless steel. J. Appl. Microbiol. 83, 335–339.

Frank, J.F., Hankin, L., Koburger, J.A., Marth, E.H., 1985. Tests for groups of microorganisms. Standard Methods for the Examination of Dairy Products. American Public Health Association, Washington, DC, pp. 189–202.

Holah, J.T., Higgs, C., Robinson, S., Worthington, D., Spencer, H.A., 1990. A conductance-based surface disinfection test for food hygiene. Lett. Appl. Microbiol. 11, 255–259.

Husmark, U., Rönner, U., 1992. The influence of hydrophobic electrostatic and morphological properties on the adhesion of *Bacillus* spores. Biofouling 5, 335–344.

Ito, K.A., 1981. Thermophilic organisms in food spoilage: flat-sour aerobes. J. Food Prot. 44, 157–163.

Johnston, M.D., Jones, M.V., 1995. Disinfection tests with intact biofilms: combined use of the Modified Robbins Device with impedance detection. J. Microbiol. Methods 21, 15–26.

Mosteller, T.M., Bishop, J.R., 1993. Sanitizer efficacy against attached bacteria in a milk biofilm. J. Food Prot. 56, 34–41.

Pless, J., Futschik, K., Schopf, E., 1994. Rapid detection of Salmonellae by means of a new impedance-splitting method. J. Food Prot. 57, 369–376.

Stadhouders, J., Hup, G., Hassing, F., 1982. The conceptions index and indicator organisms discussed on the basis of the bacteriology of spray-dried milk powder. Neth. Milk Dairy J. 36, 231–260.

Journal of Applied Microbiology 2004, **96,** 110–116 doi:10.1046/j.1365-2672.2003.02136.x

Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel

S.G. Parkar[1], S.H. Flint[2] and J.D. Brooks[1]

[1]Institute of Food, Nutrition and Human Health, Massey University, Palmerston North, New Zealand, and [2]Fonterra Research Centre, Palmerston North, New Zealand

2003/0371: received 5 May 2003, revised 15 June 2003 and accepted 26 August 2003

ABSTRACT

S.G. PARKAR, S.H. FLINT AND J.D. BROOKS. 2003.

Aims: To determine the mechanism for both the removal and inactivation of 18-h biofilms of a thermophilic *Bacillus* species that optimally grows at 55°C on stainless steel.

Methods and Results: The cleaning strategies tested were based on biofilm biochemistry and physiology, and focused on the chemistry of the cleaners, the duration and temperature of the cleaning process and a combination of various cleaners. The success of the cleaning regimes was determined based on the removal of cells and organic debris and the elimination of viable cells. The results confirmed that a caustic (75°C for 30 min) and acid (75°C for 30 min) wash, relied upon heavily in most food processing industries for cleaning-in-place systems, was successful in removing these biofilms. However, any changes in the concentrations of these cleaners or the temperature of cleaning drastically affected the overall outcome. Alternative cleaning agents based on enzymatic or nonenzymatic breakdown of cellular proteins or polysaccharides, surfactant action, use of oxidative attack and free radicals varied in degrees of their success. Combining proteolytic action with surfactants increased wetability and therefore enhanced the cleaning efficiency.

Conclusions: Several procedures, including caustic/acid and enzyme based cleaners, will be satisfactory, provided that the correct process parameters are observed i.e. concentration, time, temperature and kinetic energy (flow). Confirmation of these results should be carried out in a pilot plant through several use/clean cycles.

Significance and Impact of the Study: Confidence in standard and alternative cleaning procedures for food manufacturing plant to prevent contamination with thermophilic bacilli that threaten product quality.

Keywords: biofilm, clean in place, cleaning, milk, stainless steel, thermobacilli.

INTRODUCTION

Bacteria form biofilms on the surfaces of stainless steel equipment in food processing industries, releasing bacteria that compromise the safety and quality of the final product. Other unfavourable conditions associated with biofilms that affect food manufacturers include reduced flow through blocked tubes, reduced plant run times, corrosion of stainless steel, and reduced heat transfer through plate heat exchangers. Cleaning and disinfection have been incorporated into the cleaning-in-place (CIP) regimes in food manufacturing industries (Romney 1990; Zottola and Sasahara 1994). However, bacterial contamination and product spoilage because of biofilm formation are recurring problems (Carpentier *et al.* 1998). Cleaning and disinfection studies have focused on eliminating food-borne pathogens (e.g. *Listeria*) and have neglected other contaminating organisms (e.g. thermophilic bacilli) that threaten food quality.

Present address: S.G. Parkar, HortResearch, Private Bag 3123, Hamilton, New Zealand (e-mail: sgparkar@hortresearch.co.nz).
Correspondence to: J.D. Brooks, Institute of Food, Nutrition and Human Health, Massey University, Private Bag 11222, Palmerston North, New Zealand (e-mail: j.d.brooks@massey.ac.nz).

© 2003 The Society for Applied Microbiology

Bacterial biofilms may be defined as cells adhering to, and growing on, artificial substrates and are often associated with an extracellular polysaccharide matrix. The very nature of their structure and formation provides greater resistance to cleaning and disinfection, compared with planktonic cells (Joseph *et al.* 2001). Many agents have traditionally been used for CIP (Romney 1990) to allow cleaning to within acceptable levels.

It is difficult to find references in the literature about effective cleaning systems for sporeforming bacteria in food manufacturing plant. Studies on biofilms in dairy manufacturing plant have focused on *Pseudomonas* species and food borne pathogens (Dunsmore 1980; Frank and Koffi 1990; Zottola and Sasahara 1994). There are a few publications dealing with sporeforming bacteria (Husmark and Rönner 1992) and only limited information on thermophilic sporeforming bacilli as biofilms in dairy manufacturing plants (Parkar *et al.* 2001).

We have chosen to use the vegetative form of a thermophilic bacillus for our study because spores have not been observed within the biofilms of these organisms (Parkar *et al.* 2001). Any residual bacteria left after a cleaning cycle can potentially sporulate and therefore continue to proliferate and cause problems within the food manufacturing system. Here, we have compared for the first time, various cleaning strategies to control the formation of biofilms of sporeforming thermophilic bacilli (e.g. *Bacillus flavothermus)*.

Cleaners were selected for this study on the basis of their mechanisms of action as follows:

- **i** Alkaline cleaning, causing dissolution of cell material and removal of calcium deposits e.g. standard caustic soda and acid, and Eliminator.
- **ii** Enzyme based cleaning:
  - **a** Protease cleaners such as Paradigm which is an enzyme based cleaner combining proteolytic activity with surfactant action, and Purafect®, a serine protease.
  - **b** Polysaccharidase based agents such as Purastar™, an α-amylase, Cellulase^{L}, a polysaccharidase and mutanolysin, a cell wall peptidoglycan hydrolase.
- **iii** Oxidizing chemicals such as sodium hypochlorite, Halamid (a chloramine), Oxine® (chlorine dioxide) and Perform® (combination of peracetic acid and hydrogen peroxide). These are all general pro-oxidants generating reactive oxygen and/or chlorine species. They initiate a cascade of oxidative attack on bacterial -SH group containing moieties such as enzymes, and peroxidative attack on membrane unsaturated fatty acids (Estrela *et al.* 2002).
- **iv** A quaternary ammonium chloride, such as Bactosolve®, that causes breakdown of cell membrane followed by intracellular potassium efflux and finally cellular protein/nucleic acid damage (Romney 1990).
- **v** Detergents, such as Tween-80 (a synthetic polysorbate) and dobanic acid, have surfactant effects; they enhance access of other cleaners to the biofilms by increasing their wetability.

On the basis of these treatments this paper aims to: (i) validate and establish the correct process parameters to achieve good cleaning practices in standard commercial procedures within a laboratory scale system, (ii) estimate the effect and efficiency of other agents that target cleaning in a defined biochemical mode of action within a laboratory scale system and (iii) examine the efficiency of some of the most effective cleaning procedures in a pilot industrial scale system.

MATERIALS AND METHODS

Sources of bacterial strains and cleaning chemicals

Bacillus flavothermus (B12-C^{m}), originally isolated from a milk powder manufacturing plant, was grown in trypticase soya broth (TSB) (BBL, Becton Dickinson, Cockeysville, MD, USA) at 55°C for 18 h, from stocks stored on Microbank cryobeads (Pro-Lab Diagnostics, Austin, TX, USA) at −20°C at the Biofilm Research Unit at the Institute of Food, Nutrition and Human Health, Massey University.

Paradigm (proteolytic enzyme cleaner) was obtained from Ecolab, Hamilton, New Zealand. Eliminator (caustic based cleaner), Perform® (hydrogen peroxide/peracetic acid) and Bactosolve® (a quarternary ammonium compound) were obtained from Orica Chemnet, Mt Maunganui, New Zealand.

Purafect® (a subtilisin), Purastar™ HPAmL (an α-amylase), and Cellulase^{L} (Genencor International, Rochester, NY, USA) were supplied by Enzyme Services, Auckland, New Zealand. Oxine® was a gift from Biocide Pacific Ltd, Auckland, New Zealand. Mutanolysin was obtained from Sigma-Aldrich, Auckland, New Zealand.

Dobanic acid (a linear alkylbenzene sulphonic acid), was obtained from Chemcolour Industries NZ Ltd, Auckland, New Zealand. Tween-80 and lecithin were obtained from BDH, Palmerston North, New Zealand. All other chemicals were of analytical grade and obtained locally, unless otherwise mentioned.

Biofilm substrate

Cold rolled stainless steel, grade 316 with 2B surface finish as supplied for dairy manufacturing plant construction, was cut into coupons (1 cm × 1 cm × 1 mm) (Parkar *et al.* 2001). The coupons were then inserted into the laboratory biofilm reactor (Flint *et al.* 2001) and these were used to grow biofilms for all laboratory studies. An alternative

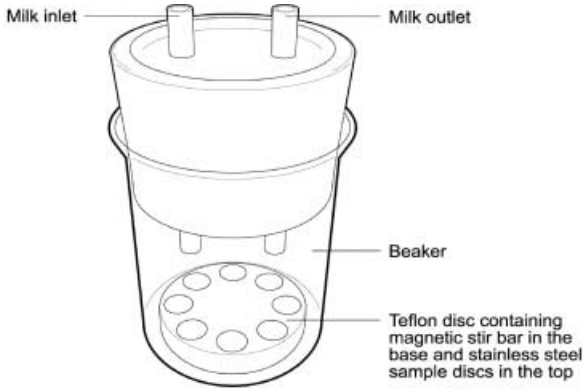


Fig. 1 Diagram of the annular reactor used for biofilm growth on stainless steel coupons (Reservoir is a 500-ml glass beaker operating with a volume of 200 ml. The sample discs are represented by the small circles in the large Teflon disc. This system was placed on a heated magnetic stirrer controlled at a temperature of 55°C)

'annular' reactor (Fig. 1) was used to grow biofilms on stainless steel discs of 1 cm diameter and 1 mm thick (designed to fit in the pilot plant), of the same grade and finish and this was used to prepare surfaces for pilot plant trials.

Biofilm generation

For the first phase of work, i.e. screening of a variety of cleaning regimes, a laboratory coupon reactor was used in the same setup as described previously (Flint *et al.* 2001). Cleaned and sterile stainless steel coupons, inserted into clean silicone tubing, were the substrates for biofilm formation. The entire system was cleaned and autoclaved before use. The planktonic cultures used to inoculate biofilm reactors were obtained from a 6-h culture of the cells in TSB grown, with agitation, at 55°C. To inoculate the substrates in the biofilm reactors, a 6-h culture, containing *ca* 8 $\log_{10}$ cells ml^{-1} was centrifuged at 3000 *g* for 10 min, and the cell pellet was washed and resuspended in sterile distilled water. These cells were used to initiate biofilm formation in the reactors by incubating them at ambient temperature with sterile stainless steel coupons for 30 min followed by five rinses with sterile distilled water.

There were two milk flows in the reactors. Pasteurized skim milk at 55°C was recirculated past the inoculated coupons in the reactor at 140 ml min^{-1} for 18 h to generate biofilms. Fresh milk was continuously added to the system (working volume 225 ml) at 4·7 ml min^{-1} and the surplus overflowed to drain. This dilution rate exceeded the growth rate of the bacteria, and avoided an increase in numbers of planktonic cells. The coupons with biofilms attached to them were aseptically cut from the tubing and rinsed five times with sterile distilled water prior to examination for biofilms.

For the second phase of work, an annular rotating disc reactor was used. In the annular reactor, eight stainless steel discs were fitted into a Teflon holder containing a bar magnet in the base. This assembly was placed in a 400 ml beaker on a magnetic stirrer. The assembly was fitted with a rubber bung that could take in two silicone tubes. One tube allowed the milk to flow in and the other tube, which had a baffle dipping into the milk to cause turbulence, passed the milk to the waste sink from the reactor (Fig. 1). The setup was autoclaved and the stainless steel discs were inoculated with washed *B. flavothermus* ($B12\text{-}C^{m}$) cells as already described. The milk flow rate was 4·7 ± 1 ml min^{-1}, determined from the doubling rate of the bacteria to avoid a net increase in the numbers of bacteria in the planktonic culture.

Cleaning regimes – laboratory trials

Once the 18-h mature biofilms were generated on the stainless steel coupons in the biofilm reactor, sterile distilled water was passed through the reactor tubing to rinse off residual milk or loosely attached cells. The coupons were aseptically removed by cutting the silicone tubing and were transferred to sterile 15-ml tubes. The coupons were subjected to cleaning by adding 2 ml of the different cleaning agents under specified conditions (Table 1) and agitated on a vortex mixer for 5 s and then

Table 1 Cleaning procedures used

1. 2% NaOH at 75°C, 30 min followed by 15 min distilled water rinse at ambient temperature and 1·8% HNO_3 at 75°C, 30 min
2. Eliminator (sodium hydroxide + sodium metasilicate) 2% at 75°C, 20 min
3. Paradigm (0·08% P2010 and 0·09% P2030) at 60°C, 30 min
4. Purafect®, 0·12 subtilisin U ml^{-1} at 37°C for 30 min
5. Purastar™ α-amylase, 0·05 amylase U kg^{-1} at 37°C for 30 min
6. $Cellulase^{L}$, 1·5 cellulase U ml^{-1} at 37°C for 30 min
7. Mutanolysin, 1 U ml^{-1} at 37°C for 1 h
8. Sodium hypochlorite 500 ppm 5 min, ambient temperature
9. Halamid (chloramine) 0·3% 5 min, ambient temperature
10. Oxine® (chlorine dioxide) 500 ppm 5 min, ambient temperature
11. Perform® (Peracetic acid/hydrogen peroxide) 0·2%, 5 min, ambient temperature
12. Quarternary ammonium chloride (Bactosolve®) 25 ppm, 30 min, ambient temperature
13. Tween-80, 6% w/v followed by wash with lecithin–Tween-80 (3%) (Eginton *et al.* 1998)
14. Dobanic acid HFP (Hydrogen Fluoride Process) 0·1%, 5 min, ambient temperature

The cleaning regime for these cleaners was based on the literature/manual from the respective companies, or chosen by us. The control coupons were rinsed five times with sterile distilled water at 22°C and used for total count or viable count.

rinsed five times with sterile distilled water at 22°C. In the control treatment, coupons were rinsed five times with sterile distilled water at 22°C and used for total count or viable count. The cleaning efficiency was assessed in terms of numbers of viable cells remaining by impedance measurement using the Bactrac 4100™ (Sy–Lab Geräte GmbH, Purkersdorf, Austria) micro-organism growth analyser (Flint and Brooks 2001) and in terms of the total cell count by using epifluorescence microscopy (Parkar *et al.* 2001). The presence of polysaccharide matrix was noted microscopically as fluorescent material of the acridine-orange stained coupons. It was expressed as percentage of total field by arbitrarily dividing the field into a grid format, and counting the squares with debris vs total number of squares.

After the initial stage, when a number of cleaners were tested individually as mentioned in Table 2, different combinations of agents, temperatures and cleaning treatments were tried as given in Table 3. Again, the cleaning efficiency was estimated in terms of viability and the total number of cells left behind.

Control preparations

Biofilms on stainless steel surfaces all reached levels of 6–7 $\log_{10}$ cells cm^{-2} and were totally covered with biofilm.

Cleaning regimes – pilot scale trials

The chemical cleaning combinations that gave the most promising results in the laboratory experiments were run on a pilot plant. Duplicate discs containing biofilm were used for each cleaning treatment viz. full strength caustic and acid cleaning at 75°C (see details later), Paradigm treatment and Perform® with 0·1% dobanic acid at 60°C. The remaining two discs served as controls. For the pilot scale trials, the biofilms were grown in the annular reactor and then transferred to a modified Robbins device (MRD). This MRD, containing eight sample ports, was designed at the Fonterra Research Centre and built at a local engineering company from grade 316 stainless steel. Biofilms were not grown in the MRD in the pilot scale cleaning rig as a large quantity of milk would have been required to grow the

Table 2 Effect of different cleaning agents on the viability of biofilms of the thermophilic bacillus *Bacillus flavothermus* (B12-C^m) (control coupon $7{\cdot}0 \times 10^7$ cells cm^{-2}) in the laboratory scale reactor

Treatment*	Total cell reduction ($\log_{10}$ reduction)†	Viable cell kill ($\log_{10}$ reduction)‡	Residue§ remaining after cleaning (0 = 0%; 1 = 1–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%
1. Alkali/acid 75°C, 30 min	7	7	0
2. Alkali/acid 60°C, 30 min	5	7	0
3. Alkali/acid 50°C, 30 min	5	7	0
4. Half strength alkali/acid 60°C, 30 min	5	7	0
5. Half strength alkali/acid 50°C, 30 min	5	5·5	0
6. Eliminator, 75°C, 20 min	2	6	1
7. Paradigm, 2010 + 2030, 60°C, 30 min	7	7	0
8. Paradigm, 2030 then 2010, 60°C, 30 min each	7	7	0
9. Paradigm, 2010 then 2030, 60°C, 30 min each	7	7	1
10. Purafect®, 0·5%, 37°C, 30 min	5	3·6	1
11. Purastar™ 0·5%, 37°C, 30 min	5	6·6	1
12. Cellulase^L, 0·3%, 37°C, 30 min	5	5·8	1
13. Mutanolysin,1 U ml^{-1}, 37°C, 1 h	4	4·3	1
14. Sodium hypochlorite, 22°C, 5 min	1	5·5	2
15. Halamid, 22°C, 5 min	2	6·8	1
16. Oxine® 22°C, 5 min	1	7	2
17. Perform® 22°C, 5 min	7	7	0
18. Bactosolve®, 22°C, 30 min	2	7	2
19. Tween-80, 6% w/v, 22°C, 5 min	0	1	4
20. Dobanic acid, 22°C, 5 min	1	4	2

*Details in materials and methods and Table 1.

†Based on epifluorescence microscopy.

‡Based on impedance microbiology.

§Polysaccharide material observed using epifluorescence microscopy.

Results based on duplicate test; mean ± SD (SD about 0·2 $\log_{10}$ cells cm^{-2}).

Table 3 The effect of alternative cleaning conditions on the inactivation of biofilms of the thermophilic bacillus *Bacillus flavothermus* (B12-C^m) in the laboratory scale reactor, based on viable cell count, using Bactrac analyser

Treatment	Time (min)	Temperature (°C)	Viable cell kill (log_{10} reduction)
1. 100 ppm Oxine®	15	22	6
2. 100 ppm Oxine®	60	22	7
3. 250 ppm Oxine®	15	60	7·8
4. 250 ppm Oxine® + 1% Dobanic acid	15	22	6·8
5. 100 ppm Oxine® + 1% Dobanic acid	15	22	6·7
6. 100 ppm Oxine® + 1% Dobanic acid	15	60	7·5
7. 0·1% Dobanic acid/100 ppm Oxine®	15	22	7
8. 100 ppm Oxine® + 0·1% Dobanic acid	15	37	8
9. 100 ppm Oxine® + 0·1% Dobanic acid	15	45	8
10. 0·2% Perform®	15	22	8
11. 0·4% Perform®	15	22	8
12. 0·1% Dobanic acid/0·2% Perform®	15	22	8
13. 0·2% Perform® + 0·1% Dobanic acid	15	22	8
14. 0·4% Perform® + 0·1% Dobanic acid	15	37	8
15. 0·2% Perform® + 0·1% Dobanic acid	15	45	8
16. 0·2% Perform® + 0·1% Dobanic acid	15	60	8

biofilms. The pilot-scale cleaning rig allowed the effect of the cleaning chemicals to be determined under turbulent flow conditions (maximum pump rate of 200 kg h^{-1}) ($Re > 2000$), similar to those in a dairy manufacturing plant (Romney 1990).

Statistical analysis

All the studies were performed at least in duplicates. Viable counts obtained from the Bactrac 4100™ cell analyser were averages of duplicate counts; and total counts obtained by cell epiflurescent microscopy were averages of five counts. Results have been expressed as mean values, the standard deviation given as required.

RESULTS

Caustic and acid cleaning involved treating the biofilm coupons with 2% NaOH at 75°C for 30 min, followed by a sterile distilled water rinse and then treating with 1·8% HNO_3 at 75°C for 30 min followed by another rinse with distilled water. This was the most effective of all the caustic and acid treatments used to kill and remove biofilms. This is referred to as full strength caustic acid at 75°C (fsca 75) in our further studies. This killed all the cells in the biofilm (Table 2) and removed practically all the cells and polysaccharide matrix from the stainless steel. Using the same concentrations, or half strengths, of alkali and acid for the same time but at lower temperatures of 60 and 50°C, the cleaning efficacy was reduced. For full strength caustic acid at both 50 and 60°C (fsca 50, fsca 60) and half strength caustic acid treatment at 60°C (hsca 60) there was a 6–7 log_{10} cells cm^{-2} reduction (100% loss) in viability but only a 2 log_{10} cells cm^{-2} reduction in total cells detected by epifluorescence microscopy. The half strength caustic acid treatment at 50°C (hsca 50) resulted in only a 5·5 log_{10} cells cm^{-2} decrease in viable cells with a total cell reduction of 2 log_{10} cells cm^{-2}. Polysaccharide matrix was not detected when the cleaning cycles were carried out at 75 and 60°C, but small amounts (about 1·5%) were detected with fsca 50, and larger amounts (about 8%) were detected with hsca 50. The commercial caustic cleaner Eliminator (2%, 75°C, 20 min) reduced cell viability in the B12-C^m biofilm by 6 log_{10} cells cm^{-2} and the total cell count by *ca* 2 log_{10} cells cm^{-2} (Table 1). Fluorescent polysaccharide remnants were also observed on the coupons.

Paradigm, a commercial proteolytic enzyme cleaner consisting of two components, was used at 0·08% P2010 (enzyme/surfactant) combined with 0·09% P2030 (alkali/chelants) at 60°C for 30 min and was successful in cleaning biofilm from the test coupons in the laboratory trials. Paradigm was also used in the following combinations (0·08% P2010 followed by 0·09% P2030, and 0·09% P2030 followed by 0·08% P2010) at the same temperature with 30 min treatments for each component, resulting in a total 1 h cleaning time. When P2030 and P2010 were used simultaneously, there was a 7 log_{10} cells cm^{-2} reduction in total and viable cells and no visible polysaccharide. When P2030 was followed by P2010, there was again a 7 log_{10} cells cm^{-2} reduction in viable biofilm cells and no visible cells or polysaccharide. However, when P2010 was followed by P2030, there was a 7 log_{10} cells cm^{-2} reduction in viable biofilm cells, but residual cell material (about 11%) was seen under epifluorescence microscopy.

Under the given conditions, Purafect®, Purastar™, $Cellulase^L$ and mutanolysin did not achieve 100% killing

of the biofilms (Table 1). The viability was decreased by 3·6, 6·6, 5·8 and 4·3 $\log_{10}$ cells cm^{-2}, respectively compared with the original biofilm. The total cell count was decreased by 5, 5, 5, and 4 $\log_{10}$ cells cm^{-2}, respectively, and residual polysaccharide was seen as well.

Of the oxygen based agents, Oxine® and Perform® caused a 7 $\log_{10}$ cells cm^{-2} loss of viability of the biofilms, followed by Halamid (6·8 $\log_{10}$ cells cm^{-2} reduction) and sodium hypochlorite (5·5 $\log_{10}$ cells cm^{-2} reduction) (Table 2). However, in terms of removing cells from the surface, Perform® was the most effective causing 100% removal of cells and attached polysaccharides. Halamid caused a 2 $\log_{10}$ cells cm^{-2} total cell reduction followed by Oxine® and sodium hypochlorite, each causing a 1 $\log_{10}$ cells cm^{-2} reduction of total cells. Halamid also removed more of the polysaccharide (0·5% polysaccharide seen) than did Oxine® and sodium hypochlorite. As the latter two had a high number of cells still attached to surface, it was difficult to estimate the polysaccharide content but one can state that the cells were obviously still attached by the polysaccharide glue.

Bactosolve® produced a 7 $\log_{10}$ cells cm^{-2} reduction in viability although only a 2 $\log_{10}$ cells cm^{-2} reduction in total cells on the stainless steel. Tween-80 was least effective of all treatments tried resulting in only a 1 $\log_{10}$ cells cm^{-2} reduction in viable cells and negligible detachment of total cells, while dobanic acid caused more cell kill (4 $\log_{10}$ cells cm^{-2} reduction in viability) and cell removal (1 $\log_{10}$ cells cm^{-2} reduction) and at least 25% of the polysaccharides were removed.

All the Oxine® combinations caused a >6 $\log_{10}$ cells cm^{-2} decrease in viability (Table 3). However, microscopic examination of the coupons showed no significant removal of the cells or breakdown of the polysaccharide matrix. The Perform® combinations were 100% successful in killing the biofilms (Table 2), although microscopic observations showed that the best clean was obtained with a combination of 0·2% Perform® with 0·1% dobanic acid at 60°C.

Three different treatments (fsca 75, Paradigm® and 0·2% Perform® with 0·1% dobanic acid) selected from successful laboratory trials were tested in the pilot plant cleaning rig. The treatments resulted in no viable cells remaining on the substrate and the complete removal of the biofilm. However, fluorescent material (about 20%) was seen along the striations (part of the surface topography) of the stainless steel disc in all three trials.

DISCUSSION

Cleaning with caustic (2% NaOH) and acid (1·8% HNO_3) at 75°C for 30 min was confirmed as a reliable cleaning protocol for stainless steel coupons with confluent 18-h biofilms of *B. flavothermus* ($B12\text{-}C^m$). Caustic and acid cleaning has been the standard method used in many food processing industries (Chisti 1999). However, this is the first report detailing the effect of a standard cleaning system on sporeforming thermophilic bacteria, and the effect of changes in concentrations or temperature on biofilm viability, detachment of cells and polysaccharide removal.

Our cleaning trials prove that it is very important to use the right concentrations of agents and the recommended temperatures. A decrease in the strengths of the agents killed the cells but failed to remove all the cells from the surface. Marshall (1994) suggests that this residue may serve as a locus for attachment of more organic remnants or bacteria, resulting in faster biofilm formation and product spoilage in subsequent runs. Polysaccharide dissolution and removal using alkali in our trials needed temperatures higher than 60°C, as some exopolysaccharide was observed after cleaning with full strengths of alkali and acid at 50°C. Temperature is a critical factor in cleaning, with many effects increasing linearly with temperature (Gibson *et al.* 1999). Eliminator, even at 75°C, was not successful in completely removing cell matter. A longer cleaning or a higher concentration of the agent may be required.

When Paradigm was used according to the manufacturers' instructions at 60°C, no viable cells or cell debris were left behind on the stainless steel. Using the alkali component to dissolve proteins and polysaccharides before proteinase action was more effective than using the proteinase before the alkali. The enzyme preparations, Purafect®, Purastar™ and Cellulase^L and mutanolysin (a polysaccharidase that breaks the glycosidic bonds in the bacterial peptidoglycan network) were not completely effective. Possible explanations may be low wetability, and the fact that these enzyme cleaners only target one part of the biofilm. The addition of surfactants or a combination of these agents may result in better cleaning.

Although kinetic energy (flow) was not specifically examined as a factor in cleaning for these trials the pilot plant cleaning rig, that operates under turbulent flow, ensured that the most promising treatments were tested under conditions of flow reflecting that seen in a manufacturing plant.

In cleaning industrial plants, even a large decrease in the percentage of cells killed and removed may be insufficient for optimum running of the manufacturing plant, as the remaining cell debris may act as an organic 'conditioning film' aiding the attachment of micro-organisms in subsequent runs (Marshall 1994).

Many factors need to be considered when judging the cleaning efficacy of various agents. Irreversible attachment of cells to a cleaned surface occurs within 30 min after exposure to a fresh suspension of cells of *B. flavothermus* ($B12\text{-}C^m$). Hence, the aim of the CIP procedure should be to achieve total cleaning in the shortest possible time, to

maximize the availability of the manufacturing plant for production. The final outcome of a cleaning procedure should be judged both by the number of cells remaining viable after a treatment and by checking for the presence of any cell residue on the cleaned surface.

The work in this trial examined the effect of different cleaners on the vegetative form of *B. flavothermus*. Although spores of this organism are a concern, the vegetative forms predominate in the manufacturing plant and are the source of spores. The origin of spores in the final product is under study.

Several procedures, including caustic/acid and enzyme based cleaners, will be satisfactory, provided that the correct process parameters are observed i.e. concentration, time, temperature and kinetic energy. Confirmation of these results should be carried out in a pilot plant through several use/clean cycles.

ACKNOWLEDGEMENTS

Tuan Truong, Fonterra Research Centre, for help with the pilot scale rig. Michael Dunne for the generous gifts of Purastar™, Purafect® and Cellulase^{L}. Graeme Ogg, from Biocide International, for the generous gifts of Oxine and dobanic acid. Dave Lowry, from Ecolab, for the supply of Paradigm. Jeff Old, from Orica Chemnet, for the supply of the other cleaning chemicals. Tim Coolbear and Kylie Walker, from the Fonterra Research Centre for proof reading this script.

REFERENCES

Carpentier, B., Wong, A.C.L. and Cerf, O. (1998) Biofilms on dairy plant surface. *Bulletin of International Dairy Federation* **329**, 32–35.

Chisti, Y. (1999) Modern systems of plant cleaning. In *Encyclopedia of Food Microbiology* ed. Robinson, R., Batt, C. and Patel, P. pp. 1086–1815. London: Academic Press.

Dunsmore, D.G. (1980) Bacteriological control of food equipment surfaces by cleaning systems. I. Detergent effects. *Journal of Food Protection* **44**, 100–108.

Eginton, P.J., Holar, J., Allison, D.G., Handley, P.S. and Gilbert, P. (1998) Changes in the strength of attachment of micro-organisms to surfaces following treatment with disinfectants and cleansing agents. *Letters in Applied Microbiology* **27**, 101–105.

Estrela, C.R.A., Barbin, E.L., Espano, J.C., Marchesan, M.A. and Pecora, J.D. (2002) Mechanism of action of sodium hypochlorite. *Brazilian Dental Journal* **13**, 113–117.

Flint, S.H. and Brooks, J.D. (2001) Rapid detection of *Bacillus stearothermophilus* using impedance-splitting. *Journal of Microbiological Methods* **44**, 205–208.

Flint, S., Palmer, J., Bloemen, K., Brooks, J. and Crawford, R. (2001) The growth of *Bacillus stearothermophilus* on stainless steel. *Journal of Applied Microbiology* **90**, 151–157.

Frank, J.F. and Koffi, R.A. (1990) Surface-adherent growth of *Listeria monocytogenes* is associated with increased resistance to surfactant sanitizers and heat. *Journal of Food Protection* **53**, 550–554.

Gibson, H., Taylor, J.H., Hall, K.E. and Holah, J.T. (1999) Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. *Journal of Applied Microbiology* **87**, 41–48.

Husmark, U. and Rönner, U. (1992) The influence of hydrophobic electrostatic and morphological properties on the adhesion of *Bacillus* spores. *Biofouling* **5**, 335–344.

Joseph, B., Otta, S.K. and Karunasagar, I. (2001) Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *International Journal of Food Microbiology* **64**, 367–372.

Marshall, K. (1994) Microbial adhesion in biotechnological processes. *Current Opinion in Biotechnology* **5**, 296–301.

Parkar, S.G., Flint, S.H., Palmer, J.S. and Brooks, J.D. (2001) Factors influencing attachment of thermophilic bacilli to stainless steel. *Journal of Applied Microbiology* **90**, 901–908.

Romney, A.D.J. (1990) *CIP: Cleaning in Place*. Cambridgeshire: Society of Dairy Technology.

Zottola, E.A. and Sasahara, K.C. (1994) Microbial biofilms in the food processing industry – should they be a concern? *International Journal of Food Microbiology* **23**, 125–148.

Journal of Applied Microbiology 2004, **96,** 894–902 doi:10.1111/j.1365-2672.2004.02218.x

Calibration of the impedance method for rapid quantitative estimation of *Escherichia coli* in live marine bivalve molluscs

J. Dupont[1], F. Dumont[1], C. Menanteau[1] and M. Pommepuy[2]

[1]Laboratoire National de Référence 'Microbiologie des coquillages', and [2]Département Microbiologie et Phycotoxines, Ifremer, Direction de l'Environnement et de l'Aménagement Littoral, Nantes, France

2003/0777: received 3 September 2003, revised 21 December 2003 and accepted 22 December 2003

ABSTRACT

J. DUPONT, F. DUMONT, C. MENANTEAU AND M. POMMEPUY. 2004.

Aim: Calibration of impedance measurement was performed vs the Association Françoise de Normalisation (AFNOR) MPN method with a view to rapid enumeration of *Escherichia coli* in live marine bivalve molluscs.

Methods and Results: Linear regression models between $\log_{10}$ MPN and detection time (DT) were adjusted for several shellfish types, growth media, and impedance instruments (BacTrac and Malthus systems). *Escherichia coli* concentrations could be estimated from DT using a single regression line for BacTrac 4100 with M1 medium ($R^2 = 87{\cdot}8\%$) and Malthus with M2 medium ($R^2 = 86{\cdot}7\%$), and two regression lines for BacTrac 4110 with M2 medium ($R^2 = 86{\cdot}4$ and $88{\cdot}2\%$). The uncertainty of the predicted bacterial concentration was around $\pm 0{\cdot}43$ log unit for duplicate sample analysis. The impedance signal was attributable to *E. coli* in 99% of cases. All cultures containing *E. coli* produced an impedance signal with BacTrac 4100 and BacTrac 4110, whereas 5·6% did not exhibit a signal with Malthus.

Conclusions: Impedance measurement is a possible alternative to the MPN method for rapid quantitative estimation of *E. coli* in live bivalve shellfish.

Significance and Impact of the Study: The impedance method reduces analysis handling time considerably and is much easier to use than the MPN method. Moreover, results can be obtained within 5–10 h, allowing rapid intervention to ensure public health protection in case of shellfish contamination.

Keywords: calibration, *E. coli*, estimation, impedance, shellfish.

INTRODUCTION

The microbiological classification of harvesting areas for live bivalve molluscs in the European Union is based on the number of *Escherichia coli* in 100 g of shellfish meat and liquor, as specified in Directive 91/492/EEC (Anon. 1991). A five-tube, three-dilution most probable number (MPN) method is recommended for enumeration of these faecal organisms, but any other method of equivalent accuracy may be used. An Association Française de Normalisation (AFNOR) MPN method (Anon. 2000a), derived from the ISO 7251 standard, is usually used in France for *E. coli* counts in shellfish. However, this method is time-consuming and labour-intensive, and results are obtained only after 3 days.

Various studies have shown that conductance or impedance measurement can be used to detect or enumerate micro-organisms in water (Silverman and Munoz 1979; Noble *et al.* 1991; Colquhoun *et al.* 1995) and food products (Martins and Selby 1980; Firstenberg-Eden 1983; Firstenberg-Eden and Klein 1983; Firstenberg-Eden *et al.* 1984; Gibson *et al.* 1984; Weihe *et al.* 1984; Ogden 1986; Nieuwenhof and Hoolwerf 1987; Cousins and Marlatt 1990; Piton and Rongvaux-Gaïda 1990; Dalgaard *et al.* 1996). In particular, the impedance technique allows rapid quantitative estimation of *E. coli* in live shellfish (Dupont *et al.* 1994, 1996) without the drawbacks of the MPN method.

Correspondence to: J. Dupont, Ifremer DEL/MP/LNR, BP 21105, 44311 Nantes Cedex 03, France (e-mail: jdupon@ifremer.fr).

© 2004 The Society for Applied Microbiology

Impedance measurement has recently been recognized formally in France and Germany as a suitable technology for microbiological examination of food products, and two general standards have been published (Anon. 1999, 2000b). This method is based on the principle that bacteria actively growing in a culture medium produce positively or negatively charged end-products that cause an impedance variation of the medium. This variation, which is proportional to the change in the number of bacteria in the culture, makes it possible to measure bacterial growth (Richards *et al.* 1978). The time at which growth is first detected, referred to as detection time (DT), is inversely proportional to the log number of bacteria in the sample, which means that bacterial counts can be predicted from DT. A calibration process is required initially to establish a mathematical relation experimentally between DT and the log number of bacteria.

Specific application of the impedance technique for enumeration of *E. coli* in live shellfish has been standardized by AFNOR (Anon. 2002), and the calibration process has been clearly defined. Calibration is based on bacteriological examination of shellfish samples in parallel with the impedance method described in the standard and the MPN reference method (Anon. 2000a), followed by statistical analysis of the results. Calibration is carried out for each type of shellfish and on at least 50 samples. The set of data pairs allows coverage of a balanced range of *E. coli* concentrations of 4 log units (e.g. from 10^2 to 10^5 *E. coli* per 100 g), which is representative of the shellfish contamination levels likely to be found in the marine environment. Artificially contaminated samples may be used if it is not possible to collect a sufficient number of naturally contaminated samples to cover the necessary bacterial concentration range. A linear regression model is adjusted between DT and *E. coli* concentration, and the specificity of impedance response with regard to *E. coli* must also be checked.

As indicated above, this type of calibration has already been performed for quantitative estimation of *E. coli* in shellfish using the Malthus AT analyser (Malthus Instruments, Bury, UK) with 100-ml measuring cells (Dupont *et al.* 1996). However, several changes have occurred since then: (i) the reference MPN method (Anon. 1988) against which the impedance method was calibrated has been modified (Anon. 2000a), (ii) a study involving commercially available impedance instruments (Dupont, J., Dumont, F., Menanteau, C., unpublished data) has shown that BacTrac analysers operating with 100 or 10-ml measuring cells can produce impedance curves from shellfish samples of as good quality as those of the Malthus 100-ml system and (iii) modifications of the coliform broth previously used (especially an increase in tryptone, lactose and/or NaCl content) can improve impedance response in some cases.

The purpose of the present study was to perform new calibrations for rapid, routine quantitative estimations of *E. coli* in live bivalve molluscs according to the updated AFNOR standardized procedures. Linear regression models were adjusted for several types of shellfish, growth media and impedance instruments. Comparisons were made for the regression lines of the different types of shellfish analysed and for the characteristics of the impedance signals generated by the different media and analysers used.

MATERIALS AND METHODS

Media

Tryptone salt water (TSW; Biokar Diagnostics, Beauvais, France) was used for sample preparation and dilutions; Malthus coliform broth (MCB; Malthus Instruments), Tryptone USP (Biokar Diagnostics) and lactose (Merck, Darmstadt, Germany) for *E. coli* detection by impedance measurement; lauryl sulphate broth (LS; Biokar Diagnostics) for enumeration of *E. coli* in shellfish by the MPN method; *Escherichia coli* (EC) broth (EC; Merck, Darmstadt, Germany) and peptone water (PW; Biokar Diagnostics) for enumeration of *E. coli* in shellfish by the MPN method and confirmation of *E. coli* growth in impedance-measuring cells and TBX agar (Oxoid, Basingstoke, UK; Bio-Rad, Marnes-la-Coquette, France) for confirmation of *E. coli* growth in impedance-measuring cells.

Shellfish samples

Samples of oysters (*Crassostrea gigas*), mussels (*Mytilus edulis*) and cockles (*Cerastoderma edule*) were collected over an 18-month period in more or less contaminated growing areas or natural beds along French coasts. Approximately one-fourth of the samples were artificially contaminated by placing shellfish in tanks containing ambient static seawater, to which were added varying doses of waste water taken in sewage treatment plants.

Sample preparation

Samples were prepared according to the NF V 08-106 standard (Anon. 2002). For each sample, animal shells were scrubbed with a stiff bristle brush under running cold drinkable tap water. Depending on the shellfish type, between 5 and 30 animals were opened using a sterile shucking knife. Approximately 100 g of meat and shell liquor (MSL) diluted 1 : 3 with TSW were homogenized in a Waring blender (Dynamics Corp., New Hartford, CO, USA) for 1 min at 15 000 rev min^{-1}. The homogenate was allowed to stand for 15–30 min at room temperature and then further diluted with TSW to obtain a 1 : 10 suspension before inoculation into culture media.

Escherichia coli counts

Enumeration of *E. coli* in shellfish was performed by the AFNOR standardized five-tube MPN method (Anon. 2000a): 10, 1 and 1 ml of consecutive decimal dilutions of the 1 : 10 MSL suspension were inoculated into each of five tubes containing 10 ml of double- or single-strength LS broth and incubated at 37°C for 48 h. The contents of each tube showing gas formation or opacity obscuring possible gas formation was subcultured into a tube containing 10 ml of single-strength EC broth and another containing 10 ml of PW and then incubated in a water bath at 44°C for 24 h. Gas formation in EC tubes and indole production in PW tubes confirmed the presence of *E. coli*. According to the number of tubes yielding *E. coli* at each dilution, the MPN of bacteria per 100 g of MSL was calculated from MPN tables (De Man 1983). Each sample was analysed in duplicate.

Impedance measurements

Impedance measurements were performed on Malthus System V (Malthus Instruments), and BacTrac 4100 and BacTrac 4110 (Sy-Lab, Neupurkersdorf, Austria) microbiological analysers. The following detection parameters were used: baseline 1·5 μS, first difference 3·5 μS, second difference 0·2 μS for the Malthus analyser; delay 2 h (Bac Trac 4100) and 3 h (BacTrac 4110), drift balance 1 h, M-value threshold 1% for the BacTrac analysers. Five millilitres of the MSL 1 : 10 suspension were inoculated in duplicate into 10-ml measuring cells containing 5 ml of double-strength selective medium for BacTrac 4100 and 10 ml into 100-ml measuring cells containing 90 ml of single-strength selective medium for Malthus and BacTrac 4110. M1 (MCB), M2 [MCB + tryptone (1 g l^{-1}) + lactose monohydrate (4·7 g l^{-1})], M3 [M1 + NaCl (4·0g l^{-1})] and M4 [M2 + NaCl (4·0 g l^{-1})] media were tested on Malthus and BacTrac 4100, and M2 and M4 media on BacTrac 4110. Seeded cells were incubated at 44°C for at least 20 h and scanned every 6 and 10 min for Malthus and BacTrac analysers, respectively. DT and total impedance change were recorded for each measuring cell (for BacTrac 4100 and BacTrac 4110, the DT indicated include initial recording delays of 2 and 3 h, respectively).

Relationship between *E. coli* counts and DT

Statistical analysis was applied only to samples which gave (i) two DT (DT_1 and DT_2) differing by not more than 1 h and corresponding to measuring cells in which the presence of *E. coli* had been confirmed and (ii) two MPN estimations (N_1 and N_2) belonging to category 1 or 2 (Anon. 2000a) and differing by not more than 1 log unit. For each sample, the arithmetic mean of DT_1 and DT_2 (DT), and the arithmetic mean of $\log_{10}N_1$ and $\log_{10}N_2$ ($\log_{10}N$) were calculated. The data pairs (DT, $\log_{10}N$) were subjected to linear regression analysis for each type of shellfish, growth medium and analyser, using Statgraphics Plus software, Version 5.0 (Magnugistics, Inc., Rockville, MD, USA). Regression lines for $\log_{10}N$ (Y) vs DT (X) were compared for the different types of shellfish.

Specificity of the impedance response

After measurements were stopped, measuring cells were subcultured in EC broth and PW tubes, as well as on TBX agar plates, at 44°C for 24 h. After incubation, tubes were examined for gas and indole production, and plates for the presence of greenish-blue colonies indicative of β-glucuronidase activity in order to confirm the presence or not of *E. coli* in cells. Bacterial identification using the API 20E system (BioMérieux, Marcy l'Etoile, France) was performed in case of discordance between the results of gas/indole and β-glucuronidase tests.

RESULTS

Relationship between *E. coli* counts and DTs

Regressions. One hundred and eighty-one to 200 exploitable data pairs were obtained from naturally and experimentally contaminated shellfish samples, depending on the analyser and growth medium used (67 to 77 for oysters, 55 to 62 for mussels and 56 to 66 for cockles). The contamination level ranged from $1{\cdot}7 \times 10^2$ to $6{\cdot}0 \times 10^5$ *E. coli* per 100 g of MSL for oysters and mussels, and from $2{\cdot}5 \times 10^2$ to $4{\cdot}5 \times 10^5$ for cockles. On the whole, the number of samples was evenly distributed over the entire *E. coli* concentration range. A linear regression model was fitted for each type of shellfish between the decimal logarithms of MPN estimations and DT for the different media and impedance systems. For each regression, the number of data pairs, the regression coefficients (slope and intercept), the standard error of the predicted value, the determination coefficient (percentage of variance explained by the model), and the result of the lack-of-fit test (verification of the linearity of the regression model) are shown in Tables 1, 2 and 3 for Malthus, BacTrac 4100 and BacTrac 4110 analysers, respectively. The regression line equation is $Y = a + bX$, where Y is the $\log_{10}$ of the MPN estimation of *E. coli* in 100g of MSL, X the DT (expressed in h), and a and b the intercept and slope, respectively.

Given the F-values calculated for each regression and the corresponding degrees of freedom, the regression tests were highly significant ($P < 10^{-4}$). In general, the lack-of-fit test was not significant, confirming that the linear model was

Table 1 Results for linear regressions between *Escherichia coli* concentrations ($\log_{10}$ MPN 100 g^{-1} of meat and shell liquor) and detection times with the Malthus analyser

Shellfish type	Growth medium	Number of data pairs	Intercept	Slope	S.E. of estimation	R^2 (%)	Lack-of-fit test
Oysters	M1	69	9·806	−0·700	0·392	76·8	NS
	M2	75	10·203	−0·823	0·338	85·2	NS
	M3	70	10·073	−0·812	0·406	77·1	NS
	M4	77	10·296	−0·854	0·355	83·4	NS
Mussels	M1	57	9·674	−0·709	0·375	80·7	NS
	M2	59	10·193	−0·832	0·309	88·2	NS
	M3	58	10·148	−0·840	0·369	85·1	NS
	M4	58	9·732	−0·782	0·370	85·1	NS
Cockles	M1	56	10·037	−0·766	0·381	80·8	NS
	M2	66	10·062	−0·821	0·313	88·1	NS
	M3	61	10·048	−0·841	0·373	82·1	NS
	M4	64	9·525	−0·768	0·362	84·2	S

S.E., standard error; R^2, determination coefficient; S, significant ($P < 0·05$); NS, not significant ($P \geq 0·05$).

Table 2 Results for linear regressions between *Escherichi. coli* concentrations ($\log_{10}$ MPN 100 g^{-1} of meat and shell liquor) and detection times with the BacTrac 4100 analyser

Shellfish type	Growth medium	Number of data pairs	Intercept	Slope	S.E. of estimation	R^2 (%)	Lack-of-fit test
Oysters	M1	69	10·541	−0·996	0·276	88·9	NS
	M2	71	10·273	−0·955	0·317	86·4	NS
	M3	72	10·458	−0·954	0·392	80·5	NS
	M4	69	10·120	−0·886	0·353	82·5	S
Mussels	M1	62	10·488	−0·986	0·297	88·9	NS
	M2	59	8·862	−0·717	0·382	82·6	NS
	M3	60	10·672	−0·993	0·368	81·7	NS
	M4	58	9·593	−0·793	0·330	85·4	NS
Cockles	M1	60	10·402	−0·991	0·318	86·5	NS
	M2	61	10·253	−0·948	0·344	84·0	NS
	M3	59	10·171	−0·914	0·376	81·1	NS
	M4	59	11·050	−1·027	0·348	84·0	NS

S.E., standard error; R^2, determination coefficient; S, significant ($P < 0·05$); NS, not significant ($P \geq 0·05$).

Table 3 Results for linear regressions between *Escherichia coli* concentrations ($\log_{10}$ MPN 100 g^{-1} of meat and shell liquor) and detection times with the BacTrac 4110 analyser

Shellfish type	Growth medium	Number of data pairs	Intercept	Slope	S.E. of estimation	R^2 (%)	Lack-of-fit test
Oysters	M2	67	10·535	−0·840	0·320	86·4	NS
	M4	71	10·760	−0·882	0·352	84·2	NS
Mussels	M2	55	10·817	−0·891	0·317	90·0	NS
	M4	55	10·749	−0·888	0·336	87·1	S
Cockles	M2	59	10·515	−0·857	0·264	90·0	NS
	M4	63	10·590	−0·881	0·337	86·6	NS

S.E., standard error; R^2, determination coefficient; S, significant ($P < 0·05$); NS, not significant ($P \geq 0·05$).

adequate. The exceptions (all with M4 medium) concern oysters (using BacTrac 4100), mussels (using BacTrac 4110) and cockles (using Malthus). Thus, the overall results for regression analyses showed that *E. coli* concentrations could be estimated from DT. The highest determination coefficient and lowest standard error of estimation were observed

Table 4 Differences between regression line intercepts and slopes for the three types of shellfish (oysters, mussels, cockles) according to the growth medium and the impedance analyser

Growth medium	Malthus analyser		BacTrac4100 analyser		BacTrac 4110 analyser	
	Intercept	Slope	Intercept	Slope	Intercept	Slope
M1	S	NS	NS	NS	–	–
M2	NS	NS	S	S	S	NS
M3	S	NS	NS	NS	–	–
M4	NS	NS	S	S	S	NS

S, significant ($P < 0{\cdot}05$); NS, not significant ($P \geq 0{\cdot}05$); –, medium not used.

with M1 medium for BacTrac 4100, and with M2 medium for Malthus and BacTrac 4110, regardless of the shellfish type.

Comparison of regression lines. The comparison of regression lines for the three types of shellfish indicated a significant difference between slopes and/or intercepts for Malthus with M1 and M3 media, and BacTrac 4100 and BacTrac 4110 with M2 and M4 media, respectively (Table 4). Conversely, no significant difference was found between slopes and intercepts for Malthus with M2 and M4 media, and BacTrac 4100 with M1 and M3 media. For BacTrac 4110 with M2 medium, no significant difference was found for slopes and intercepts between mussel and cockle regressions. The characteristics of regressions for all shellfish types for Malthus with M2 medium and BacTrac 4100 with M1 medium, and for mussel and cockle samples combined for BacTrac 4110 with M2 medium, are reported in Table 5.

Determination of E. coli concentrations. To measure *E. coli* concentrations on an arithmetic scale from the regression line equation, $Y = a + bX$, it is not sufficient simply to calculate the antilogarithm of the Y estimation. As such a procedure can lead to underestimation of the measurement, a bias correction factor needs to be applied (Beauchamp and Olson 1973). For the sample sizes considered here, the estimation of the *E. coli* concentration is expressed as $10^{(a+bX+\hat{s}^2_{yx}/2)}$, where $\hat{s}^2_{yx}$ is the unbiased estimation of the variance of Y, whose value is given in Tables 1–3 and 5 (standard error of estimation). Estimation accuracy for the 95% confidence limits (*ca* $2\hat{s}_{yx}$) ranges from 0·53 to 0·81 logarithmic unit as a function of the regressions.

Determination of maximum DT. Maximum DT is defined as the DT whose corresponding *E. coli* concentration is equal to the theoretical detection limit of the impedance method, which depends on the amount of MSL inoculated into the measuring cells. Maximum DT (Table 7) is calculated from the regression line equation considered, taking into account the bias correction factor. It is expressed as $DT_{max} = [a + \hat{s}^2_{yx}/2 - \log_{10}(EC)_{TDL}]/b$, where $(EC)_{TDL}$ is the theoretical detection limit of the impedance method for *E. coli*. Under the conditions used here, the value for the detection limit is $1{\cdot}0 \times 10^2$ *E. coli* per 100 g of MSL for Malthus and BacTrac 4110 and $2{\cdot}0 \times 10^2$ *E. coli* per 100 g of MSL for BacTrac 4100.

Characteristics of impedance response

Total change in impedance. A study of the 431 to 441 impedance curves corresponding to positive *E. coli* measuring cells obtained for each growth medium from the 240 samples analysed (all shellfish combined) showed that the total change in impedance (ΔI) depended on the medium used (Table 6). For Malthus and BacTrac 4100, the lowest mean impedance variation was observed with M3 medium (122 μS and 3·4%, respectively), whereas the highest was observed with M2 medium (244 μS and 6·5%, respectively). For BacTrac 4110, mean impedance variation was higher with M2 medium (9·8%) than with M4 medium (6·2%). The rather high coefficients of variation (16·5–18·5%) observed for the Malthus analyser were due to the presence of a small number (from 3 to 10 depending on the growth medium) of extreme ΔI values. In other respects, no notable differences in mean total impedance change were observed between shellfish types.

Detection time. DT for a given *E. coli* concentration varied according to the analyser and growth medium used. Mean

Table 5 Results for linear regressions between *Escherichia coli* concentrations ($\log_{10}$ MPN 100 g^{-1} of meat and shell liquor) and detection times for shellfish types combined

Analyser	Growth medium	Shellfish type	Number of data pairs	Intercept	Slope	S.E. of estimation	R^2 (%)	Lack-of-fit test
Malthus	M2	All	200	10·126	−0·821	0·323	86·7	NS
BacTrac 4100	M1	All	191	10·431	−0·984	0·298	87·8	NS
BacTrac 4110	M2	Mussels + cockles	114	10·659	−0·873	0·290	89·9	NS

S.E., standard error; R^2, determination coefficient; S, significant ($P < 0{\cdot}05$); NS, not significant ($P \geq 0{\cdot}05$).

Table 6 Total change in impedance for the three analysers and the four growth media used, all shellfish combined

Growth medium	Conductance change (μS)			Impedance change (%)					
	Malthus			BacTrac 4100			BacTrac 4110		
	Mean	Mini	CV	Mean	Mini	CV	Mean	Mini	CV
M1	145	52	16·5	4·6	3·0	8·5	–	–	–
M2	244	67	16·6	6·5	4·0	8·8	9·8	8·0	5·5
M3	122	51	18·5	3·4	2·0	11·2	–	–	–
M4	212	56	16·5	4·8	4·0	9·5	6·2	4·0	6·9

–, Medium not used; CV, coefficient of variation (%).

Table 7 Mean detection times (h) corresponding to a bacterial concentration of 230 *Escherichia coli* per 100 g of meat and shell liquor for the three impedance analysers and the four media used

Growth medium	Impedance analyser		
	Malthus	BacTrac 4100	BacTrac 4110
M1	10·42 (10·92)	8·23 (8·29)	–
M2	9·50 (9·94)	8·63 (8·70)	9·63 (10·05)
M3	9·39 (9·83)	8·54 (8·61)	–
M4	9·43 (9·88)	8·84 (8·91)	9·50 (9·91)

Values in brackets are maximum DT (DT_{max}) corresponding to the theoretical detection limit of the impedance method (100 *E. coli* 100 g^{-1} for Malthus and BacTrac 4110, 200 *E. coli* 100 g^{-1} for BacTrac 4100). Mean detection times were calculated from the regression line equations of the three shellfish types

–, Medium not used.

DT corresponding to a bacterial concentration of 230 *E. coli* per 100 g of MSL, calculated from the regression line equations, is given in Table 7 for each growth medium and analyser, together with mean DT corresponding to the theoretical detection limit of the impedance method (100 *E. coli* per 100 g of MSL for Malthus and BacTrac 4110, and 200 *E. coli* for BacTrac 4100). DT was 0·5 h to 2·2 h shorter with BacTrac 4100 than with the other two analysers, depending on the medium used, but approximately the same with Malthus and BacTrac 4110 for M2 and M4 media (around 9·5 h), and 1 h longer with M1 medium (10·4 h) than with the other media for the Malthus analyser.

Specificity for E. coli. Among the 463–480 measuring cells subcultured to search for gas and indole production and β-glucuronidase activity, only 0·2–1·9% produced an impedance signal within 20 h in the absence of *E. coli* (false-positive signal), whereas 0–26% containing *E. coli* failed to emit an impedance signal within 20 h (false-negative signal), depending on the growth medium and analyser used (Table 8). The lowest rate of false-positive and -negative impedance response was observed with M2 medium for Malthus (0·2 and 5·6%, respectively) and BacTrac 4110 (0·2 and 0%, respectively), and with M1 medium for BacTrac 4100 (0·9 and 0%, respectively). Some bacteria mixed with *E. coli*, such as *Citrobacter freundii*, *Enterobacter agglomerans*, *Erwinia* sp., *Klebsiella oxytoca*, *Klebsiella pneumoniae* and *Kluyvera* sp., were sometimes found in signal-emitting cultures. Impedance responses produced by cultures in which *E. coli* was not present usually gave atypical curves (low rate of impedance change and low amplitude impedance variation), with long DT ranging from 9·7 to 16·3 h. The false-negative responses observed for Malthus with M2 medium always corresponded to slightly contaminated samples.

DISCUSSION

The results obtained in this study show a significant relationship between DTs and *E. coli* concentrations as estimated by the new AFNOR standardized MPN method. Regression tests were highly significant ($P < 10^{-4}$), regardless of shellfish type or the growth medium and analyser used. The linear model was generally suitable for the data observed (the lack-of-fit test was significant for only 3/30 regressions). The percentage of variance explained by the regression models and indicated by R^2 values was generally

Table 8 Rates of false-positive and false-negative impedance responses for all shellfish samples (oysters, mussels, cockles) combined

Growth medium	False-positive responses (%)			False-negative responses (%)		
	Malthus	BacTrac 4100	BacTrac 4110	Malthus	BacTrac 4100	BacTrac 4110
M1	0·2	0·9	–	16·0	0	–
M2	0·2	1·9	0·2	5·6	0	0
M3	0	1·8	–	13·3	8·7	–
M4	0·7	1·6	0·4	26·3	11·5	0

False-positive response: presence of a detection time, but with no *Escherichia coli* found in the measuring cell; false-negative response: absence of a detection time, but with *E. coli* found in the measuring cell; –, medium not used.

high, showing that data variables were quite strongly related. In addition, standard errors of estimation, ranging from 0·264 to 0·406 according to the different regressions, were fairly low and actually lower than those previously reported for shellfish (0·44–0·50) (Dupont *et al.* 1996). This may have been, in part, because of the greater precision of the reference method used here (five-tube MPN) as compared with that used in the earlier study (three-tube MPN). The highest R^2 values were obtained with M1 medium for BacTrac 4100, and with M2 medium for Malthus and BacTrac 4110. These media showed a stronger relationship between DT and *E. coli* concentrations than the others. Moreover, the lowest values for the standard error of estimation, which determines the precision of the *E. coli* concentrations predicted from DT, were also observed with the same medium/analyser combinations. The comparison of regression lines showed that there were no significant variations for the different types of shellfish with M2 medium for Malthus and M1 medium for BacTrac 4100. Thus, a single regression line combining all observations was feasible, thereby facilitating conversion of DT into bacterial counts. According to the equation for this line, a DT of 9·5 h for Malthus and 8·3 h for BacTrac 4100 corresponds to the European Union standard of 230 *E. coli* per 100 g of MSL, which is used to classify harvesting areas in category A from which shellfish can be taken for direct consumption. For BacTrac 4110 with M2 medium, the comparison of regression lines for different types of shellfish indicated a significant difference between intercepts, which was attributable to the variation between oyster regression, on the one hand, and mussel plus cockle regression, on the other. Thus, M2 medium can be used with BacTrac 4110, in which case one regression line could relate to oysters and another to both mussels and cockles. The uncertainty of the predicted *E. coli* concentration (from a single DT) is *ca* ±0·63 and ±0·58 log unit for Malthus and Bactrac 4100, respectively, when a single regression line is used for the three shellfish types. Performance of impedance measurement in duplicate for a sample is an easy means of obtaining a more precise estimation of the *E. coli* concentration ($\pm 0{\cdot}63\sqrt{2} = \pm 0{\cdot}45$ and $\pm 0{\cdot}58\sqrt{2} = \pm 0{\cdot}41$ log unit).

The results concerning the characteristics of impedance response showed, first of all, that response intensity, as measured by the total change in impedance, depended on the growth medium. The large impedance variation observed with M2 medium and to a lesser extent with M4 medium, when compared with the smaller variations obtained with M1 and M3 media, can be explained by the greater amount of nutrients (tryptone and lactose) present in the first two media. This allowed the bacteria to multiply in larger quantities and thus produce more metabolically charged end-products, which in turn caused a greater change in impedance. M3 and M4 media gave lower impedance variations than M1 and M2 media, respectively, because of higher salt content. Notable differences in the total change of impedance were sometimes observed for a given growth medium, probably because of differences in *E. coli* strains. On the basis of the minimum values found here for total conductance or impedance variation (Table 6), it may be considered that *E. coli* generates signals with an amplitude of at least 65 μS for Malthus with M2 medium, 3% for BacTrac 4100 with M1 medium and 8% for BacTrac 4110 with M2 medium.

DTs were always shorter with BacTrac 4100 than Malthus and BacTrac 4110 for the same sample contamination level, regardless of shellfish type. These shorter DT were certainly because of higher *E. coli* concentrations in the measuring cells for BacTrac 4100. Although inoculum size was twofold smaller for Bac Trac 4100, culture volume was 10 ml when compared with 100 ml for the other two analysers. Mean DT for samples with a contamination level of 230 *E. coli* per 100 g of MSL showed that analytic response time was comparable for Malthus and BacTrac 4110 with all the media tested except M1 medium for Malthus (mean DT 1 h longer). This can be explained essentially by the lower growth rate of *E. coli* in M1 medium, as indicated by the lesser slope of the regression line between DT and *E. coli* concentration. The absolute value for this slope is actually proportional to the bacterial growth rate, as previously determined (Richards *et al.* 1978). For Malthus and BacTrac 4110, mean DT were shortened to the same extent by addition of NaCl alone or tryptone and lactose to M1 medium, whereas the addition of all three ingredients (NaCl, tryptone and lactose) did not further shorten DT. For BacTrac 4100, M1 medium seemed most appropriate for the shortest response time.

The rate of false-positive impedance responses was low for the three analysers and the four media used, although a slightly higher rate was observed for BacTrac 4100 with M2, M3 and M4 media. Impedance curves were then usually non-characteristic when compared with those produced by *E coli*. Moreover, associated DT were longer than DT_{max}, so that there were no practical consequences with regard to analytic results. The rate of false-negative impedance responses was clearly high for Malthus with M1, M3 and M4 media, and relatively high for BacTrac 4100 with M3 and M4 media. For Malthus, a conductance change actually occurred in these cases, but without a DT. In fact, the rate of conductance variation on which DT determination is based was too low to allow expression of a DT. A lower threshold value for the first difference (difference in conductance between two consecutive measurements) would have reduced the frequency of false-negative conductance responses to some extent. For BacTrac 4100, studies of impedance curves showed very slight variation, perhaps because of the relatively high NaCl content of M3 and M4

media. Conversely, the rate of false-negative impedance responses was low for Malthus with M2 medium, and no false-negative responses were observed for BacTrac 4100 with M1 and M2 media, and for BacTrac 4110 with M2 and M4 media.

The 10-ml cells gave very good results with BacTrac 4100, in contrast to the poor results obtained with Malthus (Dupont *et al*., unpublished data). In fact, preliminary trials with several impedance instruments have shown that approximately one-fourth of the conductance curves obtained with Malthus 10-ml cells from mussel samples were not interpretable because of an erratic pattern. The differences observed between the two analysers may have been due to cell geometry, the electric current frequency used and/or the magnitude actually measured (impedance or conductance).

In summary, this study confirms that impedance measurement with different instruments is a possible alternative to the MPN method for quantitative estimation of *E. coli* in live marine bivalve molluscs. In view of the results obtained for regressions between DT and *E. coli* concentrations, the specificity of the impedance response, and the intensity and earliness of the electric signal, Malthus and BacTrac 4110 (100-ml measuring cells) seem suitable choices with M2 medium, and BacTrac 4100 (10-ml cells) with M1 medium. In practical terms, the benefit of using 10-ml over 100-ml cells relates to increased analytic capacity and reduced investment and operating costs. However, the bacterial detection limit is higher with 10-ml than 100-ml cells. An additional calibration will be performed using the new BacTrac 4300 system with disposable 20-ml cells with a view to combine a low detection limit with operational requirements. The main advantages of the impedance method over the MPN method are a considerable reduction in analytic handling time and a greater facility of use. These benefits provide a quite appreciable improvement in the analytic capacity of a laboratory. Moreover, the short response time with the impedance method (between 5 and 10 h when compared with 3 days with the standardized MPN method) allows rapid intervention to ensure public health protection during potential episodes of shellfish contamination in production areas.

ACKNOWLEDGEMENTS

The authors are grateful to IFREMER coastal laboratory staffs for gathering shellfish and ensuring the transport of samples to our centre.

REFERENCES

Anon. (1988) *Méthodes d'analyse bactériologique pour le contrôle des coquillages*. Circulaire du 28 avril 1988 relative à l'harmonisation du contrôle bactériologique des coquillages. Paris: Ministère de l'Agriculture, Secrétariat d'Etat à la Mer.

Anon. (1991) *Council Directive of 15 July 1991 Laying Down the Health Conditions for the Production and the Placing on the Market of Live Bivalve Molluscs (91/492/EEC)*. Official Journal of the European Communities, no. L268 24.9.91, 1–14.

Anon. (1999) *DIN 10115 Grundlagen des Nachweises und der Bestimmung von Mikroorganismen in Lebensmitteln mittels Impedanz-Verfahren (Fundamentals for Detection and Determination of Microorganisms in Foodstuffs with the Impedance Method)*. Deutsches Institut für Normung, 6 p.

Anon. (2000a) *NF V 08-600 Microbiologie des aliments – Dénombrement des Escherichia coli présumés dans les coquillages vivants – Technique du nombre le plus probable (Microbiology of Foods and Foodstuffs Products – Enumeration of Presumptive Escherichia coli in Living Shellfish – MPN Technique)*. Association Française de Normalisation, 16 p.

Anon. (2000b) *NF V 08-105 Microbiologie des aliments – Principes de base de l'impédancemétrie appliquée aux examens microbiologiques (Microbiology for Food and Animal Feeds – Guidelines of Impedance Measurement for Microbiological Examinations)*. Association Française de Normalisation, 11 p.

Anon. (2002) *NF V 08-106 Dénombrement des Escherichia coli présumés dans les coquillages vivants – Technique indirecte par impédancemétrie directe (Enumeration of Presumptive Escherichia coli in Live Shellfish – Indirect Technique Using Direct Impedance Measurement)*. Association Française de Normalisation, 16 p.

Beauchamp, J.J. and Olson, J.S. (1973) Correction for bias in regression estimates after logarithmic transformation. *Ecology* **54**, 1403–1407.

Colquhoun, K.O., Timms, S. and Friker, C.R. (1995) Detection of *Escherichia coli* in potable water using direct impedance technology. *Journal of Applied Bacteriology* **79**, 635–639.

Cousins, D.L. and Marlatt, F. (1990) An evaluation of a conductance method for enumeration of Enterobacteriaceae in milk. *Journal of Food Protection* **53**, 568–570.

Dalgaard, O., Mejlholm, O. and Huss, H.H. (1996) Conductance method for quantitative determination of *Photobacterium phosphoreum* in fish products. *Journal of Applied Bacteriology* **81**, 57–64.

De Man, J.C. (1983) MPN Tables, corrected. *European Journal of Applied Microbiology and Biotechnology* **17**, 301–305.

Dupont, J., Ménard, D., Hervé, C. and Minier, B. (1994) Analytical procedure for use of conductance measurement to estimate *Escherichia coli* in shellfish. *Journal of Applied Bacteriology* **77**, 296–302.

Dupont, J., Ménard, D., Hervé, C., Chevalier, F., Beliaeff, B. and Minier, B. (1996) Rapid estimation of *Escherichia coli* in live marine bivalve shellfish using automated conductance measurement. *Journal of Applied Bacteriology* **80**, 81–90.

Firstenberg-Eden, R. (1983) Rapid estimation of the number of microorganisms in raw meat by impedance measurements. *Food Technology* **37**, 64–70.

Firstenberg-Eden, R. and Klein, C.S. (1983) Evaluation of a rapid impedimetry procedure for the quantitative estimation of coliforms. *Journal of Food Science* **48**, 1307–1311.

Firstenberg-Eden, R., Van Sise, L., Zindulis, J. and Kahn, D. (1984) Impedimetric estimation of coliforms in dairy products. *Journal of Food Science* **49**, 1449–1452.

Gibson, D.M., Ogden, I.D. and Hobbs, G. (1984) Estimating the quality of fish by automated conductance measurement. *International Journal of Food Microbiology* **1**, 127–134.

Martins, S.B. and Selby, M.J. (1980) Evaluation of a rapid method for the quantitative estimation of coliforms in meat by impedimetric procedures. *Applied and Environmental Microbiology* **39**, 518–524.

Nieuwenhof, F.F.J. and Hoolwerf, J.D. (1987) Impedance measurement as an alternative to the plate count method for estimating the total count of bacteria in raw milk. *Journal of Food Protection* **50**, 665–668.

Noble, P.A., Ashton, E., Davidson, C.A. and Albritton, W.L. (1991) Heterotrophic plate counts of surface water samples by using impedance methods. *Applied and Environmental Microbiology* **57**, 3287–3291.

Ogden, I.D. (1986) Use of conductance methods to predict bacterial counts in fish. *Journal of Applied Bacteriology* **61**, 263–268.

Piton, C. and Rongvaux-Gaïda, D. (1990) Estimation par conductimétric de la flore aérobie mésophile, des bactéries coliformes et de la flore psychrotrophe du lait cru (Estimation by conductimetry of mesophilic aerobic flora, coliform bacteria and psychrotrophic flora in raw milk). *Lait* **70**, 293–306.

Richards, J.C.S., Jason, A.C., Hobbs, G., Gibson, D.M. and Christie, R.H. (1978) Electronic measurement of bacterial growth. *J. Phys. E: Sci. Instrum.* **11**, 560–569.

Silverman, M.P. and Munoz, E.F. (1979) Automated electrical impedance technique for rapid enumeration of fecal coliforms in effluents from sewage treatment plants. *Applied and Environmental Microbiology* **37**, 521–526.

Weihe, J.L., Seibt, S.L. and Hatcher, W.S. (1984) Estimation of microbial populations in frozen concentrated orange juice using automated impedance measurements. *Journal of Food Science* **49**, 243–245.

Gomensoro®

instrumentación científica

MADRID (Sede Central)

Aguacate, 15
28044 Madrid
Tlf:91.508.65.86 ·
ventas@gomensoro.net

BARCELONA

Passeig de la Mare de Déu del Coll, 124
Local-3
08023 Barcelona
Tlf :93.210.82.56
ventasbcn@gomensoro.net

BILBAO

andres.carvajal@gomensoro.net

MURCIA

silvia.tunica@gomensoro.net

SEVILLA

alberto.ovelar@gomensoro.net

VALENCIA

carmen.rubio@gomensoro.net

VALLADOLID

pedro.saez@gomensoro.net