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Impedance method application for number detection *Escherichia coli* in molluscs testing by official laboratory

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ABSTRACT
Bivalve molluscan shellfish (BMS) have an important role in the transmission of some pathogens responsible for food-borne disease because they are filter-feeding animals capable of greatly concentrating certain pathogenic agents (bacteria, viruses, or parasites) present in the water column, like *Escherichia coli*. The reference method for *E. coli* testing in bivalves is the most probable number (MPN) method therefore, as this method has some disadvantages, alternative techniques of equivalent accuracy could be used without the drawbacks of the MPN method such as the direct impedance technique. This method is based on the principle that bacteria produce positively or negatively charged end products causing an impedance variation of the medium that can be used to measure their growth. The present study evaluated the efficiency of the direct impedance measuring technique through a series of laboratory tests performed on 6 kg of clams belonging to two different batches. The preliminary obtained data, compared with the MPN reference method and dd-PCR to validate the alternative method, show that the direct impedance technique has the potential to be used in place of TBX culture for confirming *E. coli* in MPN assays.

Section: RESEARCH PAPER

Keywords: Bivalve; shellfish; MPN method; indices of contamination

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1. INTRODUCTION

Bivalve molluscan shellfish (BMS) such as mussels (Mytilus spp.), clams (family Veneridae), razor clams (superfamily Solenoidea), scallops (family Pectinidae), oysters (family Ostreidae) and cockles (family Cardiidae) are filter-feeding animals and have an important role in the transmission of some pathogens responsible for food-borne disease [1].

In fact, BMS feed by filtering water through the gills and retaining plankton and organic particles necessary for their metabolism [2], so they are naturally exposed to the risk of accumulation of any biological and chemical pollutants in the aquatic environment [3]. BMS can filter varying amounts of water and their filtration capacity depends on the size, species and relaying temperature [2].

For this reason, they are capable of greatly concentrating certain pathogenic agents (such as bacteria, viruses or parasites) present in the water column [3], posing a threat to human health. In fact, BMS are considered high-risk foods whose production and marketing are regulated by various regulations, including EU Regulation 627/2019 [4] which sets out their microbiological-hygiene requirements and the health and hygiene standards for shellfish farming like tolerance limits for chemical and biological contaminants; distinction of areas of provenance; different destination for molluscs according to the area of provenance, in particular the purification and relaying of all molluscs from waters classified as unfit.

The waters destined for bivalve mollusc cultivation are classified into three zone types (A, B, C) by the Regional Health Authority through an assessment carried out every three years:

- molluscs reared in zone A may be harvested and for direct human consumption;

- molluscs reared in zone B may not be for direct human consumption unless they place a period of time in a purification or relaying centre in order to obtain the microbiological, biological, chemical and physical requirements laid down for zone A;
- molluscs reared in zone C may not be for direct human consumption until they have been in a purification or relaying centre for a period of not less than two months.

Microbial flora of a fish product is closely related to its living habits and to the microbiological characteristics of the environment in which it is grow up [2]. Since it would not be possible to search for all possible pathogenic microorganisms with routine techniques, fish product and water quality are established using so-called quality marker organisms, whose presence in concentrations above certain limits indicates the possible presence of ecologically related pathogens [5]. *Escherichia coli* is an indicator of faecal contamination (EU Regulation 2073/2005) and it is one of the most widely used indices of contamination in BMS.

There are many techniques that can be used for *E. coli's* qualitative-quantitative determination. The reference method for *E. coli* testing in bivalves, specified in both Codex Code of Practice/Standard and EU legislation (EU Regulation 2019/627), is Most Probable Number (MPN) method. This method has numerous advantages but also some disadvantages: it's labour-intensive, time-consuming (showing results only after three days), and has statistical limitations on precision. Therefore, could be used alternative methods of equivalent accuracy without the drawbacks of the MPN method such as the direct impedance technique. This method is based on the principle that bacteria produce positively or negatively charged end products causing an impedance variation of the medium, that is proportional to the change in the number of bacteria in the culture and can be used to measure bacterial growth [6]. In this way the direct impedance technique allows rapid quantitative estimation of *E. coli* in live bivalve molluscan shellfish [7], [8]. Initially for each type of shellfish, a calibration process based on bacteriological examination of samples in parallel with direct impedance technique and the MPN reference method followed by statistical analysis of the results is necessary [9]. A commonly used alternative to traditional, culture-based methods for food microbiology is Droplet Digital PCR (dd-PCR) [10]. Digital PCR is based on the principles of limited dilution, end-point PCR, and Poisson statistics, with absolute quantification as its heart [11]. Therefore, quantification is less affected by poor amplification efficiency and inhibitors of amplification that may be present in samples.

The aim of the present work was to evaluate the efficiency of the direct impedance technique through a series of laboratory tests and the comparison of results with the MPN reference method and Droplet Digital PCR (dd-PCR) to validate the alternative method.

In Section 2 we will describe the samplings carried out, the tests performed in the laboratory and the methods of analysis compared in the following study (MPN method and direct impedance technique). In Section 3 we will compare the results obtained. In Section 4 we will list the conclusions.

2. MATERIALS AND METHODS

To test the applicability of the direct impedance technique to a bivalve molluscan shellfish (BMS) matrix, 6 kg of clams (*Ruditapes philippinarum*) belonging to two different batches (batch 1 sampled the 16/01/2022 and batch 2 sampled the 17/01/2022 consist of 3 kg each one) taken from an Italian fish farm were used (Figure 1, Figure 2).

Figure 1. Batch 1 sampled the 16/01/2022.

Figure 2. Batch 2 sampled the 17/01/2022.

Clam samples, transported at refrigeration temperature and received by the laboratory in insulated boxes, were contaminated with three different concentrations (150,1500,15000 CFU/portion test) of an *E. coli* field strain isolated from mussels (*Mytilus galloprovincialis*) and cryopreserved at a temperature of -80 °C, that was maintained on non-selective solid nutrient agar (Oxoid, UK).

Following artificial contamination, samples were analysed using both the MPN method described in ISO 16649-3 and the enumeration *Escherichia coli* in live shellfish- indirect technique using direct impedance measurement (AFNOR NF V08-106).

2.1. Most Probable Number (MPN) method

In MPN method, the amount of *E. coli* present in each sample was enumerated using the European Union Reference Laboratory (EURL) reference five tube, three dilution most probable number (MPN) method based on ISO 16649-3. Briefly, from each batch three clam samples were obtained and examined by two different operators (operator A and operator B).

For each one, at least 10-25 individual shellfish weighing between 75 and 100 g were shucked and homogenised with peptone water in a 1:10 suspension. All samples were contaminated and after samples contamination, three serial ten-fold dilutions (10^{-1}, 10^{-2}, 10^{-3}) of this homogenate were prepared and inoculated into tubes containing minerals modified glutamate broth (MMGB). All tubes were incubated at 37 °C ± 1 °C for 24 ± 2 h. MMGB positive tubes, that had changed their colour from purple to yellow (Figure 3), were inoculated on

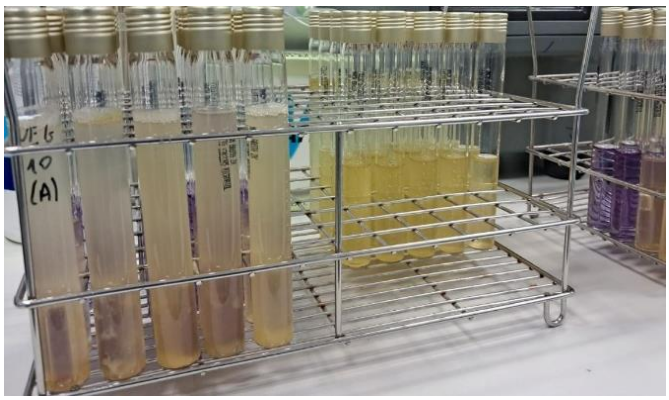

Figure 3. MMGB positive tubes that had changed their colour from purple to yellow.

tryptone bile X-glucuronide (TBX) agar and incubated for a further 18-24 h at 44 °C ± 1 °C.

Following bacterium growth in TBX agar, confirmed by the presence of blueish green colony, the combination of *E. coli* positives was used to calculate the MPN of this bacterium in 100 g of BMS.

2.2. The direct impedance technique

To carry out the direct impedance technique (AFNOR NF V08-106) from the two different examined batches (batch 1 and batch 2), 10 samples (5 from each batch) were obtained by weighing 100 g of shelled clams and diluting them with 200 ml of PSS (Peptone salt solution) in a 1:3 suspension.

Each sample was contaminated, the solution was homogenised for at least 1 minute and was decanted into sterile preserving jars for at least 15-20 minutes. After decantation, 30 ml of the homogenised sample were transferred to 70 ml of PSS diluent in a 1:10 suspension, from which 7.5 ml (for each sample) were taken and inoculated in specific cells equipped with electrodes (Figure 4), that were inserted in the empty positions of the BacTrac 4300 (SY-LAB) series analyser (Figure 5) set to the specified temperature requirements (44 °C for *E. coli*). The results were read after 24 hours (Figure 6).

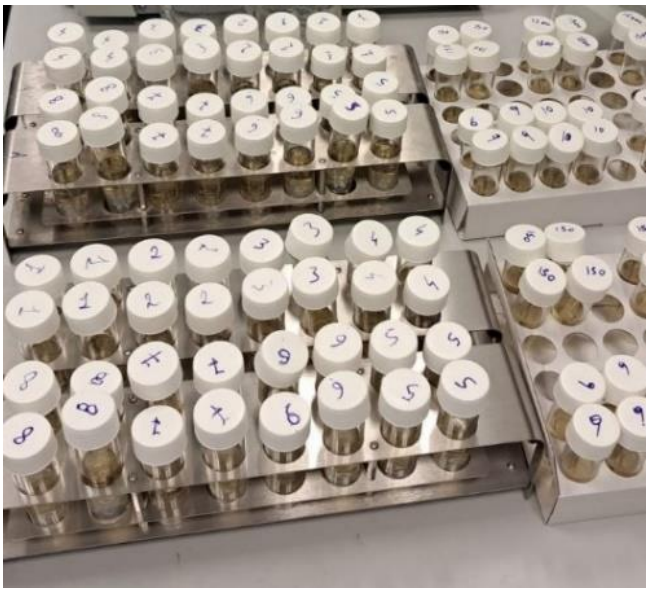

Figure 4. Cells equipped with electrodes used for performing the direct impedance technique.

Figure 5. BacTrac 4300 (SY-LAB) series analyser.

Figure 6. Results of the direct impedance technique showed by the BacTrac 4300 (SY-LAB) series analyser after 24 hours.

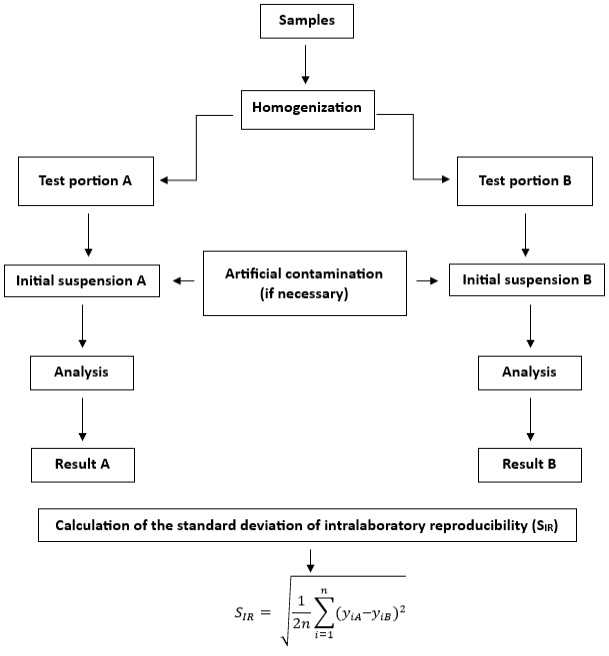

Figure 7. Experimental protocol for the estimation of the standard deviation of intralaboratory reproducibility (S_{IR}) (UNI EN ISO 16140-3:2021).

3. RESULTS AND DISCUSSION

The laboratory tests, carried out under conditions of intralaboratory reproducibility (Figure 7) produced the results necessary to calculate the standard deviation of intralaboratory reproducibility (S_{IR}) and the estimated systematic error (eBias)

Table 1. Calculation of the standard deviation of intralaboratory reproducibility (S_{IR}), Batch 1: 16th January 2022; Batch 2: 17th January 2022.

N. samples	Batch	Inoculum	Result A (x_{iA})	Result A (x_{iB})	$Y_{iA} = \log_{10}(x_{iA})$	$Y_{iB} = \log_{10}(x_{iB})$	$\lvert y_{iA} - y_{iB} \rvert$	$\lvert y_{iA} - y_{iB} \rvert^2$
1	Batch 1	150	4900	6800	3.69	3.83	0.14	0.02
2	Batch 1	150	670	1900	2.83	3.28	0.45	0.20
3	Batch 2	150	1900	2600	3.28	3.41	0.14	0.02
4	Batch 2	150	1100	1400	3.04	3.15	0.10	0.01
5	Batch 1	1500	20000	22000	4.30	4.34	0.04	0.00
6	Batch 1	1500	14000	14000	4.15	4.15	0.00	0.00
7	Batch 2	1500	12000	52000	4.08	4.72	0.64	0.41
8	Batch 2	1500	18000	17000	4.26	4.23	0.02	0.00
9	Batch 1	15000	400000	590000	5.60	5.77	0.17	0.03
10	Batch 2	15000	71000	230000	4.85	5.36	0.51	0.26
							Total	0.95
							Total/(2xn)	0.05
							S_{IR}	0.22

needed for the validation of the impedance method. To check whether the performance of the alternative method is similar to the reference method validated on that matrix, S_{IR} of the verified method (direct impedance technique) must be $\leq 2 \times S_R$ (standard deviation of interlaboratory reproducibility) as provided by the UNI EN ISO 16140-3:2021.

The acceptability limit, for the AFNOR NF V08-106 method used in this study on clams, was satisfied as the S_{IR} is 0.22 (Table 1) and it is $\leq 2 \times 0.180 = 0.36$. A comparison of the results of both methods showed that the impedance method has a higher sensitivity than the MPN method (Table 2) because the microbial concentration present in the initial inoculum is almost the same as that found in the sample (Table 3). This observation is confirmed by the eBias obtained by the absolute difference between the results of the artificially contaminated food matrix and the inoculum suspension both expressed in $\log_{10}$ cfu/portion test, which must always be lower than 0.5 $\log_{10}$ (Table 4).

The impedance method data were also compared with droplet digital PCR data that allows absolute quantification for trace nucleic acids with very high precision and sensitivity. Summary of samples analysed by both the impedance technique and the dd-PCR method had identical results (data not showed).

4. CONCLUSIONS

In this study we have shown that enumeration *Escherichia coli* in live shellfish- indirect technique using direct impedance measurement has the potential to be used in place of TBX culture for confirming *E. coli* in MPN assays. In fact, both methods produced similar results.

However, the direct impedance technique ensured a higher sensitivity, more precise enumeration of *E. coli* in BMS matrix and faster execution (showing results after 24 hours and not after 48 hours as the MPN method). In this way the proven method ensures rapid quantitative estimation of *E. coli* in live shellfish allowing a faster closure of the production area and therefore a rapid suspension of farmed mollusc harvesting.

It also makes the recall and withdrawal process of non-compliant molluscs more rapid and timely by ensuring food safety for consumer health protection, a reduction of the economic damage suffered by food industries (by decreasing the quantity of product to be withdrawn) and savings on the

Table 2. Results obtained by the MPN reference method, Batch 1: 16th January 2022; Batch 2: 17th January 2022.

Operator	Batch	Inoculum	Results
A	Batch 1	150	54000
B	Batch 2	150	92000
A	Batch 1	1500	>160000
B	Batch 2	1500	>160000
A	Batch 1	15000	>160000
B	Batch 2	15000	>160000
A	Batch 1	NEG	130
B	Batch 2	NEG	130

Table 3. Results obtained by the two operators (A and B) who performed the direct impedance technique, Batch 1: 16th January 2022; Batch 2: 17th January 2022.

N. samples	Batch	Inoculum	Operator A	
1	Batch 1	150	4800	4900
2	Batch 1	150	670	370
3	Batch 2	150	840	1900
4	Batch 2	150	140	1100
5	Batch 1	1500	12000	20000
6	Batch 1	1500	13000	14000
7	Batch 2	1500	12000	9600
8	Batch 2	1500	23000	18000
9	Batch 1	15000	240000	400000
10	Batch 2	15000	63000	71000
11	A: Batch 1; B: Batch 2	Negative	2900	600
12	Inoculum without matrix	150	29000	72000
13	Inoculum without matrix	1500	190000	670000
14	Inoculum without matrix	15000	1500000	2100000
N. samples	**Batch**	**Inoculum**	**Operator B**	
1	Batch 1	150	6800	7600
2	Batch 1	150	3000	1900
3	Batch 2	150	2900	2600
4	Batch 2	150	1400	1600
5	Batch 1	1500	22000	15000
6	Batch 1	1500	14000	12000
7	Batch 2	1500	52000	52000
8	Batch 2	1500	16000	17000
9	Batch 1	15000	590000	760000
10	Batch 2	15000	290000	230000
11	A: Batch 1; B: Batch 2	Negative	440	660
12	Inoculum without matrix	150	1300	7600
13	Inoculum without matrix	1500	17000	46000
14	Inoculum without matrix	15000	150000	550000

Table 4. Calculation of the estimated systematic error (eBias), Batch 1: 16th January 2022; Batch 2: 17th January 2022.

Inoculum without matrix

Inoculum	Results		Mean	Log_{10}
150	1300		4450	3.65
1500	17000		31500	4.50
15000	150000	550000	350000	5.54

Contamined matrices

Batch	Inoculum	Mean	Log_{10}	Log_{10} Inoculum	eBias
Batch 1	150	7200	3.86	3.65	0.21
Batch 1	150	2450	3.39	3.65	0.26
Batch 2	150	2750	3.44	3.65	0.21
Batch 2	150	1500	3.18	3.65	0.47
Batch 1	1500	18500	4.27	4.50	0.23
Batch 1	1500	13000	4.11	4.50	0.38
Batch 2	1500	52000	4.72	4.50	0.22
Batch 2	1500	16500	4.22	4.50	0.28
Batch 1	15000	675000	5.83	5.54	0.29
Batch 2	15000	260000	5.41	5.54	0.13

resources used by the competent authority to verify that recall and withdrawal actions are correctly implemented by food business operators.

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