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The species accuracy of the Most Probable Number (MPN) European Union reference method for enumeration of *Escherichia coli* in marine bivalves

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ABSTRACT

Continuous European Union programmes with specified methods for enumeration of *Escherichia coli* in bivalves for human consumption are currently running. The objective of this research was to examine the species accuracy of the five times three tube Most Probable Number (MPN) EU reference method used for detection of *E. coli* in marine bivalves. Among 549 samples of bivalves harvested from Norwegian localities during 2014 and 2015, a total number of 200 bacterial isolates were prepared from randomly selected culture-positive bivalves. These presumptive *E. coli* isolates were characterized biochemically by the Analytical Profile Index (API) 20E, as well as by Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS). The majority of isolates (90%) were identified as *E. coli*, by both API 20E and MALDI-TOF MS. Ten isolates (5%) were identified as *Klebsiella pneumoniae*, while one isolate was identified as *K. oxytoca* by both methods, whereas three isolates were identified as *Acinetobacter baumannii*, *Citrobacter braakii*, and *Enterobacter cloacae*, respectively. The identification of the remaining six isolates were not in compliance between the two methods.

1. Introduction

Bivalve molluscs, including blue mussels (*Mytilus edulis*), great scallops (*Pecten maximus*), flat oysters (*Ostrea edulis*), horse mussels (*Modiolus modiolus*), and carpet shells (*Mya arenaria*), are suspension feeders and actively filter and retain particles from their surrounding water, including free living or particle bound bacteria. Bivalves are thereby excellent bio-samplers that reflects the load of *Escherichia coli* and other microorganisms of faecal origin, such as enteric viruses (e.g., Norovirus), in the water column at a given location (Lunestad et al., 2016). These microorganisms may originate from humans and other homeothermic animals either via sewage, by runoff from land, or from the wild fauna. *E. coli* is a well-established indicator of faecal contamination, and its absence in food products indicates a manufacturing process under appropriate sanitary conditions (Baylis et al., 2011; Buttiaux and Mossel, 1961).

According to EU Directive 854/2004/EC (2004), national food safety authorities, in this case the Norwegian Food Safety Authority (NFSA), has the responsibility of monitoring and classifying production areas for bivalve molluscs (NFSA, 2013). The production areas are classified as A, B, C or prohibited areas depending on the content of *E. coli* in the soft parts and mantle water of harvested bivalves. A Class A area have an upper limit of 230 *E. coli*/100 g sample material measured as fresh weight, and such bivalves may go directly for human consumption. A Class B area has an upper limit of 4,600 *E. coli*/100 g, whereas a class C area has an upper limit of 46,000 *E. coli*/100 g. Bivalves from B and C area must be purified by resuspension at Class A area until meeting the limit of 230 *E. coli*/100 g or heat treated. Areas with samples exceeding the upper limit of a Class C area are prohibited for harvesting. According to 2015/2285 (2015) concerning bivalve product to be placed on the market, 20% of the samples may contain *E. coli* between 230 and 700/100 g sample material, while the remaining 80% of the samples must be below 230/100 g sample material.

The quantitative method for detection and enumeration of *E. coli* in bivalve molluscs are specified in EU Council Directive 91/492/EEC (1991). This method is based on a Most Probable Number (MPN) principal (Oblinger and Koburger, 1975) with five tubes, each in three dilutions. The MPN principal is based on the number of positive tubes at increasing dilutions of a sample, and further calculations are necessary to convert the results into a MPN value, with a probable range. This MPN technique is commonly used in combination with verification on chromogenic agar to calculate the number of *E. coli* in bivalves (Donovan et al., 1998). The applied MPN method utilise Minerals Modified Glutamate Broth (MMGB) as growth medium, and material from positive tubes, i.e., tubes with colour change from acid production, are confirmed on Tryptone Bile with X-glucuronide (TBX) agar for the determination of β-glucuronidase production, a common feature of *E. coli* (Donovan et al., 1998). According to the EU method, bacterial growth with colour change in MMGB and presence of β-glucuronidase

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production is considered to be *E. coli*. It is known that other members of the Enterobacteriaceae may possess the β-glucuronidase enzyme and it could be assumed/suspected that they may also give false-positive blue-green colonies on the TBX agar. To examine the species accuracy of the standardised EU MPN method, we performed further characterisation of the presumptive *E. coli* isolates by both the Analytical Profile Index (API) 20E test kit and a Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS) (Bourassa and Butler-Wu, 2015). The API 20E test kit is designed to identify members of the Enterobacteriaceae family and to differentiate between closely related, and morphologically similar, bacterial strains based on enzymatic degradation of carbohydrates, amino acids and some other reactions in 20 miniature wells, resulting in a biochemical profile specific for each species. In MALDI-TOF MS small molecules from lyophilized bacteria gives distinct spectra allowing identification into genus and specie levels. A target plate with pre-treated samples is exposed to a nitrogen laser applying short pulses of high-energy, causing desorption and ionization of each sample (Bourassa and Butler-Wu, 2015). To identify a particular bacterium a characteristic Peptide Mass Fingerprint (PMF) pattern of highly abundant peptides derived from ribosomal proteins are matched, which ionize readily and represent about 60–70% of the dry weight of a bacterial cell (Singhal et al., 2015). During PMF matching, the spectra of known bacterial species included in the database of reference spectra (MALDI Biotyper Library) are compared with the spectra of the unknown bacterial isolate (Bourassa and Butler-Wu, 2015; Singhal et al., 2015).

The objective of this research was to examine the species accuracy of the five times three tube MPN EU reference method used for detection of *E. coli* in marine bivalves assessed by API 20E and MALDI-TOF MS.

2. Materials and methods

2.1. Samples

From October 2014 to November 2015, a total of 549 samples were collected and examined, comprising 447 samples of blue mussels (*M. edulis*), 40 samples of flat oysters (*O. edulis*), 39 samples of great scallops (*P. maximus*), 12 samples of carpet shells (*M. arenaria*), and 11 samples of horse mussels (*M. modiolus*). The samples were collected at rearing localities along the coast of Norway (Fig. 1), and transported to the laboratory under chilled conditions close to 4 °C. The microbiological analyses were initiated within 24 h of sampling.

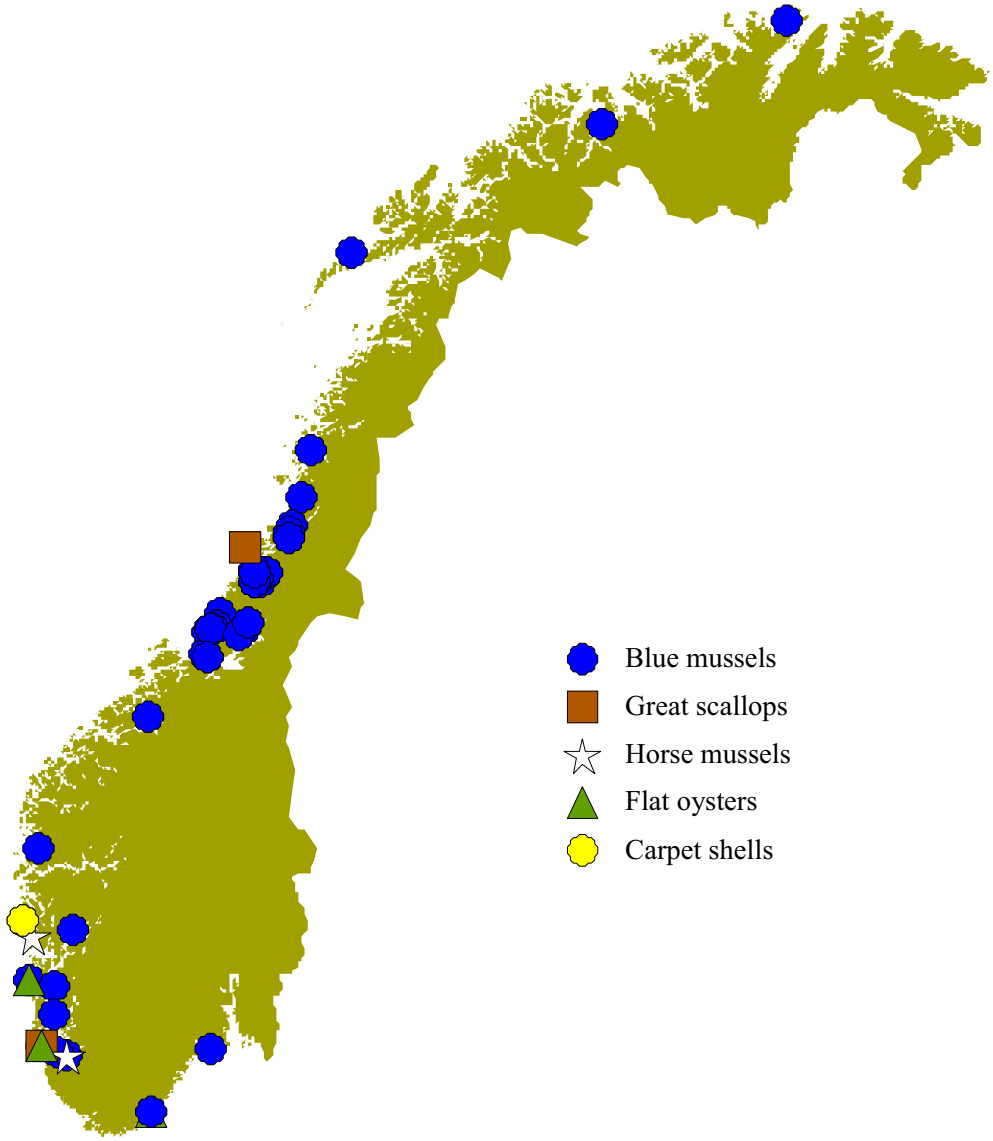

Fig. 1. Sampling sites of bivalve molluscs along the Norwegian coast in the period from 2014 to 2015.