False-negative enterococci counts in seawater with the IDEXX Enterolert-E most probable number technique caused by *Bacillus licheniformis*

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Abstract

Enterolert-E is an easy-to-use method for the enumeration of enterococci in water samples as an indicator of sewage pollution. This most probable number technique replaced the laborious and more time-consuming MEA-BEA plating method and it is used extensively in ballast water testing and monitoring. In spring 2018, the Control Union Water ballast water test facility measured high enterococci concentrations in Wadden Sea water without any correlation with polluted freshwater input. By isolating bacteria from samples incubated in Enterolert-E culture medium, followed by analyses of colony morphology and DNA, it is shown that these erroneously high concentrations were caused by *Bacillus licheniformis*, a gram-positive rod-shaped chlorine-resistant bacterium. It is concluded that control analyses or the MEA-BEA method must be performed when high enterococci concentrations are measured in water samples that are not suspected to be polluted. (135 words)

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Introduction

Enterococci are widely used as an indicator of sewage pollution in water samples. In the case of testing Ballast Water Treatment Systems (BWTSs) for type approval by the International Maritime Organization (IMO) and United States Coast Guard (USCG), enterococci are one of the several groups of organisms that are enumerated before and after water treatment, which is done chemically or by UV radiation, in order to determine treatment efficacy. Once type-approved and on board of ships, BWTSs should reduce the risk of transferring aquatic invasive species by ballast water discharges and enterococci are used as an indicator microbe. The IMO and USCG have regulated the minimum concentrations of organisms that are needed before treatment and the maximum concentrations of organisms that are allowed after treatment (Table 1).

Table 1. IMO and USCG regulations for the minimum concentrations of aquatic organisms that are needed before treatment in type approval tests and the maximum concentrations of organisms that are allowed after treatment in both test and ships' discharge water.

Organism	Before	After
≥50 µm in minimum dimension	≥100,000 per m^3	<10 per m^3
≥10-50 µm in minimum dimension	≥1,000	<10 per mL
Total Heterotrophic Bacteria	USCG: ≥1,000 IMO: ≥10,000	No criterium
Escherichia coli	No criterium	<250 per 100 mL
Enterococci	No criterium	<100 per 100 mL
Vibrio cholerae O1/O139	No criterium	<1 per 100 mL

The three indicator microbes, *Escherichia coli*, enterococci, and *Vibrio cholerae* have no minimum required concentrations before treatment in BWTS type approval tests. In other words, they do not need to be added to the test water. When a test facility, like that of Control Union Water (CUW), uses natural unpolluted water with no history of relevant indicator microbe abundances, the absence of these microbes means that their treatment efficacy cannot be established. Therefore, the discovery of enterococci in CUW test water, originating from the Wadden Sea, a nature reserve and UNESCO World Heritage, was met with some enthusiasm but also with skepticism. In addition, chlorine-treated ballast water had enterococci concentrations above the discharge standard (Table 1), which is extremely rare and suggestive of a chlorine-resistant enterococcus strain.

The skepticism led to the investigation of the positive reaction in the Enterolert-E method employed to measure enterococci concentrations. This most probable number (MPN) IDEXX method, where a 100 mL sample mixed with a proprietary culture medium is distributed over 97 wells and incubated at 41° C, is based on the metabolization of 4-methyl-umbelliferyl-β-Glucoside by enterococci β-Glucosidase to fluorescent 4-methyl-umbelliferyl. From samples incubated in Enterolert-E culture medium, one microbial species was isolated, identified and then regrown in Enterolert-E, thereby disclosing *B. licheniformis* as the cause of the false-positive enterococci enumeration. It is also shown that *B. licheniformis* does not lead to false-positive counts in the MEA-BEA selective agar plating method.

Material and Methods

Enterolert-E for enterococci was obtained from IDEXX (https://www.idexx.com/). The culture medium content of one Enterolert-E ampule was added to 100 mL sample. The sample-reagent mixture was poured in an IDEXX quanti-tray/2000 with 97 wells and sealed with the IDEXX tray sealer. The trays were incubated at 41 ± 0.5 °C for 24 hours. Under UV light yellow fluorescing (positive) wells were counted and the enterococci sample concentration was calculated from the IDEXX MPN table. A positive control for the enterococci analyses was Certified Reference Material (CRM) *E. faecalis* NCCB 100299 from Biosisto (www.biosisto.com), used in a 10,000x dilution in Normal Saline Solution (NSS, 9 gram NaCl/L).

In March and April 2018, samples were obtained from the CUW ballast water test facility (Texel, The Netherlands) during BWTS testing. Ballast water test samples before and after treatment are routinely checked for enterococci to determine BWTS efficacy (Table 1). Because elevated enterococci concentrations were discovered, that can be related to polluted freshwater input, samples were also taken in a range of salinities (10 – 30 g/kg) in the Lake IJssel-Wadden Sea estuary. If the source of the enterococci would be Lake IJssel, higher concentrations would be expected at lower salinities. Salinity and water temperature were analyzed with a Digital Conductivity Meter GMH 3400 with Pt sensor (https://www.greisinger.de/). Samples were stored cool in sterilized bottles and processed within 8 hours.

Culture medium from IDEXX-positive wells from two samples was extracted with a needle and syringe. From samples M2-T-D1 (treated with chlorine, 29-3-2018), WSN (not-treated, 28-3-

2018) and CRM *E. faecalis* NCCB 100299 one drop was plated on Tryptic Soy Agar (TSA, Biotrading, http://www.biotrading.com/) plates and incubated at 41° C for 24 hours. Colonies were observed by eye and with a Zeiss Discovery V8 stereomicroscope (https://www.zeiss.com/) and photographed.

Genomic DNA was isolated from the first two (M2-T-D1 and WSN) and six additional isolates using a commercial kit and protocol (DNeasy PowerSoil kit, https://www.qiagen.com/). Polymerase chain reactions (PCRs) were performed with 2 µL DNA template, 1 unit Qiagen Taq and the universal primers F27 (5 '-AGAGTTTGATCMTGGCTCAG-3) and R1492 (5'-TACGGYTACCTTGTTACGACTT-3') according to Lane (1991) which resulted in an amplicon of about 1500 bp. Sanger sequencing of the amplicons was performed by Baseclear B.V. (https://www.baseclear.com/). The newly derived sequences were analyzed using BLAST (Altschul et al. 1990) against the nucleotide database on the NCBI website. Subsequently, ARB (Ludwig et al. 2004) was used for constructing a dendrogram of the newly derived sequences (all *Bacillus licheniformis*) and their nearest neighbors. To confirm the presence of *Bacillus licheniformis* in seven samples with different salinities and chlorine treatments, a *B. licheniformis* colony from each sample was plated on TSA, incubated 24 hours at 41° C, and sequenced after PCR-amplification.

To confirm the false-positive reaction by *B. licheniformis* in Enterolert-E, colonies cultured on TSA were individually suspended in 100 mL sterile seawater and analyzed by the IDEXX method.

Finally, it was investigated if *B. licheniformis* also produced false-positive results in the standard enterococci mEnterococcus agar (MEA, http://www.biotrading.com/) and Bile Esculin agar (BEA, http://www.biotrading.com/) analysis. 100 mL sample was filtered on a membrane filter that was placed and incubated (48 ± 3 hours at 41 ± 0.5 °C) on a MEA plate that contains sodium azide to repress Gram-negatives. The reduction of Triphenyl Tetrazolium Chloride to red formazan is an indicator of bacterial growth (Slanetz and Bartley 1957). If red colonies are present the filter is placed on a BEA plate and incubated for 20-30 minutes at 41 ± 0.5 °C. Enterococci grow in the presence of bile and convert esculin to produce a dark brown to black precipitation. A *B. licheniformis* colony was suspended in NSS and plated on MEA plates with CRM *E. faecalis* NCCB 100299 as a control. To check the viability of *B. licheniformis* in this MEA-BEA test it was also grown on TSA agar.

Results and Discussion

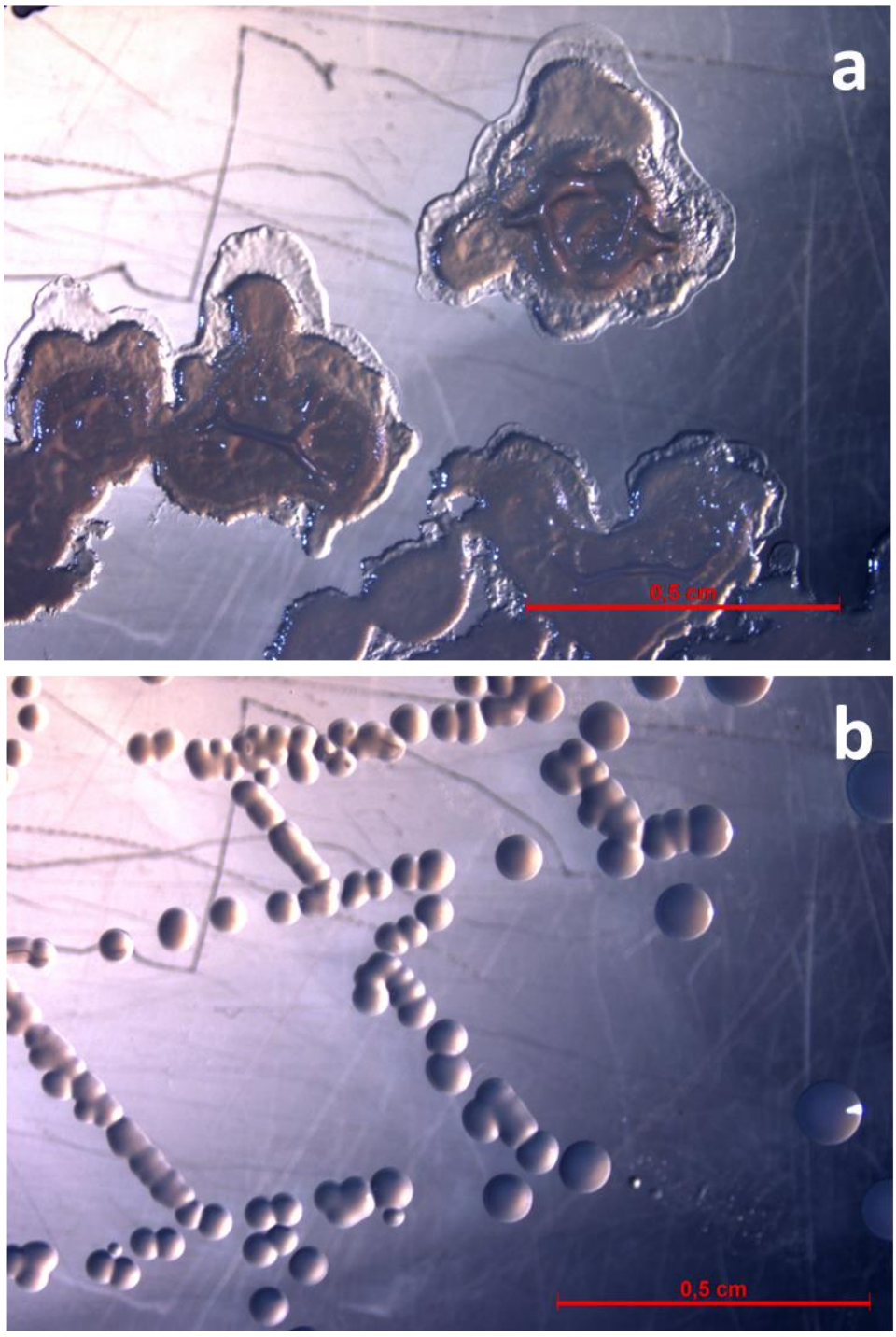
In spring 2018 samples from the Marsdiep area, the concentration of presumed enterococci as measured by the IDEXX Enterolert-E method ranged from less than 100 to nearly 2,000 per 100 mL (Table 2).

Table 2. Concentrations of IDEXX Enterolert-E measured presumed enterococci in seven Wadden Sea and BWTS chlorine-treated samples. Isolates obtained from a positive IDEXX well were sequenced and re-examined in IDEXX Enterolert-E to confirm the false-positive reaction. M2-T-D1 and M3-T-D5 are BWTS test samples originating from the Wadden Sea NIOZ harbor (WSN) after a one- or five-day chlorine (20 mg/L) treatment respectively. SS-VG-24 is a Wadden Sea location. Codes NIOZ-1 to -9 will be used in the deposition of the sequence data in GenBank®.

Sample	Date	Code	Treatment	"enterococci" per 100 mL	Sequence match *B. licheniformis*	IDEXX false positive
M2-T-D1	29-3-2018	NIOZ-1	Chlorine	340	100%	yes
WSN	5-4-2018	NIOZ-2	None	570	100%	yes
Marsdiep tidal inlet	5-4-2018	NIOZ-3	None	1990	100%	yes
SS-VG 24	9-4-2018	NIOZ-4	None	80	100%	yes
WSN	10-4-2018	NIOZ-5	None	920	99.5%	yes
Marsdiep tidal inlet	10-4-2018	NIOZ-6	None	870	100%	yes
M3-T-D5	10-4-2018	NIOZ-7	Chlorine	170	100%	yes

Plating of IDEXX-grown “enterococci” on TSA revealed a species with a colony-type that was large, lobed, wrinkly, ‘star- or lichen-like’ and grey (cf. Macauleya et al. 2006), much different from the *E. faecalis* CRM which was small, round, smooth and cream-colored (Figure 1).

DNA analysis identified all isolates as *Bacillus licheniformis* (Table 2) with as nearest neighbors: X68416 *Bacillus licheniformis* and CP000002 *Bacillus licheniformis* DSM 13 = ATCC 14580 (Figure 2). *B. licheniformis* is a Gram-positive rod-shaped bacterium and not coccoid as enterococci (Logan and De Vos, 2015).



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158 Figure 1. Plate-grown presumed enterococci, in fact, *B. licheniformis* (a) and CRM *E. faecalis* (b).

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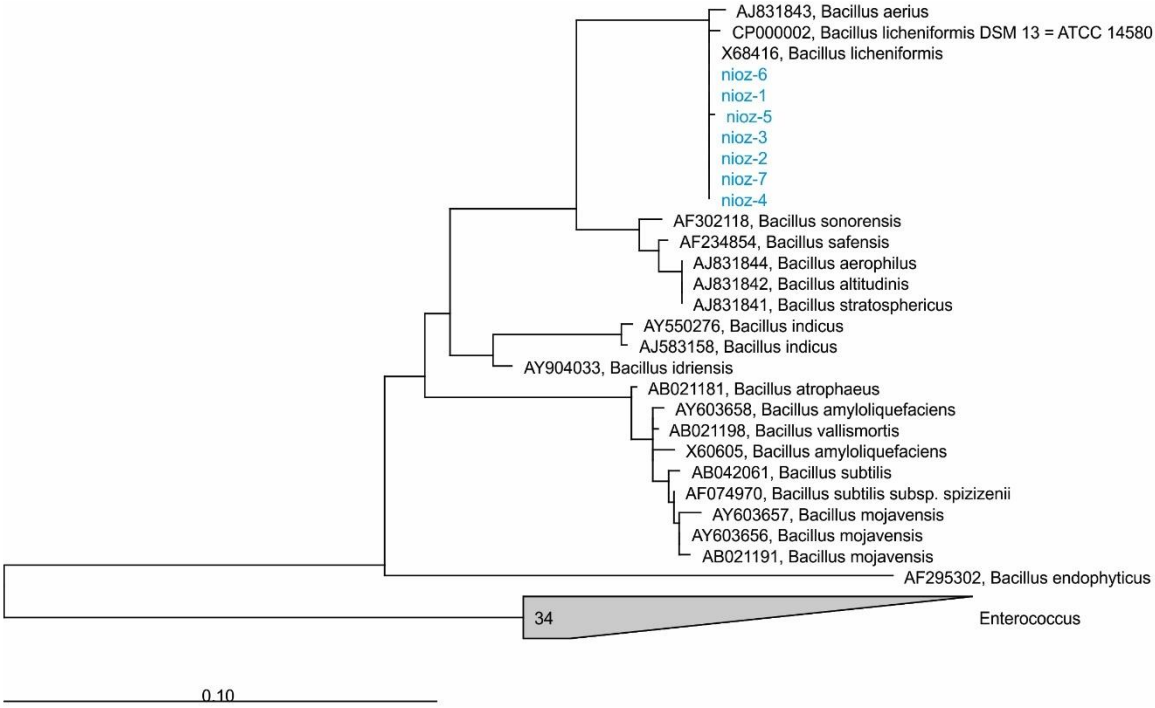


Figure 2. Sequence tree of *B. licheniformis* isolates NIOZ-1 to -7 (see Table 2).

When a *B. licheniformis* isolate was incubated in Enterolert-E, the *B. licheniformis*-fluorescent wells were indistinguishable from CRM enterococci-fluorescent wells (Figure 3).

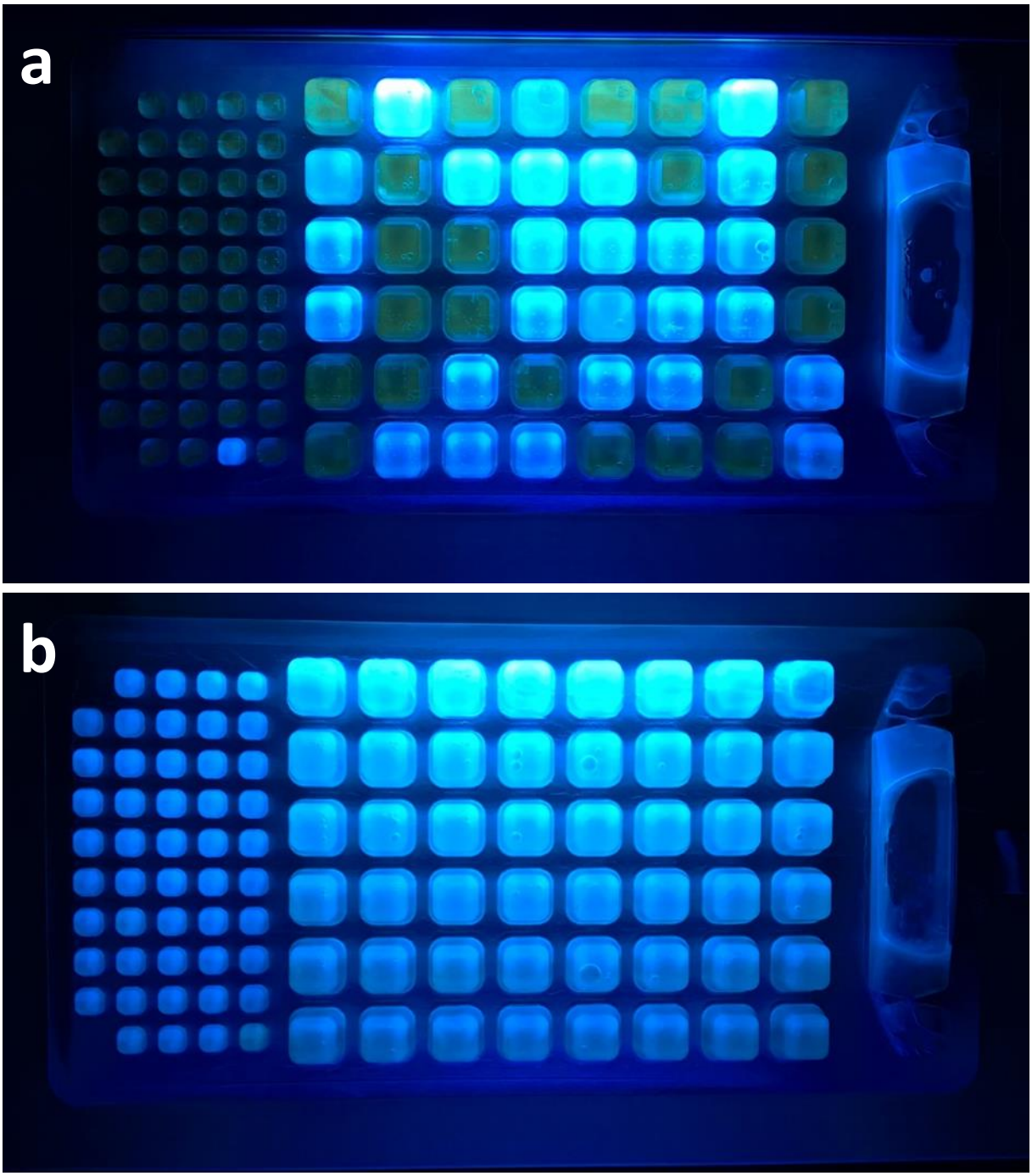
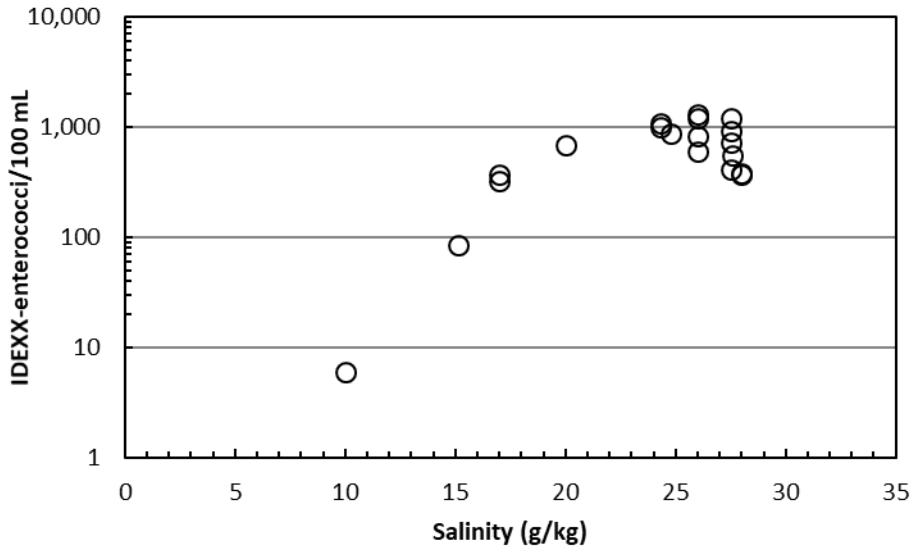


Figure 3. False-positive IDEXX reactions confirmation. Top: *E. faecalis* CRM showing several large fluorescent (positive) and one small positive well. Bottom: *B. licheniformis* isolated from sample WSN 5-4-2018 showing all positive wells. **Figure in color.**



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178 Figure 4. IDEXX Enterolert-E presumed enterococci concentrations in the Wadden Sea as a function
179 of salinity.

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181 The concentration of *B. licheniformis* increased with salinity, excluding a freshwater source
182 (Figure 4). Although generally considered a soil bacterium (Logan and De Vos, 2015), *B.*
183 *licheniformis* has been found in marine environments previously. Twenty-one strains of *B.*
184 *licheniformis* were isolated from marine sediment off the Italian west coast and all were able
185 to grow in seawater and in NaCl solutions up to 150 g/L (Manachini and Fortina 1994). In the
186 Wadden Sea, the water temperature ranged from 6 to 12°C and the highest concentrations of
187 *B. licheniformis* (ca 2,000/100 mL) were measured at <8 °C with lower concentrations at
188 elevated temperatures (12-13°C) at the end of April. This temperature range seems quite low
189 for a thermophilic species (Ronimus et al. 1997), but strains of *B. licheniformis* are known to
190 have a large thermotolerance (Logan and De Vos, 2015). The presence of *B. licheniformis* in
191 the Wadden Sea samples is perhaps more related to the presence of suspended marine
192 sediment (Manachini and Fortina 1994) due to high spring wind speeds than to temperature.

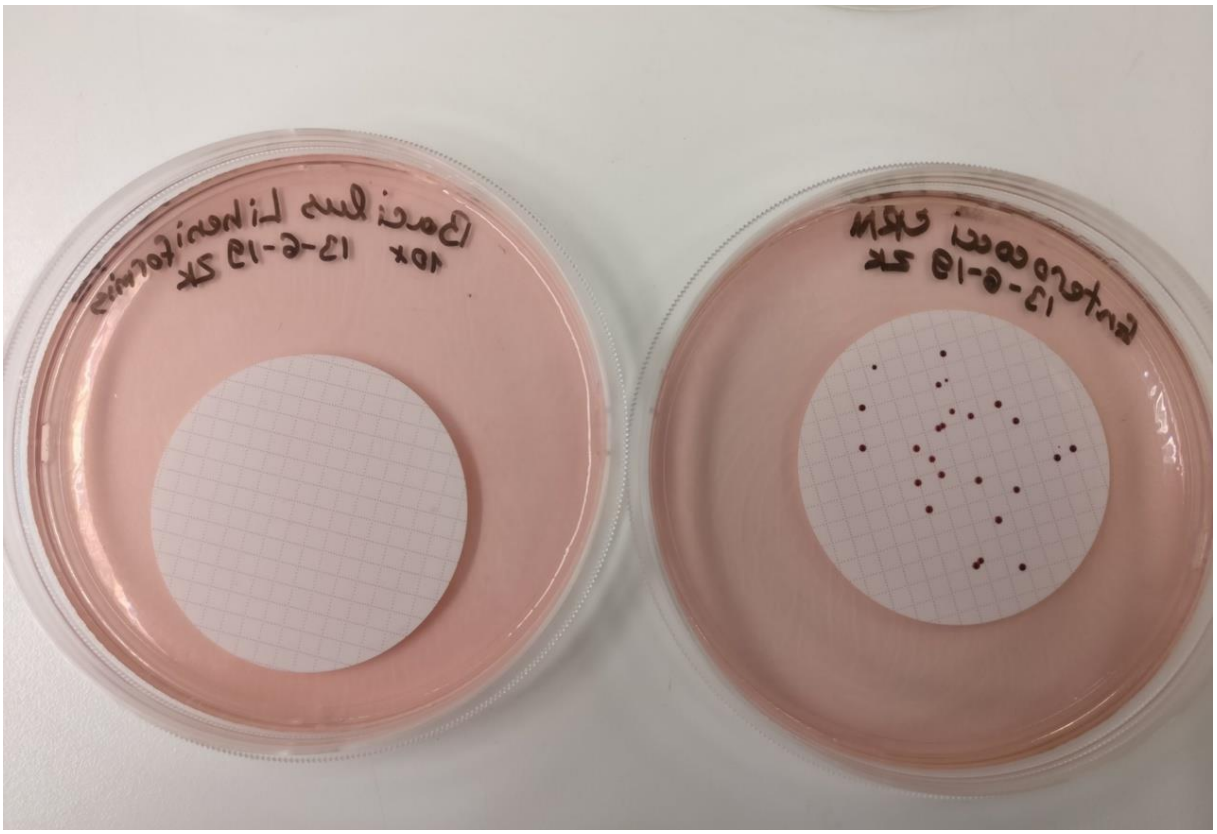

Figure 5. *B. licheniformis* (left) and *E. faecalis* CRM (right) filtered on membrane filters and incubated on MEA plates. *B. licheniformis* did not grow on the MEA plate. **Figure in color.**

B. licheniformis did not produce false-positive results in the standard enterococci MEA-BEA method (Figure 5). It failed to grow on the MEA plate so that the subsequent BEA analysis was not needed. As a simultaneous viability control, *B. licheniformis* did grow on TSA agar. This means that the MEA-BEA method can be used as an alternative to Enterolert-E when *B. licheniformis* is present in water samples.

In the type approval testing of BWTSs, it is paramount to obtain accurate concentrations of all biological components before and after treatment. Once type-approved and used on board of ships, the discharge standard requires concentrations of enterococci <100 per 100 mL and again an accurate enumeration is important. The IDEXX Enterolert-E is a relatively simple and

206 fast method, but it will grossly overestimate enterococci when *B. licheniformis* is present. The
207 problem can be worse in chlorine-treated ballast water because *B. licheniformis*
208 concentrations above the ballast water discharge standard (Table 1) were obtained after one
209 and five-day chlorine treatment (Table 2). The chlorine resistance of *Bacillus* spp. and *B.*
210 *licheniformis* in particular, have been reported previously (Macauleya et al. 2006, Paes et al.
211 2012).

212 The practical consequence of these possible false positives in the IDEXX Enterolert-E is to
213 either count the relative abundance of enterococci- and *B. licheniformis*- type colonies on TSA
214 to correct the Enterolert-E count, or to use the MEA-BEA method. The approach by the CUW
215 test facility is to test the first of a series of samples of a given salinity (marine, brackish or
216 fresh) by Enterolert-E and MEA-BEA to check if any differences occur due to false positives. If
217 these are not present, the Enterolert-E method is continued, and TSA checks are performed if
218 in time the enterococci concentrations increase considerably. If false positives are present,
219 the MEA-BEA method is used.

220 The false-positive effect of *B. licheniformis* on Enterolert-E has been communicated to IDEXX
221 and future adaptations to the method by this company may safeguard its use in the
222 enumeration of enterococci in water samples.

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