Chapter 3

Quantifying heterotrophic bacteria in ballast water treatment systems: a comparative analysis of plate counting, flow cytometry and qPCR

Quantifying heterotrophic bacteria in ballast water treatment systems: a comparative analysis of plate counting, flow cytometry and qPCR

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Abstract

To enumerate culturable heterotrophic bacteria, during ballast water treatment system verification testing, it is mandated under the ETV protocol that cells are quantified using heterotrophic plate counting (HPC) techniques using media for fresh water and seawater respectively. Yet, it is well established that HPC techniques may underestimate the number of living bacteria present in natural water samples. In the present study HPC was compared with flow cytometry (FCM) and qPCR. All three approaches were applied on a stationary natural fresh water and seawater sample-point over a consecutive 30-week period. Bacterial abundances using HPC, FCM and qPCR generally yielded concentrations in the range of 10^4, 10^6 and 10^7 cells mL^{-1}, respectively. Substantial differences in abundance patterns were observed among the three techniques over time. With respect to FCM, glutaraldehyde-fixed and formalin/hexamine-fixed samples yielded similar results. The absence of a correlation between FCM and qPCR in freshwater samples was potentially caused by variation in gene copy number among various bacterial species. In contrast, no significant differences were observed when a monoculture of *E. coli* was quantified using FCM and qPCR over a 5-day storage period. In conclusion, FCM appears the more reliable technique to detect heterotrophic bacteria in natural water compared to qPCR.

1. Introduction

In 1883, Robert Koch described the first method to count bacteria in water by introducing the Heterotrophic Plate Count (HPC) method (Koch 1912). Since then, HPC has become an important method for water quality monitoring (Payment, Sartory et al. 2003). Applications vary from monitoring hygiene to mapping bacterial communities in natural environments (Oliver 1987, Sartory 2004, Nagvenkar and Ramaiah 2009). Water samples are inoculated on agar plates enriched with organic nutrients. The plates are incubated at a specific temperature and the amount of colony forming units (CFU) is counted after a set number of days. Each CFU may have originated from a single bacterium or perhaps a cluster of thousands of bacteria (Sutton 2011). Therefore, the CFU result is a conservative estimate of the number of culturable cells in a sample.

In recent years, HPC has been used to evaluate the disinfection performance of ballast water treatment systems (BWTS) (Cangelosi, Aliff et al. 2015). The use of HPC in testing BWTS is a direct result of international legislation prescribing limitations on the number of living organisms allowed in discharged ballast water. On March 23, 2012, the United States Coast Guard (USCG) issued the Standards for Living Organisms in Ships' Ballast Water Discharged in U.S. Waters (Final Rule) (USCG 2012). Furthermore, in September 2017, the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention) of the International Maritime Organization (IMO) entered into force (IMO 2004). Both the IMO and USCG set limitations to the number of living organisms discharged in ship's ballast water in their comparable Ballast Water Discharge Standards (BWDS). For ship-owners, to comply with the BWDS, the installation of a BWTS onboard their vessel is, in most cases, the optimal way to achieve compliance. Although, the current BWDS sets no limit to the number of heterotrophic bacteria, the Final Rule describes the commitment of the USCG to periodically assess the feasibility of implementing the Phase-Two BWDS. This Phase-Two is likely to introduce limits on the number of living heterotrophic bacteria in discharged ballast water.

In order for BWTS-manufacturers to install their equipment on seagoing vessels, they have to obtain IMO type approval from an IMO-member flag state. Similarly, to discharge BWTS-treated ballast water in U.S. waters, the BWTS has to obtain USCG type approval. Both the IMO (BWMS Code) (IMO 2018) and the USCG Final Rule (USCG 2012) have adopted a mandatory testing protocol to evaluate the BWTS for Type Approval certification. In the USCG Final Rule, the Generic Protocol for the Verification of Ballast Water Treatment Technology is incorporated by reference (ETV protocol, (NSF-International 2010). The ETV

protocol prescribes that the challenge water used to test BWTS for Type Approval must be evaluated for culturable heterotrophic bacteria. Specifically, HPC techniques must be used with 2216 Marine Agar (MA) and salt-modified R2A agar (R2A) for seawater and brackish water and Plate Count Agar (PCA) and Nutrient Broth (NB) agar for freshwater, using a 5-day incubation period.

It has been recognized that the number of living water-borne bacteria derived from immunofluorescent microscopy exceeds the number of CFU's as recorded by HPC (Xu, Roberts et al. 1982, Colwell 2002). For example, Fluorescent Microscopy (FM) using nucleic acid dyes such as Acridine Orange (AO) and 4',6-diamidino-2-fenylindool (DAPI) indicate that the number of DNA-containing bacteria in natural oligotrophic samples is two orders of magnitude higher than what is recorded by HPC and that this discrepancy disappears when eutrophic bacterial cultures are assessed (Zweifel and Hagström 1995). The discrepancy is also known as the "great plate count anomaly" (Staley and Konopka 1985). Bacteria that are not culturable but are still alive are considered to be in a Viable But Non-Culturable (VBNC) state (Oliver 2005). Several hypotheses for the great plate count anomaly have been proposed. For instance, a vast number of species is present in the sample and each one requires specific environmental conditions to reproduce effectively. In addition, the eutrophic plate media contain nutrients that are orders of magnitude higher than in natural water, thereby favoring only a limited number of species most adept at utilizing these resources (Connon and Giovannoni 2002). The FM observation suggesting that the concentrations of bacteria are underestimated by HPC has been corroborated by using Flow Cytometry (FCM) (Hoefel, Grooby et al. 2003). Similar to FM, in FCM analysis the cells are stained with a fluorescent dye and detected individually by a laser. Data acquisition using FCM is achieved objectively using particle scatter and fluorescence detectors. Although FCM uses well-defined parameters in counting bacterial cells, debris contained in the sample can obscure fluorescent cell signals. Also, the fraction of the bacterial community effectively stained by the fluorescent dyes is challenging to quantify in natural water samples (Gasol and Del Giorgio 2000).

A fourth enumeration technique that yields promising results is the quantitative Polymerase Chain Reaction (qPCR). This technique combines the traditional PCR reaction with real-time fluorescence analysis. Primers are used to target a specific DNA sequence (usually a gene coding region) and PCR amplifies that particular sequence. A double-stranded DNA (dsDNA) fluorescent stain is added to the reaction mix. As a result, fluorescence increases proportionally to the amount of DNA copies formed. Combined with

markers with a known DNA content, this technique can reliably estimate, in real-time, the number of gene copies during the PCR reaction (Skovhus, Ramsing et al. 2004). Two limitations are identified when this technique is used to enumerate organisms. First, genes often have multiple copies per organism, so the number of gene copies tends to overestimates the number of organisms (Nadkarni, Martin et al. 2002). Secondly, if the aim is to exclusively quantify heterotrophic bacteria, interference of genes amplified from autotrophic bacteria (cyanobacteria) and eukaryotic cell organelles (chloroplasts) may result in substantial overestimations when investigating natural water samples (Hodkinson and Lutzoni 2009). While it is recognized that HPC solely detects viable cells, which are the cells or interest, alternative methods like FCM and qPCR are providing important insight in the (otherwise overlooked) VBNC community related to ballast water treatment. Notably, it has been shown that bacteria in the VBNC state can be resuscitated (Chaiyanan, Huq et al. 2001, Fernandez-Delgado, Garcia-Amado et al. 2015). The dispersion of VBNC bacteria by ship's discharges could therefore interfere with the objective of the BWDS to reduce the spread of aquatic invasive species through ballast water. In order to obtain quantitative information on the accuracy of plate counting in BWTS test water, a thorough comparison between HPC, FCM and qPCR was made.

First, procedural tests were performed to compare the ETV-prescribed 5-day plate-observation period with shorter as well as with longer incubation times. In addition, two types of bacterial fixatives were compared to identify the most effective one for bacterial FCM enumeration. Furthermore, an *Escherichia coli* monoculture was analyzed with FCM and qPCR over a 5-day storage period. Finally, bacteria were enumerated using marine and freshwater that was sampled from late winter to autumn with HPC plates, FCM and qPCR.

2. Methods

2.1. Sampling procedure

Surface samples were taken every other week from week 8 until week 38, 2013 from the Marsdiep tidal inlet (marine) and Lake NIOZ (fresh water) using a clean bucket. Salinity and temperature were measured directly in the bucket using a calibrated Conductivity Meter with a Pt sensor (GMH 3430, Greisinger). The sensor was calibrated with the following Laboratory Salinity References: 3, 22, and 32 g KCl kg^{-1}. One liter of the bucket sample was transferred into a polycarbonate bottle and transported to the laboratory for further processing.

2.2. Heterotrophic plate counting

Media for HPC were chosen, based on the recommendations in the ETV protocol (NSF-International 2010). For seawater samples, 27.6-g Difco™ 2216 Marine Agar (MA) was dissolved in 500-mL milli-Q™. The second seawater medium was 9.1-g Difco™ R2A agar (R2A) dissolved in 500-mL Enriched Seawater, Artificial Water (ESAW) medium excluding the ESAW-prescribed nutrients (Harrison, Waters et al. 1980), because R2A agar is an oligotrophic medium intended to cultivate stressed and poor-growing bacteria. For freshwater samples 11.8-g Difco™ Plate Count Agar (PCA) was dissolved in 500-mL milli-Q™. The second freshwater medium was prepared using 4.0-g Difco™ Nutrient Broth and 7.5-g Difco™ Nutrient Agar (NB) dissolved in 500-mL milli-Q™. All media were dissolved in 1-L autoclavable bottles using a microwave oven and subsequently autoclaved 20 minutes (120°C, 0.2 MPa) in a Laboklav (SHP Steriltechnik AG). The media were poured in petri dishes (Ø 100mm x 15mm, VWR) inside a laminar flow bench (Interflow) and stored in a SI-900R incubator (Jeiotech) at 25°C for three days to check for contamination. Positive controls for each plate were made by inoculating them with a swab from a keyboard surface. After collection, samples were diluted 10 times using 0.2-µm filtered sample water. From this dilution, a 100-µL volume was spread onto the plates within 30 minutes after sampling. Triplicate plates were incubated in a SI-900R incubator (Jeiotech) at 25°C.

In week 8, at day 4, day 5 and day 7, Colony Forming Units (CFU) were counted semi-automatically with the aid of a HG/ColonyCounter application (HyperGEAR) installed on an iPad mini (Apple, Model A1432). From week 10 onwards, colonies were solely counted after 7 days of incubation. A digital picture was made of each agar plate, placed on a black background for contrast. A standard setup was used to make sure all plates were photographed under similar circumstances, resulting in a digital database of all plates. The Colony counter software automatically detected colonies on the plate's pictures and the results were immediately subjected to a visual correction by using the add/delete option in the software. Furthermore, the plate itself was examined to verify if a putative colony on the photograph actually was a colony. A detailed comparison between automatic and corrected counting results was not performed, because substantial corrections were required in all cases.

2.3. Flow cytometry

Triplicate samples of 1.5-mL were transferred into 2-mL cryovials (Greiner Bio-One) containing 150-µL 25% (w/v) glutaraldehyde (GA; 2.3% final concentration) or 150-µL 18% (w/v) Formalin/Hexamine (FH 1.6% final concentration), incubated for 30 minutes at 4°C,

frozen in liquid nitrogen and stored at -80°C until further analysis. After thawing at room temperature, samples were diluted 10 times by adding 100-µL sample to 900-µL 0.2-µm filtered Tris-Ethylenediaminetetraacetic acid buffer (Tris-EDTA buffer, pH 8.0). Subsequently, PicoGreen® (ThermoFisher; 500 times commercial stock dilution) was added. The 'total bacteria' stain PicoGreen® was used to target all intact bacterial cells. The results of the 'dead bacteria' stain SYTOX™ Green (ThermoFisher) are not reported because in the natural samples the fluorescent signal of the dead cells stained with SYTOX™ was obscured by fluorescent debris, leading to inconclusive results. Samples were incubated for 15-30 minutes in the dark at room temperature and analyzed using a FACSCanto™ flow cytometer (Becton Dickinson) with a 488 nm laser. As particle-detection trigger, green fluorescence was used (FBG channel, 530 nm). The flow rate was monitored twice per day using Trucount™ beads (Becton Dickinson) diluted in Tris-EDTA buffer. Performance of fluorescence detectors was checked using Cytometer Setup & Tracking beads (Becton Dickinson). Coefficients of variations were maintained below 6%. Results were analyzed using FCS Express version 4 software (De Novo Software).

2.4. qPCR

Within 15 minutes after sampling, 50 mL sample was filtered over a 45-mm 0.2 µm polycarbonate filter (Millipore, Sigma-Aldrich). Filters were stored in cryovials at -80 °C. DNA extraction from the filters was performed using the PowerSoil® DNA isolation kit (MO BIO). After extraction, the resulting 100 µL DNA solution was divided over 3 aliquots: 15 µL for quality control, 20 µL as working solution and 65 µL as backup. All aliquots were stored at -20 °C.

DNA extraction performance was estimated using a NanoDrop (Thermo Fisher Scientific) assessment. Subsequently, DNA concentrations were determined in duplicate using PicoGreen® (250-times commercial stock dilution) and a fluorescence analyzer (488 -> 520 nm) (Spectramax FS2500). The resulting DNA concentration was used to add equal amounts of DNA to each PCR reaction. In addition, a gel electrophoresis including SmartLadder (Eurogentec) was performed to estimate the size and weight of the extracted DNA fragments. As loading dye, Bromophenol Blue was used. All electrophoresis gels contained 1.5% agarose and were run on 80-V for 45 minutes.

The master mix for the standard curve was produced using the following primers for the 16S rRNA gene. F: 341 (5'-CCTACGGAGGCAGCAG-3'), R: 907(A) (5'-CCGTCAATTCATTTGAGTTT-3') and R: 907(C) (5'-CCGTCAATTCCTTTGAGTTT-3'). For one reaction: H_2O (36 µL), 10X PicoMaxx PCR buffer (5 µL, Agilent Technologies),

10X dNTP (5 µL), 50X Bovine Serum Albumin to relief PCR amplification inhibitors (Kreader 1996) (BSA, 1 µL), F:341 primer (0.2 µL), R:907A primer (0.2 µL), R:907C primer (0.2 µL), 125X Taq polymerase (PicoMaxx, 0.4-µL) and template DNA (2-µL) was used. The DNA template originated from extracted DNA of sample water obtained in week 10, for both seawater and fresh water. The PCR conditions were: 95°C (4 min.); 36 cycles: 95°C (30-sec.), 55°C (30 sec.), 72°C (1 min.). Followed by 72°C (7 min.), 4°C (10 min.) and 15°C (∞). Resulting PCR products were used to make a standard curve for qPCR. The length of the standard curve bands was 566 bp which has an average weight of 373,560 Dalton, which results in $6.2 * 10^{-10}$ ng copy^{-1}. Fluorescence analysis indicated that the DNA concentration of the sea water template sample was 1.6 ng µL^{-1}. In order to obtain $1.0 * 10^9$ copies µL^{-1} in 100-µL volume, 38.76 µL sample was diluted in 64.24 µL SPUDA 10^6 + Tris. The standard curve was made by diluting 4 µL of the initial stock to 36 µL of SPUDA 10^6 + Tris and repeating this step nine times. An additional standard curve of fresh water was made in a similar fashion.

The master mix for the samples was made using the following primers targeting the 16S rRNA gene. F: arch519aS15 (5'-CAGCMGCCGCGGTAA-3') and R: bact785bA18 (5'-TACNVGGGTATCTAATCC-3'). Per sample: H_2O (14.2 µL), 10X PCR buffer (PicoMaxx, 2 µL), 10X dNTP (2 µL), 50X BSA (0.4 µL), F:arch519aS15 primer (0.2 µL), R:bact785bA18 primer (0.2 µL), 125X Taq polymerase (PicoMaxx, 0.2 µL), 50X SYBR® Green (0.4 µL) and template DNA (2 µL). As template for freshwater samples, pure sample water was used. For templates of seawater samples, 10 times diluted (milli-Q™) sample water was used to mitigate the inhibitory effect of the seawater minerals on the PCR reaction. The PCR conditions were: 95°C (2 min.), 42 cycles: 95°C (30 sec.), 48°C (40 sec.), 72°C (40 sec.), followed by a fluorescence scan. After 42 cycles: 95°C (10 sec.) followed by a melt-curve from 65°C to 95°C using increments of 0.5°C for 5 sec., including a fluorescence scan, concluded by 15°C (∞).

2.5. FCM and qPCR comparison using E.coli

To compare qPCR with FCM and to study the impact of storage time on bacterial enumeration in a controlled manner, a standardized laboratory test setup was devised. *E.coli* (Vitroids™, Sigma-Aldrich) was incubated in a culture vessel with MacConkey medium at 44°C for 24 h in a Speedy Breedy incubator (BacTest®). To enable linear regression analysis between qPCR and FCM, a serial-dilution was made in four-fold by diluting the source culture 10^1-, 10^2-, 10^3-, 10^4-, 10^5-, 10^6- and 10^7-times with MacConkey medium. Samples for qPCR and FCM were taken from the first two dilution series on day 0. The remaining two

dilution series were stored at 4°C for five days. On day 5 the stored dilution series were sampled for qPCR and FCM analysis. FCM samples were fixed with GA, samples for qPCR were filtered, stored and extracted as described above.

A qPCR analysis was performed using a NovaQUANT™ Coli qPCR Kit (Novagen®), containing the proprietary primers and *E. coli* DNA standard. The DNA standard was used to make a calibration dilution ranging from 1 ng μL^{-1} through 0.1 fg μL^{-1}. To calculate *E. coli* cell concentrations, it was assumed that 10-ng genomic *E. coli* DNA standard was equal to 2,000,000 *E. coli* cells. The mastermix consisted of 10 µL SsoFast™ EvaGreen® Supermix (Bio-Rad), 2 µL NovaQUANT™, 6 µL PCR grade water and 2 µL *E. coli* standard or sample. Samples were run on a CFX96-Real Time System (Bio-Rad) using the following protocol: 98°C (2 min.), 98°C (10 sec.), 60°C (25 sec.), measure fluorescence, for 41 cycles. The DNA melt-curve was determined using 0.2°C increments for 10 sec. from 75°C to 95°C. *E. coli* samples for FCM were processed and analyzed as described in Chapter 2.3, with the change in DNA stain to SYBR® Green in lieu of PicoGreen®.

2.6. Statistical analysis

A least squares linear regression model was used for testing the null hypothesis (H_0) that there is no significant correlation between two groups. Because there was little temporal variation in bacteria concentrations, additional single-factor ANOVAs on log(x+1) transformed data were performed to test H_0 that there is no difference between the means of two analysis methods. H_0 was rejected at $P<0.05$. All statistical analyses were performed in Microsoft Excel for Mac v16.45.

3. Results

3.1. Procedural

3.1.1. Fungal overgrowth and smear

Of the 204 petri dishes included in the HPC experiment, five plates (2%) were excluded from analysis due to fungal overgrowth. Furthermore, twelve plates (6%) were excluded from analysis due to bacterial growth smear that obscured distinct colonies. Fungal growth was considered interfering with the analysis solely when the whole plate was overgrown. Fungal interference was observed four times in PCA and once in NB medium. No fungal interference was observed in both saltwater media. The presence of smear was more or less evenly observed over the four media types.

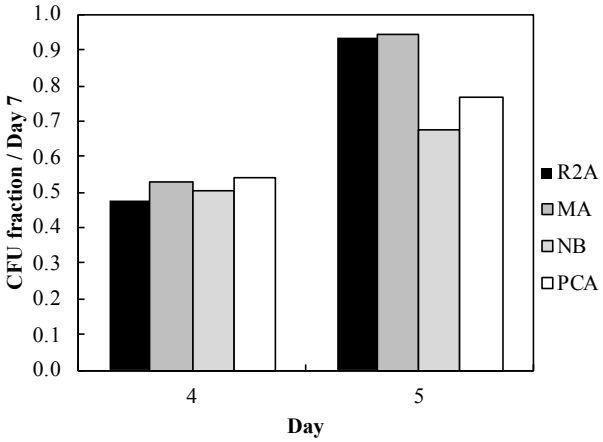

Figure 1. *Number of colony forming units (CFU) detected on four HPC media after 4 and 5 days incubation relative to a 7-day incubation period.*

3.1.2. Incubation time effect on CFU counts

In week 8, at the ETV-prescribed 5-day incubation time CFU numbers were in 84% of cases lower then after 7 days of incubation. Furthermore, CFU counts were at 50% after 4 days relative to 7 days for both freshwater and saltwater media. At day 5, the saltwater and freshwater media yielded 94% and 71% of CFU's compared to day 7, respectively (Figure 1). Subsequent HPC incubations were solely counted on day 7 to obtain the maximum number of CFU's.

Table 1. *Average bacteria concentrations during week 8 to 38, 2013.*

Method	Salinity	Sub-method	Average (mL^{-1})	CV (%)	Min – Max	
HPC	Freshwater	NB	1.5×10^4	72	0.7 – 42.0	$\times 10^3$
		PCA	8.6×10^3	45	0.0 – 1.7	$\times 10^4$
	Seawater	R2A	3.3×10^3	92	0.0 – 1.3	$\times 10^4$
		MA	3.9×10^4	91	0.1 – 14.1	$\times 10^4$
FCM	Freshwater	FH	3.8×10^6	84	0.7 – 8.9	$\times 10^6$
		GA	4.5×10^6	84	0.3 – 11.4	$\times 10^6$
	Seawater	FH	4.4×10^6	52	2.1 – 9.7	$\times 10^6$
		GA	4.5×10^6	63	1.2 – 11.9	$\times 10^6$
qPCR	Freshwater		5.2×10^7	52	0.4 – 11.7	$\times 10^7$
	Seawater		2.2×10^7	65	0.5 – 6.8	$\times 10^7$

CV = Coefficient of Variance; HPC = Heterotrophic Plate Count Agar; FCM = Flow Cytometry; qPCR = quantitative PCR; NB = Nutrient Broth; PCA = Plate Count Agar; FH = Formalin/Hexamine; GA = Glutaraldehyde; MA = Marine Agar; R2A = salt-modified R2A agar;

3.1.3. Comparing fixatives for FCM

Samples fixed with GA showed a slightly higher bacterial abundance than samples fixed with FH, both in fresh water and seawater (Table 1). However, when comparing the log(x+1) transformed data with a single-factor ANOVA, the means were not significantly different from each other (Table 2a and 2b). Regression analysis showed an obvious correlation between the two fixative results (Figure 4c and 4d) with high R^2 values of 0.99 and 0.98 in freshwater and seawater, respectively (Table 3).

3.1.4. E.coli experiment

Using an *E. coli* monoculture, cell concentrations obtained from qPCR and FCM were highly correlated. Results were log-transformed and a least squares linear regression model on Day 0 and Day 5 concluded significant correlations within and between days and methods ($p < 0.001$ in all cases) (Figure 2). An ANOVA test found no significant differences between the means of qPCR and FCM irrespective of the day of sampling ($p = 0.88$). In other words, neither the use of different detection techniques nor the 5-day storage at 4°C yielded significant differences in the abundance of *E. coli* cells.

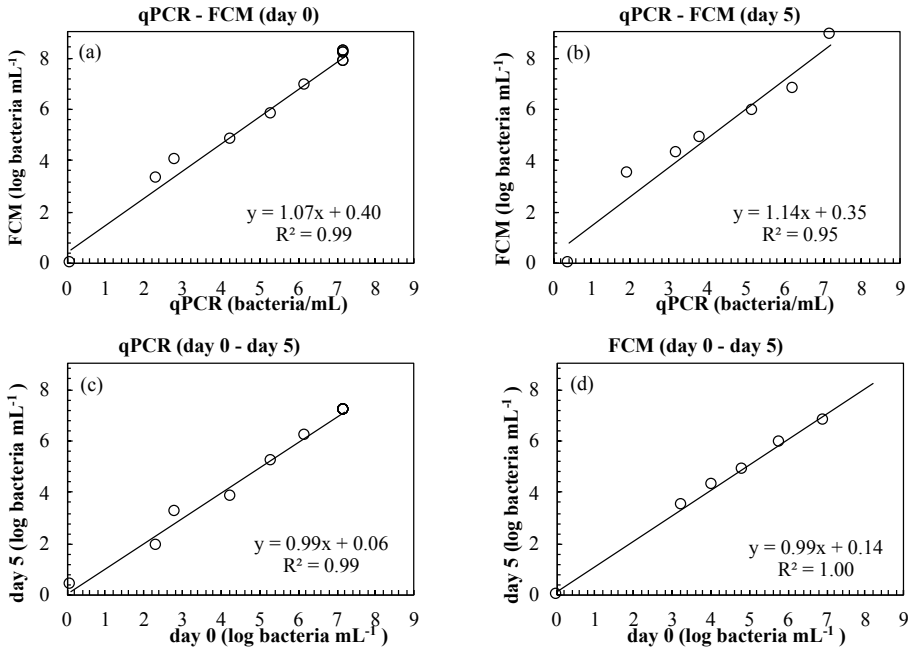

Figure 2. *E. coli experiment, comparing FCM (flow cytometry) and qPCR within (**a**, **b**) and between days (**c**, **d**).*

3.2. Experimental

3.2.1. Temporal development and technical differences

In Figure 3, Table 1 and Table 2, it is shown that bacterial concentrations obtained by the HPC, FCM and qPCR were significantly different from each other. These differences were similar between freshwater and seawater. The HPC methods yielded the lowest bacterial concentrations, ranging from 10^3 and 10^4 cells mL^{-1}. Samples analyzed using FCM yielded bacterial concentrations two to three orders of magnitude higher at 10^6 cells mL^{-1}. The highest estimates of bacterial abundances were observed using qPCR at 10^7 cells mL^{-1}. The coefficient of variation (CV) of the various techniques ranged from 45-91% (Table 1).

3.2.2. Regression models of HPC, FCM and qPCR

In general, the correlations between the three techniques were poor (Figure 4, Table 3). The techniques yielded significant differences (Table 2). The best correlations were found between FCM and qPCR, notably in seawater. (Figure 4e and h). Large positive intercepts as in the ETV-required plate count techniques indicate systematically higher bacteria concentrations in NB compared to PCA and R2A compared to MA (Figure 4a and b).

Table 2. *Results of a single-factor ANOVA on log(x+1) converted seawater and freshwater data to test the null hypothesis (H_0) that there is no difference between the means of two analysis methods in **a** freshwater and **b** seawater. $P<0.05$ (in italics) indicates a significant difference between the means.*

Table 2a

P Freshwater	NB	PCA	FH	GA
NB				
PCA	*0.03*			
FH	*0.00*	*0.00*		
GA	*0.00*	*0.00*	0.40	
qPCR	*0.00*	*0.00*	*0.00*	*0.00*

Table 2b

P Seawater	MA	R2A	FH	GA
MA				
R2A	*0.00*			
FH	*0.00*	*0.00*		
GA	*0.00*	*0.00*	0.71	
qPCR	*0.00*	*0.00*	*0.00*	*0.00*

NB = Nutrient Broth; PCA = Plate Count Agar; FH = Formalin/Hexamine; GA = Glutaraldehyde; qPCR = quantitative PCR; MA = Marine Agar; R2A = salt-modified R2A agar;

Table 3. *R^2 results of correlation plots between two datasets in **a** freshwater and **b** seawater.*

Table 3a

R^2 Freshwater	NB	PCA	FH	GA
NB				
PCA	0.11			
FH	0.01	0.01		
GA	0.00	0.02	0.99	
qPCR	0.01	0.00	0.02	0.11

Table 3b

R^2 Seawater	MA	R2A	FH	GA
MA				
R2A	0.10			
FH	0.11	0.34		
GA	0.00	0.32	0.98	
qPCR	0.01	0.02	0.40	0.48

NB = Nutrient Broth; PCA = Plate Count Agar; FH = Formalin/Hexamine; GA = Glutaraldehyde; qPCR = quantitative PCR; MA = Marine Agar; R2A = salt-modified R2A agar;

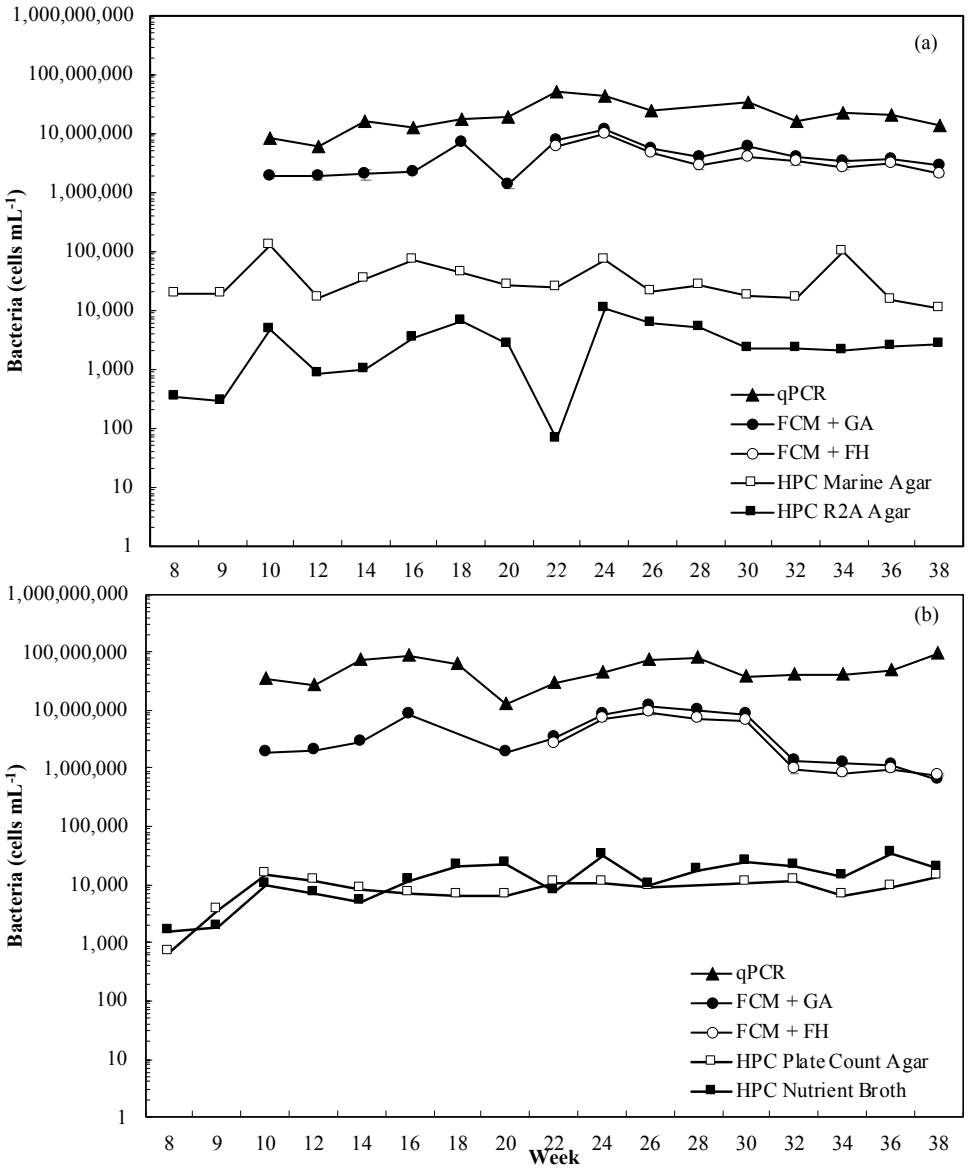

Figure 3. *Average concentrations of bacteria in* ***a*** *Seawater and* ***b*** *Freshwater from week 8 to week 38 (2013) using five different quantification techniques. qPCR in gene copy numbers* mL^{-1}*; FCM in cells* mL^{-1}*; HPC in colony forming units* mL^{-1}*.*

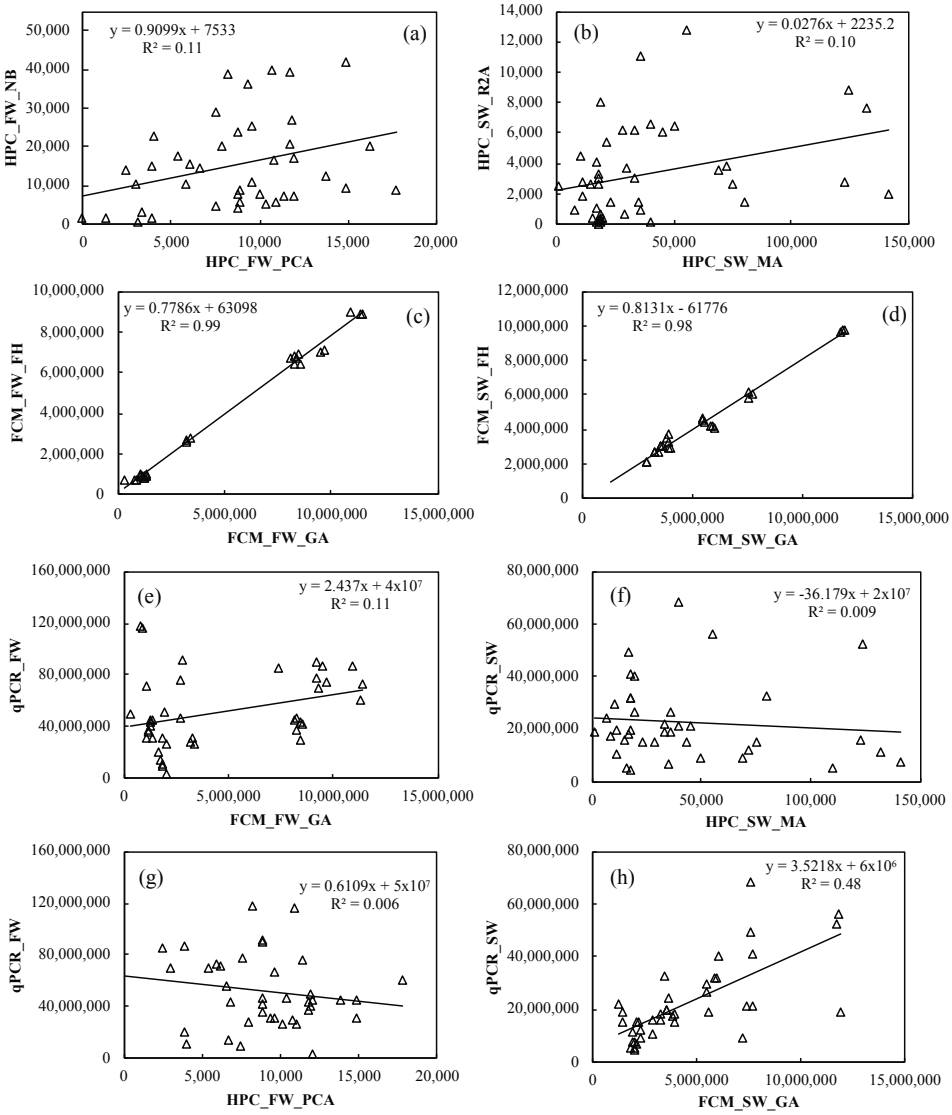

Figure 4. *Regression analysis between* ***a*** *PCA (plate count agar) and NB (nutrient broth) in freshwater.* ***b*** *MA (marine agar) and R2A (salt-modified) in seawater. FCM (flow cytometry) in* ***c*** *freshwater and* ***d*** *seawater comparing GA (glutaraldehyde) and FH (formalin/hexamine) fixative. qPCR and FCM in seawater using* ***e*** *GA and* ***f*** *FH fixative and similarly for freshwater in* ***g*** *and* ***h.***

4. Discussion

4.1. Procedural

The observed fungal interference in freshwater media was also reported in a HPC study of groundwater using the low-nutrient NWRI agar and R2A agar, but similar to the salt-modified R2A agar results reported above, the referenced study did not report fungal interference from groundwater samples on R2A agar (Lillis and Bissonnette 2001). Fungal interference could not be related to low-nutrient agar, since both R2A and NWRI are low-nutrient media. The frequent observation of fungal growth on HPC plates raises the question whether fungi should also be included in future ballast water treatment studies. The significance of fungi in water systems is still poorly understood, however it has been reported that certain fungi are known to infect phytoplankton species (Wetsteijn and Peperzak 1991).

Generally, colony count software provides a time-saving benefit in case of >100 colonies per plate. Also, the manual addition or deletion of CFU's in such software is helpful to keep track of the number of colonies. The resulting photos provide a convenient database for later reference.

In FCM analysis both GA and FH samples fixed with GA yielded results that were not significantly different from each other in both seawater and freshwater. Both GA and FH fixatives perform relatively similar to each other. Due to an apparent higher result when using GA compared to FH it is not recommended to use the fixatives interchangeably when comparing relative differences among samples.

The 3- to 5-day plate-incubation time, as prescribed in the ETV protocol for HPC, underestimates the concentration of culturable heterotrophic bacteria by 6% (saltwater) to 29% (freshwater) compared to a 7-day incubation time. Considering that culturable aerobic heterotrophic bacteria are subject to a minimum test water uptake requirement of 10^3 CFU mL^{-1}, this may lead to the erroneous conclusion that this requirement was not met. At present there is no risk of a false-negative result for discharged ballast water since the BWDS does not currently prescribe discharge limitations on heterotrophic bacteria. However, this may change in future revisions of ballast water regulations such as the USCG Phase-Two Standard (USCG 2012). In general, it appears that the minimum challenge conditions for culturable heterotrophic bacteria in the ETV protocol and the BWMS Code are too high, considering that the amount of VBNC's as measured by FCM or qPCR consistently exceed the minimum challenge conditions by orders of magnitude.

4.2. Experimental

It was noteworthy to observe low levels of correlation among HPC, FCM and qPCR

methods. Although it was anticipated that absolute quantities would be different, it was nevertheless expected that growth patterns among various methods would indicate similar trends. Instead, growth patterns were reasonably stable during the 30-week sampling period as the CV was <100% in all methods. Among techniques opposing growth trends were occasionally observed. For example, qPCR results increased from week 30 through week 38 while conversely, FCM methods showed a decline in bacterial abundance (Figure 3b). In short, growth patterns showed a completely different pattern among HPC and FCM and qPCR. The absence of meaningful correlations between different enumeration methods is in addition to the significant differences in the means. HPC, FCM and qPCR result in significantly different mean result and are not significantly correlated.

An important consideration is the presence of VBNC's. In recent years the widespread presence of these bacteria has been elucidated. Some VBNC's are normally culturable but in response to stress-factors become non-culturable. They are not dead however, and under the right circumstances they can regain their ability to grow and divide (Oliver 2010). Among the identified VBNC are all human pathogens included in the IMO and USCG ballast water discharge regulations. Most notably, VBNC *Vibrio cholerae* O1 was identified in Bangladesh in 1994 (Islam, Miah et al. 1994). And in 1996 it was observed that VBNC *V. cholera* O1 could revert to a culturable state in the human intestine (Colwell, Brayton et al. 1996). Ballast water treatment can be regarded as a stress factor for many bacterial species, potentially inducing VBNC-state. So, the presence of VBNC bacteria, which are thus undetectable by HPC methods, but remain a potential threat, is recommended to be considered in the choice of detection method. Furthermore, as long as the heterotrophic bacteria are solely monitored for the challenge they pose to a BWMS, it could be argued that the total number of bacteria is more relevant than the culturable number of bacteria, as VBNC bacteria presumably pose a similar BWMS challenge as culturable bacteria (e.g., active substance degradation or light-attenuation)

4.3. qPCR and quantifying bacteria

The high degree of correlation between *E. coli* concentrations obtained with qPCR and FCM was remarkable because in natural samples these correlations were lower or absent. This discrepancy may partly be attributed to varying 16S rRNA gene copy numbers among bacterial species. When assessing a monoculture using the NovaQUANT™ assay, calibration material based on known gene copy numbers is readily available for *E. coli*. In natural samples, the bacterial species assembly is unknown, so the average gene copy number needed to convert the gene-copy results to actual bacterial cells is not readily available. Studies have

been dedicated to estimate 16S rRNA gene copy numbers more reliably (Vetrovsky and Baldrian 2013), and some estimates, marginally improved the comparison between FCM and qPCR (data not shown). Due to the legal implications of the BWDS, to introduce qPCR as quantitative tool in ballast water compliance testing, the average gene copy number of 16S rRNA is required per sample. In order to obtain this, the bacterial species assembly of each ballast water sample should be determined using complicated and time-consuming molecular techniques. If, finally, a reliable gene copy estimate is obtained, it is expected (based on the *E. coli* experiment) that the resulting cell abundance will largely resemble cells concentrations obtained using FCM. Therefore, FCM is considered more user-friendly, cheaper and quicker than qPCR to reach a similar endpoint. Thanks to its specificity however, for the indicator microbes ballast water compliance methods based on qPCR have been developed for *E.coli, Enterococci* and *V. cholerae* (Darling and Frederick 2018).

4.4. Concluding remarks

In conclusion, FCM appears more reliable than qPCR to detect total bacterial abundance in natural water samples. Most importantly, there was no correlation between HPC and FCM results in bacterial trends over time. Therefore, these results support the notion that the prescribed HPC techniques are not predictive of the actual challenge posed by bacteria in the challenge water. More fundamentally, it is unclear whether the challenge posed by bacteria can be distinguished from other organic matter sources contained in the Dissolved and Particulate Organic Carbon (DOC, POC) pools. Organic matter poses a challenge to oxidant-based systems by reacting with hypochlorite, thus (potentially) forming disinfection byproducts and lowering the Total Residual Oxidants (TRO) available to kill the organisms in the treated water. This chemical process supposedly does not discriminate between organic matter originating from dead or living material. Compared to the challenge water POC requirements for type approval testing the amount of Carbon of 10^6 bacteria/mL is negligeable (5 mg C/L vs 26 µg C/L, respectively) (Trousselier, Bouvy et al. 1997). In UV-systems, the main challenge is low ultraviolet-transmission (UV-transmission) in the water caused by attenuating substances. The main contributors to UV attenuation are humic and fulvic acids as part of the DOC fraction. It is unclear how living bacteria contribute to the challenge posed to UV-based BWMS other than being part of the POC fraction, which has its own minimum required concentration in the challenge water. Therefore, in the absence of discharge limits, the relevance of the heterotrophic bacterial challenge requirements to the type approval process must be further investigated. At the same time, it must be noted that in the absence of regulating heterotrophic bacteria, high bacterial growth in treated ballast water

can occur as a side-effect of treatment, which impact should also be further assessed (Stehouwer, van Slooten et al. 2013).

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