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# Development of a novel automated analytical method for viability assessment of phytoplankton used for validation of ballast water treatment systems

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## Abstract

To limit the spreading of aquatic invasive species, regulations require ships' ballast water to be treated before discharge. To validate ballast water treatment system (BWTS) performance, treated water is analyzed for living organisms in different size classes. Quantitative assessment of the size class 10–50 μm (mainly phytoplankton) is carried out using the vital stain method, which requires labor-intensive manual microscope counts of fluorescent (i.e., living) cells. The method is slow, demands specialized personnel, and is challenged by subjectivity and mobile organisms. Using a high-content screening platform (HCS-Platform) and image analysis, we developed an automated, objective and faster quantification method. The automated method neutralized subjectivity by using fixed cell recognition parameters for image analysis. The implementation of membrane filters gently manipulated the organisms into a 2D plane that reduced mobility. Quantifications were performed at different concentrations using monocultures of slow-moving *Rhodomonas salina*, highly mobile *Tetraselmis suecica* and natural algae. Results were compared to the standard manual counting procedure. Automated counts of monocultures were comparable to manual counts at low and medium concentration levels. Manual counts of *T. suecica* at high concentration levels were significantly lower compared to automated counts stressing the challenge to count mobile cells in 3D. Natural algal counts were similar for both counting approaches, but accuracy was challenged by colony forming species and high number of algal species ~ 10 μm. Automated counts were significantly faster than manual counts. In conclusion, the HCS-Platform showed promising results as an alternative quantitative phytoplankton assessment method for BWTS validation.

**Keywords** Advanced microscopy · Algae · Invasive species, ballast water · Image analysis · Monitoring · Ballast water treatment systems

## Introduction

The Ballast Water Management Convention (IMO 2004) entered into force in 2017 which created an increase in the demand for the installation of ballast water treatment systems (BWTSs) in ships. The convention states that to prevent the spread of aquatic invasive species, all ships carrying ballast water (with few exceptions) are required to have a Type Approved BWTS installed that removes, kills, or renders organisms harmless (IMO 2004). In the Type Approval certification process, the performance of BWTSs are evaluated at certified land-based testing facilities through the collection of treated ballast water (BW) samples and subsequent quantification of viable organisms in different size classes. The size classes consist of plankton ≥ 50 μm, plankton ≥ 10 to < 50 μm (hereafter 10–50 μm), and some indicator microbes (Toxigenic *Vibrio cholerae*, *Escherichia coli*, and intestinal *Enterococci*) (IMO 2004).

The validation procedures for Type Approval require that a BWTS must consistently demonstrate that certain discharge standards are met in five consecutive land-based test cycles for each of three test water salinities: freshwater (< 1 psu), brackish (10–20 psu), and marine water (28–36 psu) (IMO 2016a). The land-based test cycles are followed up with a ship-board test which also consists of five consecutive test cycles. The purpose of the ship-board test is to validate the BWTS

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performance after installment on the ship. The discharge standards are outlined in IMO Regulation D-2 and state that treated BW must contain < 10 org. $m^{-3}$ for plankton ≥ 50 μm, and < 10 org. $mL^{-1}$ for plankton 10–50 μm (IMO 2016a).

Test intake water and control discharge water must fulfill certain concentration requirements. To ensure that BWTS are properly challenged in the treatment process, the concentration requirement for intake water for the size class 10–50 μm during land-based testing is ≥ 1000 org. $mL^{-1}$ (IMO 2016a). Control discharge water is a certain volume of the intake water that has bypassed the treatment system and is stored in a holding tank to simulate BW storage conditions. To confirm that an observed elimination of organisms in treated discharge water is a result of the BWTS and not environmental conditions, the concentration requirement for control discharge water for the size class 10–50 μm is ≥ 100 org. $mL^{-1}$ (IMO 2016a). The IMO G8 Guidelines for approval of ballast water management systems state that for intake, control discharge, and treated discharge water, the number of 1 mL subsamples for the size class 10–50 μm that need to be analyzed for each test cycle is 3, 6, and 6, respectively (IMO 2016a). In land-based validation procedures, a total volume of 15 mL per test cycle is therefore required to be analyzed.

For quantification of organisms in the size class 10–50 μm (mainly phytoplankton), the most widely accepted standard method is the vital stain (VS) method (Steinberg et al. 2011; IMO 2016b). When using the VS method, regulations state that BW samples must be analyzed within 6 h (IMO 2016b). The analysis involves staining of the BW samples with the fluorescent dyes: fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA) which both freely cross the cell membrane. If cells are alive, the initially quenched stains are activated by unspecific enzyme activity. The resultant cell-impermeable green fluorescent molecule allows the cells with enzyme activity and intact cell membrane to be quantified as living through direct microscopic counts (Rotman and Papermaster 1966; Steinberg et al. 2011; IMO 2016b).

The accuracy of the VS method has been questioned in a number of studies which have highlighted the problem with ambiguous staining of live and dead cells, as well as the variability in staining intensities among different algal species and growth stages (Garvey et al. 2007; Peperzak and Brussaard 2011; MacIntyre and Cullen 2016). Furthermore, the VS method involves labor-intensive direct microscope counts by specialized personnel that are challenged by fatigue and subjectivity regarding size class and live/dead classification. The VS method is typically carried out using a standard Sedgewick Rafter counting chamber holding a volume of 1 mL. The vertical water column of the counting chamber allows highly mobile algal species to move in 3D, further complicating counting performance. All together, the described issues limit the performance of the *human counting machine* and significantly reduce quantification accuracy (First and Drake 2012; Bradie et al. 2018). Despite the concerns raised about accuracy and sensitivity, several studies have described the method as a powerful and robust method for evaluating treatment performances of BWTSs (Steinberg et al. 2011; Adams et al. 2014; Baek and Shin 2015).

To address the outlined issues with manual counts, the aim of the current study was to develop an automated, faster and more objective counting approach based on the VS method which could be used as an alternative approach for BWTS validation. Here we describe an approach involving a high-content screening (HCS) platform that scans test water samples containing phytoplankton where VS have been applied. Subsequently, samples underwent image analysis for quantification of fluorescent algal cells. The automated approach was performed in combination with a filter technique that limited the mobility of highly mobile organisms. The approach was tested on fluorescent beads, monocultures of two algal species: (1) low mobility *Rhodomonas salina* and (2) high mobility *Tetraselmis suecica* and on natural algal compositions. The results obtained from automated counts were compared to standard direct manual counts using the VS method to evaluate the effects of concentration levels, mobility, cell size, and algal species composition (complexity) on quantification accuracy.

## Materials and methods

### Experimental design

The test water (20 psu, Marine SeaSalt, Tetra, Germany) was prepared to represent three composition complexity levels: (A) low complexity with fluorescent beads, (B) medium complexity with algal monocultures, and (C) high complexity with natural algae. At each composition complexity level, different algal concentrations were evaluated using manual microscope and automated counts for comparison.

#### (A) Fluorescent beads

Fluorescent beads (6 μm FocalCheck microspheres, F14807, Invitrogen) were initially used to test the manual and automated performance standard on non-mobile objects of a similar shape and size and fluorescence intensity as algal cells. This size of beads was chosen as they are below the minimum size class of 10–50 μm.

#### (B) Algal monocultures

*Tetraselmis suecica* and *Rhodomonas salina* were grown as semi-continuous cultures at 20 °C in 500 mL Erlenmeyer bottles containing 20 psu artificial seawater (Marine SeaSalt, Tetra, Germany) enriched with f/2-medium (Guillard and

Ryther 1962) under 12:12 h light/dark conditions. Three concentration levels were evaluated: (1) low level at 10–30 org. $mL^{-1}$, (2) medium level at 100–140 org. $mL^{-1}$, and (3) high level at 1100–1300 org. $mL^{-1}$. For each concentration level, a total of ten counting rounds were carried out consisting of one manual and one automated count. One round typically took 45–60 min including preparation of equipment/microscopes, rinsing, and evaluation of counts.

#### (C) Natural algal populations

Natural seawater was collected from Kerteminde Fjord, Denmark in November 2018 (17.6 psu, 10.2 °C). Approximately 10 L of seawater was subsequently filtered through a 45-μm filter device (polyamide, Ø47 mm) to remove large particles and zooplankton. The water was then transported to the laboratory and slowly (~ 2 h) acclimatized to room temperature (18 °C). For experiments, the water container homogenized by gentle inversion before transferring 100 mL to an Erlenmeyer flask that was placed on a magnetic stirrer (300 rpm). For evaluation of living organisms, eight manual and ten automated counts were carried out. In addition, a sample of 50 mL was preserved with 1% acidified Lugol's for later determination of algal species and phyla composition.

### Quantification procedures of algal cells

The number of living cells in the test water was quantified using the VS method. A combination of fluorescein diacetate (FDA) and 5-chloromethylfluorescein diacetate (CMFDA, both from Molecular Probes-Invitrogen, USA) was used. Samples were prepared by adding 5.5 μL of 500 μM CMFDA and 5.5 μL of 1000 μM FDA to 1.1 mL test water in an Eppendorf tube that was kept in a dark container and counted after 10–15 min. Classification of living and dead cells during quantification followed the criteria described in Steinberg et al. (2011) and IMO (2016b).

### Manual microscope counts

Manual counts were performed using a standard 1 mL Sedgewick Rafter counting chamber (Pyser – SGI Limited, UK). To quantify the algal concentrations (org. $mL^{-1}$), the whole stained sample was counted on a fluorescence microscope (Leica DMR) using a × 10 objective (× 10/25 PH1, HC PL FLUOTAS) (Table 1). All manual samples were counted by one analyst to keep variations in subjectivity and individual visual criteria at a minimum. In total, over a period of 8–10 h, 10 samples of 1 mL were analyzed per experiment.

### Automated counts

Due to visible obstruction caused by the filter used in this assay, samples can only be analyzed on an inverted microscope. Automated counts of algal cells were performed on a high-content screening platform (HCS-Platform) (Nikon Eclipse T*i*-E inverted microscope, Zyla sCMOS camera) with integrated image analysis software (NIS-elements imaging software). On a glass slide, 2 × 500 μL drops of stained sample were placed within two outlined circles (Fig. 1a). Two membrane filters (diameter = 47 mm, pore size = 0.8 μm) were carefully placed on top of the drops which spread out the stained sample under the membrane filters. On top of each membrane filter, a "suction" filter was placed to produce an upwards removal of water through the membrane filter. Consequently, the membrane filters were gently forced towards the glass slide and at the same time, algal cells were trapped in a 2D plane as they are unable to move through the membrane filter. The "wicking" filters were then removed, and the glass slide was placed in the stage of the HCS-Platform and covered with a black box to prevent external light from interfering with the analysis (Fig. 1b).

The glass slide was scanned using a × 4 objective to obtain multiple images of each 3 × 3 mm that in combination covered the whole area of the two circles (Table 1). The images were afterwards subjected to an *object count analysis* using the NIS-Elements imaging software with pre-set algal cell recognition parameters to estimate the number of algal cells per mL. In total, over a period of 8–10 h, 10 samples of 1 mL were analyzed per experiment.

For the two monocultures, the algal cell recognition parameters were based on *Area*, *Width*, *MinFeret* (the minimum caliper diameter), *Circularity*, *Mean intensity*, and an *Intensity threshold* (Table 2). Recognition criteria were based on the range of recognition parameter measurements of 1195 individuals of *T. suecica* and 1140 individuals of *R. salina*. The minimum and maximum values of the ranges when combining both species were selected as recognition criteria to ensure the inclusion of both species in the object count analyses. The natural algal cells were a mix of species, shapes, and sizes and the recognition criteria were based on the range of recognition parameter measurements of 140 individuals.

To evaluate the accuracy of the cell recognition criteria used for the automated approach, every image from a randomly selected high concentration level scan of monocultures (*R. salina*: run no. 5 and *T. suecica*: run no. 5) and natural seawater (run no. 5) were thoroughly evaluated manually and compared to the automated counts.

Automated counts of autofluorescent beads were performed using a Sedgewick Rafter counting chamber. An aliquot (1 mL) of sample containing beads was transferred to the counting chamber and left for 10 min to allow sedimentation of the beads. The sample was scanned using two different

**Table 1** The calculated number of "frozen" images needed to scan a full sample in a standard Sedgewick Rafter counting chamber (circular view) compared to using the filter technique (quadrangular view)

| | Sedgewick rafter, × 10 | HCS and filter platform × 4 |
|---|---|---|
| Objective magnification | × 10 | × 4 |
| Eyepiece magnification/total magnification | × 10/× 100 | – |
| Field of view diameter/square (mm) | 2.5 | 3 |
| Field of view area ($mm^2$) | 4.9 | 9 |
| Scan area of Sedgewick Rafter/filters ($mm^2$) | 1000 | 3470 |
| Number of images to ideally cover whole scan area | 204 | 475[a] |
| Scan area including "horizontal slices" factor (× 70/× 1) | 14,490 | 475 |

[a] In theory, 385 images were needed to cover the whole area but in practice 475 images were needed to make certain circle edges were covered completely

magnification objectives (× 4 and × 10) on the HCS-Platform and analyzed with the image analysis software.

## Counting times

Counting time was defined as the time from when the Sedgewick Rafter counting chamber/glass plate with filters was placed in the microscope/HCS-Platform until a final concentration was estimated by tally counter for the manual counting approach or through object count analysis software for the automated approach. Counting time is presented in min $mL^{-1}$. In addition, counting times (min $mL^{-1}$) were extracted from a number of randomly selected land-based tests of BWTSs performed at DHI, Denmark (certified by IMO and USCG to test and approve BWTS).

## Identification and quantification of natural algal populations

Samples were analyzed with an inverted microscope according to the Utermöhl method (Utermöhl 1931). An aliquot (50 mL) of the Lugol's preserved natural seawater sample was gently mixed by inversion before settling in a sedimentation chamber for 24 h. Microalgae where then identified down to species level (where possible) using a Nikon inverted microscope (Eclipse TS2-FL).

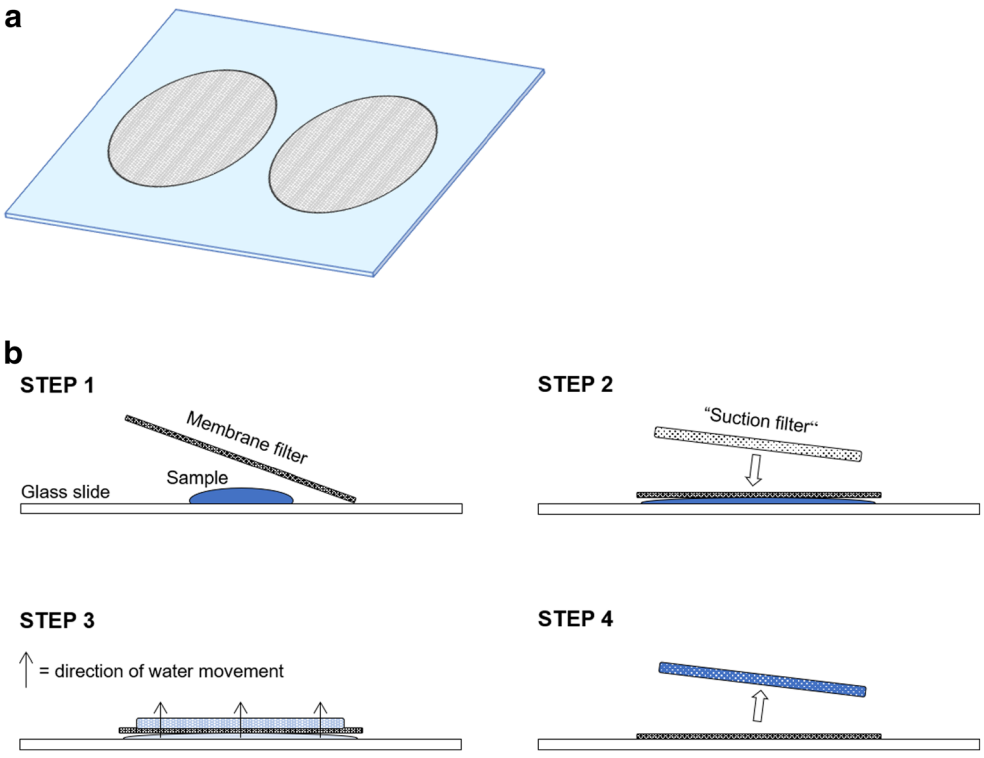


**Fig. 1** Outline of the different steps of the filter technique bringing the organisms from a 3D to a 2D area. **a** Glass slide with two marked circles for placement of 500 μL stained sample in each circle. **b** A membrane filter (0.8 μm pore size) was carefully placed on top of each drop of stained sample (Step 1). A "suction" filter was placed on top of the membrane filter to drag water upwards through the membrane filter (step 2 and step 3). This caused suction of the membrane filter to the glass slide trapping the stained cells in a 2D plane (step 4). After suction of water, there was still a thin layer of water left between the glass plate and membrane filter preventing algal cells from drying out

**Table 2** Range of cell recognition parameters based on *n* individuals of *Tetraselmis suecica*, *Rhodomonas salina* and natural algae and the selected recognition criteria to trigger detection during Object Count analysis in NIS-Elements

| | *Tetraselmis suecica* | *Rhodomonas salina* | Recognition criteria monocultures | Natural algae | Recognition criteria natural algae |
|---|---|---|---|---|---|
| *n* | 1195 | 1140 | 1195 + 1140 | 140 | |
| Area (μm) | 85.3–320 | 56.0–291 | 56–320 | 74.7–1309 | 74–2000 |
| Width (μm) | 6.16–14.9 | 5.5–13.4 | 5.5- | 6.5–26.4 | 6.5- |
| MinFeret (μm) | 8.12–18.0 | 8.16–18.0 | 8.1–50 | 9.7–41.8 | 9–50 |
| Circularity | 0.55–0.98 | 0.62–0.98 | 0.54–1.00 | 0.53–0.97 | 0.53–1.00 |
| Roughness | | | | 0.84–0.96 | 0.84–1.00 |
| Mean intensity | 2966–3910 | 2854–3787 | ≥ 2854 | 2846–3808 | 2846- |
| Intensity threshold | 1800 | 1600 | 1800 | 1800 | 1800 |

### Immobilization experiments using acetic acid

The highly mobile algal species, *T. suecica*, is pH sensitive and can be immobilized at pH of approximately 5.0 for an extended period of time (hours) without any effect on the cell membrane (data not shown). A modified procedure from Steinberg et al. (2012) was used to test immobilization efficiency of acetic acid on *T. suecica* and *R. salina* (Appendix 1). A volume of 500 μL stained sample with algal cells was mixed with 600 μL of 5 mM acetic in water (AA water). The final mix thus contained 2.73 mM AA water and stained test water. Both automated and manual counts were performed using a Sedgewick Rafter counting chamber. In addition, a Sedgewick Rafter counting chamber modified from 1 mL to 250 μL was used.

### Statistics

Statistical analysis was carried out using IBM SPSS Statistics 24. Comparison of means was done using a one-way ANOVA and a significance level of 0.05. Data was also tested for normality (Shapiro-Wilk) and homogeneity of variances. Where appropriate, tests were followed up with Tukey's post hoc test for testing multiple means in a dataset against each other. Comparison of regression lines and interaction analysis were performed using ANCOVA (Statgraphics 18) and a significance level of 0.05.

## Results

### Fluorescent beads

There were no significant differences between manual and automated counts ($n = 5$) of 6 μm fluorescent beads at the three different overall mean concentration levels of 1292 ± 45, 69 ± 3, and 3.8 ± 0.2 beads mL$^{-1}$ (Fig. 2). Overall, mean concentration levels were defined as the mean of the counted concentrations for each approach at each level.

### Comparison of manual and automated counting performances

Counts were carried out over a period of approximately 8–10 h at fixed intervals. Time did not have any effect on algal concentrations in the test water (Figs. 3 and 4). Counts for both approaches and for both monoculture species only deviated slightly above or below the mean of all count lines except for Fig. 3e, where the automated counts were consistently lower than the manual counts.

For natural algal species counts, the variations around the overall mean were larger compared to monoculture counts, but there was no significant effect of time on the variation in the test water concentration (Fig. 4).

At the low and medium algal concentrations, no significant differences were observed for *R. salina* and *T. suecica*

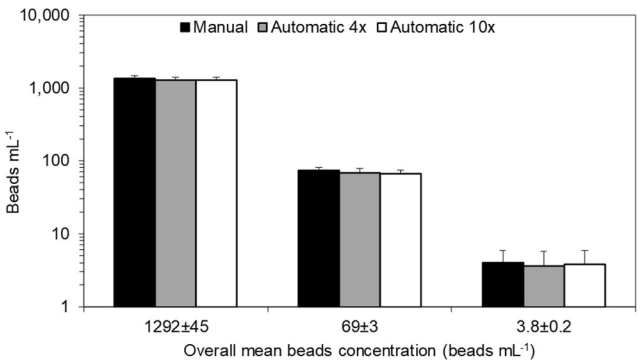


**Fig. 2** Mean ± SD concentrations (beads mL$^{-1}$) of 6 μm fluorescent beads using a standard Sedgewick Rafter counting chamber estimated manually in a fluorescence microscope (black) and automatically with × 4 (gray) and × 10 magnification objectives (white). Categories on X-axis represent overall mean beads concentration of the three counting approaches. For each column $n = 5$

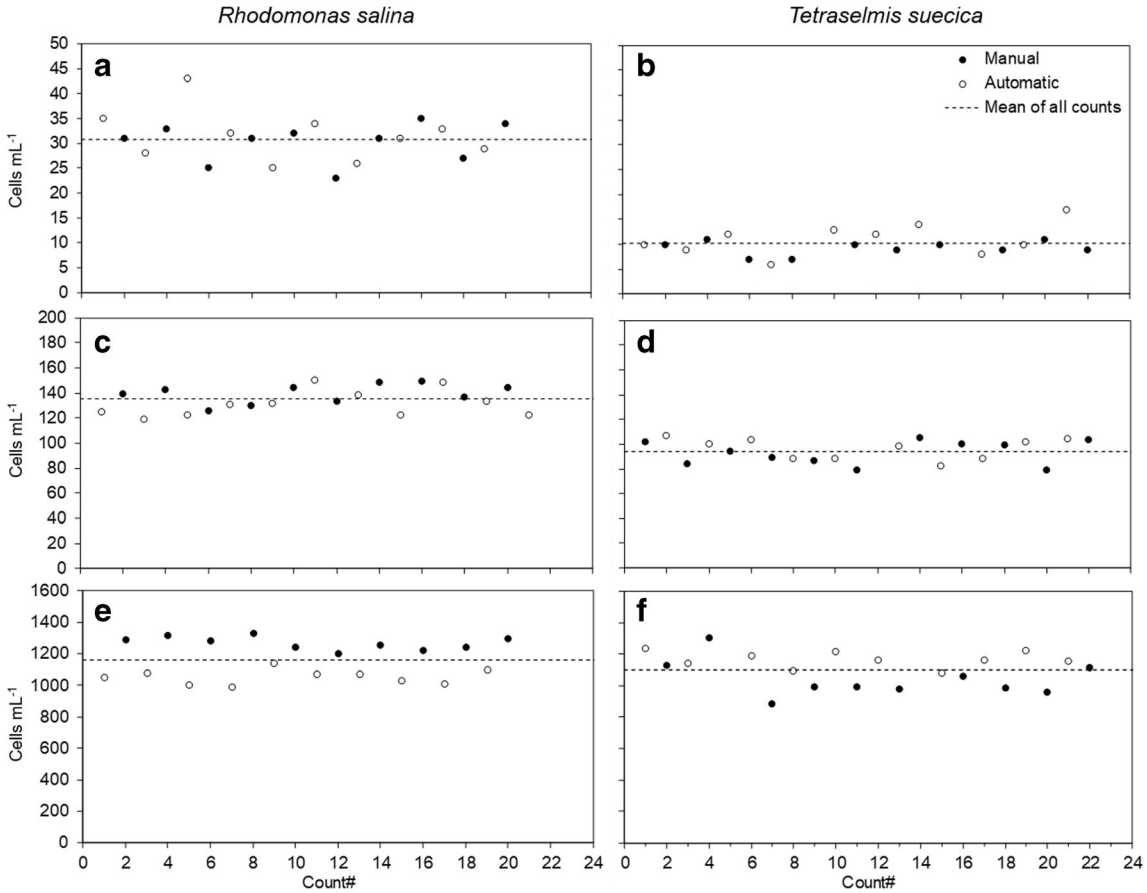


**Fig. 3** Variation of algal concentrations (cells $mL^{-1}$) in test water of *Rhodomonas salina* and *Tetraselmis suecica* in **a**, **b** low, **c**, **d** medium, and **e**, **f** high concentration conditions over a period of approximately 8–10 h with samples counted at fixed intervals. Concentrations were assessed from manual (black circle) and automated (white circle) counts using the vital stain method. The mean of the combined manual and automated counts is included and shown as a punctured line. For each experiment (**a**–**f**) $n = 10$

(Fig. 5a, b). At the highest concentration, automated counts for *R. salina* were significantly lower ($P < 0.001$) than manual counts and vice versa for *T. suecica* ($P = 0.006$) (Fig. 5c).

The mean concentration for natural algal samples was around 165 cells $mL^{-1}$. There was no significant difference between the manual and automated quantification approach when a × 4 magnification objective was used, whereas a significantly smaller concentration was counted when using a × 10 magnification objective (Fig. 6).

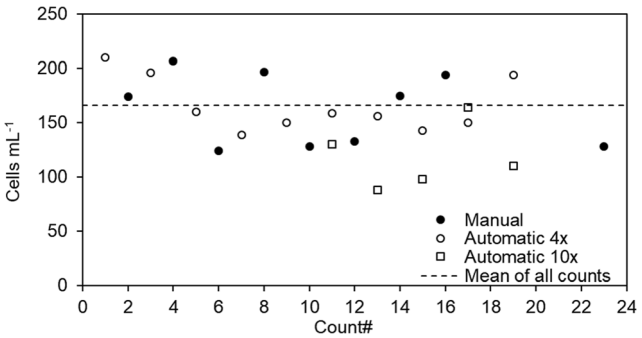


**Fig. 4** Variations in cell concentration (cells $mL^{-1}$) in natural seawater assessed from manual (black circle) and automated counts using the vital stain method. Counts were carried out over a period of approximately 8–10 h at fixed intervals. Automated counts were performed using two different magnification objectives (× 4 (white circle) and × 10 (white square)). The mean concentration of the combined manual and automated × 4 counts is included and shown as a punctured line

## Counting times

For both monoculture algal species, manual counting times were significantly higher (for all $P < 0.001$) than automated counting times (Fig. 7). Manual counting times at the low and medium concentrations were around 8 min and typically about 3 min longer than automated counts. At the high concentration levels, manual counting times were around 12–14 min and between about 7 and 9 min longer than automated counting times. While algal concentration level had no effect on automated counting times, there was a significant increase ($P < 0.001$) in counting times for both monoculture species when algal concentrations were > 1000 cells $mL^{-1}$ (Fig. 7). Counting times were not recorded for *T. suecica* at medium concentration levels.

Based on the results obtained on counting times of monocultures, the total time to count 15 mL (required by IMO for

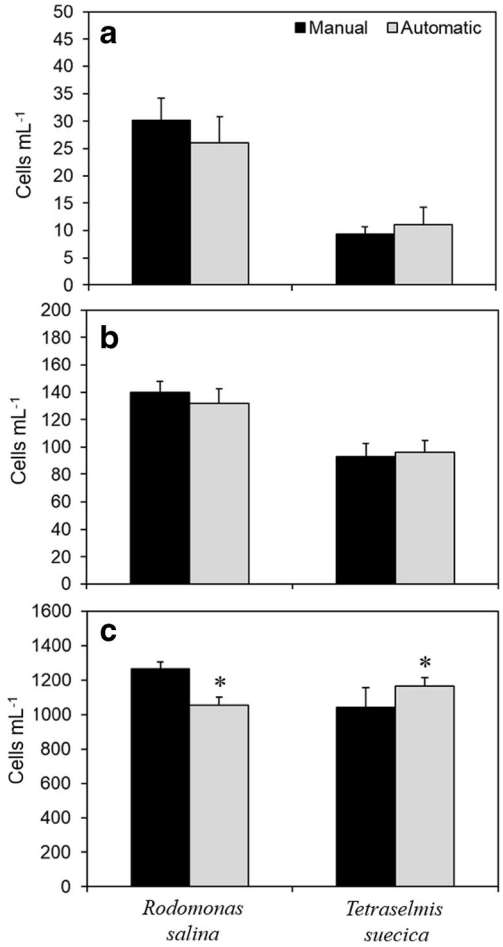


**Fig. 5** Mean ± SD algal concentrations (cells $mL^{-1}$) from manual (black) and automated (gray) counting methods of *Rhodomonas salina* and *Tetraselmis suecica* in **a** low, **b** medium, and **c** high concentration conditions. Significantly different means are marked with an asterisk. For each column $n = 10$

land-based validation) using the manual approach would approximately be (3 mL intake subsamples × 12.5 min + 6 mL control discharge subsamples × 8 min + 6 mL treated discharge subsamples × 8 min) 133.5 min (2 h 14 min) (Table 3). The total time to count 15 mL using the automated approach will be 75 min (1 h 15 min) corresponding to 44% less time per test cycle when compared to the manual approach. Adding all the test cycles together from each of the three salinities, this becomes a total volume of (15 mL × 5 test cycles×3 salinities) 225 mL (Table 3). Consequently, for land-based tests, the total time to count the volume required by the guidelines using the manual and automated approach will be 33 h 23 min and 18 h 45 min, respectively.

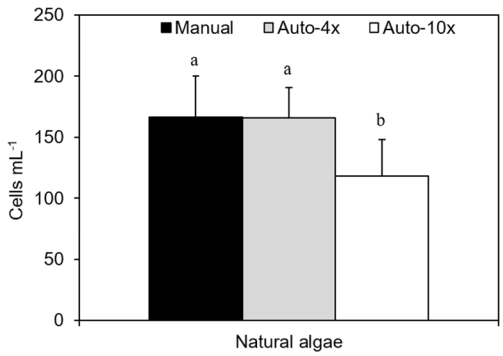


**Fig. 6** Mean ± SD of natural algal concentrations (cells $mL^{-1}$) evaluated using manual (black) and automated counts. Automated counts were performed using two different magnifications (× 4 (gray) and × 10 (white) objectives). Significantly different means between counting approaches are marked with letters. For manual counts $n = 8$, automated × 4 counts $n = 10$ and for automated × 10 counts $n = 5$

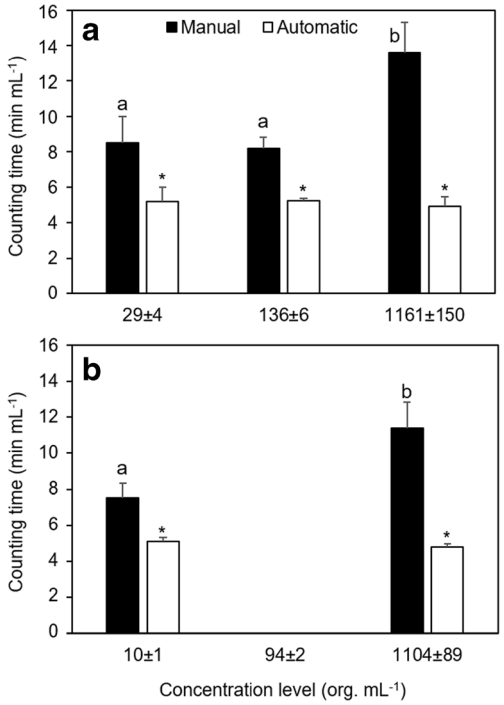


**Fig. 7** Mean ± SD counting times (min $mL^{-1}$) for manual (black) and automated (white) counts of **a** *Rhodomonas salina* and **b** *Tetraselmis suecica* at three concentration levels. Significantly different means between automated and manual counting times at the different concentration levels are marked with an asterisk. Significantly different counting times between concentration levels for each algal species are marked with letters. For each column $n = 10$

Automated counts of natural algae compositions using a × 4 magnification objective took around 6 min $mL^{-1}$ which was significantly lower ($P < 0.001$) than manual counting times of around 11 min $mL^{-1}$ (Fig. 8). Automated counts using a × 10 magnification objective took around 17 min $mL^{-1}$ and was significantly higher ($P < 0.001$) than both manual and automated × 4 counting times (Fig. 8).

Counting times (min $mL^{-1}$) obtained from DHI, Denmark, included mean organism concentrations and total counting times from inlet (3 mL), control discharge (6 mL), and treated discharge (6 mL) water samples ($n = 10$ for each) collected from freshwater (FW), brackish water (BrW), and seawater (SW) tests. Statistical analysis showed significant higher counting times with increasing concentration at all salinities ($P_{FW} = 0.0004$; $P_{BrW} = 0.026$, $P_{SW} = 0.025$). There was effect

**Table 3** The number of 1 mL subsamples of each test water type required by the regulations. From the number of test cycles per salinity and the number of salinities (freshwater, brackish water, and marine water), the total volume of all subsamples to be counted can be calculated for a land-based test. The counting times (min $mL^{-1}$) based on the results obtained on monocultures at the different concentration levels are used to calculate the total time needed to count the total volume of a land-based test

| Test water type | Intake water | Control discharge | Treated discharge | Total |
|---|---|---|---|---|
| Subsamples of 1 mL | 3 | 6 | 6 | |
| No. of test cycles per salinity | 5 | 5 | 5 | |
| No. of salinities | 3 | 3 | 3 | |
| Total volume (mL) | 45 | 90 | 90 | 225 |
| Manual counting time (min $mL^{-1}$) | 12.5 | 8 | 8 | |
| Manual total time (min) | 562.5 | 720 | 720 | 2002.5 |
| Automated counting time (min $mL^{-1}$) | 5 | 5 | 5 | |
| Automated total time (min) | 225 | 450 | 450 | 1125 |

of salinity on counting times with increasing concentration between BW and SW samples whereas counting times of FW samples were significantly different from both BrW ($P = 0.04$) and SW ($P = 0.017$).

## Natural algal composition

For algal species identification, 652 individuals were identified in a total volume of 5.1 mL which was equivalent to 128 org. $mL^{-1}$. The Lugol's counts showed that the natural seawater samples comprised of 31 species divided between nine phyla (Fig. 10). The five most dominating species were *Pseudo-nitzschia* spp. (colony forming diatom, 14%), unidentified pennate diatom (12%), unidentified cryptomonad (cryptophyte, 8%), *Chattonella* sp. (raphidophyte, 6%), and euglenoid spp. (euglenoids, 5%) which all together represented 45% of the total number of identified algal cells (Fig. 9). Diatoms and dinoflagellates were the two most dominating phyla representing 40 and 25%, respectively, of the total phytoplankton concentration.

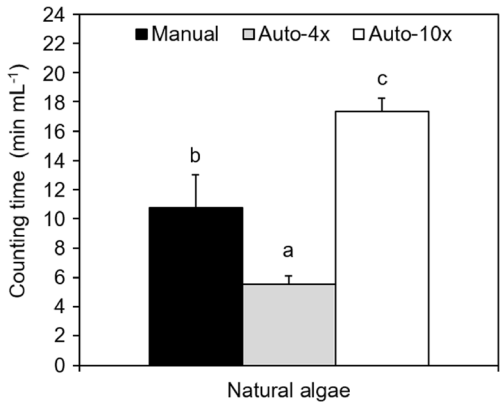


**Fig. 8** Mean ± SD counting times (min $mL^{-1}$) of natural algal composition for manual (black) and automated counts. Automated counts were performed at two different magnifications (× 4 (gray) and × 10 (white) objectives). Significantly different means between counting approaches are marked with letters. For manual counts $n = 8$, automated × 4 counts $n = 8$ and for automated × 10 counts $n = 5$

## Accuracy of the automated approach

To evaluate the accuracy of the automated approach, a "manual review" of the image analysis process was performed (Table 4). In total, 475 images were produced from each scan that when combined covered the surface area of both circles in the filter technique. Each image was then manually evaluated and compared to the data from the automated image analysis.

For *R. salina*, there were 56 cases where there was disagreement between the manual review and the automated approach. The cases include cells identified by the manual review but not the automated approach and vice versa. The overall result was a net difference of 20 cells corresponding to 1.8% deviation from the total automated count. For *T. suecica*, there were 16 cases of disagreement between the manual review and the automated approach which resulted in a net difference of 5 cells corresponding to 0.4% (Table 4).

In natural algal samples, the automated approach was not able to identify and count all cells of colony forming algal units. Therefore, there were 64 cases of disagreement between manual review counts and automated counts. The proportion of cases of disagreements is 43% when related to the automated counts of 150 identified cells. The cases of disagreements resulted in a net difference of 67 cells corresponding to a 45% higher manual review count compared to the automated count (Table 4). If excluding colony forming individuals, the net difference was reduced to 22 cells corresponding to 15% higher manual review counts. Furthermore, cases of disagreement decreased to 29%.

## Effects of acetic acid on immobilization of algal cells

Acetic acid-treated *T. suecica* showed no significant differences between the two automated counts and the manual

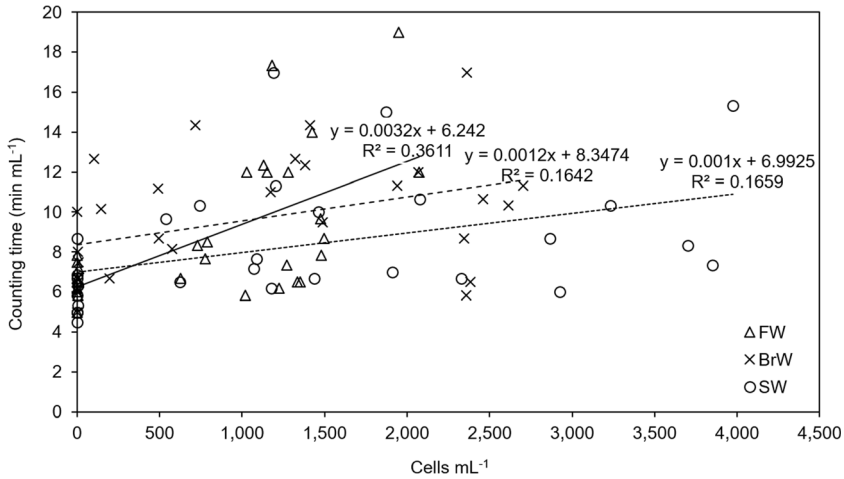


**Fig. 9** Counting times (min $mL^{-1}$) as a function of natural algal concentration (cells $mL^{-1}$) based on inlet, control discharge, and treated discharge water samples (for each $n = 10$). Data was obtained from three different salinities: freshwater, FW (white triangle) (< 1 psu); brackish water, BrW (cross mark) (10–20 psu) and seawater, SW (white circle) (>28 psu)

count (Fig. 11). Moreover, similar counts were obtained in non-treated samples when using the modified version of a Sedgewick Rafter counting chamber (250 μL volume). Significantly lower ($P < 0.001$) manual counts of non-treated *T. suecica* cells were obtained using a standard Sedgewick Rafter counting chamber.

Manual counts of acetic acid treated *R. salina* were significantly lower ($P = 0.007$) than non-treated counts. Similar significant ($P = 0.002$) outcome was observed in automated counts using × 4 magnification. There was no significant difference between using × 4 and × 10 magnification objectives for treated automated counts. However, when compared to treated manual counts, treated automated counts using × 4 and × 10 magnification objectives differed significantly ($P = 0.015$ and $P = 0.006$, respectively). All non-treated counts showed no significant difference.

## Discussion

A number of automated analysis methods for the estimation of living phytoplankton are presently available. These

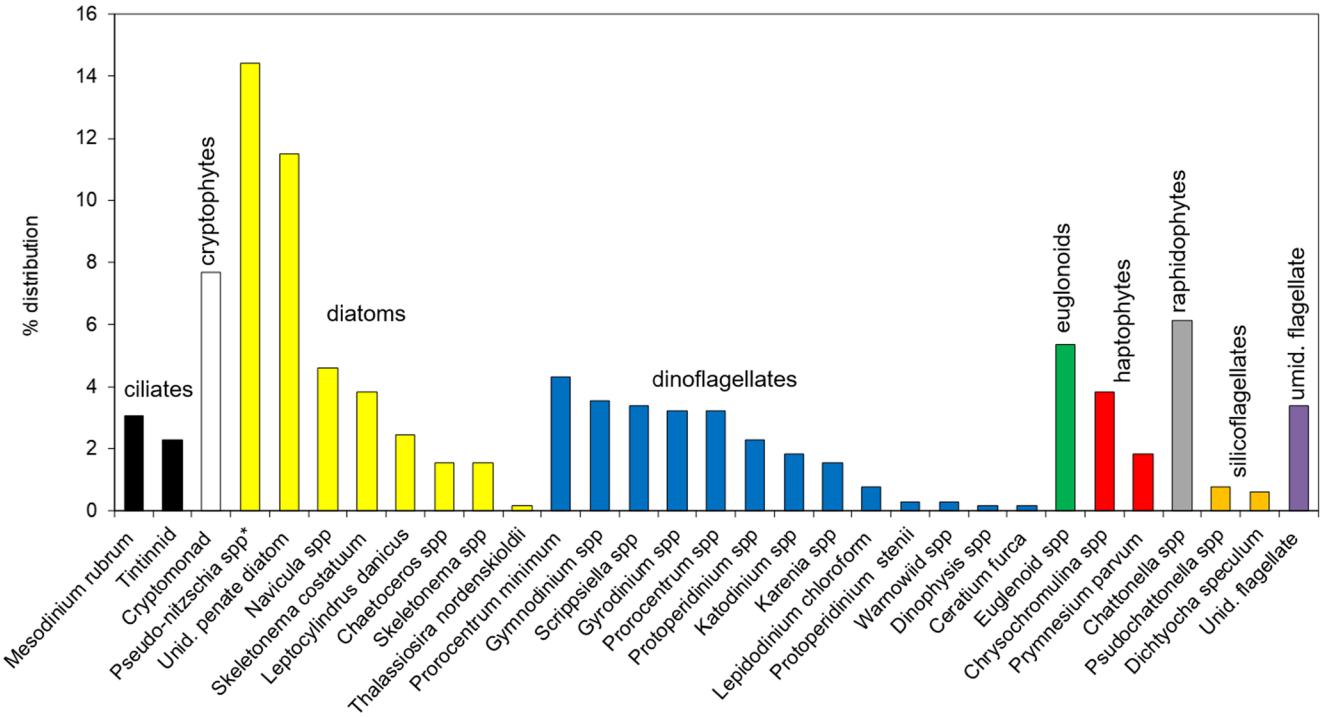


**Fig. 10** The percentage-wise distribution of the species identified in the natural seawater sample. The colors signify the different phyla of species. Black = ciliates. White = cryptophytes. Yellow = diatoms. Blue = dinoflagellates. Green = euglenoids. Red = haptophytes. Gray = raphidophytes. Orange = silicoflagellates. Purple = unidentified flagellate

**Table 4** Comparison of "manual review" counts and automated counts of *Rhodomonas salina* and *Tetraselmis suecica* monocultures (high concentration level scan) and natural algae (presented with and without colony forming algae) and their corresponding percentage-wise deviations from the automated approach

| | "Manual review" | Automated approach | % deviation |
|---|---|---|---|
| *Rhodomonas salina* | 1118 | 1138 | 1.8 |
| *Tetraselmis suecica* | 1214 | 1219 | 0.4 |
| Nat. algae (+ colony forming species) | 217 | 150 | 45 |
| Nat. algae (− colony forming species) | 172 | 150 | 15 |

methods include, among others, pulse-amplitude modulated fluorometry (PAM), ATP assays, FDA bulk analysis assays, and flow cytometry (van Slooten et al. 2015; Wright et al. 2015; Casas-Monroy et al. 2016; Outinen and Lehtiniemi 2017; Peperzak et al. 2018). The instruments for these methods are often simple to use and are designed to be handled by untrained personnel to obtain quick indicative analyses of treated BW. However, these methods only provide bulk estimates of organism concentrations. They are not directly applicable to estimate compliance with BW discharge standards regulations that are specifically related to numerical concentrations below a specified limit in defined size classes. Other automated approaches have been suggested, but most of the methods provide bulk analysis results and not a numeric outcome as required by the regulations. To our knowledge, only two automated methods are able to provide numeric outcomes: flow cytometry and FlowCAM (combination of flow cytometer and imaging microscopy). Studies performed on the use of these methods have highlighted challenges for the size class 10–50 μm which include accurate counting of colony-forming algal species as well as clogging and sedimentation issues in the flow tube (Reavie et al. 2010; Romero-Martínez et al. 2017; Kydd et al. 2018; Peperzak et al. 2018). In flow cytometry, no images are recorded for potential post-analysis and it only provides a numeric outcome. This feature is possible in FlowCAM, but often, this process is time consuming and increases overall analysis time (Reavie et al. 2010).

Therefore, to fulfill the numerical and size class requirements as well as more accurately quantify colony forming individuals, the VS method is currently the most suitable method available. It is not as fast as indicative analyses but is nevertheless able to provide results within hours which is significantly faster than regrowth assays as the MPN assay which typically take weeks. In addition, for ships to be allowed to enter US waters, the VS method is currently the only assessment method approved by the USCG for evaluation of BWTS performance. In the present study, we have addressed some of the above-mentioned quantification quality issues (fatigue, subjectivity, size identification, high algal mobility, low counting rates) linked to the VS method by applying an automated cell quantification approach in combination with a filter technique for preparing the sample.

## Performance of manual and automated counting approaches

Experiments were initially performed on 6 μm autofluorescent beads with the aims to explore the performance of × 4 and × 10 magnification, respectively. The field of view is larger when using a × 4 compared to a × 10 magnification objective which reduces the number of images needed to scan a full sample thus reducing counting rate as well as digital storage space. The results showed equal accuracy at all concentration levels as well as between manual and automated counts (Fig. 2) and therefore × 4 magnification was used for the automatic approach.

The choice to use small-sized *R. salina* (8–9 μm range) was to explore the effect of cell size around the regulatory size cut-off on quantification quality. The data showed that algae in this size class became more difficult to count using the manual approach because the algal cells could be confused with autofluorescent debris which typically was in the 6–7 μm size range (Fig. 5). Consequently, quantification accuracy was lowered. This was especially significant at concentration levels of > 1000 org. $mL^{-1}$, where a deviation of 17% was observed. The presence of a high detrital load in samples has previously been shown to decrease counting accuracy as well as counting rates during a study where the recovery success of 50 μm microbeads added to natural plankton water samples was investigated (First and Drake 2012).

The definition of the size class 10–50 μm in the regulations has been addressed and criticized in several studies (Gollasch et al. 2007, 2012; van der Star et al. 2011; Liebich 2013; Casas-Monroy et al. 2016; Liu et al. 2016; Lundgreen et al. 2018). No studies have, to the authors' knowledge, shown that species < 10 μm are less robust towards treatments and until this has been demonstrated, they should therefore be considered as potential invasion risks. A large proportion of all known algal species are < 10 μm which includes several toxic or nuisance species such as *Pseudochattonella verruculosa*, *Microcystis viridis*, *Skeletonema* sp., *Thalassiosira* sp., and

*Phaeocystis* sp. To better protect aquatic environment from invasions by phytoplankton, it would be rational to include the species < 10 μm in the validation procedures of BWTSs. The automated approach examined in this study was more accurate in enumerating *R. salina* and separating them from autofluorescent debris compared to the manual approach (Table 4; Fig. 5). Correct identification by the automated approach was confirmed by a manual review of one of the automated analyses where only a 1.8% deviation between counts was observed (Table 4). To efficiently exclude autofluorescent debris in the analyses, the MPN method is the most suitable method available as the regrowth assay does not distinguish between cell sizes and will effectively include phytoplankton species < 10 μm. Another approach to minimize the impact of autofluorescent debris could be to investigate other fluorescent stains than FDA/CMFDA. If the counting should indeed only include the 10–50 μm size class, as currently required, the automated approach is more precise than manual counts because exact cut-off criteria for organism size can be applied.

The equal deviations observed for both manual and automated counts indicate that the identification criteria remained constant in all counts for both counting approaches and suggest that the deviations could be explained by variations in sample concentrations. Furthermore, the relatively small variation between results suggests that both approaches were reliable counting methods for *R. salina*.

The highly mobile *T. suecica* cells were difficult to locate in the water column of the Sedgewick Rafter counting chamber when counted manually. This was especially evident at the high concentration level where multiple cells moved simultaneously around in the 3D plane (Fig. 5). Consequently, cells were overlooked resulting in a lower quantification quality. The filter technique developed for and used in the automated approach gently forces the highly mobile *T. suecica* cells into a 2D plane which significantly improved the quantification accuracy. Using the filter technique to immobilize the *T. suecica* cells explains the significantly higher *T. suecica* cell counts observed in the automated approach compared to the manual approach (Fig. 5). This hypothesis was further supported by the results obtained from the lowered Sedgewick Rafter counting chamber as well as the cells immobilized with acetic acid (Fig. 11). Thus, by using the filter technique, a more accurate estimation of the number of cells present in the sample was obtained. As *T. suecica* is slightly bigger than *R. salina*, judgment of cell size and separation from autofluorescent debris became less of a challenge (Table 2). The results from the manual review of the automated approach on *T. suecica* likewise support the clearer distinction as there was little disagreement in the comparative analysis (0.4%, Table 4). The larger the algal cells, the more accurately they can be distinguished from autofluorescent debris improving quantification accuracy. That larger cells increase enumeration accuracy is supported by a study by First and Drake (2012) where manual counts of 150 μm microbeads had a higher recovery than 50 μm microbeads with and without debris.

The larger deviations (17% for *R. salina* and 13% for *T. suecica*) between the mean manual and automated counts observed at the high concentration level (Fig. 5c) were caused by the manual counts suffering from a mix of identification issues, maintaining efficient counting rates and issues related to counting a volume (3D) compared to a surface (2D).

The analysis of organism composition of the natural samples showed high diversity in number of phyla (8) comprising of 29 algal and 2 ciliate species (Fig. 10). The natural samples were thus considered to be of high composition complexity to further challenge the performance of the automated approach. The use of complex natural plankton samples is central to perfect a single technique and has been highlighted as one of the most important components to include in validation of techniques (Steinberg et al. 2012). For the natural seawater samples, good agreement between manual and automated (× 4) counts was observed (Fig. 6). However, there was a 45% difference in cell numbers when performing the manual review of the automated counts (Table 4). This was largely caused by the automated approach not being able to correctly quantify individual cells of colony forming units which resulted in an underestimation of cell concentration. Furthermore, correct judgment of cell size in the manual review was difficult because the magnification was only × 4 and the dominating cell size for several species were close to the lower limit of 10 μm which resulted in a 15% overestimation compared to the automated approach.

The fact that similar counts were obtained by the automatic approach and the manual approach (Fig. 6) could be explained by a combination of lack of counts of mobile species in the 3D water column and less false positives in the manual approach because a × 10 magnification was used compared to × 4 magnification in the manual review. The study by Peperzak et al. (2018) likewise highlighted that in living samples, judgment of living (fluorescent intensity) cells as well as sizing are key issues encountered by microscopists. To overcome the challenge with colony forming individuals for the automatic counting, advanced machine learning should be investigated and implemented in future studies.

For all the comparative counting experiments, data was collected over a period of 8–10 h (Figs. 3 and 4). The plots showed that time did not have an impact on the concentration level of the organisms throughout the sampling period. Any differences between manual and automated counts could thereby be attributed to other variables such as cell concentration level, composition complexity, cell size, and/or mobility behavior.

The colony forming species encountered in the natural sample consisted of *Pseudo-nitzschia* spp. (14.4%), *Skeletonema costatuum* (3.8%), *Leptocylindrus danicus* (2.5%), and *Thalassiosira nordenskioldii* (0.2%) (Fig. 10).

In the manual approach, it was possible to determine vitality and count the individuals of these colony forming species. The variations in staining intensity of the individual cells did however challenge correct identification of the vital individuals. In the automated approach, the recognition criteria used in the image analysis process could in most cases not distinguish individual cells within colonies. Particularly, recognizing individual cells and handling varying staining intensities of colony forming individuals were the key issues. Variations in staining intensity have previously been reported as an issue when applying the VS method on natural phytoplankton populations and different algal cultures (Garvey et al. 2007; Peperzak and Brussaard 2011; MacIntyre and Cullen 2016). The diatoms *Pseudo-nitzschia* spp. and *Leptocylindrus danicus* were especially problematic, which resulted in an underestimation of cell concentrations in the automated counts. To be able to accurately count natural samples which regularly contain colony forming species, the image analysis procedure needs to be refined and could be solved by using more advanced approaches such as other automatized quantification and species identification programs (PlanktoVision, ImageJ) (Schulze et al. 2011, 2013). Similar issues are also encountered when using flow cytometry (Christaki et al. 2011; Zhou et al. 2012; Peperzak et al. 2018) and FlowCAM (Reavie et al. 2010; Camoying and Yñiguez 2016; Romero-Martínez et al. 2017).

To see if it was possible to obtain more accurate counts and increase quantification quality in the automated approach, a scan using a × 10 magnification objective was performed (Fig. 6). However, the results were significantly lower than both the manual and automated (× 4) counts. When using a × 10 objective, the depth of focus was reduced (see Berek (1927). This resulted in some algal cells being out of focus despite applying the filter technique and being consequently missed by the image analysis software. This suggests that the filter technique does not force cells into a perfectly flat 2D plane. Instead, the distance between glass plate and membrane filter is sufficiently small for the × 4 objective to capture all cells within its field of focus. A solution could be to include image stacking in the z-plane, but such operation would increase data and analysis time considerably.

Acetic acid was very efficient in immobilizing *T. suecica* cells (Fig. 11). Similar counts of non-treated cells were obtained using the lowered Sedgewick Rafter counting chamber, which suggests that cell membrane structure was not affected by the acetic acid. The observed difference in counts between non-treated *T. suecica* cells using a standard vs. a lowered Sedgewick Rafter counting chamber suggest that reducing the height of the water column significantly improves counting accuracy of highly mobile algal species. The pH sensitivity of *R. salina* was different from *T. suecica*. Acetic acid-treated *R. salina* clearly showed changes in membrane structure and decreased fluorescent intensity which resulted in

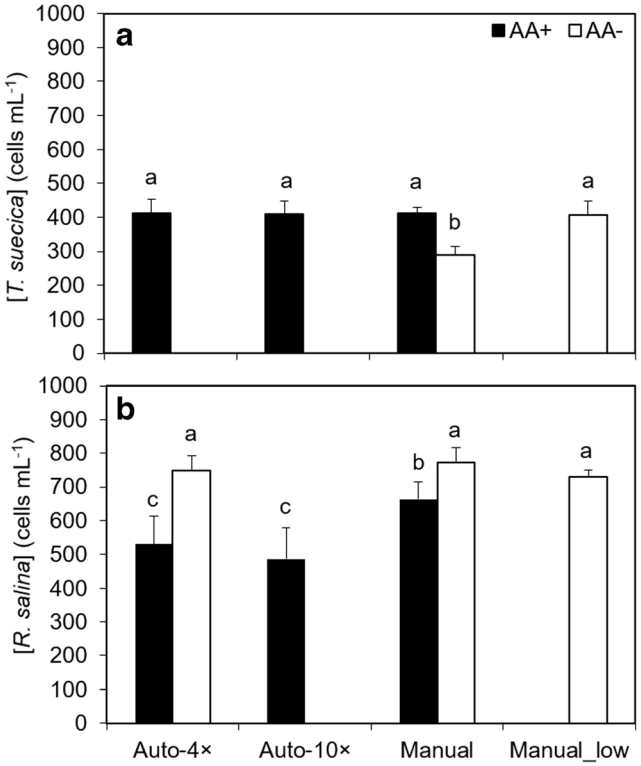


**Fig. 11** Automated and manual vital stain counts using a Sedgewick Rafter counting chamber of acetic acid treated (AA+) and non-treated (AA-) for **a** *Tetraselmis suecica* and **b** *Rhodomonas salina*. Acetic acid-treated algal cells (black columns) were assessed manually and automatically using both × 4 and × 10 objectives on the HCS-Platform. Non-treated algal cells (white columns) were assessed manually by use of a modified lower Sedgewick Rafter chamber height of 250 μm compared to a standard height of 1000 μm. In addition, non-treated *Rhodomonas salina* was assessed automatically in a Sedgewick Rafter counting chamber using a × 4 objective. For Manual_low, $n = 4$. For all other columns, $n = 5$

cells being more difficult to identify compared to non-treated cells. Furthermore, the automated approach could not accurately recognize acetic acid-treated cells using any of the magnifications. Using acetic acid as an immobilization agent seems to be highly efficient for quantification of some algal species but others, and therefore, this method is not recommended for improved counting of natural algal populations.

## Counting times

For both monoculture species, the automated counting times at all concentration levels were similar with rates around 5 min $mL^{-1}$ (Fig. 7). Since the HCS-Platform performs the same scanning and quantification procedure for each sample, no variation was expected. The small observed variation was caused by internal data storage limitations in the software that after a number of analyses had to be restarted to clear the data storage memory. This variation can be overcome by increasing storage capacity. At all concentration levels and for both species, manual counting times were significantly longer than automated

counting times (Fig. 7). The longer counting times in the manual approach can mainly be explained by two factors. First, a × 10 objective with a smaller field of view must be used for more reliable identification of cells compared to the × 4 magnification as previously discussed. Second, a Sedgewick Rafter counting chamber has a water column height of 1000 μm. Consequently, a vertical scan at each field of view must be performed to locate all the 10–50 μm cells present in the water column. The microscope settings for the manual approach gives a depth of focus of 14.4 μm (calculated from the formula in Berek 1927) which means that at any given time, only a small horizontal slice of the vertical column is in focus. In theory, this means that about 70 horizontal slices must be checked in each field of view to cover 1000 μm. However, the vertical range for each field of view can typically be covered in a few seconds by moving the stage up or down. It does not seem as a major time-consuming process, but overall, it adds up when scanning the whole volume of the counting chamber. In the automated approach, the filter technique produced a much larger surface area ($A_{\text{filter}} = \pi \times r^2 = \pi \times (½ \times 47\ \text{mm})^2 = 1735\ \text{mm}^2$, hence 3470 $\text{mm}^2$ for the two scanned filters) which needed to be scanned (Table 1). On the other hand, no vertical scans were needed.

Manual counting times at the highest concentration level for both species were significantly longer than at low and medium concentration levels (Fig. 7). This is a logical consequence of manual counts of more cells per volume. In addition, for the samples containing *R. salina*, it became more difficult to distinguish between fluorescent algal cells and autofluorescent debris when algae concentrations increased. The slightly larger *T. suecica* was easier to distinguish from autofluorescent debris, but it was more difficult to keep track of higher concentrations of the more mobile *T. suecica* which caused an increase in the time used for manual counting. The observed longer counting times at the high concentration levels in the present study was likewise observed by First and Drake (2012) in their experiment using microbeads.

Based on the counting times obtained on monocultures from the present study, the estimated time needed to count the total volume of VS samples for the size group 10–50 μm in a full land-based test (Table 3) could be calculated. Our data showed that the automated approach potentially will save a testing facility approximately 15 h of counting time per land-based test. The manual counting times obtained from DHI for different concentration levels and salinities were comparable to the manual counting times of monocultures and natural algae in the present study. Using the data from DHI, it was estimated that a full land-based test using manual counting would take 31.2 h versus 33.4 h in this study (Table 3). It is important to highlight that the calculated counting times and derived saved counting time for the automated method in the present study is based on a single analyst. Although the analyst was trained at DHI during several months, variations in counting times are expected between analysts in general. A comparison of counting times of a full land-based test between the certified analysts from DHI and the analyst in the present study showed a minor deviation of 7%.

The statistical analysis from the DHI data furthermore suggests that FW samples takes more time to count with increasing concentration compared to BrW and SW samples which is supported by statements from trained personnel on their experiences with sample counts (personal communication).

For natural seawater samples, significantly higher counting times were likewise recorded for manual counts compared to the automated counts using a × 4 magnification objective (Fig. 8). Manual counting times were about 3 min longer per mL when counting natural seawater compared to monocultures. The longer counting times are partly explained by the presence of more autofluorescent debris. In addition, counting times were affected by the large variety in size and shape of the numerous species present in the natural seawater sample. It takes more time to identify if a cell fulfills the criteria or not.

For the natural seawater samples, an automated scan using a × 10 magnification objective was additionally performed (Fig. 8). The field of view area for this approach was 1 $\mu m^2$, and consequently, twice as many images were needed compared to × 4 magnification to cover the scanning area of one circle on the glass plate (Fig. 1). The scanning time for 1 mL was > 3 times longer compared to using a × 4 magnification objective. If using a larger magnification (i.e., × 10) had improved quantification accuracy considerably then the tradeoff of a longer counting time may be considered as an overall improvement of quantification quality.

## Other methods tested to examine fixation of mobile algal species

Immobilization or fixation of highly mobile algal species can significantly reduce fatigue of the analyst, decrease counting time, and increase identification accuracy that overall can improve quality of the quantification procedures. In the present study, different approaches were tested for immobilization of algal cells without directly or indirectly decreasing the health status of the cells which would interfere with the evaluation of BWTS performances. Flocculating agents have the ability to bind to organic matter (cells) to induce sedimentation of cells which for example have been used in harvesting procedures of microalgae rich in lipids used for biofuel production (Hu et al. 2008). The technique could likewise be useful to sediment BW samples although flocculation has been reported not to be 100% efficient and that efficiency differs among microalgal species (Lertsutthiwong et al. 2009; Gerchman et al. 2017; Ummalyma et al. 2017). Apart from flocculation, the present study also examined other different immobilization techniques such as gel entrapment and coating surfaces with cell-binding agents to find the most suitable method for algal

immobilization (On-line Appendix 2). The performed pilot experiments on immobilization techniques in On-line Appendix 2 showed that all the investigated flocculation and immobilization techniques using different agents were unsuitable for algal immobilization. However, the developed filter technique proved to be the most suitable and reliable technique for algal viability assessment.

## Conclusions

The described automated method could be a remedy to overcome the main challenges experienced in microscopy and flow cytometry. In the present study, the automated method was tested using the VS method and adapted to the strengths and limitations of the FDA/CMFDA stains. Manual microscopy is slow which can affect both the vitality and fluorescent signal, making it vulnerable to subjectivity and fatigue among microscopists. The counting times of the automated approach were up to three times faster than the manual approach which significantly reduces the overall time of land-based tests. The automated approach provides fast and objective counts with the possibility of post hoc examination of the images, while flow cytometry has no visual checks which can lead to the inclusion of inanimate green autofluorescent particles in the counts (Tang and Dobbs 2007). Furthermore, the automated approach could, like the FlowCAM, be adapted for algal monitoring in connection to HABs by applying specific probes of different-colored fluorophores for toxic/non-toxic species identification.

The automated approach in combination with the filter technique was able to immobilize highly mobile species such as *T. suecica* to accurately identify samples at concentration levels from around 10 to more than 1000 cells $mL^{-1}$. Estimations of natural algal concentrations were similar for the automated and manual approach of cells within the 10–50 μm range, but the presence of colony forming algal species reduced the quantification accuracy of the automated approach because the image analysis was challenged by identification of individual colony units. The quantification accuracy of both approaches was reduced when counting cultured algal cells as well as natural phytoplankton species around or below 10 μm because the presence of autofluorescent debris interfered with accurate identification of cells. The automated method can include a precise cut-off of 10 μm to follow the IMO and USCG regulations. Such precision is not possible during manual counting.

The future development of more efficient stains—that might even be able to target and include DNA damaged cells—to replace or supplement FDA/CMFDA would further improve the automated method. In combination with the rapid developments within image analyses and machine learning, the post-analysis option could be improved further by applying machine learning for more accurate quantification (Kruk et al. 2017; Orenstein and Beijbom 2017) and species identification. Therefore, we believe that this method could potentially become a strong and reliable tool for viability assessment of phytoplankton used for validation of BWTS.

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