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Indicative ballast water analysis testing for port State control purposes

Okko Outinen & Maiiu Lehtiniemi

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Asiasanat
Painolastivesiyleissopimus, indikatiivinen analyysi, näytteenotto

Tiivistelmä
Kansainvälisen merenkulkujärjestön (IMO) kansainvälinen yleissopimus alusten painolastivesien ja sedimenttien valvonnasta ja käsittelystä (BWM-yleissopimus) tuli voimaan 8.9.2017. Sen tarkoituksena on vähentää akvaattisten vieraslajien leviäminen. Kaikkien alusten on täytettävä BWM-yleissopimuksen painolastiveden suorituskykyvaatimukset (D-2-standardi) viimeistään 8.9.2024. IMO on määritellyt BWM-yleissopimuksen mukaiset suuntaviivat satamavaltiotarkastuksia suorittaville viranomaisille. Niihin kuuluvat alustava ja yksityiskohtainen tarkastus sekä tarvittaessa näytteenotto painolastivedestä alustavien ja yksityiskohtaisten analyysien tekemiseksi aluksen vaatimustenmukaisuudesta. Suomen satamavaltiotarkastusviranomainen Liikenne- ja viestintävirasto (Traficom) voi jatkossa ottaa näytteitä painolastivedestä ja tehdä alustavia analyysejä siitä. Traficomin tilaaman tutkimuksen tarkoituksena oli testata neljän alustavan analyysin tekemiseksi tarkoitetun laitteen käyttökelpoisuutta satamavaltiotarkastuksissa. Tutkimuksen tarkoituksena oli myös tuottaa hyödyllistä tietoa erilaisista valvontaan liittyvistä aiheista IMO:n kokemuksenkeruuvaihetta varten. Tutkimuksen teki Traficomia varten Suomen ympäristökeskus (SYKE).

Testatut alustavat menetelmät olivat ATP (Adenosine TriPhosphate), PAM (Pulse Amplitude Modulation), STAF (Single Turnover Active Fluorometry) ja MFA (Motility and Fluorescence Assay) –menetelmät. Näytteet otettiin vuoden 2018 lokakuussa ja marraskuussa kahdella suomalaisella aluksella, joihin on asennettu painolastiveden käsittelylaitteisto. Alustavien analyysien tuloksia verrattiin samoista näytteistä tehtyihin yksityiskohtaisiin laboratorioanalyyseihin FDA- (fluorescein diacetate) ja epifluoresenssimikroskopiamenetelmää käyttäen.

Kaikki testatut laitteet osoittautuivat kevyiksi, kohtuuhintaisiksi ja helppokäyttöisiksi. Jokaisella laitteella saatiin esille selviä eroja elinkelpoisissa eliöpitoisuuksissa käsiteltyjen ja käsittelemättömien vesinäytteiden välillä, mikä osoitti niiden kyvyn erotella käsiteltyä ja käsittelemätöntä vettä. Laitteilla oli kuitenkin vaikeuksia määritellä tarkkoja testituloksia D-2 standardin mukaisesti. Kun standardin yhdenmukaisuusrajat on määritelty elinkelpoisten eliöiden määränä kutakin eliöluokkakohtaista näytteen tilavuutta kohden, etenkin ATP-menetelmä ei kyennyt muuntamaan ATP-pitoisuuksia elinkelpoisiksi eliöpitoisuuksiksi. Lisäksi menetelmillä, joilla mitataan elinkelpoisuutta pelkästään fotosynteettisesti aktiivisista kasviplanktonsoluista (PAM ja STAF) voi olla mahdotonta havaita niukkaravinteisten avomerinäytteiden vaatimustenmukaisuus, sillä avomerellä nämä eliöt saattavat esiintyä harvakseltaan eläinplanktoniin verrattuna. Kun laitteiden tarkoituksena on antaa alustavia tuloksia, olisi järkevämpää, että IMO erikseen määrittäisi D-2 standardin alustavien näytteiden vastaavuusrajat. Nykyisiä D-2 standardin vastaavuusrajoja on hyvin vaikea soveltaa, kun niitä verrataan alustavien analyysien alustaviin tuloksiin, jotka eivät ole yhtä tarkkoja.

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Sammandrag
Internationella sjöfartsorganisationens (IMO) internationella konvention för kontroll och hantering av fartygs barlastvatten och sediment (BWM-konventionen) från år 2004 trädde i kraft den 8 september 2017. Dess syfte är att förhindra spridningen av vattenlevande främmande arter. Alla fartyg ska uppfylla BWM-konventionens prestandanormer för barlastvatten (D-2 standarden) senast den 8 september 2024. IMO har för hamnstatskontrollmyndigheterna utarbetat riktlinjer gällande BWM-konventionen. De inbegriper inledande och detaljerad inspektion samt vid behov provtagning av barlastvattnet för indikativ och detaljerad analys av att ifrågavarande fartyg uppfyller kraven. Transport- och kommunikationsverket (Traficom), som är hamnstatskontrollmyndighet i Finland, kan framgent ta barlastvattenprover i samband med sina inspektioner. Syftet med denna av Traficom beställda studie var att testa hur väl fyra utvalda anordningar avsedda för indikativ analys betjänar hamnstatskontrollens syften. Ett annat syfte med studien var att ge information om olika ämnen med anslutning till kontrollen av överensstämmelsen med kraven för IMO:s erfarenhetsuppbyggande fas. Studien utfördes för Traficoms räkning av Finlands miljöcentral (SYKE).

De testade indikativa metoderna utgjordes av ATP (Adenosine TriPhosphate), PAM (Pulse Amplitude Modulation), STAF (Single Turnover Active Fluorometry) och MFA (Motility and Fluorescence Assay). Provtagningen skedde i oktober och november 2018 ombord på två finska fartyg som är utrustade med barlastvattenhanteringssystem. Resultaten av de indikativa analyserna jämfördes med detaljerade laboratorieanalyser av samma prov gjorda med FDA- (fluorescein diacetate) och epifluorescentmikroskopimetoden.

Alla de anordningar som testades ansågs lätta, ha rimligt pris och vara hanterliga. I fråga om vart och ett av anordningarna fanns det en klar skillnad i koncentrationerna av livskraftiga organismer i behandlade och obehandlade vattenprover, vilket bevisar anordningarnas förmåga att skilja på behandlat och obehandlat vatten. Det visade sig dock svårt för anordningarna att fastställa exakta testresultat i enlighet med D-2 standarden. Då standardens utsläppsvärden har fastställts som antalet livskraftiga organismer per volym av intresse, visade det sig att i synnerhet ATP-metoden inte förmådde konvertera de registrerade ATP-koncentrationerna till koncentrationer av livskraftiga organismer. Dessutom, kan det visa sig omöjligt för metoder som enbart mäter livskraften hos fotosyntetiskt aktiva fytoplanktonceller (PAM och STAF) att upptäcka överensstämmelsen hos oligotrofiska prover från öppet hav, där dessa organismer kan vara fåtaliga i jämförelse med djurplankton. Då anordningarna är avsedda att vara indikativa, vore det rimligt att IMO utarbetade särskilda indikativa utsläppsgränsvärden för dem. D-2 standardens nuvarande utsläppsgränsvärden är mycket svårtillämpliga i jämförelse med de preliminära resultaten av de indikativa analyserna.

Kontaktperson Ville-Veikko Intovuori	Språk engelska	Sekretessgrad offentlig	Sidoantal 53
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Abstract
The International Convention for the Control and Management of Ships Ballast Water and Sediments, 2004 (BWM Convention) of the International Maritime Organization (IMO) entered into force on 8 September 2017, aiming to prevent the spread of aquatic non-indigenous species. All ships must follow the ballast water performance standard (D-2 standard) of the BWM Convention at the latest by 8 September 2024. IMO has determined guidelines for port State control authorities under the BWM Convention that include initial and detailed inspection, as well as ballast water sampling for indicative and detailed analyses of the ships compliance, if necessary. The Finnish Transport and Communications Agency (Traficom), the port State control authority in Finland may conduct ballast water sampling and indicative analyses during inspections in the future. The aim of the study commissioned by Traficom was to test four indicative analysis devices for port State control monitoring purposes. The study equally aimed to provide useful information on various compliance monitoring related subjects for the experience-building phase of the IMO. The study was conducted for Traficom by the Finnish Environment Institute (SYKE).

The tested indicative methods were Adenosine TriPhosphate (ATP), modified Pulse Amplitude Modulation (PAM) fluorometry, Single Turnover Active Fluorometry (STAF) and Motility and Fluorescence Assay (MFA). The sampling events were conducted in October and November, 2018, on two Finnish ships that have ballast water treatment systems installed onboard. The indicative analysis results were compared to detailed laboratory analyses that were conducted for the same samples with fluorescein diacetate (FDA) and epifluorescent microscopy method.

All devices tested were considered portable, reasonably priced and manageable for port state control officers to use. There was a clear difference in the estimated viable organism concentrations between the treated and untreated water samples using each device, showing their capability to differentiate between untreated and treated waters. The devices had difficulties in referring their results to the compliance limits of the D-2 standard. As these compliance limits are defined as number of viable organisms per volume of interest, especially the ATP method does not convert the recorded bulk ATP values into viable organism concentrations. In addition, methods that measure viability only from photosynthetically active phytoplankton cells (PAM and STAF) might be unable to detect compliance status for oligotrophic open sea samples, where these organisms can be rarely present. As the devices are designed to be indicative, it would be more reasonable if the IMO provided indicative compliance limits for the D-2 standard separately. The present D-2 standard compliance limits are inapplicable when compared to the method-specific thresholds of the indicative analysis devices and their accuracy.

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ALKUSANAT

Liikenne- ja viestintävirasto (Traficom) on teettänyt tutkimuksen painolastivesien alustavasta analyysimenetelmistä satamavaltiotarkastuksia varten. Tutkimuksen tulokset on koottu tähän raporttiin. Neljää erilaista alustavaan analyysin käytettävää laitetta testattiin kahdella aluksella ja tarkoituksena oli löytää laite, joka sopisi parhaiten Suomessa tehtäviin satamavaltiotarkastuksiin ja Itämeren olosuhteisiin.

Tutkimuksen teki apulaistutkija Okko Outinen ja tutkimusprofessori Maiju Lehtiniemi Suomen ympäristökeskuksesta (SYKE). Tutkimuksen ohjausryhmään kuuluivat erityisasiantuntija Ville-Veikko Intovuori, johtava asiantuntija Anita Mäkinen ja yksikönpäällikkö Mirja Ikonen Traficomista sekä Okko Outinen ja Maiju Lehtiniemi SYKEstä.

Traficom haluaa esittää suuren kiitoksensa kaikille alustavaan analyysiin käytettävien laitteiden valmistajille eli Luminultralle, bbe Moldaenke GmbH:lle, Chelsea Technologies Group Ltd:lle ja Microwiselle, jotka ystävällisesti lainasivat laitteitaan tutkimusta varten. Haluaisimme myös esittää erityisen kiitoksemme kahdelle varustamolle, Arctia Shipping Oy:lle ja VG-Shipping Oy:lle, joiden aluksilla testit suoritettiin. Ilman heitä tutkimusta ei olisi ollut mahdollista toteuttaa.

Helsingissä, 31. lokakuuta 2019

Ville-Veikko Intovuori
erityisasiantuntija
Liikenne- ja viestintävirasto Traficom

FÖRORD

Transport- och kommunikationsverket (Traficom) har låtit utföra en undersökning av metoderna för indikativ analys av barlastvatten med tanke på hamnstatskontrollen. Resultatet av undersökningen presenteras i denna rapport. Fyra olika anordningar avsedda för indikativ analys testades ombord på två fartyg och avsikten var att finna en anordning som lämpar sig bäst för hamnstatskontrollen i Finland och för de förhållanden som råder på Östersjön.

Undersökningen utfördes av biträdande forskare Okko Outinen och forskningsprofessor Maiju Lehtiniemi vid Finlands miljöcentral (SYKE). Styrgruppen bestod av specialsakkunnig Ville-Veikko Intovuori, ledande sakkunnig Anita Mäkinen och enhetschef Mirja Ikonen från Traficom och Okko Outinen och Maiju Lehtiniemi från SYKE.

Traficom riktar ett stort tack till alla de fyra tillverkarna av anordningar för indikativ analys dvs. Luminultra, bbe Moldaenke GmbH, Chelsea Technologies Group Ltd och Microwise, som vänligen lånade ut sina anordningar för undersökningen. Vi vill också rikta ett speciellt tack till rederierna Arctia Shipping Oy och VG-Shipping Oy, på vars fartyg testningen skedde. Utan dem skulle undersökningen inte ha varit möjlig.

Helsingfors, den 31 oktober 2019

Ville-Veikko Intovuori
specialsakkunnig
Transport- och kommunikationsverket Traficom

FOREWORD

The Finnish Transport and Communications Agency (Traficom) has conducted a study on the indicative ballast water analysis testing for port State control purposes. The results of the study have been compiled in this report. Four different indicative analysis devices were tested on board two ships and the purpose was to find a device that would be most suitable for the port State control inspections in Finland and for the Baltic Sea conditions.

The study was carried out by Assisting Researcher Okko Outinen and Research Professor Maiju Lehtiniemi of the Finnish Environment Institute's (SYKE). The steering group of the study consisted of Special Adviser Ville-Veikko Intovuori, Chief Adviser Anita Mäkinen and Head of Unit Mirja Ikonen of Traficom, and Okko Outinen and Maiju Lehtiniemi of SYKE.

Traficom wants to return great thanks to all indicative analysis device manufacturers, i.e. Luminultra, bbe Moldaenke GmbH, Chelsea Technologies Group Ltd and Microwise who kindly lent their devices for the study. We would also like to address our special thanks to the two shipping companies Arctia Shipping Oy and VG-Shipping Oy, on whose ships the tests were conducted. Without them the study would not have been possible.

Helsinki, 31 October 2019

Ville-Veikko Intovuori
Special Adviser
Finnish Transport and Communications Agency Traficom

Sisällysluettelo

1 Introduction

The spread of aquatic non-indigenous species (NIS) and their impacts to marine environment, human health and local resources (IMO, 2009) have initiated a chain of actions towards mitigation and prevention of the issue. The International Convention for the Control and Management of Ships Ballast Water and Sediments, 2004 (BWM Convention) was adopted in 2004 by the International Maritime Organization (IMO), and it entered into force on 8 September 2017 after the BWM Convention was ratified by 52 States representing 35.14% of the World's merchant shipping tonnage a year earlier (IMO, 2019). Currently, the number of contracting States is 79 and they represent approximately 80.94 % of the World's merchant shipping gross tonnage (IMO, 2019).

The BWM Convention obligates ships to comply with ballast water exchange standard (regulation D-1) or ballast water performance standard (regulation D-2), depending on the building or renewal survey date of the ship (Figure 1) (MEPC, 2018). According to the BWM Convention (IMO, 2009), the D-1 standard requires ships to exchange at least 95% of the ballast water volume at open sea during the voyage, whereas the D-2 standard determines viable organism concentration limits in discharged ballast water for ships conducting ballast water treatment as follows;

- Less than 10 viable organisms/m^3 of size ≥50µm in minimum dimension,
- Less than 10 viable organisms/ml of size ≥10 - <50µm in minimum dimension, and
- Less than 1 colony forming unit (CFU)/100 ml of Toxicogenic *Vibrio cholerae*, 250 CFU/100ml of *Escherichia coli* and 100 CFU/100ml of intestinal *Enterococci*.

In short, ships built on 8th of September 2017 or after must follow the regulation D-2 standard immediately, whereas ships built before this date must follow the D-2 standard after their first or second renewal survey of the International Oil Pollution Prevention (IOPP) certificate, or at the latest, by 8th of September 2024 (MEPC, 2018). Until meeting with the D-2 standard, ships must follow the D-1 standard. As the main focus in the future is the compliance of ships' ballast waters with the regulation D-2 standard of the BWM Convention, the remainder of the report will focus on the requirements of sampling and analyses of this regulation.

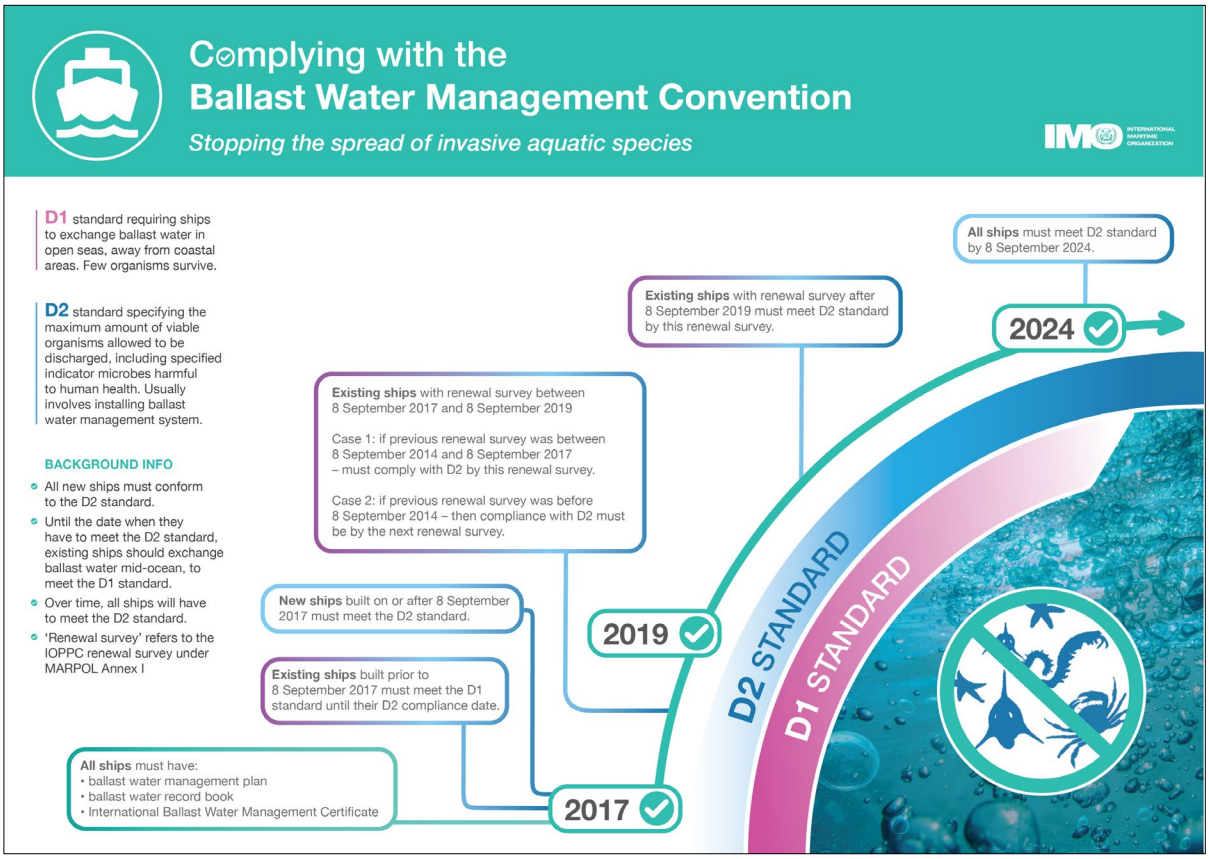

Figure 1. Implementation of the BWM Convention (IMO, 2018a).

IMO established ballast water experience-building phase (EBP) in July 2017 to monitor the implementation of the BWM Convention (MEPC, 2017). The EBP is a three-stage period that includes data gathering, data analysis and the BWM Convention review stages (Figure 2). The purpose of EBP is to gather experience around the procedures assigned by the BWM Convention, and a specific timeline for the EBP will be determined after the data analysis stage (MEPC, 2017). The EBP includes a non-penalization policy, where port State control (PSC) authorities can exempt ship owners from penalties in a situation where the ship fails to meet the D-2 standard even though they have conducted ballast water treatment properly.

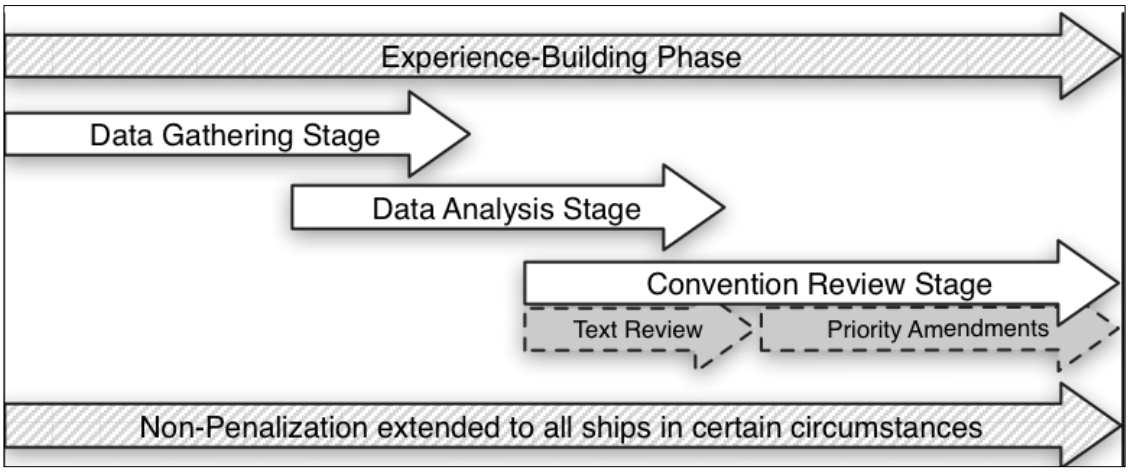

Figure 2. IMO's ballast water experience-building phase (MEPC, 2017).

IMO has also determined guidelines for PSC authorities under the BWM Convention (MEPC, 2014). The four-stage PSC inspection includes initial inspection and detailed inspection, as well as indicative and detailed analyses if necessary. The purpose of initial inspection is to make sure that a ship officer onboard is nominated as responsible for the ballast water management system (BWMS) and the required documentation is appropriate and up to date. The essential documents include International Ballast Water Management Certificate, Ballast Water Management Plan and Ballast Water Record Book. If any documentation is missing or invalid, or if the ship has no officer responsible of the BWMS, detailed inspection can be conducted, which includes further questions to the ship crew and examination of the BWMS functionality (MEPC, 2014).

The detailed inspection may lead to indicative and detailed analyses of the compliance, which includes sampling of the ships' ballast water (MEPC, 2014). Principles for ballast water sampling, as well as indicative and detailed analyses of the ships' compliance have been covered relatively well by earlier research (e.g. David and Gollasch, 2015; IMO, 2015; Outinen and Lehtiniemi, 2017), but to summarise, in-line sampling is generally preferred over in-tank sampling to get a more representative sample of the whole discharge. Sampling should aim to obtain a series of 10 minute samples during the discharge and avoid the first and last five minutes of the de-ballasting period, as it may result to under- or overestimation of present organism concentrations. Sampling for ≥50µm organisms requires at least 300 litres of sample water, whereas sufficient sample volume is approximately one litre for indicator microbes and five to six litres for ≥10 - <50µm organisms (David and Gollasch, 2015). IMO (2015) has defined indicative analysis as a relatively quick, indirect or direct measurement of the present viable organism concentration, and detailed analysis as generally a more complex compliance test that provides a direct and precise measurement of viable organism concentration in the ballast water discharge.

The current study was assigned by the Finnish Transport and Communications Agency (Traficom). The maritime section of Traficom is the Finnish national maritime administration and they are responsible for PSC inspections and ship surveys in Finland. The main purpose of the study was to test different indicative analysis devices and recommend the most suitable method for PSC compliance monitoring purposes. The recommendation will be provided exclusively to Traficom and is therefore not presented in the current report. Additionally, the report aims to provide useful practices and potential improvements as general suggestions to the implementation of the BWM Convention during the IMOs EBP.

The report is a follow-up study to the literature review of the indicative ballast water analysis methods (Outinen and Lehtiniemi, 2017). The tested indicative analysis methods were Adenosine TriPhosphate (ATP) method, modified Pulse Amplitude Modulation (PAM) fluorometry, Single Turnover Active Fluorometry (STAF) and Motility and Fluorescence Assay (MFA). The first three viability methods were chosen because they were considered most suitable methods for indicative analysis by the literature review. The MFA method was included as a recommendation by Traficom, since the new method was not featured in the literature review. The suitability of the methods referred to indicative analysis requirements described in the literature review, such as cost of the device, analysis time, portability, ease of use and accuracy.

2 Materials and methods

2.1 Indicative analysis methods

Based on the previous literature review (Outinen and Lehtiniemi, 2017), it was recommended that organism size fraction ≥10 - <50µm in minimum dimension is the most suitable organism group for indicative compliance analyses. This was the main reason why sampling in the present study concentrated on this size fraction. However, as some of the tested indicative analysis devices also measured viability for ≥50µm organisms and bacteria, sampling of these organism groups were also included.

The tested indicative analysis methods included Adenosine TriPhosphate (ATP, Luminultra, B-Qua BW monitoring kit), modified Pulse Amplitude Modulation (PAM) fluorometry (algae luminescence, equivalent to PAM, bbe-Moldaenke, 10Cells), Single Turnover Active Fluorometry (STAF, Chelsea Technologies Group [CTG], FastBallast) and motility and fluorescence assay (MFA, Microwise, BallastWISE) (Table 1). All indicative analysis devices measure the ≥10 - <50µm organism size group. In addition, BallastWISE measures viability for the largest organism size group (≥50µm), and the Luminultra ATP kit measures viability for all three organism groups. Number of analysis replicates varied between the devices due to their wide range of analysis times. MFA and ATP analyses were done once each (20-40 min per analysis), CTG FastBallast twice (8 min per analysis) and 10cells three times (2-4 min per analysis). One additional replicate was conducted for MFA (≥10 - <50µm) and ATP analyses (≥10 - <50µm and bacteria) each during the second sampling event, as there was only one set of samples being analysed (treated samples).

Table 1. Indicative analysis device specifications.

Device	Size fractions measured	Sample volume	Weight	Cost	Total analysis time (min)
BBE 10cells	≥10 - <50µm	10 ml	5 kg	5,000 – 10,000 €	<5
CTG FastBallast	≥10 - <50µm	20 ml	5 kg	9600 £	<10
Microwise BallastWISE	≥50µm, ≥10 - <50µm	900-1000 ml, 200 ml	8 kg (+external computer)	18,900 €	20-30
Luminultra B-QUA ATP	≥50µm, ≥10 - <50µm, bacteria	300-1000 l, 200 ml, 100 ml	12 kg	5,000 € (+consumables 76€ per analysis)	30-40

2.1.1 ATP method

The Luminultra B-QUA ATP method estimates viable organism concentration within a sample through the extraction of Adenosine TriPhosphate, a molecule

produced by all viable organisms (Hodson et al., 1976; Bakalar, 2014). Luminultra is a global microbial monitoring company with 16 years of experience in the monitoring and microbe control of drinking water, manufacturing industries, energy applications and wastewater treatment (Luminultra, 2019).

The B-QUA kit enables the detection of organism viability for all three organism size groups separately within approximately 30 to 40 minutes (Luminultra, 2018). The analysis time depends primarily on the experience of the person conducting the analysis. Anyhow, the minimum analysis time is approximately 30 minutes since the methodology includes several mixing and filtration steps (Appendix 1, detailed chemical quantities not presented as desired by the manufacturer). Different organism size groups are separated with filters and the ≥50µm, as well as the ≥10 and <50µm organisms are analysed from the filters, whereas bacterial ATP is analysed from <0.7µm filtrate after additional filtration steps (Figure 3). In short, the method is a bulk ATP measurement for each organism group and the compliance is evaluated from the amount of intracellular ATP (cATP) extracted per sampled volume. Therefore the methodology is not able to determine the size of individual organisms within the analysed sample. The results are displayed as concentrations of cATP per volume (Table 2). There is no conversion to viable organism concentrations, as the manufacturer does not consider conversion factor necessary for indicative analyses.

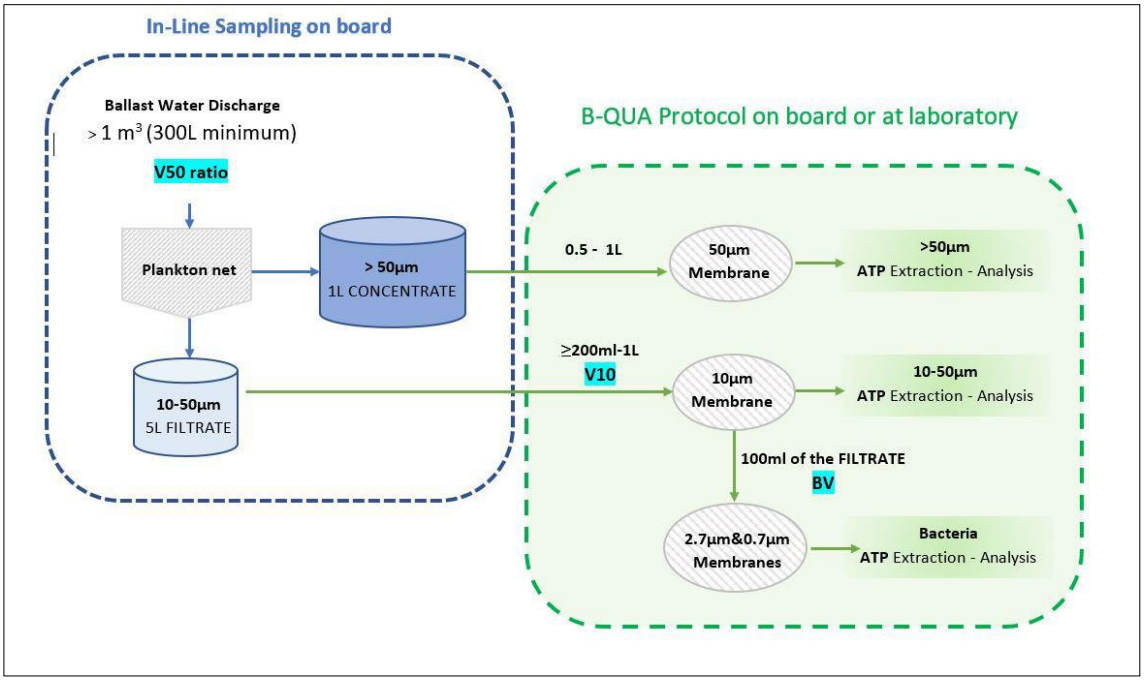

Figure 3. Sampling options for the Luminultra B-QUA ATP kit organism group analyses (Luminultra, 2018).

Table 2. Compliance limits for the Luminultra ATP analyses (Luminultra, 2018). *Values under green limits indicate compliance, red non-compliance and orange values recommend improving and/or repeating the measurement.*

Size fraction	**Most likely compliant**	**Signal close to the limit**	**Most likely not compliant**
≥50µm (cATP pg/m³)	<10,000	10,000-750,000	>750,000
≥10 and <50µm (cATP pg/ml)	<500	500-1500	>1,500
Bacteria (cATP pg/100ml)	<1000	1000-5000	>5000

2.1.2 Modified PAM method

PAM fluorometers measure photosystem II (PSII) efficiency, the photosynthetic activity of autotrophic organisms within a sample (Schreiber, 2004). The detection of PSII differentiates photosynthetic variable fluorescence (Fv) from other sources of fluorescence that generate only constant fluorescence and do not react to light (Kromkamp and Forster, 2003). PAM method utilizes multiple turnover (MT) method to detect Fv emitted by viable phytoplankton cells. Generally, the MT method uses relatively long (50 - 1000ms) light pulses to generate maximum fluorescence emission from the viable phytoplankton cells within a sample, and therefore the recording and recovery time of the pulses are also longer (Kromkamp and Forster, 2003).

The modified PAM method (10Cells) measures delayed fluorescence, which is emitted when electrons that were released from the photosystem recombine (Moldaenke et al., unpublished). This emission occurs only in living algae cells, and its signal can be detected for a few milliseconds up to a few hours after the excitation source is turned off. Delayed fluorescence is unaffected by interfering fluorescence from dead cells and inorganic matter.

BBE Moldaenke is a German spectrofluorometer and toximeter manufacturer with over 20 years of experience in the environmental monitoring industry (BBE Moldaenke, 2019). The 10cells fluorometer predisposes the samples to excitation light pulses (excitation and recording time 300 ms) at 650 nm and the emission is measured approximately at 685 nm (Moldaenke et al., unpublished). The viable organism concentration is converted from the luminescence signal (equivalent to Fv signal) recorded throughout the measurement. The analysis steps include two filtration steps, and the fluorometer detects the signal from the second 8µm filter (Figure 4, Appendix 2). 10cells conducts 5-10 measurements during the analysis (adjustable), records the average bulk fluorescence signal of the ≥10 - <50µm sized phytoplankton cells and converts this signal into an organism concentration by using a predetermined fluorescence-per-cell value within the ≥10 - <50µm size class. Therefore, even though the results are displayed as organism concentrations, it is an indirect measurement and the device is not able to measure whether the Fv signal has been emitted by a few large cells or several smaller cells within the size group.

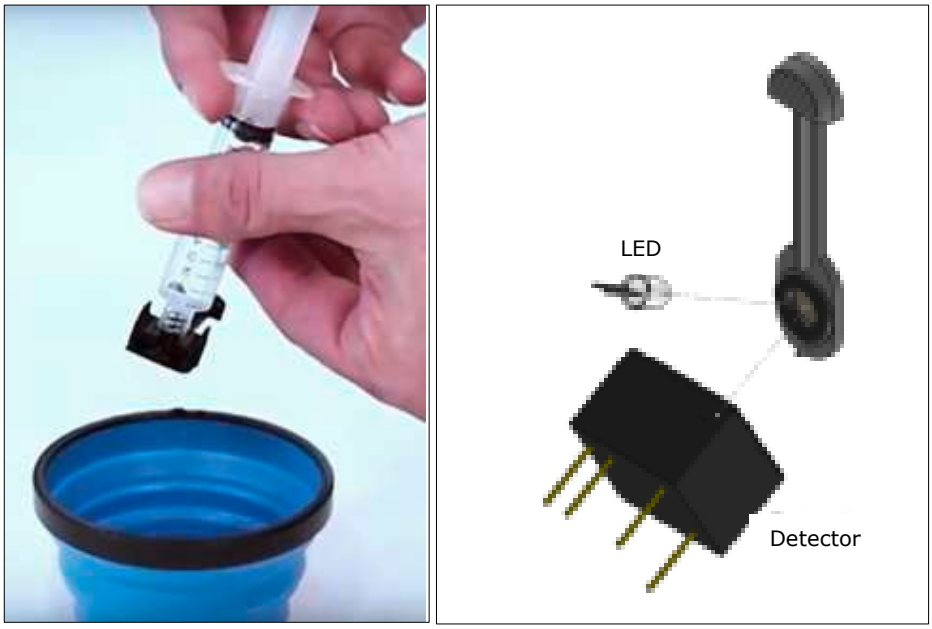

Figure 4. Syringe filter and the BBE 10cells detection unit (Moldaenke et al., unpublished).

2.1.3 STAF method

Similarly to PAM fluorometry, the STAF method measures PSII efficiency of the ≥10 and <50µm-sized phytoplankton cells. Anyhow, in contrast to the PAM method, STAF is based on single turnover (ST) method of variable PSII fluorescence. The ST method utilizes brighter and shorter excitation flashes (usually between 100 - 400µs) than the MT method, enabling the execution of more single measurements per analysis due to shorter recovery times between the saturation pulses (Oxborough, 2019). Because of the longer excitation flashes and inflection that are associated with the PAM method, maximum PSII efficiency measured with the MT method can be up to 16% higher than the maximum PSII efficiency measured using the ST method, which varies on the measured phytoplankton taxa (Kromkamp and Forster, 2003). Fast Repetition Rate (FRR) fluorometers can be adjusted to utilize both, ST and MT flashes (Kolber et al., 1998), and the STAF method has been developed from FRR fluorometry to obtain higher sampling frequency in comparison to PAM and FRR methodologies (K Oxborough, personal communication, 1 February 2019).

Chelsea Technologies Group Ltd (CTG) has 53 years of experience as a global manufacturer for wide range of sensors and systems in environmental, maritime and process control industries (CTG, 2019). The FastBallast fluorometer uses four excitation LEDs (at 455, 470, 530 and 624 nm wavelengths) and sends 400µs light pulses to saturate photosynthesis (Oxborough, 2019). The use of LEDs can be adjusted and default settings utilise royal blue and blue wavebands (455 and 470 nm). Fluorescence emission is detected at 682 nm.

The FastBallast STAF method includes inserting a sample (20ml) to the sample chamber and the analysis itself has two tests (Level 1 and 2) (Oxborough, 2019). If the measured Fv signal from the sample is so low that the result "pass" would become inevitable, level 1 test provides the result in just over a minute. For the differentiation, level 1 test uses a numeric of 0.08 as a threshold value. If >0.08 of Fv is detected, the analysis automatically continues to level 2 test. The level 2 test runs for approximately six minutes on top of the level 1 test. FastBallast has a stirring unit that mixes the 20ml sample slowly during the tests and the device takes several hundred individual measurements during the analysis (0.5 ml per semi-discrete measurement). The analysis calculates standardized cell fluorescence (SCF) value of the sample using these individual measurements and presents the result (cells/ml) based on the Fv and the SCF values. The total analysis time is approximately eight minutes and on top of the estimated cell concentration, the analysis provides the SCF value, which refers to an average cell size measured during the analysis (Figure 5).

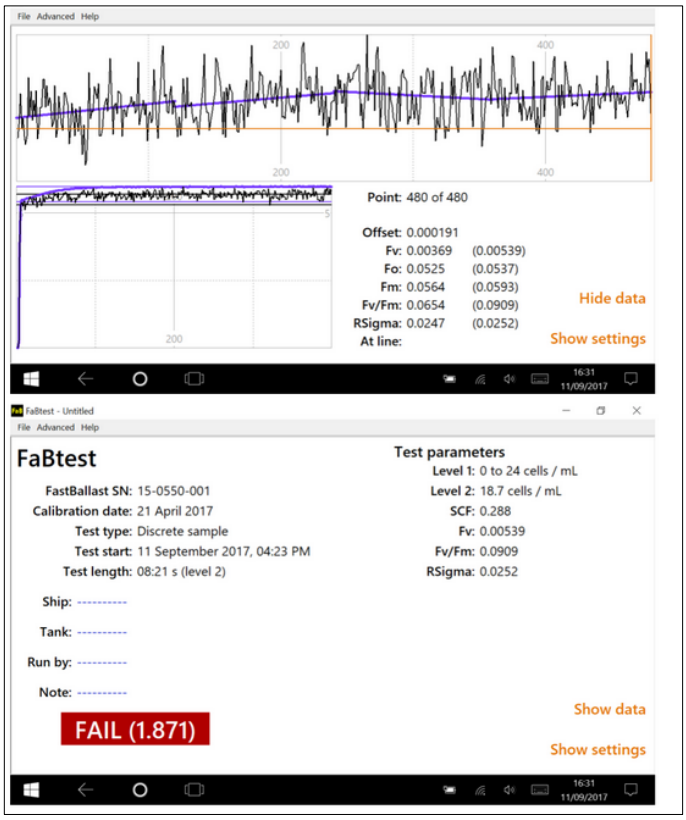

Figure 5. FastBallast analysis result presenting Fv from the individual measurements, cell concentration and the SCF value (Oxborough, 2019).

2.1.4 MFA method

The BallastWISE Motility and Fluorescence Assay (MFA) method was not featured in the previous literature review by Outinen & Lehtiniemi (2017), since the methodology was released in 2018. Microwise is a newly founded company concentrating solely in developing technology and equipment to measure living organisms in water samples (Microwise, 2019). The BallastWISE MFA device has two sample chambers separately for the ≥50µm and ≥10 - <50µm organism groups and both analyses can be run simultaneously. The analysis time is approximately 30 minutes, but the ≥10 - <50µm analysis is slightly faster and can be run separately in 20 – 25 minutes. The sample chambers used for the analyses are 60ml and 24µl for the ≥50µm and ≥10 - <50µm organism groups respectively. As the MFA ≥50µm analysis evaluates viable organism concentrations per litre, this value has to be divided by the sample volume (150 or 300 l in this study), if concentrated samples have been applied.

The device utilises cameras to track and measure individual organisms. Each camera has a high pass filter at 590nm, which allows chlorophyll-*a* fluorescence to pass through, while blocking the original light source. Each subsample is first illuminated with red light above 590nm in order to track the motility of organisms that do not necessarily contain chlorophyll. The subsample is subsequently illuminated with violet light (420nm) in order to detect fluorescence emitted from autotrophic cells containing chlorophyll-*a*. Motile organisms are tracked by the computer in real time. Chlorophyll-*a* fluorescence is detected only from intact cells, regardless of their motility. Therefore, Ballastwise counts the total number of viable organisms separately for motile organisms, fluorescing organisms, as well as motile and fluorescing organisms.

The Ballastwise compares the tracked motile organisms to its database of tracks and size measurements, as well as swimming velocity and acceleration

parameters for the final enumeration. The device records raw video sequences and organism size and track logs, which allows the MFA analyses to be re-examined if there are uncertainties about the analysis results regarding to organism motility. Visualisation of data in the form of track plots enables more elaborate examination of whether clear swimming patterns of motile organisms can be identified.

However, at the time of the study the re-examination of the analyses was not possible on site, and it was done by sending the analysis files to the manufacturer. These settings need to be permanently adjusted in the future, but they are useful for future development of the device by providing more detailed documentation of the analyses. There are no distinct analysis steps, but before it can be started, the samples, sample chambers and rubber tubes need to be placed to the device (Figure 6).

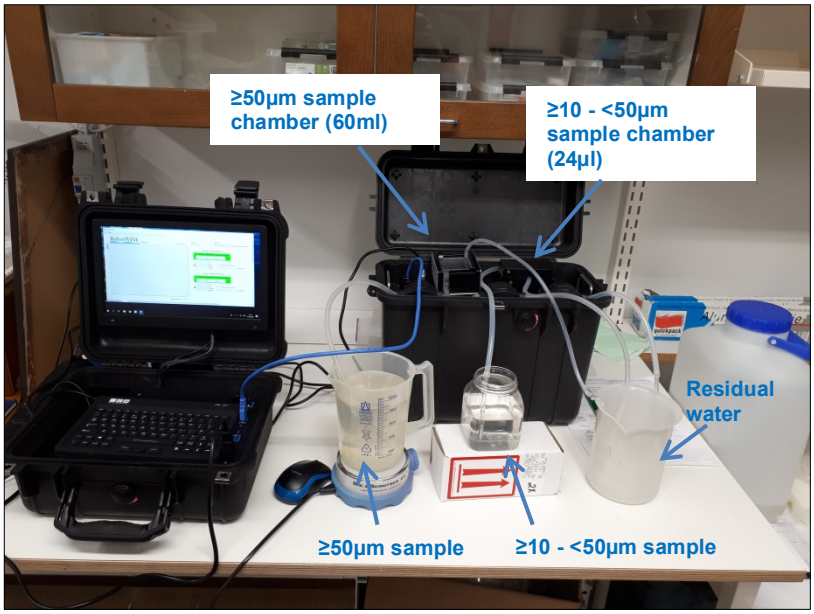

Figure 6. BallastWISE detection unit connected to an external computer.

2.2 Experimental design

The study included two separate onboard samplings. The first sampling event was carried out on 25 October, 2018, at M/S Polaris, an icebreaker (Arctia Shipping) that has Auramarine CrystalBallast ultraviolet (UV)-filtration ballast water treatment system (BWTS) installed onboard (Figure 7). M/S Polaris was located at its home dock, the Katajanokka quay (Port of Helsinki), wherefrom the state-owned company operates icebreaking services, oil spill prevention and other offshore services with nine ships (Arctia Shipping, 2019). The sampled water was ballasted fresh of the dock, treated and sampled immediately after treatment. Therefore the first sampling was conducted under less realistic circumstances regarding to what would be a typical port State control (PSC) ballast water compliance monitoring event, as there was practically no holding time for the ballasted water in the tanks after treatment. In addition, UV treatment systems generally utilize the UV-treatment twice for ballast water, during ballasting and de-ballasting. As the ballast water was sampled immediately after treatment however, the water was treated only once with UV in the present study. The indicative analyses were conducted immediately after sampling. After the sampling and indicative analyses of the UV-filtrated ballast water, untreated seawater samples were taken from the dock where the ship was standing, to compare if the devices were able to differentiate organism viability between treated and untreated samples.

Figure 7. Auramarine CrystalBallast treatment system and monitoring unit at M/S Polaris.

The second sampling was conducted on 5 November, 2018, at Mirva VG, a dry cargo carrier (VG-Shipping) equipped with OceanGuard 3-phase BWTS utilising filtration, electro catalysis and ultrasonic treatment (EUT) system onboard (Eureka Marine Engineering, 2014, Figure 8). This sampling event aimed to mirror a real-time PSC inspection event and the viability analyses were conducted only for the treated ballast water samples. VG-Shipping is a private Finnish ship owner and management company that also provides cargo delivery services (VG-Shipping, 2019). Mirva VG sampling was conducted at the Port of Inkoo, approximately 50 km west from Helsinki, where the ship visited to unload cargo. The sampled ballast water was ballasted in 2 November, 2018, at Sillamäe, North-eastern coast of Estonia. Therefore the holding time of the ballast water was approximately 72 hours after treatment, as the treatment was conducted during ballast water intake.

Figure 8. Oceanguard Ballast Water Management System at Mirva VG.

2.3 Onboard sampling

300 litres of ballast water was sampled during both sampling events. Both ships had isokinetic sampling valve including a tap as a sampling point (Figure 9). The sampled water was led from the tap with a hose through a water flow meter (Gardena Water Smart, paddle wheel) and 50µm plankton net to 80 l water containers (Figure 10). Therefore organisms ≥50µm concentrated to the cod-end of the plankton net and <50µm organisms filtrated through the net to the water containers.

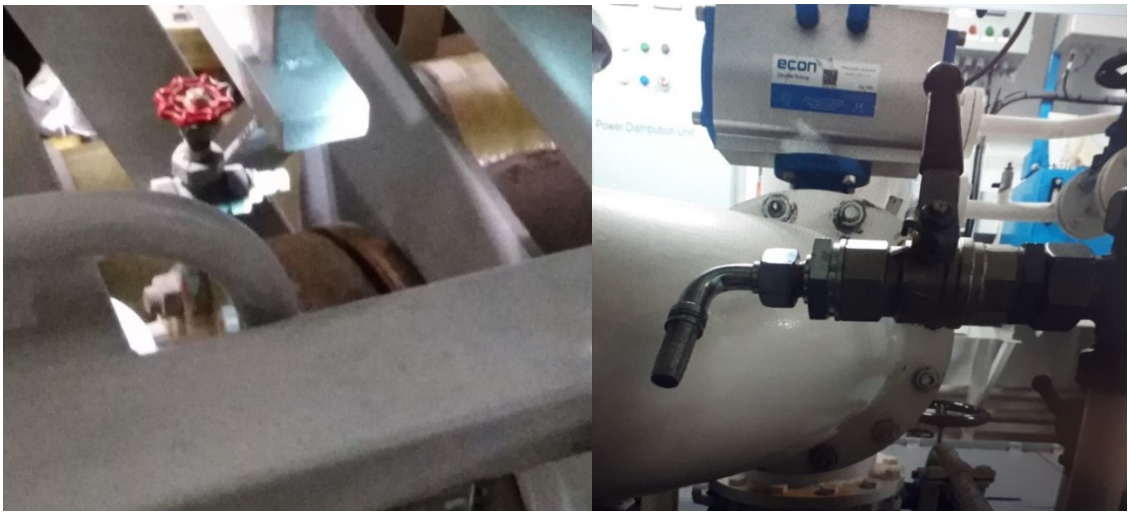

Figure 9. Sampling tap at M/S Polaris (left) and Mirva VG (right).

Figure 10. Water flow meter and other sampling equipment set up at M/S Polaris.

The sampling tap was opened five minutes after the de-ballasting started and closed between every 30-50 l for a few minutes to collect 1-2 l of the <50µm filtrate to a 10 l water container in order to get a representative sample and to switch the 80 l water container when needed. The excessive filtrate was poured into ships' bilge. The ≥50µm concentrate was collected from the cod-end of the plankton net to a 5 l water container after 150 litres of sampling, and at the end of the sampling to another container. Therefore, the ≥50µm concentrate was divided equally between Luminultra ATP and BallastWISE ≥50µm analyses (150 l

volume per analysis), as they were the only indicative devices that measured the largest organism size fraction. Overall, the sample volumes were 300 l for ≥50µm organisms and 10 l for ≥10 - <50µm organisms. Organisms <10µm were not filtrated out as the indicative analysis devices are expected to take this into consideration during the analysis stages. The recorded water flow velocity did not exceed 20 l per minute at any point during sampling.

Untreated water samples were included to the first experiments at M/S Polaris and they were taken of the dock with a Limnos water sampler (10 l), as well as with a 50 µm plankton net (2 x 300 l) (Table 3). The 300 l (x2) samples of ≥50µm organisms were obtained by one tow with the plankton net at 3.51 m depth of the dock (plankton net diameter 33cm). Untreated samples were analysed in the laboratory after the analyses of treated water samples. The 10 l of untreated sample water collected with the Limnos sampler was filtrated through 50µm sieve in the laboratory before the indicative analyses. In contrast to the treated samples, the untreated ≥50µm concentrate (2 x 300 l) was taken separately for Luminultra ATP and BallastWISE analyses (300 l volume per analysis). The completion of each sampling and analysis event took approximately 8-12 hours (Table 3).

Table 3. Approximate timetable for the conducted sampling events, as well as indicative and detailed analyses.

Sampling phase	Time frame
Equipment preparation & sample collection	60-75 min
Indicative analyses (all devices, 5 – 40 min per device) of treated water onboard	2-3 hours
Sampling for untreated sea water (Polaris only)	30 min
Sample transportation to the laboratory	30-60 min
FDA + microscopy (Preparation + staining + microscopy)	2-4 hours
Indicative analyses to untreated water (Polaris only)	2-3 hours

2.4 FDA staining and epifluorescent microscopy

Detailed analyses were conducted using Fluorescein diacetate (FDA) staining and epifluorescent microscopy method, as it is considered one of the most suitable methods to determine viable organism concentrations for the ≥10 - <50µm organisms (David and Gollasch, 2015). Furthermore, as there is no clear evidence that the use of additional staining chemicals would stain viable organisms more efficiently (MacIntyre and Cullen, 2016), other stains, such as 5-chloromethylfluorescein diacetate (CMFDA) were not applied. FDA detects viability by diffusing through cell membrane of viable organisms. Enzymatic activity within intact cell membrane results in accumulation of fluorophore, which can be detected as green fluorescent emission and quantified with an epifluorescent microscope (Rotman and Papermaster, 1966; Welschmeyer and Maurer, 2011; MacIntyre and Cullen, 2016).

FDA staining and epifluorescent microscopy analyses were conducted only for the ≥10 - <50µm organism group in three replicates per sample, to compare organism concentrations between detailed and indicative analysis samples. The FDA staining and epifluorescent microscopy analyses were conducted in accredited (ISO 17025) Marine Research Centres' laboratory. The method included concentrating samples (5 l of <50µm filtrate with a 10µm plankton net, Figure 11), preparation of the FDA solutions, sample staining and cell counting under epifluorescent microscope (100x magnification, Appendix 3). All FDA analyses were conducted within six hours from sample collection and each sample was analysed for 20 minutes to limit the impact of potential stain leakage (Adams et al., 2014).

Figure 11. The 10µm plankton net used for the concentration of the detailed samples.

2.5 Data analysis

The data was tested for normality with Levene's homogeneity of variances test (SPSS 23). As the data did not fulfil the requirements of parametric tests (Levene = 3.292, df = 44, p <0.01), non-parametric Mann-Whitney U test was used to compare the viable organism concentrations between detailed analysis and 10cells indicative analysis. Due to the uneven and insufficient number of replicates conducted with the rest of the indicative analysis devices, statistical analyses could not be conducted for these data. Instead, the data of the other devices was compared to the organism concentrations of the detailed analyses with figures. Indicative analysis results for ≥50µm organisms and bacteria were only compared directly with IMOs' compliance limit values.

3 Results

All viability assessment methods detected different numbers of viable organisms between the treated and untreated water samples (Table 4). The untreated water samples were consistently non-compliant between the methods, whereas some variation was detected between the methods in the treated samples. Most of the treated samples at M/S Polaris (Filtration+UV) were also considered non-compliant by the analysis methods as only the ATP analyses for the ≥10 - <50µm organisms and bacteria indicated compliance. More inconsistency in compliance determination was detected between the methods for the treated samples at Mirva VG (Filtration+EUT) (Table 4).

Table 4. Compliance analysis results for the viability assessment methods. Green colour indicates compliant result, red non-compliant, and orange indicates that the analysis must be repeated with enhanced sample volume.

Treatment	Method	Size fraction	Replicate 1	Replicate 2	Replicate 3	Unit
UV+ filtration (Polaris)	10cells	≥10 - <50µm	62	89	72	org/ml
	FastBallast	≥10 - <50µm	610.2	588.7		org/ml
	BallastWISE	≥10 - <50µm	45.833			org/ml
		≥50µm	113.7			org/l
	B-Qua ATP	≥50µm	4361431.59			ATP pg/m^3
		≥10 - <50µm	270.03			ATP pg/ml
		Bact.	896.46			ATP pg/100 ml
	FDA (detailed)	≥10 - <50µm	73.44	69.408	64.8	org/ml
Untreated	10cells	≥10 - <50µm	406	490	481	org/ml
	FastBallast	≥10 - <50µm	4188	3524		org/ml
	BallastWISE	≥10 - <50µm	372.8			org/ml
		≥50µm	1386			org/l
	B-Qua ATP	≥50µm	53388644.42			ATP pg/m^3
		≥10 - <50µm	1797.72			ATP pg/ml
		Bact.	5839.75			ATP pg/100 ml
	FDA (detailed)	≥10 - <50µm	196.84	206.64	216.16	org/ml
EUT+ filtration (Mirva VG)	10cells	≥10 - <50µm	7.4	5.4	5.9	org/ml
	FastBallast	≥10 - <50µm	77.5	46.1		org/ml
	BallastWISE	≥10 - <50µm	10.43	4.167		org/ml
		≥50µm	242.2			org/l
	B-Qua ATP	≥50µm	384770.91			ATP pg/m^3
		≥10 - <50µm	38.67	41.32		ATP pg/ml
		Bact.	178.17	200.56		ATP pg/100 ml
	FDA (detailed)	≥10 - <50µm	1.484	1.68	2.016	org/ml

In general, there was variation between the detailed analysis and indicative device analysis results in the ≥10 - <50µm size fraction (Figure 12). However, the organism concentrations did not differ significantly between 10cells and the detailed analyses (Mann-Whitney U test: $U \leq 4$, $p \geq 0.1$). The BallastWISE results indicated less viable organisms than the detailed analysis results for the UV-treated samples, and more viable organisms for the other samples. The FastBallast results showed consistently higher viable organism concentrations than the detailed FDA analyses. ATP analysis results for the ≥10 - <50µm organisms were reported as intracellular ATP (cATP) concentrations as described in the method section. The recorded cATP concentrations showed a similar pattern between different samples than the FDA counts with the lowest cATP concentrations being recorded from the EUT-treated samples and highest from the untreated samples (Figure 13).

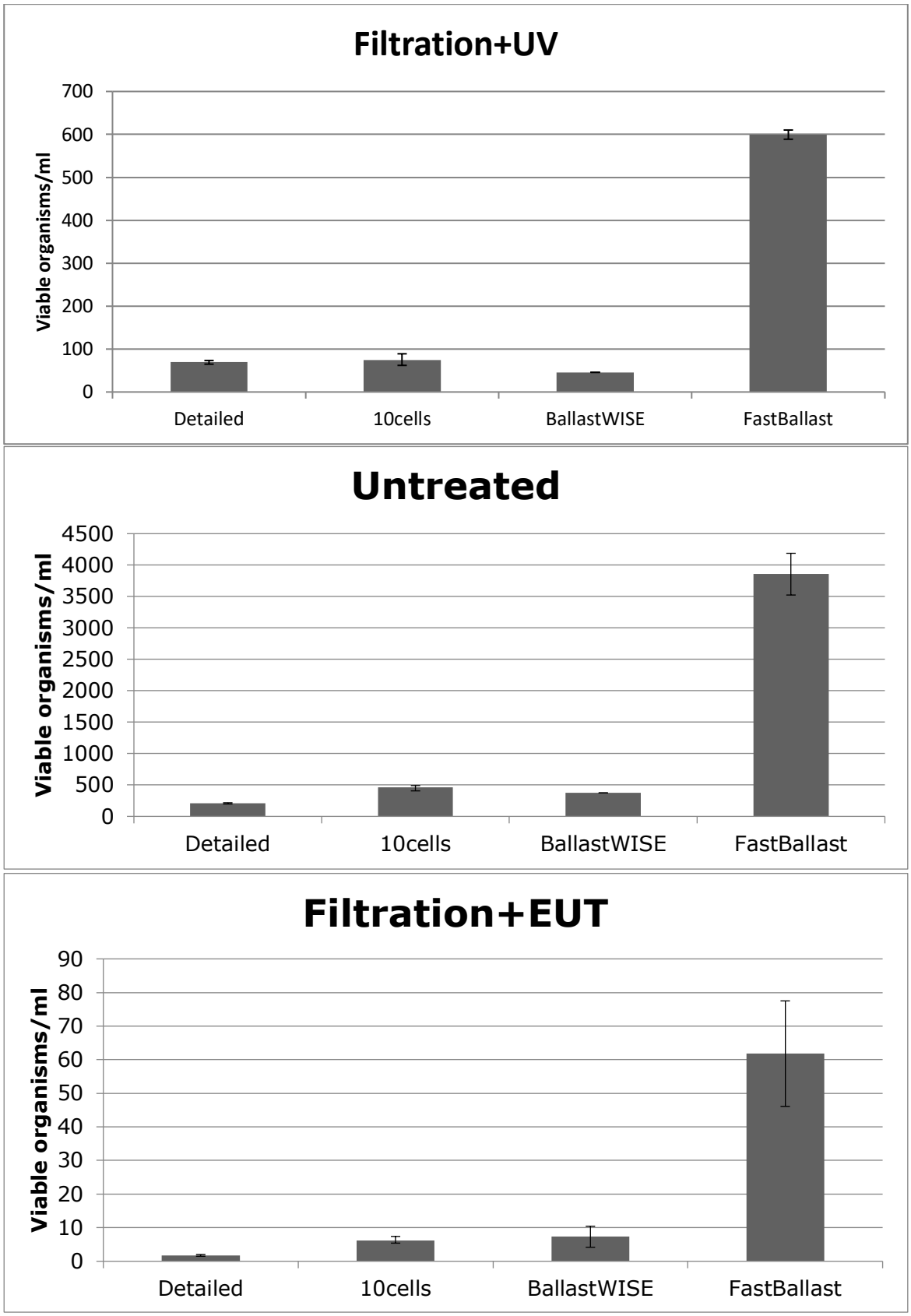

Figure 12. The ≥10 - <50µm viable organism concentrations between the detailed analyses and the indicative analysis devices (except Luminultra ATP).

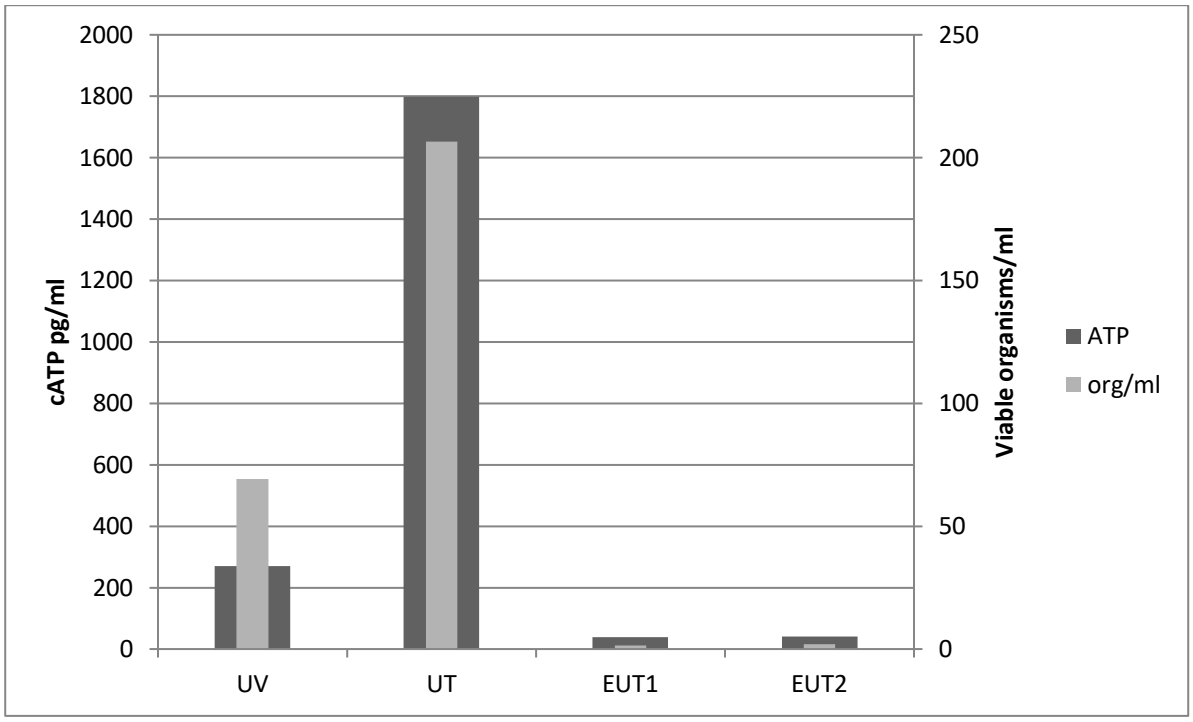

Figure 13. The ≥10 - <50µm analysis results between the Luminultra B-Qua kit in intracellular ATP concentrations and the detailed analyses in viable organisms.

The ATP analyses for ≥50µm organisms did not indicate compliance directly for any samples (one sample was in the 'close to the limit' range), whereas bacteria analyses indicated non-compliance only for the untreated water (Table 4). The ≥50µm analyses conducted with BallastWISE indicated heavy non-compliance for all samples. Final BallastWISE ≥50µm viable organism concentrations were converted to correspond the IMO ≥50µm limit value volume (m3), as the original analysis results were given in organisms per litre (Table 5).

After re-examination of the results by the manufacturer (Microwise), the viable organism concentration for the treated water at M/S Polaris decreased from 113,7 to 13 organisms/l (Figure 14). Similar pattern was detected for the treated water at Mirva VG, where the viable organism concentration decreased from 242,2 to 18 organisms/l after the re-examination (Figure 15). The BallastWISE ≥50µm analysis for the untreated water got overloaded with moving particles and motile organisms. Therefore, the manufacturer advised to triple the number of viable organisms as the analysis was able to handle only approximately third of the camera view (Figure 16). Therefore the viable organism concentration for the untreated water was 4158 organisms/l according to the analysis re-examination.

*Table 5. Final viable organism concentrations for BallastWISE ≥50µm analyses after conversions. The re-examined organism concentrations were divided by sample volume and multiplied by 1000 to make them comparable with the IMO limit value. *The organism concentration of the analysis tripled after re-examination.*

Treatment	Original result (viable organisms/l)	Result after re-examination by the manufacturer (organisms/l)	Final organism concentration after conversions (Viable organisms/m^3)
Filtration+UV (150 l)	113.7	13	86.667
Untreated (300 l)	1386	4158*	13860
Filtration+EUT (150 l)	242.2	18	120

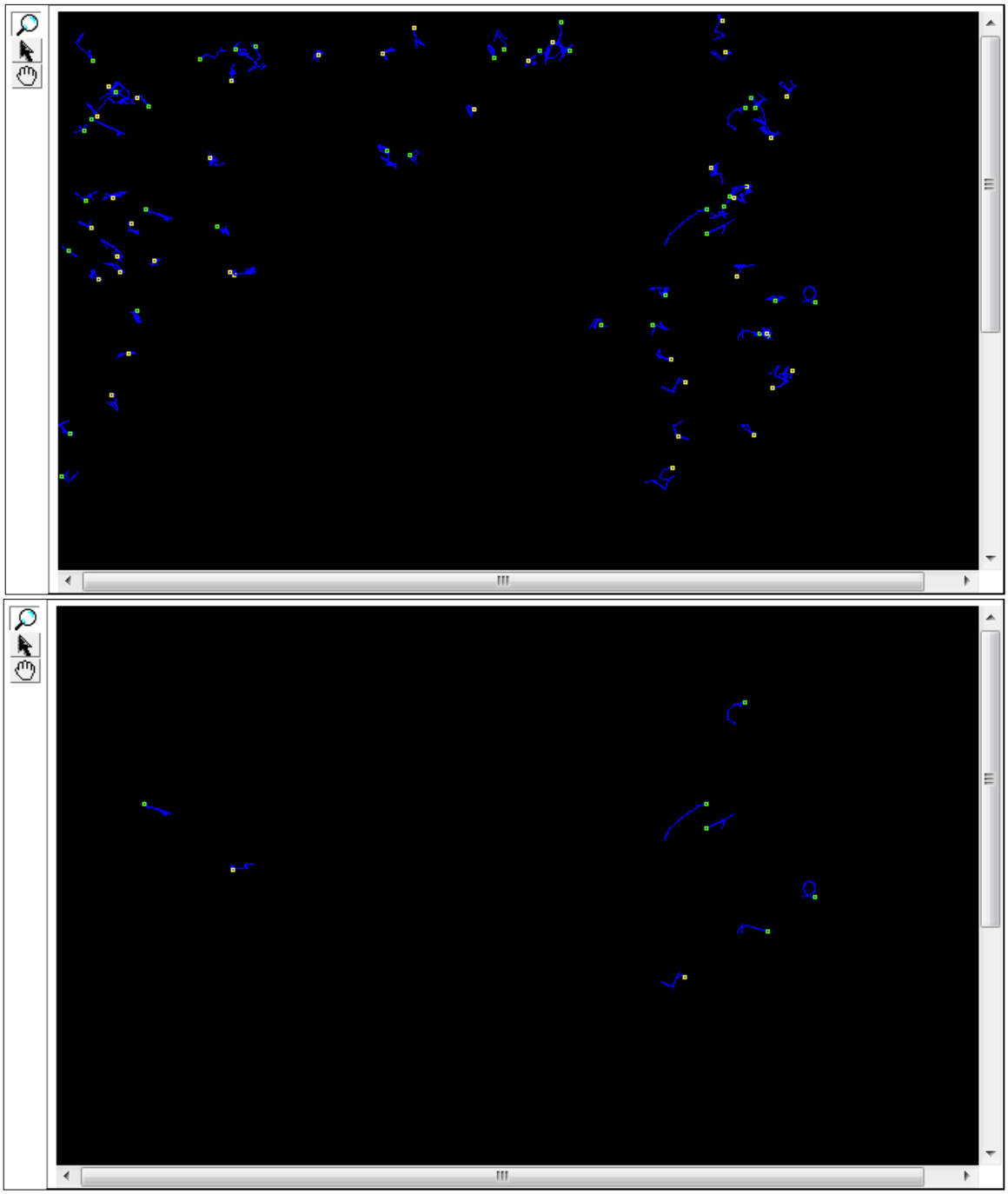

Figure 14. BallastWISE ≥50µm analysis for the UV-treated water at M/S Polaris showing motile viable organisms before and after re-examination by the manufacturer (Acceleration setting changed from 5000 to 2000).

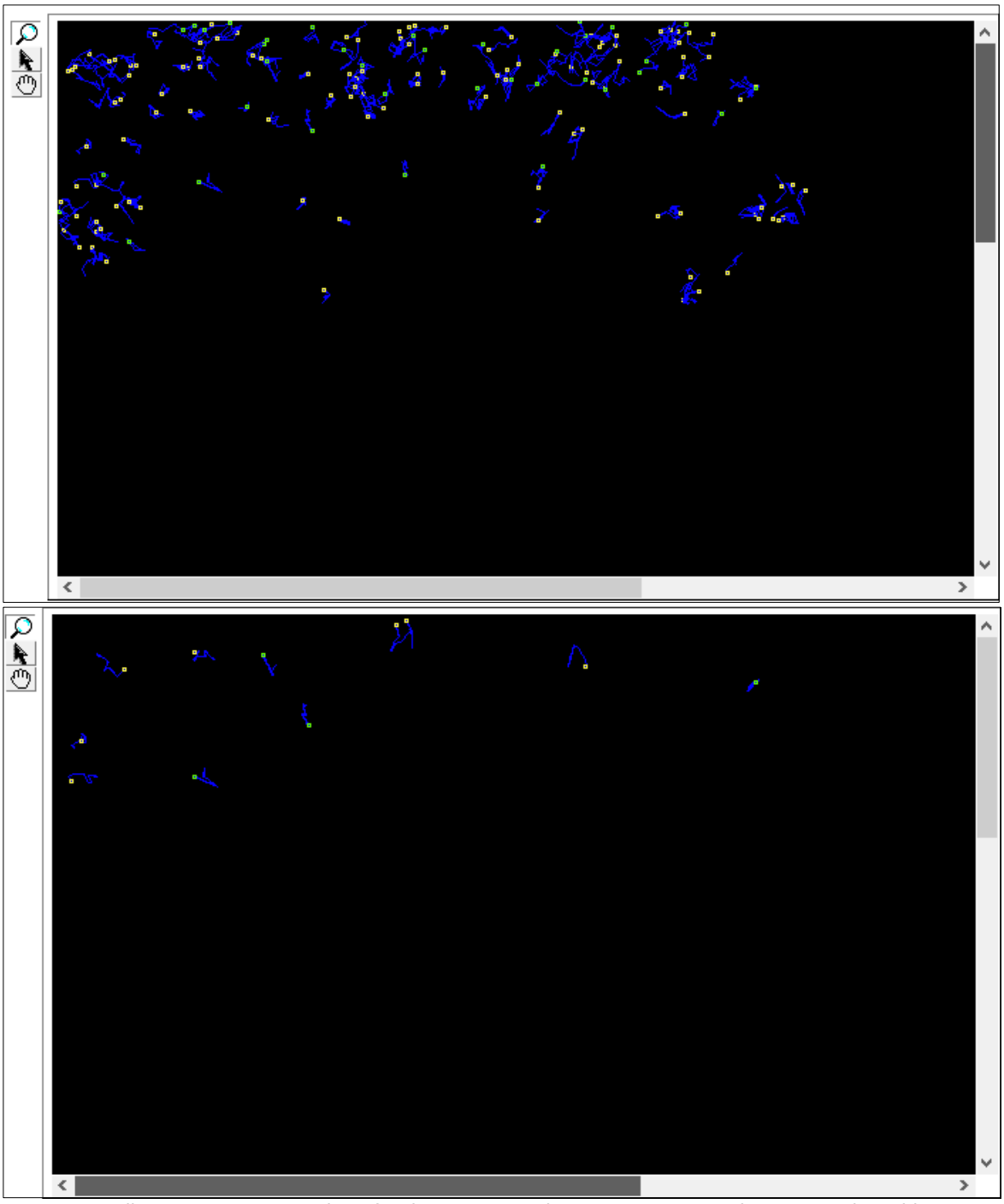

Figure 15. BallastWISE ≥50µm analysis for the EUT-treated water at Mirva VG showing motile viable organisms before and after re-examination by the manufacturer (Acceleration setting changed from 5000 to 2000).

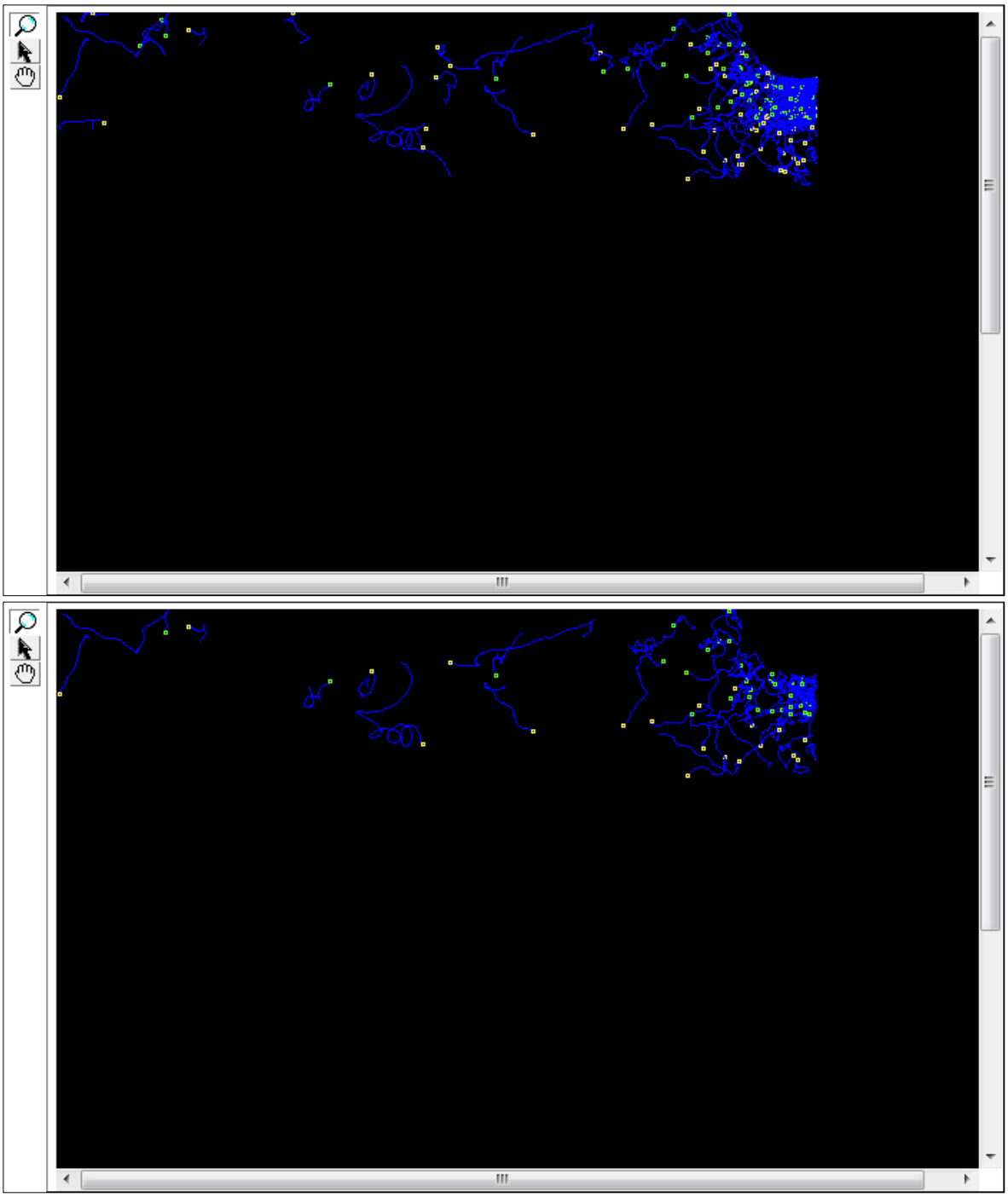

Figure 16. BallastWISE ≥50µm analysis for the untreated water showing motile viable organisms before and after re-examination by the manufacturer (Acceleration setting changed from 5000 to 2000, only third of the camera view assessed).

4 Discussion

4.1 Ballast water sampling

If the port State control (PSC) inspection proceeds to indicative and detailed analysis stages of the inspection, representative sampling of the ships' ballast water has to be conducted prior to the viability assessments (MEPC, 2014). IMO (2009) has stated in the G2 guidelines for ballast water sampling that sampling from the ballast water discharge line should be conducted with isokinetic sampling facility, where the inlet pipe of the sampling tool can be installed parallel to the ballast water discharge line (Figure 17). In addition, further sampling equipment includes a net for sample concentration, water containers for samples and surplus water, funnel, toolkit to set up plankton net and tape (IMO, 2009).

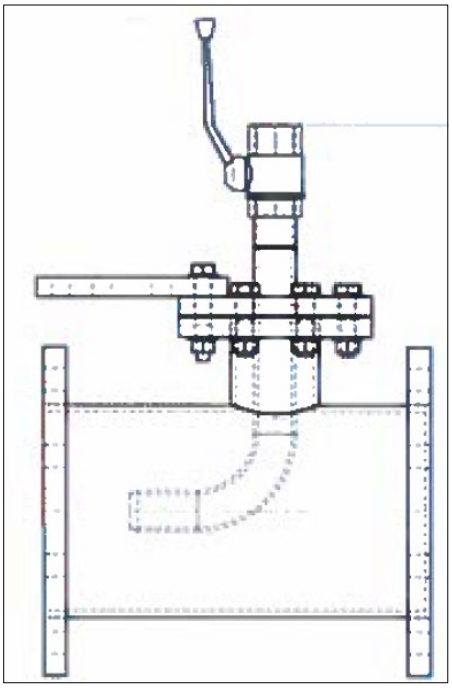

Figure 17. Guidance for isokinetic sampling facility (Auramarine, 2015).

According to present knowledge, there are only a few available isokinetic sampling tools for ballast water sampling, such as the ones provided by SGS and Triton (Schillak, 2014; Bradie et al., 2018b), but other tools can be similarly produced for ballast water sampling (Moser et al., 2018). The ships sampled in the present study were visited prior to the actual sampling events to ensure the operability of sampling. M/S Polaris had an installed outflow pipe in the discharge line, connected to a tap as a sampling point, whereas Mirva VG had no sampling point installed and the piping led straight towards the discharge outlet after treatment. After the pre-visit at Mirva VG, similar sampling point was installed onboard as in the piping system of M/S Polaris.

Sampling with previously mentioned commercial tools would have not been possible at Mirva VG as there was no side valve available at the discharge line, where the sampling tool could have been applied. Therefore, further standardization needs to be implemented during the International Maritime Organizations' (IMO) experience-building phase (EBP). The present G-2 (Guidelines for ballast water sampling) and G-8 (Guidelines for approval of ballast water management systems) guidelines by IMO (IMO, 2009; MEPC, 2016) are inadequate in terms of conducting successful sampling on treated ballast water at all ships. Handling of the sampling issue is under progress with the development

of new ISO standard for ballast water sampling, and new requirements of the commissioning testing that was accepted by the IMO in November, 2018 (Bailey and Rajakaruna, 2017; IMO, 2018b). The following remarks and experiences from the present sampling events will be nevertheless valuable, even though the ISO standard may change sampling requirements significantly in the near future.

Ballast water sampling at M/S Polaris and Mirva VG was relatively straightforward after the pre-visits, as a water hose was connected to the sampling tap with suitable connectors and the sampled water was led through the hose and a flow meter to the 50µm plankton net. The standardization of the sampling point itself could potentially improve the sampling conditions across all types of ships significantly.

Sampling of different organism size fractions, as well as duration and complexity of sampling are all closely related to one another. IMO (2015) has defined representative sampling in Annex 1 of the G2 guidelines as *"relative concentrations and composition of the populations in the volume of interest"*. In addition, earlier research by David (2013), David and Gollasch (2015) and Gollasch and David (2017) suggests that sufficient amount of sample water is 300-500 litres for ≥50µm organisms, 5-6 litres for ≥10 - <50µm organisms and one litre for the indicator microbes. David and Gollasch (2015) also mentioned that ≥10 - <50µm organisms are the most suitable organism group to detect ships' compliance in indicative manner, since the sampling for larger organisms and the bacterial analyses in colony forming units (CFU) can be too time-consuming during a PSC inspection.

Finnish Transport and Communications Agency advised in the previous literature review that the maximum analysis time for an indicative analysis onboard would be approximately two hours (Outinen and Lehtiniemi, 2017). Even though it was presented as 'analysis time', the sampling time has to be included into this timeframe. Measuring bacterial concentrations with the preciseness of the D-2 standard (in CFUs) is never shorter than four hours with commercially available methods (IMO, 2015), and sampling for hundreds of litres is not only more time-consuming, but also more complicated than collecting 5-6 litres of water in sequences and just pouring the water through a 50µm sieve afterwards. The plankton net for concentrating samples is only needed if ≥50µm organisms are sampled. Furthermore, if the indicative analysis indicates non-compliance, detailed analysis can be conducted to the same size fraction. Therefore, sampling of at least 600 – 1000 litres of ballast water would be necessary for ≥50µm organisms (both analyses) and this would be just enough to conduct one replicate per each analysis. Even more sample water is needed if more replicates are being analysed, as would be ideal. The sample water required for the ≥10 - <50µm organisms (5-6 litres) is sufficient for indicative and detailed analyses including several replicates per analysis.

4.2 Indicative and detailed viability assessments

As indicative analysis devices are expected to be accurate, quick and easy to use, these attributes are discussed specifically for each tested device and method in the following sections. In terms of portability and cost, all devices can be considered portable and reasonably priced even though some differences were noted in both categories. Rest of the device attributes can be considered almost equally important.

Generally, the accuracy of a device should weigh more than analysis time and ease of use, but as these tests will be carried out by PSC officers in Finland, if a method is too time-consuming or difficult for a PSC officer to conduct, it is not considered preferable for indicative analysis. The accuracy of a device or method can be evaluated in two ways, the accuracy of the methodology itself and the

accuracy of the measurements recorded by each device in the present study. Accuracy of the methodology refers to the analysis algorithm that determines the viable organism concentrations in a sample, because indicative analysis can be indirect or direct measurements of the present viable organism concentration (IMO, 2009, 2015). Direct conversion to viable organism concentrations is not strictly necessary, but according to IMO (BWM.2/Circ.42, 2015) also indirect measurements of viable organisms should refer to the D-2 standard, which is expressed as viable organisms per volume of interest.

4.2.1 Detailed analysis

Detailed viability assessments for ≥50µm and ≥10 - <50µm organism size fractions can be done with microscopy analyses, even though mechanical methods such as flow cytometry also exist (David and Gollasch, 2015; Peperzak et al., 2018). Organism motility alone is not considered as a reliable indicator for organism viability (Schillak, 2016), and especially for the ≥10 - <50µm size fraction, staining methods are advised to separate dead and viable organisms (David and Gollasch, 2015).

Detailed analyses for bacteria usually require laboratories with accredited methods and this may not be provided even by commercial laboratories in some countries for toxicogenic strains of *Vibrio cholerae*. For example, after recent discussions with the National Institute for Health and Welfare in Finland, there are no laboratories in Finland that are analysing toxicogenic strains of *Vibrio cholerae* in CFUs from natural water samples, as the analytical methods of such measure are expensive and rarely requested. Laboratory analyses of *V. cholerae* in Finland are most commonly conducted to classify strains from already detected infections. Accredited methods for this analysis are most likely provided in some countries, but it would require substantial efforts and resources from the PSC authorities to conduct them in a reasonable manner.

Fluorescein diacetate (FDA) assays express organism viability by staining organisms with intact cell membrane and active intracellular esterases into green fluorescence emission (Garvey et al., 2007). Viable organisms can be counted under an epifluorescent microscope after staining. Even though various studies (e.g. Selvin et al., 1989; Garvey et al., 2007; Reavie et al., 2010; Adams et al., 2014; MacIntyre and Cullen, 2016) have reported that FDA fails to stain certain organisms, including also some species within the same taxonomic group, the method has been considered useful and reliable for the ≥10 - <50µm sized organisms (Reavie et al., 2010; Adams et al., 2014).

These conclusions can be somewhat supported by the findings of the present study. The viable organism counts indicated that there was a very clear difference in viable organism concentrations between the untreated and treated samples. The differences were also observed under the microscope, as the untreated samples contained brightly fluorescing motile and non-motile organisms within frequent intervals. The treated samples in turn, contained only a few brightly fluorescing cells, relatively lot of weakly or partially fluorescing organisms and no motility in any replicates.

The number of partially fluorescing cells was clearly higher in the ultraviolet (UV)-treated samples than in the electro catalysis and ultrasound (EUT)-treated samples. This outcome was not particularly surprising, as the holding time for the EUT-treated samples was approximately 72 hours before the sample collection, whereas the UV-treated water at M/S Polaris was sampled right after treatment. In addition, studies by Tobiesen et al. (2011) and First and Drake (2013; 2014) reported that organism viability determination after UV-treatment can be problematic due to the ability of certain micro-organisms to resist high UV doses. Viability assessments related to UV-treatment can be also complicated by the

delayed effect of the treatment, as it tends to damage organisms instead of instantly killing them (van Slooten et al., 2015). Furthermore, Wright and Welschmeyer (2015) reported that the FDA method may not be the most suitable method to detect viability after UV-treatment. Based on their data, 89% of the UV-treated samples indicated compliance when analysed with FDA, whereas 100% of these samples indicated non-compliant when assessed with the most probable number (MPN) method.

Even though the FDA analyses showed clear differences in viable organism concentrations between the untreated and treated water samples, especially the concentrations in untreated and EUT-treated samples were almost consistently lower than the concentrations detected by the indicative analysis devices. Some of this may be due to the method of concentrating samples for the FDA assays, as some of the 10-14 µm sized organisms may have gone through the 10µm plankton net being used. Furthermore, several previous studies (e.g. Reavie et al., 2010; Adams et al., 2014; Schillak, 2016) have used magnifications up to 200x or even 400x during the counting process under epifluorescent microscope, whereas 100x magnification was used in this study. 100x magnification was considered sufficient for untreated samples to detect ≥10 - <50µm organisms, and also to ease the detection of the grid during counting. The magnification was not considered as a problem with brightly fluorescing organisms, but might have complicated the detection of weakly fluorescing organisms that were close to the 10µm size limit.

4.2.2 Luminultra ATP

The Adenosine TriPhosphate (ATP) method has been widely considered as a suitable indicative analysis method for viability assessments in terms of analysis time, ease of use and accuracy (Hwang et al., 2010; Penru et al., 2012; van Slooten et al., 2015; Welschmeyer and Kuo, 2016). Even though some of these studies applied different ATP assays than the Luminultra B-QUA kit, the method was considered more complicated than other tested indicative analysis devices. The completion of the assay (Appendix 1) within a reasonable time requires conducting several analysis steps simultaneously for different size fractions, and the numerous filtration steps and sample extractions expose the method to errors if the protocol is not followed carefully.

The method is not considered too difficult for PSC officers to conduct after profound training, but it is not considered particularly simple either. After all, PSC officers may have relatively lengthy periods without performing ballast water sampling and indicative analyses between inspections, since these steps are conducted only if initial and detailed inspections are inadequate (MEPC, 2014). As a result, PSC officers may lose the benefit of training to conduct the ATP analysis rapidly. Furthermore, after the analysis of approximately 30-40 minutes, the assessment provides result for all organism size fractions but only one replicate per each fraction. If uncertainties arise, the analysis needs to be conducted again for another replicate, which can be too time-consuming especially if more ballast water needs to be sampled. The manufacturer has indicated that some of the analysis steps will be most likely automated in the future versions of the kit.

Results of the present study regarding to the interpretation of ATP analyses were also somewhat ambiguous from the PSC point of view. Only the analyses for the untreated water indicated non-compliance consistently to all organism size fractions, whereas the compliance results between the organism size fractions differed for the UV- and EUT-treated samples, although ballast water treatment systems (BWTS) may impact differently on different sized organisms. According to the Luminultra ATP protocol, if any of the size fraction analyses indicates non-compliance, the whole sample can be considered non-compliant. Close to the limit results (yellow colour) refer to conducting further replicates or improving the

measure. The analyses for all D-2 standard organism size fractions may provide valuable information that the BWTS has impacted differently on different sized organisms. However, this may leave PSC officers into difficult situations if analyses indicate compliance for one size fraction and close to the limit for the other, especially when these results cannot be directly compared to the D-2 standard limit values. In these situations it is not always possible to sample more ballast water and conduct analyses again.

ATP was the only method that indicated compliance for the ≥10 - <50µm organisms in the UV-treated samples. This may be due to methodological differences as intracellular ATP (cATP) can degrade relatively quickly after treatment (IMO, 2015). Nevertheless, the UV-treated sample was non-compliant for the ≥50µm analysis, indicating that the filtration of the BWTS unit failed or the cATP did not degrade as much and as quickly from the larger organisms.

The measured cATP concentrations were relatively low throughout the study for the ≥10 - <50µm size fraction. It can be seen as a benefit of the method that cATP degraded quickly from samples after treatment at least for this size fraction, and therefore would have confirmed compliance to the PSC inspectors. The 'close to the limit' range for this size fraction is 500-1500 cATP pg/ml and even the cATP value measured for the untreated water was relatively close to this range (1797,72 cATP pg/ml). In comparison, the viable organism counts of the detailed analysis of this size for the untreated samples contained over 200 viable organisms per ml on average, which is 20 times higher than the D-2 standard compliance limit (10 viable organisms per ml).

One of the major advantages of the Luminultra method is the ability to detect compliance for all three organism size fractions. IMO (2018b) accepted guidance for the commissioning testing of ballast water management systems (BWM.2/Circ.70) in November, 2018 and it obliges the completion of indicative analysis for all D-2 standard organism size fractions after installation of the BWTS onboard. Therefore, the Luminultra ATP can be used for commissioning testing, since it was the only tested indicative analysis device that measures compliance for all size fractions. Alternatively, commissioning testing can be done by using a combination of devices to validate compliance, but this may be more time-consuming and less cost-efficient. Commissioning testing will be very important to ensure that the BWTS operates efficiently on all organism size fractions, especially when some analyses also in this study indicated different compliance status for different organism size fractions after treatment.

However, the ATP analysis for bacteria is a very indirect measurement of the D-2 standard indicator microbe viability. The bacteria analysis includes filtration down to 0,7µm and the cATP analysis is conducted on this filtrate. Therefore, if the sample water contains other bacteria than the indicator microbes (Toxicogenic *Vibrio cholerae*, *Escherichia coli* and Intestinal *Enterococci*) or other organisms <0,7µm, the ATP analysis for bacteria may indicate non-compliance for this size fraction even though the sample does not contain any of the D-2 indicator microbes.

The method is not linked to the D-2 standard of the indicator microbes since it is not targeted to detect their presence. Therefore, it does not indicate the compliance of a ship appropriately. If the D-2 standard for bacteria was changed to detect the presence of all bacteria of certain size in the future, the method would be more appropriate. On the flip side, as the indicator microbes are rarely present even in untreated ballast water in large enough concentrations as described by the D-2 standard (Welschmeyer and Kuo, 2016), the method can be useful to detect the BWTS efficiency towards all smaller organisms during the commissioning testing, when the cATP concentrations are compared between untreated and treated ballast waters.

Some previous studies (e.g. Penru et al., 2012; First and Drake, 2014; van Slooten et al., 2015; Hyun et al., 2018) have used also alternative ATP assays for the detection of ballast water treatment efficacy, but the Luminultra ATP kit is considered the only ATP assay designed primarily to ballast water compliance monitoring. The Luminultra ATP is a bulk ATP analysis for different size fractions and it is able to detect degradation of viability after treatment. As an indirect measurement of the organism viability, Luminultra have developed method-specific thresholds to detect compliance/non-compliance. Therefore, the accuracy of the ATP method results is difficult to evaluate because the measured cATP concentrations are not directly comparable with the viable organism concentration limits described in the D-2 standard. ATP assays have also proven accurate when tested on controlled cultures (Hyun et al., 2018), but these cATP/organism –ratios cannot be applied when heterogeneous ballast water samples are being analysed.

This is also most likely the reason why the result is presented in cATP concentrations and the compliance limits are presented as cATP concentration ranges (most likely compliant, close to the limit and most likely non-compliant). For example, some data provided by Lo Curto et al. (2018) indicates that the cATP/viable organism -ratio is very inconsistent between different heterogeneous samples of treated and untreated water. Therefore, it is very difficult to compare the results to the present D-2 standard from the obtained cATP measurements. The level of uncertainty for each organism size fraction can be roughly evaluated from the 'close to the limit' compliance ranges provided by Luminultra (2018) (presented in method section), which is very large especially for the ≥50µm organisms (10,000-750,000 cATP pg/m3). In addition, 'close to the limit' range estimated by Hyun et al. (2018) for the ≥10 - <50µm organisms (788 – 98,610 cATP pg/ml) indicates that there can be significant variation in the cATP/organism –ratio also in this size fraction.

4.2.3 BBE 10Cells

Pulse Amplitude Modulation (PAM) fluorometry method has proven suitable for indicative analyses of ballast water compliance for the ≥10 - <50µm organisms by previous research (David and Gollasch, 2015; Gollasch et al., 2015; van Slooten et al., 2015; Bradie et al., 2018a). These studies have also reported that on top of short analysis time and user-friendliness, PAM fluorometers have been found correlating particularly well with FDA stained microscopy counts for organisms of this size, even though the method does not count cells during the analysis (Gollasch and David, 2011).

The 10Cells modified PAM fluorometer was considered easy to use and had the fastest analysis time of the indicative analysis devices tested. The sample preparation process included two filtration steps, but they were considered relatively easy and their completion will not cause difficulties to PSC officers. The sample preparation and analysis time together was less than five minutes, which is short enough to enable the analysis of several replicates during a PSC inspection, adding reliability to the final result (compliant or non-compliant).

According to the statistical analysis, there were no significant differences in detected organism concentrations between the 10Cells and detailed analysis, even though the 10Cells viable organism counts were clearly higher than the counts of the detailed analysis for the untreated and EUT-treated samples. This may be due to the inability of FDA to stain certain organisms (MacIntyre and Cullen, 2016; Vanden Byllaardt et al., 2018), or differences in filtration, as the detailed samples were concentrated with 10µm plankton net, whereas the 10Cells device utilizes 8µm filters and conducts the measurements from these filters.

Further evaluation on the accuracy of the 10Cells methodology is needed. Bbe 10Cells, similarly to other PAM fluorometers, estimates the viable cell

concentration using a conversion factor that is applied to the variable fluorescence (Fv) reading recorded from the sample (Casas-Monroy et al., 2016; Castro et al., 2018; Moldaenke et al., unpublished). The conversion factors are based on fluorescence-per-cell estimates that are generally conducted device-specifically with cultured phytoplankton species depending on the size class of interest. For improved and more precise measurements, the manufacturer suggests two measurements for the ≥10 - <50µm size fractions to determine the concentration of viable cells. This includes measurements from 20µm and 8µm filters and requires utilization of different conversion factors for each measurement (Moldaenke et al., unpublished). After the measurements, estimation of the final viable cell count can be calculated for the ≥10 - <50µm size category.

However, 20µm filters were not provided for the present study, and the viable cell counts for 10Cells originated from single measurements conducted on the 8µm filter. In this case the device assumes a predominant cell size of 15µm within the ≥10 - <50µm category and the conversion factor is based on this assumption (Casas-Monroy et al., 2016; Moldaenke et al., unpublished). The analysis is a bulk measurement of Fv for the ≥10 - <50µm sized phytoplankton, where the viable organism concentration is estimated from this bulk value assuming that the bulk fluorescence signal originates from 15µm-sized cells.

Another issue with methods detecting only photosynthetic activity from viable phytoplankton cells may be oligotrophic open sea waters with low biological variation of the species present (Bradie et al., 2018a). This concerns especially ships that conduct ballast water exchange at oceanic locations during the voyage, where concentrations of certain organisms may be below the D-2 standard limits even in untreated water. Sampling for larger quantities of ballast water and potentially the ≥50µm organisms would be more appropriate for such ships, but further research is needed to discuss this in more detail.

The methodology of the 10Cells using a fixed conversion factor cannot be considered highly accurate, but a relatively reliable numerical correlation between Fv measurements and number of viable phytoplankton cells, particularly close to the compliance limit (10 viable organisms/ml) in the ≥10 - <50µm size class has been detected (Gollasch and David, 2011; Gollasch et al., 2015; Bradie et al., 2018a). The accuracy of this correlation decreases when samples contain more viable cells per ml (e.g. hundreds of cells), but an accurate number of viable cells in this case is irrelevant from compliance monitoring point of view, if the sample is clearly non-compliant. All things considered (analysis time, user-friendliness and accuracy), the 10Cells modified PAM fluorometer performed relatively well during the measurements in the present study and this can be also supported by previous literature as mentioned above.

4.2.4 Chelsea Technologies Group FastBallast

Although the Fast Repetition Rate (FRR) method has been used for decades (Kromkamp and Forster, 2003), FRR-based methods, such as the FastBallast utilizing Single Turnover Active Fluorometry (STAF) have been quite rarely applied to ballast water-related viability assessments. van Slooten et al. (2015) and Castro et al. (2018) compared single turnover (ST) and multiple turnover (MT) based fluorometers on untreated and treated ballast water samples and detected relatively similar results between the two methods.

The viable organism concentrations measured with FastBallast were consistently higher by a relatively large margin in the present study in comparison to the detailed analysis counts. The analyses for all ≥10 - <50µm size fraction measurements were conducted on <50µm filtrate, and no further filtration was applied to the FastBallast analyses since it was not part of this analysis steps and not provided by the manufacturer.

The FastBallast methodology determines a standardized cell fluorescence (SCF) value from several hundred 0,5ml semi-discrete measurements during the analysis. This SCF value originates from the strength of individual Fv signals and refers to average cell size of the sample (Oxborough, 2019). The test algorithm estimates viable cell count for the sample based on this value and it is developed to distinct viable cells within the ≥10 - <50µm size range from <10µm-sized cells. The analysis is therefore able to indicate an outcome called 'PASS (small cells)', when the SCF value of the analysis is below a threshold value of 0,2 (assessed by the manufacturer), even if the sample contains over 10 viable cells per ml. The test algorithm has been developed with measurements on cultures of several phytoplankton species (Oxborough, 2019).

Castro et al. (2018) applied further filtrations on their samples and obtained viable cell counts separately for ≥10 - <50µm and >2 - <10µm size fractions. As one would expect, the numbers of viable cells within the >2 - <10µm range were significantly higher. Even though the FastBallast algorithm aims to differentiate viable cells within the ≥10 - <50µm range from smaller viable cells, this task may be more complicated when applied to heterogeneous ballast water samples. Each of the several hundred semi-discrete measurements during the FastBallast analysis are targeted to 0,5 ml volume at a time, which may contain anything from hundreds to millions (during blooms) of cells in natural water samples (Ryther, 1954; Smayda, 1957). Therefore, the test algorithm that is based on the SCF value can most likely make a differentiation between the presence of micro- and pico-sized phytoplankton within a sample but the differentiation between ≥10 - <50µm and <10µm cells may not be as straightforward. This may be due to SCF values not correlating directly with cell size in minimum dimension, as <10µm cells with relatively high number of PSII complexes can emit similar amounts of fluorescence than some cells within the ≥10 - <50µm size range based on Fv measurements for different phytoplankton species in Oxborough (2019).

The consistently higher viable organism concentrations recorded by FastBallast in comparison to detailed analysis is likely due to proportion of cells <10µm in high enough numbers to generate a high SCF value (K Oxborough, personal communication, 1 February 2019). Furthermore, concentrating detailed analysis samples with 10µm plankton net had probably some impact as well, but this impact was noted also in relation to other indicative analysis results. To clarify the matter slightly further, additional tests were done on non-filtrated natural sea water samples using FastBallast and the detailed FDA analysis protocol. All fluorescing organisms (also <10µm) were counted after staining under epifluorescent microscope and they correlated relatively well with the FastBallast viable cell counts (Table 6, Appendix 4).

The accuracy of FastBallast to measure viability for ≥10 - <50µm phytoplankton cells would most likely benefit from an additional filtration step, as also a study by Castro et al. (2018) indicated. Filtration can result in large error margins (as in Castro et al. 2018), but if the methodology is not able to make a clear distinction between the size fractions defined by the IMO, the filtration step is very likely needed. If the D-2 standard is changed in the future to cover also viable organisms <10µm, the FastBallast methodology can become highly useful for compliance monitoring of these organisms.

Altogether, the FastBallast STAF is a sensitive fluorometer by its' measuring algorithm, instead of using fixed conversion factors on bulk values. The fluorometer itself can detect the presence of various viable phytoplankton taxa with four LED arrays set to different wavebands. The approach can be considered suitable for compliance monitoring, if further filtration steps are included in the future versions of the device. FastBallast, as well as other devices detecting viability only from autotrophic organisms, has the same limitation with oligotrophic open sea samples that may contain relatively low numbers of these organisms. In terms of other requirements of indicative analysis devices,

FastBallast analysis was relatively fast (approximately 8 minutes) and the simplest to use of all devices tested in the study, as the analysis procedures included only inserting a 20 ml sample into the sample chamber and pressing 'start' on the touch screen.

4.2.5 Microwise MFA

The Motility and Fluorescence Assay (MFA) methodology includes two indications of organism viability; motility and chlorophyll fluorescence, detected with cameras pointing towards the sample chambers. The BallastWISE device determines compliance separately for ≥50µm and ≥10 - <50µm sized organisms. The motility and fluorescence attributes can be applied to both analyses. The main viability indicator for the ≥50µm organisms is motility but chlorophyll fluorescence detection via violet light attribute can be added to the analysis from device settings. The device was considered relatively easy to use, and fast enough with an analysis time of 20-30 minutes. The deployment of rubber tubes and sample chambers appropriately into the device will be manageable with some training.

There are no reference studies using BallastWISE for compliance monitoring thus far, since the device is relatively new. The device operated fairly well in the study and there was a clear difference in viable organism concentrations between the treated and untreated samples. Especially the analysis for ≥10 - <50µm organisms estimated similar viable organism counts than the detailed analysis. The methodology is very promising for compliance monitoring in the future as the device enumerates viable organisms with cameras. Therefore it is not a bulk method and the results do not need conversions to be comparable with the D-2 standard limits.

Nevertheless, certain factors during the analysis steps indicated that the device is still at development stages to some extent. This became evident when the device was connected to the external computer with a cable and the BallastWISE program on the computer showed error messages even though everything was connected appropriately. The error messages disappeared after restarting the computer and re-connecting the cables and power cords.

In addition, the BallastWISE analysis for the ≥50µm organisms requires potentially further development. As mentioned in the introduction, the viable organism limit for this size fraction is less than 10 viable organisms per cubic metre, whereas the BallastWISE ≥50µm analysis (version 3.13) reports the result as viable organisms per litre. Moreover, the ≥50µm analysis sample volume is approximately 1 litre and the analysis does not seem to take larger sample volumes (concentrated samples) into account, as there is no setting in the program where to apply sample volume for this analysis. Sampling of one random litre of ballast water is obviously a very insufficient amount of water when the volume of interest for this organism size fraction is a cubic metre. The final organism concentration can be calculated afterwards as conducted in the results section, but it needs to be done by the PSC officers after the analysis. However, the manufacturer has expressed that this factor will be updated for the newer versions of the software.

In addition, the ≥50µm analysis seems to have difficulties differentiating between drifting particles and motile organisms. This was noted from treated and untreated samples after the re-examination by the manufacturer (represented in the results). By changing the acceleration setting of the analysis, the viable organism concentrations changed remarkably as not all motile particles detected by the device as living organisms in the original analysis indicated clear swimming patterns. It can be seen as a benefit that the video-tracked analyses can be reviewed, but this is something that PSC officers will not be able to conduct onboard. The parameters related to organism motility need to be optimized in the

future to increase accuracy and reliability of the ≥50µm analysis. As the results of the ≥10 - <50µm analyses indicated, the device has good potential for being a reliable indicative analysis device, but the final product needs to be unambiguous without any uncertainties regarding to sample volume and the detection of viable organisms.

4.3 General suggestions to the IMO experience-building phase

In order to justify and rationalize recommendations for the most suitable indicative analysis devices for PSC authorities, it is essential to remember the definition of indicative analysis and requirements for the devices;

"An indicative analysis means a compliance test that is a relatively quick indirect or direct measurement of a representative sample of the ballast water volume of interest. A direct measurement, which is directly comparable to the D-2 standard may also be indicative if it has large confidence intervals or high-detection limits" (IMO, 2015).

The study revealed clear differences in all of the indicative analysis measurements between the treated and untreated water samples, so at least referring to the definition, one might assume that all devices are highly suitable for compliance monitoring. However, IMO (2009) has set relatively detailed limit values (D-2 standard) of ships compliance for all three organism size fractions as presented in the introduction. Based on these limit values that determine ships compliance, indicative analysis device manufacturers aim to make a differentiation between the compliance and non-compliance status. There is great discrepancy between these definitions. One allows indicative analysis devices to measure compliance indirectly or with large confidence intervals or high-detection limits, while the other determines compliance in a very detailed manner.

This inconsistency has resulted in a wide range of studies (e.g. Bradie et al., 2018a; Hyun et al., 2018; Lo Curto et al., 2018; Vanden Byllaardt et al., 2018) stating that several methodologies are suitable for indicative analyses, and simultaneously, discussing that the accuracy limitations associated with the devices have to be tested and studied further. There is also evidence (e.g. van Slooten et al., 2015; Hyun et al., 2018) that some devices correlate very accurately with viable organism counts from cultured samples of individual species. Cultured samples can be useful for the testing and calibration of the devices, but ballast water samples are generally natural sea water and have heterogeneous species composition (Olenin et al., 2000), and the viability determination for such samples differs greatly from tests conducted on monocultures.

Therefore, it would make more sense if IMO set wider compliance ranges for indicative analysis results, since the devices are allowed to have large confidence intervals. Accuracy and suitability of indicative analysis devices are difficult to evaluate if the allowed confident intervals have not been defined. The compliance limits or ranges for indirect methods can be method-specific and without conversions to viable organism concentrations, but they should be universally set by the legislative organization, in this case, the IMO. It can be considered questionable if an international agreement, such as the BWM Convention relies on limit values set by parties with commercial interests.

In general, the studied devices and methodologies were able to differentiate untreated samples from the treated ones. Each method had their advantages and disadvantages regarding to being comparable to the current D-2 standard compliance limits, but this was expected as the methods are indicative. As the data gathering and analysis stages of the EBP will likely impact the future form of the BWM Convention, the comparability of their results to the present D-2 standard will not be evaluated further.

It is also important to remember that the present study and all analyses were conducted only on ballast water from the Gulf of Finland (Northern Baltic Sea), which differs greatly from oceanic conditions (Leppäkoski and Olenin, 2000). The same analyses conducted on other sea areas with different species composition could have resulted in different outcomes, although the indicative analysis methods should be able to determine compliance status of all ballast waters regardless of the location.

Principles of representative sampling to all organism size fractions have been well-established by e.g. Gollasch and David (2011), David (2013), Gollasch and David (2017), Bradie et al. (2018b) and Moser et al. (2018). The forthcoming ISO standard for ballast water sampling will be more than needed to harmonize sampling procedures across all ship types, treatment systems and organisms of interest. Sampling for larger quantities of ballast water (hundreds of litres) will be most likely too challenging for PSC officers within a reasonable timeframe. Furthermore, if the indicative analysis device is preferred being portable and backpack-sized, it cannot be considered appropriate for the additional sampling tools to cover half of a vehicle either.

If some countries wish to utilize experts for occasional compliance monitoring visits on ships, it would be ideal to monitor at least the ≥50µm and ≥10 - <50µm size fractions to see whether the ballast water treatment has been efficient on both size fractions. The treatment efficiency on different size fractions was also partially evidenced by the present study results, as both indicative analysis devices detecting compliance for the ≥50µm size fraction (BallastWISE and Luminultra ATP) showed either 'close to the limit' or 'non-compliant' to all treated and untreated ≥50µm samples. Testing for the indicator microbes does not represent ships compliance appropriately, as these microbes are often present in relatively low concentrations even in untreated ballast waters (Welschmeyer and Kuo, 2016). In the commissioning testing all size fractions have to be sampled and regarding to indicator microbes, it would be more sensible to test the BWTS efficacy on all smaller organisms to verify the operability of the treatment system.

The requirements of ballast water treatment efficacy should be reconsidered in the future. Previous studies by Tobiesen et al. (2011), First and Drake (2014) and van Slooten et al. (2015) have reported issues in viability determination after UV-treatment. This can be supported by the findings of the present study, since the UV-treated samples were almost consistently non-compliant. The UV-treated ballast water was treated with UV only once and sampled right after treatment without a holding time, which very likely impacted these analyses and resulted in this outcome. However, some relatively short international sea voyages do not always enable significant holding times for ballast waters after treatment, and if similar issues are noted in the future, it can be worth considering that the treatment method has to be developed further. If the impact of the ballast water treatment cannot be detected onboard, PSC officers can end up in difficult situations even though the treatment would be able to eventually eliminate organism viability.

The arrangements of detailed analysis equally require further clarification. Microscopic assessments and flow cytometry together with staining can be considered as valid methods for viability determination of ≥10 - <50µm and ≥50µm organisms (David and Gollasch, 2015; Peperzak et al., 2018). After consulting local health authorities here in Finland, now there is real evidence that accredited methods for the viability assessment of all regulation D-2 indicator microbes with the precision requested by IMO is not provided in all countries. There is also great uncertainty in terms of the organisation or laboratory that will conduct the detailed analyses of the ≥10 - <50µm and ≥50µm organisms in the future. At least in Finland, the PSC authority does not have laboratories accessible, nor expertise for such analyses and the responsible organisation would

have to be prepared to conduct the analyses seven days a week and most likely on a relatively short notice.

5 Conclusions

The increased knowledge around ballast water management and compliance monitoring has simultaneously revealed new issues related to sampling and viability determination. Continuous efforts are therefore needed during the International Maritime Organizations (IMO) experience-building phase (EBP) to establish harmonized implementation of the Ballast Water Management Convention (BWM Convention). Re-evaluation of certain guidelines within the BWM Convention may become useful, including distinct indicative compliance limits or ranges for the D-2 standard. The present D-2 standard is far too detailed for such analyses. Promising efforts have been already made to enhance compliance monitoring, such as the commissioning testing, introduced in November, 2018. In addition, hopefully the upcoming ISO standard for ballast water sampling manages to harmonize equal sampling conditions for different types of ships. The present type approval guidelines are evidently insufficient for sampling of all ships with one set of sampling equipment. The indicative analysis methods studied here have all potential for being reliable indicative compliance tools and adjustments to the BWM Convention will determine the suitability of each method in the future.

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Appendix 1. Luminultra ATP analysis steps

1) Some preparation can be done beforehand.
    a. Label 3 ultralute tubes (>50µm, 10-50 µm and bacteria).
    b. Label 2 beads tubes (>50µm and 10-50µm).
2) Preparation of luminase – enzyme rehydration (also before entering the ship)
    a. Take one luminase bottle and one buffer bottle, open luminase bottle, remove red rubber cap and throw it away. Add buffer, screw the white cap, gently swirl and invert twice, wait 5 min.
3) Take Ultralyse30 with pipet and drop it to bead tubes (>50µm and 10-50µm)
4) Take Ultralute with pipet to both bead tubes (>50µm and 10-50µm)
5) Take one 50µm filter with forceps and set it to filtering bottle.
6) Pour sample to filtering bottle and pump water through the filter.
7) Take filter from filtering bottle with forceps and bend it half so that the filter fits to bead tube (>50µm).
8) Put the filter in the bead tube (>50µm) and set it to the grinder.
9) Run the grinder 3*2 min.

While grinder is running you can prepare the second bead tube (10-50µm).

10) Take one 10µm filter with forceps and set it to filtering bottle (10-50µm).
11) Pour 200 ml of sample water from <50µm filtrate and pump the water through the filter.
12) Take filter from filtering bottle with forceps and bend it twice so that filter fits to bead tube (10-50µm).
13) Put the filter in the second bead tube (10-50µm) and set the bead tube to grinder.
14) Run the grinder 3*2 min.

While grinder is running you can start with bacteria analysis.

15) Insert one syringe filter 2,7µm and after that one bacteria filter 0,7µm to syringe.
16) Push 2*50 ml of sample water (<10µm) from filtering bottle (10-50µm) gently through the syringe filters.

17) Remove the filters and insert the second (0,7µm) bacteria filter back to syringe.

18) Set Ultralyse7 to empty syringe.

19) Push the Ultralyse through the 0,7µm syringe filter to the Ultralute tube.

20) Let the first bead tube (>50µm) rest 5 min for settling down after running the grinder.

21) Take dilution from the bead tube (>50µm) and set it to Ultralute tube (>50µm).

22) Let the second bead tube (10-50µm) rest also 5 min after running the grinder.

23) Take dilution from the bead tube (10-50µm) and set it to Ultralute tube (10-50µm).

24) Start the calibration of luminometer (Luminomaster).

25) Take 5 Luminometer tubes ready.

26) Set Luminase and Ultracheck1 to one Luminometer tube.

27) Run the Luminometer and write down the value (>5 000) to app. →UC1

28) Take on empty Luminometer tube, run the Luminometer and write down the value.

29) Set Luminase to Luminometer tube, run the Luminometer and write down the value.

30) Set Ultralute to same Luminometer tube where you already set Luminase. Run the Luminometer and write down the value to app. →RLU BN

31) Set Luminase to three Luminometer tubes (>50µm, 10-50µm and bacteria).

32) Take dilution from Ultralute tube (>50µm) with a pipette and push it to one Luminometer tube.

33) Run the luminometer and write down the value to app.

34) Take dilution from Ultralute tube (10-50µm) with a pipette (new tip after every use) and push it to one Luminometer tube.

35) Run the Luminometer and write down the value to app.

36) Take dilution from Ultralute tube (bacteria) with a pipette and push it to one Luminometer tube.

37) Run the Luminometer and write down the value to app.

38) Calculate the results with the app.

39) Save the results in app and send them to your personal email.

Method reference;

Aqua-tools, 2018. B-QUA Ballast Water Monitoring Solution, Instruction Protocol. Aqua-tools, France.

Appendix 2. BBE 10Cells analysis steps

1) Pour sample water through pre-filter (50µm) into the rubber plastic cup.
2) Take 10ml of the pre-filtered sample water with a 12 ml syringe.
3) Place the smaller 8µm filter to the tip of the syringe.
4) Push the 10ml sample water gently through the 8µm filter.
5) Place the filter into the detection unit.
6) Press "start".
7) After few minutes of analysis, result is displayed on the screen as organisms per ml and saved automatically to the device.

   Method reference;

   BBE ,2015.10 cells User Manual, Version II. BBE Moldaenke Gmbh, Germany.

Appendix 3. FDA staining and epifluorescent microscopy analysis steps

1) Primary FDA solution was made by mixing 50 mg of FDA powder and 10 ml of reagent grade dimethyl-sulfoxide (DMSO) (pipetted with XXX pipette) into a glass beaker.

2) FDA working solutions (new working solution was made for each replicate) were made by mixing 1 ml of FDA primary solution and 100 ml of distilled water into another glass beaker.

3) Samples were stained by pipetting 5 ml of concentrated sample and 417 µl of FDA working solution into a 20 ml scintillation vial.

4) Stained samples were incubated in dark at room temperature for 10 minutes.

5) 1ml of stained sample was loaded into Sedgewick-rafter counting chamber and allowed to settle for 2 min.

6) Fluorescing organisms were counted at 100X magnification, using Leica DMI 3000b inverted microscope with a blue light excitation-green bandpass emission (FDA, excitation 450 – 490 nm, dichoric 510 nm, suppression filter LP 515 nm) for 20 minutes. Partially fluorescing organisms were counted as viable. Fluorescing organisms were counted if any dimension was >10µm using the grid line thickness (18µm) of the counting chamber as a size reference. Cells within colonies were counted only when size of an individual cell within the colony was >10µm.

7) Fluorescing organisms were counted from every second vertical grids within 20 minutes and the final organism counts were doubled to correspond the 1 ml volume.

8) Final viable organism counts were calculated using the total sample volume and the volume of the concentrate.

   Final organism count calculations

   Polaris treated samples (UV+filtration):

   Original volume: 5 litres of <50µm filtrate →concentrated to 720 ml→1ml loaded into Sedgewick-Rafter chamber, 0.5 ml counted.

   Viable organism counts:

   Replicate 1: 255

   Replicate 2: 241

   Replicate 3: 225

   Therefore:

$$x\ (viable\ organisms\ ml^{-1}) = \frac{count \times 2 \times 720}{5000}$$

   Polaris untreated samples:

   Original volume: 5 litres of <50µm filtrate →concentrated to 700 ml→1ml loaded into Sedgewick-Rafter chamber, 0.5 ml counted.

Viable organism counts:

Replicate 1: 703

Replicate 2: 738

Replicate 3: 772

Therefore:

$$x\ (viable\ organisms\ ml^{-1}) = \frac{count \times 2 \times 700}{5000}$$

Mirva VG treated samples:

Original volume: 5 litres of <50µm filtrate →concentrated to 70 ml→1ml loaded into Sedgewick-Rafter chamber, 0.5 ml counted.

Viable organism counts:

Replicate 1: 53

Replicate 2: 60

Replicate 3: 72

Therefore:

$$x\ (viable\ organisms\ ml^{-1}) = \frac{count \times 2 \times 70}{5000}$$

Method references;

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Appendix 4. Additional FastBallast testing

FastBallast and FDA detailed analysis comparison on natural sea water samples;

*Table 6. Additional testing for FastBallast on natural sea water samples. *FastBallast indicated that at sites 3 and 4, a small number of large cells decreased the estimate of viable cell counts. Site 4 sample was run third time with FastBallast after 30µm filtration, which resulted in a clearly higher cell count (295 viable cells/ml).*

	Site 1	**Site 2**	**Site 3**	**Site 4**
FDA count (org./ml)	196	289	360	336
FastBallast count (org./ml)	175	236	184	139
	170	296	237	179
				295*

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