Management of Biological Invasions (2017) Volume 8 Article in press

Open Access

Review

CORRECTED PROOF

Application of flow cytometry in ballast water analysis—biological aspects

Ingunn Alne Hoell[1,*], Ranveig Ottoey Olsen[1], Ole-Kristian Hess-Erga[3], Gunnar Thuestad[1] and Aud Larsen[2]

[1]*Western Norway University of Applied Sciences, Postboks 7030, 5020 Bergen, Norway*
[2]*Hjort Centre for Marine Ecosystem Dynamics, Uni Research Environment, Nygårdsgaten 112, N-5008 Bergen, Norway*
[3]*Norwegian Institute for Water Research, Thormoehlensgt, 53 D, 5006 Bergen, Norway*

**Corresponding author*

E-mail: ingunn.hoell@hvl.no

Received: 22 December 2016 / Accepted: 22 May 2017 / Published online: 26 June 2017

Handling editor: Alisha Davidson

Abstract

Ballast water may, when discharged, cause the spread of nonindigenous and potentially invasive species. International ballast water treatment regulations have accelerated the development of new methods to detect, enumerate and assess the status of organisms in the water to be discharged. Flow cytometry (FCM) is a powerful technique with a broad range of applications with the possibility for multi-parametric analysis and the potential of combining it with other techniques being two strong advantages. This review will discuss whether FCM is suitable for ballast water analysis according to international ballast water regulations, and sum up the advantages and disadvantages. It will also give an overview of available labeling techniques. Finally, a discussion on the knowledge gaps and future potential for FCM within ballast water analysis is presented.

Key words: invasive species, biological invasions, compliance control, water analysis, IMO, US Coast Guard

Ballast water—a vector for spreading of organisms in marine environments

In ports, water is pumped into the ballast tanks of ships with little cargo to ensure stability and trim during the voyage, and to maintain structural integrity and safety. Ballast water is then discharged again when cargo is loaded in a new port. Thus organisms are transported across nature's own barriers, including variable salinities and temperatures, with the risk of spreading non-native and possibly invasive species (i.e. species that are established outside of theirs natural past or present distribution, whose introduction and/or spread threaten biological diversity). Many organisms do not survive in the ballast tank because of suboptimal and variable conditions. Several studies have shown that the abundance and diversity of microorganisms like plankton, bacteria and viruses, decreases over time in the ballast tanks (Williams et al. 1988; Lavoie et al. 1999; Gollasch et al. 2000; Drake et al. 2002). Temperature, low oxygen saturation, and the presence of chemical pollutants all play a crucial role in selectivity of organisms in the ballast tank (López-Amorós et al. 1997; Joachimsthal et al. 2003; Drillet et al. 2013; Zaiko et al. 2015). Sometimes, non-native species possess a competitive advantage in their recipient environment. It has been proposed that bloom forming species are those able to escape predation pressure at the beginning of the bloom by utilizing a so called loophole—a set of deterrence or avoidance mechanisms (e.g., toxin production, increasing large body size to prevent ingestion, etc.) (Irigoien et al. 2005). The environmental status of the receiving area is another important factor for the colonization success of invasive species. Natural stress (e.g. variable salinities), or stress caused by human influence, like organic enrichment, pollution, physical habitat alterations etc., favors the spread of invasive species, whereas a robust native ecosystem represent a natural impediment to bioinvasions (Occhipinti-Ambrogi and Savini 2003). Even though successful invasions are rare, they are difficult to control once they have occurred (Hoddle 2004), leading to biodiversity loss, ecosystem imbalance, and fishery and tourism impairment.

Research on ballast water introduced organisms has focused mostly on metazoans, especially the zebra mussel's invasion of the Great lakes of North America (Griffiths et al. 1991; Strayer 2010). However, microorganisms and viruses are numerically dominant in the environment, found in densities up to 10^{11} L^{-1} in sea water (Delong 1992; Wommack and Colwell 2000; Curtis et al. 2002). They are therefore most likely to arrive in the highest number in the ballast tanks (Ruiz et al. 2000). Of particular concern are ballast water dispersed pathogenic bacteria that may affect human health directly. Historically there are several examples of presence of *Vibrio cholerae*, the causative agent of human cholera, in ship's ballast water (McCarthy and Khambaty 1994; Ruiz et al. 2000), although the concentration of bacteria in general is lower in ballast water samples than in coastal water samples (Ruiz et al. 2000). Another concern is Harmful Algal Blooms (HABs). Such blooms can cause widespread harmful impact, including anoxic conditions (Tango et al. 2005; Nelson et al. 2008) and the release of toxic compounds (Anderson 2009).

It is well known that microorganisms can employ survival strategies to withstand periods of unfavorable conditions such as a dark ballast tank or starvation. These strategies include formation of cysts (Grigorszky et al. 2006) and the ability to enter a viable but non-culturable (VBNC) state. Both indicator bacteria and pathogenic species have been recorded in a VBNC state in marine and aquatic environments (Barcina et al. 1997; Liu et al. 2009; Fernandez-Delgado et al. 2015; Kaberdin et al. 2015), potentially posing a health risk by remaining contagious, producing toxins (Krebs and Taylor 2011), or by recovering from the VBNC state as a result of altered environmental conditions (Liu et al. 2009; Fernandez-Delgado et al. 2015) upon discharge. Another survival strategy in a ballast tank can be the formation of biofilms, also referred to as "interior hull fouling" (Drake et al. 2005). Such biofilms establishments can offer the bacterial cells protection against physical, chemical or biological stress (Decho 2000), and thus may represent an additional risk of microbial invasion when released into water during normal ballasting operations (Drake et al. 2005).

Ballast water regulations and compliance

In February 2004 the International Maritime Organization (IMO) adopted "the International Convention for the Control and Management of the Ships' Ballast Water and Sediments (IMO 2004)", or the "IMO convention". The IMO convention was ratified the 08th of September 2016, entry into force will take place the 08th of September 2017. Once entered into force, ballast water will need to be treated before discharge, or otherwise managed, as formulated in annex section D-1 and D-2 (IMO 2008a). According to the D-2 standard the discharged ballast water must contain less than:

1. A total of 10 viable organisms per m^3 (for organisms ≥ 50 μm)
2. A total of 10 viable organisms per ml (for organisms ≥ 10–<50 μm)
3. A total of 1 colony forming unit per 100 mL of *Vibrio cholerae* (serotypes O1, O139)
4. A total of 250 colony forming unit per 100 mL of *Escherichia coli*
5. A total of 100 colony forming unit per 100 mL of intestinal Enterococci

The D-2 standard is based partly on size; organisms ≥ 50 μm include macroalgae, large protists, zooplankton, and fish larvae, whereas the ≥ 10–<50 μm size group is dominated by phytoplankton. Individual species identification is not required with the exception of a few indicator bacteria with a human health impact; *V. cholerae* (O1 and O139), *E. coli*, and intestinal Enterococci. Notice that apart from the indicator bacteria the standard does not address organisms < 10 μm, even though several bloom-forming harmful algae can be found in this size-class, e.g. *Phaeocystis* spp., and *Chrysochromulina* spp. (Seoane et al. 2012). Viruses are also excluded from the performance standard.

To comply with the D-2 standard, most ships will need to install ballast water treatment systems (BWTS) to purify their ballast upon discharge. UV-irradiation is a popular disinfection technology, used by about 30% of today's BWTS (Delacroix et al. 2013). Other technologies used for ballast water disinfection include chemical treatments (e.g. chlorination, ozonation and electrolysis) and/or physical (e.g. heat and cavitation) treatment technologies. IMO has provided guidelines for the approval of BWTS, the G9 and G8 guidelines, with and without the use of active substances, respectively (IMO 2008a, IMO 2008c). Several BWTS have been evaluated on both land based test facilities and on shipboard trials (Veldhuis et al. 2006; Echardt and Kornmueller 2009; Wright et al. 2010; Delacroix et al. 2013; Bakalar 2016) and in 2012 the US Coast Guard (USCG) published a final rule in the Federal Register (USCG 2012) which comprises a separate certification program for ships entering U.S. waters. Verification testing of technologies is described in the ETV protocol, but different certification protocols have created uncertainty on how to simultaneously meet

U.S. and IMO regulations and testing regimes. There has been some criticism to the IMO guidelines for approval of BWTS for not taking into account water temperature differences among testing sites and seasons, however the G8 guidelines are now being revised to also include issues like temperature. Temperature may affect grazing, growth, reproduction rates, and natural decay amongst various organisms, but may also have an impact on the treatment technologies e.g. the efficacy of chemical treatments and the decay of disinfectants (Drillet et al. 2013). Various treatment technologies should therefore be clarified in relation to the temperature of the water.

Once the IMO Convention enters into force, ships may be subjected to inspections by port state controls for compliance control of the discharged ballast water. Compliance control can be performed in two steps; an indicative test and a detailed compliance test (IMO 2008b). Indicative tests are used to identify potential non-compliance at an early stage. Non-compliance or doubts whether the discharged ballast water is compliant will require a detailed compliance test. A detailed compliance test may be performed without a prior indicative test.

There has been some irregularity in terminology affecting the evaluation of discharged ballast water. The IMO Convention and the revised G8 guidelines refers to "viable" cells, where viable organisms are organisms that are reproductively viable, however the USCG also use the term "living" in their final rule. For owners and producers of BWTS based on UV technology, the difference between living and viable is of major importance. UV-induced DNA-damages sometimes cause cells that are vital but non-viable (Olsen et al. 2015). Photoreactivation and dark repair mechanism can counteract such DNA lesions, but DNA damages may escape without repair.

Available methods for ballast water compliance control

Microscopy is a sensitive method that is frequently used for detailed compliance testing today. It not only allows for quantification of microorganisms and for characterization of cell morphology, but can also account for cellular features that can inform about viability, or be combined with staining techniques for live/dead evaluations. The method is however, time-consuming and only small volumes can be analyzed, and it requires a high level of expertise (Stehouwer et al. 2013). Other methods for quantification of microorganisms, such as the plate count technique and the most-probable number (MPN), rely on growth, which can also be time consuming, especially for slow growing species. Selective growth media can allow some degree of microbial differentiation but species- or strain-identification will require additional analysis. Moreover, growth based methods will often underestimate the number of living cells since many organisms are unable to grow and reproduce under laboratory conditions (Amann et al. 1995; Rappe and Giovannoni 2003; Allen et al. 2004; Tyson and Banfield 2005), as well as the VBNC cells. Discrepancies between laboratory results and reality is likely when using solid media (plate counts) as the ballast water organisms originate from liquid medium (sea-, brackish- or fresh water). Recently, the USCG concluded that the most probable number (MPN) method was not a satisfactory method for determining the number of living organisms in the ≥ 10–<50 μm size class during type approval of BWTS. The regulations require BWTS to be evaluated based on their ability to remove or kill certain organism (EPA 2010), whereas the MPN method according to the USCG assess the viability of an organism to colonize after treatment and is hence a different standard than that required.

Due to the above mentioned shortcomings of traditional methods to assess the effectiveness of a BWTS, cell counts detecting living, damaged, dormant, VBNC, and dead cells are better obtained by direct single cell measurements for the ≥ 10–<50 μm size class. Flow cytometry (FCM), a technique that provides high precision detection and analysis of live and dead microorganisms by light scattering and/or fluorescence, represents such methodology. FCM is applied to liquid samples for detection and analyses of particles hydrodynamically or acoustically focused in a stream so that they, one by one, pass light beams from one or more lasers. The scatter and fluorescence intensity is measured by sensitive photomultiplier tubes. This allows individual measurements according to size (forward scatter), surface/granularity/complexity (side scatter), and fluorescence either caused by own pigments or by fluorescent dye markers. The FCM technology can also be used for cell counting, cell sorting and biomarker detection.

FCM was first developed in the 1960s (Fulwyler 1965; Fulwyler 1968; Dittrich 1971) and initially applied in clinical microbiology for detection of bacteria in blood (Mansour et al. 1985). FCM has rapidly become an essential tool in microbiology with increased popularity and widespread applications. Today FCM is routinely used in the diagnosis of health disorders such as cancer (Racila et al. 1998). Other applications of FCM in basic research vary from studies of cell cycles (Muller et al. 2010), microbial community analysis (Zubkov et al. 2000; Muller and Nebe-von-Caron 2010), microbial moni-

toring of sea- and drinking water (Joachimsthal et al. 2003; Berney et al. 2008; Hammes et al. 2008), to evaluations of bacterial susceptibility to antibiotics (Pore 1994; Roth et al. 1997). Phytoplankton are particularly suited for FCM studies due to their auto-fluorescence as demonstrated by the discovery of the presence of genus *Prochlorococcus* (Campbell and Vaulot 1993; Campbell et al. 1994) as well as the smallest eukaryote in the ocean, *Osterococcus tauri* (Courties et al. 1994; Chretiennotdinet et al. 1995).

Reasons for FCM's popularity include the possibility for rapid and multi-parametric analysis and for detecting microbial cells irrespective of their cultivability. The technology offers opportunities for analysis at both community and single-cell levels. Also, it is possible to combine FCM with various stains or labels, thereby marking target cells with fluorescent molecules that separate them from abiotic particles. FCM is considered a promising tool for type approval testing and possibly for detailed ballast water analysis, since it offers the possibility to measure relative size of organisms, the number of organisms, and the vitality of organisms in a sample (Bakalar 2014; Olsen et al. 2015; Olsen et al. 2016a; Olsen et al. 2016b).

Evaluating methods for ballast water compliance control—FCM a promising tool

Primarily, a ballast water compliance method must differentiate cells into size according to the D-2 standard. The size of the organisms should be documented according to their minimum dimension (the smallest part of their body). FCM can estimate particle size based on scattering signals and compare with calibrated spherical microbeads with a known diameter. For FCM instruments detection of minimum dimension is challenging, and it is easier to measure the maximum dimension of an organism (Gollasch and David 2015). For organisms in the ≥ 50 μm size-group, FCM instruments with a wide size range must be used, as organisms ≥ 50 μm can block the fluidics system of many FCM instrument. Detection can also be difficult due to limitations in the forward scatter. For organisms in the ≥ 10–<50 μm size group, on the other hand, FCM is more appropriate. Chlorophyll *a* auto-fluorescence is used to identify phytoplankton (Veldhuis and Kraay 2000), the dominant organisms of this size group. Previous studies have shown that FCM is a well suited method for measuring the number of phytoplankton cells, providing comparable numbers to microscopy (Stehouwer et al. 2013). It is important to be aware, however, that single cell suspensions are essential for accurate enumeration of cells with FCM, as cell aggregates give rise to a single event only and thus cause problems for cell enumeration of colony forming algae and bacteria (Veldhuis et al. 2005; Christaki et al. 2011; Zhou et al. 2012).

Secondly, a compliance method must be able to detect the viability of cells. Often, fluorescent stains are used to evaluate cell *vitality*. Table 1 gives an overview of some available dyes to stain intact cells, dead cells and total cells, respectively. When choosing a stain, excitation and emission maximum has to be considered according to available lasers and detectors in the flow cytometer. Detection of a cell's *viability* is complex, and it is therefore increasingly common to use indirect single cell measurements, like metabolic activity, membrane potential, oxidative stress, and membrane permeability, to analyze one or more cellular functions (see details in Box 1). A common approach for classifying live cells is to subtract dead cells from total cells. Exclusion dyes are used to label cells with compromised membranes, an indicator of dead cells since these cannot maintain their membrane potential, e.g. propidium iodine (PI) (Berney et al. 2007; Schenk et al. 2011), SYTOX Green (Brussaard et al. 2001; Steinberg et al. 2012) and SYTOX Blue (Olsen et al. 2016a). For ballast water analysis PI fluorescence cannot be detected together with chlorophyll *a,* since their emission spectra overlaps. PI is therefore not applicable for analysis of phytoplankton species but is suitable for analysis of bacteria and other heterotrophic organisms. Alternatively, permeable vital stains (like FDA, CMFDA or CFDA-AM) can be used directly to enumerate living cells (Lee et al. 2015; Olsen et al. 2015). Today, the USCG follows the Environmental Technology Verification (ETV) protocol during approval of BWTS, where the stains FDA and CMFDA in combination are used to evaluate viability. Recently, a study of 24 different phytoplankton species from seven divisions were analyzed, where living and heat-killed cells were stained by FDA/CMFDA. The results revealed acceptable accuracy for only 10 out of 24 species, and combining CMFDA with FDA did not improve the performance of FDA alone (MacIntyre and Cullen 2016), in contrast to other reports (Peperzak and Brussaard 2011; Steinberg et al. 2011). One problem using the vital stains is the likelihood of overestimating living cells in UV-irradiated samples since damaged and non-viable cells will be detected as living (Olsen et al. 2015; Olsen et al. 2016a). This will, however, most likely not affect the assessment of BWTS that use other disinfection technologies than UV-irradiation. Studies that combine dyes have improved evaluations of UV-irradiated samples by generating more information of each cell. FCM analysis can at an early stage after UV-

Table 1. Some available dyes for vitality analysis by FCM.

Target	Cell functionality	Dye	Flourescence excitation/emission maxima (in nm)	Reference
Intact cells	Metabolic activity	ChemChrome dyes (CY, CB, CV6) (esterase activity)	488/520	Diaper and Edwards 1994; Porter et al. 1995a; Catala et al. 1999; Parthuisot et al. 2000
		CMFDA, FDA, CFDA, CFDA-AM (esterase activity)	493/517 (CMFDA) 495/517 (FDA, CFDA) 485/535 (CFDA-AM)	Breeuwer et al. 1994; Peperzak and Brussaard 2011; Schenk et al. 2011; Lee et al. 2015; Olsen et al. 2015; MacIntyre and Cullen 2016; Olsen et al. 2016a,b
		Calcein dyes (esterase activity)	493/514 (Calcein-AM) 360/455 (Calcein Blue-AM) 400/452 (Calcein Violet-AM)	Brussaard et al. 2001; Peperzak and Brussaard 2011
		CTC (respiratory activity)	450/630	del Giorgio et al. 1997; Joux et al. 1997; Yamaguchi and Nasu 1997; Rezaeinejad and Ivanov 2011
	Membrane integrity/ membrane potential/pump activity	Rh123 (mitochondrial membrane pot.)	507/529	Diaper and Edwards 1994; López-Amorós et al. 1995
		$DiOC_n$ (mitochondrial membrane pot.)	484/501 ($DiOC_6(3)$) 482/497 ($DiOC_2(3)$)	Zuliani et al. 2003; da Silva et al. 2005; Reis et al. 2005; Novo et al. 1999; Novo et al. 2000
		JC-1 (mitochondrial membrane pot.)	498,593/525,585	Zuliani et al. 2003.
		$DiBAC_4(3)$ (plasma membrane pot.)	493/516	López-Amorós et al. 1997; Berney et al. 2006; Peperzak and Brussaard 2011; Rezaeinejad and Ivanov 2011
		Indo-1 (cytoplasmic Ca^{2+})	350/405	Bailey and Macardle 2006
	Oxidative stress	CellROX Oxidative Stress reagent	644/665 (CellROX Deep Red) 545/565 (CellROX Orange) 485/520 (CellROX Green)	Davila et al. 2015; Tormos et al. 2015
Dead cells	Membrane permeability	SYTOX dyes	504/523 (SYTOX green) 444/480 (SYTOX blue) 547/570 (SYTOX orange) 640/658 (SYTOX red)	Veldhuis et al. 2006; Steinberg et al. 2012; Martinez et al. 2013; Olsen et al. 2016a
		Propidium iodide (PI)	538/617	López-Amorós et al. 1997; Williams et al. 1998; Lehtinen et al. 2004; Berney et al. 2007; Shi et al. 2007; Schenk et al. 2011
		7AAD	546/647	Herault et al. 2002; Quinn et al. 2007
Total cells	Intact and permeabilized cells	SYTO	485/498 (SYTO 9) 488/506 (SYTO 13)	Guindulain et al. 1997; Lebaron et al. 1998b; Lebaron et al. 2001; Lehtinen et al. 2004; Berney et al. 2006
		Hoechst 33342	352/455	Marie et al. 1996; Joux et al. 1997; Shi et al. 2007
		DRAQ5	647/681	Edward 2012
		DAPI	358/463	Marie et al. 1996; Shi et al. 2007
		Ethidium bromide	524/605	Berney et al. 2006
		LDS751	543/712	Bischoff et al. 1998; Mundle et al. 1999
		SYBR green	497/520	Marie et al. 1999; Berney et al. 2008; Hammes et al. 2008
		Fluorescein (FITC)	490/525	Hedhammar et al. 2005; Canovas et al. 2007

irradiation separate cells that are UV-damaged from live cells (Olsen et al. 2016).

Thirdly, a detailed compliance test must be able to identify certain species; *V. cholerae* (O1 and O139), *E. coli*, and intestinal Enterococci. Stehouwer et al. (2013) performed analysis using cluster software on FCM data, but concluded that species identification is not straight forward. Physiological changes within a species over time, for example changes in size and fluorescence under nutrient limitation, complicated the cluster analysis (Stehouwer et al. 2013). A more promising approach is the use of species-specific

Box 1. Cell viability - target sites for FCM analysis.

Metabolic activity. Enzyme activities, such as esterase, provide indication of metabolic activity in a cell. Non-fluorescent substrates diffuse into the cells and are converted into fluorescent products by intracellular enzymes. However, detection of fluorescent products only demonstrates the cells ability to synthesize enzymes in the past (and to maintain them in an active form), since enzyme reactions usually are energy independent. Importantly, enzyme activity might not be detectable temporarily, for examples in cases of cell damage, dormancy or starvation; or it may remain below the detection limits. Also, presence of active ion pumps can interfere in metabolic activity evaluations (Breeuwer et al. 1994; Amor et al. 2002; Hoefel et al. 2003).

Membrane integrity / membrane potential / pump activity. Membrane integrity demonstrates the protection of cell constituents. Ion concentration gradients and active transport of ions across the cytoplasmic membrane create a difference in voltage across the membrane. In microorganisms it is typically in the order of 100 mV, with the interior negative. Only live cells are able to maintain this membrane potential. The membrane potential decreases in cells with damaged membranes, whereas dead cells cannot generate or maintain a membrane potential since the ions move freely across the membrane. Membrane potential can be detected using dyes that accumulate in the cells according to their charge. Their fluorescent signal can be directly related to the cell energy levels. Since membrane integrity studies do not require cell activity, it is suitable for detection of starved, dormant or injured cells (Vives-Rego et al. 2000).

Oxidative stress. In live cells, reactive oxygen species (ROS) are generated at controlled rates. However, under conditions of oxidative stress, production of ROS increases. Special fluorogenic probes have been developed for measuring oxidative stress in cells. These dyes are non-fluorescent in a reduced state and emit fluorescence upon oxidation.

Membrane permeability. Exclusion dyes are used to detect permeable membranes. Cells with intact membranes are impermeable to these dyes, whereas the dyes enter cells with damaged membranes and fluoresce upon nucleic acid binding. It is important to be aware that dead cells can be underestimated when DNA is degraded, or altered to such a degree that the dye is unable to bind (Lebaron et al. 1998a; Olsen et al. 2016a). Membrane permeability can also be reversed or just a temporary condition (Duffy et al. 2000; Shi et al. 2007; Davey and Hexley 2011). It is therefore important to allow sufficient time for membrane repair before analysis.

Multicolor approaches. When using a single dye, overestimation of subpopulations (metabolic active or dead) can occur (Olsen et al. 2015; Olsen et al. 2016b). Combining dyes allows for differentiation based on cellular functions. Dual-staining protocols that combine permeable and impermeable dyes have been able to distinguish between active, damaged and dead cells (López-Amorós et al. 1997; Lehtinen et al. 2004; Herrero et al. 2006; Quiros et al. 2007; Olsen et al. 2016a). For bacteria analysis, a total cell count dye is commonly added to distinguish between cells and noise.

Limitation: None of the staining techniques can give a definite answer about the cells reproductive growth.

fluorescent antibodies, immuno FCM (Peperzak et al. 2000), for identification. The technique has been used successfully to identify various species; for example, the brown tide algae *Aureoumbra lagunensis* (Koch et al. 2014), *Cryptosporidium parvum* (Barbosa et al. 2008), and *Legionella pneumophila* (Fuechslin et al. 2010). But the technique is not well established in ballast water analysis. Probes used for immuno FCM are designed for a specific species or group, and can be applied to any microorganism if specific antibodies are available, but require *a priori* knowledge of which species to look for in a sample. One general problem with immunofluorescence detection of microorganisms, is that the antibodies often only covers some subgroups within the indicated species. Alternative methods for species identification include combining FCM with fluorescent in situ hybridization (FISH) (Joachimsthal et al. 2004), using rRNA-targeted oligonucleotide probes (Simon et al. 1997; Joachimsthal et al. 2004), FCM cell sorting followed by identification of groups or single cells (Wallner et al. 1997), or combining FCM with digital photography of particles

(such as FlowCam, CytoSense and ImageStream flow cytometers) (Zetsche et al. 2014). It is important to bear in mind that species identification must be combined with proofs that the cells are alive.

The most obvious advantages of using FCM for ballast water analysis are: (1) Rapid analyzes with thousands of events being detected per second enabling large(r) volumes to be analyzed (from rates of µL/min up to mL/min) and reducing fading of the fluorescent signals as dyes are prone to degradation over time when exposed to light (Johnson and Araujo 1981). (2) Analyzes of microorganisms irrespectively of their cultivability enabling detection of e.g. viable but non-culturable (VBNC) cells (Porter et al. 1995b). (3) Limited sample handling; filtration to prevent clogging when the ballast water contains large particles or organisms and/or addition of fluorescent stains when appropriate being the only. (4) High sensitivity; particles down to 50 nm can be detected (Steen 2004), meaning even marine viruses can be detected (Marie et al. 1999; Brussaard et al. 2000; Larsen et al. 2001; Marie et al. 2001).

FCM is indeed a promising tool for assessment of BWTS but there are still some disadvantages: (1) Apparatus costs are still somewhat expensive (although prices are decreasing). (2) Enumeration of rare events can be extremely difficult (Joux and Lebaron 2000; Lemarchand et al. 2001) (although rare events are also problematic with other analysis methods like microscopy). Methods used for ballast water analysis must be able to detect a few living organisms amongst high concentrations of dead cells. It is therefore essential to validate the sensitivity of the method to ensure that low densities of variable organisms will be detected. (3) Most FCM apparatus are not applicable for detection of organism in the ≥ 50 µm size-group, the exception is instruments with digital photography options which can detect particles up to mm size. Also, another issue is the volume for this size class (less than 10 organisms per cubic meter). Even with concentration of volume, the analysis would take a long time.

Knowledge gaps and future applications for flow cytometry within ballast water analysis

As of today, FCM is a promising technology for detection and quantification of microorganisms according to the IMO convention, and is also included in the IMO "Circular 42" document containing a list of available methods for ballast water compliance monitoring. However, most FCM research has focused on the ≥ 10–<50 µm size group, and rational monitoring may result in different protocols for the various groups in the convention. For example, to circumvent clogging of the FCM apparatus when analyzing larger organisms (≥ 50 µm), protocols using special flow cytometers, like e.g. the FlowCam, must be developed. But when using such instruments, a database of reference pictures is required to distinguish between organisms and particles. Furthermore, it is uncertain whether FCM is the best option for identifying bacterial species, since a protocol must separate one species from its close relatives, and at the same time combine this with information of the cells viability. To do that with FCM, it is possible to combine either immuno FCM or FISH (Joachimsthal et al. 2004) with live/dead staining (López-Amorós et al. 1997; Lehtinen et al. 2004), however this approach has not been applied to ballast water samples, to our knowledge. Also, in the D-2 standard, concentration of bacteria are given in cfu/100 mL, so methods for detection are based on growth and not single cell analysis.

Most research relevant for FCM analysis of ballast water is so far performed on species representative for a size group, like the phytoplankton *Tetraselmis* sp. (Steinberg et al. 2012; Carney et al. 2013; Olsen et al. 2016a,b), or indicator bacteria like *E. coli* and *Vibrio* sp. (López-Amorós et al. 1997; Joachimsthal et al. 2004; Lehtinen et al. 2004) (for overview see Table 2). *Tetraselmis* sp. represents marine organisms in the ≥ 10–<50 µm size category in the D-2 standard and is sometimes used in test water to fulfill the biological water quality criteria during approval of BWTS. *Tetraselmis* sp. is, however, not as abundant in coastal waters as diatoms, dinoflagellates and prymnesiophytes species and little research is performed on FCM analysis of natural water samples after treatments simulating that of BWTS. In natural waters, the microbial community is diverse and varies according to location, season and environmental conditions (Barcina et al. 1997; Zubkov et al. 2000; Drillet et al. 2013). Such variations can influence cell activities between species, or within a single species. Also, organisms occurring in natural waters are sometimes associated with other organisms (Khandeparker and Anil 2013), or particles, which may affect the resistance to UV-radiation, heat or chemicals (Hess-Erga et al. 2010; Tang et al. 2011). Further research should therefore be focused on validating FCM protocols for more species, as well as for natural waters containing different organisms. Even though microorganisms smaller than 10 µm and viruses are not included in the D-2 standard today, they may be important in the future, not the least as pathogen carriers. FCM is a good candidate for their detection.

Table 2. Overview of publications relevant for FCM analysis of ballast water.

Target organisms	Method of choice	Reference
Dinoflagellates, diatoms, green algae and microalgae	Green auto-fluorescence detection by FCM	Tang and Dobbs 2007
Cysts of dinoflagellate (*Alexandrium catenella*)	Viability analysis using SYTOX green stain and FCM	Binet and Stauber 2006
Phytoplankton	Clustering analysis by FCM based on size/forward scatter, and various fluorescence signals (green 525 nm, yellow/orange 575 nm, and red 620 nm).	Stehouwer et al. 2013
Phytoplankton (*Chaetoceros calcitrans*, *Chlorella autotrophica* and *Phaeocystis globose*)	Enumeration and size detection by forward scatter, green auto-fluorescence detection by FCM, and viability analysis using SYTOX green stain and FCM	Martinez et al. 2013
Phytoplankton	Viability analysis using SYTOX green stain and FCM	Veldhuis et al. 2006
Phytoplankton (*Tetraselmis impelludicida*)	Viability analysis using SYTOX green stain and FCM, as well as SYTOX green stain and FlowCam analysis	Steinberg et al. 2012
Phytoplankton (*Tetraselmis suecica*)	Viability analysis using CFDA-AM staining and FCM, and CFDA-AM/CYTOX Blue dual-staining and FCM analysis	Olsen et al. 2015; Olsen et al. 2016a; Olsen et al. 2016b
Phytoplankton (40 different strains)	Viability analysis using various stains (Calcein-AM, CMFDA, $DiBAC_4(3)$, FDA, H_2DCFDA, and SYTOX-Green) and FCM	Peperzak and Brussaard 2011
Seawater algae (*Isochrysis galbana* and *Phaeodactylum tricornutum*) and freshwater algae (*Selenastrum capricornutum* and *Scenedesmus obliquus*)	Viability analysis using FDA stain and FCM	Lee et al. 2015
Brown tide causing pelagophyte (*Aureoumbra lagunensis*)	Immune FCM (fluorescently labeled antibodies against *A. lagunensis*)	Koch et al. 2014
Prokaryotic and eukaryotic cells	Enumeration and size detection by forward scatter	Joachimsthal et al. 2003
Bacteria (*E. coli*)	Viability analysis using SYTO9/PI dual staining and FCM, and green-fluorescent protein (GFP)/PI analyzed with FCM	Lehtinen et al. 2004
Bacteria (*E. coli* and *Salmonella typhimurium*)	Viability analysis using various stains (Rh123, $DiBAC_4(3)$, PI, and CTC) and FCM	López-Amorós et al. 1997
Bacteria (total bacteria count, *Enterobacteria*, *Vibrio* spp., and *Escherichia coli*	FISH and FCM	Joachimsthal et al. 2004

Some studies have looked at the ability of different species to regrow after UV-irradiation (Hess-Erga et al. 2010; Martinez et al. 2012; Martinez et al. 2013; Stehouwer et al. 2013). Analysis at the NIOZ testing facility (Texel, The Netherlands) proved that the genera *Thalassiosira*, *Skeletonema*, *Pseudo-nitzscia* and *Chaetoceros* are able to survive harsh UV-treatments (some even double UV-treatments) and regrow afterwards (Stehouwer et al. 2013). Identification of such resistant species can be used to develop more robust testing regimes for BWTS when desirable. A few studies have identified the lethal UV-doses for specific species (Ou et al. 2012; Olsen et al. 2016b). FCM has the advantage of giving rapid feedback whether the UV-dose applied is immediately lethal or not. However, variable results especially when low UV doses are applied, demonstrates the challenge of giving definite recommendations for UV-treatments.

Introducing irradiated species into rich growth medium after irradiation simulate the conditions arising when ballast water is discharged and has shown to affect the recovery of cells after UV-irradiation. Cell recovery was slower (due to acclimatization) but in total higher (greater) in a rich growth medium compared to the original environment (Martinez et al. 2013). Identification of regrowing species can provide important information of potential future invaders. A second factor that may influence the recovery of cells after irradiation is the introduction of irradiated cells into light. Recovery and regrowth are expected to increase under such conditions, due to the photo-reactivation mechanisms (Carney et al. 2011). FCM is therefore well suited to analyze cell recovery after UV-irradiation, which is not sufficiently considered in the IMO guidelines today. More research focus in this field will provide important information on the likelihoods of DNA repair.

FCM analysis has been used to study water treated with UV-irradiation (Berney et al. 2006; Schenk et al. 2011; Ou et al. 2012; Martinez et al. 2013; Olsen et al. 2015). Other studies have applied FCM to

analyse water treated with chlorination (Wang et al. 2010), electrolysis (Song et al. 2012), and ozonation (Bai et al. 2016). However, still more research should focus on validating FCM protocols for various water treatment technologies, also for BWTS installed onboard ships.

FCM already holds several advantages for ballast water analysis and the development will continue. Further advances of fluorescent dyes and probes, as well as progress in labeling protocols, is expected. Indeed, functional probes which can detect the physiological and metabolic status of the cells will improve the FCM analysis. Methods using immunofluorescent technology or fluorescence labeled oligonucleotide probes, and FCM cell sorting technologies, will improve species identification. Also, FCM can be combined with other technologies, including genomics and proteomics and in the future, possibly have integrated protein analysis or DNA sequencing options. For field biosafety analysis, a more user-friendly instrument would be desirable; such as a portable or even online flow cytometer combined with straightforward interpretation. A few portable/online instruments are available today, but as far as we know continuous monitoring of ballast water by FCM have yet to be achieved. Vibrations onboard that potentially effects the alignment of lasers, or clogging of the device, are potential obstacles. However, such real-time methods could also be useful for drinking water monitoring (Berney et al. 2008). Real-time FCM was first applied in 1980 (Martin and Swartzendruber 1980), and recently a series of demonstrative examples of potential applications was performed. Some relevant suggested applications were: "(1) fluorescent labeling of heat-induced membrane damage in a autochthonous freshwater bacterial community, (2) initial growth response of late stationary *E. coli* cells inoculated into fresh growth media, and (3) oxidative disinfection of a mixed culture of auto-fluorescent microorganisms" (Arnoldini et al. 2013). Real-time monitoring of treated ballast water with FCM could discover potential problems or failings with BWTS, preventing the release of potentially invasive species.

Conclusion

In summary, FCM is a powerful technique with a great potential for ballast water monitoring as well as detailed compliance testing. As of today, FCM is best suited for analysis of the ≥ 10–<50 size class in the D-2 standard, and samples should be brought to labs onshore for analysis. Sample analysis is fast, so results should be available the same day (assuming lethal doses of treatment).

Acknowledgements

This research was founded by the Norwegian Research Council (project BallastFlow, project no. 208653) and Knutsen OAS Shipping AS, and supported by Stord/Haugesund University College, Solstad Shipping, VRI Rogaland, UH-nett Vest and TeknoVest. We thank the reviewers of *Management of Biological Invasions* for the important job they are doing, helping us to improve this paper.

References

Allen MJ, Edberg SC, Reasoner DJ (2004) Heterotrophic plate count bacteria - what is their significance in drinking water? *International Journal of Food Microbiology* 92: 265–274, https://doi.org/10.1016/j.ijfoodmicro.2003.08.017

Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. *Microbiological Reviews* 59: 143–169

Amor KB, Breeuwer P, Verbaarschot P, Rombouts FM, Akkermans ADL, De Vos WM, Abee T (2002) Multiparametric flow cytometry and cell sorting for the assessment of viable, injured, and dead bifidobacterium cells during bile salt stress. *Applied and Environmental Microbiology* 68: 5209–5216, https://doi.org/10.1128/AEM.68.11.5209-5216.2002

Anderson DM (2009) Approaches to monitoring, control and management of harmful algal blooms (HABs). *Ocean & Coastal Management* 52: 342–347, https://doi.org/10.1016/j.ocecoaman.2009.04.006

Arnoldini M, Heck T, Blanco-Fernandez A, Hammes F (2013) Monitoring of Dynamic Microbiological Processes Using Real-Time Flow Cytometry. *PloS ONE* 8: 11, https://doi.org/10.1371/journal.pone.0080117

Bai M, Zheng Q, Tian Y, Zhang Z, Chen C, Cheng C, Meng X (2016) Inactivation of invasive marine species in the process of conveying ballast water using center dot OH based on a strong ionization discharge. *Water Research* 96: 217–224, https://doi.org/10.1016/j.watres.2016.03.056

Bailey S, Macardle PJ (2006) A flow cytometric comparison of Indo-1 to fluo-3 and Fura Red excited with low power lasers for detecting Ca^{2+} flux. *Journal of Immunological Methods* 311: 220–225, https://doi.org/10.1016/j.jim.2006.02.005

Bakalar G (2014) Review of interdisciplinary devices for detecting the quality of ship ballast water. *Springerplus* 3: 8, https://doi.org/10.1186/2193-1801-3-468

Bakalar G (2016) Comparisons of interdisciplinary ballast water treatment systems and operational experiences from ships. *Springerplus* 5: 12, https://doi.org/10.1186/s40064-016-1916-z

Barbosa JMM, Costa-de-Oliveira S, Rodrigues AG, Hanscheid T, Shapiro H, Pina-Vaz C (2008) A flow cytometric protocol for detection of *Cryptosporidium* spp. *Cytometry Part A* 73A: 44–47, https://doi.org/10.1002/cyto.a.20502

Barcina I, Lebaron P, Vivesrego J (1997) Survival of allochthonous bacteria in aquatic systems: A biological approach. *Fems Microbiology Ecology* 23: 1–9, https://doi.org/10.1111/j.1574-6941.1997.tb00385.x

Berney M, Hammes F, Bosshard F, Weilenmann HU, Egli T (2007) Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Applied and Environmental Microbiology* 73: 3283–3290, https://doi.org/10.1128/AEM.02750-06

Berney M, Vital M, Hulshoff I, Weilenmann HU, Egli T, Hammes F (2008) Rapid, cultivation-independent assessment of microbial viability in drinking water. *Water Research* 42: 4010–4018, https://doi.org/10.1016/j.watres.2008.07.017

Berney M, Weilenmann HU, Egli T (2006) Flow-cytometric study of vital cellular functions in *Escherichia coli* during solar disinfection (SODIS). *Microbiology-Sgm* 152: 1719–1729, https://doi.org/10.1099/mic.0.28617-0

Binet MT, Stauber JL (2006) Rapid flow cytometric method for the assessment of toxic dinoflagellate cyst viability. *Marine Environmental Research* 62: 247–260, https://doi.org/10.1016/j.marenvres.2006.03.011

Bischoff FZ, Lewis DE, Nguyen DD, Murrell S, Schober W, Scott J, Simpson JL, Elias S (1998) Prenatal diagnosis with use of fetal cells isolated from maternal blood: Five-color fluorescent in situ hybridization analysis on flow-sorted cells for chromosomes X, Y, 13, 18, and 21. *American Journal of Obstetrics and Gynecology* 179: 203–209, https://doi.org/10.1016/S0002-9378(98)70273-X

Breeuwer P, Drocourt JL, Rombouts FM, Abee T (1994) Energy-dependent, carrier-mediated extrusion of carboxyflourescein from *Saccharomyces cerevisiae* allows rapid assessment of cell viability by flow-cytometry. *Applied and Environmental Microbiology* 60: 1467–1472

Brussaard CPD, Marie D, Bratbak G (2000) Flow cytometric detection of viruses. *Journal of Virological Methods* 85: 175–182, https://doi.org/10.1016/S0166-0934(99)00167-6

Brussaard CPD, Marie D, Thyrhaug R, Bratbak G (2001) Flow cytometric analysis of phytoplankton viability following viral infection. *Aquatic Microbial Ecology* 26: 157–166, https://doi.org/10.3354/ame026157

Campbell L, Vaulot D (1993) Photosynthetic picoplankton community structure in the subtropical North Pacific Ocean near Hawaii (station Aloha). *Deep-Sea Research Part I-Oceanographic Research Papers* 40: 2043–2060, https://doi.org/10.1016/0967-0637(93)90044-4

Campbell L, Nolla HA, Vaulot D (1994) The importance of *Prochlorococcus* to community structure in the central north pacific ocean. *Limnology and Oceanography* 39: 954–961, https://doi.org/10.4319/lo.1994.39.4.0954

Canovas M, Garcia V, Bernal V, Torroglosa T, Iborra JL (2007) Analysis of *Escherichia coli* cell state by flow cytometry during whole cell catalyzed biotransformation for L-carnitine production. *Process Biochemistry* 42: 25–33, https://doi.org/10.1016/j.procbio.2006.07.027

Carney KJ, Basurko OC, Pazouki K, Marsham S, Delany JE, Desai DV, Anil AC, Mesbahi E (2013) Difficulties in obtaining representative samples for compliance with the Ballast Water Management Convention. *Marine Pollution Bulletin* 68: 99–105, https://doi.org/10.1016/j.marpolbul.2012.12.016

Carney KJ, Delany JE, Sawant S, Mesbahi E (2011) The effects of prolonged darkness on temperate and tropical marine phytoplankton, and their implications for ballast water risk management. *Marine Pollution Bulletin* 62: 1233–1244, https://doi.org/10.1016/j.marpolbul.2011.03.022

Catala P, Parthuisot N, Bernard L, Baudart J, Lemarchand K, Lebaron P (1999) Effectiveness of CSE to counterstain particles and dead bacterial cells with permeabilised membranes: application to viability assessment in waters. *Fems Microbiology Letters* 178: 219–226, https://doi.org/10.1111/j.1574-6968.1999.tb08680.x

Chretiennotdinet MJ, Courties C, Vaquer A, Neveux J, Claustre H, Lautier J, Machado MC (1995) A new marine picoeucaryote: *Ostreococcus tauri* gen. et sp. nov. (Chlorophyta, Prasinophyceae). *Phycologia* 34: 285–292, https://doi.org/10.2216/i0031-8884-34-4-285.1

Christaki U, Courties C, Massana R, Catala P, Lebaron P, Gasol JM, Zubkov MV (2011) Optimized routine flow cytometric enumeration of heterotrophic flagellates using SYBR Green I. *Limnology and Oceanography-Methods* 9: 329–339, https://doi.org/10.4319/lom.2011.9.329

Courties C, Vaquer A, Troussellier M, Lautier J, Chretiennotdinet MJ, Neveux J, Machado C, Claustre H (1994) Smallest eukaryotic organism. *Nature* 370: 255–255, https://doi.org/10.1038/370255a0

Curtis TP, Sloan WT, Scannell JW (2002) Estimating prokaryotic diversity and its limits. *Proceedings of the National Academy of Sciences of the United States of America* 99: 10494–10499, https://doi.org/10.1073/pnas.142680199

da Silva TL, Reis A, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ (2005) Stress-induced physiological responses to starvation periods as well as glucose and lactose pulses in *Bacillus licheniformis* CCMI 1034 continuous aerobic fermentation processes as measured by multi-parameter flow cytometry. *Biochemical Engineering Journal* 24: 31–41, https://doi.org/10.1016/j.bej.2005.01.013

Davey HM, Hexley P (2011) Red but not dead? Membranes of stressed *Saccharomyces cerevisiae* are permeable to propidium iodide. *Environmental Microbiology* 13: 163–171, https://doi.org/10.1111/j.1462-2920.2010.02317.x

Davila MP, Munoz PM, Tapia JA, Ferrusola CO, da Silva CB, Pena FJ (2015) Inhibition of Mitochondrial Complex I Leads to Decreased Motility and Membrane Integrity Related to Increased Hydrogen Peroxide and Reduced ATP Production, while the Inhibition of Glycolysis Has Less Impact on Sperm Motility. *PloS ONE* 10(9): e0138777, https://doi.org/10.1371/journal.pone.0138777

Decho AW (2000) Microbial biofilms in intertidal systems: an overview. *Continental Shelf Research* 20: 1257–1273, https://doi.org/10.1016/S0278-4343(00)00022-4

Delacroix S, Vogelsang C, Tobiesen A, Liltved H (2013) Disinfection by-products and ecotoxicity of ballast water after oxidative treatment - Results and experiences from seven years of full-scale testing of ballast water management systems. *Marine Pollution Bulletin* 73: 24–36, https://doi.org/10.1016/j.marpolbul.2013.06.014

del Giorgio PA, Prairie YT, Bird DF (1997) Coupling between rates of bacterial production and the abundance of metabolically active bacteria in lakes, enumerated using CTC reduction and flow cytometry. *Microbial Ecology* 34: 144–154, https://doi.org/10.1007/s002489900044

Delong EF (1992) Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America* 89: 5685–5689, https://doi.org/10.1073/pnas.89.12.5685

Diaper JP, Edwards C (1994) The use of fluorogenic esters to detect viable bacteria by flow-cytometry. *Journal of Applied Bacteriology* 77: 221–228, https://doi.org/10.1111/j.1365-2672.1994.tb03067.x

Dittrich WGW (1971) Flow-through chamber for photometers to measure and count particles in a dispersion medium. USA Patent 3761187, http://patent.ipexl.com/US/3761187.html

Drake LA, Meyer AE, Forsberg RL, Baier RE, Doblin MA, Heinemann S, Johnson WP, Koch M, Rublee PA, Dobbs FC (2005) Potential invasion of microorganisms and pathogens via 'interior hull fouling': biofilms inside ballast water tanks. *Biological Invasions* 7: 969–982, https://doi.org/10.1007/s10530-004-3001-8

Drake LA, Ruiz GM, Galil BS, Mullady TL, Friedman DO, Dobbs FC (2002) Microbial ecology of ballast water during a transoceanic voyage and the effects of open-ocean exchange. *Marine Ecology Progress Series* 233: 13–20, https://doi.org/10.3354/meps233013

Drillet G, Schmoker C, Trottet A, Mahjoub MS, Duchemin M, Andersen M (2013) Effects of temperature on type approval testing of ballast water treatment systems. *Integrated Environmental Assessment and Management* 9: 192–195, https://doi.org/10.1002/ieam.1394

Duffy G, Kilbride B, Sheridan JJ, Blair IS, Mcdowell DA (2000) A membrane-immunofluorescent-viability staining technique for the detection of *Salmonella* spp. from fresh and processed meat samples. *Journal of Applied Microbiology* 89: 587–594, https://doi.org/10.1046/j.1365-2672.2000.01151.x

Echardt J, Kornmueller A (2009) The advanced EctoSys electrolysis as an integral part of a ballast water treatment system. *Water Science and Technology* 60: 2227–2234, https://doi.org/10.2166/wst.2009.676

Edward R (2012) Red/far-red fluorescing DNA-specific anthraquinones for nucl:cyto segmentation and viability reporting in cell-based assays. In: Conn PM (ed), Methods in Enzymology, Vol 505: Imaging and Spectroscopic Analysis of Living Cells: Live Cell Imaging of Cellular Elements and Functions. San Diego: Elsevier Academic Press Inc, pp 23–45

EPA (2010) Generic Protocol for the Verification of Ballast Water Treatment Technologies, version 5.1. In: National Service Centre for Environmental Publications, https://nepis.epa.gov/

Fernandez-Delgado M, Garcia-Amado MA, Contreras M, Incani RN, Chirinos H, Rojas H, Suarez P (2015) Survival, induction and resuscitation of *Vibrio cholerae* from the viable but non-culturable state in the Southern Caribbean Sea. *Revista Do Instituto De Medicina Tropical De Sao Paulo* 57: 21–26, https://doi.org/10.1590/S0036-46652015000100003

Fuechslin HP, Koetzsch S, Keserue HA, Egli T (2010) Rapid and quantitative detection of *Legionella pneumophila* applying ommunomagnetic separation and flow cytometry. *Cytometry Part A* 77A: 264–274

Fulwyler MJ (1965) Electronic separation of biological cells by volume. *Science* 150: 910–911, https://doi.org/10.1126/science.150.3698.910

Fulwyler MJ (1968) Particle separator. USA Patent US3380584 A, https://www.google.com/patents/US3380584

Gollasch S, David M (2015) Ballast water sampling and sample analysis for compliance control. In: Global Maritime Transport and Ballast Water Management, Issues and Solutions. Invading Nature - Springer Series in Invasion Ecology: Springer, Vol. 8, pp 171–223, https://doi.org/10.1007/978-94-017-9367-4_8

Gollasch S, Lenz J, Dammer M, Andres HG (2000) Survival of tropical ballast water organisms during a cruise from the Indian Ocean to the North Sea. *Journal of Plankton Research* 22: 923–937, https://doi.org/10.1093/plankt/22.5.923

Griffiths RW, Schloesser DW, Leach JH, Kovalak WP (1991) Distribution and dispersal of the Zebra mussel (*Dreissena polymorpha*) in the Great Lakes region. *Canadian Journal of Fisheries and Aquatic Sciences* 48: 1381–1388, https://doi.org/10.1139/f91-165

Grigorszky I, Kiss KT, Beres V, Bacsi I, M-Hamvas M, Mathe C, Vasas G, Padisak J, Borics G, Gligora M, Borbely G (2006) The effects of temperature, nitrogen, and phosphorus on the encystment of *Peridinium cinctum*, Stein (Dinophyta). *Hydrobiologia* 563: 527–535, https://doi.org/10.1007/s10750-006-0037-z

Guindulain T, Comas J, Vivesrego J (1997) Use of nucleic acid dyes SYTO-13, TOTO-1, and YOYO-1 in the study of *Escherichia coli* and marine prokaryotic populations by flow cytometry. *Applied and Environmental Microbiology* 63: 4608–4611

Hammes F, Berney M, Wang YY, Vital M, Koster O, Egli T (2008) Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Research* 42: 269–277, https://doi.org/10.1016/j.watres.2007.07.009

Hedhammar M, Stenvall M, Lonneborg R, Nord O, Sjolin O, Brismar H, Uhlen M, Ottosson J, Hober S (2005) A novel flow cytometry-based method for analysis of expression levels in *Escherichia coli*, giving information about precipitated and soluble protein. *Journal of Biotechnology* 119: 133–146, https://doi.org/10.1016/j.jbiotec.2005.03.024

Herault O, Domenech J, Georget MT, Clement N, Colombat P, Binet C (2002) All-trans retinoic acid prevents apoptosis of human marrow CD34(+) cells deprived of haematopoietic growth factors. *British Journal of Haematology* 118: 289–295, https://doi.org/10.1046/j.1365-2141.2002.03573.x

Herrero M, Quiros C, Garcia LA, Diaz M (2006) Use of flow Cytometry to follow the physiological states of microorganisms in cider fermentation processes. *Applied and Environmental Microbiology* 72: 6725–6733, https://doi.org/10.1128/AEM.01183-06

Hess-Erga OK, Blomvagnes-Bakke B, Vadstein O (2010) Recolonization by heterotrophic bacteria after UV irradiation or ozonation of seawater; a simulation of ballast water treatment. *Water Research* 44: 5439–5449, https://doi.org/10.1016/j.watres.2010.06.059

Hoddle MS (2004) Restoring balance: Using exotic species to control invasive exotic species. *Conservation Biology* 18: 38–49, https://doi.org/10.1111/j.1523-1739.2004.00249.x

Hoefel D, Grooby WL, Monis PT, Andrews S, Saint CP (2003) A comparative study of carboxyfluorescein diacetate and carboxyfluorescein diacetate succinimidyl ester as indicators of bacterial activity. *Journal of Microbiological Methods* 52: 379–388, https://doi.org/10.1016/S0167-7012(02)00207-5

International Maritime Organization (2004) International Convention for the Control and Management of Ships' Ballast Water and Sediments. International Maritime Organization, London, http://www.classnk.or.jp/HP/pdf/activities/statutory/ballastwater/convention_ballast.pdf

International Maritime Organization (2008a) Guidelines for Approval of Ballast Water Management Systems (G8). Resolution MEPC.174(58), http://www.classnk.or.jp/hp/pdf/activities/statutory/ballastwater/guideline_g8_rev.pdf

International Maritime Organization (2008b) Guidelines for ballast water sampling (G2). Resolution MEPC.173(58), http://www.classnk.or.jp/hp/pdf/activities/statutory/ballastwater/guideline_g2.pdf

International Maritime Organization (2008c) Procedures for approval of ballast water management systems that make use of active substances (G9). Resolution 169(57), http://www.classnk.or.jp/hp/pdf/activities/statutory/ballastwater/guideline_g9_rev.pdf

Irigoien X, Flynn KJ, Harris RP (2005) Phytoplankton blooms: a 'loophole' in microzooplankton grazing impact? *Journal of Plankton Research* 27: 313–321, https://doi.org/10.1093/plankt/fbi011

Joachimsthal EL, Ivanov V, Tay JH, Tay STL (2003) Flow cytometry and conventional enumeration of microorganisms in ships' ballast water and marine samples. *Marine Pollution Bulletin* 46: 308–313, https://doi.org/10.1016/S0025-326X(02)00401-0

Joachimsthal EL, Ivanov V, Tay STL, Tay JH (2004) Bacteriological examination of ballast water in Singapore Harbour by flow cytometry with FISH. *Marine Pollution Bulletin* 49: 334–343, https://doi.org/10.1016/j.marpolbul.2004.02.036

Johnson GD, Araujo G (1981) A simple method of reducing the fading of immunoflourescence during microscopy. *Journal of Immunological Methods* 43: 349–350, https://doi.org/10.1016/0022-1759(81)90183-6

Joux F, Lebaron P (2000) Use of fluorescent probes to assess physiological functions of bacteria at single-cell level. *Microbes and Infection* 2: 1523–1535, https://doi.org/10.1016/S1286-4579(00)01307-1

Joux F, Lebaron P, Troussellier M (1997) Succession of cellular states in a *Salmonella typhimurium* population during starvation in artificial seawater microcosms. *Fems Microbiology Ecology* 22: 65–76, https://doi.org/10.1111/j.1574-6941.1997.tb00357.x

Kaberdin VR, Montanchez I, Parada C, Orruno M, Arana I, Barcina I (2015) Unveiling the metabolic pathways associated with the adaptive reduction of cell size during *Vibrio harveyi* persistence in seawater microcosms. *Microbial Ecology* 70: 689–700, https://doi.org/10.1007/s00248-015-0614-7

Khandeparker L, Ani AC (2013) Association of Bacteria with Marine Invertebrates: Implications for Ballast Water Management. *Ecohealth* 10: 268–276, https://doi.org/10.1007/s10393-013-0857-z

Koch F, Kang Y, Villareal TA, Anderson DM, Gobler CJ (2014) A novel immunofluorescence flow cytometry technique detects the expansion of brown tides caused by *Aureoumbra lagunensis* to the Caribbean sea. *Applied and Environmental Microbiology* 80: 4947–4957, https://doi.org/10.1128/AEM.00888-14

Krebs SJ, Taylor RK (2011) Nutrient-dependent, rapid transition of *Vibrio cholerae* to coccoid morphology and expression of the toxin co-regulated pilus in this form. *Microbiology-Sgm* 157: 2942–2953, https://doi.org/10.1099/mic.0.048561-0

Larsen A, Castberg T, Sandaa RA, Brussaard CPD, Egge J, Heldal M, Paulino A, Thyrhaug R, Van Hannen EJ, Bratbak G (2001) Population dynamics and diversity of phytoplankton, bacteria

and viruses in a seawater enclosure. *Marine Ecology Progress Series* 221: 47–57, https://doi.org/10.3354/meps221047

Lavoie DM, Smith LD, Ruiz GM (1999) The potential for intracoastal transfer of non-indigenous species in the ballast water of ships. *Estuarine Coastal and Shelf Science* 48: 551–564, https://doi.org/10.1006/ecss.1999.0467

Lebaron P, Catala P, Parthuisot N (1998a) Effectiveness of SYTOX green stain for bacterial viability assessment. *Applied and Environmental Microbiology* 64: 2697–2700

Lebaron P, Parthuisot N, Catala P (1998b) Comparison of blue nucleic acid dyes for flow cytometric enumeration of bacteria in aquatic systems. *Applied and Environmental Microbiology* 64: 1725–1730

Lebaron P, Servais P, Agogue H, Courties C, Joux F (2001) Does the high nucleic acid content of individual bacterial cells allow us to discriminate between active cells and inactive cells in aquatic systems? *Applied and Environmental Microbiology* 67: 1775–1782, https://doi.org/10.1128/AEM.67.4.1775-1782.2001

Lee J, Choi EJ, Rhie K (2015) Validation of algal viability treated with total residual oxidant and organic matter by flow cytometry. *Marine Pollution Bulletin* 97: 95–104, https://doi.org/10.1016/j.marpolbul.2015.06.029

Lehtinen J, Nuutila J, Lilius EM (2004) Green fluorescent protein-propidium iodide (GFP-PI) based assay for flow cytometric measurement of bacterial viability. *Cytometry Part A* 60A: 165–172, https://doi.org/10.1002/cyto.a.20026

Lemarchand K, Parthuisot N, Catala P, Lebaron P (2001) Comparative assessment of epifluorescence microscopy, flow cytometry and solid-phase cytometry used in the enumeration of specific bacteria in water. *Aquatic Microbial Ecology* 25: 301–309, https://doi.org/10.3354/ame025301

Liu YM, Wang CA, Tyrrell G, Hrudey SE, Li XF (2009) Induction of *Escherichia coli* O157:H7 into the viable but non-culturable state by chloraminated water and river water, and subsequent resuscitation. *Environmental Microbiology Reports* 1: 155–161, https://doi.org/10.1111/j.1758-2229.2009.00024.x

López-Amorós R, Castel S, Comasriu J, Vivesrego J (1997) Assessment of *E. coli* and *Salmonella* viability and starvation by confocal laser microscopy and flow cytometry using rhodamine 123, DiBAC4(3), propidium iodide, and CTC. *Cytometry* 29: 298–305, https://doi.org/10.1002/(SICI)1097-0320(19971201)29:4<298::AID-CYTO6>3.0.CO;2-6

López-Amorós R, Comas J, Vivesrego J (1995) Flow cytometric assessment of *Escherichia coli* and *Salmonella thyhimurium* starvation - survival in sea water using rhodamine123, propidium iodide, and oxonol. *Applied and Environmental Microbiology* 61: 2521–2526

MacIntyre HL, Cullen JJ (2016) Classification of phytoplankton cells as live or dead using the vital stains fluorescein diacetate and 5-chloromethylfluorescein diacetate. *Journal of Phycology* 52: 572–589, https://doi.org/10.1111/jpy.12415

Mansour JD, Robson JA, Arndt CW, Schulte TH (1985) Detection of *Escherichia coli* in blood using flow-cytometry. *Cytometry* 6: 186–190, https://doi.org/10.1002/cyto.990060303

Marie D, Brussaard CPD, Thyrhaug R, Bratbak G, Vaulot D (1999) Enumeration of marine viruses in culture and natural samples by flow cytometry. *Applied and Environmental Microbiology* 65: 45–52

Marie D, Partensky F, Vaulot D, Brussaard C (2001) Enumeration of phytoplankton, bacteria, and viruses in marine samples. Current protocols in cytometry, Chapter 11, https://doi.org/10.1002/04711142956.cy1111s10

Marie D, Vaulot D, Partensky F (1996) Application of the novel nucleic acid dyes YOYO-1, YO-PRO-1, and PicoGreen for flow cytometric analysis of marine prokaryotes. *Applied and Environmental Microbiology* 62: 1649–1655

Martin JC, Swartzendruber DE (1980) Time - new parameter for kinetic measurements in flow cytometry. *Science* 207: 199–201, https://doi.org/10.1126/science.6153131

Martinez LF, Mahamud MM, Lavin AG, Bueno JL (2012) Evolution of phytoplankton cultures after ultraviolet light treatment. *Marine Pollution Bulletin* 64: 556–562, https://doi.org/10.1016/j.marpolbul.2011.12.021

Martinez LF, Mahamud MM, Lavin AG, Bueno JL (2013) The regrowth of phytoplankton cultures after UV disinfection. *Marine Pollution Bulletin* 67: 152–157, https://doi.org/10.1016/j.marpolbul.2012.11.019

McCarthy SA, Khambaty FM (1994) International dissemination of epidemic *Vibrio cholerae* by cargo ship ballast and other nonpotable waters. *Applied and Environmental Microbiology* 60: 2597–2601

Muller S, Harms H, Bley T (2010) Origin and analysis of microbial population heterogeneity in bioprocesses. *Current Opinion in Biotechnology* 21: 100–113, https://doi.org/10.1016/j.copbio.2010.01.002

Muller S, Nebe-Von-Caron G (2010) Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. *Fems Microbiology Reviews* 34: 554–587, https://doi.org/10.1111/j.1574-6976.2010.00214.x

Mundle S, Venugopal P, Shetty V, Ali A, Chopra H, Handa H, Rose S, Mativi BY, Gregory SA, Preisler HD, Raza A (1999) The relative extent and propensity of CD34(+) vs. CD34(-) cells to undergo apoptosis in myelodysplastic marrows. *International Journal of Hematology* 69: 152–159

Nelson TA, Haberlin K, Nelson AV, Ribarich H, Hotchkiss R, Van Alstyne KL, Buckingham L, Simunds DJ, Fredrickson K (2008) Ecological and physiological controls of species composition in green macroalgal blooms. *Ecology* 89: 1287–1298, https://doi.org/10.1890/07-0494.1

Novo D, Perlmutter NG, Hunt RH, Shapiro HM (1999) Accurate flow cytometric membrane potential measurement in bacteria using diethyloxacarbocyanine and a ratiometric technique. *Cytometry* 35: 55–63, https://doi.org/10.1002/(SICI)1097-0320(19990101)35:1<55::AID-CYTO8>3.0.CO;2-2

Novo DJ, Perlmutter NG, Hunt RH, Shapiro HM (2000) Multiparameter flow cytometric analysis of antibiotic effects on membrane potential, membrane permeability, and bacterial counts of *Staphylococcus aureus* and *Micrococcus luteus*. *Antimicrobial Agents and Chemotherapy* 44: 827–834, https://doi.org/10.1128/AAC.44.4.827-834.2000

Occhipinti-Ambrogi A, Savini D (2003) Biological invasions as a component of global change in stressed marine ecosystems. *Marine Pollution Bulletin* 46: 542–551, https://doi.org/10.1016/S0025-326X(02)00363-6

Olsen RO, Hess-Erga O-K, Larsen A, Thuestad G, Tobiesen A, Hoell IA (2015) Flow cytometric applicability to evaluate UV inactivation of phytoplankton in marine water samples. *Marine Pollution Bulletin* 96: 279–285, https://doi.org/10.1016/j.marpolbul.2015.05.012

Olsen RO, Hess-Erga O-K, Larsen A, Hoffmann F, Thuestad G, Hoell IA (2016a) Dual staining with CFDA-AM and SYTOX Blue in flow cytometry analysis of UV-irradiated *Tetraselmis suecica* to evaluate vitality. *Aquatic Biology* 25: 39–52, https://doi.org/10.3354/ab00662

Olsen RO, Hoffmann F, Hess-Erga O-K, Larsen A, Thuestad G, Hoell IA (2016b) Ultraviolet radiation as a ballast water treatment strategy: Inactivation of phytoplankton measured with flow cytometry. *Marine Pollution Bulletin* 103: 270–5, https://doi.org/10.1016/j.marpolbul.2015.12.008

Ou H, Gao N, Deng Y, Qiao J, Wang H (2012) Immediate and long-term impacts of UV-C irradiation on photosynthetic capacity, survival and microcystin-LR release risk of *Microcystis aeruginosa*. *Water Research* 46: 1241–1250, https://doi.org/10.1016/j.watres.2011.12.025

Parthuisot N, Catala P, Lemarchand K, Baudart J, Lebaron P (2000) Evaluation of ChemChrome V6 for bacterial viability assessment in waters. *Journal of Applied Microbiology* 89: 370–380, https://doi.org/10.1046/j.1365-2672.2000.01126.x

Peperzak L, Brussaard CPD (2011) Flow cytometric applicability of fluorescent vitality probes on phytoplankton. *Journal of Phycology* 47: 692–702, https://doi.org/10.1111/j.1529-8817.2011.00991.x

Peperzak L, Vrieling EG, Sandee B, Rutten T (2000) Immuno flow cytometry in marine phytoplankton research. *Scientia Marina* 64: 165–181, https://doi.org/10.3989/scimar.2000.64n2165

Pore RS (1994) Antibiotic suseptibility testing by flow-cytometry. *Journal of Antimicrobial Chemotherapy* 34: 613–627, https://doi.org/10.1093/jac/34.5.613

Porter J, Diaper J, Edwards C, Pickup R (1995a) Direct measurements of natural planctonic bacterial community viability by flow-cytometry. *Applied and Environmental Microbiology* 61: 2783–2786

Porter J, Edwards C, Pickup RW (1995b) Rapid assessment of physiological status in *Escherichia coli* using flourescent probes. *Journal of Applied Bacteriology* 79: 399–408, https://doi.org/10.1111/j.1365-2672.1995.tb03154.x

Quinn J, Fisher PW, Capocasale RJ, Achuthanandam R, Kam M, Bugelski PJ, Hrebien L (2007) A statistical pattern recognition approach for determining cellular viability and lineage phenotype in cultured cells and murine bone marrow. *Cytometry Part A* 71A: 612–624, https://doi.org/10.1002/cyto.a.20416

Quiros C, Herrero M, Garcia LA, Diaz M (2007) Application of flow cytometry to segregated kinetic modeling based on the physiological states of microorganisms. *Applied and Environmental Microbiology* 73: 3993–4000, https://doi.org/10.1128/AEM.00171-07

Racila E, Euhus D, Weiss AJ, Rao C, McConnell J, Terstappen L, Uhr JW (1998) Detection and characterization of carcinoma cells in the blood. *Proceedings of the National Academy of Sciences of the United States of America* 95: 4589–4594, https://doi.org/10.1073/pnas.95.8.4589

Rappe MS, Giovannoni SJ (2003) The uncultured microbial majority. *Annual Review of Microbiology* 57: 369–394, https://doi.org/10.1146/annurev.micro.57.030502.090759

Reis A, da Silva TL, Kent CA, Kosseva M, Roseiro JC, Hewitt CJ (2005) Monitoring population dynamics of the thermophilic *Bacillus licheniformis* CCMI 1034 in batch and continuous cultures using multi-parameter flow cytometry. *Journal of Biotechnology* 115: 199–210, https://doi.org/10.1016/j.jbiotec.2004.08.005

Rezaeinejad S, Ivanov V (2011) Heterogeneity of *Escherichia coli* population by respiratory activity and membrane potential of cells during growth and long-term starvation. *Microbiological Research* 166: 129–135, https://doi.org/10.1016/j.micres.2010.01.007

Roth BL, Poot M, Yue ST, Millard PJ (1997) Bacterial viability and antibiotic susceptibility testing with SYTOX Green nucleic acid stain. *Applied and Environmental Microbiology* 63: 2421–2431

Ruiz GM, Rawlings TK, Dobbs FC, Drake LA, Mullady T, Huq A, Colwell RR (2000) Global spread of microorganisms by ships - Ballast water discharged from vessels harbours a cocktail of potential pathogens. *Nature* 408: 49–50, https://doi.org/10.1038/35040695

Schenk M, Raffellini S, Guerrero S, Blanco GA, Alzamora SM (2011) Inactivation of *Escherichia coli*, *Listeria innocua* and *Saccharomyces cerevisiae* by UV-C light: Study of cell injury by flow cytometry. *Lwt-Food Science and Technology* 44: 191–198, https://doi.org/10.1016/j.lwt.2010.05.012

Seoane S, Puente A, Guinda X, Juanes JA (2012) Bloom forming and toxic phytoplankton in transitional and coastal waters of Cantabria region coast (Southeastern Bay of Biscay, Spain). *Marine Pollution Bulletin* 64: 2860–2866, https://doi.org/10.1016/j.marpolbul.2012.08.023

Shi L, Guenther S, Huebschmann T, Wick LY, Harms H, Mueller S (2007) Limits of propidium iodide as a cell viability indicator for environmental bacteria. *Cytometry Part A* 71A: 592–598, https://doi.org/10.1002/cyto.a.20402

Simon N, Brenner J, Edvardsen B, Medlin LK (1997) The identification of *Chrysochromulina* and *Prymnesium* species (Haptophyta, Prymnesiophyceae) using fluorescent or chemiluminescent oligonucleotide probes: a means for improving studies on toxic algae. *European Journal of Phycology* 32: 393–401, https://doi.org/10.1080/09670269710001737339

Song Y, Wang J, Yang J, Wu Y, Li N, Gong N, Pan X, Sun Y, Li D (2012) Algae detection and ship's ballast water analysis by a microfluidic lab-on-chip device. *Instrumentation Science & Technology* 40: 305–315, https://doi.org/10.1080/10739149.2012.673199

Steen HB (2004) Flow cytometer for measurement of the light scattering of viral and other submicroscopic particles. *Cytometry Part A* 57A: 94–99, https://doi.org/10.1002/cyto.a.10115

Stehouwer PP, Liebich V, Peperzak L (2013) Flow cytometry, microscopy, and DNA analysis as complementary phytoplankton screening methods in ballast water treatment studies. *Journal of Applied Phycology* 25: 1047–1053, https://doi.org/10.1007/s10811-012-9944-8

Steinberg MK, First MR, Lemieux EJ, Drake LA, Nelson BN, Kulis DM, Anderson DM, Welschmeyer NA, Herring PR (2012) Comparison of techniques used to count single-celled viable phytoplankton. *Journal of Applied Phycology* 24: 751–758, https://doi.org/10.1007/s10811-011-9694-z

Steinberg MK, Lemieux EJ, Drake LA (2011) Determining the viability of marine protists using a combination of vital, fluorescent stains. *Marine Biology* 158: 1431–1437, https://doi.org/10.1007/s00227-011-1640-8

Strayer DL (2010) Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. *Freshwater Biology* 55: 152–174, https://doi.org/10.1111/j.1365-2427.2009.02380.x

Tang KW, Dziallas C, Grossart HP (2011) Zooplankton and aggregates as refuge for aquatic bacteria: protection from UV, heat and ozone stresses used for water treatment. *Environmental Microbiology* 13: 378–390, https://doi.org/10.1111/j.1462-2920.2010.02335.x

Tang YZ, Dobbs FC (2007) Green autofluorescence in dinoflagellates, diatoms, and other microalgae and its implications for vital staining and morphological studies. *Applied and Environmental Microbiology* 73: 2306–2313, https://doi.org/10.1128/AEM.01741-06

Tango PJ, Magnien R, Butler W, Luckett C, Luckenbach M, Lacouture R, Poukish C (2005) Impacts and potential effects due to *Prorocentrum minimum* blooms in Chesapeake Bay. *Harmful Algae* 4: 525–531, https://doi.org/10.1016/j.hal.2004.08.014

Tormos AM, Talens-Visconti R, Bonora-Centelles A, Perez S, Sastre J (2015) Oxidative stress triggers cytokinesis failure in hepatocytes upon isolation. *Free Radical Research* 49: 927–934, https://doi.org/10.3109/10715762.2015.1016019

Tyson GW, Banfield JF (2005) Cultivating the uncultivated: a community genomics perspective. *Trends in Microbiology* 13: 411–415, https://doi.org/10.1016/j.tim.2005.07.003

United States Coast Guard (2012) Standards for living organisms in ships' ballast water discharged in US waters. Department of Homeland Security, Federal Register, No. 57

Veldhuis MJW, Brussaard CPD, Noordeloos AAM (2005) Living in a *Phaeocystis* colony: a way to be a successful algal species. *Harmful Algae* 4: 841–858, https://doi.org/10.1016/j.hal.2004.12.013

Veldhuis MJW, Fuhr F, Boon JP, Ten Hallers-Tjabbers C (2006) Treatment of ballast water; How to test a system with a modular concept? *Environmental Technology* 27: 909–921, https://doi.org/10.1080/09593332708618701

Veldhuis MJW, Kraay GW (2000) Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. *Scientia Marina* 64: 121–134, https://doi.org/10.3989/scimar.2000.64n2121

Vives-Rego J, Lebaron P, Nebe-Von Caron G (2000) Current and future applications of flow cytometry in aquatic microbiology. *Fems Microbiology Reviews* 24: 429–448, https://doi.org/10.1111/j.1574-6976.2000.tb00549.x

Wallner G, Fuchs B, Spring S, Beisker W, Amann R (1997) Flow sorting of microorganisms for molecular analysis. *Applied and Environmental Microbiology* 63: 4223–4231

Wang Y, Claeys L, Van Der Ha D, Verstraete W, Boon N (2010) Effects of chemically and electrochemically dosed chlorine on *Escherichia coli* and *Legionella beliardensis* assessed by flow cytometry. *Applied Microbiology and Biotechnology* 87: 331–341, https://doi.org/10.1007/s00253-010-2526-2

Williams RJ, Griffiths FB, Vanderwal EJ, Kelly J (1988) Cargo vessel ballast water as a vector for the transport of non-indigenous marine species. *Estuarine Coastal and Shelf Science* 26: 409–420, https://doi.org/10.1016/0272-7714(88)90021-2

Williams SC, Hong Y, Danavall DCA, Howard-Jones MH, Gibson D, Frischer ME, Verity PG (1998) Distinguishing between living and nonliving bacteria: Evaluation of the vital stain propidium iodide and its combined use with molecular probes in aquatic samples. *Journal of Microbiological Methods* 32: 225–236, https://doi.org/10.1016/S0167-7012(98)00014-1

Wommack KE, Colwell RR (2000) Virioplankton: Viruses in aquatic ecosystems. *Microbiology and Molecular Biology Reviews* 64: 69, https://doi.org/10.1128/MMBR.64.1.69-114.2000

Wright DA, Gensemer RW, Mitchelmore CL, Stubblefield WA, Van Genderen E, Dawson R, Orano-Dawson CE, Bearr JS, Mueller RA, Cooper WJ (2010) Shipboard trials of an ozone-based ballast water treatment system. *Marine Pollution Bulletin* 60: 1571–1583, https://doi.org/10.1016/j.marpolbul.2010.04.010

Yamaguchi N, Nasu M (1997) Flow cytometric analysis of bacterial respiratory and enzymatic activity in the natural aquatic environment. *Journal of Applied Microbiology* 83: 43–52, https://doi.org/10.1046/j.1365-2672.1997.00165.x

Zaiko A, Martinez JL, Ardura A, Clusa L, Borrell YJ, Samuiloviene A, Roca A, Garcia-Vazquez E (2015) Detecting nuisance species using NGST: Methodology shortcomings and possible application in ballast water monitoring. *Marine Environmental Research* 112: 64–72, https://doi.org/10.1016/j.marenvres.2015.07.002

Zetsche EM, El MallahI A, Dubois F, Yourassowsky C, Kromkamp JC, Meysman FJR (2014) Imaging-in-Flow: Digital holographic microscopy as a novel tool to detect and classify nanoplanktonic organisms. *Limnology and Oceanography-Methods* 12: 757–775, https://doi.org/10.4319/lom.2014.12.757

Zhou Q, Chen W, Zhang HY, Peng L, Liu LM, Han ZG, Wan N, Li L, Song LR (2012) A flow cytometer based protocol for quantitative analysis of bloom-forming cyanobacteria (*Microcystis*) in lake sediments. *Journal of Environmental Sciences* 24: 1709–1716, https://doi.org/10.1016/S1001-0742(11)60993-5

Zubkov MV, Sleigh MA, Burkill PH, Leakey RJG (2000) Picoplankton community structure on the Atlantic Meridional Transect: a comparison between seasons. *Progress in Oceanography* 45: 369–386, https://doi.org/10.1016/S0079-6611(00)00008-2

Zuliani T, Duval R, Jayat C, Schnebert S, Andre P, Dumas M, Ratinaud MH (2003) Sensitive and reliable JC-1 and TOTO-3 double staining to assess mitochondrial transmembrane potential and plasma membrane integrity: Interest for cell death investigations. *Cytometry Part A* 54A: 100–108, https://doi.org/10.1002/cyto.a.10059