6th IMarEST Ballast Water Technology Conference, 12-13 January 2017, London, UK
https://doi.org/10.24868/bwtc6.2017.005

CMOS-based Image Cytometry for Detection of Phytoplankton in Ballast Water

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ABSTRACT

An image cytometer (CYT) for the analysis of phytoplankton in fresh and marine water environments is introduced. A linear quantification of the number of cells over several orders of magnitude of concentrations was observed using cultures of *Tetraselmis* and *Nannochloropsis* measured by autofluorescence of the chlorophyll in a laboratory environment. The functionality of the system outside the laboratory was analysed by phytoplankton quantification of samples taken from marine water environment (Dutch Wadden Sea, The Netherlands) and fresh water environment (Lake Ijssel, The Netherlands). The CYT was also employed to study the effects of two ballast water treatment systems (BWTS), based on chlorine electrolysis and UV sterilisation by determining the vitality of the phytoplankton. In order to ensure the detection limit, a large volume (1l) of samples was collected and concentrated to 3 ml using Celltrap™ filters. The results were compared to benchmarked flow cytometer and PAM Fluorometry at Marine Eco-Analytics (MEA-NL). The image cytometer reached a 10 cells/ml limit of detection (LoD) with an accuracy between 0.7 and 0.5 log, and a correlation of 88.29% in quantification and 96.21% in vitality, when compared to benchmarked monitoring techniques.

Keywords: Ballast Water, image Cytometer, Cytometry, Phytoplankton, Compliance Monitoring, Maritime

1. Introduction

Globalisation has become a primary driver of one of the most prevalent forms of environmental degradation, i.e. marine and aquatic invasive species. As trade continues to flourish, the prevention of bio-invasion has grown to become an immense challenge (Kannan, 2015; Bax et al., 2003). Microorganisms carried in ballast water can easily spread into a new habitat, which can generate a potentially devastating impact threatening ecosystems and economies (Moreno-Andrés et al., 2016; Brussaard et al., 2016)

Ballast water from ships is considered to be the most important vector in dispersing invasive species throughout the world (Seebens et al., 2013) since up to 150000 metric tons of fresh/marine water can be pumped in or out in only one ballast/de-ballast operation (Dunstan & Bax, 2008). In response to the threats from continued introductions of aquatic invasive species, the United Nations – International Maritime Organization (IMO) has adopted the International Convention for the Control and Management of Ship's Ballast Water and Sediments (IMO, 2004). Its compliance standards require testing for phytoplankton,

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zooplankton, toxicogenic *Vibrio cholera*, *Escherichia coli* and intestinal Enterococci upon discharge of the ballast water in the harbour.

Ballast water treatment systems (BWTS) disinfect ballast water in order to reduce the number of viable organisms to low risk levels for the ecosystem and human health; BWTS are either on board or port-based systems which are able to clean all ballast water before it is released into the harbour (Delacroix et al., 2013; Rivas-Hermann et al., 2015; Steuhouwer et al., 2015; Fernandes et al., 2015)

The main on board and port-based treatment technologies used today are ultraviolet (UV) sterilisation (Liltved et al., 2011; Stehouwer et al., 2015) and chlorine electrolysis (Maranda et al., 2013).

A variety of analytical methods have been used to identify changes in populations of marine organisms ranging from large to small scale such as remote spectrometry from satellites and airplanes, in situ spectrometry, (laser)-induced fluorescence, microscopy and flow cytometry (Golden et al., 2012). Other biosensing systems for on-board analysis of ballast water and quantification of the living organisms have been developed and reported. For example, sensors based on molecular and genetic engineering methods (Sanchez-Ferandin et al., 2013; Wollschläger et al., 2014) or relying on the photosynthetic properties universally present in phytoplankton (Meneely et al., 2013) have been developed.

In a previous paper (Pérez et al., 2014), the authors introduced an optical reader based on angular spatial frequency processing and incorporating consumer electronics complementary-metal-oxide semiconductor (CMOS) image sensor array for the detection of waterborne microorganisms. By leveraging this optical reader, a portable image cytometry system for the rapid detection and quantification of phytoplankton is hereby presented.

2. SixSenso Image cytometer (CYT)

The CYT is an opto-electronic reader comprised of a CMOS image sensor array as detector and a band-limited light emitting diode (LED) source centred at an excitation wavelength of 466 nm. The excitation beam illuminates the sample volume which is contained in a disposable Poly (methyl methacrylate) (PMMA) cuvette with a capacity of up to 3 ml. An interference optical multiple bandpass filter allows the simultaneous detection of two fluorescent channels centred at 512 nm +/- 25 nm and 630 nm +/- 36 nm, respectively. Phytoplankton species exhibit auto-fluorescence above 610 nm (red fluorescence), because of their chlorophyll *a* protein complex. This autofluorescence can be used for both quantification and measurement of vitality by size discrimination.

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Figure 1. CYT instrument and operating system displayed on a computer monitor. The system is composed of: a light emitting diode (LED) light source; an optical lens to collimate the excitation beam; an optical filter to block the excitation signal after the sample and to select two fluorescent channels centred at 512 nm and 630 nm; a parabolic mirror which acts as an optical transforming element onto the CMOS image sensor array, that captures the light sample to process.

Testing procedure

Unialgal cultures of *Tetraselmis* and *Nannochloropsis* were measured to evaluate the linearity, repeatability, reproducibility, dynamic range, and LoD of the CYT reader. These were determined by means of serial dilutions of two phytoplankton species: *Tetraselmis* (14µm cell diameter) and *Nannochloropsis* (5µm cell diameter).

Next, the system functionality was tested by quantifying phytoplankton species in samples from fresh and marine waters. CYT capabilities were further examined using water samples collected from full scale BWTS subjected to UV sterilization or chlorine electrolysis. Phytoplankton numbers were quantified and the vitality of the cells was measured before and after the treatment.

For comparison, field samples were analysed also with a standard flow cytometer (Beckman Coulter EPIC-XL-MCL) (Veldhuis & Kraay, 2000). The vitality of the phytoplankton was measured as the efficiency of the photosynthetic system of the phytoplankton (Schreiber, 1998). For this analysis the WALZ-Water-PAM was used to measure bulk fluorescence properties of the phytoplankton (Veldhuis et al., 2006).

Sample collection and preparation

Controlled cultures of *Tetraselmis* and *Nannochloropsis* were performed in a laboratory environment (ICFO-The Institute of Photonic Sciences, Barcelona). Both were obtained from concentrated stock, the former of the *Tetraselmis chuii* species and the latter of the *Nannochloropsis oculata* species. Both were purchased from Acuinuga (A Coruña, Spain).

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Dilutions of the samples (1/10 v/v) were made with marine water medium filtered using a 0.2-µm hollow membrane CellTrap™ filter.

Samples of both marine and fresh water were measured for reader validation in an on board ballast water treatment environment. Both marine and fresh water (aquatic) samples were subject to BWTS chlorine electrolysis, and only aquatic samples were exposed to BWTS UV sterilisation.

These measurements were performed on board the Marine Eco Analytics Innovator Test Barge in the Netherlands. Aquatic samples were collected from Lake Ijssel, and marine water samples from brackish water off the coast of Den Oever, in the Dutch Wadden Sea. One of the aquatic samples was concentrated from its original volume of 400 ml down to 3 ml using the CellTrap™ filtering unit. In this case, the CYT intensity recorded by the reader was corrected using the volume eluted from the filter (3 mL), the original sampled volume (400 ml), and the recovery rate (RR). The RR of the CellTrap™ is 0.98 as reported by the manufacturer.

In the case of the UV BWTS, the treatment included two different steps. The first consisted in exposing the water sample to UV light followed by a 24 hour holding period; the second one included an additional UV exposure step after the 24 hour holding period.

3. Calculation

The quantification accounting for size of organisms in cells per ml is achieved by transforming the total fluorescence angular distribution intensity into a probability size density (PSD) curve to outweigh the cubic-law size-dependent fluorescence intensity from different phytoplankton species present in the sample. Then, the total phytoplankton concentration is estimated using a 4 parameter logistic (4PL) regression (O'Connel et al, 1993), as Equation 1.

$$\boldsymbol{Concentration}\left[\frac{\boldsymbol{cells}}{\boldsymbol{ml}}\right] = \boldsymbol{C}\left(\frac{\boldsymbol{A-D}}{\boldsymbol{I_{CYT}-D}}\right)^{\boldsymbol{1/B}} \qquad \textbf{Equation 1}$$

Where (A,B,C,D) refer to the four parameters of the regression, with values (2.17×10^{-8}, 1.31×10^{3}, 3.33×10^{7}, 4×10^{-2}) respectively, and CYT refers to the fluorescence intensity measured.

For the vitality index measurement, the light source in the system was controlled by a pulse width modulation (PWM) signal. For the maximum fluorescence (F_m) the sample was excited by a high intensity PWM; for the minimum fluorescence (F_0), the sample was excited by a low intensity PWM signal. The vitality index was calculated as follows, Equation 2.

$$\boldsymbol{Vitality} = \mathbf{1} - \frac{\boldsymbol{F_0}}{\boldsymbol{F_m}} \qquad \textbf{Equation 2}$$

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4. Results and discussion

Detection and quantification of Tetraselmis and Nannochloropsis

The initial tests were performed in a laboratory environment where two independent series of both *Tetraselmis* and *Nannochloropsis* cultures were measured over five orders of magnitude in 1/10 (v/v) dilutions of the cell number.

Figure 2 compiles the results for the *Tetraselmis* and *Nannochloropsis* measurements. Figure 2a displays the results for *Tetraselmis*, of both series and all repetitions. At the highest concentration measured, the CYT reports a concentration of 10^4 cells/ml; the lowest concentration detected was 18 cells/ml. Figure 2b displays the *Nannochloropsis* results for both series and all repetitions. At the highest concentration measured, the reader reports a concentration of $3 \cdot 10^3$ cells/ml; the lowest concentration detected was 6 cells/ml.

Figure 2. Results for the *Tetraselmis* and *Nannochloropsis* samples. (a) Tetraselmis results, with concentrations measured by the CYT from 10^4 cells/ml at a dilution from the stock of 10^{-2}, to <10 cells/ml at a dilution from the stock of 10^{-6}. (b) *Nannochloropsis* results, with concentrations measured by the CYT from $3 \cdot 10^3$ cells/ml at a dilution from the stock of 10^{-3}, to <10 cells/ml at a dilution from the stock of 10^{-7}.

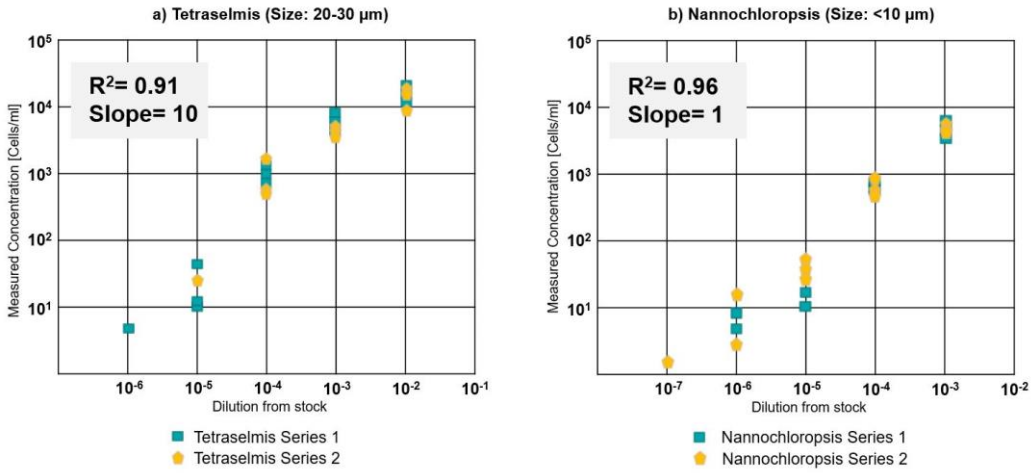

Table 1 shows the intra-assay and inter-assay logarithmic deviations for both species at all concentrations measured. On average the platform exhibits an intra-assay deviation of 0.32 and an inter-assay deviation of 0.1; this translates into accuracy between 0.7 and 0.5 log. The initial phytoplankton stock samples' concentration was measured using a microscope; counts in the field-of-view where transformed into total concentration per ml. The *Tetraselmis* and *Nannochloropsis* stock solutions showed a concentration of 10^6 cells/ml and $7 \cdot 10^6$ cells/ml, respectively.

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Table 1. Intra-assay and inter-assay deviation for both species at all concentrations measured.

	Dilution from stock	Intra-Assay Deviation		Inter-Assay Deviation
		Series 1	Series 2	
Nannochloropsis	10^{-3}	0.08	0.01	0.06
	10^{-4}	0.02	0.07	0.05
	10^{-5}	0.18	0.12	0.20
	10^{-6}	0.11	0.51	0.41
	10^{-7}	0	0.59	0.47
Tetraselmis	10^{-2}	0.06	0.08	0.07
	10^{-3}	0.07	0.04	0.08
	10^{-4}	0.08	0.16	0.14
	10^{-5}	0.26	1.11	1.32
	10^{-6}	1.91	0.18	1.49

To evaluate the LoD, a total of 10 independent samples per organisms were measured at the lowest detected concentrations; 18 cells/ml for Tetraselmis and 6 cells/ml for *Nannochloropsis*. The *Tetraselmis* samples had an inter-assay deviation of 0.20; the *Nannochloropsis* samples had an inter-assay deviation of 0.38. Deviations are taken as the standard deviation of the base 10 logarithm of the measurements. Our results prove a 10 cells/ml limit of detection (LoD) with accuracy between 0.7 and 0.5 log, and a correlation of 88.29% in quantification and 96.21% in vitality, compared to gold standard.

Marine water and fresh water (aquatic species) analysis, and BWTS validation

Samples collected from the field consist of a larger variety of phytoplankton differing in size (2 - > 50 micron) and chlorophyll content. These samples were therefore analysed by the reader accounting for size variations. Both marine and aquatic species were analysed. But only aquatic species were compared to standard measurements techniques (flow cytometer and PAM fluorometer) due to equipment availability.

Figure 3 displays the marine water samples measured before and after the disinfection step using a chlorine electrolysis BWTS. Samples 1 and 3, the decrease in cell numbers was one order of magnitude with respect to untreated samples. In sample 2, the BWTS has a lower impact: the reduction was only 50% numerical reduction below the size threshold and even smaller above the 10 µm size range.

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Figure 3. Marine water samples measured before and after chlorine electrolysis BWTS. The phytoplankton population was quantified in the two windows of interest; above and below the 10 µm threshold. The effect of the electrolysis by chlorine, reduces the phytoplankton population in both regions. This can be specially noted in samples 1 and 3, were the decrease in concentration is of one order of magnitude. In sample 2, the system has a lower impact, were it reduced the population in half an order of magnitude below the size threshold, while remain similar above the size threshold.

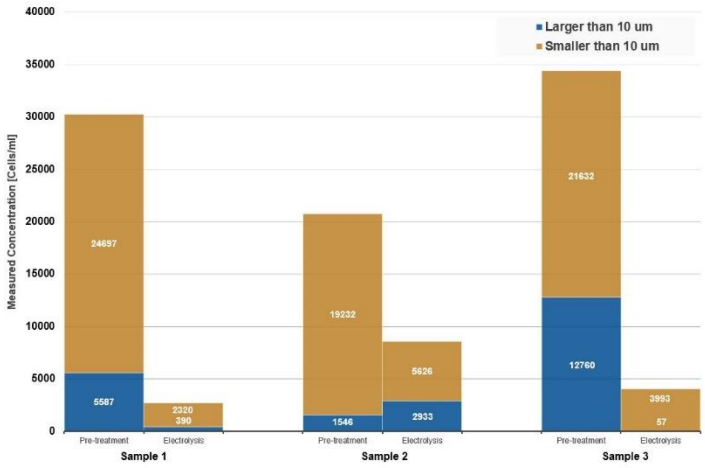

Figure 4 displays the concentration results for aquatic samples before and after three different treatment protocols of the BWTS (chlorine electrolysis, UV sterilization with 1-day holding, and UV disinfection with one day holding and a second UV exposure after holding). Four samples are assessed in this study; sample 1 underwent no treatment, and samples 2 through 4 was submitted to each of the three different BWTS protocols. It is of relevant importance to ballast water application to monitor the phytoplankton concentration at significant times because the biological evolution of the samples is time-dependent.

Sample 2 in Figure 4 was treated with chlorine electrolysis, the results show a decrease in cell numbers of at least one order of magnitude, similar to the effects of BWTS in marine water samples (Figure 4). Samples 3 and 4 show the results after UV sterilization and 1-day holding (sample 3), and second UV exposure after holding (sample 4). Phytoplankton concentration was reduced in both treatments and for both size regions. The data obtained by the CYT reader was corroborated by flow cytometry and PAM fluorometer results.

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Figure 4. Aquatic samples were tested before and after three different protocols of BWTS (chlorine electrolysis, UV sterilization with 1 day holding, and UV sterilisation with one day holding and a second UV exposure after holding). Phytoplankton population was measured for the two windows of interest; above and below the 10 µm size threshold. CYT samples were measured against a gold standard reference flow cytometer. The image cytometer has correlation factors of 94.89% and 81.70% above and below the 10 µm size threshold, respectively.

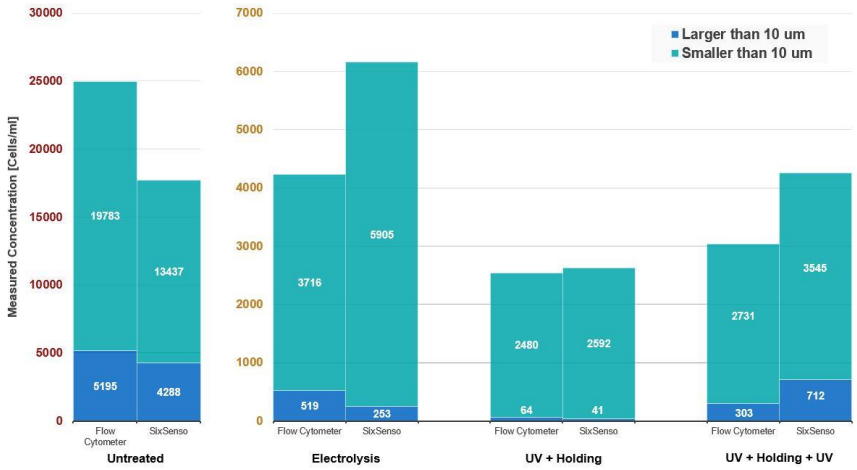

Figure 5. Fresh water (aquatic) samples were tested for vitality index before and after three different BWTS protocols (chlorine electrolysis, UV sterilisation with 1 day holding, and UV sterilisation with one day holding and a second UV exposure after holding). The samples were also measured with PAM fluorometer, for reference. The correlation with the PAM is 92.43%. The CYT reader measured the vitality index both below and above the 10 µm size threshold whereas the PAM data could only be provided above the 10 µm size threshold. Therefore, only the vitality index data above the 10 µm size threshold is shown.

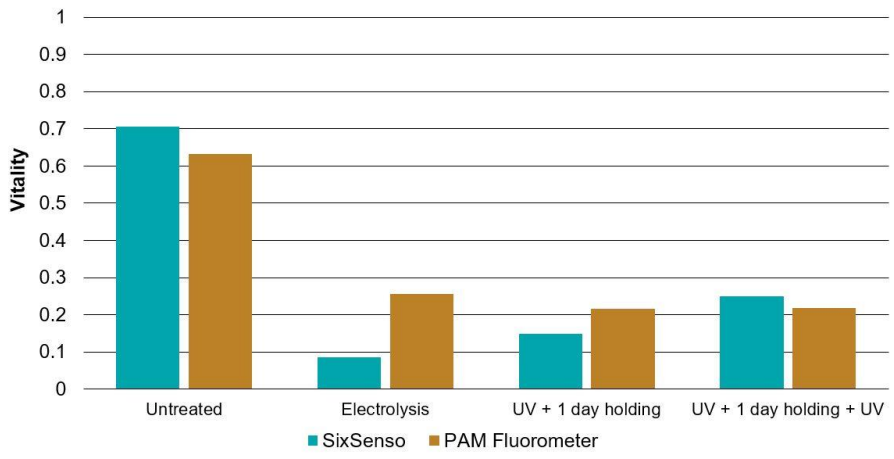

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Figure 5 summarises the vitality index measurements of non-treated and treated samples. The vitality index of the sample after treatment was 0.01 below the size threshold and 0.08 above it. For comparison, the latter was measured at 0.25 using the PAM fluorometer.

In terms of quantification, compared to the flow cytometer, the CYT reader has correlation factors of 94.89% and 81.70% above and below the 10 µm size threshold, respectively. In terms of vitality, compared to the PAM fluorometer, the reader has correlations factors of 92.43% and 100% above and below size threshold, respectively.

5. Conclusion

The results and their reproducibility demonstrated the high level of performance of the proposed a new image cytometer (CYT) for the quantification of phytoplankton in fresh and marine water, both inside and outside a laboratory environment.

In the present study, the efficacy of BWTS was also quantified. To this end, accurate phytoplankton vitality measurements were achieved by using PAM fluorometer techniques (the gold standard for vitality index in phytoplankton).

The quantification accounting for size of organisms in cells per ml is achieved by transforming the total fluorescence angular distribution intensity into a probability size density (PSD) curve to out-weight the cubic-law size dependent fluorescence intensity from different phytoplankton present in the sample. Then, the total phytoplankton concentration is estimated using a 4 parameter logistic (4PL) regression.

Future work will include the quantification of waterborne bacteria; resulting in a complete analysis of the sample.

Acknowledgements

This work was funded by the European Union's Horizon 2020 research and innovation program under grant agreement no. 642356 (CYTOWATER project), by Fundació Privada Cellex, by the Spanish MINECO (Severo Ochoa grant SEV-2015-0522), by the European Regional Development Fund (FEDER) through grant TEC2013-46168-R, by AGAUR 2014 SGR 1623. Funded by the Spanish Ministry of Economy and Competitiveness (TEC2016-75080-R, OPTO-SCREEN). The research leading to these results has received funding from the European Union Seventh Framework Programme under grant agreement n°696656 Graphene Flagship.

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