Diatom Research, 2018
Vol. 33, No. 3, 405–411, https://doi.org/10.1080/0269249X.2018.1523231

Taylor & Francis
Taylor & Francis Group

Check for updates

Monitoring *Amphora* sp. growth by flow cytometry

NICOLINA DIAS*, **DAVID MOTA, ANA NICOLAU & MANUEL MOTA**

CEB – Centre of Biological Engineering, University of Minho, Braga, Portugal

A protocol on flow cytometry (FC) was developed for monitoring the growth of *Amphora* sp. in non-axenic and near-axenic (< 15% of bacteria) cultures, enabling the discrimination of aggregates and suspended bacterial cells. Autofluorescence emitted by diatoms was gated on the FL3 red channel and bacteria stained with SYTO-BC were detected on the FL1 green channel. In the FL3 positive region, two subpopulations were gated, one corresponding to a homogeneous high-density region, consisting mostly of single diatoms (R1), and a subpopulation of events with up to one log decade fold of FL3 fluorescence intensity and increased relative cell size, suggesting the presence of aggregates of two or more diatoms in the culture (R2). Percentages of single diatoms, diatoms with attached bacteria and free-suspended bacteria were determined in each quadrant (A, B, C) at different growth phases. The aggregates of diatoms with attached-bacterial cells increased with culture age, and the percentage of free-suspended bacteria accounted for less than 10% in this controlled experiment. It is expected that growth monitoring by FC will allow the detection of bacterial contamination or overgrowth at early stages of *Amphora* sp. cultures.

Keywords: *flow cytometry, benthic microorganisms, diatoms, growth monitoring, autofluorescence*

Introduction

Amphora sp. is a benthic diatom commonly found in a wide variety of surface coastal waters, and has been widely tested for growth rate and nutritional value (De la Peña 2007). As they are commonly mass-cultured (De Pauw et al. 1984) and settled onto plates as diet for grazing juvenile abalones, these photosynthetic microorganisms play a strategic role in seawater aquaculture, as feed and settlement induction of advanced larval stages (Avendaño-Herrera & Riquelme 2007, Capinpin Jr. 2007, Colen et al. 2011).

Changes during phytoplankton growth may have wide-ranging ecological consequences (Peperzak & Brussaard 2011). In this regard, fast and reliable methods to detect early alterations in different growth phases of cultured diatoms are required.

Flow cytometry (FC) has been universally accepted as a method for phytoplankton analysis (Dorsey et al. 1989, Phinney & Cucci 1989, Veldhuis et al. 1997, Vives-Rego et al. 2000, Franklin et al. 2005) due to the distinctive red autofluorescence emitted by chlorophyll, and because less sub-sampling is required to obtain good statistical significance (Dubelaar & Jonker 2000). Furthermore, the characterization of the functional properties of individual cells by FC offers important information on the dynamics of the analysed populations (Davey & Kell 1996). Studies have shown that FC offers a way to look at cell cycle and cell phase of growth changes over time, and at cell signalling events (Dubelaar & Jonker 2000, Gerashchenko et al. 2010). The strong relationship between diatoms and bacteria has been widely studied (Bruckner et al. 2011, D'Costa & Anil 2011), and it is well known that the coexistence between algae and bacteria can be beneficial, but also have harmful effects on growth in culture. When using FC with non-axenic cultures, where bacteria and diatoms coexist, each cluster represents a specific population, gated in different regions and subsequently analysed. The authors aim to address problems that arise when non-axenic benthic diatoms are cultured, namely distinguishing between single cells and aggregates in different growth phases, and the possibility of obtaining results shifted by the fluorescence emitted by diatoms and bacteria.

We aimed to develop a simple, rapid, reliable and inexpensive FC protocol that can be standardized for the quantification of diatom cells in the various stages of exponential and plateau growth, as well as to discriminate subpopulations of individual diatoms, suspended bacteria and aggregates of diatoms with attached bacteria in non-axenic cultures of *Amphora*. For this purpose, a non-axenic culture of *Amphora* sp. was used as a model for the early detection of bacterial contamination, enabling the follow-up of bacteria overgrowth in aquaculture systems.

*Corresponding author. E-mail: nidias@deb.uminho.pt
Associate Editor: Peter Kroth

(Received 15 May 2018; accepted 1 August 2018)

© 2018 The International Society for Diatom Research

Published online 20 Nov 2018

Materials and methods

Strain cultures and cultivation conditions

The marine diatom *Amphora* sp. was obtained from the Culture Collection of Algae and Protozoa (CCAP 1001/3). Non-axenic stock cultures were grown in f/2 + Si enriched seawater as recommended by CCAP (Guillard's medium for diatoms: http://www.ccap.ac.uk/media/documents/f2_Si.pdf) in cell culture flasks with ventilation caps (Orange Scientific, Braine-l'Alleud, Belgium). Sterile filtered seawater (0.2 µm membrane, Millipore Co, Bedford, MA, USA) was used for f/2 + Si medium preparation. Batch cultures were incubated on an orbital shaker Type DOS-20L (SkyLine, ELMI ltd, Latvia) at 50 rpm with a light:dark cycle of 12:12 h, at 20°C.

Near-axenic cultures ($< 15\%$ of diatom with attached bacteria) were obtained following the method described by Gerashchenko et al. (2010) with some modifications. A final concentration of 100 U penicillin mL^{-1}, 100 µg streptomycin mL^{-1} and 200 µg neomycin mL^{-1} were used and the cells were incubated under the above conditions for 24 h. About 10 mL of *Amphora* sp. culture were transferred from the antibiotic-treated medium to a 75 cm^3 culture flask filled with 40 mL of sterile filtered f/2 + Si medium enriched with 2 mL of Luria-Broth medium (LB: Liofilchem ® Roseto degli Abruzzi (TE), Italy), and incubated under the same culture conditions.

Flow cytometry

Dye and staining conditions

SYTO-BC dye (Molecular Probes, Invitrogen, Eugene, OR, USA) is a non-fluorescent cell-permenant dye that shows fluorescence enhancement upon binding nucleic acids of live or dead cells. The dye is excited at 485 nm and emits at 500 nm. The SYTO-BC stock solution was prepared at 5 μM in pre-filtered (0.2 μm) distilled water. Before staining, all samples were sonicated for disaggregation in a Sonorex ultrasonic bath (Bandelin, Berlin, Germany) at an ultrasonic frequency of 35 kHz for 1–3 min, to disperse large diatom aggregates, thus preventing cell clogging in the fluid system. Bright-field microscopic observation was performed before and after sonication, in order to evaluate the efficiency of the disaggregation procedure and its safety for diatom integrity (Figs S1a, S1b). Before sonication, large aggregates of diatoms with clumps of bacteria were abundant. After sonication, diatoms appeared mostly singly or in pairs with dispersed bacteria, and without visible disruption of their delicate structure.

For the FC analysis, 500 μL sonicated sample was taken from the culture flask, stained in the dark with 5 μL of SYTO-BC for 5 min at a final concentration of 0.5 μM.

Setting parameters and data analysis

FC acquisition was carried out using an EC800 Flow cytometer analyser (Sony Biotechnology Inc., Champaign, IL, USA). The forward angle light scatter (FS) detector provided a rough measure of size. Photomultiplier tubes (PMTs) equipped with bandpass filters (BP) – FL1, BP 525/50 nm and FL3, BP 665/30 nm – detected the green and red fluorescence signals, respectively. Green fluorescence was detected in FL1 after staining with SYTO-BC, and red autofluorescence was recorded in FL3 for the presence of chlorophyll pigments in diatoms. All fluorescence emissions were collected in log mode. Events were acquired without compensation. All gates applied for population discrimination were set manually based on control samples.

For diatom enumeration, a volume of 50 µL was analysed at a flow rate of 40 µL min^{-1}. A total number of 15,000–25,000 events were acquired for each sample, providing good statistical analysis. Every sample was run in triplicate to assess the variability within the sample. The experiment was repeated in three different batches for reproducibility.

Acquisition of data was carried out with the EC800 software version 1.3.6. (Sony Biotechnology Inc., Champaign, IL, USA) and data were analysed with the FC software FCS Express 6 – RUO – version 6.05.0028 (de Novo Software, Glendale, CA, USA).

Estimation of diatom density, growth rate and doubling time by FC

Autofluorescence (FL3 +) was used to detect the presence of diatoms and to exclude non-algal particles (FL3 −) (Franqueira et al. 2000). The density of *Amphora* sp. was determined at days 0, 2, 5, 7, 9, 11, 13 and 16.

A standard method was selected to validate counting by FC. A preliminary experiment was carried out involving two different methods (direct count with a Fuchs-Rosenthal chamber and spectrofluorometry), which are in common use for counting phytoplankton. Those were compared with absolute counts obtained by FC during *Amphora* sp. growth. Table S1 compares the two methods in order to choose the best for the cross-validation of the FC method. Despite not being the method showing the best correlation with FC count, and since both values were highly correlated, spectrofluorometry was selected as the standard method because it was less time-consuming.

A Spectrofluorimeter (Horiba Aqualog 800, Kyoto, Japan) and a quartz cell (QS High Precision cell, Art. N° 140-F-10-40, Hellma Analytics, Müllheim, Germany) were used to assess culture emissivity. The full spectrum of the non-axenic culture was first obtained and highest peaks were observed at 456 nm (excitation) and 681 nm (emission). *Amphora* sp. autofluorescence, measured by spectrofluorometry, was converted to diatom

density (diatoms mL^{-1}) with interpolation of the unknown values from the standard curve, and the Spearman correlation coefficient was calculated (Table S2).

Growth rate (GR) was estimated during the exponential phase with measurements performed at day 2 and day 7 according to the regression model equation:

$$Y = Y0e^{(Kt)}, \quad (1)$$

where Y is the diatom density on day 7 of culture growth, $Y0$ is the diatom density on day 2 of culture growth (as this is day 0 of exponential growth), t is the time expressed in days (d) and K is the growth rate constant, which is expressed as day^{-1}.

Doubling time was computed as ln (2)/K. The same instrument settings were used for the entire duration of the experiments to allow comparison between days.

Monitoring growth of Amphora *sp. by FC*

Percentage of diatom *vs.* bacteria present in non-axenic and near-axenic cultures of *Amphora* sp. in different growth phases (exponential and stationary phases) was assessed. Bacteria stained with SYTO-BC were detected on FL1 with the same FC settings used for diatoms. Negative controls of live, unstained diatoms were applied to determine PMT and voltage settings. Therefore, live diatoms stained with SYTO-BC and isolated bacteria stained with SYTO-BC were used to clarify the contribution of nucleic acid staining from diatoms and bacteria. An overlay histogram plotted on FL1 showed that diatoms emitted weaker green fluorescence than bacteria (Fig. S2). The threshold for FL1−/FL1+ was set at log 2, so the contribution of SYTO-BC to live diatoms was considered negligible.

Epifluorescence microscopic observations

Epifluorescence microscopy was used as an alternative approach to cross-validate the cytometry data, by visualizing *Amphora* sp. cultures under particular conditions, namely, a near-axenic, a non-axenic culture of diatoms, and a culture of isolated bacteria. For microscopic analysis, 500 μL of sonicated sample taken from each culture flask was stained, in the dark, for 5 min with 5 μL of SYTO-BC at a final concentration of 0.5 μM. When necessary, the stained sample was diluted in sterilized hypertonic NaCl solution (previously 0.2 µm filtered). Ten microlitres of sample was then placed on a glass slide coated with a drop of glycerol-based anti-fading mounting reagent, Citifluor AF1 (Hatfield, PA, USA). The samples were visualized with an epifluorescence microscope BX51 (Olympus, Shibuya-ku, Tokyo, Japan) equipped with FITC filter (EX 470 nm − EM BP 490/520). The epifluorescence images were taken with a DP71 digital camera (Olympus, Shibuya-ku, Tokyo, Japan) with 60× and 100× magnification objectives.

Statistical analysis

Statistical inference using Wilcoxon signed rank test for comparisons between paired groups was accomplished with GraphPad Prism 5.03 software for Windows 64 bit, San Diego, CA, USA. Statistical significance was determined at $P < 0.05$.

Results and discussion

Since the late 1970s, FC has driven scientists' interest in its potential application for algal studies (Paau et al. 1978). The precise, simultaneous measurements of individual particle volume, fluorescence and light scatter properties that can be made at rapid rates are unique features of the technique that allowed FC to be established as a useful tool for the majority of aquatic particles (Phinney & Cucci 1989). Another obvious advantage is the analysis of small samples containing thousands of cells in a few seconds, thereby providing powerful statistical confidence.

Some authors have reported that FC is more accurate than conventional microbiological methods for obtaining information from heterogeneous, complex samples (D'Costa & Anil 2011, Peniuk et al. 2016).

In this study, FC was used to assess the biological characteristics of non-axenic and near-axenic *Amphora* sp. as a fast, inexpensive and reliable tool for monitoring growth of benthic diatoms in culture. The high precision syringe delivery mechanism of the flow cytometer allowed diatom density to be accurately estimated. FC was shown to be a rapid counting technique for suspensions of benthic diatoms in near-axenic or non-axenic cultures, allowing the analysis of a large number of diatoms and consequently improving statistical interpretation.

Estimation of diatom density, growth rate and doubling time by FC

Red fluorescence was the discriminating feature for detecting diatoms, estimating diatom density (diatoms mL^{-1}) by selecting all the events gated on FL3+. Consequently, no staining with SYTO-BC was required. Afterwards, a growth curve was displayed (Fig.1). A lag phase of 2 days was followed by an exponential phase up to day 7. Growth rate for *Amphora* sp. and the number of times that the diatom population doubled per day during the exponential phase were determined. An average growth rate of $0.6221 \pm 0.02\ d^{-1}$ was obtained with a corresponding doubling time of 1.41 days. A maximum diatom density of 1.09×10^6 cells mL^{-1} was found at day 11, after which the culture reached the stationary phase.

Monitoring growth of* Amphora *sp. by FC

Benthic diatoms have the ability to form small aggregates, so a linear scale for FS measurements was used to better differentiate individual cells from aggregates (Fig.2).

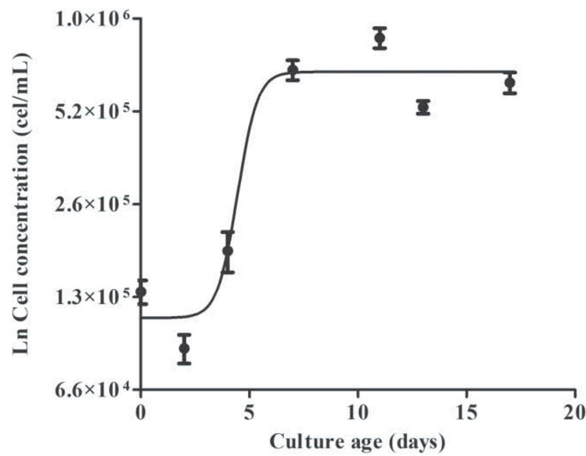

Fig. 1. *Amphora* sp. growth curve (n = 3; mean ± SD). Density of *Amphora* sp. (diatoms mL^{-1}) estimated up to 16 days of culture.

Diatoms were selected according to their FS-lin/FL3 signal (Fig.2a) and two distinct gates (R1 and R2) were set: R1 – a homogeneous subpopulation characterized by high density of small red autofluorescent diatoms, and R2 – a heterogeneous subpopulation characterized by lower diatom density, showing a higher relative size than in R1. On average, diatom density in gate R1 was about four-fold higher than in R2. Although the separation of R1 and R2 was arbitrary, the subpopulations were consistent for all samples. Hence, a colour dot-plot was displayed to improve the visualization of the two gates: R1 was coloured red and R2 was coloured purple (Fig.2b). An overlay histogram of the diatom population, gated on FL3 +, confirmed the differences between R1 and R2 with respect to relative size (Fig.2c) and the autofluorescence signal (Fig.2d), suggesting that diatoms in R1 were mostly single cells, and R2 consisted of small aggregates of two or more diatoms. Free-suspended bacteria were identified as blue events.

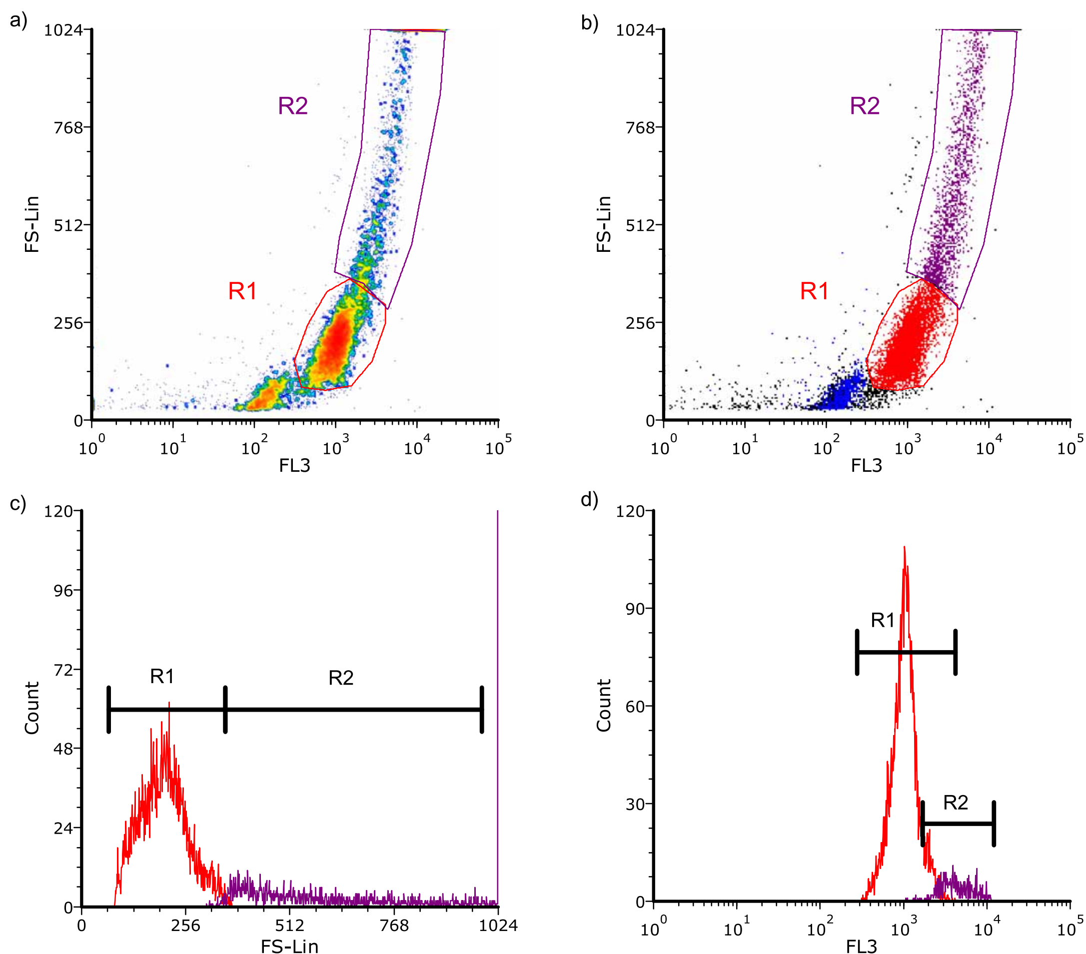

Fig. 2. Gating for characterization of *Amphora* sp. culture. (a) Bivariate dot-plot of the forward light scatter (FS-linear scale) and red autofluorescence (FL3-log scale) of diatoms. Homogeneous subpopulation was gated as R1. Heterogeneous subpopulation was gated as R2; (b) Same bivariate dot-plot converted into a coloured dot-plot. (c) Overlay of the single-parameter histogram (FS-lin) comparing the relative size of diatom population gated in R1 and R2. (d) Overlay of the single-parameter histogram (FL3) comparing the intensity of fluorescence (665/30 nm) emitted by diatoms gated in R1 and R2.

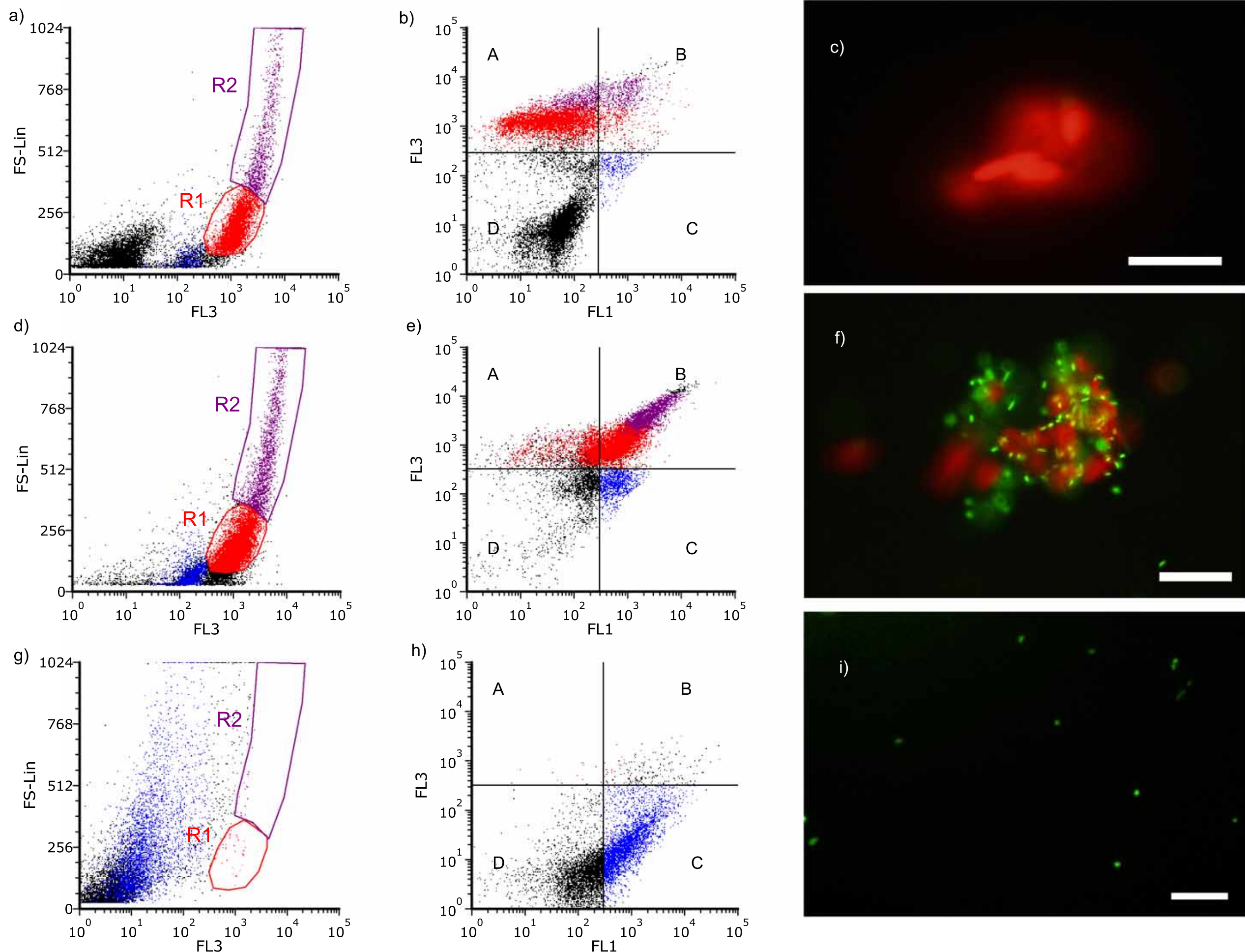

Fig. 3. FC analysis of biological controls for cross-validation of FC data. A suspension of bacteria-free diatoms (a–c), a mixed suspension of diatoms with bacteria (d–f), and a suspension of diatom-free bacterial cells (g–i) were used as controls. Images represented the epifluorescence microscopic observations of the different suspensions. Scale bar = 10 μm.

Cross-validation of data obtained by FC and gating strategy

A combined gating strategy was applied for monitoring the growth of *Amphora* sp. by FC. The first step was staining the sample with the acid nucleic dye SYTO-BC, critical for the separation of diatoms and bacteria. The gates R1 (red) and R2 (purple) were set on the FS-lin/FL3 plot to gate the diatoms. Then, a bivariate dot-plot (FL1 *vs.* FL3) was displayed. The next step was to set the thresholds to split the dot-plot into four quadrants (A, B, C, D) for discrimination of single positive FL1 − /FL3 + , double positive FL1 + /FL3 + , single positive FL1 + /FL3 − , and double negative FL1 − /FL3 − populations, respectively. To accomplish this, a suspension of bacteria-free diatoms (Fig.3a–c), a mixed suspension of diatoms with bacteria (Fig.3d–f) and a suspension of diatom-free bacterial cells (Fig.3g–i) were used as biological controls.

The gates R1 (red) and R2 (purple) were highlighted on the bivariate dot-plot showing the presence of different subpopulations: single diatoms free from bacteria (R1; FL1 − ; FL3 +), single diatoms with attached bacteria (R1; FL1 + ; FL3 +), small aggregates of diatoms free from bacteria (R2; FL1 − ; FL3 +) and small aggregates of diatoms with attached bacteria (R2; FL1 + ; FL3 +).

Epifluorescence microscopy was performed for cross-validation of the FC data. Microscopic observations showed that events gated in quadrant A were mostly single and small aggregates of diatoms free of bacteria (Fig.3c), while quadrant B was characterized by small clumps of diatoms with attached green bacteria (Fig.3f). In B, each dot represented one or more bacterial cells attached to individual diatoms. Consequently, percentages in each quadrant of the dot-plot were not for single cells, whereas the total number of events represents either single diatoms, or diatoms attached with bacteria. Free-suspended bacteria

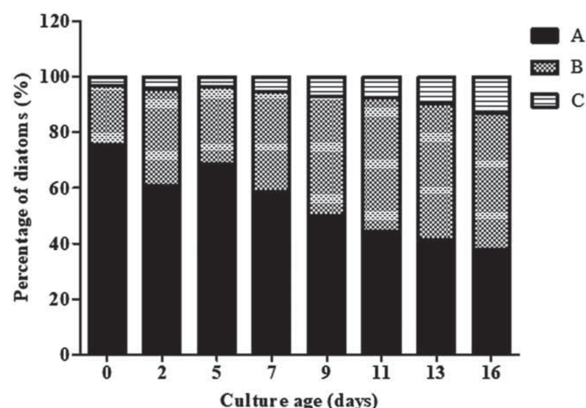

Fig. 4. Stacked-bar graph representing the percentages (%) of events gated in quadrants A, B, and C, for up to 16 days of *Amphora* sp. culture.

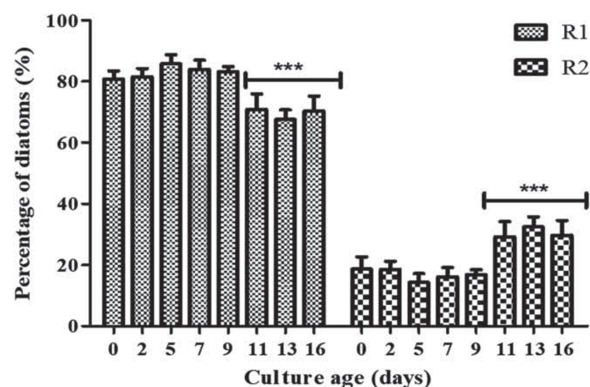

Fig. 5. Bar graph representing the percentage (%) of events gated in R1 and R2 in quadrant B for up to 16 days of *Amphora* sp. growth ($n = 3$; average ± SD).

positive for FL1 and negative for red fluorescence were gated in quadrant C (Fig.3i). Double negative events, in quadrant D, were considered as non-living particles (black events) and were not counted for the event total.

Monitoring of non-axenic cultures of Amphora *sp. vs. culture age*

Different percentages of events gated in each of the quadrants (A, B, and C) were obtained in different growth phases of the culture (Fig.4). Lag and exponential phases were characterized by a high percentage of diatoms (> 60%) gated in A. The stationary phase was characterized by a reduction of 20% of diatoms gated in A, with a corresponding increase in B. A slight increase in suspended (C) bacteria was also observed in this late phase.

A more detailed analysis of the diatom/bacteria in quadrant B showed two distinct subpopulations (R1 and R2), which allowed the percentage of single diatoms and small aggregates of diatoms with attached bacteria to be estimated, respectively (Fig.5). The graph shows a significant decrease in single diatoms ($P < 0.0001$) and a corresponding increase in aggregates ($P < 0.0001$) in the stationary phase.

Monitoring of non-axenic cultures vs. near-axenic cultures by FC

Bacteria are frequently associated with benthic diatoms, interacting in the micro-zone surrounding diatom cells, both contributing to the build-up of phototrophic biofilms (Affan et al. 2006). Bacteria may be free-living or attached to the diatom surface, showing tight interaction with microalgae (Olson et al. 1983). Near-axenic cultures (< 15% of bacteria) were compared with non-axenic cultures of *Amphora* sp., and the percentage of events gated in quadrants A, B and C was calculated.

Table 1 summarizes the results obtained (in percentages) for A, B and C from three independent assays of non-axenic cultures and near-axenic cultures in exponential growth. Significant differences were found between the percentage of events in non-axenic and near-axenic cultures in quadrants A ($P = 0.005$) and B ($P = 0.01$). In non-axenic cultures, less than 60% diatoms were free of bacteria (A) and nearly 40% were single or small aggregates of diatoms with attached bacteria (B). Free-suspended bacteria (C) accounted for less than 10% of the diatom culture. On the other hand, in near-axenic cultures, single diatoms gated in A, small aggregates of diatoms and bacteria in B, and free-suspended bacteria (C) represented roughly 80%, 15% and 5% of the diatom culture, respectively.

Conclusions

A simple, rapid, reliable and inexpensive FC protocol is proposed for the quantification of cells that are in various stages of exponential and stationary growth. Staining bacteria with SYTO-BC enabled four quadrants to be discriminated based on the combination of two bivariate dot-plots (FS-lin/FL3) and (FL1/FL3). Exponential phase was characterized by a high percentage of single diatoms.

Table 1. Percentage of events (%) gated in quadrants A, B, and C.

	A	*P*	B	*P*	C	*P*
Non-axenic	54.2 ± 6.7	0.005*	37.7 ± 6.1	0.01*	8.1 ± 2.1	*n.s.n.s.*
Near-axenic	81.2 ± 5.8		13.3 ± 5.6		5.5 ± 3.0	

*Significant values ($P < 0.05$); *n.s.*: not significant.

Small aggregates with attached bacteria were present in all growth phases, but the percentage of these aggregates increased in stationary phase. Although the study was conducted in a controlled setting, monitoring the percentage of diatoms and suspended bacteria enabled the culture conditions to be controlled, with respect to bacterial overgrowth or aggregation that might affect early stages of the culture.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This study was supported by the Portuguese Science and Technology Foundation (FCT) under the scope of the strategic funding of UID/BIO/04469/2013 unit, COMPETE 2020 (POCI-01-0145-FEDER-006684) and the BioTecNorte operation (NORTE-01-0145-FEDER-000004) funded by the European Regional Development Fund under the scope of Norte2020 – Programa Operacional Regional do Norte.

Supplemental data

Supplemental data for this article can be accessed at https://doi.org/10.1080/0269249X.2018.1523231.

References

AFFAN A., KARAWITA R., JEON Y.-J., KIM B.-Y. & LEE J.-B. 2006. Growth characteristics, biochemical composition and antioxidant activities of benthic diatom *Grammatophora marina* from Jeju Coast, Korea. *Algae* 21: 141–148.

AVENDAÑO-HERRERA R.E. & RIQUELME C.E. 2007. Production of diatom-bacteria biofilm in a photobioreactor for aquaculture applications. *Aquacultural Engineering* 36: 97–104.

BRUCKNER C.G., REHM C., GROSSART H.-P. & KROTH P.G. 2011. Growth and release of extracellular organic compounds by benthic diatoms depend on interactions with bacteria. *Environmental Microbiology* 13: 1052–1063.

CAPINPIN JR E.C. 2007. Feeding, growth, and survival of post-larval abalone *Haliotis asinine* on different benthic diatoms. *Science Diliman* 19: 49–59.

COLEN C.V., LENOIR J., DE BACKER A., VANELSLANDER B., VINCX M., DEGRAER S. & YSEBAERT T. 2009. Settlement of *Macoma balthica* larvae in response to benthic diatom films. *Marine Biology* 156: 2161–2171.

D'COSTA P.M. & ANIL A.C. 2011. The effect of bacteria on diatom community structure - the 'antibiotics' approach. *Research in Microbiology* 162: 292–301.

DAVEY H.M. & KELL D.B. 1996. Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. *Microbiology and Molecular Biology Reviews* 60: 641–696.

DE LA PEÑA M.R. 2007. Cell growth and nutritive value of the tropical benthic diatom, *Amphora* sp., at varying levels of nutrients and light intensity, and different culture locations. *Journal of Applied Phycology* 19: 647–655.

DE PAUW N., MORALES J. & PERSOONE G. 1984. Mass culture of microalgae in aquaculture systems: progress and constraints. In: *Eleventh international seaweed symposium. Developments in hydrobiology* (Ed. by C.J. BIRD & M.A. RAGAN), Vol. 22. pp. 121–134. Springer, Dordrecht.

DORSEY J., YENTSCH C.M., MAYO S. & MCKENNA C. 1989. Rapid analytical technique for the assessment of cell metabolic activity in marine microalgae. *Cytometry* 10: 622–628.

DUBELAAR G.B.J. & JONKER R.R. 2000. Flow cytometry as a tool for the study of phytoplankton. *Scientia Marina* 64: 135–156.

FRANKLIN N.M., STAUBER J.L. & ADAMS S.A. 2005. Improved methods of conducting microalgal bioassays using flow cytometry. In: *Techniques in aquatic toxicology* (Ed. by G.K. OSTRANDER), pp. 735–755. CRC Publishers, Boca Raton, FL.

FRANQUEIRA D., OROSA M., TORRES E., HERRERO C. & CID A. 2000. Potential use of flow cytometry in toxicity studies with microalgae. *Science of the Total Environment* 247: 119–126.

GERASHCHENKO B.I., TAKAHASHI T., KOSAKA T. & HOSOYA H. 2010. Life cycle analysis of unicellular algae. *Current Protocols in Cytometry* 52: 11.19.1–11.19.6.

OLSON R.J., FRANKEL S.L., CHISHOLM S.W. & SHAPIRO H.M. 1983. An inexpensive flow cytometer for the analysis of fluorescence signals in phytoplankton – chlorophyll and DNA distributions. *Journal of Experimental Marine Biology and Ecology* 68: 129–144.

PAAU A.S., URO J. & COWLES J.R. 1978. Applications of flow cytometry to the study of algal cells and isolated chloroplasts. *Journal of Experimental Botany* 29: 1011–1020.

PENIUK G.T., SCHNURR P.J. & ALLEN D.G. 2016. Identification and quantification of suspended algae and bacteria populations using flow cytometry: applications for algae biofuel and biochemical growth systems. *Journal of Applied Phycology* 28: 95–104.

PEPERZAK L. & BRUSSAARD C.P.D. 2011. Flow cytometric applicability of fluorescent vitality probes on phytoplankton. *Journal of Phycology* 47: 692–702.

PHINNEY D.A. & CUCCI T.L. 1989. Flow cytometry and phytoplankton. *Cytometry* 10: 511–521.

VELDHUIS M.J.W., CUCCI T.L. & SIERACKI M.E. 1997. Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological implications. *Journal of Phycology* 33: 527–541.

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.