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Original Article

Using in situ flow cytometry images of ciliates and dinoflagellates for aquatic system monitoring

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(With 4 figures)

Abstract

Short-period variability in plankton communities is poorly documented, especially for variations occurring in specific groups in the assemblage because traditional analysis is laborious and time-consuming. Moreover, it does not allow the high sampling frequency required for decision making. To overcome this limitation, we tested the submersible CytoSub flow cytometer. This device was anchored at a distance of approximately 10 metres from the low tide line at a depth of 1.5 metres for 12 hours to monitor the plankton at a site in the biological reserve of Barra da Tijuca beach, Rio de Janeiro. Data analysis was performed with two-dimensional scatter plots, individual pulse shapes and micro images acquisition. High-frequency monitoring results of two interesting groups are shown. The abundance and carbon biomass of ciliates were relatively stable, whereas those from dinoflagellates were highly variable along the day. The linear regression of biovolume measures between classical microscopy and in situ flow cytometry demonstrate high degree of adjustment. Despite the success of the trial and the promising results obtained, the large volume of images generated by the method also creates a need to develop pattern recognition models for automatic classification of in situ cytometric images.

Keywords: aquatic systems, microbial ecology, in situ flow cytometry, method.

Usando imagem de citometria de fluxo in situ de ciliados e dinoflagelados para o monitoramento de sistemas aquáticos

Resumo

A variabilidade de curto período em comunidades do plâncton é pouco documentada, especialmente as variações que ocorrem em grupos específicos das assembleias por causa das análises tradicionais serem muito trabalhosas e demoradas. Além disso, não permitem que a alta frequência amostral necessária para a tomada de decisão. Para superar esta limitação, nós testamos o CytoSub, um citômetro de fluxo submersível. Este aparelho foi ancorado a aproximadamente 10 metros de distância da linha de maré baixa a uma profundidade de 1,5 metros por 12 horas para monitorar o plâncton em um sítio da reserva biológica da praia da Barra da Tijuca, Rio de Janeiro. A análise dos dados foi realizada a partir de gráficos de dispersão bidimensionais, pelas assinaturas ópticas individuais escaneadas (*pulse shape profile*) e aquisição de micro imagens. Resultados do monitoramento de alta frequência de dois grupos interessantes são apresentados. A abundância e a biomassa de carbono de um grupo de ciliados foram relativamente estáveis, ao passo que o grupo de dinoflagelado, foi altamente variável ao longo do dia. O modelo de regressão linear das medidas de biovolume entre a clássica microscopia e a citometria de fluxo in situ apresentou alto grau de ajustamento. Apesar do sucesso deste ensaio e dos resultados promissores obtidos, o grande volume de imagens geradas por este método também gerou a necessidade de se desenvolver modelos de reconhecimento de padrões para a classificação automática de imagens de citometria in situ.

Palavras-chave: sistemas aquáticos, ecologia microbiana, citometria de fluxo in situ, método.

1. Introduction

The distribution of marine plankton is highly variable in space and time (Bricaud et al., 2012; Batchelder et al., 2012). The occurrence of geographical variation in nature and the habitat factors determining community assemblages contribute to this variability. Informations about such variability are obtained from different sampling approaches.

These approaches involve a range of classical techniques (Utermöhl, 1958; Hobbie et al., 1977 or Brito at al., 2013) and devices, including vessel-based equipment, anchored instruments used for in vivo chlorophyll measurements and satellite observations from which the colour of the sea can be determined (Del Giorgio et al., 1996; Dickey, 2001). Phytoplankton populations, for example, display metabolic diversity and a high degree of physiological plasticity (Platt, 1981), but their buffering capacity is low (Smayda, 1998). Thus, these populations respond quickly to external forcing factors. This responsiveness allows them to serve as good indicators of environmental conditions. However, our understanding of phytoplankton population dynamics and the factors regulating their populations at meso and micro scales remains limited due to our inability to measure the species composition, size distribution and growth rates of phytoplankton (Sosik et al., 2003). The composition and size structure of plankton, for instance, helps to determine the carbon and nutrient flows through the ecosystem and can be an important indicator of the response of coastal environments to anthropogenic disturbances, such as nutrient or pollutant inputs. Harmful algal blooms or outbreaks of toxic organisms (Anderson et al., 2002) commonly coexist with species that have similar ecological requirements and environmental tolerances, later called functional groups (Nair et al., 2008).

However, with the exception of the information furnished by several laboratory experiments (Tillmann, 2004; Rosetta and McManus, 2003; Alonso et al., 2000), little is known about the functional characteristics of protozooplankton (ciliates and microflagellates) and of metazoans or the interactions occurring in the overall assemblage, e.g., the interactions among zooplankton, phytoplankton, bacteria and viruses. The first prerequisite for a more realistic understanding of the interactions among the factors that regulate species composition is the availability of detailed and continuous long-term observations. This type of observation has become possible because new instruments were developed and made available for use in the continuous monitoring of specific organisms or groups (Pereira et al., 2016; Bergkvist et al., 2012, Campbel et al., 2010). However, these instruments produced substantial amounts of data. In this context, models based on cytometric signatures have been successfully developed and applied (Embleton et al., 2003; Kenneth et al., 2008; Pereira and Ebecken, 2011).

The in situ scanning flow cytometry (SFC) technique, in conjunction with an add-on image-in-flow module, represents one of the most promising technologies for addressing the gap of knowledge in this area. This approach can be used to study the microscopic constituents of marine and freshwater ecosystems. So, the purpose of this study was (*i*) to test this technology under real conditions and (*ii*) to produce a short time series of high-frequency monitoring data for two specific groups, e.g. ciliates and dinoflagellates, occurring in the surf zone, and (*iii*) collect samples to perform the classical measures of microscopy in order to determine their biomass and (*iv*) compare both approaches. These data shed light on the diel abundance and biomass variation of both groups given the great importance in trophic dynamics of pelagic systems, as key organisms connecting the microbial loop with the classical food chain.

2. Material and Methods

2.1. Study site

The study was conducted at a fixed site (23°00`51.62``S, 43°23`45.10``W) in the biological reserve of Barra da Tijuca beach, western of Rio de Janeiro city. It is located within the boundaries of the Marapendi ecological park. This park offers a very attractive natural landscape (Figure 1) displaying white sandbars deposited during the past 5100 years (Roncarati and Menezes, 2005) covered by restinga fixing vegetation. The beach, used recreationally by the population, is on a spit is crossed by Sernambetiba Avenue, which provides access to the area. The coastal seawater is always green. Due to the coastal drain and the effects of submarine outfalls, the water shows a degree of eutrophication. The seawater temperature, measured by a reversing thermometer, ranged from 19.3 °C to 20.5 °C during the day, whereas the salinity remained at 35.7 ppm. The salinity was measured with a Milwaukee MA887 digital refractometer (Milwaukee, WI, USA).

2.2. Data acquisition

For cytometric data acquisitions, the standard CytoSense flow cytometry instrument (CytoBuoy bv, Worden, The Netherlands) was be made submersible (CytoSub) by adding a specially designed stainless steel housing. The device was connected to a unit converter for power and data transfer from a simple kiosk on the shore in the late summer (March). A long, high-quality cable is used to attach the device to the converter (Figure 2a). The instrument can be connected to the Internet for remote operation and can detect and record large suspended particles (> 1-1000 mm diameter) in relatively large volumes of water (more than 4 cm^3 per sample). The water is pumped into a flow chamber to avoid external turbulence. The flow is controlled by a peristaltic pump. The speed of the pump is adjustable and was set at 7.8 mm^3 s^{-1} for this experiment. The sheath fluid "runs" at a rate of 80 cm^3 min^{-1}. Each suspended particle crosses a 5 µm blue laser beam (Coherent Saphyre, 488 nm, 15 mW). The laterally scattered light signals (side scatter, SWS) and the signals from fluorescence within the chamber are dispersed by a concave holographic grating and collected with a hybrid photomultiplier device (HPMT). This optical design offers the possibility of extending the dynamic range to high values (large cells) with fixed parameters. The signals from the two sources (one right, one left) of forward scattering (FWS) are collected by photodiodes (PIN). The red (FLR), orange (FLO) and yellow (FLY) spectra are collected at wavelengths of 668-734 nm (chlorophyll-a), 601-668 nm (phycocyanin) and 536-601 nm (phycoerythrin), respectively. In addition to these 5 basic parameters (FWS, SWS, FLR, FLO and FLY), some simple

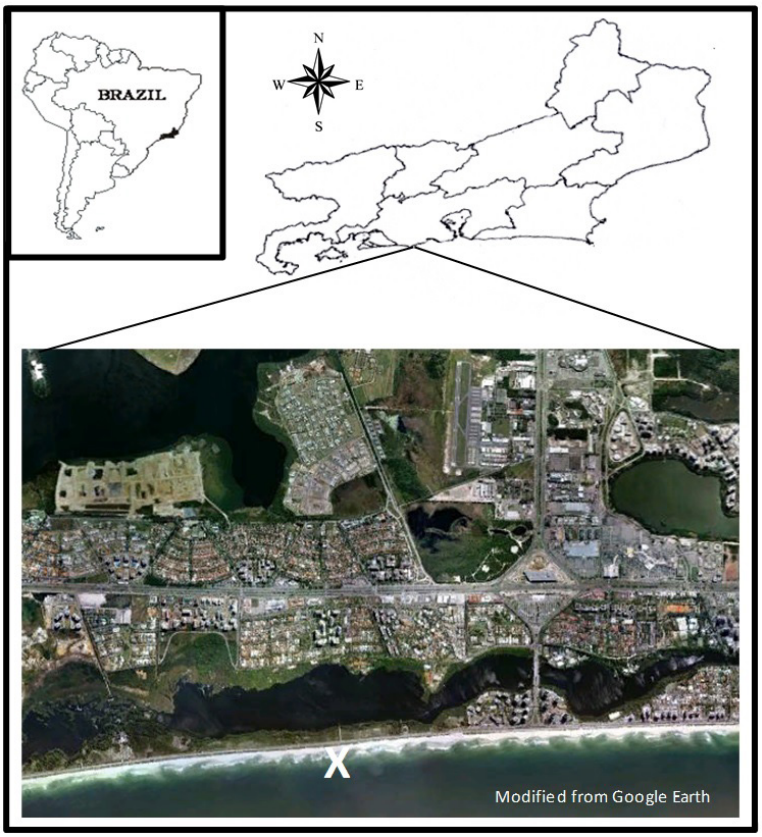

Figure 1. The state of Rio de Janeiro and a section of the Barra da Tijuca beach showing the Marapendi coastal lagoon. The cross indicates the study site.

mathematical models were assigned to each signal shape: inertia, fill factor, asymmetry, number of peaks, length, and apparent size (FWS size). All these values were summarised in cytograms to facilitate the identification of groups of cells or organisms with similar optical properties derived from these models. The instrument was pre-programmed (triggered) on the FWS channel. The data were collected on a logarithmic scale and presented as arbitrary units (au). The signal shapes were encoded at a frequency of 4 MHz, and 64 kbits were saved in grabbers. The particles flowed at a rate of 2 m^{-1} past a 5 nm laser beam. A total of approximately 12 points were defined for each nm. This information facilitates access to the optical signatures (pulse shapes profile defined in real time). The seawater and sheath fluid were mixed together in the flow from the chamber outlet and filtered through a 0.1 mm membrane (Nucleopore) to be recycled. The CytoSub can be operated at depths up to 200 m. The uncertainty in the counts is 5%. For a sampling time of 1 second, the overestimation of the examined volume due to truncation by the peristaltic pump is +5%. For a 5 second sample, the error is +1%.

On the other hand, sea water samples (200 mL) were collected, fixed in-site (2% buffered formaldehyde - final concentration) and placed on ice and in the dark for further analysis in the lab. The site was revisited and resample in the early autumn for confirmation.

2.3. Image in flow

The CytoSense used in this study is equipped with an "image-in-flow" device controlled by the CytoUSB software (CytoBuoy b.v.). This principle is based on video microscopy in combination with a very fast flash (pulse) illuminator. The technique provides still images of moving particles. The criteria for online image-in-flow acquisition are determined on the basis of each pulse shape signal. After selecting (gating) a cluster on the cytogram (a two-parameter scatter plot), the coordinates of the cluster are sent to trigger the CCD camera. Events with similar optical characteristics are photographed and stored digitally. Each sequence is then used for species recognition in combination with its respective pulse shape. The images are coupled individually to the laser scans to allow further analysis.

2.4. Biomass estimation

Many authors (Dugenne et al., 2014; Chioccioli et al., 2014; Liu et al., 2013; Graff et al., 2012; Boucher et al., 1991 among others) have developed and used different methods for estimating biomass from flow cytometers. However, due

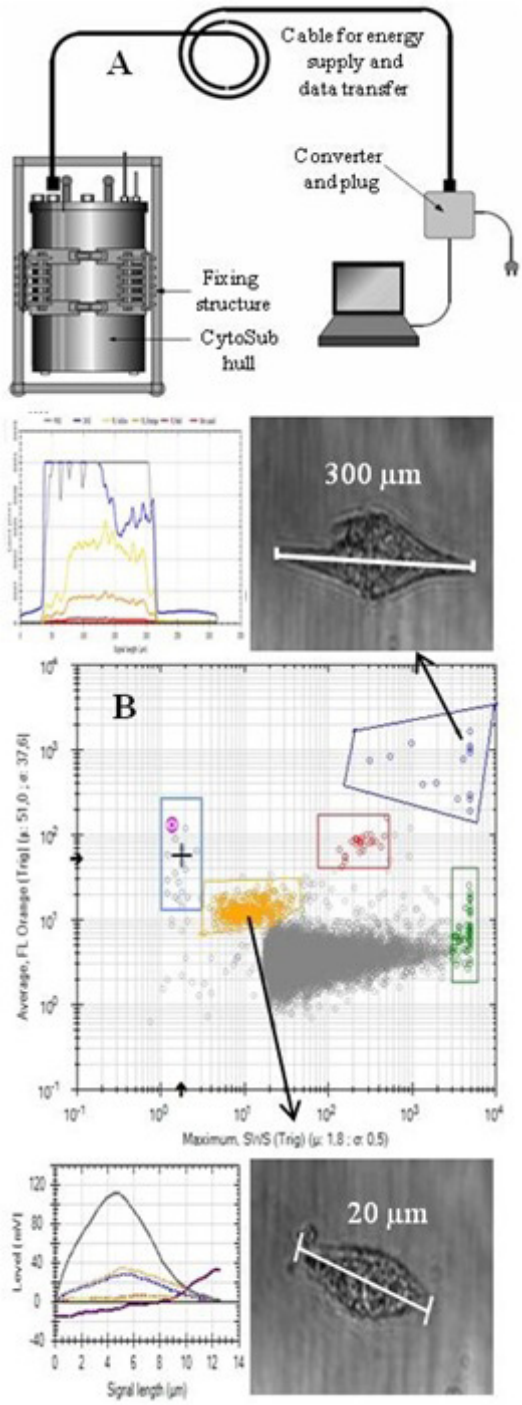

Figure 2. In (A), a schematic view of the CytosSub instrument; In (B), a citogram showing the distribution of suspended particles according the average values of the SWS sensor (side scatter) and the maximum values of the orange fluorescence sensor (FLO). Gates allow us to check individually each particle shape (pulse shape/optical signature) and size by its signal length on x axis and intensity in the y axis in milivolts (mV) and respective in-silico images.

to the skepticism of many yet, the biomass determinations here were based on linear size measurements obtained from cytometric images of living cells. These linear measurements were used to calculate the cell volume. For ciliates, the carbon biomass was calculated indirectly from the mean cell volumes (MCV) obtained by linear measurements of the cell width as the minimum axis (a) and the cell length as the maximum axis (b). These values were referred to the standard geometric shape that best resembled the shape of the cell. In particular, the ciliate biovolume (V) was calculated in terms of a shape equivalent to an ellipsoid of revolution (Davey et al., 1993) (Equation 1):

$$V = \frac{1}{6}\pi b a^2 \quad (1)$$

The calculated ciliate biovolume was then converted to carbon content. A conversion factor of 110 fgC μm^{-3} (Weisse, 1991) was applied.

For Ceratium sp., an approach based on the revolution of a standard geometric shape is not applicable. Measurements of cells based on two-dimensional images require assumptions about the third dimension. To overcome this constraint, cells were measured to determine the aspect ratio (i.e., the length: width: depth ratio) for the species. These ratios were used to estimate the third dimension in the volume calculations of Ceratium. A conversion factor of pgC $cell^{-1} = 0{,}760 \times (volume\ \mu m^3)^{0.819}$ was applied.

The total biomass ($\mu gC\ l^{-1}$) of each group was determined as the population abundance multiplied by the cells carbon content.

On the other hand, the analyses of the preserved samples were made as follow. After gentle mixing, a 50 mL subsample was removed and concentrated by settling for 48 h prior to analysis by microscopy (Utermöhl, 1958). Thirty individual cells of the ciliate and dinoflagellate groups were measured (length and diameter) at × 500 magnification using a calibrated ocular micrometer on a Wild M40 inverted microscope. So, the classical cell biovolume was calculated according Hillebrand et al. (1999) to compare both methods.

In the present work, a linear regression model was performed in Microsoft Excel 7 (Microsoft Corporation, Redmond, Washington, USA) as descriptive method aiming to estimate the curve fitting (flow cytometry / microscopy) since any assumptions about the processes which have produced the data is required.

3. Results and Discussion

Figure 2a shows a schematic view of the CytoSub flow cytometry. The analysis performed by this instrument illustrates several advantages of this technique relative to conventional flow cytometers and traditional microscopy. During the study period, the phytoplankton community was dominated by nanophytoplankton (2-20 µm) cells (52%), followed by small picophytoplankton (≤ 2 µm) cells (48%) and microphytoplankton (> 20 µm) cells (10%). The mean forward scattering values of each of

these populations, measured on the basis of individual cells, increased during the day (data not shown).

3.1. Image and pulse shapes

Figure 2b shows a scatter plot produced by the CytoClus 3 software provided by the manufacture. This example includes 5 well resolved gated clusters (coloured). We show the two most interesting groups (our opinion) in the scatter plot and consider their amplitudes and pulse shapes. First, an image of a free-living marine ciliate appears towards the bottom of the plot. This cell shows nearly 40 mV of orange fluorescence. A possible explanation of this result is that the ciliate had ingested smaller particles with this trait (Not et al., 2007). Organisms belonging to this group are considered ubiquitous and are often abundant in marine and freshwater environments, where they play an important role in the transfer of carbon from picoplankton and nanoplankton to metazoans (Fenchel, 1987). According to Urrutxurtu et al. (2003) these protists consume a wide spectrum of particle sizes from bacteria to diatoms and dinoflagellates, as well as other ciliates. However, the ecological role of these protists in Rio de Janeiro's coastal waters is poorly understood. The body of the ciliate is approximately elliptical, with tapered ends. These cells range in size between 4.2 and 5 µm wide (the minimum axis) and between 14 and 20 µm long (the maximum axis). Second, an image of Ceratium sp. (Dinophyta) appears towards the top of the plot. This genus seems to be common in Brazilian coastal waters (Cardoso, 1995; Koening and Lira, 2005). Its members can be mixotrophs, obtaining food from photosynthesis and phagocytosis. According to Suk and Lee (2009), several invasive species are known and have been detected in ballast waters (Botes, 2003; Senanayake et al., 2010). Like other dinoflagellates, the members of this genus are very heavily pigmented. The pulse shape of the dinoflagellate shows high levels of orange (1000 mV) and yellow (3000 mV) fluorescence. The arms or horns represent the most distinctive characteristic of these cells. The shape and size of the cells varies from species to species, and environmental conditions, primarily temperature, are also known to affect morphological variation in the group. This specimen, with 300 µm in length, shows a large and straight body. The cell is widest on both sides of the girdle. It has also two unequal, parallel or slightly divergent hypothecal horns and an epitheca tapering gradually into an apical horn.

3.2. Abundance and biomass variation

The ciliates abundance was relatively stable during the day, ranging from 1,13 E+4 cells mL^{-1} in the evening to 1,59 E+4 cells mL^{-1} at 11 pm, which is in agreement with the values found by Rezende et al. (2015) near the entrance to the Guanabara bay. In contrast, the population of Ceratium sp. was highly variable throughout the monitoring period, ranging from 5 to 168 cells/mL^{-1} and presenting two peaks, a lower peak in the morning (7:30 h) and a higher peak immediately after midday (12:30 h).

Table 1 presents the estimated average values of carbon biomass (cell count multiplied by average cell biovolume based on the assumed carbon:biovolume conversion factors). These means were calculated from linear measurements of 30 individuals from each population. This information is essential for evaluating the role of the organisms in food webs and in the cycling of materials. Ciliates have often been thought of as the 'typical' microheterotrophic protist grazers on phytoplankton even though heterotrophic dinoflagellate biomass often equals that of ciliates in the plankton (Sherr and Sherr, 1994), but here, it is not the case since the estimated average biomasses were 0.0281 µgC l^{-1}, 0,0019 µgC l^{-1} for ciliate and Ceratium populations respectively. Cell volume varied to a certain extent within the two species. The estimated cell volumes were 18 μm^3 for ciliates and 42,415 μm^3 for Ceratium sp. The latter shows great variability of shape and size giving a major challenge because the high morphological complexity. However, our data about Ceratium are consistent with those reported by Queiroz et al. (2014). The coefficients of variation of cell volume were 0.27 and 0.24, respectively. It is probable that this size variability is due to differences in the growth status of the individual cells. The estimated carbon content should be considered in the context of our methodology and may reflect differences in the nutritional state of the organisms. We found a carbon content of 2.016,26 pgC $cell^{-1}$ and 2.955,83 pgC $cell^{-1}$ for ciliates and Ceratium, respectively. These results show that the Ceratium cell is slightly denser than the ciliate cell. In this study, the ciliate group were by far the most abundant group comprising up to 15 times more biomass when compared to the Ceratium group. In this way, Figure 3 shows the diel variations in the carbon biomass of the two populations at 30 minute intervals during the monitoring period. The biomass curve of the ciliates showed little fluctuation, with a maximum value of 0.29 µgC l^{-1} at 11,00 h and a minimum of 0.21 µgC l^{-1} at 18.00 h. In contrast, the biomass curve

Table 1. The main average features of the two populations.

Population	Mean width µm	Mean length µm	Volume μm^3	C content pgC $cell^{-1}$	Mean Pop. *n*	Mean pop. biomass µgC l^{-1}
Ciliates	4.6	17	18.29 ± 0.27	2,016.26 ± 0.06	13.97	0.0281
Ceratium	25	291	42,415 ± 0.24	2,955.83 ± 0.04	31	0.0019

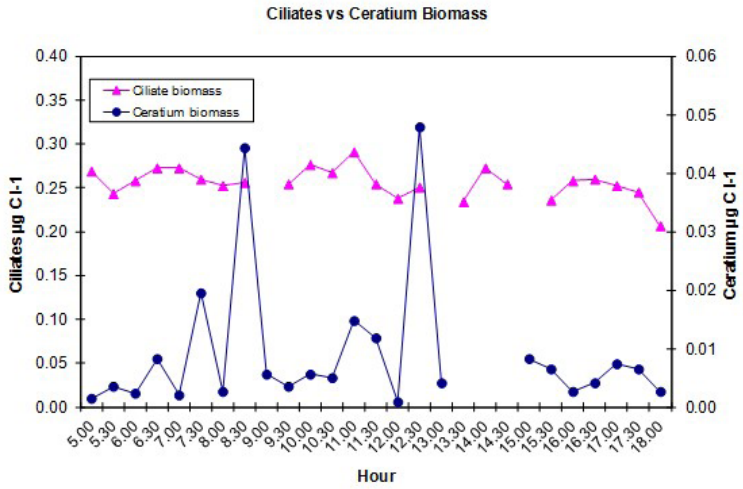

Figure 3. Diel distribution of the abundance of Ceratium (filled circles), and a not identified ciliate protozoa (triangles).

of Ceratium sp. was extremely variable. Its curve includes two substantial peaks. One of the peaks corresponded to the maximum value of the curve, 0.048 µgC l^{-1} at 12.30 h. The lowest value of Ceratium sp. biomass was observed at dawn (0.001 µgC l^{-1} at 6.00h). The abundance and biomass curves had the same shape (not shown). Both curves have gaps in particular times because the organism was not detected at those times. Nevertheless, the Figure 3 offers a useful indication of the role and contribution of these groups within the community and food web.

Figure 4 shows the results of a linear regression model of cellular biovolume measurements obtained from in situ flow cytometry and those obtained by microscopy in the laboratory considering p-value < 0.05 for statistical significance. The high values of r^2 shown in Figures 4a ($r^2 = 0.8874$ for ciliates) and 4b ($r^2 = 0.8504$ for Ceratium) show the high degree of adjustment of the statistical model and coherence between the two approaches.

3.3. Early warning

The early warning principle is based on the detection of a combination of numerically dominant clustered events by the flow cytometer and on the use of the image-in-flow technique to identify the organisms involved in the events. A possible example is furnished by Ceratium sp. This relatively harmless group of organisms plays an important role in the environment as predators and as prey. Although the toxicity of Ceratium has not been demonstrated, fish mortality was observed in association with Ceratium blooms in Thailand and Japan and attributed to oxygen depletion (Taylor et al., 1995). In addition, mass mortality of *Ostrea lurida* (Pacific oyster) larvae was reported in Mexico in association with Ceratium blooms (Landsberg, 2002).

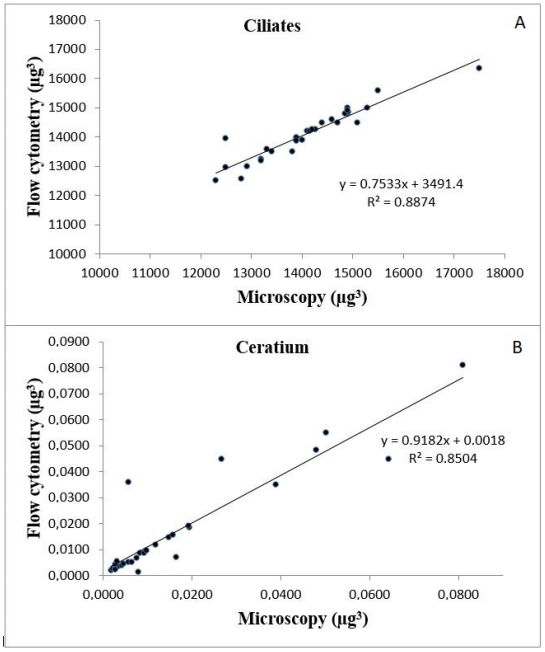

Figure 4. Linear regression of the biovolume measures ($p < 0.05$) between in situ flow cytometry and microscopy. In A, measures from the ciliate group and in B, from the dinoflagellate (Ceratium sp.) group.

4. Conclusions

The test of the CytoSense/CytoSub instrument appeared to be successful. This instrument represents a viable alternative for plankton monitoring, water quality assessment and management. Its operator is able to identify and track the dynamics of the target individual species or clusters. Since the traditional techniques commonly used to count and calculate the biomass of aquatic microorganisms

(bacteria, ciliates, flagellates and algae) require a lot of time and professional expertise, the adoption of innovative technologies like in situ flow cytometry, as proposed in this article, will help to fill this gap, thus contributing to a better decision making process in environmental monitoring and management. The use of "imaging in flow" validates the scanned pulse shape profiles ending the problem of possible overlaps and false positives. However, depending on the selected group (gated cluster), this device can generate a large number of images in very short time. It makes the visual or manual inspection of these files impossible. Therefore, it is necessary to develop computational methods for pattern recognition and automatic classification of cytometric images. This reflects the direction of our future studies, the search for such methods.

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