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Digital holographic microscopy for microalgae biovolume assessment

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ABSTRACT

The relative amount of biomass in a body of water is one of the various indicators widely used in water quality evaluation. This implies complex tasks such as identification and characterization of microorganisms and measurement of their biovolume. Particularly, the latter is estimated by assuming simple geometrical shapes for the microorganism and by calculating its dimensions from images taken with a conventional microscope. In order to have a more precise and automatic method for biovolume evaluation, a hybrid methodology based on digital holographic microscopy and image processing is proposed. The whole volume of a specimen under study is obtained combining the phase contrast image of an off-axis hologram with the thickness-profile data of the specimen extracted from the cell silhouette. This technique has been used for determining the biovolume of *Ceratium Hirundinella* cells in water samples. The methodology proposed also shows that it is possible to estimate accurately an *effective refractive index* of the microorganism. Experimental results have shown that this technique is not only an efficient and fast alternative, but also suitable for automatizing the entire process.

1. Introduction

In Biology and Medicine, identification, quantification, characterization and detection of microorganisms such as parasites, bacteria, and microalgae, are particularly important as diagnosis tools [1–6]. Generally, these observations are carried out under a microscope by highly specialized technicians. Many times, the morphology of these specimens is complex and the performance of the analysis strongly depends on its correct identification and quantification, which is a tiring and time-consuming task. Particularly, the estimation of microalgae biovolume is one of the most widely studied morphometric descriptors and it is important for the study of phytoplankton ecology. Related parameters, such as cell size, carbon content and physiology functions are also important for marine ecosystem studies. This calculation is performed by associating microalgae with simple geometrical shapes and by determining their volume measuring linear dimensions with an ordinary microscope [7–10]. Briefly, this procedure consists of measuring at least twenty individual cell biovolume for each of the species. After that, the average biovolume is multiplied by the number of cells/ml in order to obtain its relative abundance. In addition, this complex methodology contributes with approximate values only.

In the last decades, taking advantage of digital cameras equipped with CCD or CMOS sensors, and digital image processing tools, holography field has gained new interest due to the so-called digital holography (DH) [11]. Since 1999, digital holographic microscopy (DHM) has emerged as an interesting alternative to conventional microscopy [12–16]. A digital hologram, consisting of the interference between an object and a reference beam, is recorded by a digital camera and the holographic image is numerically reconstructed using diffraction theory basis. Calculation of the complex optical field allows direct access to quantitative amplitude and phase information [10]. Moreover, numerical focusing is possible by reconstructing the single recorded hologram at several distances, emulating the focusing control of a conventional microscope. In all these approaches, the phase signal provided by DHM is obtained using a transmission configuration and it is proportional to the integrated optical path length (OPL) along the optical axis through the specimen, depending on both, morphology and mean intracellular refractive index. Although methods to decouple both variables exist [13], the measurement remains as an integrated value. Recently, multiple angles digital holographic tomography and tomographic phase microscopy have rapidly evolved to recover a full three-dimensional (3D) refractive index map of intracellular structures, or to estimate the three-dimensional morphology and shape of microsamples. Nevertheless, these techniques rely on some mechanical scanning achieved either by rotating the object [15,17,18] or varying the illumination

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beam angle [19], or involve the use of continuous tunable laser [20,21].

In the present paper, we propose a simple methodology that combines the information obtained from only two holograms with image processing tools, to determine biovolume of *Ceratium Hirundinella*. These microalgae are dominant members of the summer phytoplankton and can develop algal blooms as a result of cell rapid growth. As a consequence, they affect the ecosystem of water bodies causing mass mortalities in fish and taste and odor problems in drinking water. These events have occurred recently in Argentina, dating just from the last two decades, thus the interest in studying this species has increased. In addition, it is important to emphasize the fact that it is not only important to estimate the number of microorganisms in a sample, but also to devote more attention to the estimation of biovolume and biomass of individual species [10]. In this sense, because of *C. Hirundinella* large size, they might actually contribute a major fraction of the overall biomass in mixed-species samples.

The present study is performed in water samples containing *C. Hirundinella* collected from the La Cienega dam, Jujuy Province, Argentina. *C. Hirundinella,* are dinoflagellates microorganisms of 80–400 μm in length. They are strongly compressed dorsoventrally, they have an apical long and narrow horn with a blunt tip and antapical horns straight with pointed closed tips. The antapical horns are normally slightly diverged from each other distally; they also present an excavation in the ventral side, providing an additional geometrical complexity. The quite variable shape of the cell requires complicated equations for the biovolume calculations by geometrical approximations, demanding several lengths and diameters measurements per cell [8]. These properties are evidenced out in the photograph of a conventional microscope field in Fig. 1, taken with a 10 × Microscope Objective (MO). Moreover, a certain degree of uniformity of cell size distribution is observed. As it can be seen in Fig. 1, the largest number of *C. Hirundinella* cells in a sample lie on frontal position, while some others lie in dorsoventral position, as shown in the highlighted area of the same figure.

Taking advantage of this fact and of the size uniformity, the calculation of biovolume is performed using a single hologram of the specimen at each of these positions. From holograms of the cell in frontal position, phase delay introduced is obtained. This information is combined with an average cell thickness extracted from the silhouette measured from holograms of the individuals that appear in dorsoventral position. The calculation, which does not require any sophisticated devices, is part of an automated system for determination of the relative abundance of microorganisms in water samples, whose main aim is to provide a portable tool that can be used by specialist on the sampling site to perform biovolume measurements *"in situ"*.

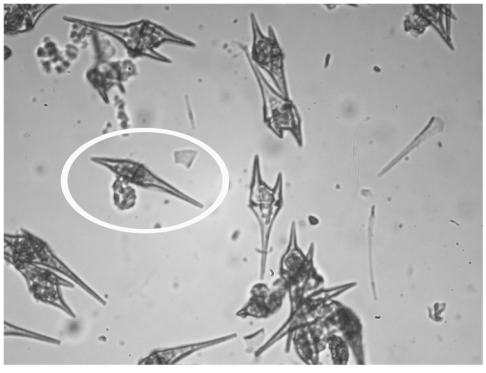

Fig. 1. A typical field of view of a water sample containing *C. Hirundinella*, taken with a conventional microscope.

2. Instruments and methods

2.1. Holograms registration and reconstruction

The transmission DHM and phase image reconstruction techniques used for the present study have been described in Refs. [11,12,21]. Briefly, they consist of recording a hologram by means of an interferometric set-up, onto a solid state array detector such as a CCD or CMOS sensor and, subsequently, numerically reconstructing the information by means of a computer. A layout of DH Microscope prototype constructed for this purpose, is depicted in Fig. 2(a).

Essentially, it is a Mach–Zehnder interferometer, whose object arm is fitted with a small microscope built by inserting an *X*–*Y* microscope stage to put the sample and a microscope objective (MO) which acts as a magnifying lens and forms a real image of the specimen, as it is illustrated in Fig. 2(b). To facilitate the automatic search of a field containing microalgae, the plate of the DH microscope is driven by two step motors to orderly scan consecutive fields of view.

The most remarkable feature of this architecture, early proposed by Van Ligten and Osterberg [22], is that the hologram plane (sensor plane) is located between the MO and the image plane at a distance *d* from the latter. This is equivalent to a holographic arrangement without lenses, with an object wave emanating directly from the magnified image instead of the object itself. Therefore, the hologram consists of an unfocused image of the sample modulated by the interference fringes formed by the object and the reference beams, as shown in Fig. 3(a). The polarized collimated light beam originated at a He–Ne laser of wavelength $\lambda = 632.8$ nm is divided by a beam splitter BS_1 in order to obtain the object and the reference beams. The plane wave that travels through the object arm, after being reflected by the mirror M_1, illuminates the sample to be analyzed. The wave diffracted by the specimen, is collected and magnified by a 20 × /0.40 NA or a 10 × / 0.25 NA objective, MO_1. At the same time, the plane wave that travels through the reference arm is reflected by the mirror M_2, and magnified by MO_2, with the same characteristics of MO_1, to match the curvatures of the two wave fronts. The object beam and reference beam are recombined in the second beam splitter, BS_2, and interfere at the output of the interferometer. A TV camera, with a CMOS Bayer Array 2592 × 1944 pix^2, 1.75 μm square pixels, 8 bit deep and a frame rate up to 25 Hz is used to record this interference pattern (digital holograms). A grabbed frame of this sequence cropped at 512 × 512 pix^2 size is temporarily stored in the buffer of an image digitizer board for further processing.

The reconstruction of the original microscopic field of view of the sample is performed digitally on a computer. This procedure simulates the reconstruction process in conventional holography, which consists of illuminating the hologram with a replica of the reference beam *R* used in the registration stage. Literature describes several methods to retrieve the complete information of the object wave in both, amplitude and phase [11,12]. In this application, the reconstruction of holograms is carried out by using the angular spectrum propagation method. The reconstructed wave front, both in amplitude and phase, are shown in Fig. 3 (b) and (c) respectively. The latter is a two-dimensional phase distribution called the wrapped phase image. Since this wrapped phase suffers from 2-π phase jumps, it is unusable until the phase discontinuities are removed. Therefore, a procedure of phase unwrapping must be performed in order to recover the true continuous phase values to denote real physical quantity. Many phase unwrapping algorithms have been developed during the last three decades [23–27]. In this application, the quality guide phase unwrapping algorithm [27], is used to retrieve the continuous phase