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A protocol for the simultaneous identification of chitin-containing particles and their associated bacteria

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ABSTRACT

Chitin is the second most abundant polymer on Earth, playing a crucial role in the biogeochemical cycles. A core issue for studying its processing in aquatic systems is the identification and enumeration of chitin-containing particles and organisms, ideally in a manner that can be directly linked to bulk chitin quantification. The aim of this study was the development of such a technique. We successfully combined the methodology of bulk chitin determination using wheat germ agglutinin (FITC-WGA) for staining chitin-containing particles and organisms along with CARD-FISH staining of either chitin-containing eukaryotic cells or bacteria associated with them. Environmental chitin staining was successfully applied to natural water samples. Fungal hyphae, diatoms, and dinoflagellates, sestonic aggregates and chitin-containing structures derived from metazoa were observed. Also, hybridized bacteria attached to chitinaceous debris were clearly visualized. Finally, as proof of principle, cultured yeast cells were simultaneously-targeted by FITC-WGA and the EUK516 probe without exhibiting any interference between both stains. The presented approach appears as a powerful tool to evaluate the contribution of different size classes and organisms to chitin production and consumption, opening the possibility for application of single-cell approaches targeting the ecophysiology of chitin transformations in aquatic systems.

Introduction

Chitin is a natural polymer widely distributed in nature, which is mostly found in fungi and invertebrates [22]. In marine systems, euphausiids and copepods are the main chitin producers [26,38], since it is a component of their exoskeletons, exuviae and faecal pellets [28,60]. In addition, there are also some diatom genera, such as *Thalassiosira* and *Skeletonema*, which produce chitin as a significant constituent of their biomass [54]. The polymer is also present in the cyst walls of some ciliates, flagellates and amoebae, in the lorica walls of certain ciliates, and in the cell walls of fungi [22,59].

Chitin plays a crucial role in the carbon and nitrogen biogeochemical cycles [55]. A continual rain of chitin is deposited on the ocean floor that is formed by dynamic processes such as molting cuticles and the senescence of planktonic organisms. This chitin supply forms part of the particulate aggregates known as "marine snow" [3]. These aggregates harbour rich detrital communities of eukaryotic and prokaryotic organisms, which represent an important site for the biological processes of production, decomposition and nutrient recycling in the water column [2].

Despite the continuous deposition of this highly insoluble polymer, low quantities of chitin are found in ocean sediments [43], a phenomenon which mainly results from the activity of chitin-degrading bacteria [7,22]. In addition, certain fungi and diatoms are also capable of chitin hydrolysis [41,56,58]. Although it has been estimated that only a very low fraction of bacterial communities is chitinolytic, a much higher proportion is able to utilize chitin hydrolysates [47]. Despite the long-standing recognition of the specific interaction of certain members of the aquatic bacterial communities with chitin particles (*i.e. Vibrio cholera* [44]), the production of extracellular chitinases has been detected in other bacterial species [29]. In fact, it has recently been proposed that bacterial community composition might actually play a significant role in chitin degradation rates modulated by environmental

factors such as temperature and abundance of zooplankton and phytoplankton [8]. Therefore, the identification and quantification of chitin-containing particles, and their associated bacteria, is necessary for a deeper understanding of chitin processing in aquatic environments.

Typically, chitin-containing cells have been enumerated by employing the fluorescence brightener Calcofluor [15,24,25,46], which interacts with β-linked D-glucopyranose polysaccharides, including chitin. However, it preferentially stains cellulose, which is another polysaccharide frequently found in the particles and organisms of aquatic systems [31]. More recently, to overcome this specificity issue, and to be able to combine chitin staining using fluorescent *in situ* hybridization together with oligonucleotide probes (FISH), Würzbacher and Grossart [59] proposed the use of the fluorescently labeled chitin-binding domain (CBD) to detect and identify aquatic fungi. In addition, fungi have also been quantified in aquatic samples using CARD-FISH, without specific chitin counter staining [27].

However, none of these techniques provides a direct link for the identification and quantification of chitin-containing particles with bulk estimates of chitin concentration. A simple method for bulk chitin determination in water and sediment samples relies on the staining of chitin-containing particles with fluorescein succinylated wheat-germ agglutinin (FITC-WGA) [34]. WGA is a sugar-binding protein that has a high affinity for *N*-acetylglucosamine residues [4], which is the major component of chitin. WGA has been shown to bind specifically to this polymer, even when samples contain high concentrations of cellulose, clay and bacteria [34], and it is more specific than Calcofluor for the determination of chitin [32].

Despite the fact that WGA can also react weakly with *N*-acetyl-neuraminic acid residues [14,21], which are found in fungi and other biological samples [5,10], Monsigny et al. [33] reported that, in contrast to the native lectin, succinylated-WGA did not recognize *N*-acetyl-neuraminic acid residues, but still maintained the same affinity for *N*-acetylglucosamine residues as the native lectin. Nevertheless, due to its affinity for *N*-acetylglucosamine residues, WGA could also potentially stain the thick peptidoglycan layer of Gram-positive bacteria [50,57].

In histological studies, this lectin has been widely used to localize chitin in the cell walls of fungi [19,32,52], as well as in molluscs and insects [40]. It is also employed to enumerate diatoms and crustacean eggs containing chitin [17,42].

Although FITC-WGA staining of chitin to date has not been used together with CARD-FISH, Bennke et al. [9] proposed a technique combining CARD-FISH and staining with (other) fluorescent lectins, demonstrating the potential of such combined methods to identify bacteria associated with extracellular polymeric substances.

In this study, a novel combined protocol was developed for FITC-WGA staining of chitin-containing particles and organisms together with CARD-FISH staining of chitin-containing eukaryotic cells and/or their associated bacterial cells.

Materials and methods

A set of samples from Bahia Blanca Estuary (BBE, Argentina) was used for developing the protocol. Water was pre-filtered with a 20 μm net, in order to obtain the size fraction ≤20 μm that previous studies had shown contains the major bulk fraction of chitin in certain marine environments, including BBE [11,22,30].

Pre-filtered samples were fixed with freshly prepared buffered paraformaldehyde solution (PFA) at a final concentration of 1% at 4 °C for 24 h. Portions of 10 mL were then filtered through 25 mm diameter type GTTP polycarbonate filters with a 0.2 μm pore size

(GAMAFIL). The filters were rinsed twice with sterile phosphate buffered saline (PBS) and stored at −20 °C until further analysis.

In order to provide a known positive control for the technique, a culture of commercial baker's yeast (*Saccharomyces cerevisiae*) was established in sterile water amended with saccharose. After visible cell growth, and within 24 h of starting the cultivation, the yeast cells were fixed with PFA at a final concentration of 1% for 1 h at room temperature.

Once established, the protocol was also performed on samples from the Atlantic Uruguayan coast, in order to check its applicability to a different system, as well as samples from BBE that had not been pre-filtered previously through 20 μm mesh. Samples from the Atlantic Uruguayan coast were also fixed with 1% PFA, and filtered through 0.2 μm pore size 47 mm diameter polycarbonate filters (Millipore). Whole water samples from BBE were fixed and filtered as described above.

The new protocol was set up by combining the methodology of chitin determination using wheat-germ agglutinin (FITC-WGA) [34] and the protocol for bacterial identification using *in situ* hybridization with horseradish peroxidase (HRP)-labeled probes linked to catalyzed reporter deposition (CARD-FISH) [39]. The reagent solution for FITC-WGA was prepared by adding 8 μL FITC-WGA (Vector Laboratories) to 2 mL phosphate buffer (1 M Na_2HPO_4, pH 9.25). For CARD-FISH, the following HRP-labeled probes (biomers.net, Ulm, Germany) were used: EUB338 I-III (most *Bacteria*) [16] for hybridization of natural water samples, and EUK516 (most *Eukarya*) [6] for staining the yeast culture. Between 1 and 3 μL of a 50 ng μL^{-1} stock solution of each HRP probe were employed for every 100 μL of hybridization buffer [0.9 M NaCl, 20 mM Tris–HCl, pH 8.0, 0.02% SDS, 1% blocking reagent (Roche), 55% formamide]. After hybridization, unbound probe was removed by incubating the samples in washing buffer [20 mM Tris–HCl, pH 8.0, 5 mM EDTA (pH 8.0), 0.003 M NaCl, 0.01% SDS]. Samples were then incubated in 1X PBS [0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4]. Tyramide conjugated with red-fluorescing Alexa 594 (molecular probes) was used to reveal the hybridizations in order to distinguish them from green-fluorescing WGA chitin staining. Following washing, the samples were incubated with tyramide-containing amplification buffer [2 M NaCl, 10% dextran sulfate, 0.1% blocking reagent (Roche), 1X PBS, 0.15% H_2O_2, 1 μg μL^{-1} tyramide]. A final wash in 1X PBS was carried out for another 15 min. CARD-FISH + WGA-stained preparations were counterstained with DAPI at a final concentration of 1 μg mL^{-1}, in order to check for possible artifacts in their respective signals.

Microscopic images were captured by a Leica DMLS2 fluorescence microscope with a Nikon DXM1200F camera, as well as an Olympus IX81 with a CCD Model DP71 camera, and a confocal Olympus BX61 laser microscope. Microscopic images were processed with the J Image program [18].

Different tests were carried out in order to establish the final protocol. In particular, the following were tested:

i) Whether common cell permeabilization protocols for CARD-FISH interfered with FITC-WGA staining. Assayed permeabilization protocols were: a) treatment with 10 mg mL^{-1} lysozyme (AppliChem, Darmstadt, Germany) for 1 h at 37 °C for natural water samples, or b) treatment with 1 mg mL^{-1} proteinase K (Ambion, USA) for 1 h at 37 °C for natural samples, and for one and four hours at 37 °C for the yeast culture.

ii) Different temperatures and times for FITC-WGA staining and washing. Staining was performed on natural samples at: a) 35 °C for two hours, b) 35 °C for three hours, and c) overnight at 30 °C. Washing after staining was undertaken in 1X PBS at room temperature for either 15 or 30 min in the dark. The assayed temperatures and times for staining were chosen taking into account the protocol for chitin determination [34] and CARD-
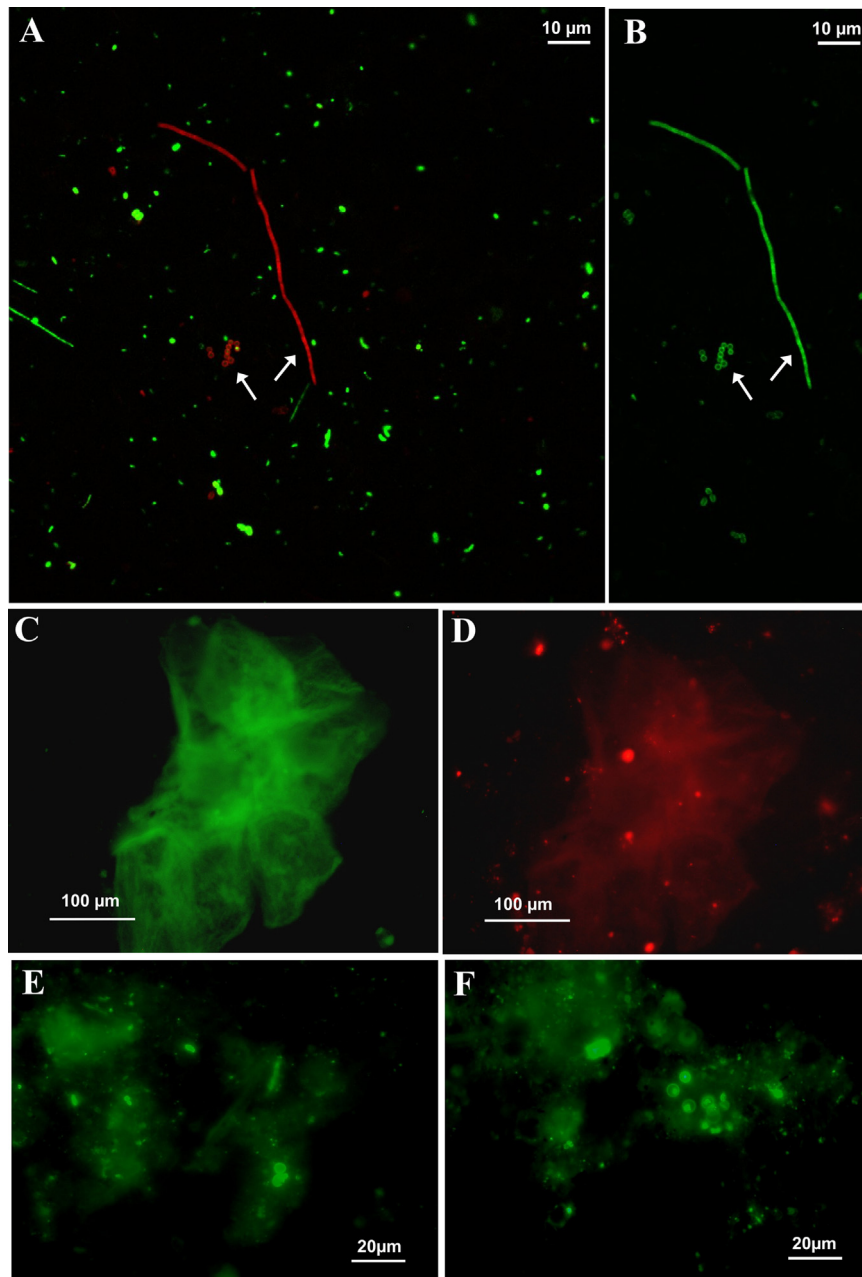
Fig. 1. (A and B) Sample treated with lysozyme. Microscopic images captured by an Olympus BX61 confocal laser microscope (100×) from brackish water samples (Uruguayan coastal lagoon-Rocha). The arrows in panel A highlight cyanobacterial-like cells visibly stained with FITC-WGA in panel B. (C–F) Microscopic images captured by a Leica DMLS2 fluorescence microscope (100×). Samples treated with proteinase K. (C) Particle (possibly remains of an exoskeleton) stained with FITC-WGA (green) and associated bacteria (EUB I-III hybridization-D-red) (BBE sample). (E and F) Metazoan structure (agglomerate vesicles) stained with FITC-WGA (green) captured by an Olympus IX81 fluorescence microscope (100×) from a Uruguayan coastal lagoon. The two panels show the same sample either stained with WGA at 35 °C for 2 h (E) or at 30 °C overnight (F). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

FISH [28]. A temperature of 30 °C overnight and, at most, 33 °C for 4 h were optimal for the determination of chitin in aquatic samples [34], whereas standard CARD-FISH protocols were run at 35 °C. On this basis, an increase in temperature and a reduction in the time were tested for staining with WGA, discarding the combinations 30 °C for two hours and 35 °C overnight.

iii) Whether hybridization with HRP-probes interfered with FITC-WGA staining of the same sample and *vice versa*. To check whether hybridization with HRP-probes interfered with FITC-WGA staining, hybridization was performed before and after FITC-WGA staining. This assay was carried out on natural samples.

iv) Whether hybridization and FITC-WGA staining could be simultaneously carried out. The yeast culture was used to check if hybridization and FITC-WGA staining could be simultaneously performed on the same cell, and the order of the procedures was also tested.

Furthermore, the capability of visualization and enumeration of FITC-WGA stained particles with flow cytometry was tested for both the yeast culture and the natural samples. For this purpose, fixed liquid samples of either yeast culture or natural planktonic communities were stained with FITC-WGA at 35 °C for two hours. After staining, samples were centrifuged at 12,000 rpm and 15 °C for 5 min, and washed twice with sterile 1X PBS. After the sec-
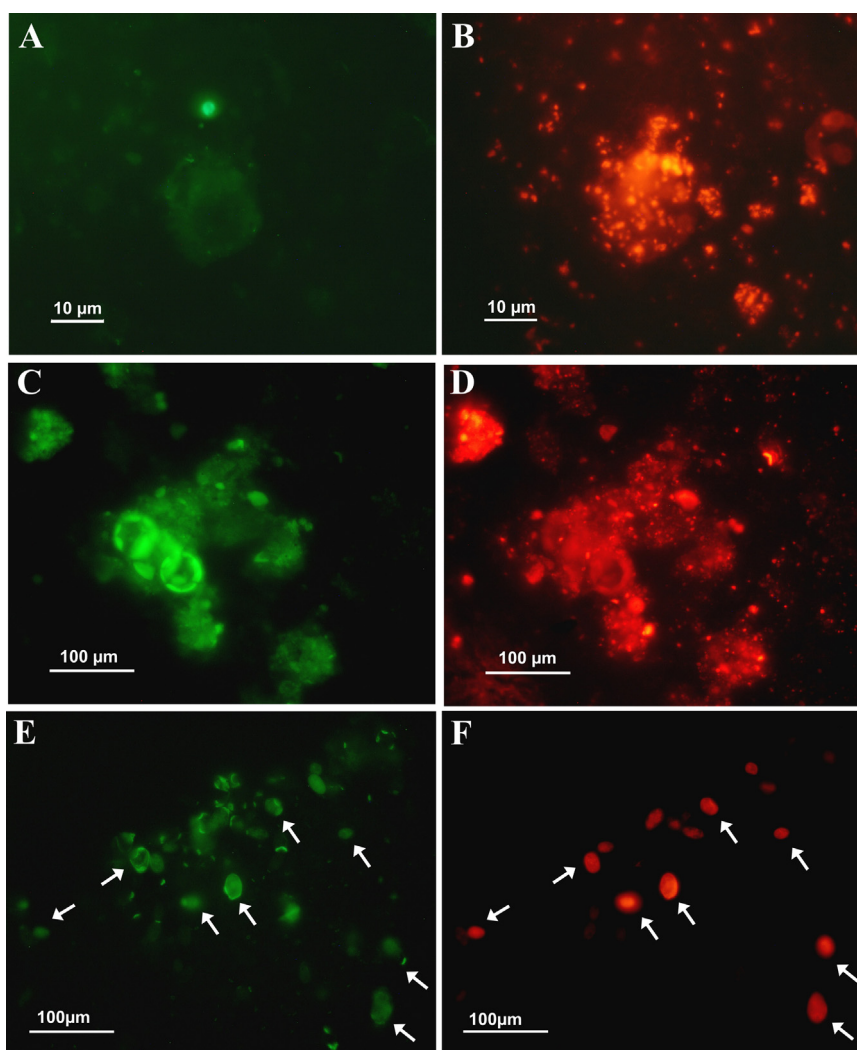
Fig. 2. Microscopic images captured by a Leica DMLS2 fluorescence microscope (100×). (A–D) Sestonic aggregate from the BBE sample composed of diatom debris colonized by bacteria stained with FITC-WGA either before (A and B) or after (C and D) the bacterial hybridization procedure. (E and F) Yeast cells stained with FITC-WGA and hybridized with EUK 516.

ond wash, the sample was re-suspended with sterile Milli-Q® (MQ) water and analyzed in a flow cytometer (MicroA50, Apogee Flow Systems) according to its green fluorescence and side scatter.

Results and discussion

The CARD-FISH lysozyme permeabilization protocol did not interfere with FITC-WGA staining (Fig. 1A and B). In fact, samples treated with lysozyme showed more permanent and less photosensitive staining than those not previously treated. Furthermore, permeabilization with proteinase K did not interfere with WGA staining either (Fig. 1C and D).

An improvement in FITC-WGA staining after the lysozyme treatment agreed with the observation that some lysozymes (*i.e.* the egg-white lysozyme used here) may also hydrolyze chitin [35], although at a lower level than chitinases [51]. On the other hand, chitin is generally associated with proteins or with other polysaccharides in nature [12,20,37], and proteinase K treatment might have improved the accessibility of WGA to chitin in particles and cell walls. Moreover, the use of proteinase K to remove protein partially from fungal spore walls has been shown to result in a substantial improvement in the percentage of stained spores [45,48]. Thus, treatment with any of the enzymes typically used in the

CARD-FISH permeabilization step is in fact advantageous for staining chitin-containing particles.

Regarding the FITC-WGA staining temperature and time, the optimal settings were either two hours at 35 °C or overnight at 30 °C (Fig. 1E and F). Washing samples with 1X PBS at room temperature was equally suitable for 15 or 30 min when little chitinaceous material was found in the sample, while washing for 30 min was preferable when the samples were charged with chitin-containing particles, which helped to ensure low-level background.

Hybridization with HRP-labeled probes did not interfere with FITC-WGA staining, and *vice versa*. FITC-WGA stained particles were perfectly visible after hybridization (Fig. 2A and B) and hybridized bacteria were equally visible after FITC-WGA staining (Fig. 2C and D). Thus, FITC-WGA staining could be applied before or after hybridization without any noticeable change in the quality of the images obtained.

Bennke et al. [9] suggested that fluorescent lectin binding analysis (FLBA) was better performed after the hybridization procedure because the lectin-specific signals were stronger compared to labeling carried out before CARD-FISH. However, in our final protocol, it was recommended to perform FITC-WGA staining prior to hybridization in order to reduce the time of total processing, allowing hybridization to proceed overnight. In addition, re-staining with

Table 1
Detailed protocol for the FITC-WGA and CARD-FISH combination.

Procedure	Step	Description
Preparation of samples	1	Embed filters in 0.1% agarose
	2	Air dry the filters
Permeabilization alternatives:		
A—with lysozyme	3	Incubate filters in lysozyme solution (10 mg mL^{-1}) (37 °C, 1 h)
	4	Wash with MQ water several times (room temperature—RT)
B—with proteinase K	3	Incubate filters in proteinase K solution (1 mg mL^{-1}) (37 °C, 1 h for natural samples, 4 h for yeast culture)
	4	Wash with MQ water several times (1 min at RT)
Inactivation of peroxidases	5	Incubate filters in 0.01 M HCl (1 mL/filter) (20 min at RT)
	6	Wash filters with MQ water several times (RT)
	7	Air dry the filters
Staining with FITC-WGA	8	Incubate filters with a drop of 10–30 μL FITC-WGA at 30 °C overnight or at 35 °C for 2 h
	9	Incubate filters with 1X PBS for 15–30 min at room temperature (RT)
	10	Wash filters with MQ water three times (1 min each at RT)
	11	Air dry the filters
Hybridization	12	Mix hybridization buffer and probe working solution and add to filter sections
	13	Incubate filters for at least 2 h or overnight at 35 °C
	14	Wash filters in pre-warmed washing buffer (37 °C, 15 min,)
	15	Incubate filters in 1X PBS for 15 min at RT
Amplification and washing	16	Incubate filters in substrate mix with Alexa 594-conjugated tyramide for 15 min at 37 °C in the dark
	17	Incubate filters in 1X PBS for 15 min at 37 °C in the dark
	18	Wash filters with MQ several times
	19	Air dry the filters in the dark
Staining with DAPI	20	Stain with DAPI (1 μg mL^{-1} solution) for 3 min in the dark
	21	Rinse with MQ water, air dry and mount the filter on a microscope slide

FITC-WGA was also possible prior to DAPI staining, if needed, without resulting in an increased intensity green background (Fig. 2C).

Finally, it was also possible to combine chitin staining and phylogenetic staining of the same organism (baker's yeast); therefore, opening up the possibility of further identification of chitin-containing cells (Fig. 2E and F).

A detailed protocol of all the steps for sample processing of simultaneous FITC-WGA and CARD-FISH is provided in Table 1

Environmental chitin staining was successful for natural water samples, and there was no interference with the observation of different structures and organisms even in samples exhibiting high background fluorescence (Fig. 3). Background fluorescence has been described for some lectins after performing fluorescent lectin staining on agarose-embedded samples [9]. Although in the current study this was not specifically tested for, there were no empirical clues that this could be the case for this lectin. In most of the samples, the background was very clean and dark (Fig. 3C, D and F), and even when samples showed a brighter background, the target signals (*i.e.* found in recognizable organisms) were still much brighter than the background (Fig. 3A, B and E). Thus, it was found that interference with agarose embedding was not a problem for this lectin, at least in the set of samples used.

Individual chitin-containing organisms, such as fungal hyphae, as well as diatoms and dinoflagellates, were identified in the samples (Fig. 3A–C). In addition, chitin-containing structures derived from metazoans were visible (Figs. 1E–F, 3D).

Unexpectedly, it was possible to observe WGA staining of the peripheries of cells that seemed to be *Cyanobacteria* (Fig. 1A and B). Although chitin is known as a widespread polymer that accounts for an important biomass fraction of different organisms [17,22,53,54,59], these result highlighted how direct visualization of natural samples can certainly provide new insights into its distribution among planktonic cells. Nevertheless, the results should be carefully interpreted with regard to possible cross-reactivity. As mentioned in the introduction, the use of the derivative succinylated-WGA improves specificity to sialic acid residues found in fungi and other organisms [14,21], but the affinity for the exposed peptidoglycan layer of Gram-positive bacteria is still an option,

which is particularly relevant in freshwaters where these organisms can constitute a high fraction of the bacterial community [49].

In addition to individual organisms, it was possible to stain sestonic aggregates composed of diatoms, bacteria, fungal hyphae and other chitin-containing cells (Figs. 2A–D, 3C, E and F). Although in this set of samples the exclusively chitin-made debris was not extensively colonized, it was still possible to visualize hybridized bacteria associated with them (Fig. 1C and D). In contrast, presumed senescent chitin-containing cells were heavily colonized (Fig. 2A–D).

Finally, it was possible to detect FITC-WGA stained cells using flow cytometry, which were seen to form distinctive cytometric populations (data not shown), therefore, providing an opportunity for counting and sorting different chitin-containing particles in the pico- and nanoplankton size range.

The results of this study indicated that the new protocol simultaneously using FITC-WGA and CARD-FISH had great potential for the identification and quantification of organisms producing and/or degrading chitin in aquatic systems, both under fluorescence microscopy and, potentially, by flow cytometry. In combination with bulk measurements of chitin obtained by FITC-WGA staining [34], it therefore represents a powerful tool for evaluating the contribution of different size classes and organisms to chitin production and consumption.

Moreover, the possibility of simultaneous identification of chitin-containing organisms and their associated bacteria represented an excellent complementary approach to recent metagenomic-based studies analyzing the diversity of chitinolytic members of microbial communities [8]. Direct microscopic visualization of these associations can provide further insights into the key players involved in chitin processing, as has been successfully demonstrated for other relevant biogeochemical processes, such as anaerobic methane oxidation [13].

Microscopy can also reveal the extent of the specificity of associations between chitin producers and consumers, as already shown for the phytoplankton-flavobacterial associations based on cell-surface molecule interactions [9]. Furthermore, in combination with isotopic tracer-based techniques, it can be used to estimate
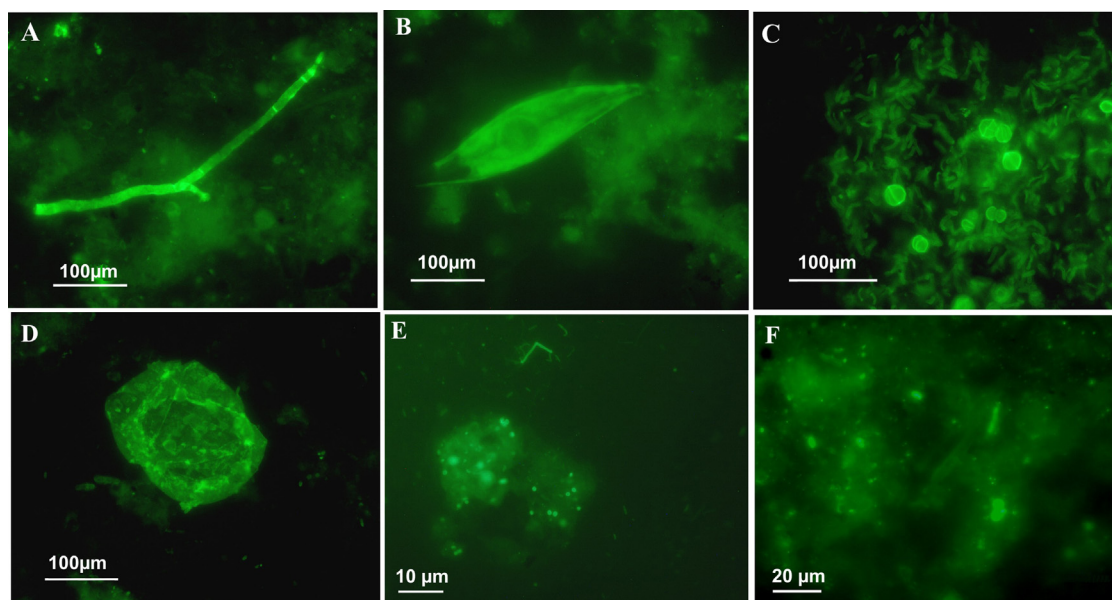
Fig. 3. (A–E) Microscopic images captured by a Leica DMLS2 fluorescence microscope (100×) of FITC-WGA stained particles and cells. (A) Fungi (hypha) stained in brackish water samples (Uruguayan coastal lagoon). (B) Diatom in the BBE sample. (C) Dinoflagellates and possible hyphae aggregated in a marine sample (BBE). (D) Particle (possibly metazoan remains) from the BBE sample. (E and F) Sestonic aggregates apparently composed of diatom debris, fungal hyphae and other cells (such as cyanobacteria) from BBE (E) and a Uruguayan coastal lagoon (F).

the rates of chitin synthesis and hydrolysis at the single-cell level, shedding light on the eukaryotic-prokaryotic interactions involved [1], and even leading to a quantitative assessment of the contribution of target taxa [36]. Altogether, the new protocol represents a further contribution to the understanding of the tight coupling between chitin production and consumption, as shown by the rapid turnover of this abundant polymer in aquatic systems [23].

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