



# Phylogenetic and morphological characterisation of the green algae infesting blue mussel *Mytilus edulis* in the North and South Atlantic oceans

Francisco Rodríguez<sup>1,5</sup>, Stephen W. Feist<sup>2</sup>, Laure Guillou<sup>1</sup>, Lisbeth S. Harketstad<sup>3</sup>, Kelly Bateman<sup>2</sup>, Tristan Renault<sup>4</sup>, Stein Mortensen<sup>3,\*</sup>

<sup>1</sup>Station Biologique de Roscoff, UMR 7144 CNRS et Université Pierre et Marie Curie, BP74, 29682 Roscoff, Cedex, France

<sup>2</sup>Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Road, Weymouth, Dorset DT4 8UB, UK

<sup>3</sup>Institute of Marine Research, PO Box 1870, Nordnes, 5817 Bergen, Norway

<sup>4</sup>IFREMER, Laboratoire de Génétique et Pathologie, 17390 La Tremblade, France

<sup>5</sup>Present address: Centro Oceanográfico de Canarias (IEO), Ctra. San Andrés 45, 38180 S/C Tenerife, Spain

**ABSTRACT:** Blue mussels *Mytilus edulis* with shell deformations and green pustules containing parasitic algae were collected at 3 coastal sites (Burøy, Norway; Bockholm, Denmark; Goose Green, Falkland Islands). A comparative study, including mussel histopathology, algal morphology, ultrastructure and phylogenetic position was performed. Green pustules were mainly located in the posterior portion of the mantle and gonad tissues and the posterior adductor muscle. Electron microscopy confirmed the presence of algal cells with similar morphology to *Coccomyxa parasitica*. Algae were oval shaped with a single nucleus and chloroplast, 1 or 2 mitochondria and a dense granular cytoplasm with a lipid inclusion body, Golgi apparatus and small vesicles. Partial small subunit (SSU) rRNA phylogeny confirmed the inclusion of parasitic algae into the *Coccomyxa* clade. However, the sequence identity between almost full SSU rRNA sequences of parasitic algae and others in this clade yielded an unexpected result. Green algae from mussels were distant from *C. parasitica* Culture Collection of Algae and Protozoa (CCAP) strain 216/18 (94 % identity), but very similar (99 % identity) to *C. glaronensis* (a lichen endosymbiont) and green endophytes from the tree *Ginkgo biloba*. The CCAP strain 216/18 was a sister sequence to *Nannochloris* algae, far from the *Coccomyxa* clade. These results suggest a misidentification or outgrowth of the original CCAP strain 216/18 by a different 'Nannochloris-like' trebouxiophycean organism. In contrast, our sequences directly obtained from infested mussels could represent the true *C. parasitica* responsible for the green pustules in blue mussels.

**KEY WORDS:** Blue mussels · *Coccomyxa parasitica* · *Mytilus edulis* · *Mytilus edulis chilensis* · Parasitic alga · Phylogeny

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## INTRODUCTION

The green algae genus *Coccomyxa* (Trebouxiophyceae, Chlorococcales, Coccomyxaceae) includes both free-living planktonic marine and freshwater species (Guiry et al. 2005), epiphytic (Lamenti et al. 2000) and symbiotic species with lichens (Lohtander et al. 2003), protozoans (Hoshina & Imamura 2008) or even trees (e.g. in *Ginkgo biloba*; Trémouillaux-Guiller et al. 2002). Two marine species, *Coccomyxa ophiurae* and *C. para-*

*sitica*, have been described as parasitic, the former in starfish (Mortensen & Rosenvinge 1933) and the latter in giant scallops *Placopecten magellanicus* (Naidu & South 1970, Stevenson & South 1974, 1975) and blue mussels *Mytilus edulis chilensis* (Boraso de Zaiexo & Zaiexo 1979, Bala 1995, Gray et al. 1999).

The first description of *Coccomyxa parasitica* was made by Naidu & South (1970) in *Placopecten magellanicus* from insular Newfoundland, Canada. The presence of *C. parasitica* in the Northern Hemisphere

was later reported in the West Kattegat, a bay of the Baltic Sea bounded by Denmark and Sweden (Nielsen et al. 1995). Infestation of bivalves by such green algae have been regularly reported in the literature, and several hosts are suspected to be infected by *C. parasitica*, including oysters and heart cockles *Clinocardium nuttalli* (Hartman & Pratt 1976). Main characteristics for *C. parasitica* are an absence of flagella and pyrenoid, a single set of chloroplasts, mitochondrion and Golgi apparatus, and reproduction by autospore formation. Most of these features are in fact common for numerous other trebouxiophycean coccoid species (Henley et al. 2004).

*Coccomyxa parasitica* was initially considered as parasitic because it may cause severe damage in heavily affected individuals, in particular important shell deformities and significant loss of dry weight compared with healthy hosts (Naidu 1971, Gray et al. 1999). The prevalence of *C. parasitica* in mussels from the Falkland Islands can reach 23%, and was considered to reduce the reproductive rate in infected populations (Gray et al. 1999). The parasitic relationship was previously reported as facultative, based upon the culture of *Coccomyxa parasitica* (Culture Collection of Algae and Protozoa [CCAP] strain 216/18) on an inorganic medium by Stevenson & South (1974). The observation of digested algae inside mussel haemocytes aggregating around algal colonies suggests that the mussel can counteract the infections (Gray et al. 1999, Mortensen et al. 2005).

Mortensen et al. (2005) reported the presence of green pustules in soft tissues of *Mytilus edulis*, resembling the earlier descriptions of *Coccomyxa parasitica* in *M. edulis chilensis* in the South Atlantic Ocean (Gray et al. 1999). This and previous studies on bivalves infested by *C. parasitica* were, however, only supported by morphological features. Published small subunit (SSU) rRNA phylogenies of *Coccomyxa* isolates include only lichen, protozoan photobionts and the green endophyte in *Ginkgo biloba* (Trémouillaux-Guiller et al. 2002, Lohtander et al. 2003, Karsten et al. 2005, Hoshina & Imamura 2008). The phylogenetic relationship with their marine counterpart, *C. parasitica*, has not been determined. In the present article we report data on the SSU rRNA phylogeny, histopathology, ultrastructure and pigments of the green algae infesting blue mussels in the North Sea and the Falkland Islands. Finally, SSU rRNA was also obtained for the unique available strain labelled as *C. parasitica* (CCAP 216/18) for comparison purposes.

## MATERIALS AND METHODS

**Sample collection.** Blue mussels showing shell deformations as described by Gray et al. (1999) and

Mortensen et al. (2005) were collected at 3 sites. The Norwegian *Mytilus edulis* sample was collected from a shallow water site at Burøy, near Kragerø, southern Norway (58° 50' N, 9° 35' E) (see Mortensen et al. 2005). The Danish *M. edulis* sample was collected from the lower part of the intertidal zone at Bockholm, Flensburg Fjord (54° 51' N, 09° 35' E), where mussels with green pustules were reported by Meixner (1984). Mussels were transported live to the Institute of Marine Research (IMR), Bergen, Norway, and placed in a quarantine tank with aerated, recirculated water at 7°C. Blue mussels *M. edulis chilensis* in the Falkland Islands were collected at Goose Green (51° 58.49' S, 58° 58.07' W) (see Gray et al. 1999). The mussels were packed on ice in a container and transported by air to Oxford, UK, transferred by car and plane by a commercial transport service to IMR and held as described previously. All mussels were acclimated to the same temperature and salinity conditions prior to further transport and sampling. Mussels for pigment analysis and phylogenetic studies were wrapped in humid paper attached to a cool block and transported live to Roscoff, France, by overnight mail for further sampling.

**Examination of infested mussels.** From each sample, 20 affected specimens were opened and examined for macroscopic lesions. The locations of green pustules were noted. For light microscopy, 5 × 10 mm samples from tissues with green pustules were removed with dissection scissors and fixed in Davidson's fixative (Shaw & Battle 1957), embedded in paraffin, sectioned at 3 µm thickness, stained with hematoxylin and eosin and observed using a Leica DMRBE microscope at 100 to 1000× magnification.

For transmission electron microscopy, mantle fragments containing green pustules were fixed in Karnovsky's fixative, and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer before dehydration through a graded acetone series. Specimens were embedded in epoxy resin and semi-thin sections stained with toluidine blue were prepared for light microscope evaluation for selection of areas for ultrastructural analysis. Ultrathin sections (70 to 90 nm thickness) were mounted on copper grids and stained with uranyl acetate and Reynolds' lead citrate (Reynolds 1963). Grids were examined using a JEOL JEM 1210 transmission electron microscope and digital images captured using a Gatan Erlangshen ES500W camera and Gatan Digital Micrograph™ software.

**Pigment analysis.** Green pustules from infested mantle and/or adductor muscle sections were thawed and centrifuged, and the green coloured supernatant filtered onto 25 mm diameter GF/F Whatman filters and stored at -20°C until further analysis. In addition, we tried to grow free-living cell cultures of *Coccomyxa* from infested mussel tissues of each geographical site

using F/2 and Keller media in 10 ml volume tubes. Unfortunately, we were unable to cultivate the parasitic green algae and only 2 strains (a small diatom and a filamentous alga containing a siphonaxanthin-like carotenoid) were isolated. HPLC pigment separations were performed following the method of Zapata et al. (2000). Frozen filters were thawed in 1 ml of 95% methanol and the extract was refiltered through 25 mm diameter GF/F Whatman filters to remove cell and filter debris. Pigment extracts (200  $\mu$ l) were mixed with 40  $\mu$ l Milli-Q ultrapure water to avoid peak distortion (Zapata & Garrido 1991) and immediately injected into a Hewlett–Packard HPLC 1100 Series System equipped with a quaternary pump and diode array detector. All sample preparations were made under subdued light. Chlorophylls and carotenoids were detected by absorbance at 440 nm and identified by diode array spectroscopy (wavelength range: 350 to 750 nm, 1 nm spectral resolution). Pigments were identified from retention time and comparison of their visible UV light ( $UV_{vis}$ ) spectrum with those stored on a pigment database developed using previous analyses of phytoplankton cultures.

**DNA extraction and phylogenetic analysis.** Algal pustules were cut off from mantle and adductor muscle pieces and broken with a spatula. The green supernatant was taken with a syringe and centrifuged in Eppendorf microcentrifuge tubes. After discarding the supernatant, concentrated dark green pellets were used for molecular analyses. DNA was extracted using a modified 1% N-cetyl N,N,N-trimethylammonium bromide (CTAB) protocol (Ishida et al. 1999). The entire SSU rRNA gene was amplified using the oligonucleotide primers 5'-ACCTGGTTGATCCGCC AG-3' and 5'-TGATCCTTCYGCA GGTTAC-3', as described by Moon-van der Staay et al. (2001). PCR products were cloned using the TOPO<sup>®</sup> Taq amplified (TA) cloning kit (Invitrogen) following the manufacturer's recommendations. PCR products were screened by restriction fragment length polymorphism (RFLP) to assess genetic polymorphism, as both the DNA from the host and the parasite could be amplified. For this purpose, PCR products (10  $\mu$ l) were digested with 1 U  $\mu$ l<sup>-1</sup> *Hae*III (GIBCO BRL) for 3 h at 37°C and separated by electrophoresis. SSU rRNA sequences were determined using the Plateforme de Séquençage-Génotypage at the Station Biologique of Roscoff, France. Some chimeras with SSU rRNA regions from *Mytilus edulis* were obtained and eliminated prior to subsequent analyses. Finally, almost complete SSU rRNA for the green algae in Flensburg fjord (1767 and 1778 bases for clones 1 and 2, respectively) and partial sequences for Kragerø (1450 bases) and Falkland Islands (544 bases both clones) sites were obtained. Sequences of SSU rRNA of the green algae infesting

mussels from the 3 sampling sites were deposited in GenBank under accession numbers EU127470 to EU127474. Full SSU rRNA sequence from the reference culture *Coccomyxa parasitica* CCAP 216/18 was also obtained for the first time in this study (accession no. EU127469). The phylogenetic analysis included partial length SSU rRNA from other trebouxio-phyceans deposited in GenBank for a total of 35 sequences in the final data set. Two outgroup trebouxio-phycean sequences, *Trebouxia impressa* and *T. asymmetrica*, were used to root the trees. Partial SSU rRNA sequences (corresponding to sites 61 to 557 in the full 18S alignment) were aligned using CLUSTALW multiple alignment in BioEdit (Hall 1999). Poorly aligned positions and divergent regions were checked using the GBLOCKS software (Castresana 2000). A final number of 485 bases (93% from the original 519 characters) was kept by GBLOCKS indicating that a good set of sequences was included in our analysis. To assure the quality of this method, we compared the phylogenies obtained after using (or not) GBLOCKS. Both partial trees were almost identical (slightly modifying a few bootstrap values, but not the branching pattern) and the original tree based on 519 characters was kept. We have previously compared the results obtained with the partial SSU rRNA against a phylogenetic tree using almost complete sequences (~1270 positions from 1450 available ones after GBLOCKS analysis). That tree showed an almost identical topology, but was less informative because it included a shorter data set (only 3 green algae from mussels and fewer similar sequences from GenBank); therefore, we included only the partial phylogeny using 519 bases. Different nested models of DNA substitution and associated parameters were estimated using Modeltest 3.06 (Posada & Crandall 1998). The Akaike information criterion (AIC) in Modeltest selected GTR+I (General Time Reversible, proportion of invariable sites = 0.5144) and distribution of rates at variable sites with shape parameter ( $\alpha$ ) = 0.8502. Relative base frequencies were  $\pi_A$  = 0.2903,  $\pi_C$  = 0.2128,  $\pi_G$  = 0.2522 and  $\pi_T$  = 0.2447. The maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML) methods were used for the phylogenetic analysis (PAUP\*4.0b10 version, Swofford 2002) and bootstrap values were estimated from 1000 replicates.

## RESULTS

### Gross morphology

Shell and soft part morphology of the 3 batches of affected blue mussels appeared similar, with a dominance of old animals (3+ yr) being affected, exhibit-

ing a blunt shell shape and various degrees of malformation at the posterior end. Green pustules were located in the posterior end of the mantle and gonad tissues and the posterior adductor muscle, as described by Mortensen et al. (2005). Mussels from the Falkland Islands differed from the others by additionally exhibiting infections in the dorsal connective tissue covering the pericardium. These specimens also had a high number of pearls in mantle tissues (Fig. 1A).

### Histopathology

Microscopy of affected mantle tissues from the 3 batches of blue mussels showed similar features. In most infected mussels, the mantle edge was most severely affected. Additionally, large infected areas were observed around pearls and in gonadal acini. Pustules consisted of dense aggregations of

algae, haemocytes and cell debris, with a variable algal:haemocyte ratio and variable amount of debris. In the Danish samples some pustules were filled with a relatively high number of haemocytes and necrotic material, and fewer intact algae cells were seen than in the Norwegian samples. Algae were also observed freely (Fig. 1D) and as small aggregates within connective tissues and within vacuoles in the cytoplasm of affected cells (Fig. 2A). Electron microscopy confirmed the presence of multiple algal cells in the tissues of mussels from all 3 locations. In samples from Denmark, tissue and cell necrosis was moderate and algal cells were generally present within host haemocytes, many of which appeared degenerate (Fig. 2A). Infected haemocytes became rounded, losing pseudopodial extensions, and contained condensed pyknotic nuclei and numerous cytoplasmic vacuoles and secondary lysosomes. In several cases, mitochondria demonstrated hydropic swelling and contained angular inclusions lacking a laminated crystalline struc-

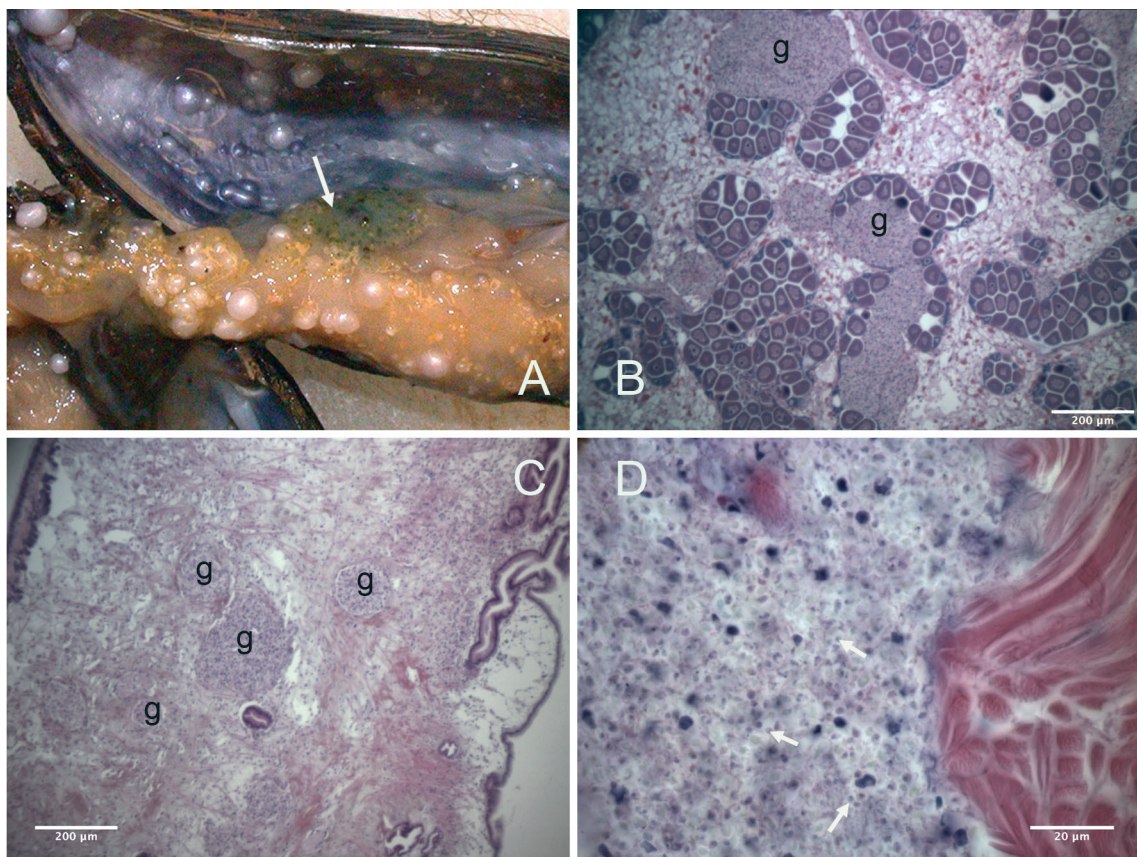


Fig. 1. *Mytilus edulis*. (A) *M. edulis chilensis* from the Falklands Islands, with a high number of pearls. Area infected with algae is indicated (arrow). (B) Histological section showing part of the mantle and gonad from a heavily infected *M. edulis* from Kragerø, revealing the green pustules as large granulomatous lesions (g) filled with algae, haemocytes and cell debris, both in the connective tissue and in female acini. (C) Part of the mantle from *M. edulis* collected at Flensburg Fjord, showing similar granulomatous lesions (g). (D) Area in the posterior adductor muscle of a *M. edulis chilensis* specimen from the Falklands Islands filled with algae, haemocytes and cell debris. Algae can be observed freely in affected areas (arrows)

ture. Affected tissues in mussels from Norway and the Falklands appeared largely necrotic with numerous algal cells distributed amongst the cellular debris. In most cases the algal cells appeared to be intact with a small proportion showing evidence of degenerative changes.

### Ultrastructure of algae

Algae identified in electron micrographs prepared from each of the different samples were intracellular and appeared very similar in size and structure (Table 1, Fig. 2B–D). Thirty algae were measured from

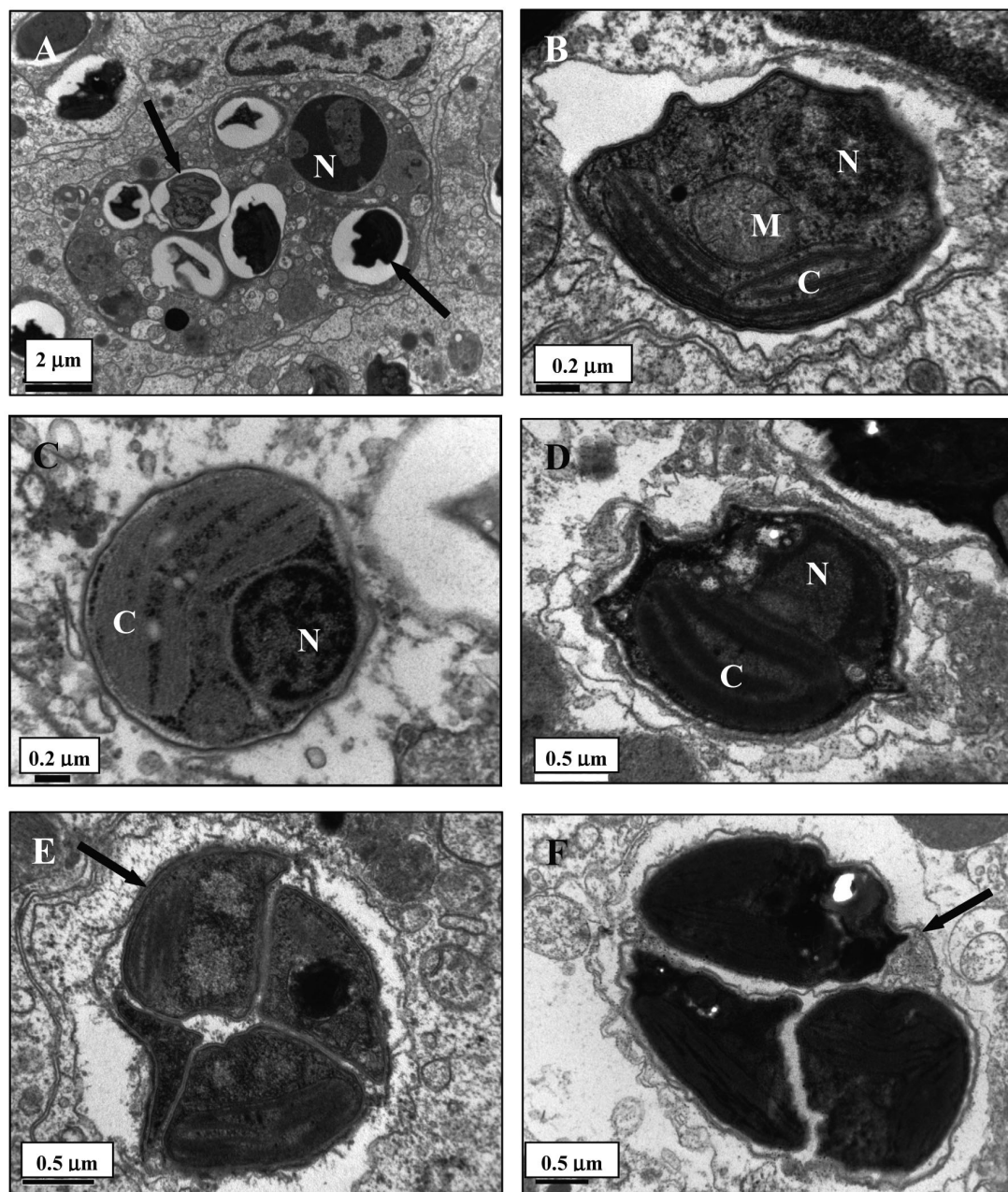


Fig. 2. Electron micrographs of *Coccomyxa*-like algae in pustules from blue mussels collected in Norway, Denmark and the Falkland Islands. (A) Affected cell, presumed to be a moribund mussel haemocyte with several unicellular algae within intracytoplasmic vesicles (arrows). An electron-lucent area is observed around algae. The affected cell contains a pyknotic nucleus (N) with a highly condensed chromatin and dense cytoplasm containing numerous vesicles. (B, C & D) Unicellular algae in samples from Denmark, Norway and the Falkland Islands, respectively. The oval algae cells clearly display a dense cytoplasm containing one chloroplast (C), nucleus (N) and a mitochondrion (M). (E & F) Algae dividing to form 4 unicellular daughter cells surrounded by a common membrane (arrows); (F) only 3 daughter cells are visible in the plane of section

Table 1. Comparison of the morphological characteristics of algae from affected blue mussels *Mytilus edulis* from Norway and Denmark and *M. edulis chilensis* from the Falkland Islands, based on measurements from electron micrographs. Check mark (✓) indicates presence

	Denmark	Falkland Islands	Norway
Length ( $\mu\text{m}$ , mean $\pm$ SE)	2.26 $\pm$ 0.05	2.45 $\pm$ 0.04	2.17 $\pm$ 0.05
Width ( $\mu\text{m}$ , mean $\pm$ SE)	1.43 $\pm$ 0.03	1.59 $\pm$ 0.04	1.48 $\pm$ 0.03
Nuclei	1	1	1
Chloroplast no.	1	1	1
Mitochondria no.	1–2	1–2	1–2
Golgi	✓	✓	✓
Vesicles	✓	✓	✓
Lipid inclusion body	✓	✓	✓
Daughter cell no.	4	3–4	4

each sample; algae had an oval shape with projections and measured 2.1 to 2.4  $\mu\text{m}$  in length and 1.4 to 1.5  $\mu\text{m}$  in width. Algae contained 1 nucleus and chloroplast, 1 or 2 mitochondria and a dense granular cytoplasm, which contained a lipid inclusion body, the Golgi apparatus and small vesicles. These features were identified in each of the 3 samples. All samples contained algae dividing to form 4 unicellular daughter cells (Fig. 2E,F).

### Pigment profile

HPLC pigment analysis of samples from the North and South Atlantic Ocean samples are shown in Fig. 3. Typical carotenoid composition from Chlorophyta was obtained including neoxanthin, violaxanthin and lutein, with traces of zeaxanthin in the green algae isolated from mussels of Flensburg Fjord (Fig. 3B). Additional unknown peaks of carotenoids (absorbance maximum, 454 and 484 nm) were observed, but their elution time did not match with any algal carotenoid, at least to our knowledge, which suggests they originated from the mussels' tissues. Chlorophyll *b* was the sole accessory chlorophyll detected.

### Phylogenetic analysis

The phylogenetic analysis (ML, MP and NJ methods) based on partial SSU rRNA sequences (Fig. 4) placed all the green algae infesting blue mussels in a subgroup within a major clade including *Coccomyxa* species, other green photobionts and the freshwater free-living chlorophyte *Paradoxia multiseta*. This clade contained 3 *Coccomyxa* isolates, and 5 sequences of green photobionts retrieved from lichens, the protozoan *Paramecium bursaria*, and *Ginkgo biloba*. Based on a larger SSU rRNA alignment (~1270 bases) an

almost identical tree topology was obtained (not shown), but it was less informative as a shorter data set and only 3 sequences from mussels could be included. The 5 sequences of green algae from mussels exhibited low variability despite their distant geographical source (range of sequence identity, 0.972 to 0.992 over a common 519 bases alignment). The closest sequences to the green algae from blue mussels in Flensburg Fjord (the ones with almost full SSU rRNA) were those of *Coccomyxa glaronensis* and endophytes from *G. biloba* (sequence identity, 0.988 to 0.992; 14 to 15 different

nucleotides excluding gaps over a common 1755 bases alignment; 0.984 sequence identity over 1450 bases alignment for Kragerø sequence). In comparison, their sequence identity against *C. parasitica* was remarkably low (0.938 to 0.943) for Flensburg Fjord isolates (107 to 110 different nucleotides) and 0.936 for Kragerø

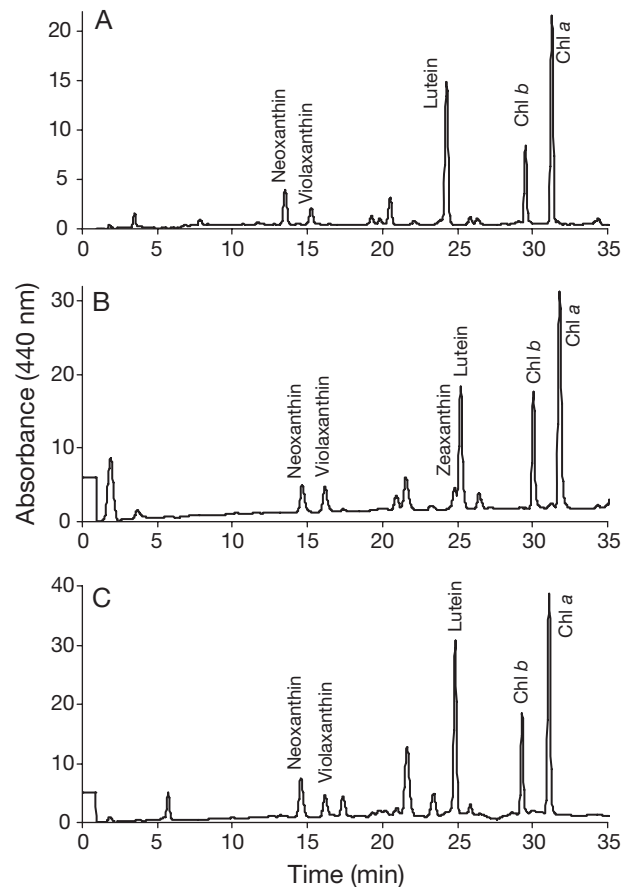


Fig. 3. HPLC pigment profile from the green algae infesting blue mussels. (A) Kragerø, (B) Flensburg Fjord, (C) the Falkland Islands

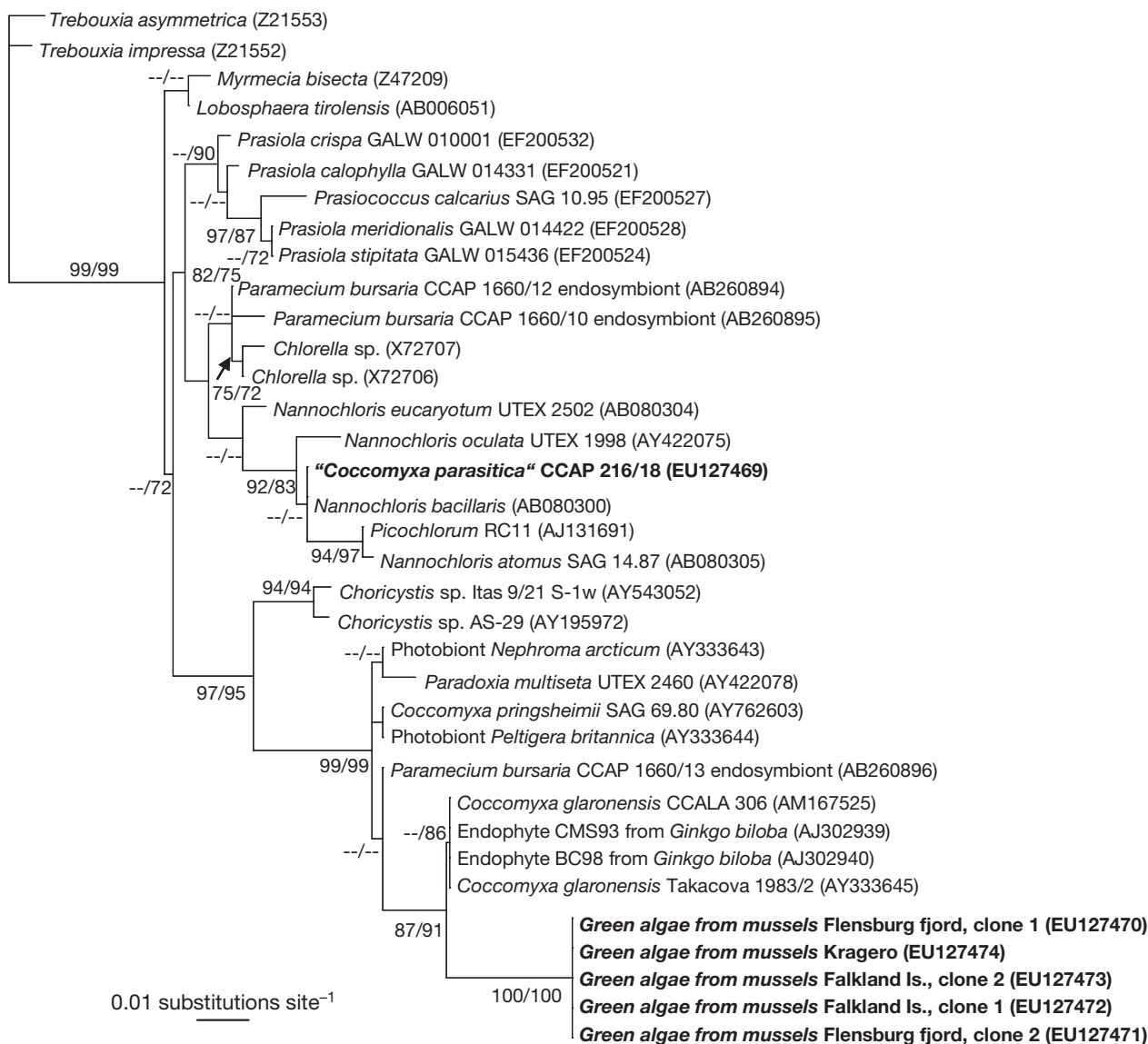


Fig. 4. Phylogenetic position of the parasitic green algae found in blue mussels. The phylogenetic tree was derived from partial SSU rRNA sequences (519 bases) from trebouxiophyceans (Chlorophyta) including free-living species and endosymbionts in land plants, lichens and protozoan and marine invertebrates. The phylogenetic tree shown was inferred by the maximum likelihood method based on a GTR+I model of DNA substitutions. New sequences obtained from this study are highlighted in **bold**. GenBank accession numbers are given in parentheses beside strain names. Bootstrap values for major clades are indicated above internodes and correspond to NJ and MP methods, respectively. Bootstrap values <70% are indicated by hyphens

sequence. SSU rRNA from *C. parasitica* CCAP 216/18 (originally isolated from the giant scallop) was sequenced for the first time in this study. Surprisingly, it was placed in our analysis as a sister sequence to several *Nannochloris* and *Picochlorum* strains, indicating that it did not belong to the *Coccomyxa* clade. The SSU rRNA from the green endosymbiont ('Zoochlorelae'; Lewis & Muller-Parker 2004) in the anemone *Anthopleura elegantissima* was not included in our phylogenetic analysis because it only overlapped 270 nucleotides in our alignment. However, NJ and MP

trees based on these characters (not shown) yielded a similar overall phylogeny and placed the 'Zoochlorelae' as a separate sister sequence to the major clade of *Coccomyxa* and green photobionts.

## DISCUSSION

Some previous publications describe the presence of green algae in blue mussel soft tissues: 3 in the South Atlantic Ocean (the Falkland Islands, Gray et al. 1999;

the Gulf of San José, Argentinean Patagonia, Boraso de Zaixso & Zaixso 1979, Bala 1995) and 2 in the North Atlantic Ocean (Flensburg in the Baltic Sea, Meixner 1984; the southern Norwegian coast, Mortensen et al. 2005). Algae isolated from the South Atlantic Ocean mussels were reported as *Coccomyxa parasitica* based on microscopical observations, while the taxonomic position of the others remained unclear.

The present sampling of live blue mussels from 3 of the described coastal sites (Goose Green, Falkland Islands; Burøy, Norway; Bockholm, Denmark) has enabled us to perform a comparative study of these cases about the taxonomic relationship between the algal strains and other green endosymbionts. Our examinations showed that affected mussels from the 3 sites appeared similar. Samplings were not strictly standardised, and differences in morphology and pathology observed on a histopathological level could, thus, be due to both speciation of mussels and conditions at the sampling points and time. However, examinations showed that both shell morphology and algae pustules had the same appearance, although the mussels from the Falkland Islands differed from the others by additionally exhibiting infections in the dorsal connective tissue covering the pericardium. The histopathological examination of affected tissues and pustules revealed similar images of the infection and to those described previously in *Coccomyxa parasitica* in mussels (Gray et al. 1999). There were also morphological similarities between the algae described in our study and the original *C. parasitica* described from the giant scallop (Stevenson & South 1974). However, they reported a *C. parasitica* that was both larger (mean dimensions  $5.5 \times 3.0 \mu\text{m}$ ,  $n = 50$ ) and more variable in size (range, 1 to  $11 \mu\text{m}$ ) and autospore formation (2, 4 and 8-celled autosporangia in the host) than the algae described in our study.

A comparison of the 3 samples showed infections in different stages, adding some information to the understanding of the progress of the infection. In the Danish sample we observed a stage where pustules were filled with a large numbers of haemocytes associated with necrotic material, and where fewer intact algae cells were seen than in the Norwegian sample. Together with the observation of disintegrating haemocytes, this indicates that there may be a balance between the propagation of the algae and the mussels' defense mechanisms, resulting in a dynamic process where the severity of algal infections could vary over time.

Electron micrographs verified the histopathological findings, revealing necrotic material inside the pustules, with phagocytosed algae inside haemocytes. Several haemocytes appeared to be disintegrating. The ultrastructure of algae within mussel tissues and

cells from the 3 locations were remarkably similar. Unicellular alga and dividing stages appeared identical, supporting the molecular and pigment profile evidence and indicating a close relationship among the 3 algal isolates examined in this study.

Other endophytic trebouxiophyceans from lichens, protozoa and land plants (e.g. *Ginkgo biloba*), including probably the genus *Coccomyxa*, are known to establish a symbiotic relationship with their hosts. Although the type of association between *Coccomyxa* and their invertebrate marine hosts is not fully clear, the damaged shells and reduced body condition in *Placopecten magellanicus* suggested its parasitic nature (Naidu 1971). Interestingly, *Coccomyxa* algae have strong hydrophobicity on their cell surface, which facilitates adhesion to surfaces (Lamenti et al. 2000). This mechanism could help to initiate the colonisation of mussels by *Coccomyxa*.

In the South Atlantic Ocean (Gulf of San José, Argentina) Bala (1995) reported green algae infesting only the blue mussels *Mytilus edulis platensis* (Trucco 2000, also cited as *M. edulis chilensis*) at 8% prevalence ( $n = 4504$ ), while other sympatric mytilids and pectinids ( $n = 14092$ ) remained unaffected. The marked similarity among the SSU rRNA sequences from green pustules in our study suggests that the same species was responsible for mussel infestation in the North and South Atlantic oceans.

The 94% similarity between almost full SSU rRNA alignments (1755 bases) of *Coccomyxa parasitica* CCAP 216/18 and the green algae infesting mussels from Flensburg Fjord was well under the 97% cutoff traditionally used at the species level in rRNA studies (Hagström et al. 2000). To explain the unexpected phylogenetic position of CCAP 216/18 as a 'Nannochloris-like' alga in our study we hypothesize that either this strain was originally misidentified or that the initial *C. parasitica* culture was substituted by a different organism, namely the monospecific strain now labelled as CCAP 216/18. The culture of CCAP 216/18 is now maintained in F/2 medium by the CCAP while originally Stevenson & South (1974) reported its isolation using Erdschreiber medium (which includes a soil extract), among others. Similar media (e.g. modified Erdschreiber and Bold's basal medium) are used for all non-marine *Coccomyxa* CCAP strains (freshwater, lichen phycobiont or soil origin,  $n = 32$ ), while the 2 available marine strains (CCAP 216/17 and 216/18, the former isolated from a rock pool) are maintained in F/2. Our several attempts to isolate *C. parasitica* from mussels using F/2 and K media were unsuccessful. This could suggest that F/2 and K recipes do not fully meet the nutritional requirements of *C. parasitica*, in contrast with other media including soil extracts, reinforcing the phylogenetic evidence about the taxonom-



ical placement of CCAP 216/18 out of the *Coccomyxa* clade. In contrast, the high sequence identity of our green algae from mussels relative to *C. glaronensis* and the green endophytes from a *Ginkgo biloba* sample would correspond with the true *C. parasitica* responsible for the green pustules in *Mytilus edulis*. These results emphasize the importance of phylogenetic studies in order to obtain solid conclusions about the taxonomic position of microalgae. This is particularly crucial in the case of small picoplanktonic algae such as those considered in the present work, which lack distinctive morphological characters. Finally, despite the SSU rRNA similarities among *C. glaronensis*, green endophytes in *G. biloba* and *Coccomyxa* in mussels, there are important ecophysiological differences in these organisms. In *Ginkgo biloba* green endophytes exist in an immature precursor form with a non-functional plastid (Trémouillaux-Guiller & Huss 2007) while *C. glaronensis* and *Coccomyxa* in blue mussels are fully developed cells retaining photosynthetic capacities.

## CONCLUSIONS

The present study has documented a similar histopathology in blue mussels *Mytilus edulis* from the North Sea and *M. edulis chilensis* from the Falkland Islands, infected by parasitic *Coccomyxa*-like algae. Different stages of infection were found, but overall, the same tissues were affected and similar morphological appearances (size and structure) of the parasitic algae were observed. Molecular results, however, indicated a distant relationship between the reference culture belonging to *Coccomyxa parasitica* CCAP 216/18, originally isolated from the giant scallop, and *Coccomyxa*-like algae infesting mussels from all 3 sites. Morphological characters from reference algal isolates deposited in culture collections are the unique features upon which most species are described. These criteria establish recognizable but dynamic boundaries between the various taxonomic levels. However, in the case of small algal cells with a limited set of morphological characters, ultrastructure, pigments and molecular markers (e.g. SSU rRNA) provide crucial taxonomic information. The *Coccomyxa* genus represents a perfect example, as it comprises several picoplanktonic species including free-living and parasitic forms in marine invertebrates, previously only characterised by morphology. This study outlines the importance of obtaining DNA sequence data to confirm the taxonomic assignment of small coccoid organisms, which, as in the case of *C. parasitica* CCAP 216/18 we presume to represent no longer the original parasitic algae, but a different trebouxiophycean species (a

*Nannochloris*- or *Picochlorum*-like organism). Our sequences obtained in the present study could represent the true *C. parasitica* responsible for the green pustules in blue mussels present in both the northern and southern hemispheres.

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## LITERATURE CITED

- Bala LO (1995) Specificity and prevalence of the endobiosis of *Coccomyxa parasitica* (Chlorophyta: Chlorococcales) in *Mytilus edulis platensis* (Mollusca: Bivalvia). *Nat Patagonia* 3:1–9
- Boraso de Zaixso A, Zaixso H (1979) *Coccomyxa parasitica* Stevenson & South endozoica en *Mytilus edulis*. *Physis* 38:131–136
- Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 17:540–552
- Gray AP, Lucas IAN, Seed R, Richardson CA (1999) *Mytilus edulis chilensis* infested with *Coccomyxa parasitica* (Chlorococcales, Coccomyxaceae). *J Molluscan Stud* 65: 289–294
- Guiry MD, Rindi F, Guiry GM (2005) AlgaeBase version 4.0. National University of Ireland, Galway. Available at: [www.algaebase.org](http://www.algaebase.org), accessed 27 Feb 2008
- Hagström Å, Pinhassi J, Zweifel UL (2000) Biogeographical diversity among marine bacterioplankton. *Aquat Microb Ecol* 21:231–244
- Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Hartman AC, Pratt I (1976) Infection of the heart cockle, *Clinocardium nuttalli*, from Yaquina Bay, Oregon, with an endosymbiotic alga. *J Invertebr Pathol* 28:291–299
- Henley WJ, Hironaka JL, Guillou L, Buchheim MA, Buchheim JA, Fawley MW, Fawley KP (2004) Phylogenetic analysis of the 'Nannochloris-like' algae and diagnoses of *Picochlorum oklahomensis* gen. et sp. nov. (Trebouxiophyceae, Chlorophyta). *Phycologia* 43:641–652
- Hoshina R, Imamura N (2008) Multiple origins of the symbioses in *Paramecium bursaria*. *Protist* 159:53–63
- Ishida K, Green BR, Cavalier-Smith T (1999) Diversification of a chimaeric algal group, the Chlorarachniophytes: phylogeny of nuclear and nucleomorph small-subunit rRNA genes. *Mol Biol Evol* 16:321–331
- Karsten U, Friedl T, Schumann R, Hoyer K, Lembcke S (2005) Mycosporine-like aminoacids and phylogenies in green algae: *Prasiola* and its relatives from the Trebouxiophyceae (Chlorophyta). *J Phycol* 41:557–566
- Lamenti G, Tiano P, Tomaselli L (2000) Biodeterioration of ornamental marble statues in the Boboli Gardens (Florence, Italy). *J Appl Phycol* 12:427–433
- Lewis LA, Muller-Parker G (2004) Phylogenetic placement of 'Zoochlorellae' (Chlorophyta), algal symbiont of the temperate sea anemone *Anthopleura elegantissima*. *Biol Bull (Woods Hole)* 207:87–92
- Lohtander K, Oksanen I, Rikkinen J (2003) Genetic diversity

- of green algal and cyanobacterial photobionts in *Nephroma* (Peltigerales). *Lichenologist* 35:325–339
- Meixner R (1984) On a microalgal infection of *Mytilus edulis*. Shellfish Committee, Ref: Marine Environmental Quality Committee, Internal Council for the Exploration of the Sea, 1984/K:30
- Moon-van der Staay SY, De Watcher R, Vaulot D (2001) Oceanic 18S rDNA sequences from picoplankton reveal unsuspected eukaryotic diversity. *Nature* 409:607–610
- Mortensen T, Rosenvinge LK (1933) Sur une nouvelle algue, *Coccomyxa astericola*, parasite dans une asterie. *K Danske Vidensk Selsk Biol Meddel* 10:2–8
- Mortensen S, Harketstad LS, Stene RO, Renault T (2005) Picoeucaryot alga infesting blue mussel *Mytilus edulis* in southern Norway. *Dis Aquat Org* 63:25–32
- Naidu KS (1971) Infection of the giant scallop, *Placopecten magellanicus* (Gmelin), from Newfoundland with an endozoic alga. *J Invertebr Pathol* 17:145–157
- Naidu KS, South GR (1970) Occurrence of an endozoic alga in the giant scallop *Placopecten magellanicus* (Gmelin). *Can J Zool* 48:183–185
- Nielsen R, Kristiansen A, Mathiesen L, Mathiesen H (1995) Distributional index of the benthic macroalgae of the Baltic Sea area. *Acta Bot Fenn* 155:1–51
- Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics* 14:817–818
- Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J Cell Biol* 17:208–212
- Shaw BL, Battle HI (1957) The gross and microscopic anatomy of the digestive tract of the oyster *Crassostrea virginica* (Gmelin). *Can J Zool* 35:324–347
- Stevenson RN, South GR (1974) *Coccomyxa parasitica* sp. nov. (Coccomyxaceae, Chlorococcales), a parasite of giant scallops in Newfoundland. *Br Phycol J* 9:319–329
- Stevenson RN, South GR (1975) Observations on phagocytosis of *Coccomyxa parasitica* (Coccomyxaceae; Chlorococcales) in *Placopecten magellanicus*. *J Invertebr Pathol* 25:307–311
- Swofford DL (2002) PAUP\*. Phylogenetic analysis using parsimony (\*and other methods), v4. Sinauer Associates, Sunderland, MA
- Trémouillaux-Guiller J, Huss VA (2007) A cryptic intracellular green alga in *Ginkgo biloba*: ribosomal DNA markers reveal worldwide distribution. *Planta* 226:553–557
- Trémouillaux-Guiller J, Rohr T, Rohr R, Huss VAR (2002) Discovery of an endophytic alga in *Ginkgo biloba*. *Am J Bot* 89:727–733
- Trucco MI (2000) Diferenciación genética con polimorfismos alozímicos de *Mytilus* spp. del Atlántico Sudoccidental. PhD dissertation, University of Vigo
- Zapata M, Garrido JL (1991) Influence of injection conditions in reversed phase high-performance liquid chromatography of chlorophylls and carotenoids. *Chromatographia* 31:589–594
- Zapata M, Rodríguez F, Garrido JL (2000) Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C8 column and pyridine-containing mobile phases. *Mar Ecol Prog Ser* 195:29–45

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