Protists, Cyanobacteria, Rotifers and Crustacea from the hypersaline ponds of Messolonghi Saltworks (W. Greece)

Salt and the environment

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Keywords: Protists; cyanobacteria; rotifers; crustacea; hypersalinity; Messolonghi saltworks

Abstract

During a survey in 2015 an impressive assemblage of organisms were found in the hypersaline ponds of the Messolonghi saltworks. The salinity ranged between 50 and 210 ppt and the organisms recorded fell in the categories of Cyanobacteria (22 species), Chlorophytes (5 species), Dinoflagellates (1 species), Protozoa (51 species), Diatoms (27 species), Rotifers (9 species), Copepods (1 species), *Artemia* sp. and one nematode. *Fabrea salina* was the most prominent protist in all samples and salinities. This ciliate has the potential to be a live-food candidate for marine fish larvae. *Asteromonas gracilis* proved a sturdy microalga performing excellently in a broad spectrum of culture salinities. Most of the specimens were identified only to the genus level and, based on their morphology, as there are no relevant records in Greece, there is a possibility for some of them to be either new species or strikingly different strains of certain species recorded elsewhere.

Introduction

Considering the lack of concise information on the organisms other than microbes from hypersaline environments in Greece, a preliminary survey in the salterns of Messolonghi (W. Greece) was made during spring and summer of 2015. The aim was to identify at least to the genus level all visible by optical microscopy organisms in order to get an idea of their presence and abundance, to be used as guide for future more elaborated studies in this biotope. Further more to test the maintenance and culture of as many of them as possible in laboratory conditions, in order to be used as live food for cultured fishes and other use in general. There is a rather annoying confusion concerning especially the exact identification of cyanobacteria and protists in the way they are presented in various papers. Scanning the literature there is a scarcity of usable picturing material to be used as a guide for similar works. In fact it is impossible for a young researcher to be guided through the species mentioned in scientific papers unless there are representative pictures given. The situation is much perplexed considering cyanobacteria and protists (algae and protozoa) from hypersalinity, a topic only scarcely studied. This is the reason why in the present study it was implemented a general scouting as a first attempt, with the aim to gain experience and to plan future monitoring in a more sound way. Pictures and live videos were taken by microscopy and a representative material is presented here. For identification, various studies were used as guides (Fontaneto & De Smet, 2015; Lee et al, 2000; Ricard, 1987; Sournia, 1986; Tomas, 1996; Foissner & Berger, 1996).

Materials & Methods

The water samples were taken from a particular evaporation pond of the Messolonghi saltern during March-September 2015 on a monthly basis thus following the salinity range (50-210 ppt) of the changing water condition. The samples were only water with no benthos included. A 2 L plastic beaker was used and the surface of the bottom was just touched in order to be disturbed just enough for its surface layer to be included in the water taken. Salinity and temperature were recorded and the samples were immediately transported to the laboratory where the temperature was kept at 21 ^{o}C. The samples were kept in 2 L Erlenmeyer conical flasks, lit with ambient light of about 1000 lux and aerated using pumped air via a pipette. 2

sub-samples of 50 ml were taken and centrifuged mildly at 3000 rpm for 3 min after which the sediment with 2 ml of water was kept for microscopical examination. The decanted supernatant was free of organisms as all of them were sedimented and then kept in the re-suspended 2 ml of the vial. There was no adverse effect of the centrifugation on the motility and viability of the organisms. The procedure of the 50 ml sub-samples was repeated for 3 successive days in order to strengthen the detection. The 2 ml concentrated samples were apportioned to 0,1 ml sub-samples as droplets in shallow glass petri dishes and examined stereomicroscopically in order to count organisms of a size bigger than 30 μm in general. That includes all protozoa, rotifers, copepods, Artemia and nematodes. After a thorough counting assessment of live specimens, a drop of Lugol was poured and the immobilized creatures were again counted. The other 2 ml sample was kept intact (no Lugol) in order to be examined microscopically. Additionally, from the live samples, organisms were removed by micropipette-suction and placed in 50 ml conical flasks with nutrient fertilized water of similar salinity in order to roughly check their suitability for growth in culture conditions. The abundance of the various organisms (less than 30 μm in size) in the sub-samples mixtures taken from the various salinities samples was calculated as the counted individuals of each species in a 1 mm^2 area of the microscopy vision field. Counts were used for comparisons among salinities for a rough estimation of abundance. All photos presented here were taken from live specimens (Figures 1-7).

Results & Discussion

The organisms found (Table 1) can be categorized as Cyanobacteria, Protozoa, eucaryotic microalgae, rotifers, copepods, Artemia and a nematode. The salinity range clearly demarcated the presence of some organisms from other. In particular, at salinities over 160 ppt only *Artemia* sp., *Dunaliella salina*, *Asteromonas gracilis, Fabrea salina* and cocciform cyanobacteria were detected and were able to stay alive and grow at similar (with their occurrence) salinities in laboratory conditions. A peculiar finding was that although the cyanobacteria were massively detected at those elevated salinities their subsequent attempted culture at similar salinities in the laboratory gave poor results. It seems that a combination of elusive parameters in their particular natural habitat fullfil their needs. In the salinity range of 110-160 ppt much more organisms (included those previously mentioned) were recorded with representatives of all categories except rotifers and copepods. At salinities of 60-110 ppt cyanobacteria, rotifers and protozoa were most abundant compared to their presence in higher salinities. *Fabrea salina* dominated in all salinities, it was easily mass cultured at almost every salinity in the range of 35-150 ppt, being thus a candidate live food for larval marine fish. At salinities higher than 160 ppt, *F. salina* encysts and can remain viable for long time, reviving again after lowering the salinity below 50 ppt. The copepod *Tisbe* sp. also exhibited a remarkable viability at a wide range of salinities (35 – 90 ppt) and was easily cultured with high reproduction rate, feeding avidly on a wide spectrum of microalgae. Its culture can remain viable even in water with a heavy organic load, with no addition of food, thus considered to be a hardy species for larval aquaculture. The green Chlorophytes (*A. gracilis*, *Tetraselmis marina* and *D. salina*) were easily mass cultured with a preference for better growth at salinities over 100 ppt. *T. marina* was the most sensitive of the three as for unknown reasons, its cells often lose all 4 flagella and are transformed to palmelloid cells. Nevertheless those three halotolerant microalgae proved to be an excellent food for the rotifer *Brachionus plicatilis*, for copepods, for Artemia and for protozoa as *F. salina*. Considering the scarcity of information in the literature on the presence of all the above categories of organisms in hypersalinity, a wide field waits to be studied in detail. The spectrum of the existed species of cyanobacteria and protists in particular may be much broader than we thought. Endemicity also may be much more intense than conservatively thought. The species in Greece may be different from even adjacent countries saltworks (there are no natural hypersaline lakes in Europe). The same holds true especially when more remote areas on Earth are considered. Because saltworks are not naturally formed and evolved biotopes but rather reflect the extreme edge of acclimation and adaptation in

extreme conditions of the marine organisms that constantly are transported from the sea to the saltpans, the endemicity theory actually refers to the sea habitat. In that sense, Foissner's (2008) moderate endemicity distribution model in protists as opposed to the ubiquity distribution model, seems to explain the findings of the present study as of the difficulties of recognition in the species level of the encountered organisms. It seems that apart from protists, this hypothesis applies also to hypersaline cyanobacteria, thus a whole unexplored eco-habitat awaits multidisciplinary approach.
The present study is nothing more than an initial attempt to outline the wealth of micro-biota in a particular hypersaline environment, with the aim to intrigue interest for thorough studies, something that is already happening in the Plankton Culture Laboratory of T.E.I. W. Greece.
The organisms presented in Figures 1 - 7 are representatives of the whole collection and especially in Fig. 7 are depicted two specimens that cannot be assigned to established species of diatoms according to the existed atlases.

Salinity range (ppt)	**50-80**	**81-110**	**111-130**	**131-160**	**>160**	**Culture response**
CYANOBACTERIA (5 unidentified)						
Synechococcus	+++	++++	++++	++	++	+
Aphanothece	++	+++	++++	+++	+	+
Microcystis	++++	+++	++	-	-	
Cyanothece	+	++	++++	+++	+	+
Oscillatoria	++++	+++	++	-	-	+
Lyngbya	++++	++	+	-	-	+
Aphanizomenon	+++	++++	++	+	-	+
Cylindrospermopsis	++	+++	++++	+	-	
Anabaena	+++	+	-	-	-	
Arthrospira	+++	++++	++++	++	-	+++
Beggiatoa	++	+	-	-	-	
Scytonema	++	+	-	-	-	
Prochlorothrix	+	-	-	-	-	
Microcoleus	+	-	-	-	-	
Tychonema	+	-	-	-	-	
Pseudoanabaena	++	+	-	-	-	
Phormidium	+++	++	+	-	-	
PROTOZOA (11 unidentified)						
Euplotes	++++	++++	++	+	-	++++
Uronychia	++++	+	-	-	-	+
Diophrys	++++	+	-	-	-	
Frontonia	++++	++	+	+	-	++
Dysteria	+					
Aspidisca	++++	++++	++	+	-	
Paramecium	++++	+++	+	-	-	++
Euglena	++	-	-	-	-	++
Paraurostyla	+++	++	+	-	-	
Colpoda	++++	+++	++	-	-	
Coleps	++	-	-	-	-	+
Amphileptus	+++	+	+	-	-	
Condylostoma	++++	+++	++	+	-	++++
Amoeba	++++	++++	++++	+++	+	++
Holophrya	++++	++	+	+	-	
Halteria	++	+	-	-	-	+
Pleuronema	++++	++	++	+	-	+
Cyclidium	++++	++++	+++	++	-	+++
Loxodes	++	++	+	-	-	
Litonotus	++	+	+	-	-	+
Chaetospira	+++	+	+	+	-	
Stichotria	+++	+	+	-	-	
Bursaridium	++	+++	-	-	-	
Climacostomum	++++	+++	++	+	-	++
Blepharisma	++++	+++	++	-	-	
Holosticha	++++	++	+	-	-	
Vorticella	++++	+++	++	+	-	+++
Remanella	++++	++	+	+	-	
Lembandion	++	-	-	-	-	
Strobidium	++	+	-	-	-	+
Uronema	++++	++++	++	+	-	+++
Bursaria	++	-	-	-	-	

Tracheloraphis	++	-	-	-	-	
Lacrymaria	+	-	-	-	-	
Hemiophrys	++	+	-	-	-	
Fabrea salina	++++	++++	++++	++++	++	++++
Dileptus	++++	+	-	-	-	+
Colpodella	++++	+++	++	-	-	++++
Phialina	+++	++	+	-	-	
Choanoflagellates	++	+	-	-	-	
MICROALGAE						
Asteromonas gracilis	+	++++	++++	++++	++++	++++
Dunaliella salina	++	++++	++++	++++	++++	++++
Dunaliella sp.	++++	++++	++++	++++	++++	++++
Tetraselmis marina	++	++++	++++	++	-	++
Hymenomonas sp.	++++	++	+	+	-	+
DIATOMS (4 unidentified)						
Cymbella	++++	+++	+++	+	-	+++
Caloneis	++	+	-	-	-	
Cyclotella	++++	+	-	-	-	+++
Craticula	++	+	-	-	-	
Navicula	++++	++++	+++	++	-	
Nitzschia	++++	++++	++++	+++	-	++++
Pleurosigma	++++	+++	++	-	-	
Entomoneis	+++	+	-	-	-	
Encyonema	++	+	-	-	-	
Ulnaria	+	-	-	-	-	
Pinnularia	++	+	-	-	-	
Surinella	+	+	-	-	-	
Neidium	++	-	-	-	-	
Synendra	++++	++	+	+	-	
Stauroneis	+	+	-	-	-	
Gyrosigma	++++	++	+	-	-	+++
Amphiprora	+	-	-	-	-	
Eunotia	++	-	-	-	-	
Epithemia	+	-	-	-	-	
Diatoma	+	-	-	-	-	
Cymatopleura	++	-	-	-	-	
Cocconeis	++++	+	+	-	-	+++
Cylindrotheca	++	++	+	+	-	+
DINOFLAGELLATES						
Gymnodinium	++++	++	+	+	-	
ROTIFERS (1 unidentified)						
Hexarthra	++	-	-	-	-	
Pleurotrocha	++++	+++	-	-	-	
Epiphanes	++	-	-	-	-	
Encentrum	+++	-	-	-	-	
Lindia	++++	+++	-	-	-	++
Colurella	+++	++	-	-	-	+
Testudinella	++	+	-	-	-	
Brachionus plicatilis	++	-	-	-	-	++++
COPEPODS						
Tisbe	++++	+++	-	-	-	++++
ARTEMIA	++++	++++	++++	++++	++++	++++
NEMATODE	++++	++++	++++	+++	+	++++

Table 1: The organisms recorded in hypersalinity at Messolonghi saltworks. "+" stands for the least presence, "++++" for maximum and "-" for absence in relation to the counts sum of each particular organism in all salinities and in combination of a rather rough estimation of their abundance among all other organisms in each particular sample examined. Concerning their response to the culture trials: "+" stands for "poor", "++" for "fair", "+++" for "good" and "++++" for "excellent".

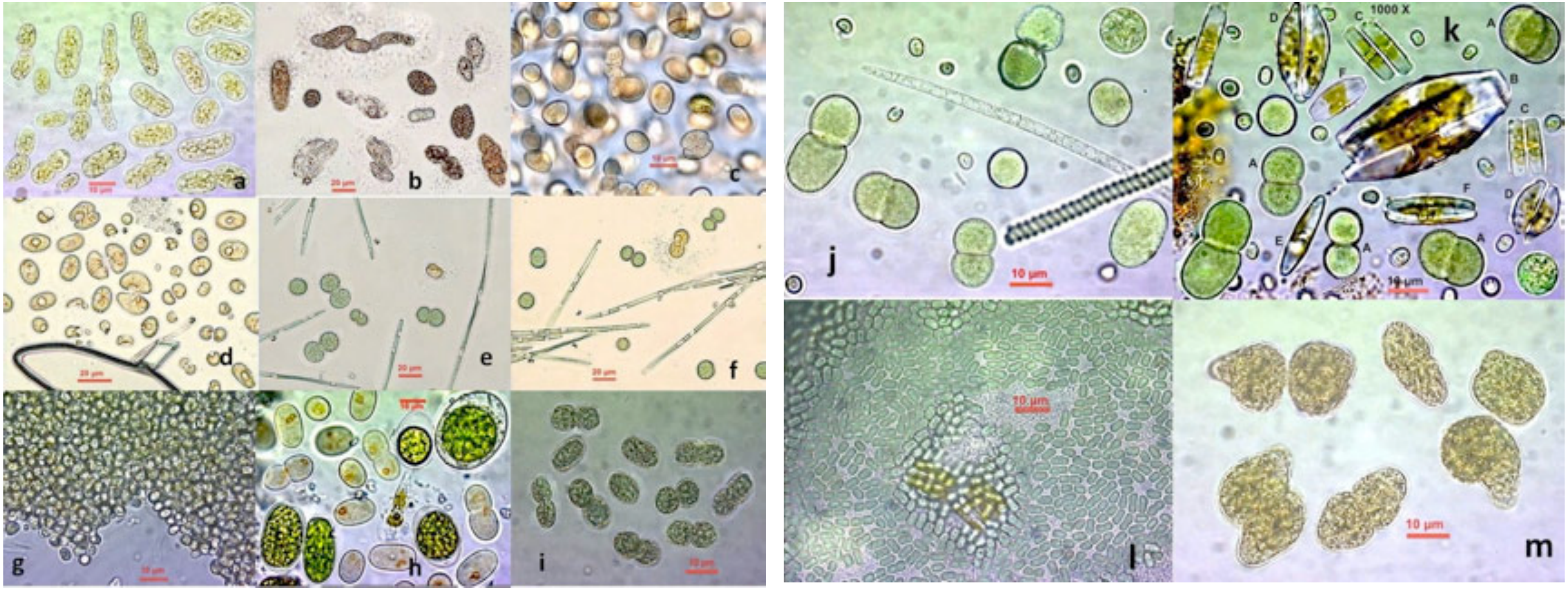

Figure1: Cocciform Cyanobacteria from hypersalinity at Messolonghi saltworks. a) Peculiar involuted cells of an unidentified species, b) variously shaped cells, sole, involuted and dividing, probably of genus *Synechococcus* and in some of them with a mucilage layer around cells, c) totally unknown species, d) kidney shaped cells of an unknown species, e) & f) various cells of genus *Aphanothece* in division state, g) *Microcystis* sp. colony, h) *Synechococcus*-like cells among normal and palmelloid cells of the chlorophyte *Tetraselmis marina*, i) probably *Synechococcus* sp., j) *Cyanothece* sp. cells at various stages of division along with an *Arthrospira* sp. filament, k) *Aphanothece* sp. and *Cyanothece* sp. cells along with pennate diatoms, l) dense colony of small greenish cells of the *Synechococcus* type, m) peculiar involuted cells of probably *Synechococcus* sp.

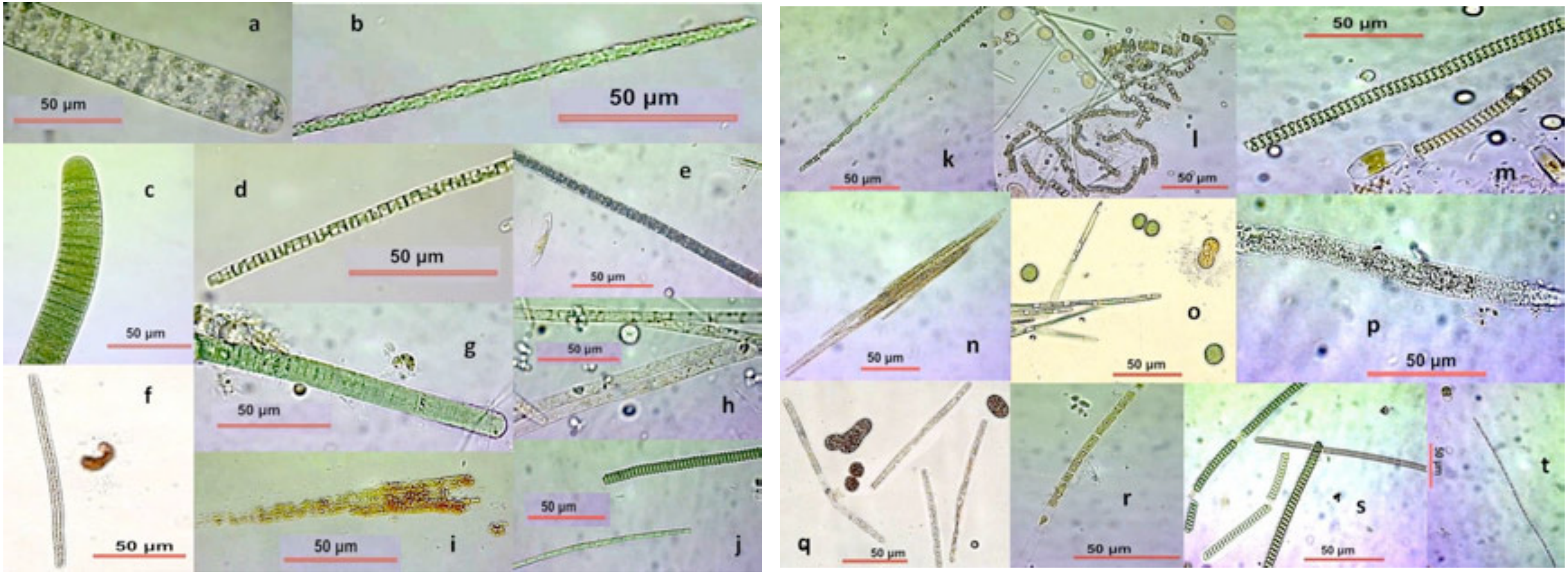

Figure 2: Filamentous Cyanobacteria from hypersalinity ponds. a) *Oscillatoria* sp., b) unidentified trichome, c) *Oscillatoria* sp., d) unidentified trichome, e) *Beggiatoa* sp.?, f) Unidentified, g) *Lyngbya* sp., h) *Tychonema* sp. i) *Aphanizomenon* sp., j) *Pseudoanabaena* sp.? and *Arthrospira* sp., k) *Prochlorothrix* sp., l) *Anabaena* sp., m) *Arthrospira* sp. thick and thin filaments, n) *Aphanizomenon* sp., o) *Prochlorothrix* sp.?, p) *Cylindrospermopsis* sp.?, q) *Beggiatoa* sp. among *Synechococcus*, r) *Cylindrospermopsis* sp., s) fragmented *Arthrospira* filaments of various thickness, t) *Cylindrospermopsis* sp.?

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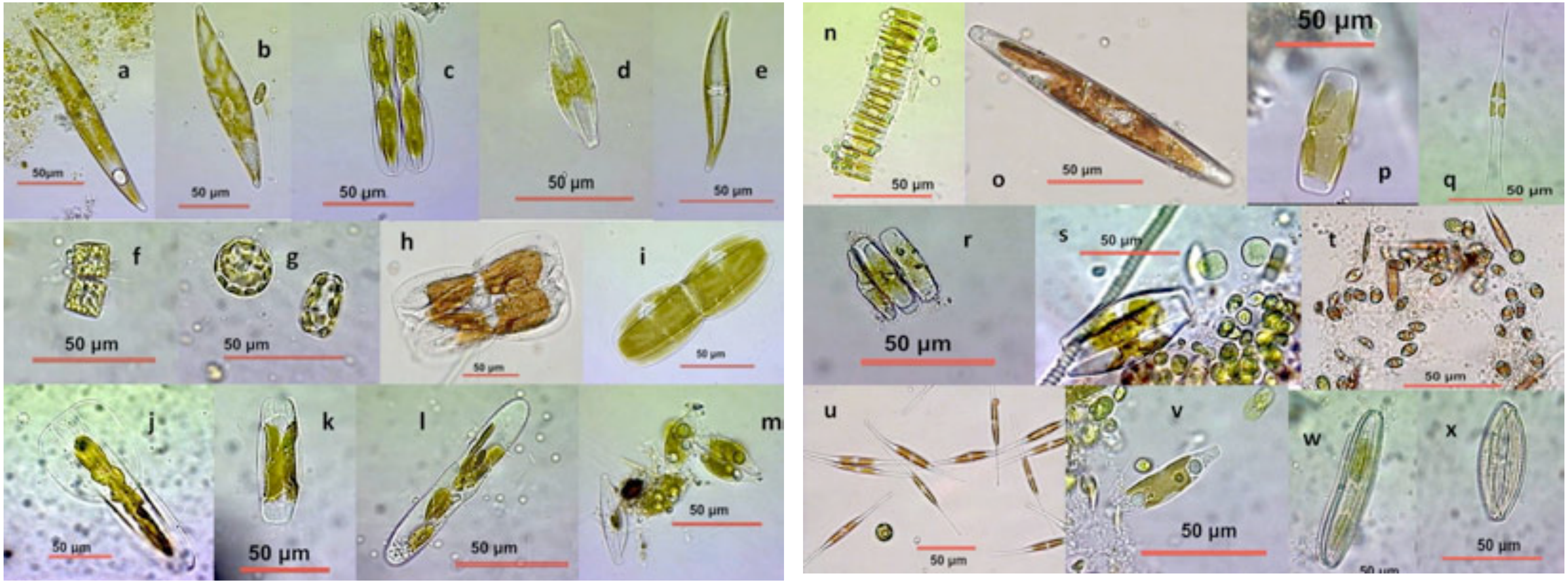

Figure 3: Diatoms from hypersalinity. a) *Pleurosigma* sp. lateral view, b) *Pleurosigma* sp., girdle view, c) *Entomoneis* sp., d) *Navicula* sp., e) *Gyrosigma* sp., f) *Cyclotella* sp. dividing, g) *Cyclotella* sp. round and elongated form, h) *Entomoneis* sp., i) *Amphiprora* sp.? j) *Gomphonema* sp.? k) unidentified Cymbelloid species, l) *Pinnularia* sp.?, m) *Cymbella* sp., n) *Eunotia* sp.?, o) *Nitzschia* sp., p) unidentified diatom, q) *Nitzschia* dividing, r) *Eunotia* sp.?, s) *Cymbella* sp., t) *Cocconeis* sp., u) *Cylindrotheca* sp., v) *Craticula* sp., w) *Epithemia* sp.?, x) *Diatoma* sp.

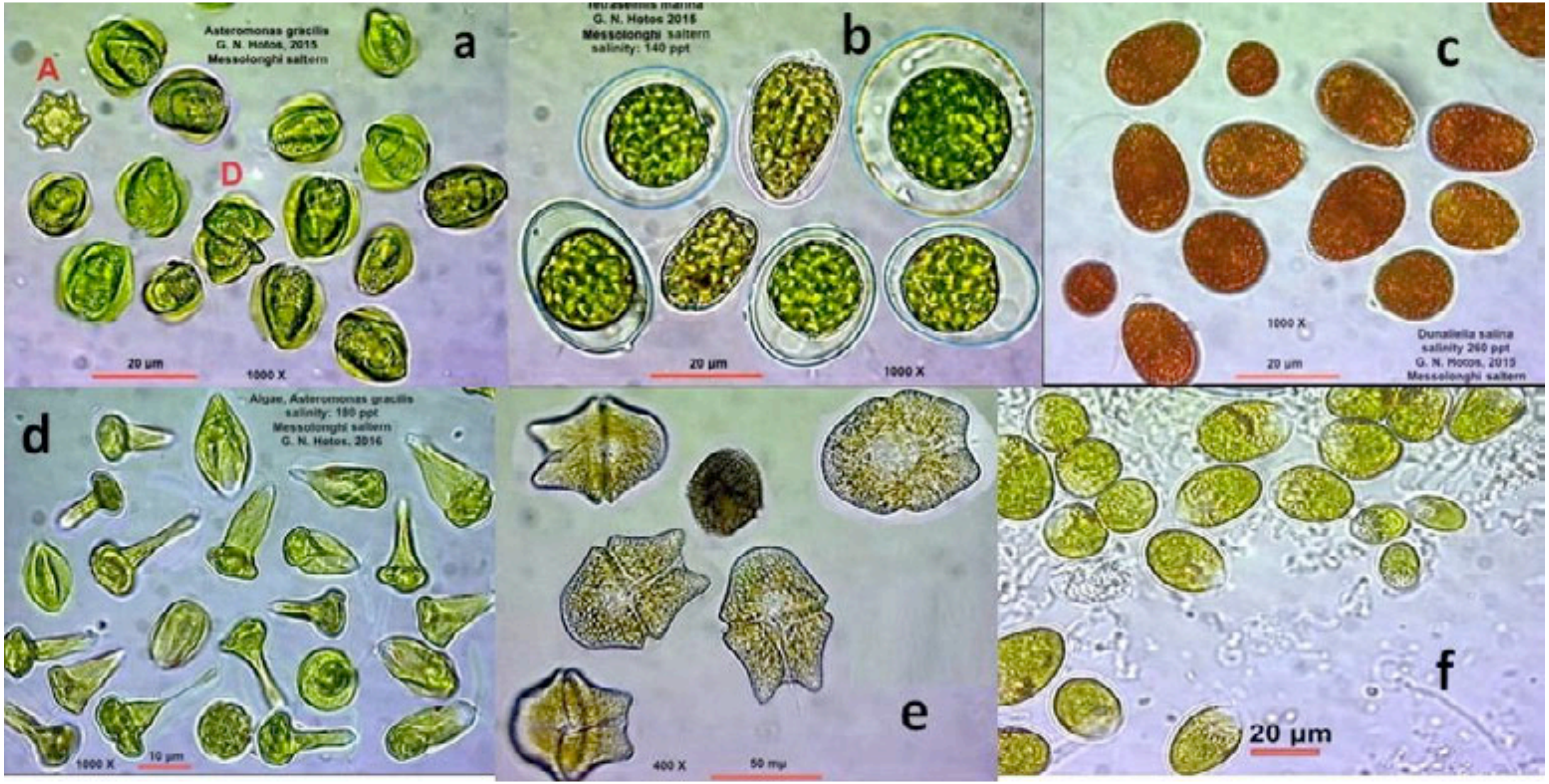

Figure 4: The dominant chlorophytes in hypersalinity. a) *Asteromonas gracilis*, b) *Tetraselmis marina*, normal and palmelloid cells, c) *Dunaliella salina,* reddish cells full of carotenoids at 180 ppt salinity, d) *Asteromonas gracilis* in peculiar cell shapes, e) The dinoflagellate *Gymnodinium* sp., f) *Dunaliella salina*, green cells at 100 ppt salinity.

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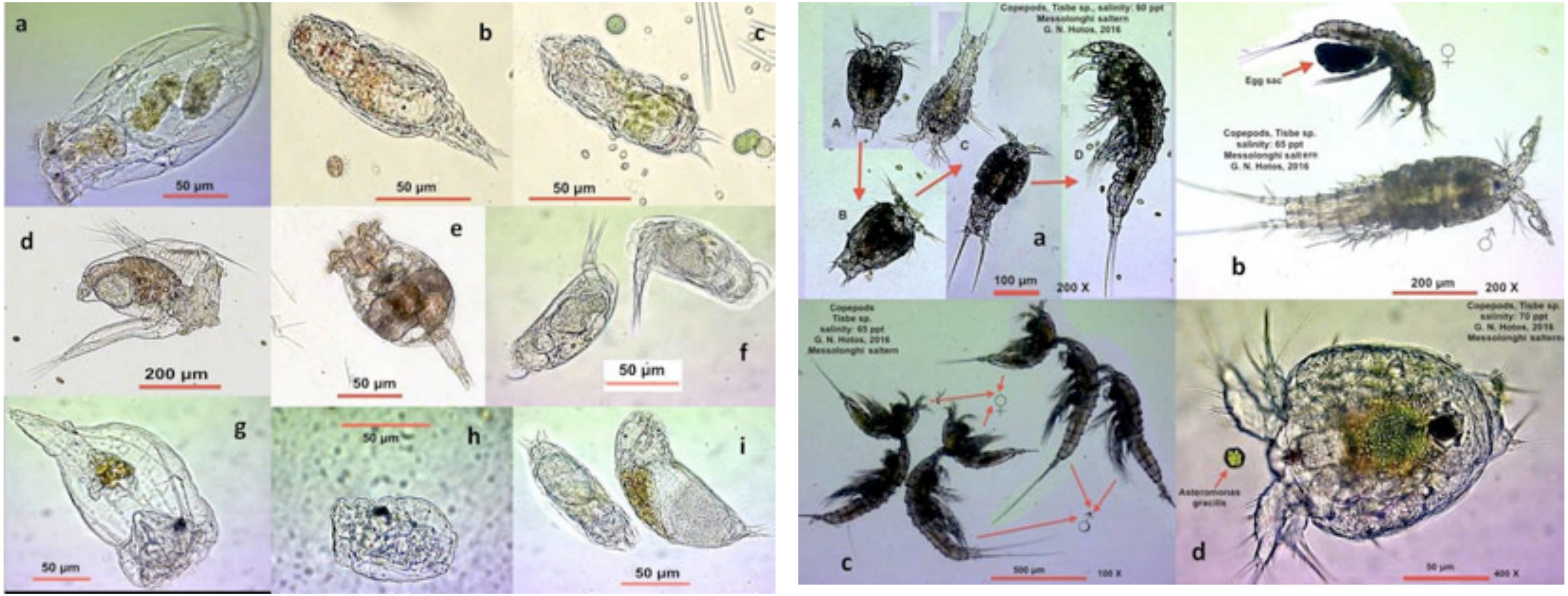


Figure 5: Metazoa from hypersalinity. Left assembly: Rotifers, a) *Testudinella* sp.?, b) *Pleurotrocha* sp., c) *Lindia* sp., d) *Hexarthra* sp., e) *Brachionus plicatilis*, f) *Colurella* sp., g) *Epiphanes* sp.?, h) unidentified marine rotifer, i) *Encentrum* sp.
Right assembly: The copepod *Tisbe* sp. a) various ontogenic stages, b) male and female individuals, c) copulation captured photo, d) *Tisbe* nauplius fed *Asteromonas* cells.

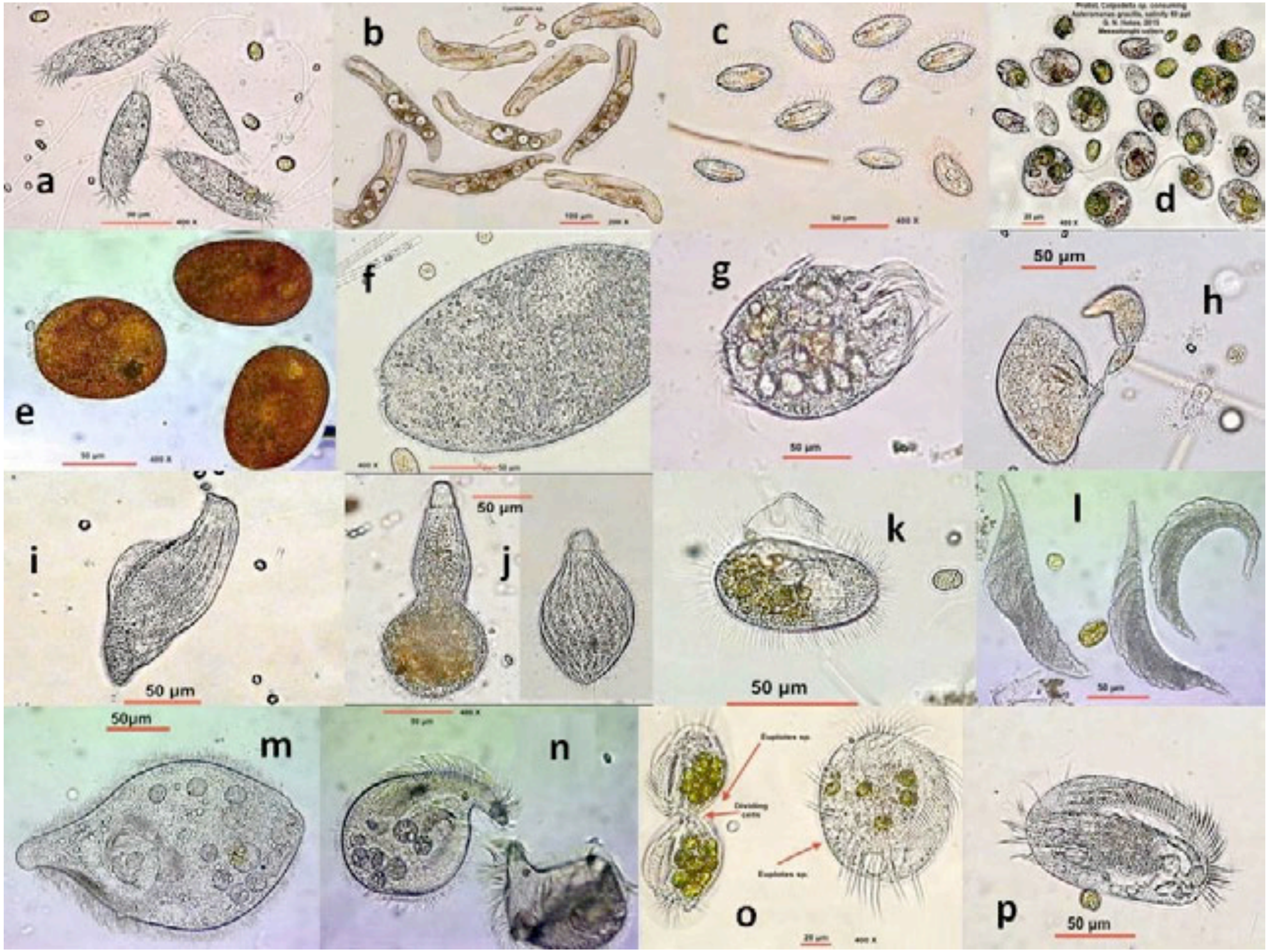
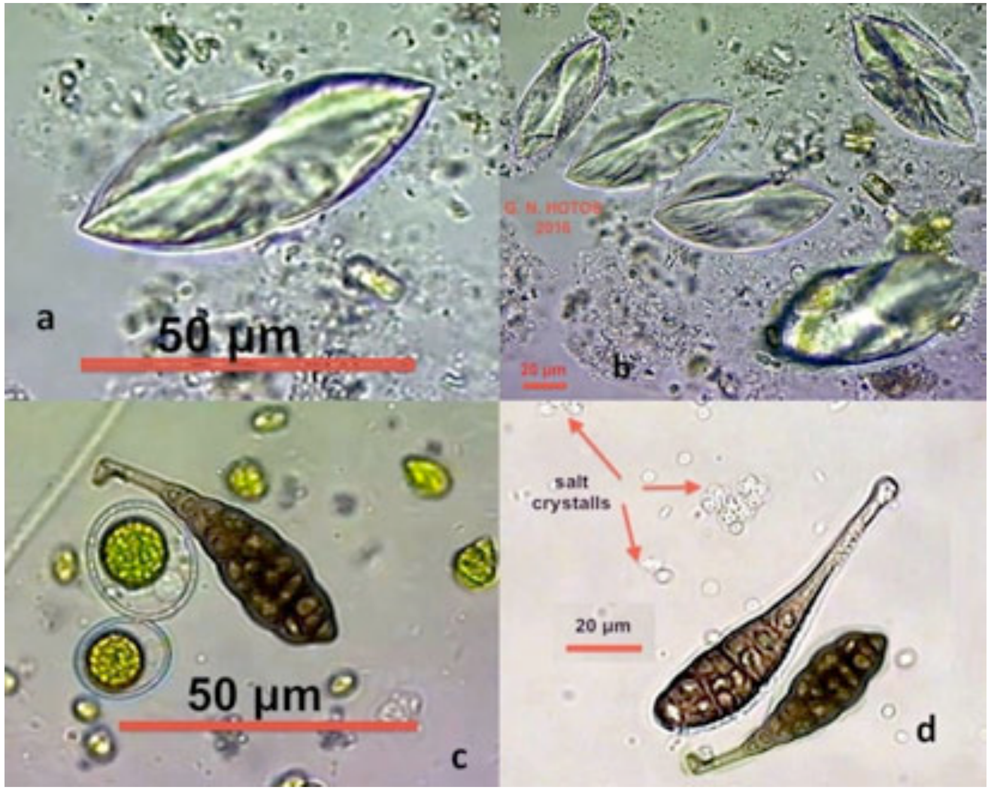


Figure 6: Representative ciliate Protozoa from a hypersaline pond of Messolonghi saltworks. a) *Litonotus* sp.?, b) *Condylostoma* sp., c) *Cyclidium* sp., d) *Colpodella* sp., e) *Frontonia* sp., f) *Climacostomum* sp., g) *Uronychia* sp., h) *Holophrya* sp. in buddying reproduction, i) *Loxodes* sp., j) *Phialina* sp., k) *Pleuronema* sp., l) *Amphileptus* sp., m) *Fabrea salina* at 90 ppt, n) *Fabrea salina* at 170 ppt, o) *Euplotes* sp. in division, full of *Asteromonas* cells (left), p) *Euplotes* sp.



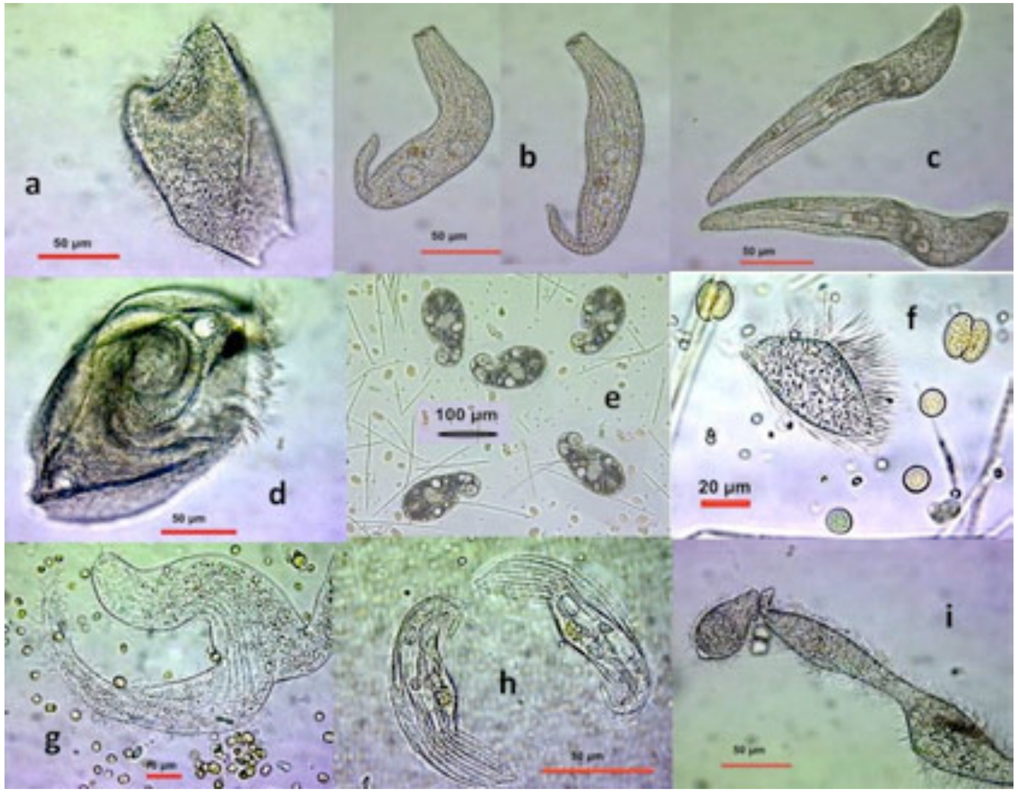


Figure 7: Unidentified organisms from the hypersalinity ponds of Messolonghi saltwork. Left plate: a & b, peculiar pennate diatom at 70 ppt, c & d, peculiar *Licmophora* resembling diatom? at 90 ppt. Right plate: a-i, ciliates not resembling anything known from Protozoan atlases.

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