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A preliminary survey on the planktonic biota in a hypersaline pond of Messolonghi Saltworks (W. Greece)

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Abstract: During a survey in 2015 an impressive assemblage of organisms were found in a hypersaline pond of the Messolonghi saltworks. The salinity ranged between 50 and 180 ppt and the organisms recorded fell in the categories of Cyanobacteria (17 species), Chlorophytes (4 species), Diatoms (23 species), Dinoflagellates (1 species), Protozoa (40 species), Rotifers (8 species), Copepods (1 species), *Artemia* sp., one nematode and *Alternaria* sp. (Fungi). *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 ies. 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.

Keywords: Protists; cyanobacteria; rotifers; crustacea; hypersalinity; Messolonghi salt-works

1. Introduction

It is well known that saltwork waters support high algal densities due to the abundance of nutrients concentrated by evaporation ([1-3]. Apart from the fact that such ecosystems are of paramount ecological value, there are also a potential source for tolerant biota that can be exploitable in terms of aquaculture [4] or other uses [5, 6]. Generally in hypersalinity the microbial life in the prokaryotic level (halophilic archaea and bacteria) has been extensively described (e.g. [7, 8]) emphasizing their role (and viruses as well) as highly essential in the biogeochemical processes. The eukaryotic invertebrate biota of the hypersalinity in terms of diversity and interaction with all the elements of this environment lagged considerably, resulting in poor understanding of its role in the dynamics of food web. In the majority of works concerning protists or crustacea the halotolerant green alga *Dunaliella* spp. (e.g. [9, 10] occupies the bulk of research for algae and the anostacan Artemia (e.g. [11, 12] for planktonic invertabrates.

Considering the scarcity of adequate information on the organisms other than bacteria from hypersaline environments in Greece [13], 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. Furthermore to test the potential for maintenance and culture of as many of them as possible in laboratory conditions, in order to be used as live food for aquaculture and other use in general. The situation is much perplexed considering cyanobacteria and protists (algae and protozoa) from hypersalinity, a topic highly varied in the literature. In fact it is very difficult for a young beginner researcher to be guided through the species mentioned in scientific papers unless there are representative pictures given. 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 of the organisms, various studies were used as guides [14-19].

2. Materials and Methods

The water samples were taken from a particular pond of the Messolonghi saltworks lying between the co-ordinates 38° 23' 47'' to 38° 23' 31'' N and 21° 24' 17'' to 21° 24' 33'' E, during April - September 2015 on a monthly basis thus following the salinity range (50-180 ppt) of the changing water conditions. This pond (~12 ha) is located at the periphery of the saltworks complex and is connected by a narrow channel with the Messolonghi lagoon the water of which is manageably diverted to fill the evaporation ponds of the saltworks. In contrast to the main evaporation ponds that produce salt, this pond is kept all year-round filled with water and although evaporation gradually increases its salinity from April onwards never becomes dry keeping its water level between 0.4 to 1.7 m at its deepest central area. 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 top layer to be included in the water taken (from ~0.7 m from the shore). Salinity and temperature were recorded and the samples were immediately transported to the laboratory where the temperature was kept at 22 °C. The samples were kept in 2 L Erlenmeyer conical flasks, lit with ambient light of about 20 μmol $m^{-2} s^{-1}$ and aerated using pumped air via a pipette. 2 sub-samples of 50 ml were taken and centrifuged mildly (SIGMA 3K10) 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 not any 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 microscopically (Leica Labovert FS inverted microscope and Leika Leitz DMRB) 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 for microalgae using Leica DM-RB microscope at 400X magnification.

Additionally, from the live samples, organisms were targeted and removed by micropipette-suction and placed in 6well multi-chamber plates (SARSTEDT) with 4 ml of Walne's nutrient fertilized water (enriched with silica in case of diatoms) of similar salinity with that of their most abundance in order to roughly check their suitability for growth in culture conditions. The culture plates were left to mature in the laboratory (at 22-23 °C, 12:12 h light:dark, illumination at 50-60 μmol $m^{-2}s^{-1}$ without aeration). The plates were examined after 10 days for population increase. When the cultures were algae, the density was calculated using a Fuchs-Rosenthal haematocytometer. If the cultures were protozoa, rotifers, copepods or Artemia then at the beginning 0.2 ml of dense phytoplankton comprised of *Asteromonas gracilis* and *Dunaliella* previously cultured was added in order to supply adequate food. After 10 days any increase in the population was recorded in a dissecting microscope (Nikon SMZ-U). 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. Notations in Table 1 in the column "culture response" mean: "-" = no change, "+" = at least 2X the initial number of organisms, "++", = between 2X-3X, "+++" = between 3X-4X, "++++" = over 4X. All photos presented here were taken (Jenoptik Progres Gryphax Arktur digital microscopy camera) from live specimens after immobilization in the freezer for about 1 hour (Figures 1-7).

3. Results & Discussion

The organisms found (Table 1) can be categorized as Cyanobacteria, Protozoa, eucaryotic microalgae, rotifers, copepods, *Artemia* a nematode and a fungi. The salinity range clearly demarcated the presence of some organisms from other. In particular, at salinities over 160 ppt only *Artemia* sp., *Dunaliella* sp., *Asteromonas gracilis, Fabrea salina* and some 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 [20], reviving again after lowering the salinity below 50 ppt (unpublished data). *F. salina* plays a crucial role in the food web in hypersaline waters in terms of a consumer of *Dunaliella* spp. [13, 21] and also in the quality of the salt production [22]. However the quotation in [13] that *F. salina* produces slime must be rejected as this is rather the result of mucus excretion of several cocciform cyanobacteria (personal observations, unpublished) or glycerol overproduction of *Dunaliella*, a genus notorious for this in high salinities [23]. The copepod *Tisbe* sp. also exhibited a remarkable viability in 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 *Dunaliella* sp.) 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 [24]. Nevertheless these three halotolerant microalgae proved to be an excellent food for the rotifer *Brachionus plicatilis*, for copepods, for *Artemia* and for the ciliate *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 awaits to be studied in detail. The spectrum of the existed species of cyanobacteria and protists in particular may be much broader than that presented here. 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) [25] moderate endemicity distribution model in protists as opposed to the ubiquity distribution model, seems to explain the findings of the present study even if here recognition based on morphology was confined to the genus and not to 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 should be considered as just a preliminary attempt to outline the wealth of micro-biota in a particular hypersaline local environment, with the aim to intrigue interest for future more elaborated studies, The organisms presented in Figures 1 - 7 are representatives of the whole collection.

Table 1. The organisms recorded in hypersalinity at Messolonghi saltworks identified to the Genus level. "+" 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".

Salinity range (ppt)	50-80	81-110	111-130	131-160	>160	Culture response
CYANOBACTERIA						-
Synechococcus	+++	++++	++++	+	+	+
Aphanothece	++	+++	++++	+	-	-
Microcystis	++++	+++	++	-	-	-
Cyanothece	+	++	++++	+++	+	+++
Oscillatoria	++++	+++	++	-	-	+
Lyngbya	++++	++	+	-	-	-
Aphanizomenon	+++	++++	++	-	-	-
Cylindrospermopsis	++	+++	+	+	-	-
Anabaena	+++	+	-	-	-	+
Arthrospira	+++	++++	++++	++	-	+
Beggiatoa	++	+	-	-	-	-
Scytonema	++	+	-	-	-	-
Prochlorothrix	+	-	-	-	-	-
Microcoleus	+	-	-	-	-	-
Tychonema	+	-	-	-	-	-
Pseudoanabaena	++	+	-	-	-	-
Phormidium	++++	+	-	-	-	++++
PROTOZOA						
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 (Chlorophytes)						
Asteromonas gracilis	++	++++	++++	++++	++++	++++
Dunaliella	++++	++++	++++	++++	++++	++++
Tetraselmis marina	++	++++	+++	+	-	++
Hymenomonas	++++	++	-	-	-	-
MICROALGAE (Diatoms)						
Cymbella	++++	+++	+++	+	-	+
Caloneis	++	+	-	-	-	-
Cyclotella	++++	+	-	-	-	+++
Craticula	++	+	-	-	-	-
Navicula	++++	++++	+++	++	-	-
Nitzschia	++++	++++	++++	+++	-	++++
Pleurosigma	++++	+++	++	-	-	-
Entomoneis	+++	+	-	-	-	-
Encyonema	++	+	-	-	-	-
Ulnaria	+	-	-	-	-	-
Pinnularia	++	+	-	-	-	-
Surinella	+	+	-	-	-	-
Neidium	++	-	-	-	-	-

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Synendra	++++	++	+	+	-	-
Stauroneis	+	+	-	-	-	-
Gyrosigma	++++	++	+	-	-	+++
Amphiprora	+	-	-	-	-	-
Eunotia	++	-	-	-	-	-
Epithemia	+	-	-	-	-	-
Diatoma	+	-	-	-	-	-
Cymatopleura	++	-	-	-	-	-
Cocconeis	++++	+	+	-	-	+++
Cylindrotheca	++	++	+	+	-	+
DINOFLAGELLATES						
Gymnodinium	++++	++	-	-	-	-
ROTIFERS						
Hexarthra	++	-	-	-	-	-
Pleurotrocha	++++	+	-	-	-	-
Epiphanes	++	-	-	-	-	-
Encentrum	+++	-	-	-	-	-
Lindia	++++	+++	-	-	-	-
Colurella	+++	++	-	-	-	-
Testudinella	++	+	-	-	-	+
Brachionus plicatilis	++	-	-	-	-	++++
COPEPODS						
Tisbe	++++	+++	-	-	-	++++
ANOSTRACA						
Artemia	++++	++++	++++	++++	++++	++++
NEMATODE						
Mesacanthoides	++++	++++	+	+	-	++++
FUNGI						
Alternaria	+	+	-	-	-	-

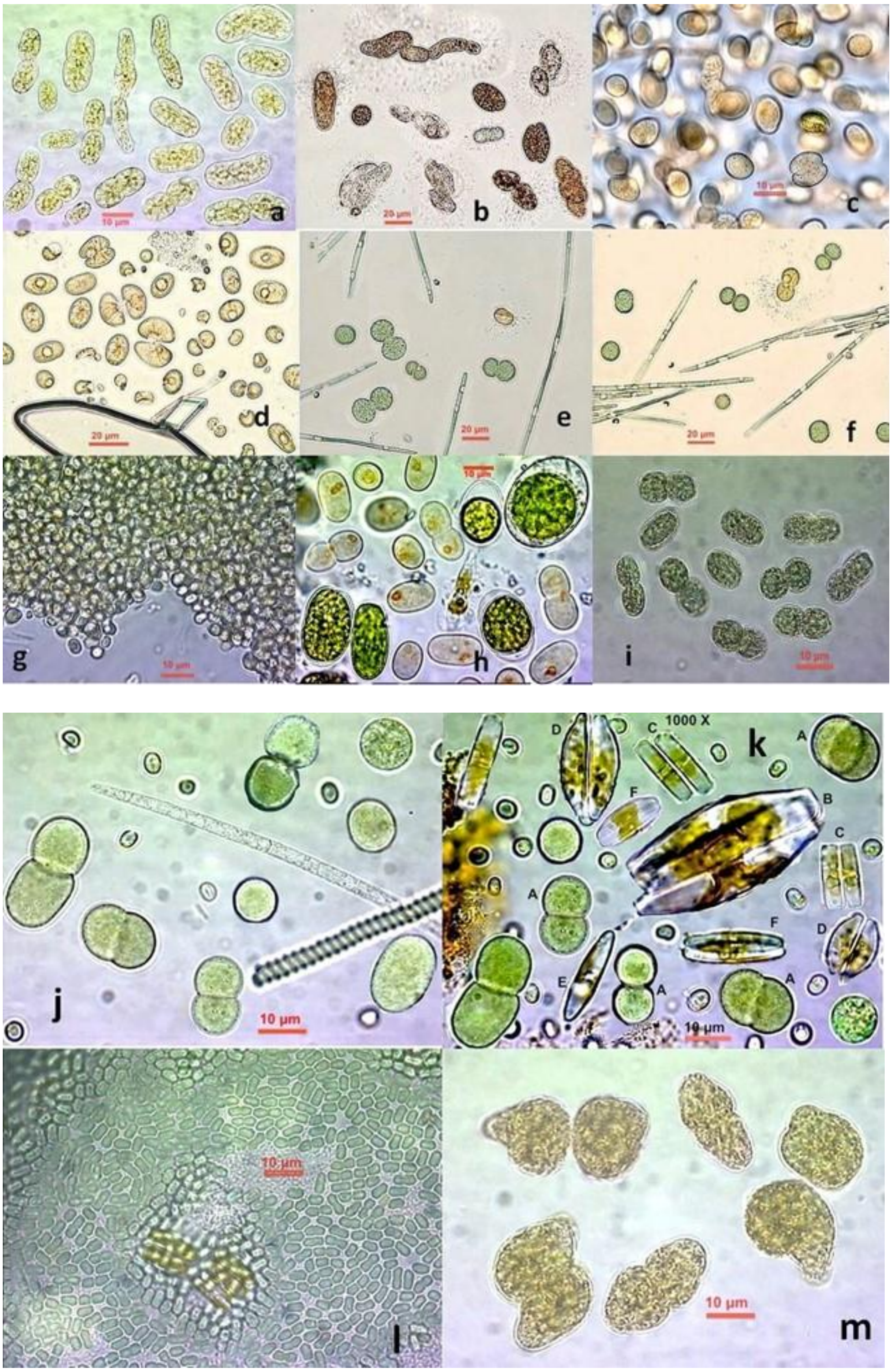

Figure 1. Cocciform Cyanobacteria from hypersalinity at Messolonghi saltworks. a) Peculiar involuted cells of an unidentified species, b) variously shaped cells, sole, involuted and dividing, probably of genus *Synechococcus* and in some of them with a mucilage layer around cells, c) totally unknown species, d) kidney shaped cells of an unknown species, e) & f) various cells of genus *Cyanothece* 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.

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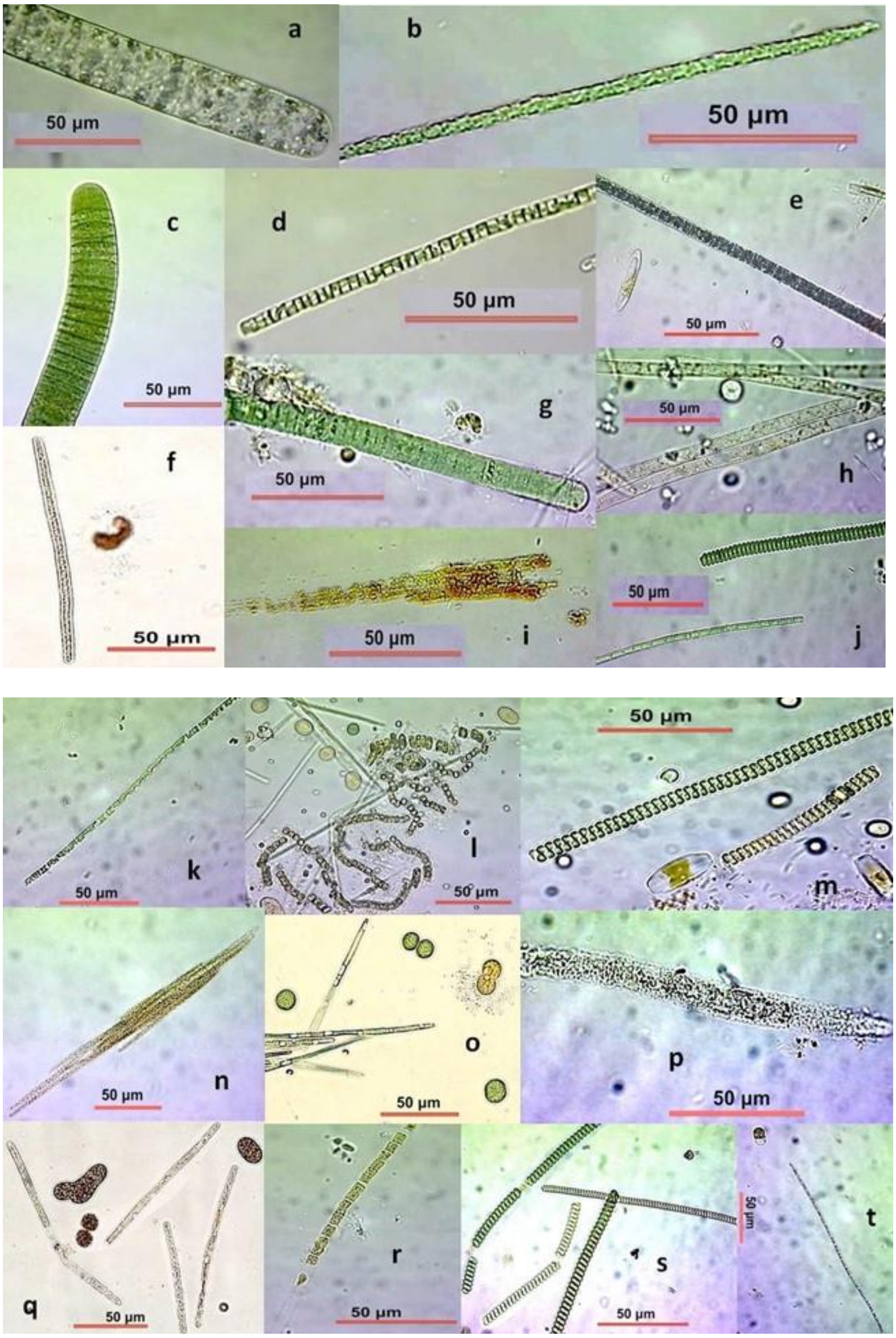

Figure 2. Filamentous Cyanobacteria from hypersalinity ponds. a) *Oscillatoria* sp., b) unidentified trichome, c) *Oscillatoria* sp., d) unidentified trichome, e) *Beggiatoa* sp.?, f) Unidentified, g) *Lyngbya* sp., h) *Tychonema* sp. i) *Aphanizomenon* sp., j) *Pseudoanabaena* sp.? and *Arthrospira* 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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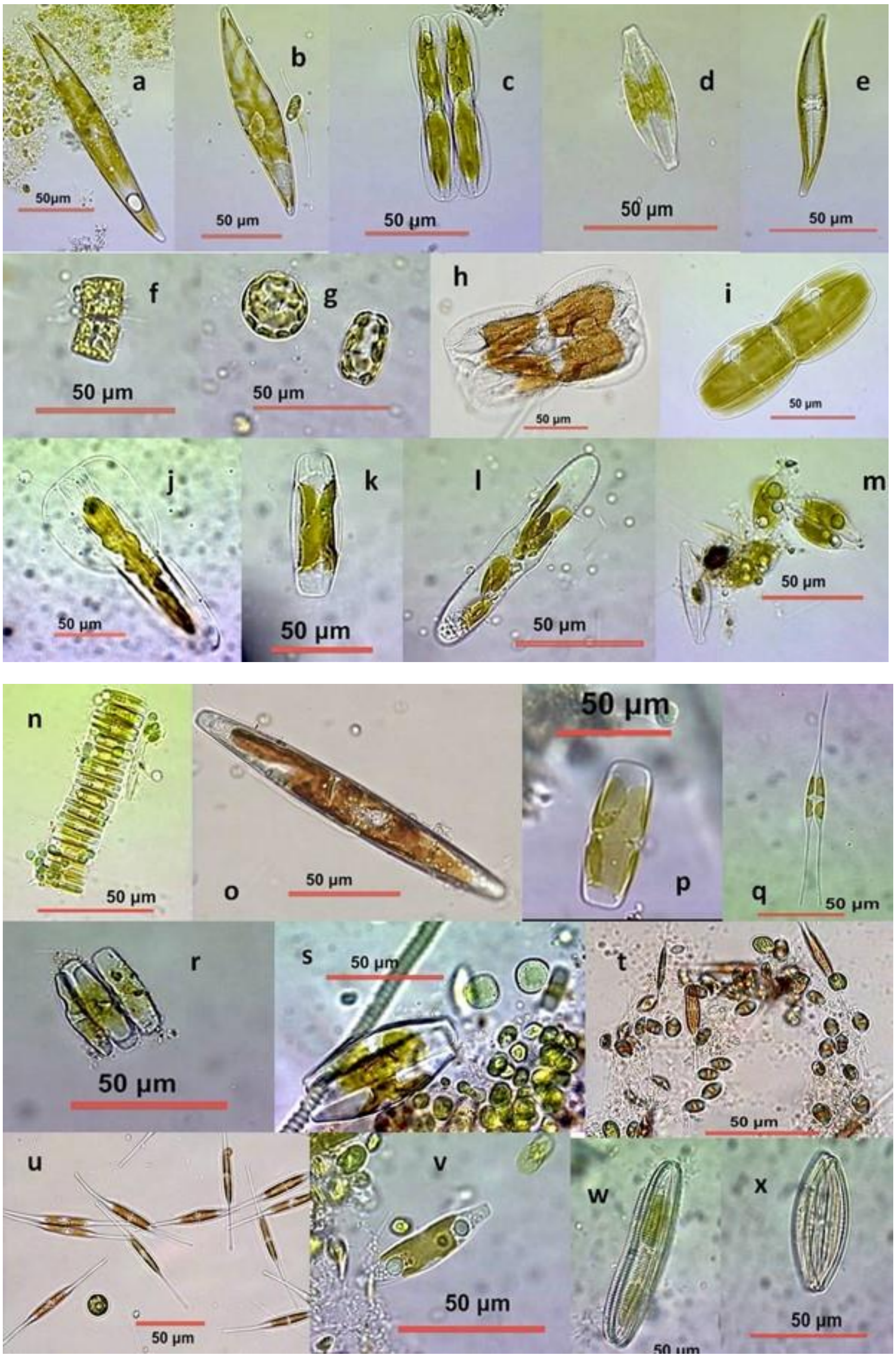

Figure 3. Diatoms from hypersalinity. a) *Pleurosigma* sp. lateral view, b) *Pleurosigma* sp., girdle view, c) *Entomoneis* sp., d) *Navicula* sp., e) *Gyrosigma* sp., f) *Cyclotella* sp. dividing, g) *Cyclotella* sp. round and elongated form, h) *Entomoneis* sp., i) *Amphiprora* sp.? j) *Gomphonema* 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.

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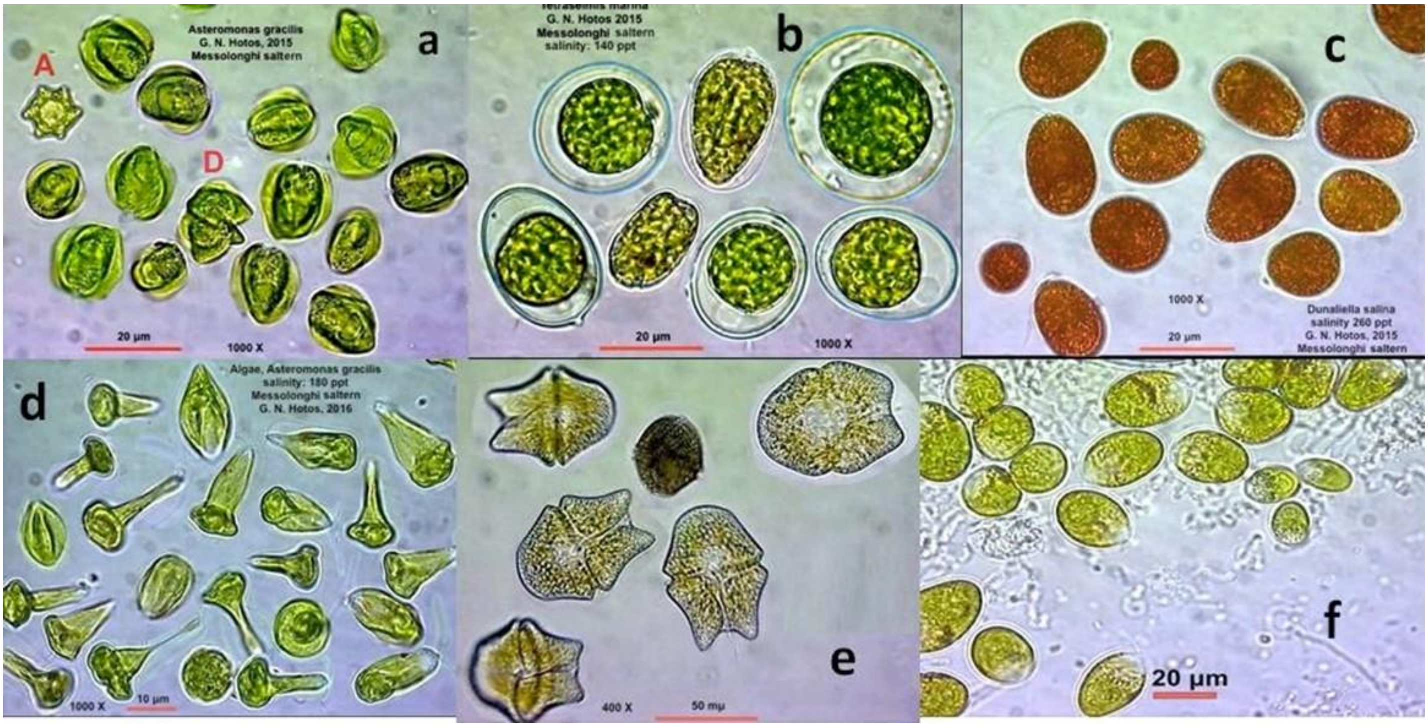

Figure 4. The dominant chlorophytes and dinophytes in hypersalinity. a) *Asteromonas gracilis*, b) *Tetraselmis marina*, normal and palmelloid cells, c) *Dunaliella* sp., reddish cells full of carotenoids at 180 ppt salinity, d) *Asteromonas gracilis* in peculiar cell shapes, e) The dinoflagellate *Gymnodinium*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

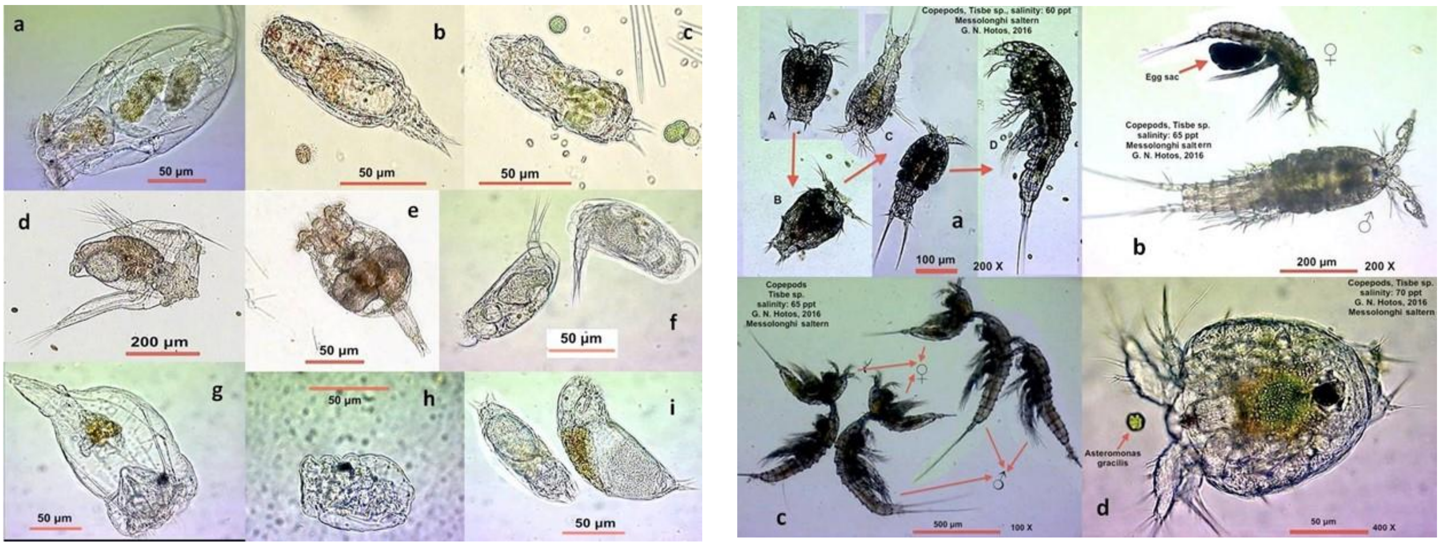

Figure 5. Metazoa from hypersalinity. Left assembly: Rotifers, a) *Testudinella* sp. b) *Pleurotrocha* sp., c) *Lindia* sp., d) *Hexarthra* sp., e) *Brachionus plicatilis*, f) *Colurella* sp., g) *Epiphanes* sp.?, h) unidentified marine rotifer, i) *Encentrum* sp. Right assembly: The copepod *Tisbe*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

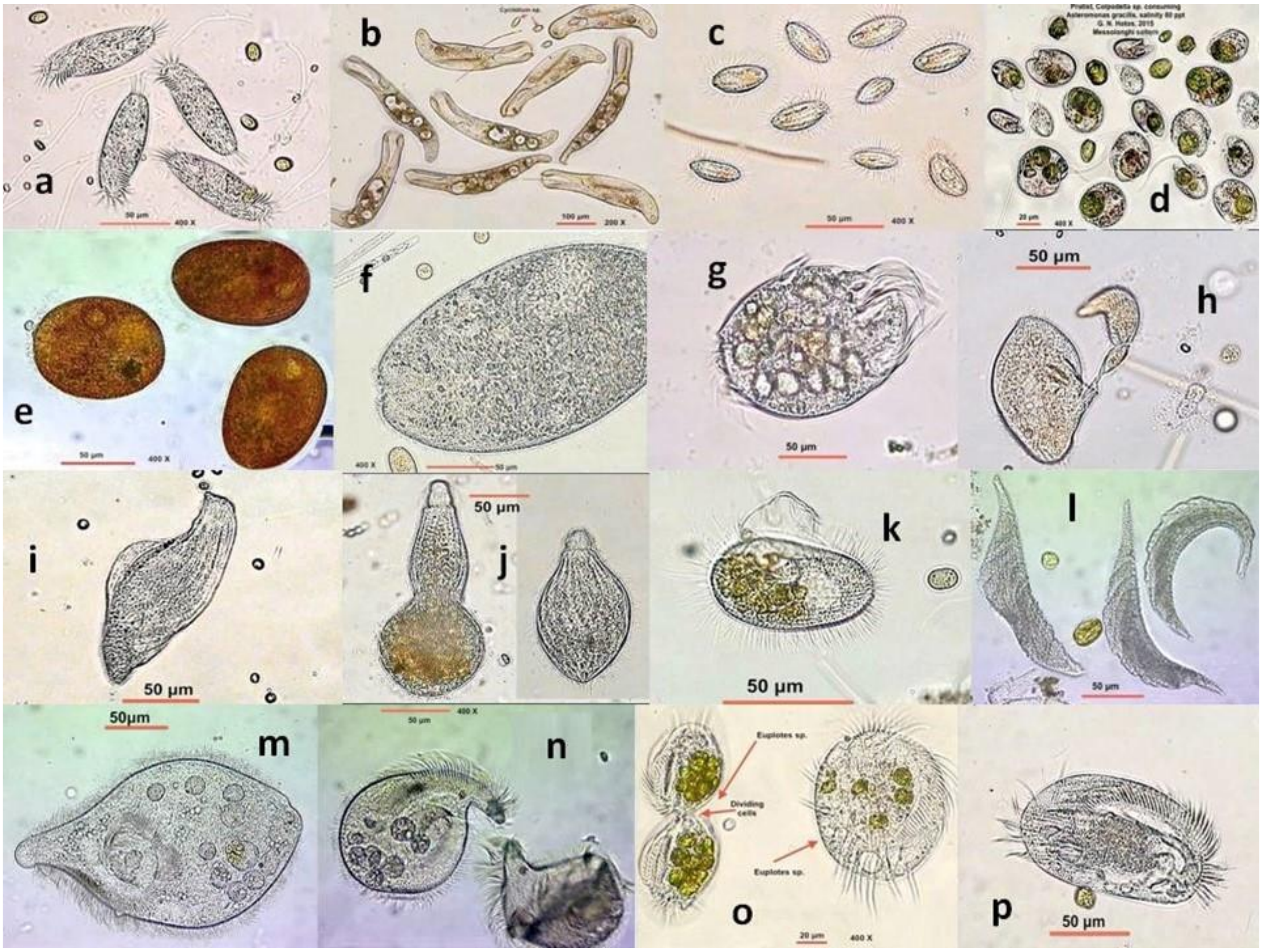

Figure 6. Representative ciliate Protozoa from a hypersaline pond of Messolonghi saltworks. a) *Litonotus* sp.?, b) *Condylostoma* sp., c) *Cyclidium* sp., d) *Colpodella* sp., e) *Frontonia* sp., f) *Climacostomum* sp., g) *Uronychia* sp., h) *Holophrya* sp. in buddying reproduction, i) *Loxodes* sp., 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.

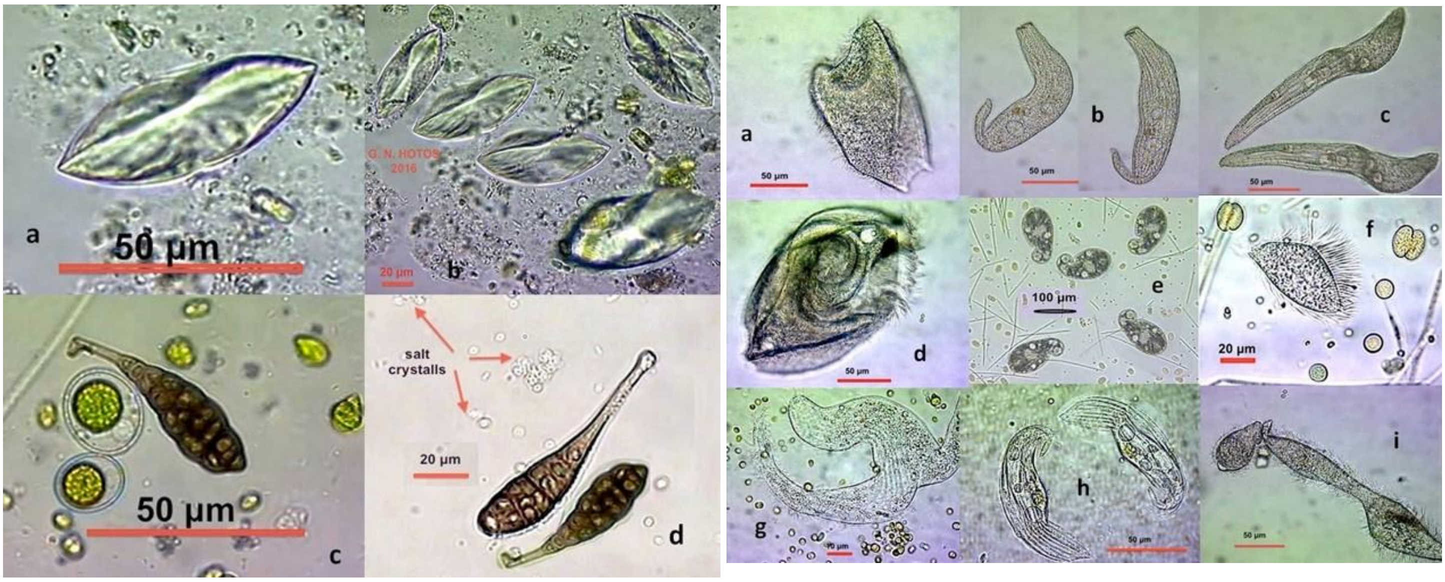

Figure 7. Unidentified organisms and fungi spores from the hypersalinity ponds of Messolonghi saltwork. Left plate: a & b, unidentified microbes among peculiar salt crystals at 175 ppt, c & d, *Alternaria* sp. (fungi) spores at 90 ppt. Right plate: a-i, ciliates not resembling anything known from Protozoan atlases.

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