Phylogenetic characterization and morphological and physiological aspects of a novel acido- and halotolerant microalga *Coccomyxa onubensis* sp. nov. (Chlorophyta, Trebouxiophyceae)

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The genus *Coccomyxa* comprises green microalgae, which can be found worldwide in remarkably versatile aquatic and terrestrial ecosystems including symbiotic associations with a number of different hosts. In this study, we describe a new species, *Coccomyxa onubensis*, based on 18S and ITS rDNA sequence data. *C. onubensis* was isolated from acidic water, and its ability to adapt to a wide range of

acidic and alkaline pH values and to high salinity was analyzed. The long-term adaptation capacity of the microalga to such extreme conditions was evaluated by performing continuous repeated batches at selected salt concentrations and pH values. Adapted cultures of *C. onubensis* were found to yield high biomass productivities from pH 2.5 to 9, with a maximum at pH 4. Moreover, *C. onubensis* was also found to adapt to salinities as high as 0.5 M NaCl, reaching biomass productivities that were similar to those of control cultures. Ultrastructural analysis by transmission electron microscopy of *C. onubensis* cells adapted to high salinity showed a robust response to hyperosmotic shock. Thus, *C. onubensis* was found to be acido- and halotolerant. High biomass productivity over a wide range of pH and salinities denotes *C. onubensis* as an interesting candidate for various biotechnological applications including outdoor biomass production.

Keywords: *Coccomyxa*, Trebouxiophyceae, green algae, 18S rDNA, ITS rDNA, phylogeny, acidotolerance, halotolerance, biotechnology

Introduction

Mass cultivation is the first step in the biotechnological use of microalgae at large scale. Suitable environmental and cultivation conditions to speed up biomass production are crucial for developing production processes that are efficient in costs and yield (Forján et al. 2015). Massive production of microalgae is challenging due to limitations to grow under harsh outdoor conditions. Extremophile microalgae have the ability to cope with such harsh conditions. These microalgae have developed the ability to grow under high or low temperature, under high or low pH, in the presence of metal ions, under high irradiance and also in the presence of a number of other extremophile microorganisms in the same habitats (Varshney et al. 2015). Despite the natural abilities of these microalgae that are promising for biotechnological applications, little attention has so far been paid to the potential of extremophile microalgae.

The conditions at which extremophile microalgae develop in their natural extreme habitats are mostly far from being optimal for growth. In the laboratory, extreme conditions can be simulated which are tuned with local climate and water conditions and simultaneously lead to high productivities of extremophile microalgae while preventing growth of competitive contaminants (Varshney et al. 2015). Salinity, pH, light irradiance and solved metals concentrations are among such physicochemical conditions. For instance, Río Tinto, a river in Southwestern Spain, is an example of a highly acidic aquatic environment that contains solved metals at concentrations which are commonly toxic to life and hosts a wide diversity of microorganisms with several microalgal species among them (Amils & Fernández-Remolar 2014). The microalga *Coccomyxa* sp. ACCV1, isolated from the acidic waters of Río Tinto (Garbayo et al. 2012), has been reported to develop better under cultivation conditions that are different from those typical of the natural acidic aquatic habitat which, for instance, include nitrogen limitation and toxic levels of solved iron and copper (Garbayo et al. 2012, Vaquero et al. 2012, Ruiz-Domínguez et al. 2015). *Coccomyxa* sp. ACCV1 grew well at pH values as low as 2.5 (Garbayo et al. 2012), but the optimal pH for high biomass production was not determined. The plasma membrane of extremophile microalgae from low pH environments shows very low permeability to protons, which allows the cytoplasmatic pH to be kept neutral (Gimmler et al. 1988, Gross 2000). Therefore, the proton concentration in the culture medium causes significant osmotic pressure, which makes microalgae from acidic habitats moderately halotolerant (Albertano et al. 1990, Gross 2000). As a consequence, microalgae from acidic habitats might be more applicable for outdoor biomass production, as seawater could eventually become a suitable resource for the algal production process.

Recently, the taxonomy of the genus *Coccomyxa* was revised using an integrative approach and DNA barcoding (Darienko et al. 2015). The ITS2 rDNA barcode allowed them to distinguish a total of seven species; three of them were newly described. Their study included far more than hundred partial or complete rDNA sequences from uncultured strains as well as several sequences from cultured strains deposited in GenBank. Among the latter was our sequence from *Coccomyxa* sp. isolated from Río Tinto. Darienko et al. (2015) recognized this strain as a yet to describe new species with a distinct barcode. Genus affiliation of our strain was already confirmed by a partial 18S rDNA sequence (GU265559; Garbayo et al. 2012). Ruíz-Dominguez et al. (2015) tentatively designated this strain as *Coccomyxa* sp. (strain onubensis) without actually performing phylogenetic analyses.

In this paper, we describe the Río Tinto isolate as a new species, *Coccomyxa onubensis*, and analyze its ability to adapt to a wide range of pH values and to high salinity. The long-term adaptation capacity of the microalga to such extreme conditions is evaluated by performing continuous repeated batches at different salinities and pH values.

Materials and methods

Microorganism and standard culture conditions

Coccomyxa sp. ACCV1 was isolated from acidic waters of the Tinto River (Huelva, Spain). *Coccomyxa* cells were grown in 1 L Erlenmeyer flasks in batch mode. Unless otherwise indicated, the cultures were grown at pH 2.5 in a culture medium based on K9 mineral medium (Silverman & Lundgren 1959), modified according to the following

96 composition: 3.95 g K_2SO_4, 0.1 g KCl, 0.5 g K_2HPO_4, 0.41 g $MgCl_2$, 2.29 g KNO_3, 0.01
97 g $CaCl_2$ and 5 mL Hutner solution (Hutner et al. 1950) in distilled water up to a final
98 volume of 1 L. Hutner solution was prepared as described in Garbayo et al. (2012). The
99 cultures were incubated at 27°C, illuminated at 150 μE m^{-2} s^{-1} with white fluorescent
100 lamps, and bubbled with air containing 5% (v/v) CO_2. Bacterial contamination in the
101 cultures was prevented by using 0.45 μm air filters in the air supply line.

102

103 *DNA extraction, PCR amplification, cloning, and sequencing*

104 Cells were harvested by centrifugation and mechanically ground with tiny glass beads
105 with a diameter of about 0.2 mm and extraction buffer (200 mM Tris-HCl, pH 7.5, 250
106 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate) in 1.5 mL Eppendorf tubes in
107 a cell mill (Qiagen, Hilden, Germany) for 10 min at 30 Hz. DNA purification and
108 precipitation was done by standard procedures. The precipitate was air dried and
109 resuspended in 50 μL of Milli-Q sterilized water for further use.

110 For amplification of the 18S rDNA, two conserved eukaryote-specific primers
111 (forward primer, 5' WACCTGGTTGATCCTGCCAGT 3', 5' PCR: Huss et al. (1999);
112 reverse primer, 5' ATATGCTTAAATTCAGCGGGT 3', NLR 204/21: Van der Auwera
113 et al. (1994)) were used to amplify the complete 18S and ITS rDNA. Thirty-five cycles
114 were run in a Biometra T3000 thermal cycler (Labrepco, Horsham, USA) using Phusion
115 High Fidelity DNA Polymerase (2U/μL) with 5 s of denaturation at 98 °C (30 s for the
116 first cycle), 20 s annealing at 55 °C, and 120 sec extension at 72 °C followed by another
117 300 s after the last cycle. The amplified products were analyzed on a 1.8% agarose gel
118 stained with ethidiumbromide. The PCR products were purified with a QIAquick PCR

purification kit (Qiagen, Hilden, Germany) and cloned with a Zero Blunt Topo Cloning Vector Kit (Invitrogen, Carlsbad, CA). The insert nucleotide sequences were determined with the universal primers M13 forward and reverse as well as some intern sequencing primers listed in Huss et al. (1999) by GATC-Biotech (Constance, Germany). The sequence was submitted to GenBank with the accession number HE617183.

Phylogenetic analyses

The 18S + ITS rDNA sequence (without intron) of *C. onubensis* ACCV1 was manually aligned on a MicroVAX computer (Digital Equipment Corporation, Maynard, MA) using the sequence editor program by Olsen et al. (1992) with representative sequences of all currently described *Coccomyxa* species (Darienko et al. 2015). For the phylogenetic analyses, ITS1 was excluded, as this region could not be unambiguously aligned with all reference sequences. For the alignment of the ITS2 region, the secondary structure was used according to Darienko et al. (2015). Phylogenetic trees were inferred from all positions available for 18S, 5.8S and ITS2 rDNA (2249 nt) by the neighbor-joining (NJ), the maximum parsimony (MP), and the maximum likelihood (ML) method using the PAUP program (Swofford 2002). 1,000 bootstrap replicates were carried out for each method. ML was used to infer the tree topology shown in Fig. 2. by selecting empirical base frequencies, setting the transition/transversion (ti/tv) ratio to 2, and assuming a gamma distribution of 0.5. For the NJ bootstrap analysis, the HKY85 correction was used to convert pair-wise sequence similarities into evolutionary distances, starting trees were obtained via NJ, and the tree-bisection-reconnection (TBR) branch-swapping algorithm was selected. In the MP analysis, starting trees were

obtained via random stepwise addition of taxa repeated 10 times, gaps were trated as "fifth base", and TBR was selected.

Repeated-batch cultures at different pH values

Experiments at different pH values were performed in 1 L flasks in repeated-batch mode. The algal cultures were grown in repeated cycles (a total of 5) for 72h/cycle. After each cycle, the cultures were diluted with fresh culture medium to 0.23 g L^{-1} biomass concentration. The pH of each culture was monitored and corrected by addition of either HCl or NaOH. The cultures were incubated in a culture room under the standard culture conditions described above. To calculate the average biomass productivity (g L^{-1} d^{-1}) of each 72h cycle in repeated-batch mode, the increase in biomass per liter at the end of a cycle was divided by the cycle duration (3 days).

Repeated-batch cultures for algal adaptation at increasing salinity

Experiments at different salinities were performed in 1 L flasks in repeated-batch mode. The culture media were prepared at pH 2.5 with different salinities, 100 mM, 300 mM, 400 mM, and 500 mM NaCl. The growth experiments were performed in: (a) non-adapted batch cultures, to which NaCl was added at the selected concentration just at the start of the experiment; (b) adapted cultures, in which NaCl was gradually increased up to the selected concentration. For that, the NaCl concentration of the repeated-batch culture was held constant until constant productivity was reached. Then the salinity was increased and the process repeated again. A culture was considered "adapted" once the biomass productivity was constant and the quantum yield (Qy) was at least 0.6 (Zijffers

et al. 2010). The algal cultures were incubated in a culture room under the standard culture conditions described above. Average biomass productivity was calculated as described before.

Light and electron microscopy

Photomicrographs of *C. onubensis* were taken from both control culture cells and NaCl adapted culture cells using an Olympus BX-61 microscope (Olympus, Tokyo, Japan) with a CCD Colour-View-II camera (Soft Imaging System, Münster, Germany) and the CellSens analysis imaging system (Olympus, Tokyo, Japan). For transmission electron microscopical observations, the algal cells were collected by centrifugation (5,000 rpm at 1,957 *g*, 1 min) from each culture (repeated-batch cultures treated with 400 mM NaCl and untreated cultures). The algal cells were fixed with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h at 4 ºC. The cells were then washed three times for 5 min using the same buffer. The samples were postfixed with 1% osmium tetroxide in 0.2 M cacodylate buffer at 4 ºC for 1 h. Samples were washed with the same buffer, dehydrated in a graded ethanol series, and embedded in Epon 812 (Electron Microscopy Science, Hatfield, USA). Ultrathin sections of 80–90 nm obtained by an ultramicrotome (Leica, Wetzlar, Germany) and placed on copper grids, were stained with aqueous 1% (w/v) uranyl acetate and lead citrate. Transmission electron micrographs were observed with a JEM 1011 (JEOL Ltd., Tokyo, Japan) electron microscope using an accelerating voltage of 80 kV. Several photographs of entire cells and of local detailed structures were taken at random, analyzed, and compared to investigate the effect of NaCl on different subcellular structures. All chemicals used for histological preparation were purchased from Electron Microscopy Sciences.

190

191 *Dry weight measurements*

192 To measure dry weight, 10 mL samples of each culture were used. The samples were
193 filtered through Whatman filters of 47 mm diameter and 0.7 μm pore size. The filters
194 containing the wet algal biomass were dried at 100 ºC in a stove for 24h (Vaquero et al.
195 2014).

196

197 *Maximal photosynthetic efficiency*

198 Maximal photosynthetic efficiency of Photosystem II (PSII), also named as Quantum
199 yield (Qy, Fv/Fm), was measured to evaluate the viability of the cells. Qy was
200 determined using the pulse amplitude modulation (PAM) technique. This technique
201 measures the chlorophyll fluorescence in dark adapted cells. The chlorophyll
202 fluorescence was measured using an AquaPen AP-100 (Photon Systems Instruments,
203 Czech Republic) device. The measurements were performed with 2 mL samples of algal
204 culture. The samples were dark-adapted for 15 min (F_o) prior to the application of a
205 saturating pulse of actinic light (F_m). The maximum quantum yield was calculated
206 according to Cuaresma et al. (2011). Qy is equivalent to F_v/F_m in dark-adapted samples
207 with $F_v = F_m-F_0$.

208

209 *Statistics*

210 Unless otherwise indicated, figures show means and standard deviations (SD) of three
211 independent experiments.

Results

Morphology

The morphology of *C. onubensis* was investigated by light and transmission electron microscopy for cells cultivated under standard conditions and under different salinities as described in Materials and Methods. Light microscopy of exponentially growing cells cultivated under standard conditions revealed the typical shape characteristic for the genus *Coccomyxa*: most mature vegetative cells were of elongated-ovoid shape with a large chloroplast that occupied more than half of the cell volume (Figs. 1a,b). The average size of mature vegetative cells cultivated under standard conditions was 6.9 ± 1.1 μm x 3.0 ± 0.3 μm. A few vacuoles were observed in the cytoplasm of each cell grown under standard conditions (Figs. 1a,b). Cultivation under high salinity (400 mM NaCl) resulted in the following morphological changes: (a) Slight increase in cell size; (b) Increase in the number of vacuoles; (c) More spherical appearance of cell shape (Figs. 1c,d); (d) Protrusion at one cell end (Figs. 1c,d). The size of cells cultivated under high salinity increased significantly up to 9.6 ± 1.5 μm x 4.7 ± 1.1 μm.

To study the phenotypic plasticity of *C. onubensis*, the effect of high salinity on the ultrastructure was determined by transmission electron microscopy and compared to control culture cells (Fig. 2). Figs. 2a and 2b were derived from longitudinal and cross-sections through cells from control batch cultures and show a unicellular microalga with a typical large chloroplast that surrounds the nucleus and occupies approximately half of the total cell volume. Fingerprint-like thylakoids and several starch bodies are obvious. The microalga was ellipsoidal in shape (Fig. 2c), and showed a distinct, but apparently thin cell wall (Fig. 2b; see also Garbayo et al. 2012). Cells from cultures grown under

236 salt stress (Figs. 2d-f) contained lipid droplets and occasionally electron-dense deposits
237 in the cytoplasm were observed (Figs. 2d,e). A protrusion at one cell end was observed
238 in some of the cells under high salinity (Fig. 2f). Typical reproduction with two to four
239 autospores formed by oblique division within the mother cell was observed (Fig. 2d).

240

241 *Phylogenetic analyses*

242 The complete 18S and ITS rDNA sequence of *Coccomyxa onubensis* was determined
243 (HE617183). The 18S rDNA contained 1801 nt excluding a 437 nt long group I intron
244 located at position 1512 (*E. coli* numbering). The ITS1, 5.8S, and ITS2 region was 643
245 nt long. The sequence was aligned with representatives of all *Coccomyxa* species
246 recently defined by Darienko et al. (2015) and a maximum likelihood phylogenetic tree
247 was inferred (Fig. 3). This tree shows, that our isolate from Río Tinto together with an
248 isolate from a tree bark (deposited in GenBank as HE586515) constitutes a new species
249 *C. onubensis* with a distinct ITS2 barcode defined as BC-10 by Darienko et al. (2015).
250 Although species delimitation of morphologically simple and asexually reproducing
251 green microalgae like *Coccomyxa* or *Chlorella* is now completely defined on the basis
252 of compensating base changes (CBC's) in conserved regions of ITS2, expressed as ITS2
253 DNA barcodes (Darienko et al. 2015 and references therein), we found four more
254 strains in the database, for which no ITS sequence is available, but which most likely
255 also belong to *C. onubensis*. Table 1 shows, that the 18S rDNA sequences of these
256 strains display less nucleotide differences to *C. onubensis* than to all other next closely
257 related *Coccomyxa* species.

258

Formal taxonomic description

***Coccomyxa onubensis* Fuentes, Huss, Montero, Torronteras, Cuaresma, Garbayo & Vílchez sp. nov.** (Fig. 1).

Diagnosis**:** Mature vegetative cells solitary or occasionally assembled in star-like structures, elongated-ovoid, asymmetric, cell size 6.9 ± 1.1 μm x 3.0 ± 0.3 μm. Chloroplast lateral, trough-shaped, covering more than half of the cell volume. Lipid droplets present in the cytosol. Starch grains present in the chloroplast. Cell wall apparently thin. Reproduction by 2-4 autospores. Protoplast division is oblique. Morphologically similar to *Coccomyxa galuniae*. Exact identification possible only using phylogenetic markers.

Habitat: acidic waters.

Type locality: Tinto River, Huelva, Andalucía, Southwestern Spain.

Holotype: (We are still waiting for the conformation of the SAG to include strain ACCV1 into their culture collection as a cryopreserved strain in a metabolically inactive state).

Iconotype: (designated here in support of the holotype): Fig. 1.

Etymology: The species name was chosen in relation to Onuba, the latin name for the Spanish City Huelva.

Adaptation of Coccomyxa onubensis *to acidic, neutral, and alkaline pH*

279 *Coccomyxa onubensis* ACCV1 was isolated from an acidic environment with a pH
280 between 2 and 3 (Garbayo et al. 2012) and was able to adapt to a wide range of pH from
281 acidic to alkaline values and to high salinity (approximately 85 % of that of seawater,
282 599 mM). Cultures were prepared to study the pH-dependence of biomass productivity
283 as described in Materials and Methods. In the adaptation process to each selected pH
284 value, only those cultures shifted from pH 2.5 to pH 0.5 and 1.5 did not grow. Cultures
285 shifted from pH 2.5 to higher pH values showed immediate adaptation in terms of cell
286 density and dry weight, until constant biomass productivities were reached in the
287 repeated-batch cultures at each selected pH value. The biomass productivity at different
288 pH values, calculated as described in Materials and Methods, is given in Fig. 4a as
289 percentage of the maximal value at pH 4. The results show that *C. onubensis* is
290 productive at acidic, neutral, and alkaline pH, if the cultures were previously adapted.
291 The algal biomass productivity was higher at acidic pH above 2.5, with a maximum of
292 0.22 g L^{-1} d^{-1} at pH 4. The difference between the maximal and the minimal biomass
293 productivity of the cultures adapted to acidic pH is only about 10 %. The biomass
294 productivity of cultures adapted to neutral and alkaline pH accounted for approximately
295 80% of the maximal value at pH 4. The slight differences in biomass productivity of the
296 cultures within the pH range from 2.5 to 9 were consistent with the slight differences in
297 the maximal photosynthetic efficiency (quantum yield, Qy) of these cultures as shown
298 in Fig. 4b. All cultures from pH 2.5 to 9 showed Qy values above 0.6, which indicates a
299 highly active photosynthesis.

300

301 *Adaptation to salinity*

Standard cultures of *C. onubensis* were adapted to increasing salinity following the procedures described in Materials and Methods. At each salinity, the biomass productivity became constant mostly after 3 or 4 72h-growth cycles of the corresponding repeated-batch culture. Fig. 5a shows the biomass productivity obtained during the steady-state once the repeated-batch cultures adapted to salinities of 100 to 500 mM (so-called "adapted" cultures, A.). Moreover, productivities from standard batch cultures with the respective amount of NaCl just added before the growth experiments (so-called "non-adapted" cultures, N.A.) are also shown. The latter data therefore reflect the saline stress experienced by non-adapted cells. The direct addition of NaCl to non-adapted batch cultures resulted in a decreased biomass productivity, which became as low as 27 % of that of standard cultures. However, the gradual adaptation to increasing salinities of adapted cultures resulted in biomass productivites similar to those reached by standard control cultures. *C. onubensis* even adapted to 500 mM NaCl, which is close to seawater salinity (599 mM), and still reached a biomass productivity of 0.17 g L^{-1} d^{-1}, approximately 80 % of that of standard cultures.

The adaptation to high salinity of *C. onubensis* requires the cells to retain functional photosynthetic activity at PSII. In Fig. 5b, the impact of the increased osmotic pressure on non-adapted cultures is evident by the immediate decrease of the chlorophyll fluorescence measured as Qy, which represents the maximal PSII efficiency. Each Qy value shown in Fig. 5b for non-adapted cultures was recorded 72h after addition of the corresponding amount of NaCl. Therefore, each Qy value represents the PSII efficiency of each batch culture, once the impact of the added NaCl had settled. In non-adapted cultures with 100 mM or 300 mM NaCl concentrations the Qy decreased after 72h by about 15% and 20%, respectively, but in cultures with 400 mM or 500 mM NaCl, the PSII efficiency decreased by approximately 50% within the

same time. In contrast, as also shown in Fig. 5b, the adaptation to increasing NaCl concentrations of repeated-batch cultures allowed the full recovery of PSII efficiency in terms of Qy values.

Discussion

Morphology

The general characteristics of *C. onubensis* cells observed under the light microscope including a large chloroplast, visible presence of vacuoles, elongated-ovoid shape, cell size, and oblique cell division (Figs. 1,2) are in good agreement with the observations reported by Darienko et al. (2015) for most *Coccomyxa*-like species. The typical reproduction with 2-4 cells formed by oblique division (Fig. 2d) was also described by Darienko et al. (2015) for other *Coccomyxa* strains, who also reported some atypical autosporangia with 16-32 daughter cells for *C. dispar* (SAG 49.84) and *C. viridis* (SAG 216–4) photoautotrophically grown in nutrient-rich BBM medium. Such atypical autosporangia were not found in photoautotrophically grown *C. onubensis* cultures.

Cultivation of *C. onubensis* under high salinity produced significant changes in the cell morphology both inside the cell and in the cell shape. The slight increase in cell size and number of vacuoles observed for *C. onubensis* adapted to high salinity is consistent with observations for other *Coccomyxa* strains (Darienko et al. 2015). *C. onubensis* was able to grow under high salinity (500 mM), almost seawater salt concentration (Fig. 5). Therefore, the morphological changes observed under such conditions prove the high adaptation capacity of *C. onubensis* to those harsh cultivation conditions.

The electron microscopy studies showed that *C. onubensis* cells adapted to salt stress differed in their general morphology from control culture cells, becoming slightly more spherical in appearance and with a protrusion at one cell end (Fig. 1), in accordance with observations of Darienko et al. (2015) and Muscatine et al. (1994) for other *Coccomyxa* strains. A viscous material was present outside some salt-adapted cells (Fig. 2c). This material might be of polysaccharide nature and might play a relevant role in regulating the ionic balance around the cells (Bérubé et al. 1999, Ferroni et al. 2007). The increase of lipid droplets due to salt stress has been previously described in the halotolerant green alga *Dunaliella tertiolecta* (Takagi et al. 2006, Goyal 2007), in *Chromochloris* (formerly *Chlorella*) *zofingiensis* and *Haematococcus pluvialis*, in which salt stress induced the production of secondary carotenoids (Orosa et al. 2001, Pelah et al. 2004).

The increase of lipid droplets found in *C. onubensis* adapted to high salinity (400 mM NaCl) is also consistent with recently published data of Darienko et al. (2015) for other *Coccomyxa* species. They studied the morphological and physiological plasticity of *Coccomyxa* for more than 40 strains and reviewed the biodiversity and biogeographical distribution of *Coccomyxa* species. According to the algal response to different salinities in terms of cell size and shape, they suggested to classify strains into three types: sensitive, intermediately sensitive and robust. Compared to salinity resistance of other *Coccomyxa* strains, *C. onubensis* might be considered as a salinity-robust species. Interestingly, all *Coccomyxa* strains found by Rodriguez et al. (2008) and Vázquez et al. (2010) as parasites of marine mussels, belong to *C. viridis*, the most salinity-robust species in the study of Darienko et al. (2015).

374 *Phylogeny*

375 The species concept for the genus *Coccomyxa* was recently evaluated by Darienko et al.
376 (2015) using integrative taxonomy and DNA barcoding. They recognized the 18S + ITS
377 sequence of our strain ACCV1 as a yet to describe new species with a distinct barcode
378 (BC-10) for ITS2. Our phylogenetic tree in Fig. 3 confirms the distinct position of
379 ACCV1 together with *C*. spec. KN-2011-T3 (HE586515), next closely related to *C.*
380 *polymorpha* (BC-2). We therefore describe here strain ACCV1 and other strains with
381 the same barcode as *Coccomyxa onubensis* spec. nov. Moreover, one of us (V.A.R.H.)
382 has determined a 18S rDNA sequence from an uncultured environmental sample
383 (RL75K2, deposited as HE617184 in GenBank), which most likely also belongs to the
384 new species. Although no ITS sequence is available for this strain, its 18S sequence
385 differs by just one nucleotide from the sequence of ACCV1 (Table 1). RL75K2 was
386 contained in a water sample taken from "Restloch" 75, an acidic mining lake in Lusatia
387 (Germany) with a pH of 2.4 (51°31'0,7.50''N, 13°42'57.34''E). According to Table 1,
388 three more strains may be assigned to *C. onubensis* based on their 18S rDNA
389 sequences: strains AH4 (KC155324; no difference to ACCV1 within 1526 determined
390 nucleotides) and AC1 (KC155323; three differences to ACCV1 within 1516
391 nucleotides) were found in Guadiana pit lake (Spain) with a pH of 2.9 (Falagan et al.
392 2014), and "*Pseudococcomyxa simplex*" Rsa3 (KM016993) was detected in an acidic
393 copper mine draining stream (Falagan et al., unpublished). Thus, all strains except *C.*
394 spec. KN-2011-T3 (Fig. 3), which was isolated from a tree bark in Java, were found in
395 extremely acidic environments. This implies, that *C. onubensis* preferentially grows
396 under acidic conditions, but due to its high adaptability may also be found in various
397 other habitats.

398

399 *Adaptation of* Coccomyxa onubensis *to acidic and alkaline pH*

400 Highly acidic aquatic environments host photosynthetic eukaryotic life. Acidophilic
401 algae are adapted to pH values as low as 0.05 and unable to grow at neutral pH, while
402 acidotolerant algae are also able to grow at neutral or even higher pH (Gross 2000). As
403 *C. onubensis* grows over a wide pH range from 2.5 to 9, it has to be considered as
404 acidotolerant microalga.

405 Considering that the intracellular pH of microalgae from acidic environments
406 has been found to be nearly neutral (Beardall & Entwisle 1984, Gimmler et al. 1988),
407 life at such conditions depends on the cell capacity to reduce the proton influx and to
408 increase the proton pump efficiency that keeps the proton efflux highly active. Several
409 acidophilic and acidotolerant microalgae have been shown to keep a very low proton
410 permeability coefficient across the plasma membrane which avoids acidification of the
411 cytosol (Gross 2000). To keep an active, intense proton efflux is an ATP-dependent
412 process, which consumes metabolic energy of approximately at least 7% of the total
413 ATP generated by photosynthesis (Messerli et al. 2005), therefore reducing the energy
414 available for growth. This is consistent with the low growth rate values found for
415 acidophilic and acidotolerant microalgae if compared to the so-called “common”
416 microalgae (Gross 2000, Vaquero et al. 2014).

417 The growth of acidophilic and acidotolerant microalgae depends on the
418 availability of dissolved inorganic carbon, CO_2 and HCO_3^-. The bioavailability of
419 dissolved inorganic carbon differs enormously depending on the pH of the culture
420 medium. By far most of the dissolved inorganic carbon at acidic pH is available in the
421 form of CO_2 . Depending on the species, acidophilic and acidotolerant microalgae have
422 been found to express high affinity carbon concentration systems that are able to capture
423 inorganic carbon at very low concentrations and convert it again into CO_2 at the active

site of Rubisco (Cuaresma et al. 2006, Verma et al. 2009, Vaquero et al. 2014). Particularly, Verma et al. (2009) proposed the presence of an external carbonic anhydrase (CA) in *Coccomyxa*, although with a carbon transport facilitating role rather than a concentrating function. This would partly explain the moderate productivity values obtained for *C. onubensis* cultured at highly acidic pH (Fig. 4a), and therefore suggests that culturing *C. onubensis* in photobioreactors under intensive control of CO_2 supply should be promising for achieving high biomass productivities.

Adaptation of Coccomyxa onubensis *to salinity*

Salinity is a stress factor for most microalgae. The capacity of microalgae to cope with increased salinity levels in the culture medium depends on the species. Depending on their adaptability to salinity, microalgae are considered as halophilic or halotolerant (Richmond 2004). *Coccomyxa onubensis* was found to grow in culture media that contain up to 0.5 M NaCl, and can therefore be considered as halotolerant. As the concentration of salt in the seawater is on average 35 g L^{-1}, *C. onubensis* might even be able to grow on seawater, thus saving water resources and costs for biomass production.

The capacity of *C. onubensis* to grow at approximately seawater salinity together with its natural ability to grow at very low pH such as 2.5, allow culture conditions, which are highly unfavorable for competing microalgae. Consequently, both halotolerance and optimal growth at acidic pH of this alga are promising properties to prevent contamination also in outdoor mass production.

It is widely accepted that the Qy value ranges from 0.6 to 0.8 in healthy microalgal cultures adapted to darkness (Zijffers et al. 2010). Thus, according to Fig. 5

447 *Coccomyxa* cultures adapted to high salinity can be considered photochemically highly
448 active. The electron transport in photosynthesis is affected by the oxidative action of
449 ROS and by the high concentrations of Na^+ which alter the D1 proteins and the water
450 complex oxidation at PSII (Sudhir et al. 2005, Sudhir & Murthy 2004). High ionic
451 strength induces the intracellular accumulation of osmo-regulant molecules in
452 microalgae that protect proteins and membranes against chemical damage produced by
453 inorganic ions, including the oxidative damage from ROS (Varshney et al. 2015).
454 However, there is little information available on the biochemical mechanisms by which
455 halotolerant microalgae cope with high osmotic pressure. Chen et al. (2015) suggested
456 the involvement of a Na^+-transporting ATPase in Na^+-transfer through the plasma
457 membrane and proposed that the halotolerance of intracellular *Dunaliella* proteins might
458 play a role in fighting against the transient intracellular salt fluctuations during
459 hyperosmotic shock. These findings are consistent with the fact the PSII efficiency in *C.*
460 *onubensis* recovers well from the hyperosmotic shock produced by the addition to the
461 culture medium of a high amount of NaCl (Fig. 5b). This might explain the halotolerant
462 behaviour of the alga in addition to the accumulation of osmo-regulant molecules,
463 whose nature and applications have still to be investigated.

464 The phenotypic changes of *C. onubensis* cells adapted to high salinity (Fig. 2)
465 reinforce the idea of a strong adaptation response, in good agreement with the
466 adaptation concept stated by Darienko et al. (2015). Such a strong adaptation response
467 enables the microalga to reach biomass productivities similar to those of control
468 cultures, which might be of biotechnological interest.

469

470 **Acknowledgements**

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590

591 **Captions to Figures**

592 Fig. 1. - Morphology of *Coccomyxa onubensis* sp. nov. Large chloroplast and
593 elongated-ovoid shape of control culture cells (a,b). Higher cell size, protrusion at one
594 cell end and spherical-like cell shape (c,d) of cells grown under high salinity.

595 Fig. 2. - Transmission electron microscopy of *Coccomyxa onubensis*. (a-c) Control cells
596 from cultures without added NaCl. (d-f) Cells adapted to salt stress (400 mM NaCl)
597 with lipid droplets (d,e) and electron-dense inclusion (e). Scale bars: (a, b, d, e, f) 1 μm
598 (x 30,000); (c) 5 μm (x 6,000). Abbreviations: Ch, chloroplast; Th, thylakoids; L, lipid
599 droplets; St, starch grains; EI, electron-dense inclusion; CW, cell wall.
600

601 Fig. 3. – Maximum likelihood tree based on 18S, 5.8S, and ITS2 rRNA gene sequences
602 of the genus *Coccomyxa*. A sister group of *Coccomyxa* (*Hemichloris antarctica* and
603 *Elliptochloris bilobata*) was chosen as an outgroup. Bootstrap values of each 1,000
604 replications of ML/NJ/MP are indicated at the branches if greater than 50%. Thick lines
605 represent nodes with 100% bootstrap support for all three methods. Branch lengths
606 reflect the evolutionary distance indicated by the scale. The different *Coccomyxa*
607 species are defined by distinct ITS2 barcodes (BC) as published by Darienko et al.
608 (2015).

609

610 Fig. 4. - Effect of pH on biomass productivity (A) and maximal photosynthetic
611 efficiency (Qy) (B) of *Coccomyxa onubensis*. Aliquots of equal biomass from a mother
612 culture were adapted to different pH values (from 2.5 to 9) through repeated-batch
613 cultivation. A repeated-batch mother culture was used as control at pH 2.5. Biomass
614 productivity of each culture is shown as percentage of the maximal productivity found

615 at pH 4 (100 % biomass productivity = 0.22 g L^{-1} d^{-1}). Details are described in Materials
616 and Methods.

617

618 Fig. 5. - Effect of salinity on biomass productivity (A) and photosynthetic efficiency
619 (Qy) (B) of *Coccomyxa onubensis*. Aliquots of equal biomass prepared from a mother
620 culture were adjusted to different NaCl concentrations (from 100 to 500 mM) and
621 incubated either as batch cultures (N.A., non-adapted cultures), or adapted to increasing
622 salinities as repeated-batch cultures (A., adapted cultures). A repeated-batch mother
623 culture was used as control. Details are described in Materials and Methods.

624

625

Figure 1

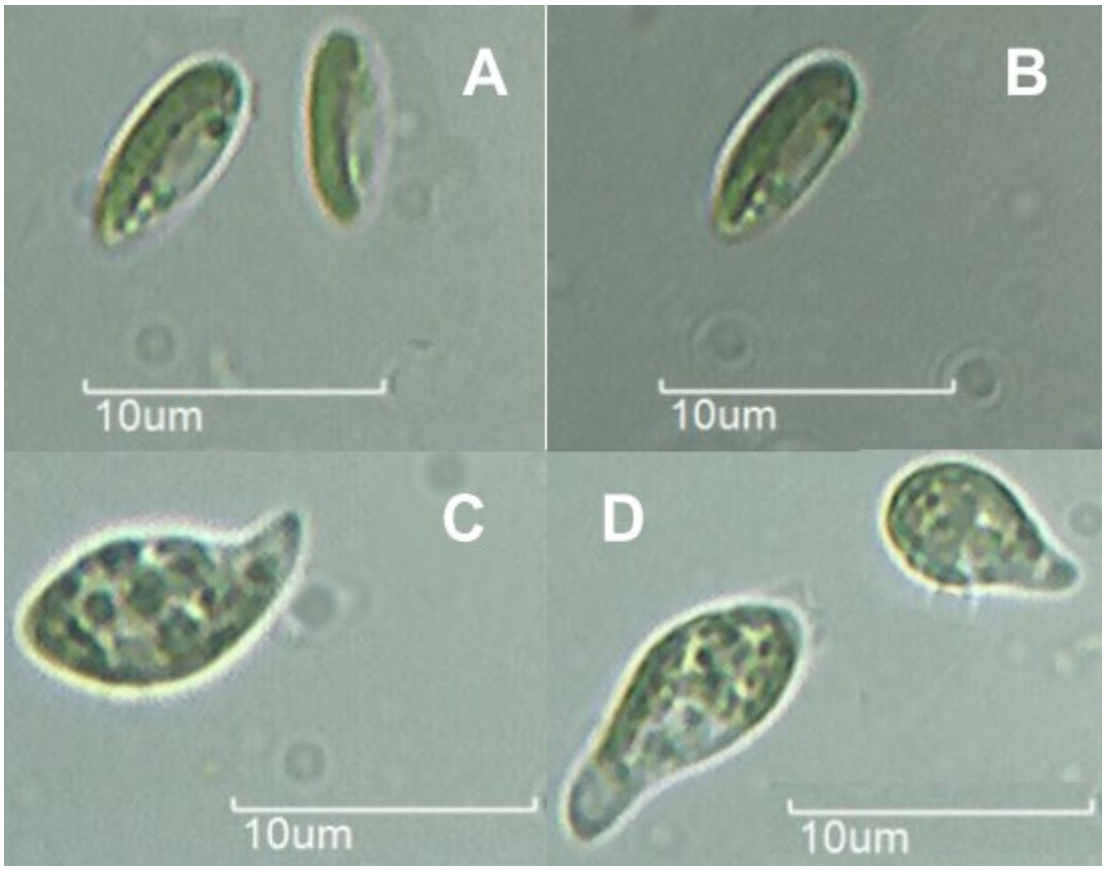
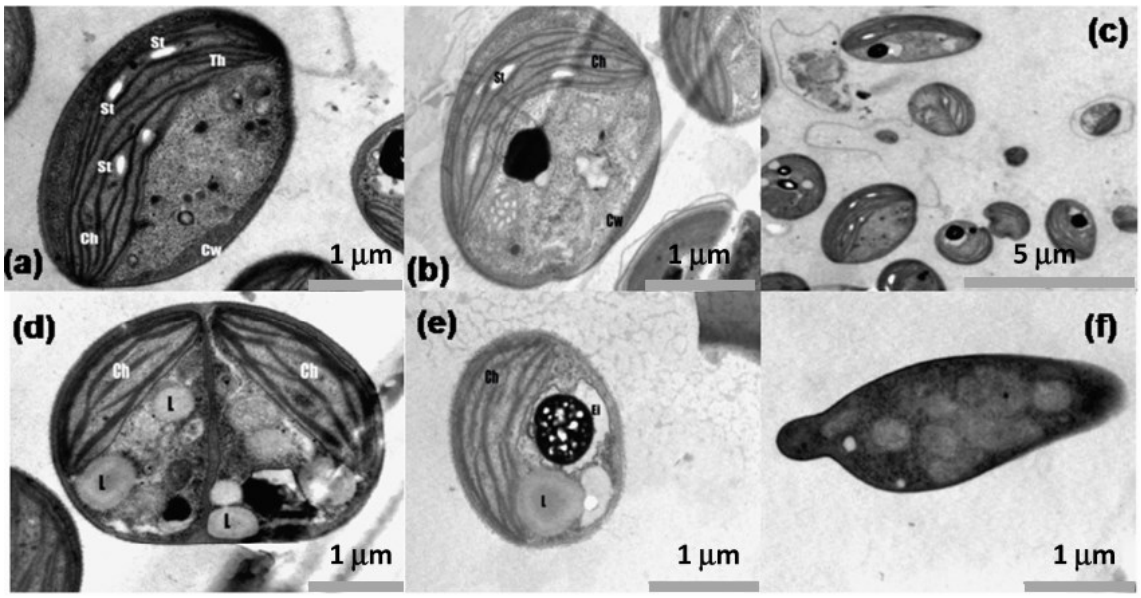


Figure 2


(a)
St
Th
St
St
Ch
Cw
1 µm
(b)
St
Ch
Cw
1 µm
(c)
5 µm
(d)
Ch
Ch
L
L
L
1 µm
(e)
Ch
El
L
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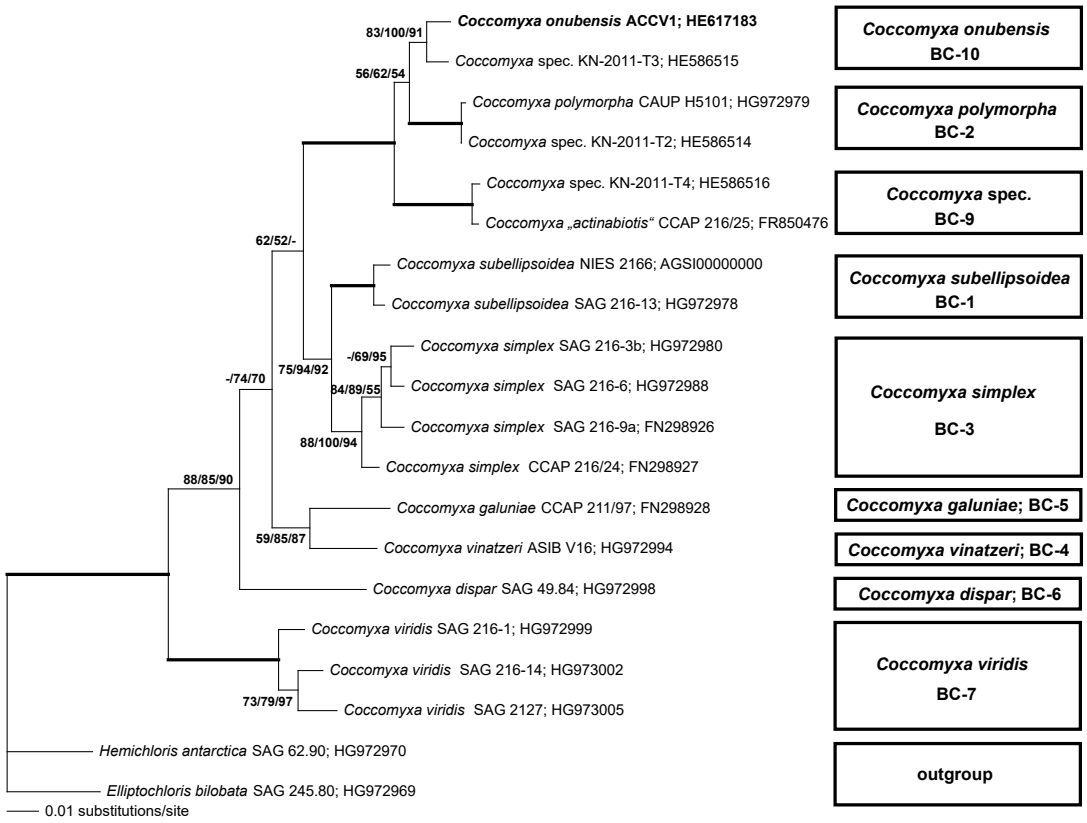
(f)
1 µm

656

657 **Figure 3**

658



659

Figure 4

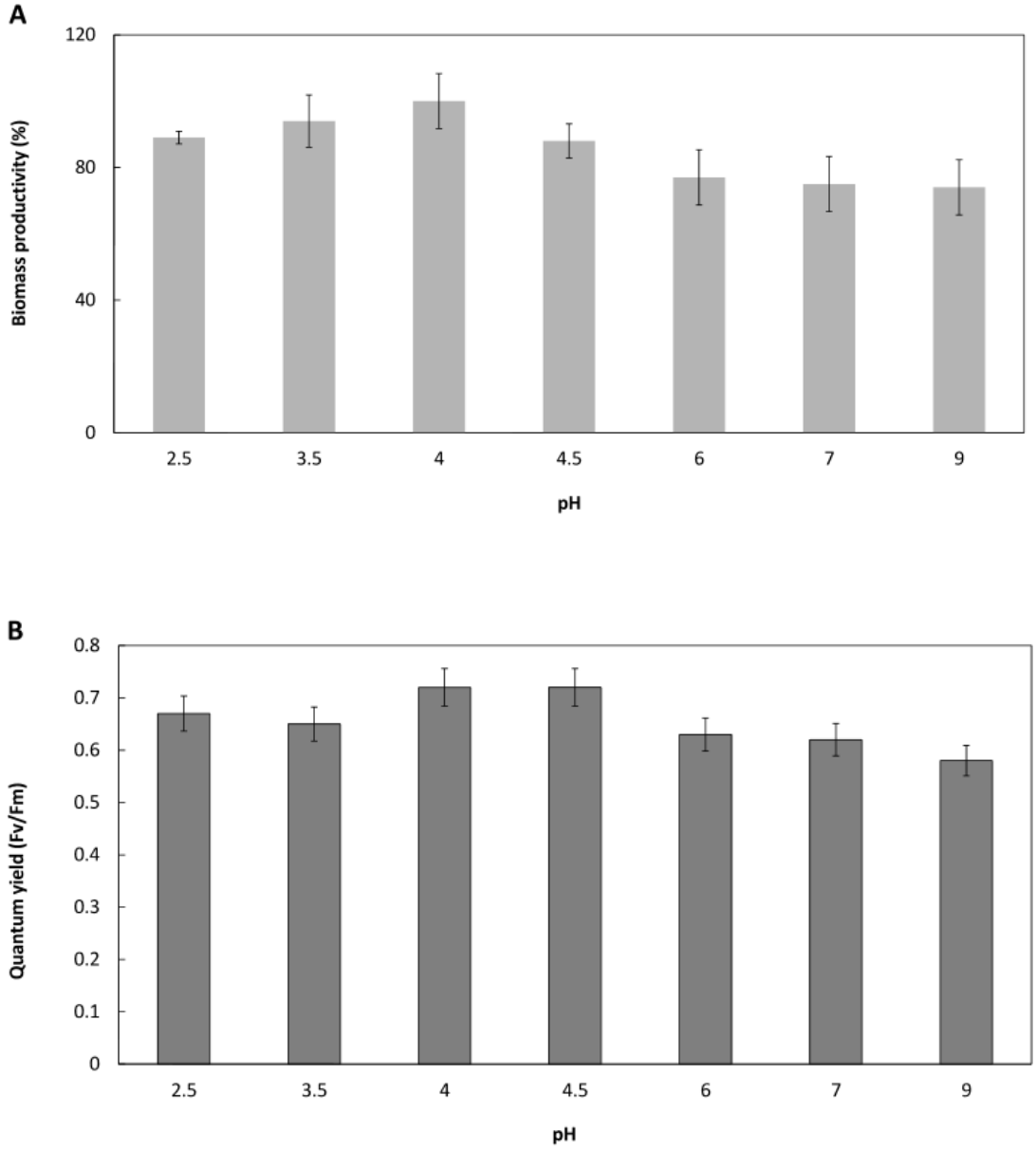
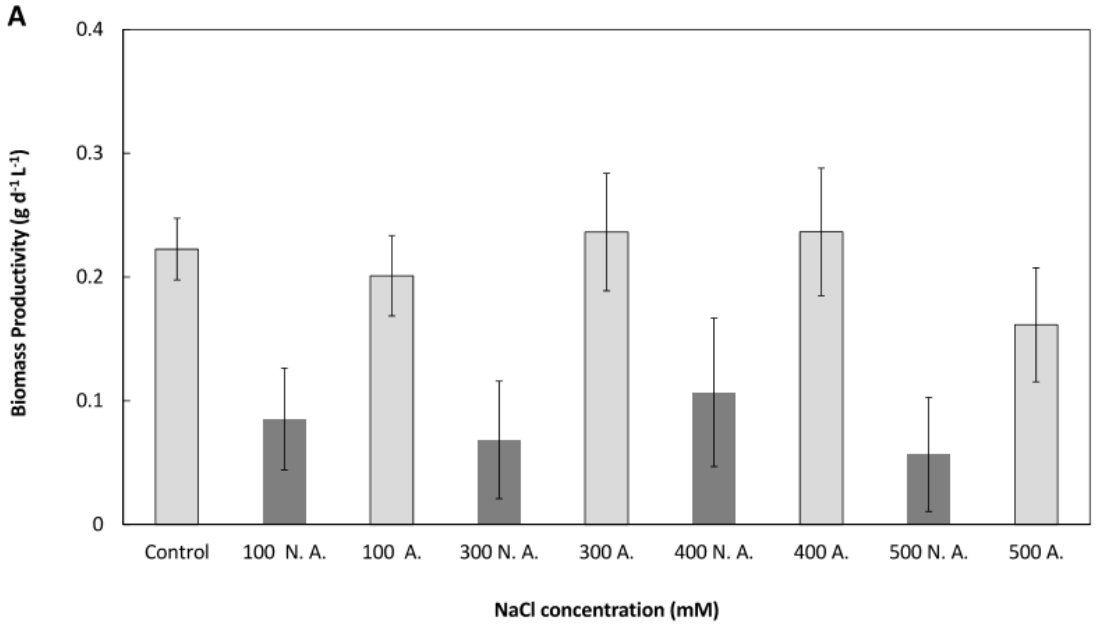
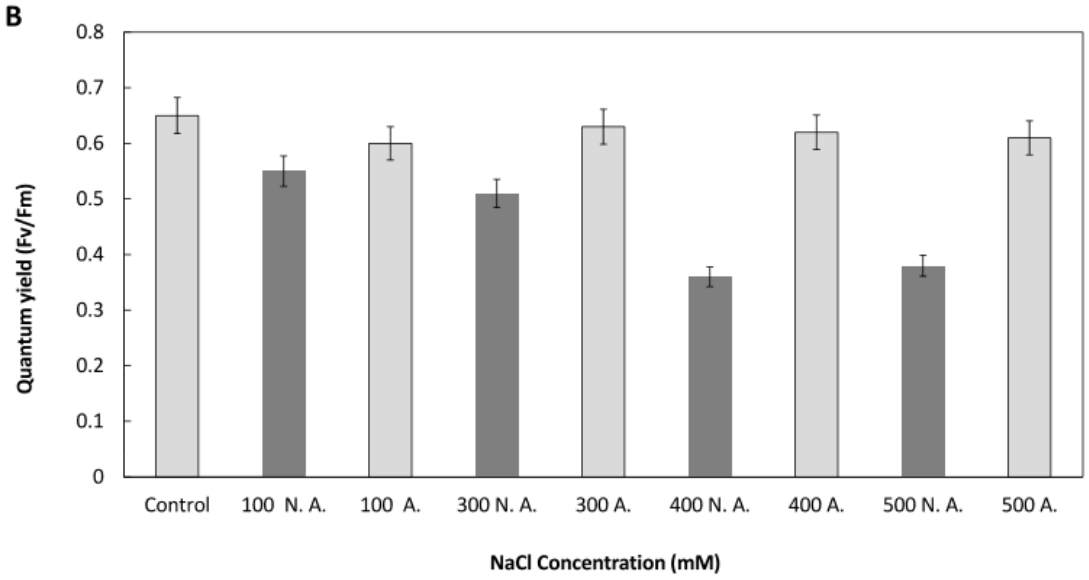

A
120
80
40
0
Biomass productivity (%)
2.5
3.5
4
4.5
6
7
9
pH
B
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Quantum yield (Fv/Fm)
2.5
3.5
4
4.5
6
7
9
pH

Figure 5


A
Biomass Productivity (g d-1 L-1)
0.4
0.3
0.2
0.1
0
Control
100 N. A.
100 A.
300 N. A.
300 A.
400 N. A.
400 A.
500 N. A.
500 A.
NaCl concentration (mM)


B
Quantum yield (Fv/Fm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
100 N. A.
100 A.
300 N. A.
300 A.
400 N. A.
400 A.
500 N. A.
500 A.
NaCl Concentration (mM)

685 Table 1: 18S rDNA nucleotide differences between *Coccomyxa onubensis* ACCV1, closely related strains, and representatives of next closely related described species. Strains enclosed in the box most
686 likely belong to *C. onubensis*.

687

Strain; GenBank No./ Sequence length (nt)	*C. onubensis* ACCV1 (1797)	*C.* spec. AH4 (1526)	*C.* spec. RL75K2 (1797)	*C.* spec. AC1 (1516)	„*Pseudoc. simplex*“ Rsa3 (1707)	*C. polymorpha* CAUP H5101 (1777)	*C. subellipsoidea* NIES 2166 (1777)	*C. simplex* SAG 216-9a (1777)
C. onub. ACCV1; HE617183	-	0	1	3	3	6	29	29
C. spec. AH4; KC155324		-	1	3	3	6	22	23
C. spec. RL75K2; HE617184			-	4	4	5	29	31
C. spec. AC1; KC155323				-	2	9	19	20
„*Pseudoc. simplex*“ Rsa3; KM016993					-	9	27	29
C. polym. CAUP H5101; HG972979						-	28	28
C. subellips. NIES 2166; AGSI00000000							-	7
C. simplex SAG 216-9a; FN298926								-

688

689