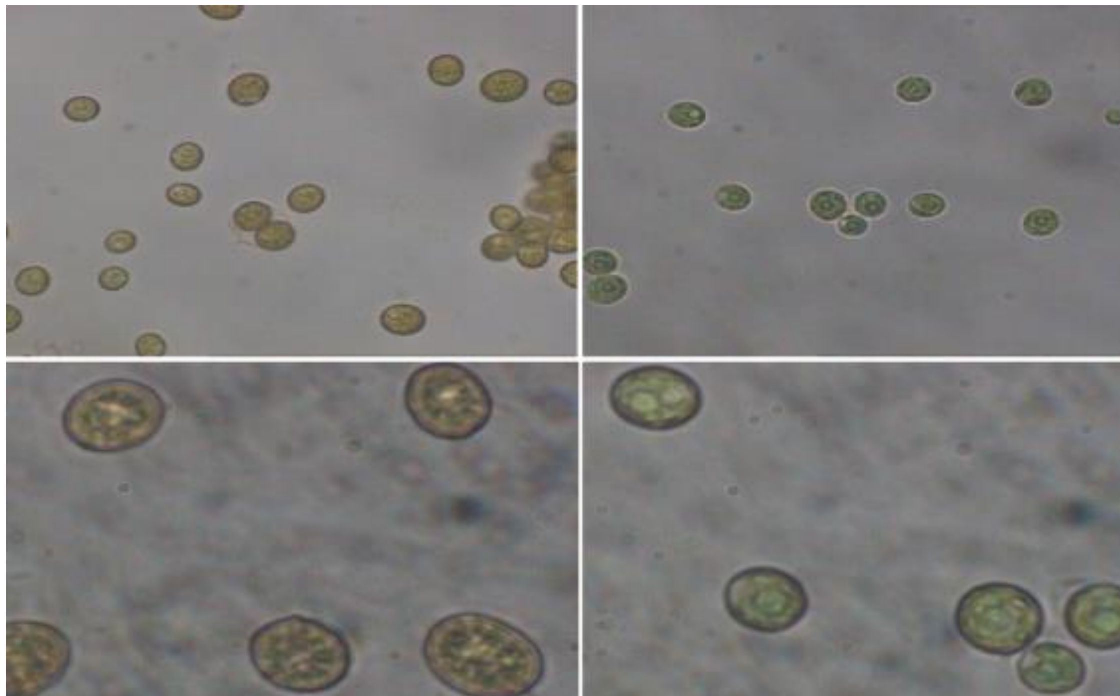
MORPHOLOGICAL AND MOLECULAR METHODS TO SELECT MICROALGAE STRAINS PRESENT IN SOLID URBAN WASTE LEACHATES



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ABSTRACT

The high metabolic capacity of microalgae coupled with a lack of total diversity and evolutionary history studies make essential the prospecting of whole environments. In the present study, microalgae diversity within the Asturian landfill was investigated. Fourteen strains were isolated and scaled-up in liquid mono-cultures. They were identified through a combination of morphologic features with molecular assignation by DNA barcoding via 18S and ITS1-5.8S-ITS2. A new specie named *Coelastrella cogersae* was discover. Moreover, it showed a caretogenic behavior during the culture. To summarize, the first screening of microalgae within a landfill has shed successful results based on morphological and molecular approaches. In addition, biotechnological capacities were found inside isolates.

RESUMEN

La alta capacidad de adaptación metabólica de las microalgas unida la falta de estudios sobre su diversidad total y su historia evolutiva hace imprescindible la exploración en todo tipo de ambientes. En este trabajo se estudió por primera vez la diversidad de microalgas existente en un vertedero asturiano. Se aislaron 14 cepas de diferentes localizaciones, las cuales fueron escaladas hasta mono-cultivos líquidos estables. Fueron identificadas mediante una combinación de caracteres morfológicos y código de barras del DNA *barcoding* de dos genes marcadores, 18S e ITS1-5.8S-ITS2. Dentro de los aislados, se descubrió una especie nueva, denominada *Coelastrella cogersae*. A su vez, la nueva especie poseía capacidad carotenogénica en cultivo. En conclusión, la primera prospección de microalgas en un vertedero ha arrojado resultados exitosos sobre la metodología combinada de identificación, así como potenciales capacidades biotecnológicas en las especies aisladas.

No data can be used without the permission of the author and/or his tutors: Yaisel Juan Borrell Pichs and José Manuel Rico Ordás.

1. Introduction

During the last century, technological and cultural development of the society has modified different aspects which must be vastly monitored. Nutritionally, there is a relationship between the decrease on the consumption of fruits and vegetables and the increase in the mortality of people with coronary heart disease and cancer, probably related with a lack of antioxidant compounds in the diet (Li *et al*., 2007). Alternative compounds called nutraceuticals have gained a lot of limelight. Among them, carotenoids have been the most important. Its great antioxidant potential as scavengers of ROS (Reactive Oxygen Species) provides a biotechnological application in human health (Guedes *et al*., 2011). Environmentally, significant attention has had greenhouse emissions, being the CO_2 the most emitted and dangerous gas among them (Chiu *et al*., 2011). Due to human activities (Liu *et al*., 2013; Arbib *et al*., 2014) levels of CO_2 has increased in the atmosphere, mainly caused by a fast exploitation of fossil fuels such as petroleum and coal (Abinandan & Shanthakumar, 2016). Flue gases from steel plants, electric power plants and cement manufacturing represent also a great percentage of emissions sources (Li *et al*., 2015).

Microalgae are a widely distributed group of microorganisms (Qiao *et al*., 2015) which take efficiently sunlight energy and CO_2 generating carbon compounds in primary metabolism (Lynch *et al*., 2015). Approximately, 50% of organic carbon fixation is carried out by microalgae (De la Vega *et al*., 2011) and, despite the complexity in defining the number of taxonomic groups in algae, it is suggested a number between 70.000 and 1 million of total (De Clerck, 2013). Surprisingly, some groups of green algae such as *Chlorella* sp. (Ramanan *et al*., 2010; Gonçalves *et al*, 2016) and cyanobacteria *Arthrospira* sp. (de Morais & Costa, 2007) have been identified not only with the CO_2-high uptake ability, but with a high tolerance to CO_2 concentration.

Among compounds which are generated by microalgal metabolism, carbohydrates can be generally used as feedstock for different microorganisms (Yen *et al*., 2013). Specifically, sulfated polysaccharides possess anti-inflamatory (Matsui *et al*., 2003) and antiviral effects (Kim *et al*., 2012). However, lipids are the most suitable group of compounds related to microalgae. Certain lipids accumulated under specific stress conditions could be advantaged for biodiesel production (Li *et al*., 2015). On the other hand, carotenoids such as astaxanthin from *Haematococcus pluvialis* (Machado Jr. *et al*., 2014) or β-

carotene from *Dunaliella salina* (Ben-Amotz *et al*., 1991) are being produced by few companies and they are established in nutraceutical market. Important substances as long-chain polyunsaturated fatty acids are desirables in human health because of their help in cognitive development and the decreased risk of chronic disease (Mühlroth *et al*., 2013).

Furthermore, their varied metabolic features make microalgae a ubiquitous group which is able to colonize all sorts of environments. For instance, these organisms have been found in environment with extreme pH, temperature, alkalinity and salinity (Varshey *et al*., 2015). Increasing the prospection and screening of particular habitats could lead the discovering of new species with an improved or even new biotechnological potential.

However, a precise identification of microalgae found in the environment is an important key in order to know the possibilities which they have. Traditionally, microalgae have been characterized based on morphological features and life cycles via light or scanning/transmission electron microscopy (Carmelo & Grethe, 1997; Fon-Sing & Borowitzka, 2013). Nevertheless, environmental parameters and their changes may cause an alteration in morphology and physiology making difficult the distinction among groups which have similar characters (Darienko *et al*., 2015). Molecular techniques such as DNA barcoding have helped to build phylogenies and identify problematic groups removing morphological mistakes (Harper & Saunders, 2001; Li *et al*., 2008; Radha *et al*., 2013;). Among gen markers which have been using for species identification (COI-5P, *rbcL*, *tufA*…), sequences belonged to ribosomal subunits are the most used in microalgal characterization. More specifically, some authors have identified microalgal groups based on 18S gen marker (Li *et al*., 2008; Durvasula *et al*., 2015) and ITS1-5.8S-ITS2 fragment (Demchenko *et al*., 2012; Liu *et al*., 2014) obtaining extraordinary results. Moreover, it can be carried out a complete study combining both morphological and molecular studies (Qiao*et al*., 2015).

Under theses premises, Spanish project "ReCO_2very" was born in 2014 (Figure 1) with the challenge of using indigenous microalgae isolated from different locations in the Asturian landfill in order to mitigate CO_2 emitted from incinerator plant of solid urban solid wastes. In turn, it will be studied the uses of microalgal biomass.

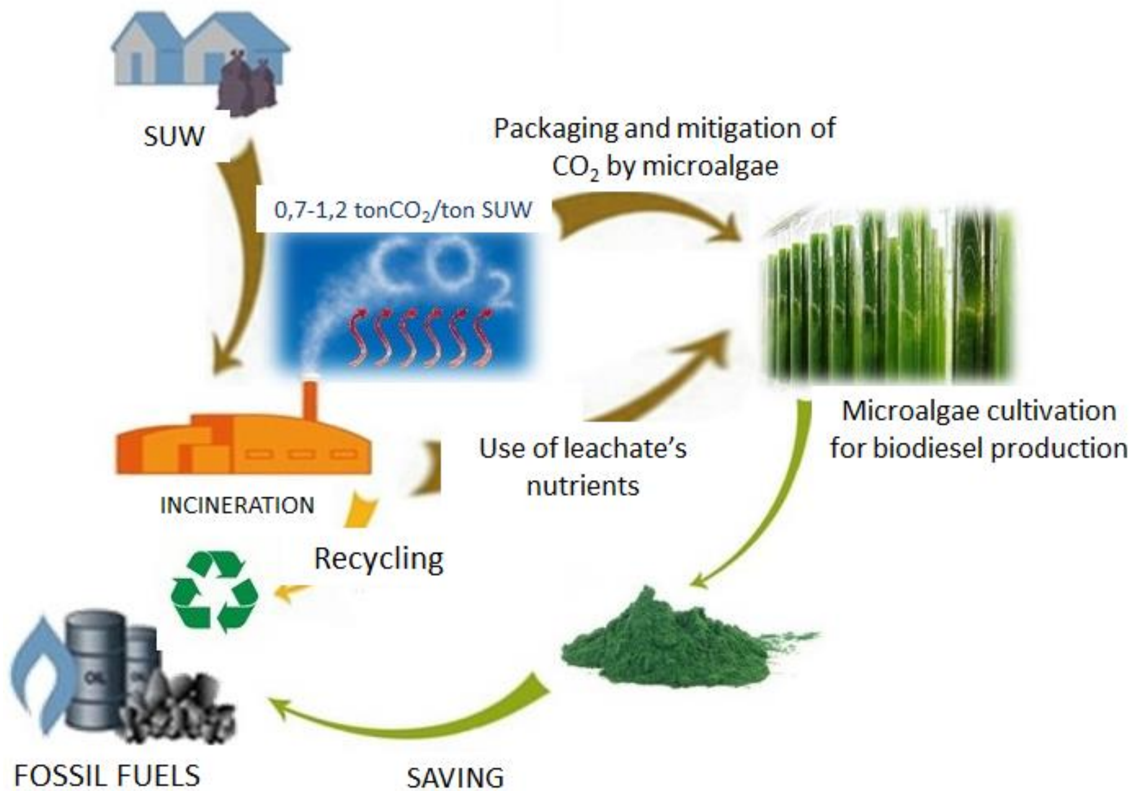

Figure 1. Scheme of "ReCO_2very" project. Solid urban wastes are burned producing CO_2 which can be fixated by a microalgal culture. Resultant biomass could be useful in different fields. Indirectly, the project will produce a save in fossil fuels exploitation.

AIMS OF STUDY

Specifically, the aims of this study are:

1- Sampling and isolation of microalgae strains from different locations around the landfill.
2- Scaling-up and maintenance of each isolate.
3- Morphological characterization through light microscopy and scanning electron microscopy.
4- Molecular identification by DNA barcoding.

2. Materials and methods

2.1 Sampling site

Samples were taking from different locations inside the Asturian central landfill (COGERSA) whose main function is the sustainable management of solid urban wastes. Sampling design pretended the integration of all types of water bodies present in the landfill in order to find out greater microalgal/cyanobacteria richness.

Table 1
Type and location of sampling sites during the study.

Name	Origin	Latitude (N)	Longitude (O)	Code
Seasonal pond 1	Natural	43º29'32.6028"	5º49'6.8353"	CE1
Seasonal pond 2	Natural	43º29'31.1883"	5º49'12.4417"	CE2
Permanent pond 1	Artificial	43º29'50.2496"	5º48'57.5999"	CP1
Permanent pond 2	Artificial	43º29'16.0390"	5º49'4.6490"	CP2
Artificial container 1	Artificial	43º29'57.2808"	5º49'3.3986"	CA1
Artificial container 2	Artificial	43º29'57.9675"	5º49'3.9152"	CA2
Raft G	Artificial			BG

Water bodies (Table 1) were classified based on their natural or artificial origin. In proper conditions, samples of 100 ml were collected in sterile test tubes and subsequently stored until their processing in the laboratory (twice per place)(Figure 2). In addition, photographs were taken for the purpose of documenting specifically the sample sites.

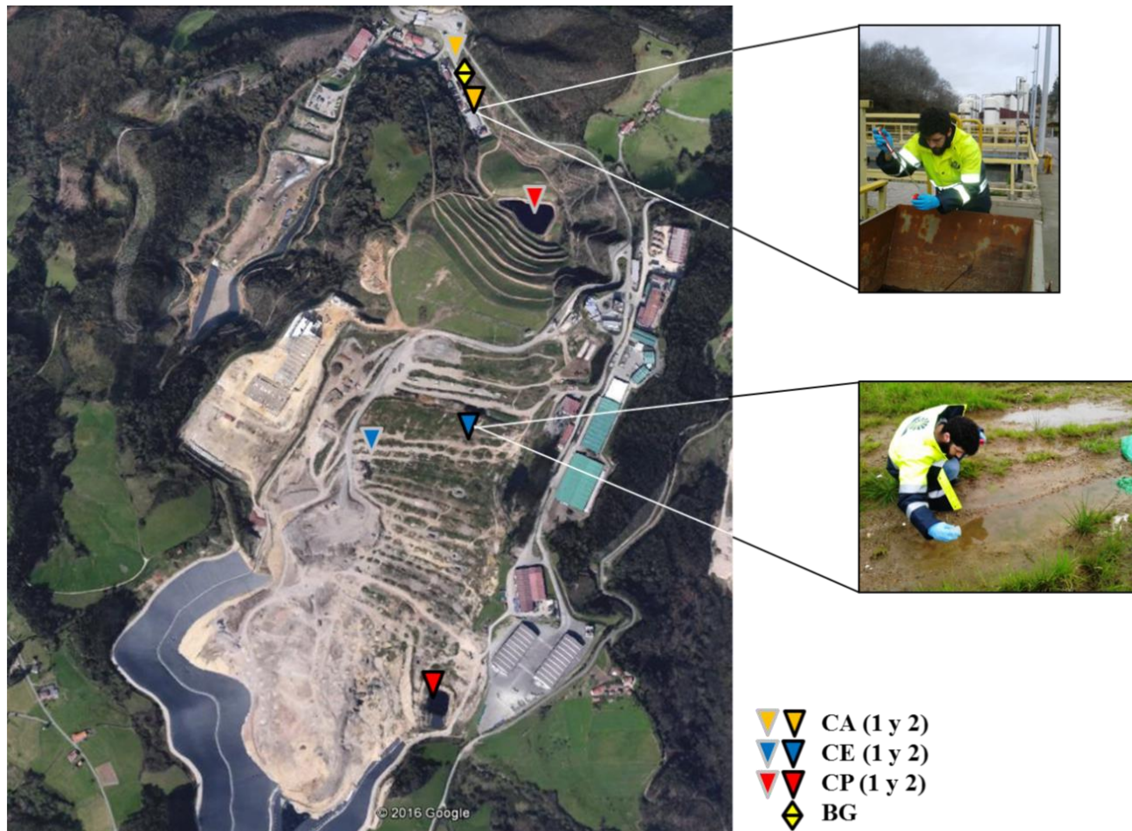

Figure 2.Map of Asturian landfill showing sampling locations by different colored triangles/rhombus.

2.2 Microalgae isolation process

Stored samples were prepared for serial dilutions from 10^{-1} to 10^{-5} and, subsequently, streaked in Petri plates of 100x15 mm with agar solid medium. Approximately, plates were inoculated with 100µl of each dilution (Andersen, 2005).

Agar solid medium was performed following the protocol proposed by Allen (1968) with few modifications. Nutrient mediums used were BG-11 (Allen, 1968) and F/2 (Guillard & Ryther, 1962) as the general mediums for microalgae and cyanobacteria respectively (Tables 2 and 3). Plates were placed in a chamber with standard conditions: 25-30 $\mu E/m^2/s^{-1}$ of irradiance, 25±2ºC of temperature and a 16:8 photoperiod (16h of light: 8h of dark). Approximately 9 days after inoculation small colonies were grown in Petri plates, being re-streaked every 15 days until the cultures were lacking in contaminations.

Table 2: BG-11 medium formulation

Table 3: f/2 medium formulation

Component	Quantity used
Fe Citrate solution	1ml
Citric acid	1ml
Ferric ammonium citrate	1ml
$NaNO_3$	1.5g
$K_2HPO_4 . 3H_2O$	1ml
$MgSO_4 . 7H_2O$	1ml
$CaCl_2 . 2H_2O$	1ml
Na_2CO_3	1ml
$MgNa_2EDTA . H_2O$	1ml
Trace metals solution	1ml
H_3BO_3	2.860g
$MnCl_2 . 4H_2O$	1.810g
$ZnSO_4 . 7H_2O$	0.220g
$CuSO_4 . 5H_2O$	1ml
$Na_2MoO_4 . 2H_2O$	0.391g
$Co(NO_3)_2 . 6H_2O$	1ml

Component	Quantity used
$NaNO_3$	1ml
$NaH_2PO_4 . H_2O$	1ml
$Na_2SiO_3 . 9H_2O$	1ml
Trace metals solution	1ml
$FeCl_3 . 6H_2O$	3.15g
$Na_2EDTA . 2H_2O$	4.36g
$MnCl_2 . 4H_2O$	1ml
$ZnSO_4 . 7H_2O$	1ml
$CoCl_2 . 6H_2O$	1ml
$CuSO_4 . 5H_2O$	1ml
$Na_2MoO_4 . 2H_2O$	1ml

2.3 Morphological identification by microscopy

Preliminary observations of cultures were carried out by an inverted light microscope with the help of phase contrast. One drop was taken and characterized based on different morphological features described before (Qiao *et al.*, 2015) with logic modifications. All images were made with 1000X magnification and it was taking in a count diverse botanical aspects: shape, size (width and length), pyrenoid position, flagella (motility or not) and cell arrangement. Therefore, images (with or without phase contrast) were modified by Photoshop program using tools such as cloning buffer, plaster and selective focus. On the other hand, Lightroom program was used to adjust clarity, exposure and light as well as to change the contrast.

In order to support the features mentioned above, scanning electron microscopy photomicrographs were taken. Specifically, external ultrastructure of cells was determined for helping in the shape and texture analysis. Pre-treatment of samples were done following Collins *et al.*, 1993 protocol with few modifications. Culture volumes between 300-500 µl (depending on the apparent cellular density) were filtered in Whatman GF/F filters of 25 mm of diameter and 0,22µm of pore size. Continually, filters

were subjected to a serial acetone:water gradient during 10 min for the dehydration. Percentages of dilutions were 25, 50, 75, 95 y 100% v:v. and test tubes were stored at 4ºC. After dehydration, filters were dried by CPD (Critical Point Dry) methodology in order to eliminate the acetone and to avoid cell damages. Furthermore, it can be achieved the total extension of filters without any wrinkle. Then, filters were sputtered by a gold cover to start with the morphological analysis.

For the purpose of obtaining a tentative identification of isolates, this morphological study was encouraged AlgaeBase database (Guiry & Guiry, 2012) and the taxonomic key developed by Bellinger & Sigee in 2015.

2.4 Scaled-up and maintenance of specific cultures (mono-cultures)

After 8 renewals, single microalgal colonies were appeared. Few of these (2-4 colonies) were picked up with a modified Pasteur pipette and put in 30-ml glass vessels (three replicates/culture) with 10ml of liquid culture. It was used both f/2 and BG-11 media. Approximately, cultures reached stationary phase in 18 days.

Likewise, 10ml were scaled-up in 250-ml Erlenmeyer flasks (three replicates/culture) with 50ml and 200ml of liquid culture. The average inoculum/medium was 1:5 (1 liquid culture:5 medium) or 1:10 depending on culture growth. Flasks were placed in a chamber with standard conditions: 70-100 µE/m2/s-1 of irradiance, 25±1ºC of temperature and a 16:8 photoperiod (16h of light: 8h of dark) (Andersen, 2005).

In order to determine the stationary phase on cultures, NO_3^- dissolved in cultures was measured with a simple test (NO_3^- Test SERA, GmbH, Berlin, Germany). Previously, 5-10 ml of liquid cultured were taken and centrifuged during 7 min at 4320 *g* in order to submit the supernatant to kit's reagents.

2.5 DNA extraction

After a centrifugation of 30 ml of liquid culture (4320 *g*, 7 min), supernatant were discarded and pellets were stored at -20ºC if it was necessary. Usually, the quantity of raw material stored as pellet was around 20-40 mg in every isolate. DNA was extracted with GeneMATRIX Plant and Fungi DNA purification kit (Roboklon GmbH, Berlin, Germany) kept at -20ºC until analysis.

Small volumes of extractions (5µl) were taken and put in an electrophoresis gel (1% w/v) in order to verify the presence of DNA. SimplySafe dye were added in order to see bands pattern under UV (UltraViolet) light (5µl of dye/100g agarose) as well as blue bromophenol for visualization of bands under natural light.

2.6 Primers and PCR conditions

In molecular identification of isolates, it was chosen five different gene markers: COI-5P, *rbcL,* 18S, ITS (ITS1-5.8S-ITS2) and *tuf A* and two pairs of primers of each one except *tuf A* gene with one pair (Table 4).

Table 4
Tested primers during the study

Gene marker	Primer name	Sequence (5'-3')	Tm	Reference
COI-5P	LCO1490	F 5'-GGTCAACAAATCATAAAGATGTTGG-3'	55,6	Gundup et al., 2012
	HCO2198	R 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'	59,7	
	GWSFn	F 5'-TCAACAAAYCAYAAAGATATYGG 3'	45,5	Saunders & McDevit, 2012
	GWSRx	R 5'-ACTTCTGGRTGICCRAARAAYCA 3'	46,7	
rbcL	Form 1D	F 5'-GATGATGARAYYATTAACTC-3'	37,5	Ghosh & Love, 2011
	Form 1D	R 5'-ATTTGDCCACAGTGDATACCA-3'	44,7	
	Form 1B	F 5'-TCIGCIAARAACTAYGGTCG-3'	44,3	
	Form 1B	R 5'-GGCATRTGCCAIARCTGRAT-3'	45,3	
18S	T18S	F 5'-CCAACCTGGTTGATCCTGCCAGTA-3'	60,6	Tale et al., 2014
	T18S	R 5'-CCTTGTTACGACTTCACCTTCCTCT-3'	58,2	
	H18S	F 5'-GGTGATCCTGCCAGTAGTCATATGCTTG-3'	63,3	Radha et al., 2013
	H18S	R 5'-GATCCTTCCGCAGGTTCACCTACGGAAACC-3'	75,3	
ITS1-5.8S-ITS2	Hits2	F 5'-AGGAGAAGTCGTAACAAGGT-3'	47,7	Radha et al., 2013
	Hits2	R 5'-TCCTCCGCTTATTGATATGC-3'	52,1	
	ITS-2	F 5'-ATGCGATACTTGGTGTGAAT-3'	48,5	Sanitha et al., 2014
	ITS-2	R 5'-GACGCTTCTCCAGACTACAAT-3'	49,5	
tufA	tufGF4	F 5'-GGNGCNGCNCAAATGGAYGG-3'	47	Saunders & McDevit, 2012
	tufAR	R 5'-CCTTCNCGAATMGCRAAWCGC-3'	49,5	

PCR was carried out in a final volume of 20µl composed (final concentrations) by a specific 1X reaction buffer, 2,5 mM of $MgCl_2$, 0,5 mM of dNTPs, 0,2 µM of each primer and 0,5 U of *Taq* polymerase. The remaining volume of mixture was filled with distilled water and an adequate quantity of extracted DNA was added in each PCR tube. The conditions for PCR used were initial denaturation of double strand at 95ºC for 5 min and 35 cycles divided in 30” at 95ºC, an annealing temperature of 55ºC for 30” and 60” at 72ºC. A final extension of 5’ at 72ºC was carried out as last step. Annealing temperatures were performed by Oligos free-software.

All reactions were verified by an electrophoresis gel (2% of agarose w/v) along with SimplySafer in order to see bands pattern under UV (UltraViolet) light (5µl of dye/100g agarose).

2.7 Sequence analysis and phylogenetic trees.

PCR bands were purified from electrophoresis gel by GeneMATRIX Agarose-Out DNA purification kit (Roboklon GmbH, Berlin, Germany). Then, they were analyzed in a new gel (2% of agarose w/v) after the purification process in order to know two features: how much DNA quantity had remained after the process and even to detect any sample lost. Mass Ladder band pattern was used for the purpose of knowing the amount of DNA (ng). It was mixed 2µl of DNA plus 2µl of Mass Ladder.

Fourteen samples of each marker gene were sent to MacroGen for sequencing using standard Sanger sequencing method. Sequences were revised and cleaned through BioEdit programme (Hall, 1999) and aligned by ClustalW multiple alignment (Thompson *et al*., 1994). Subsequently, sequences from GenBank database were obtained and put against study sequences in order to identify all isolates. After a second alignment of the whole sequence cluster, analysis and phylogenetic tree constructions were carried out by MEGA 6v software (Tamura *et al*., 2013). Neighbour-Joining method (NJ) (Saitou *et al*., 1987) with a bootstrap of 10000 (Felsenstein, 1985) was used to infer the evolutionary history. Models were carried out by ModelTest application inside MEGA 6v. Phylogenetic trees were modified by CorelDRAW x8.

3. Results

3.1 Isolation, scale-up and maintenance of cultures

In the present study, samples from 7 different places inside Asturian landfill were collected. They were plated in solid agar with a specific nutritional medium: f/2 for green algae and BG-11 adequate for both, green algae and cyanobacteria. However, it was necessary a total of 9 re-inoculations in fresh medium because of contamination, above by bacteria and yeast.

After the big effort, 14 mono-culture-plates were achieved and maintained in specific conditions explained before. Results of cultures' scaled were successful with 14 liquid cultures in different volumes: 10ml, 50ml and 200ml (Figure 3).

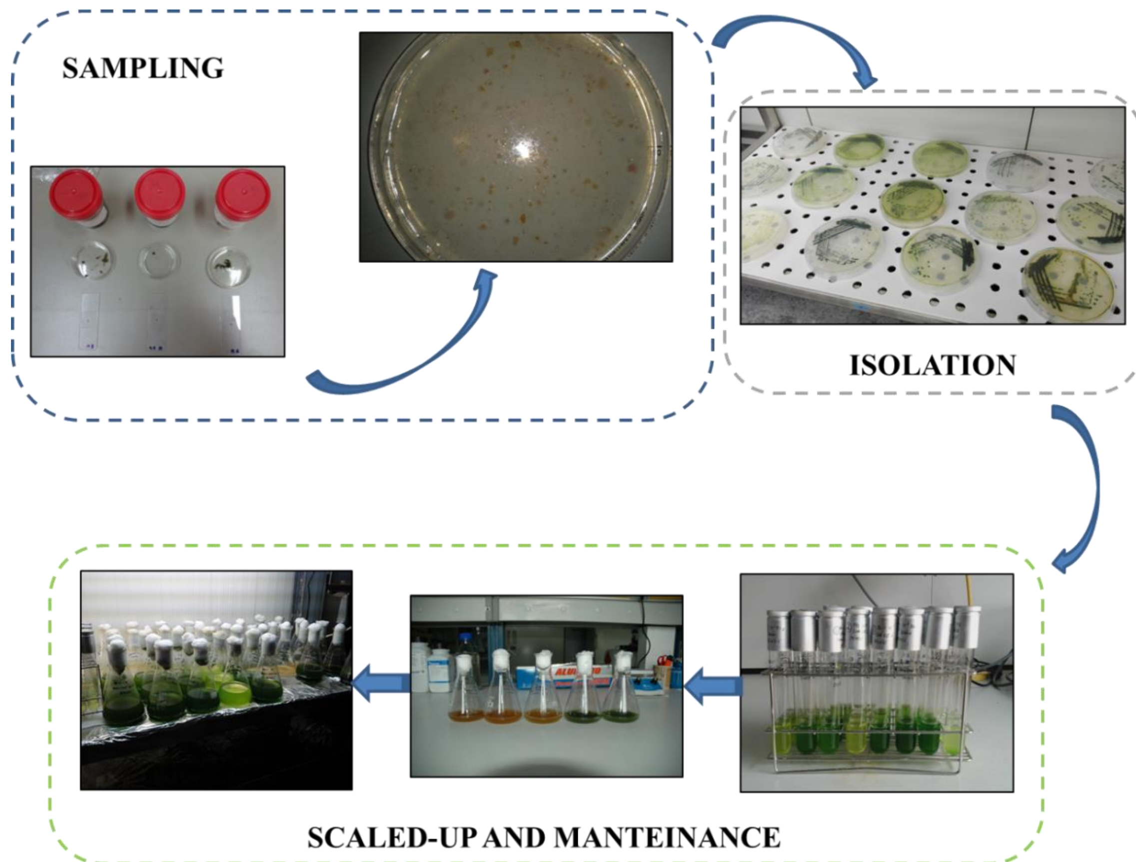

Figura 3. Sampling, isolation, scaled-up and maintenance of fourteen isolates.

With all cultures growing in Erlenmeyer flasks, a preliminary experiment with de added volume of BG-11 medium was done. Instead of the recommend quantity, cultures in stationary phase were refreshed with a quantity 20-fold less medium. Surprisingly, one isolate changed its color from green to orange in a period of 7 days (Figure 4). Moreover, it can be observed microscopically the modifications in cytoplasm of both orange and green cells. For instance, pyrenoid position (White arrow, Figure 5.d) is totally hidden by intracellular granules in orange cells as it is indicated by black narrows (Figure 5.c).

Figure 4. 50ml Erlenmeyer flasks with a culture in both phase green and orange.

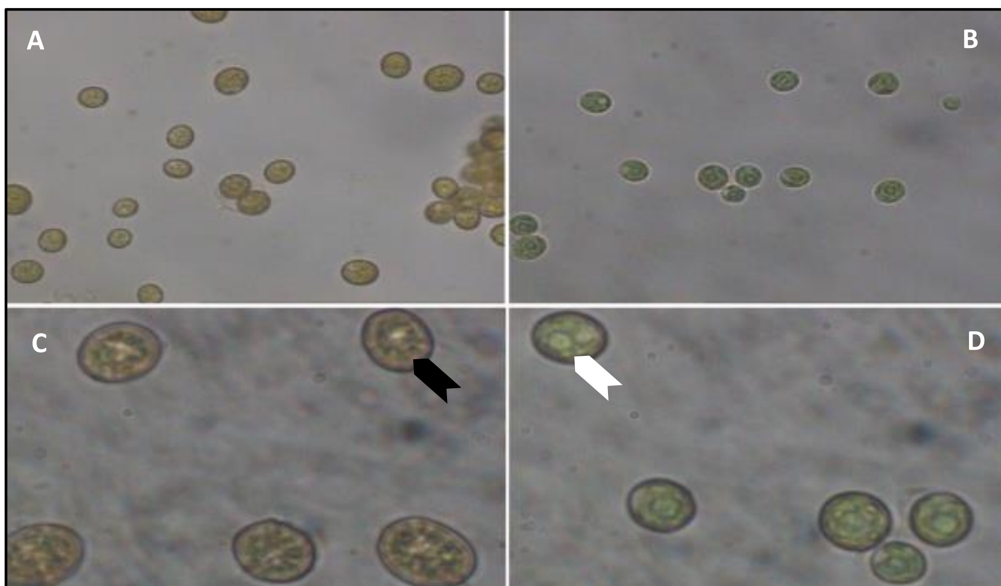

Figure 5. Two different cultures under light microscopy. A) Orange cells (x400 of magnification). B) Green cells (x400 of magnification). C) Hidden cytoplasm of orange cells (x1000 of magnification). D) Green cells showing pyrenoid position.

3.2 Morphological identification

Morphological characterization of 14 isolates was carried out mainly by light microscopy in order to achieve a group of diagnostic features which allow the tentative or preliminary identification of isolates (Table 4). However, it was not possible to isolate any microalga from CP1, CP2 and CA2 locations as well as any cyanobacteria in all sampling sites.

As it can be seen in Figure 6, there were found a moderate diversity inside the sampling sites but different structures and cellular arrangement such as solitary cells and coenobic forms. Resulted from morphology data in Table 5 obtained under light microscopy, four

different groups or genus could be identified. BG.600 and BG.602 isolates were shallowly assigned inside *Chlorococcum* genus because of their spherical shape, solitary forms, a great amount of cytoplasm starch granules and the size. Secondly, CA1.321 and CA.322 isolates were consistently related to *Chlorella* genus based on the small size, special green color and spherical shape. However the most specific features were the girdle-, cup or saucer-shape chloroplast and the parietal pyrenoid. BG.601 could be included in *Chlorella* group, but the great size made difficult its assignment. BG.603 isolate was especially difficult to include in an own group, because of the amount of morphological changes which it suffered during the acclimation from the landfill environment to the lab. Despite of that, it was assigned within *Chlamydomonas* genus. The rest of isolates were properly encompassed in *Scenedesmus*/*Desmodesmus* genus based on cell arrangement in different coenobia forms, oval or spindle-shape and size.

Furthermore, SEM (Scanning Electron Microscopy) technique shed important data about surface characteristics (Figure 7). As it was include in Table 5, there were longitudinal grooves (one or more), rifts and star-shape structures over the surface which helped in the intrinsic identification *Scenedesmus*-related isolates, though it was not determinant.

3.3 Molecular identification

A total of nine different PCR amplifications from five genes were attempted in this work in a preliminary assessment. Two genes fragments, the 18S rRNA gene and the ITS1-5.8S-ITS2 gene cluster were finally used for the molecular identifications of the fourteen isolates previously since they shed consistent and reliable PCR results. The genetic identifications were carried out using comparisons based on BLAST procedures (option highly similar sequences, MegaBLAST) to find the best matched sequences (highest alignment scores after evaluating the E-value, query cover and the percentages of identity) in the GenBank database sequences. Results of the matched database sequences, its accession numbers and the identities percentages for each of the isolates are shown in Table 6. True genetic assignments were considered only when identities percentages were above 97% for the best hits found. Another consideration used for assignments validations was that the matched database sequences (best hit) found must be already published and thus under a serious peer-paired scientific revision.

Table 5

Preliminary identification of 14 microalgae isolates from Asturian landfill.

Code	Shape	Widht (µm)	Lenght (µm)	Motility	Pyrenoid	Cell Arrangment	Special feature (SEM)	Tentative identification
BG.600	Sphaerical	13-15	13-15	No	No	No		*Chlorococcum* sp.
BG.601	Ovoid	10,89	13,18	No	No	No		"*Chlorella*" sp.
BG.602	Sphaerical	13-15	13-15	No	No	No		*Chlorococcum* sp.
BG.603	Ovoid	9,5-10,8	9,5-9,8	Yes (but lost)	No	No		*Chlamydomonas* sp.
CE1.500	Ovoid	4,2-4,3	5,7-6	No	Yes	Yes (Sometimes, 2-3-4-cell coenobia)	Longitudinal grooves	*Scenedesmus* sp.
CE1.501	Ovoid	2,4-2,7	3,1-3,3	No	Yes	Yes (2-4-cell coenobia or free cells)		*Desmodesmus* sp./*Scenedesmus* sp.
CE2.319	Spindel-acute	2,4-2,8	3,8-3,9	No	Yes	Yes (4-cell coenobia or free cells)	One longitudinal wrinkle	*Scenedesmus* sp.
CE2.320	Ovoid	6,7-8,2	8,6-9,1	No		Yes (Sometimes, 2-cell coenobia)	Longitudinal soft grooves	*Scenedesmus* sp.
CE2.401	Ovoid	3,22	5,2-5,4	No	Yes	Yes (Sometimes, 2-3-4-cell coenobia)	Longitudinal grooves	*Scenedesmus* sp.
CE2.402	Spindel-acute	2,3-2,8	3,5-3,6	No	Yes	Yes (4-cell coenobia or free cells)	One longitudinal wrinkle	*Scenedesmus* sp.
CA1.122	Ovoid	2,3-2,8	3,4-3,6	No	Yes	Yes (2-4-cell coenobia or free cells)		*Desmodesmus* sp./*Scenedesmus* sp.
CA1.123	Ovoid	2,1-2,3	3,2-3,3	No	Yes	Yes (2-4-cell coenobia or free cells)		*Desmodesmus* sp./*Scenedesmus* sp.
CA1.321	Sphaerical	2,5-3,5	2,5-3,5	No	Yes	No		*Chlorella* sp.
CA1.322	Sphaerical	2,5-3,5	2,5-3,5	No	Yes	No		*Chlorella* sp.

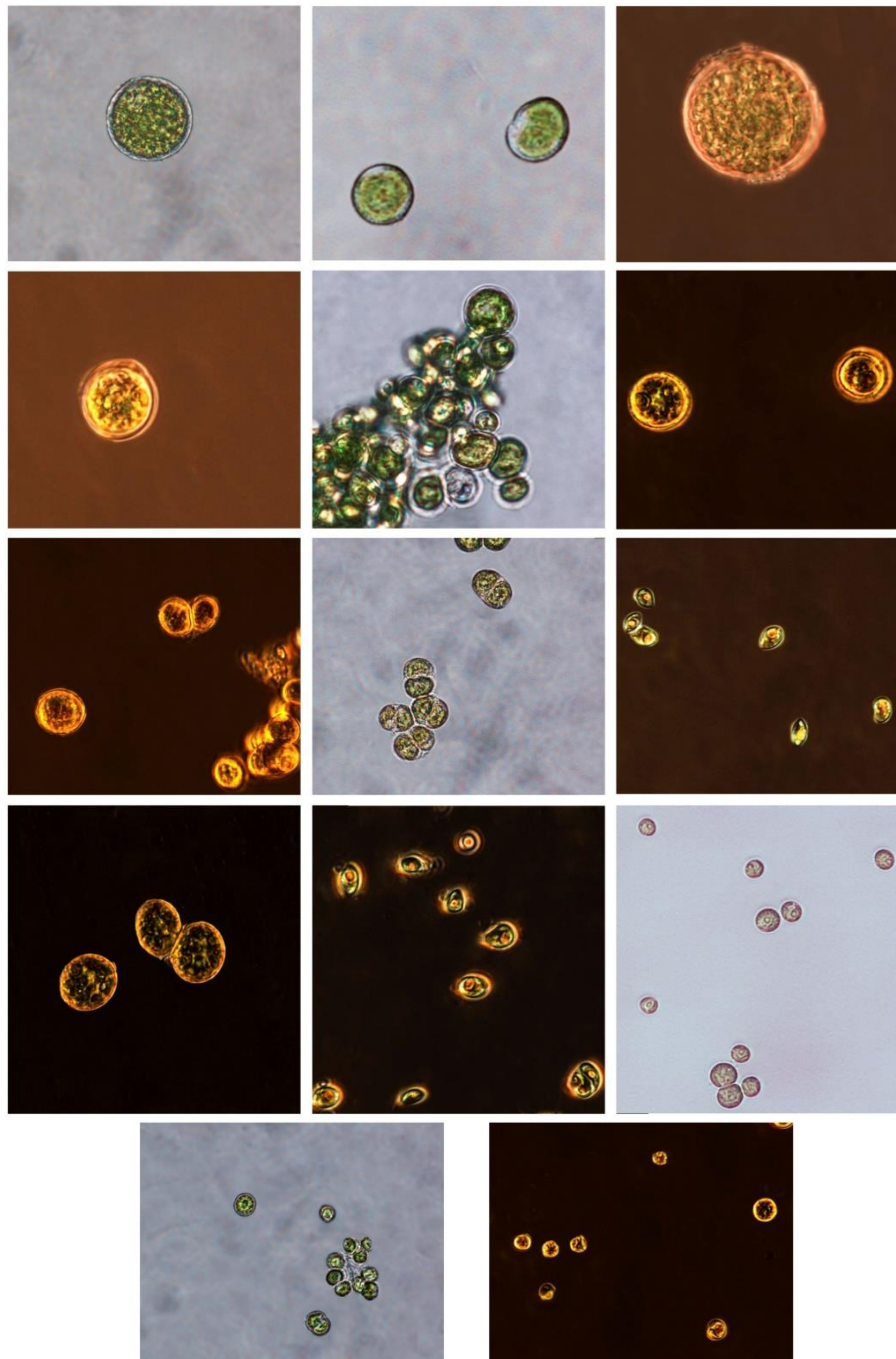

Figura 6.Images of fourteen isolates under light microscope (x1000 maginification) with and without contrast.

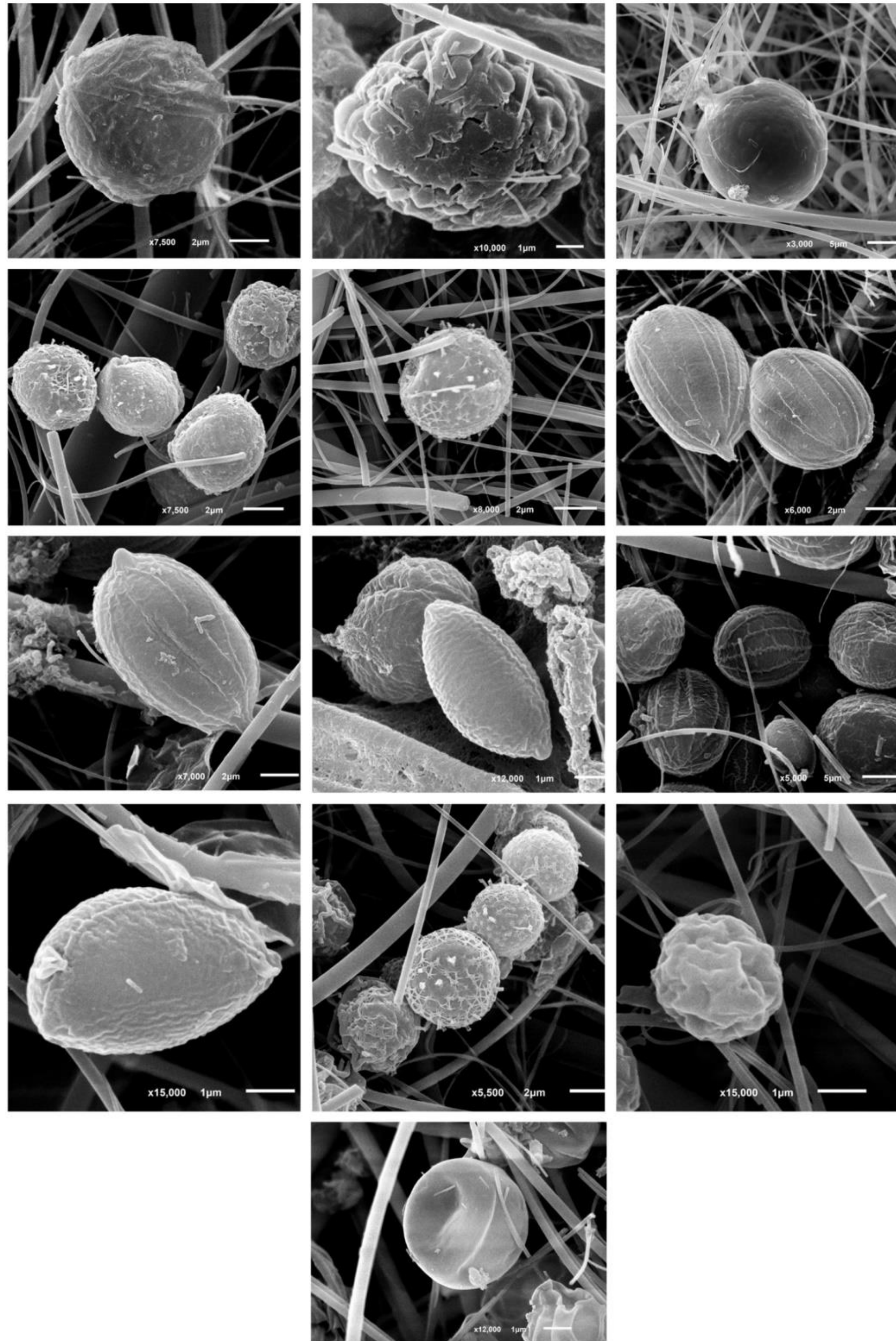

Figure 7.Images of thirteen isolates under Scanning Electron Microscopy.

Almost all the isolates showed high identities percentages to some of the GenBank database sequences using both genes. The isolates BG.601, CA1.321 and CA1.322 were matched to the same reference sequence of *Chlorella sorokiniana* with 99% of identity using the 18S gene marker. Species delimitation was not always possible using this gene. The CE2.320 isolate was only related to *Chlorella* sp. genus (99%). Much more consistent results were obtained for the CA1.122 and CA1.123 isolates showing a 100% of identity with referenced sequences of *Desmodesmus* sp. and *Desmodesmus abundans* respectively. Similar results were found for *Chlorella sorokiniana*-related isolates (BG.600 and BG.602 strains) since they were related to the same sequence of *Tetracystis tetraspora* (99%). The CE1.500 and CE2.401 isolates were found close to a *Coelastrella* sp. database sequence (99%). A high level of correlation was also found between morphological and molecular assignments of the CE2.319 and CE2.402 isolates which were related to two different *Acutodesmus obliquus* reference sequences (100 and 99% of similarity respectively). Finally, the BG.603 isolated also had a high percentage of identity (99%) with *Chlamydomonas orbicularis* database sequence. It was not possible to find any specific match for the case of the CE1.501; it was just assigned to a shallow and unspecific group (Uncultured Chlorophyte) (Table 6).

On the other hand, sequences of the ITS1-5.8S-ITS2 gene cluster obtained here for twelve isolates showed a slight fall of efficacy regarding identity percentages and thus less reliable genetic assignments. Despite this, the correspondence between best hits using ITS gene with the previous 18S results was high (Table 6). It was even new isolates assignments to a species level since the CE1.501 isolate was matched now to a *Desmodesmus multivariablisis* sub. *turkiensis* reference sequence with a high identity percentage (99%). The CA1.321 and CA1.322 isolates were found again close to *Chlorella sorokiniana* reference sequences but with lower identity percentages (95%). Surprisingly, the BG.601 isolate was related to a new reference sequence, *Chlorella vulgaris* (99%) and the CE2.320 isolate had some kind of confusion about assignments being near to both *Chlorella* sp. and *Scenedesmus* sp. database sequences (Table 6).

Genetic assignments using BLAST against the Genbank microalgae sequences revealed the identification, at the level of species, of nine out of the fourteen isolates under study (64%) using the 18S gene (Table 6). Lower percentages (58%) were found using instead the ITS cluster. The global picture showed eight cases where one of the markers failed to allow species classification, two contradictory results (same genus but different species)

and four matching results (isolates CE2.319 and CE2.402 (*Acutodesmus obliquus*), and isolates CA1.321 and CA1.322 (*Chlorella sorokiniana*)) (Table 6).

3.3.1 Phylogenies based on 18S gene sequences.

Phylogenetic trees were constructed using all the isolates' sequences and published reference sequences from each of the main taxonomic groups of microalgae. Mostly all those reference sequences belonged to prestigious culture collections such as CCAP (The Culture Collection of Algae and Protozoa), UTEX (University of TEXas Culture Collection of Algae) or SAG (The Culture Collection of Algae at Göttingen University).

The 18S NJ tree from microalgae clearly distinguished two big clades which were well supported by bootstrap values (Figure 8). The clade at the top of the tree encompasses two subclades and three orders, where 12 out of the 14 isolates were nested: Chlorellales (Class Trebouxiophyceae) Sphaeropleales and Chlamydomonadales (Class Chlorophyceae). The Chlorellales order clustered CA1.321, CA1.322 (both of them in the same branch) and BG.601 isolates. The Sphaeropleales order was divided in two main groups. The first one covered the *Desmodesmus* genus, with 3 isolates (CE1.501, and CA1.122, CA1.123) being allocated near each other. The second one predominantly included *Coelastrella* and the *Scenedesmus* (*Acutodesmus*) genus. The last order (Chlamydomonadales) comprised the *Chlamydomonas* genus where BG.603 fell into. The second big clade covered Chlorococcales order with a relative high robustness among their subclades and branches. Two isolates (BG.600, BG.602) were located inside this group, although far from the main *Chlorococcum* and *Tetracystis* genus (Figure 8).

Table 6
Molecular identification of strains isolated in the Asturian landfill including both gene markers.

Strain (isolate)	18S (GenBank)			ITS1-5.8S-ITS2 (GenBank)		
	Closest match specie	% homology	Accesion number	Closest match specie	% homology	Accesion number
BG.600	*Tetracystis tetraspora*	97	JN968582.1	(not assayed)		
BG.601	*Chlorella sorokiniana*	99	KP726221.1	*Chlorella vulgaris*	99	KP645229.1
BG.602	*Tetracystis tetraspora*	97	JN968582.1	(not assayed)		
BG.603	*Chlamydomonas orbicularis*	99	AB511839.1	*Chlamydomonas zebra*	91	AF033294.1
CE1.500	*Coelastrella* sp.	99	KM020087.1	*Coelastrella* sp.	99	KM061472.1
CE1.501	Uncultured chlorophyte	99	No clear	*Desmodesmus multivariabilis* var. *turkiensis*	99	DQ417525.1
CE2.319	*Acutodesmus obliquus*	100	KP726267.1	*Acutodesmus obliquus*	100	KP645234.1
CE2.320	*Chlorella* sp. (not published)	99	KM985412.1	*Scenedesmus* sp./*Chlorella* sp.	97	KJ676125.1/KJ696124
CE2.401	*Coelastrella* sp.	99	KM020087.1	*Coelastrella* sp.	99	KM061472.1
CE2.402	*Acutodesmus obliquus*	99	KP726267.1	*Acutodesmus obliquus*	99	KP645234.1
CA1.122	*Desmodesmus* sp.	100	AB917128.1	*Desmodesmus* sp.	98	AB917128.1
CA1.123	*Desmodesmus abundans*	100	KF673371.1	*Desmodesmus* sp.	96	AB917128.1
CA1.321	*Chlorella sorokiniana*	99	KP726221.1	*Chlorella sorokiniana*	95	KP645222.1
CA1.322	*Chlorella sorokiniana*	99	KP726221.1	*Chlorella sorokiniana*	95	KP645222.1

3.3.2 Phylogenies based on the ITS1-5.8S-ITS2 gene cluster sequences.

Published reference ITS1-5.8S-ITS2 sequences from each of the main taxonomic groups of microalgae were downloaded and aligned with the twelve sequences obtained in this work. Unfortunately, the BG.600 and BG.602 sequences were not achieved. The ITS1-5.8S-ITS2 NJ tree was supported by low bootstrap values but its topology matched the 18S gene tree (Figure 9). In it is lacking the Chlorococcales clade showing again the previously seen two subclades and three microalgae groups. Basically, there was again subclade at the top clustering the Chlamydomonadales order (Chlorophyceae class). The BG.603 isolate fell into this group with high bootstrap values. The rest of the isolates (11) were allocated in another subclade including the two other groups; on the top covering the Sphaeropleales order (8 isolates) again divided in three clear genus: *Scenedesmus* (with CE2.319, 402 isolates and questionably CE2.320 isolate), *Coelastrella* (with CE1.500 and CE2.401 isolates) and *Desmodesmus* (with CA1.122, 123 and CE1.501). The second group belonged to Chlorellales order grouped three isolates inside the genus *Chlorella.* The CA1.321, 322 isolates were joined together in this cluster with the BG.601 isolate (Figure 9).

The genetic procedures (BLAST assignments and the 18S and ITS1-5.8S-ITS2 genealogies) showed that 10 out of the 14 isolates assayed here were indeed identified as (at species level). Available genetic data was not enough for species classifications in the rest of isolates but it seems clear that in CE1.500, CE2.401 and CA1.122 isolates the genus is clear (*Coestastrella* sp. and *Desmodesmus* sp.) and more work is needed to assess the taxonomical and genetic characteristics of the genus. More relevant, CE2.320 and BG.600, BG.602 could be even new species not yet studied/described before.

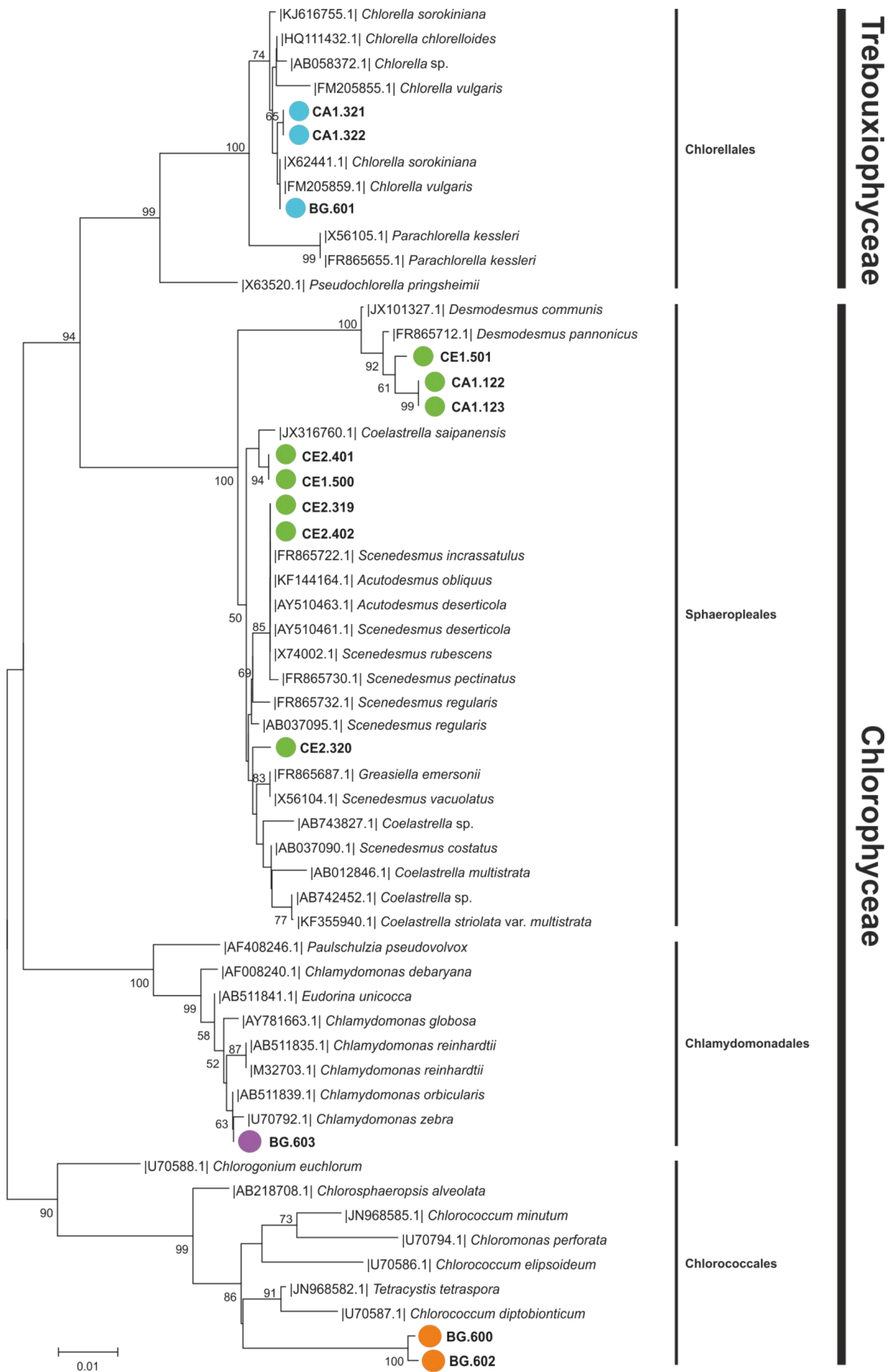

Figure 8.NJ tree for 18S analysis. Strains isolated in this study appear in colors depending on the order.

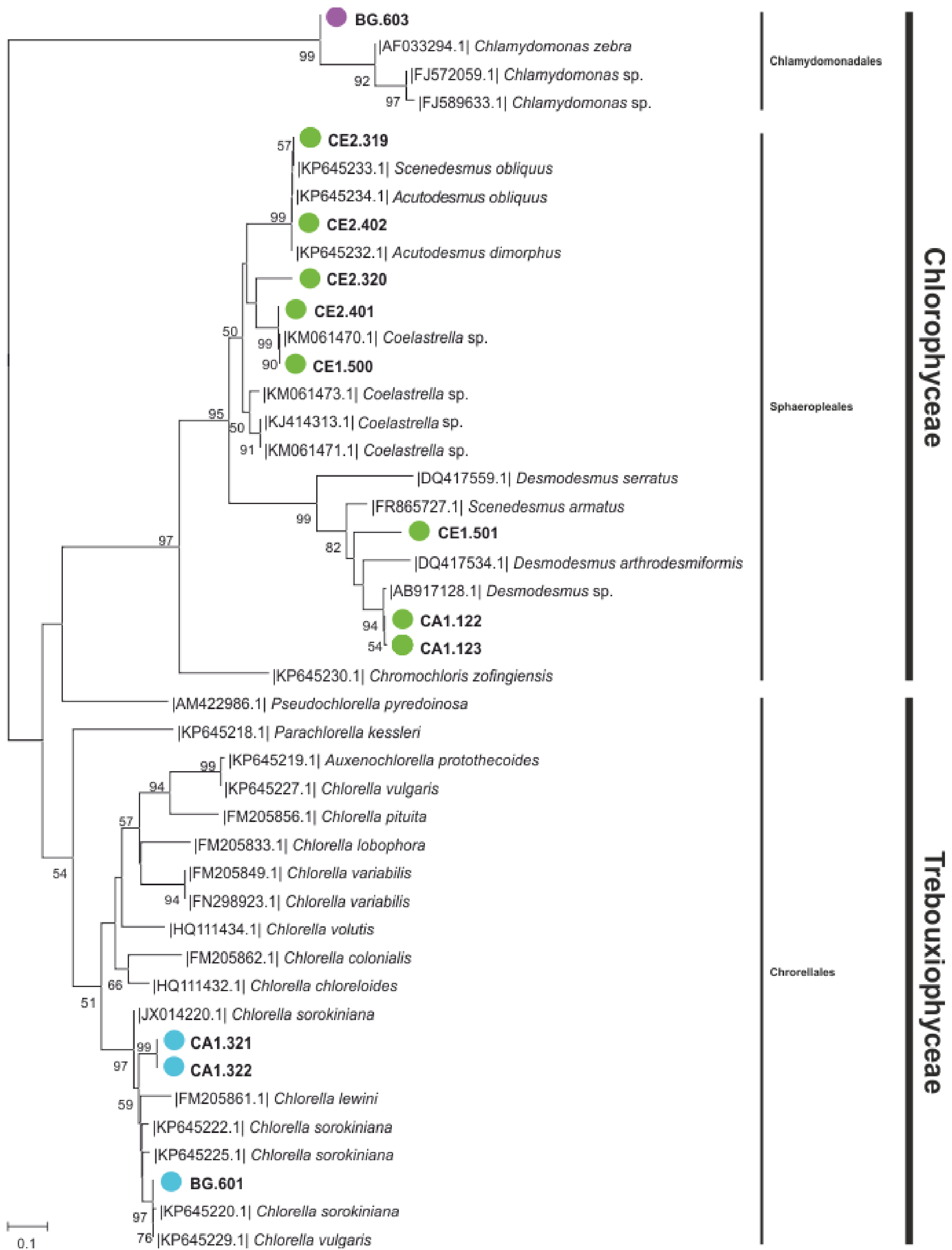

Figure 9.NJ tree for ITS1-5.8s-ITS2 analysis. Strains isolated in this study appear in colors depending on the order.

4. Discussion

Microalgae are a large group of photosynthetic microorganisms which have reached market and research attention because of their potential applications in practically all the fields such as food/feed, biomedicine, cosmetics, energetics and environmental engineering. Furthermore, its ubiquitous nature and capacity to adapt to different environments and physicochemical conditions (Varshney *et al.,* 2014) make microalgae a model group in basic research and water ecology (Toplin *et al.,* 2008). Main aims in this study have been the isolation and culture maintenance of 14 microalgae from different locations around the landfill to discover strains which would be useful for CO_2 mitigation and additional biotechnology applications such a carotenoid production. Morphological and molecular identification based on microscopy and DNA barcoding of isolates were successfully obtained. All these facts convert the study into the first which has characterized the microalgal diversity within a landfill in order to apply them to biotechnology.

4.1 Isolation, scale-up and maintenance of cultures

The use of a traditional protocol of serial dilution of raw samples followed by agar Petri dishes plating (Anderson, 2005; de la Vega *et al.,* 2011) was the most cost-effective decision despite the time consumed until the final isolation. Alternative ways are being used to overcome time spent during isolation. For instance, enrichment cultures can be performed before plating the sample in solid agar in order to increase the dominance of microalgae over the rest of organisms (Toplin *et al.,* 2008; Pryvil *et al.,* 2015). More sophisticated techniques reduce drastically the time of isolation, such as automated single cell isolation through flow cytometry (Reckermann, 2000) or using fluorescence aided cell sorting (Neofotis *et al.,* 2016). However, the experience level must be adequate and the materials are highly expensive. Moreover, environmental samples are normally in different life cycle stages, which imply several sizes and shapes, and this makes the technique less reliable.

Isolation of cyanobacteria and filamentous microalgae was not achieved in spite of their presence in raw samples. In the case of cyanobacteria, there are three possibly reasons. First of all, there are two groups of cyanobacteria based on the Nitrogen metabolism (Rippka *et al.,* 1979): one (named as diazothrophic organisms) has the capability to fix aerobically atmospheric Nitrogen in a direct way. For that purpose, they have a modified

organelle called heterocyst which metabolizes the atmospheric gas via nitrogenase enzyme reaction. N_2 is directly converted to NH_3 (i.e. *Anabaena* sp.). The rest of groups assimilate Nitrogen from inorganic salts through special nitrate transporter located in plasmatic membranes. Probably, one of the main reasons to have not any cyanobacteria isolate could be the presence of Nitrogen salt (usually $NaNO_3$) in BG-11 medium which could be toxic for heterocyst-related cyanobacteria. Thus, a new BG-11 culture medium without inorganic Nitrogen salts could be prepared in order to promote heterocytous cyanobacteria (Lee *et al.,* 2015).

The size of Cyanobacteria is several times higher than coccoid microalgae, being macroscopically distinguished. As it was explained in Materials and Method section, plate streak or inoculation were made by a serial dilution protocol with successful results with regard to green coccoid algae. Nevertheless, great sizes of cyanobacteria and filaments in general could interfere in the development of the protocol breaking the filaments or even not to be taken. Another reason could be related to agar solid medium. It is known that there are different growth inhibitors inside gelled agar (Carmichael & Gorhan, 1974) which make the development of continuous cyanobacteria solid cultures more difficult. Potential isolates in raw samples may not grow because of the absence of accommodation to the new environment which sometimes is really dry depending of the amount of agar per liter of water (Castenholz, 1988).

Scaled-up liquid cultures were successfully done in each volume being BG-11 and f/2 culture media a satisfactory choice. This result was in accordance with several authors who had isolated microalgae from various environments (Lynch *et al.,* 2015; Lee *et al.,* 2015; Neofotis *et al.,* 2016).

4.2 Identification of microalgal isolates and potential applications

A great problem which concerns microalgal taxonomy has been the difficulty to well-understand life cycles and the different cell shapes and sizes they can adopt depending on environmental changes (Radha *et al.,* 2013; Qiao *et al.,* 2015). Therefore, big efforts have been focused on DNA barcoding development to support morphological data in microalgal identification (Skaloud *et al.,* 2012; Darienko *et al.,* 2015). While BLAST assignment are just based on matching scores (identity) to sequences previously deposited in databases, genealogies can give a more complete picture about the origin and evolution in this complex organisms. We have detected serious gaps in knowledge about microalgae

taxonomy taking into account genetic divergences. The molecular phylogeny of this group is incomplete and a lot of records reached just to genus levels. Errors in species classification in the Genebank database are something usual (Kwong *et al*., 2012). Lack or ambiguity of reference sequences in databases can seriously difficult species identifications (Kwong *et al.,* 2012; Ardura *et al.,* 2013). In this work we have combined morphological and genetic results to classify and identify as farther as possible our new microalgae isolates.

4.2.1 Chlamydomonadales order (Chlorophyceae class)

The BG.603 was the only isolate related to *Chlamydomonas* genera found after genetic analyses using two different gene markers (18S and ITS1-5.8S-ITS2). In the first observation of BG location sample, the most abundance morphotype was an active, flagellated cell morphologically close to *Chlamydomonas*-relatives. However, environmental changes from the raft to laboratory conditions during isolation and scaled-up processes produced alterations in morphology and physiology (e.g. loss of flagella and larger size). These kinds of conflicts have been previously discussed in other studies (Ahmad *et al.,* 2013). In this work, DNA barcoding was necessary to identify the BG.603 isolate. It seems to belong clearly to *Chlamydomonas*. Genetic evidences showed it is probably *Chlamydomonas orbicularis* (99% of 18S identity). In any case, it seems, from our phylogenetic trees, that *Chlamydomonas zebra* and *Chlamydomonas orbicularis* are probably the same species. Errors in species classifications or lack or representation for some species/groups within the data deposited in databases such as Genbank are a usual issue (Collins *et al*., 2012). The need for combined studies to clearly assess the species taxonomy within this genus is paramount. Unfortunately, no publications related to CO_2 tolerance/mitigation or carotenoid production were found in *Chlamydomonas* genera.

4.2.2 Sphaeropleales order (Chlorophyceae class)

With a total of eight isolates assigned to Sphaeroplelales, this order was the most important inside the sampling locations. They appeared in all types of sites except raft G (BG). Firstly, morphological studies elucidated the dominance of *Scenedesmus* type morphotypes with a lower presence of *Desmodesmus* types in the case of CE1.501, CA1.122 and CA1.123 isolates. Few diagnostic characteristics (Krienitz & Bock, 2012; Qiao *et al.,* 2015) such as specific coenobia structures for both *Scenedesmus* (Spindle-shape without spines) and *Desmodesmus* (Ovoid- cylindric-shape with spine

ornamentations) forms had been found in raw samples and after the isolation process. Nevertheless, cells lost their coenobia forms (free cells) during re-inoculations in liquid cultures. Specifically, *Desmodesmus* types altered their morphology acquiring ovoid shape and bigger size. Subsequently, SEM images showed a great difference among CE1.500, CE2.401 and CE2.320 isolates surface. Series of grooves/ribs longitudinally disposed were observed. This special feature had been noticed by Uzunov *et al.,* (2008) in the first European description of *Coelastrella* sp. strains being possible that analysis performed under light microscopy were mistaken. A more precise assignation of isolates was achieved during DNA barcoding procedure. Preliminary results using BLAST application differentiated three groups or genus when using two different gene markers showing high identity percentages (96-100%, see Table 6): *Desmodesmus* sp., *Coelastrella* sp. and specially *Acutodesmus* (Scenedesmus) *obliquus.*

The CA1.122, CA1.123 isolates could be identified as *Desmodesmus* sp. based on BLAST and on the phylogenies results. These are the same results that we found for the CE1.501 isolate. It seems also clear that they are two different species. Probably CA1.122, CA1.123 are *Desmodesmus abundans* (100% of identity using 18S gene). Again we have found few reference sequences and not clear taxonomical status for the genus. The *Acutodesmus* (*Scenedesmus)* group had a high percentage of homology (99-100%) as a result of BLAST analysis in both CE2.319 and CE2.402. Moreover, they fell into the same cluster inside the two phylogenetic trees even though the lower support of its branches. Consequently, they belong to *Acutodesmus obliquus*. Finally, the third group was *Coelastrella* sp. with three representatives. Based on genetic results, CE2.401 and CE1.500 seem to be *Coelastrella saipanensis*. The CE2.320 seems to be a special case. Genetic analyses located it in the *Coelastrella* subclade in both phylogenetic trees, but totally isolated in an independent group. Fortunately, SEM morphological observations in its surface elucidated longitudinal ribs as a specific feature. Thus, CE2.320 isolate is proposed as new specie inside de *Coelastrella* genus: *Coelastrella cogersae*.

Regarding to biotechnological applications, the Sphaeroplelales strains isolated in this study have shed promising results related to CO_2 fixation. The species *Scenedesmus* (*Acutodesmus*) *obliquus* has performed very good results in different cultivation system such as glass-made vessels (Ho *et al.,* 2010) and air lift photobiorreactors (Li *et al.,* 2011) tolerating an initial gas concentration of 10 and 20% (v/v) respectively. Alternative

research was done using thermoelectric flue gas (very close to the burning plant in ReCO_2very proyect) on *Scenedesmus obsticulosus* cultures, with successful results (Toledo-Cervantes *et al.,* 2013). Microalgae could grow in perfect conditions while they were subjected to a 10% of flue gas. On the other hand, it has been reported carotenoid production accumulation in *Coelastrella* and *Scenedesmus* genus. Aburai et al. (2015) have published that high irradiance and salinity had a drastically effect in carotenoid accumulation in *Scenedesmus* sp. KGU-Y002. Moreover, different class of carotenoids was found (e.g. astaxanthin, lutein, zeaxanthin, etc.). Similarly, another strain of *Scenedesmus* sp. has yielded high accumulation of total carotenoids without any treatment (Pribyl *et al.,* 2015). *Coelastrella* genera was the less studied among isolates of this study. Surprisingly, 75% of publications explain its high capacity to accumulate carotenoid compounds (Katsuya *et al.,* 2007; Hu *et al.,* 2013; Neofotis *et al.,* 2016). In addition, the CE2.320 strain isolated in this study was able to change its color from green to strong orange. The *Coelastrella* genus is not only capable to produce and accumulate carotenoid, but it could be a relevant diagnostic feature of the group.

4.2.3 Chlorococcales order (Chlorophyceae class)

Morphologically, BG.600 and BG.602 had various forms in both solid and liquid medium (including 4 or 8 aplanospores formations). The most abundant was a big and spherical cell with a lot of cytoplasm granules of starch, very close to *Haematococcus*. However, flagellate morphotypes, which are the active form in this group, were not found. There is plenty of combination research in AlgaeBase and Bellinger & Sigee (2015) about microalgae freshwater key related to *Chlorococcum* sp. forms. Genetic studies using the 18S sequences showed strong identity (97%) of these isolates with the species *Tetracystis tetraspora* specie, other authors had found similar results (Qiao *et al.,* 2015). The 18S phylogenetic tree was slightly uncertain since both isolates seems to be isolated units close to both *Chlorococcum* and *Tetracystis* genus. A further analysis of life cycle is needed, because the main difference is the ability of *Tetracystis* to produce tetrads based on AlgaeBase data. From this study we can conclude that more information is needed within the group (our isolates can be new, and different, from what have been previously described in this group).

There are many references about biotechnological applications of *Chlorococcum* genera. The group is able to tolerate high CO_2 concentrations (Schnackenberg *et al.,* 1996;

Iwasaki *et al.,* 1996). Moreover, promising results related to carotenoid production from *Chlorococcum* sp. have been achieved (Yuan *et al.,* 2002). Astaxanthin (ester form) and adonixanthin carotenoids were detected in high values. No data of biotechnological applications of *Tetracystis* group have been found.

4.2.4 Chlorellales order (Trebouxiophyceae class)

Three isolates were well-defined inside *Chlorella* genus based on small size, spherical shape and, specially, chloroplast morphology (cup-, girdle-shaped). BLAST results confirmed morphological identification with a high percentage of homology (99%) in both gene markers. Distribution inside phylogenetic trees was precise, being clustered close to *Chlorella sorokiniana* reference sequences (CA1.321 and CA1.322). There was a doubt with BG.601 sequence because its higher size under light microscope, but molecular analysis related it to *Chlorella* species (not so precise as in the other two isolates). Due to its importance in lots of research fields, *Chlorella* is the most studied microalgae, having and important evolutionary diversity discussion (Heeg & Wolf, 2015). Fortunately, it can be concluded that CA1.321 and CA1.322 correspond to *Chlorella sorokiniana* specie while BG.601 will need a deeper analysis to assign it inside the latter species or the *Chlorella vulgaris*.

Carotenoid production in *Chlorella* genera is possible. For instance, *Chlorella zofingiensis* was proposed as an alternative source of astaxanthin instead of main producer *Haematococcus pluvialis* (Liu *et al.,* 2014). However, under the number of studies and researches focus on environmental application, it could be said that *Chlorella* genera is the best CO_2 fixator among eukaryotic microalgae group. Data showed a high percentage of removal (80%) in a *Chlorella* sp. strain growing al laboratory scale photobioreactor (Ramkrishnan *et al.,* 2014) and 85,5% in another *Chlorella* sp. strain (Cheng *et al.,* 2013) in a sequential bioreactor. Even further, a strain called *Chlorella* sp. KR-1 (Sung *et al.,* 1999) could tolerate 70% of CO_2 with a little growth yield. The revolutionary study was carried out with flue gas intake.

CONCLUSIONS

1- A moderate number of microalgae were successfully isolated in mono-cultures. These fourteen strains were also scaled-up in liquid cultures and maintained during the study. Therefore, the whole protocol from the sampling site to the laboratory was very suitable.

2- A caretogenic activity was detected in CE2.320 isolate (from green to orange color) due to a lower amount of nutrient medium. This fact would open a research area to study carotenoids production from microalgae.

3- Morphology features were important to assignation to a genus level. Moreover, SEM technique allowed the cell surface analysis of 13 out 14 isolates and helped to solve a molecular indetermination.

4- The use of 18S gene marker has allowed the molecular identification of nine out fourteen isolates to a specie level. On the other hand, ITS1-5.8S-ITS2 cluster gene marker had a lower efficiency, with an identification of seven isolates out twelve. However, only three isolates were not assigned to species.

5- CE2.320 strain is proposed as the new specie *Coelastrella cogersae* based on both morphological and molecular analysis.

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