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An integrated novel plasma-microalgae approach for landfill leachate treatment using a high-ammonia tolerant strain of *Chlorella vulgaris*

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ABSTRACT

Landfilling is the most common method worldwide to deal with the produced municipal solid wastes (MSW). Nonetheless, one major drawback of landfilling is the production of landfill leachate (LL). Various methods for LL treatment exist which can mainly be divided into biological and physical-chemical methods, however integrating both approaches result in more effective treatment. In this study, an indigenous *Chlorella vulgaris* was isolated from a landfill leachate treatment site, purified, and genetically identified for leachate treatment purposes. One replicate, C.V.M* (CCAP 211/141), of the tested *Chlorella vulgaris* showed an outstanding growth in 20 % LL (*v*/v) with a significant ammonia removal ($p < 0.05$). To confirm these findings, both of the *Chlorella vulgaris* replicates (C.V.M* and C.V.N) were further tested in 20 % LL (*v*/v). A dramatic increase in the growth of C.V.M* by 19-fold over its peer C.V.N with a 75 % ammonia removal (starting from concentration 290.73 mg/L) was observed. This percentage is further improved when injecting plasma activated microbubbles as a pre-treatment step. The plasma pre-treatment resulted in LL decolourisation after 3 h and reduced ammonia-N concentration by 1.9-fold. C.V.M* showed the highest growth in 20 % LL pre-treated by plasma compared to the untreated LL with a final biomass yield of 0.38 g/L, increase in the total ammonia-N removal (79 %) and a significant decrease in the pH value (8.6–6.67). Whole genome sequencing for both replicates revealed differences in genotypes between them which might indicate the possibility of a developed mutation or sexual reproduction that might have increased the ability of replicate/strain C.V.M* to tolerate harsher conditions and higher ammonia-N concentrations in 20 % diluted LL. These results might pave the way for a possible powerful candidate in LL treatment using a high-ammonia tolerant algal strain which when coupled with plasma pre-treatment might provide an effective, eco-friendly, and sustainable LL treatment approach.

1. Introduction

It is believed that urbanisation and increased generation of municipal solid wastes (MSW) are concomitant on a global level. The majority of the produced MSW worldwide (almost 95 %) is landfilled, as landfilling is considered an affordable, widely applicable and environment friendly technology (if engineered landfills are implemented) compared to other technologies such as compositing and incineration, however landfilling still pose the threat of landfill leachate production. Landfill leachate is a highly polluted liquid with different proportions of various undesirable/toxic compounds, it is produced as a result of decomposition of different wastes in the landfill together with percolating rainwater as well as the water content already inherent in the landfill wastes. Landfill leachate represents an environmental burden, it is estimated that one tonne of solid wastes generates 0.2 m^3 of landfill leachate during the decomposition process. Landfills are also reported to continue producing leachate for more than fifty years after their closure. Although engineered landfills are usually provided by liners and leachate collecting systems, however leachate treatment still represent a necessity before being discharged into the environment, this represents a greater burden and a major problem in low- and middle-income countries where open dumps and/or non-engineered landfills represent the most common practice [1–4]. Therefore, treatment of landfill leachate before being discharged into natural water bodies in an

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effective and sustainable way represents an extremely important pre-requisite [1,5,6].

Different treatment approaches/technologies for landfill leachate are reported. Methods for LL treatments could be classified into four main categories: conventional methods, biological methods, physical-chemical methods, and integrated biological-physical-chemical methods. Integrated approaches for landfill leachate treatments are attracting a growing attention as it has been demonstrated that no single method solely is capable of treating LL in an effective and cost-effective way enough to meet the discharge standards set by different authorities [7,8]. Common biological processes involving aerobic and anaerobic approaches although being simple, reliable, and usually cost-effective, nonetheless they have some major drawbacks such as high production of sludge (in aerobic treatments) as well as insufficient removal of some of the most significant contaminants in LL such as ammonia-N (in anaerobic treatments with the exception of Anammox) and this might be attributed to the lack of NH_3-N degradation in the anaerobic system [2,3], which in turn increase the urgency to search for alternative efficient biological treatments methods. In this context, LL treatment using microalgae represents a promising, relatively novel, eco-friendly, and efficient (with varying degrees) to the existing aerobic and anaerobic biological treatments. Microalgae were reported to grow in LL on both lab-scale and larger-scale studies with varying, yet promising, removal efficiencies of different contaminants in leachate [7,9]. Ammonia is one of the most common constituents of LL, which results from the process of biological degradation of amino acids and other nitrogen-containing organic matter found in the leachate [10]. Despite being a highly toxic component of LL (especially in high concentrations), considerable removal efficiencies were exhibited by different tested microalgae in diluted LL and were reported in several studies in the literature [10–13]. Ammonium (under most conditions) is considered a preferred nitrogen source for green microalgae. It is usually assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle or in other cases via NADP-dependent glutamate dehydrogenase (GDH) [14]. The presence of ammonia in leachate in the form of ammonium (NH_4^+)/free ammonia (NH_3) is a temperature and pH dependent process. Ammonia in its unionised free form (NH_3) is reported to be more toxic to different living organisms especially in high concentrations, its acute toxicity and sometimes lethal effect to fish, duckweed, algae, and other microorganisms is well reported in the literature [4,10,15,16].

Regarding physical-chemical treatments of LL, although being reported for its efficiency in treatment of LL (especially old/stabilised LL), however its relatively high cost and/or environmental impact risks still represent a major concern [3,4,7]. Few studies have reported the efficiency of utilising Plasma/UV technologies as physical methods in LL treatment, although scaling up is still a challenge in this aspect, however being clean eco-friendly methods greatly encourage exploring these technologies as complementing steps to the microalgal treatments [17–20].

Several studies highlighted the efficiency of combining biological methods and physical-chemical methods, hence the scope of this study focused on this integration approach, however there was no reports in the literature (as far as the authors know) on integrating plasma /UV as a pre-treatment step with microalgal treatment for LL, this novel integrated approach is believed to benefit from coupling efficient LL treatment with the production of valuable algal biomass thus represents an addition to the sustainability, efficiency and possibly cost-effectiveness of the LL treatment process for the possibility of wider application in the future. This study aims for two main objectives:

1- Implementing plasma/UV technologies as a pre-treatment step prior to the microalgal treatment to determine it's efficiency for landfill leachate treatment.
2- Comparing both growth and leachate biotreatment capability (with and without prior plasma/UV pre-treatment) for both replicates of the isolated indigenous strain of *Chlorella vulgaris* (C.V.M* and C.V. N).

2. Materials and methods

2.1. Sample collection and algae isolation

Soil samples were collected in May 2018 from Erin landfill leachate treatment site in Chesterfield, UK, owned by Viridor, a British waste management company (53°14′42.6"N 1°19′45.9"W). Soil samples are then divided into separate lots. Each lot is used to inoculate 250 mL Erlenmeyer flasks containing 100 mL fresh liquid BG11 medium [21]. The flasks are then incubated in a temperature-controlled growth room (25 ± 2 °C) in shaking incubators (80 rpm, Stuart orbital shaker). Cultures were grown autotrophically under continuous illumination provided by daylight fluorescent lamps with a photon flux density of 25 ± 5 μmol photons m^{-2} s^{-1}. The grown liquid cultures are then used to inoculate BG11 solid medium (1.5 % *w/v* Bacteriological Agar) by streaking the inoculum across the agar surface [22]. The plates are then incubated under the same conditions mentioned above. This step is repeated periodically until pure cultures were obtained. Purity of the obtained isolates is checked using microscopic examination.

2.2. Molecular identification and phylogenetic analysis

Five to ten milliliters of homogenized pure algal cultures (OD_{595} for algal cultures used were all above 1) at their exponential phase are centrifuged for 1 min at 1500 ×*g* (Ohaus, Germany). The supernatant is discarded, and the pellet is used for further DNA extraction. DNA extraction method using DNeasy® Plant Pro kit (50) (QIAGEN, Germany) is carried out as detailed in the manufacturer's instructions. The extracted genomic DNA is then used for further PCR reactions using five different primers (18S Lim, 18S Huss, 5.8S, ITS1 and ITS2), each to amplify a certain region in the ribosomal DNA of the tested green microalgal genomes. The produced PCR products are purified using QIAquik® PCR purification Kit (Qiagen, Germany) to clean up the amplified PCR products from any impurities. The manufacturer's instructions are followed. The purified PCR products are then sent to Eurofins Genomics (Koln, Germany) for sequencing. The produced sequences are assembled using BioEdit Sequence Alignment Editor software (Version 7.0.5.3) [23]. The resulting sequences are compared against other closely resembling sequences (maximal score, identity and query coverage) in the National Centre for Biotechnology Information (NCBI) database [24] using the Basic Local Alignment Search Tool (BLAST) [25]. The produced DNA sequences are aligned automatically using MUSCLE alignment provided by MEGA X software (Molecular Evolutionary Genetic Analysis) version 10.1.7 [26] under default parameters. Phylogenetic trees for the isolated strains are constructed using Neighbour-joining method. The best mathematical model to compute the evolutionary distance for each tree is determined using MEGA X and is mentioned in the description box under each tree, it was either Tamura-nei or Kimura-2-parameters model. The robustness of the statistical confidence for the constructed trees is determined using bootstrap tests based on 500 replicates and values are shown next to the tree branches.

2.3. Screening algal strains for the highest growth and ammonia-N removal in 20 % LL

Growth and ammonia-N removal capacity of the isolated strains grown in 20 % LL concentration (*v*/v) (individually and collectively) were measured throughout a 30-day experiment to determine the most potent strain/s in LL biotreatment. The landfill leachate used in this study was diluted with sterile distilled water to a concentration of 20 % (*v*/v). Microalgae strains were cultivated in 1 L autoclave-sterilised Duran bottles. Each bottle is filled with 500 mL diluted leachate with

sterile distilled water (20 % v/v), supplemented with 5 mL of an inorganic phosphate source (5.2 g/L $K_2HPO_4.3H_2O$) which is equivalent to the same amount added for preparation of 500 mL BG11. Each Duran bottle is inoculated with 20 % (*v*/v) algal inoculum (100 mL) of 29 days old algal culture. Regarding the group culture, which contains all the tested algal strains together, it was set up as 5 % (*v*/v) for each alga so the total algal inocula is 20 % (v/v). All inocula are measured at 595 nm using a spectrophotometer (Jenway 6715 UV/Vis., UK) and their OD measurements are adjusted to 0.36–0.39. The Experiment is set up at room temperature and the temperature is monitored continuously (22 °C ± 2). Autoclave-sterilised magnets were placed into the tested Duran bottles which were then each placed onto a magnetic stirrer plate to be continuously stirred and mixed during the duration of the experiment (30 days) at medium speed. The bottles were subjected to continuous light from fluorescent light tubes (to support autotrophic growth of algae) at surface intensity of 1272.5 ± 19 Lux, measured by a Luxmeter (Fisher Scientific) throughout the duration of the experiment. Growth of different green microalgae at 20 % LL is estimated by optical density (OD) measurements every five days, using a Spectrophotometer (Jenway 6715 UV/Vis., UK) at 595 nm. Ammonia-N removal was determined every five days as well according to modified Nessler method [27,28]. The most potent strain was then selected for further experiment.

2.4. Landfill leachate pre-treatment

The 20 % diluted LL used in this experiment was divided into three groups; one group was pre-treated by plasma, one group was pre-treated by UV and one group was left untreated.

2.4.1. Plasma pre-treatment

A dielectric barrier discharge reactor for plasma generation was used which incorporates the novel fluidic oscillator microbubble technology [29]. The plasma pre-treatment experimental set up was prepared as follows:

Compressed air was blown into a glass tube at 0.2 bar gauge pressure, the flow rate was measured by rotameter and was estimated to be 10 L/m ± 1. A steel rod 1 mm in diameter was positioned down the middle of the glass tube. This was connected to the live output of a high voltage amplifier, driven by a sinusoidal waveform of approximately 50 kHz. Aluminium tape was applied to the outer surface of this glass tube, and this was connected to the neutral cable of the high voltage amplifier. The peak voltages given out by the high voltage amplifier were around 2 kV (0 to peak). The plasma voltage was measured by a Tektronix high voltage probe, and the plasma current was measured by taking the voltage across a monitoring capacitor (of 6.8 nF capacitance) using a standard voltage probe. Both these probes were connected to Picoscope USB oscilloscope, which was set up to continuously capture the waveform data every few microseconds. When inserted into the leachate (Fig. 1) the plasma was observed to be present right up to the end of the glass tube where the outlet of the tube would be in contact with the liquid. This suggests that a greater number of short-lived plasma species would reach the liquid than would be that case if the plasma was positioned further away from this point of contact.

The plasma pre-treatment for the diluted LL in the Drechsel bottle, as shown in Fig. 1, was carried out for 3 h until a change in the colour of the leachate from brown to yellow was evident. The amount of diluted LL that was subjected to plasma pre-treatment was then filtered using 0.2 μm disposable sterile filters (Steritop, Millipore) driven by a vacuum pump N840FT.18 (KNF Laboport, Freiburg, Germany) and the filtrate

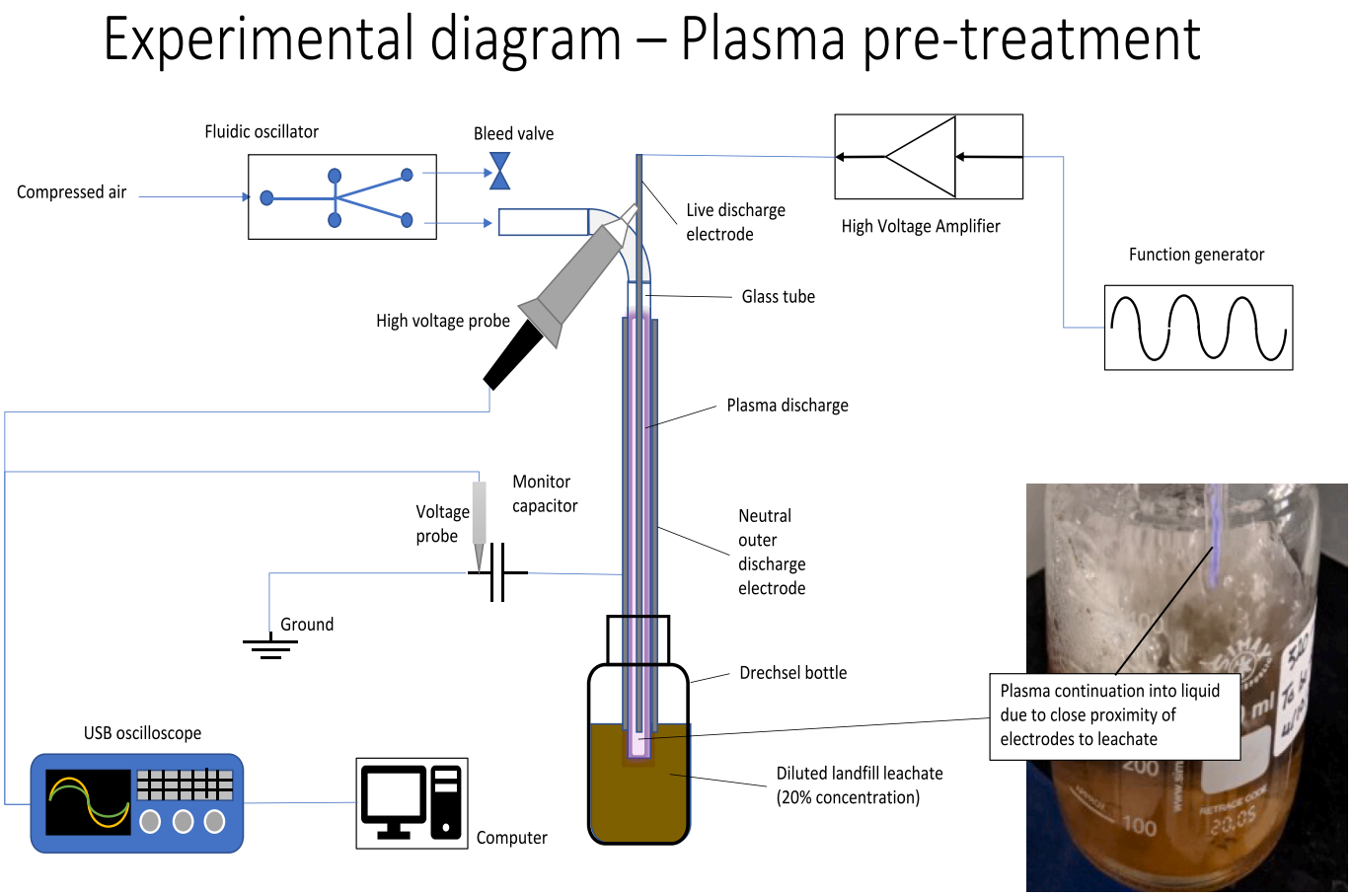

Fig. 1. Experimental diagram showing the plasma set up for the pre-treatment of the 20 % LL.

was collected in autoclave-sterilised Duran bottles. Plasma pre-treated filtered LL was then used for the experiment, where part of it was used as a control (no algae were inoculated) and the rest was used for algal inoculation by *Chlorella vulgaris* replicates (C.V.M* and C.V.N).

2.4.2. UV pre-treatment

This lot of diluted LL (20 %) (*v*/v) was pre-treated by UV prior to the experiment. The set up for the UV pre-treatment was carried out as follows:

A box was used for the apparatus to be enclosed in. A 250 mL beaker containing 200 mL of the diluted LL to be pre-treated was used. The beaker was placed on a magnetic stirrer operating at a medium speed. The UV device used is a purchased UV sterilisation apparatus, Smart UV Sterilizer U80 (CE FC RoHS, China) that uses both UVC and ozone for sterilisation. This device is equipped with a low-pressure mercury lamp design, it produces UV light (185–254 nm, Power 5 W) together with ozone which is generated at the same time when the lamp works. The lamp was positioned on the top of the beaker (containing LL) at 3.4 ± 1 cm, as measured by a ruler. The diluted LL to be pre-treated was subjected to a total of 3 h of UV/ozone lamp exposure, after which it was filtered using 0.2 μm disposable sterile filters (Steritop, Millipore) driven by a vacuum pump N840FT.18 (KNF Laboport, Freiburg, Germany) and the filtrate was collected in autoclave-sterilised Duran bottles. The filtered UV/ozone pre-treated LL was then used for the experiment where part of it was used as a control (no algae were inoculated) and the rest was used for algal inoculation. Experimental diagram for the UV pre-treatment apparatus could be found at Supplementary S1.

2.4.3. No treatment (untreated LL)

The final lot/group of diluted LL 20 % (*v*/v) was not subjected to any pre-treatments and was considered as a control group. It was filtered by passing through 0.2 μm disposable sterile filters (Steritop, Millipore) driven by a vacuum pump N840FT.18 (KNF Laboport, Freiburg, Germany) and the filtrate was collected in autoclave-sterilised Duran bottles. The filtered untreated LL was then used for the experiment where part of it was used as a control (no algae were inoculated) and the rest was used for algal inoculation.

2.5. Experimental set up for algal growth in pre-treated/untreated LL

The experiment was set up in duplicate 250 mL autoclave sterilised Duran bottles. Three bottles were used as control i.e., one for each treatment with no algal inoculation. The two tested algae were two replicates/strains of *Chlorella vulgaris* (C.V.M* and C.V.N). Each bottle was filled with 100 mL diluted leachate (20 % *v*/v) with sterile distilled water, supplemented with 1 mL of an inorganic phosphate source (5.2 g/L $K_2HPO_4.3H_2O$). Each Duran bottle was inoculated with 20 % (*v*/v) algal inoculum (20 mL). The OD for all the inocula were measured at 595 nm using a spectrophotometer (Jenway 6715 UV/Vis., UK) and the measurements were adjusted at 0.24–0.25. Autoclave-sterilised magnets were placed into the tested Duran bottles which were then each placed onto a magnetic stirrer plate to be continuously stirred and mixed during the duration of the experiment (30 days) at medium speed. The experiment was set up at room temperature and the temperature was monitored continuously (22.45 °C ± 0.85), the Duran bottles were subjected to continuous light from fluorescent light tubes (to support autotrophic growth of algae) at surface intensity of 968.5 ± 42.8 Lux, measured by a Luxmeter (Fisher Scientific) throughout the duration of the experiment. Every five days, samples (6 mL) were collected from the experimental Duran bottles to determine algal growth as described below and then filtered by 0.2 μm syringe filters, where the filtrate is used for chemical analysis of the leachate.

2.6. Algal growth measurements & chemical analysis of LL

2.6.1. Algal growth measurements

Algal growth was monitored by optical density (OD) determination every five days, using a Spectrophotometer (Jenway 6715 UV/Vis., UK) at 595 nm. At the end of the experiment (after 30 days), the dry biomass for the algal cultures was determined. 25 mL of each Duran bottle was collected (after being well homogenized) and centrifuged at 3900 rpm for 10 min using a centrifuge (Eppendorf 5810R) after which the pellet was left to dry on a pre-weighed Petri dish overnight at 50 °C.

2.6.2. Chemical analysis of landfill leachate (pH, COD and NH_3-N)

Aliquots of leachate collected every five days were filtered through 0.2 μm syringe filters after algal growth determination to be analysed for pH using a pH electrode; FiveEasyTMFE20 (Mettler-Toledo AG, Schwerzenbach, Switzerland) and COD using COD cuvette test kit LCK014 for the raw landfill leachate and LCK514 for the diluted landfill leachate samples (HACH Lange GMBH, Düsseldorf, Germany) following the instructions written in the manual provided by the company. The COD readings are done using HACH DR2800 Laboratory Spectrophotometer (HACH Lange, Germany). Filtered samples were stored at -20 °C until the end of experiment after which they were analysed for ammonia-N content using the modified Nessler method [27,28]. Samples from days 0, 5, 10, 15, 20, 25 and 30 were diluted 50× prior to the NH_3-N analysis by the Nessler method.

2.7. Genome sequencing for C.V.M* & C.V.N

DNA extraction for the two replicates/strains (C.V.M* and C.V.N) were carried out as explained earlier in detail, after which the extracted genomic DNA of the two samples was sent to the sequencing facility Novogene (Cambridge, UK). The library was checked with Qubit and real-time PCR for quantification and bioanalyzer for size distribution detection. Quantified libraries were pooled and sequenced on Illumina platforms, according to effective library concentration and data amount required.

The strain C.V.M* was submitted to the Culture Collection of Algae and Protozoa (CCAP) SAMS Limited, UK and was given an accession number: CCAP 211/141 *Chlorella vulgaris*.

2.8. Statistical analysis

Statistical analysis was carried out using R v.4.0.5 and R studio v.1.2.5. Data were presented as mean and standard deviation. Significance testing for the different measurements was carried out using paired *t*-test when comparing day 0 and 30 for each treatment, while ANOVA and post Hoc tests were used when comparing between the different treatments. A p value <0.05 was considered statistically significant.

3. Results

3.1. Sample collection and algae isolation

To identify some local indigenous microalgae for the possibility of future use in landfill leachate treatment, soil samples were collected in May 2018 from a landfill leachate treatment site in Chesterfield, UK (Fig. 2) and four green microalgal strains were successfully isolated, purified and genetically identified.

3.2. Molecular identification and phylogenetic analysis

Two primers (18S Lim and 18S Huss) in addition to another three primers (ITS1, 5.8S & ITS2) were collectively used to amplify the 18S and ITS regions of the rDNA for each algal strain, respectively. The resulted contig sequences were searched using NCBI BLAST after which

Fig. 2. Pictures for the landfill leachate treatment site from which soil samples were collected. Samples were collected from the green areas shown in the pictures (A, B, C and D).

they were submitted to the NCBI GenBank database and were given accession numbers. The identification revealed two strains of *Chlorella vulgaris* (MT137379 and MT137382), one strain of *Chlorococcum* sp. MT152906 and one strain of *Scotiellopsis reticulata* MT151679 (Fig. 3). The resulting phylogenetic trees for each strain are shown in Supplementary S2, S3 and S4.

3.3. Screening algal strains for the highest growth and ammonia-N removal in 20 % LL

3.3.1. Algal growth measurements

Growth of different algal strains individually and collectively is measured every five days by spectrophotometry as optical density (OD_{595}) to determine the strain with the highest growth at the tested 20 % LL concentration (Fig. 4). The growth of strains C.v.1.1 (C.V.M*) and C.v.1.2 (C.V.N) increased significantly from day 0 to day 30 ($p < 0.01$). However, the highest growth was observed for C.v.1.1 (C.V.M*), as it was significantly higher than C.v.1.2 (C.V.N), C.v.2, *Chlorococcum* sp., *S. reticulata* and all four algae grown together (Gp) ($p < 0.01$).

Microscopic examination for both cultures of C.v.1.1 (C.V.M*) and C.v.1.2 (C.V.N) at Days 23–24 of the experiment showed no evidence of contamination by other microorganisms as shown in Supplementary S5.

3.3.2. NH_3-N removal throughout the time interval of the experiment

The removal of NH_3-N is recorded during the screening experiment as one of the most important pollutants in the LL (Fig. 5). The highest reduction in the ammonia-N concentration was recorded for C.v.1.1 (C.V.M*), where concentration decreases from 435.9 mg/L (Day 0) to 57.9 mg/L (Day 30) as shown in Fig. 5.

3.4. Landfill leachate pre-treatment

The chemical characters of the plasma pre-treated LL, UV pre-treated LL and untreated LL were measured at the start of the experiment (before algae inoculation) and recorded in Table 1. There was no significant change in the pH values between the untreated and the plasma/

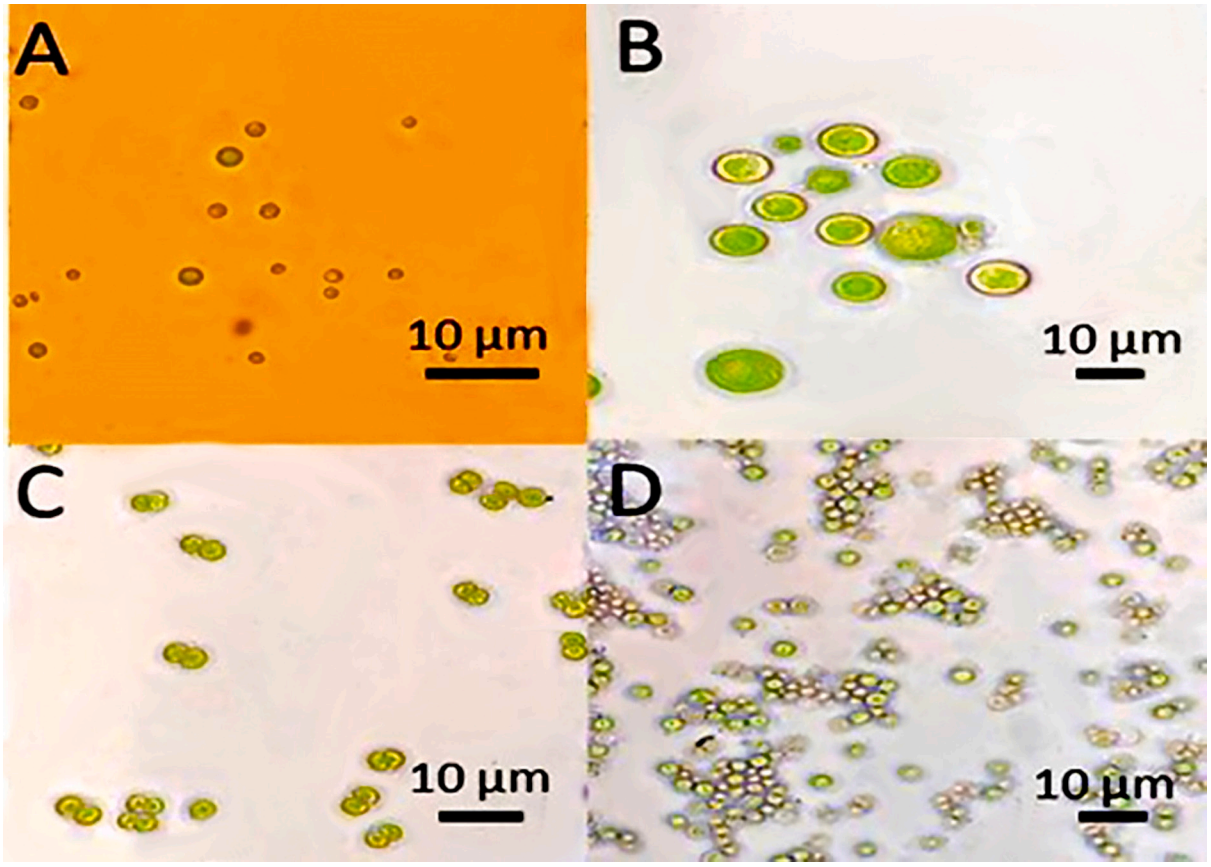

Fig. 3. Light microscope pictures for the four strains identified in this study. A) *Chlorella vulgaris* ATFG1 MT137379. B) *Chlorococcum* sp. ATFG MT152906. C) *Scotiellopsis reticulata* ATFG MT151679. D) *Chlorella vulgaris* ATFG2 MT137382. Magnification: 40 X.

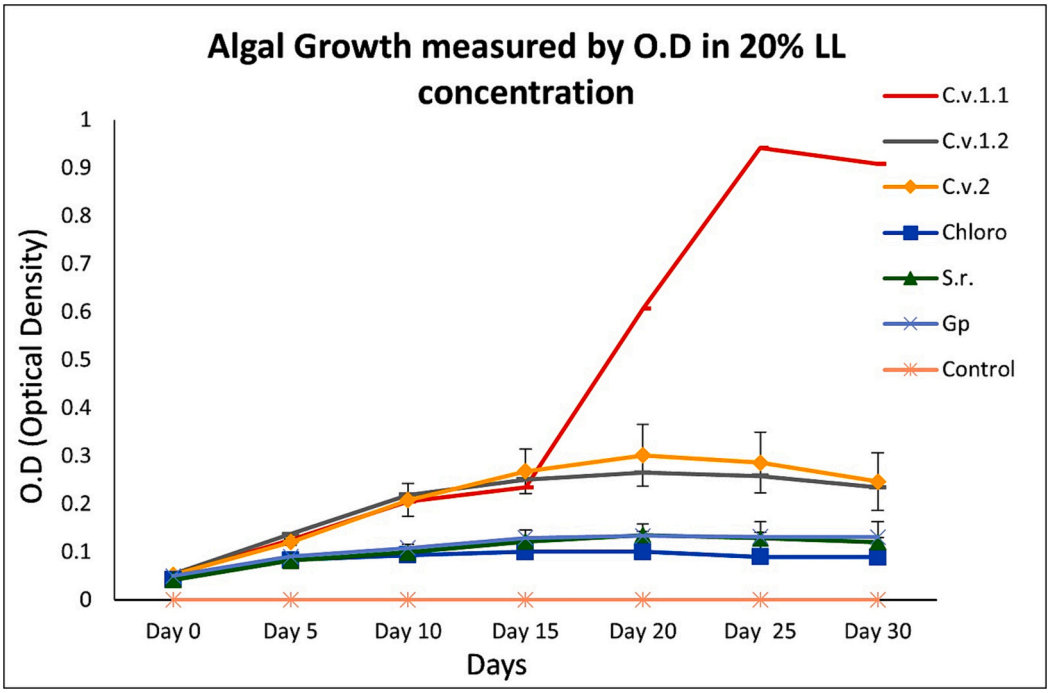

Fig. 4. Comparing growth of different algal strains tested individually and collectively in 20 % LL concentration with C.v.1.1 (C.V.M*) showing the highest growth. C.v.1.1 (C.V.M*) (*Chlorella vulgaris* 1.1), C.v.1.2 (C.V.N) (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

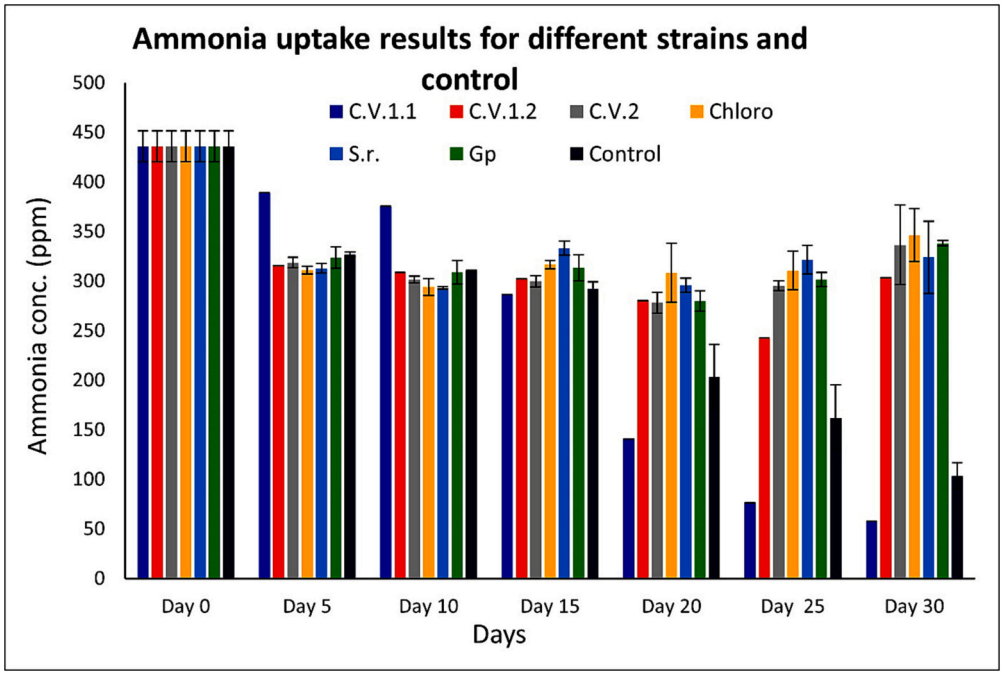

Fig. 5. Ammonia-N removal throughout the screening experiment by the different algal treatments in 20 % diluted LL. C.v.1.1 (C.V.M*) (*Chlorella vulgaris* 1.1), C.v.1.2 (C.V.N) (*Chlorella vulgaris* 1.2), C.v.2 (*Chlorella vulgaris* 2), Chloro (*Chlorococcum* sp.), S.r. (*Scotiellopsis reticulata*), Gp (group of all algal strains together), Control (control with no algae). Mean and standard deviation values of two biological replicates are plotted.

Table 1
Comparing chemical characteristics of landfill leachate with and without pre-treatments at Day 0 of the experiment.

Parameter	Plasma pre-treated LL (20 %)	UV pre-treated LL (20 %)	Untreated LL (20 %)	*P* value
pH	8.6 ± 0.015	8.6 ± 0.015	8.4 ± 0.015	0.982
COD	514.5 ± 1.5 mg/L	578 ± 1 mg/L	576 ± 1 mg/L	<0.001[a]
NH_3-N	151.38 ± 0 mg/L	219.43 ± 0 mg/L	290.73 ± 0 mg/L	<0.001[a]

Values are presented as mean ± standard deviation. ANOVA and Tukey post Hoc test were used for significance testing. A p value < 0.005 is considered statistically significant.
[a] significantly different between all groups.

UV pre-treated LL. COD change showed statistically significant difference between all the different treatments, with the lowest value recorded for the plasma pre-treated LL (514.5 mg/L). The ammonia-N content in all treatments showed a significant difference, where the highest ammonia concentration was recorded for the untreated LL (290.7 mg/L), the concentration decreased significantly after UV pre-treatment (219.4 mg/L). Moreover, the lowest concentration was recorded in the case of plasma pre-treatment (151.4 mg/L).

3.4.1. Plasma pre-treatment

The generated plasma was used as a pre-treatment step for the 20 % diluted LL (*v*/v) for 3 h, after which a visible change in the colour of LL from dark brown to yellow was evident (Fig. 6).

3.4.2. UV pre-treatment

The batch of diluted 20 % LL (*v*/v) to be pre-treated by UV/Ozone lamp was subjected to a similar three-hour pre-treatment step, yet no evident change in the dark brown colour of the LL was observed (Fig. 7).

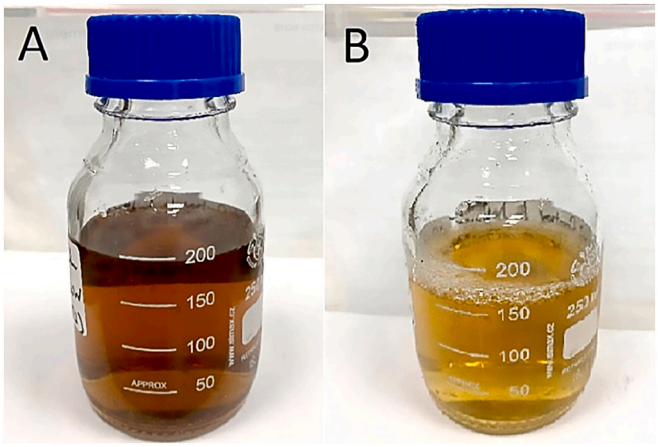

Fig. 6. A picture showing the pre-treated LL before and after plasma pre-treatment: A) before plasma pre-treatment B) after three hours of plasma pre-treatment.

3.5. Algal growth and chemical analysis of pre-treated/untreated LL

The two replicates of *Chlorella vulgaris* ATFG1 (C.V.M* & C.V.N) were selected for further experiment after C.V.M* showed the highest growth and ammonia-N removal compared to the rest of the tested algal strains in the screening experiment. LL pre-treatment using plasma/UV was applied before algal inoculation to test the effect of plasma/UV pre-treatment vs no treatment on both algal growth and nutrients/pollutants uptake efficiency in 20 % LL (*v*/v).

3.5.1. Algal growth measurements

The growth of both tested strains of the green microalga *Chlorella vulgaris* (C.V.M* & C.V.N) was determined by OD measurements every five days as well as by dry weight determination at the end of the experiment (after 30 days) and the results are shown below.

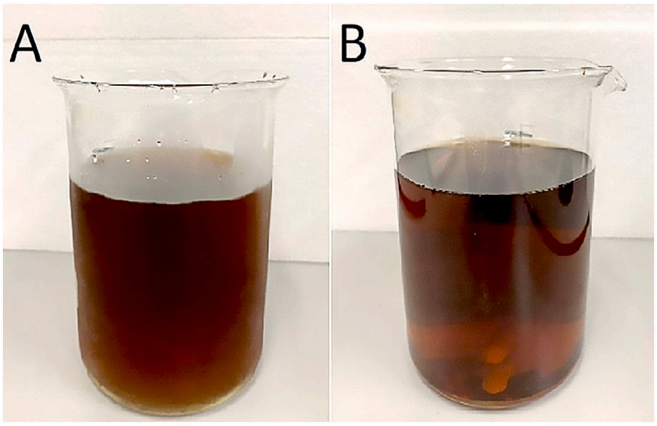

Fig. 7. A picture showing the UV pre-treated diluted LL before and after treatment: A) before UV pre-treatment B) after three hours of UV pre-treatment.

3.5.1.1. Growth measurements by OD. By comparing the growth of the two strains in different treatments at the end of the experiment (Fig. 8), it is found that the highest growth was achieved by the strain C.V.M* in plasma pre-treated LL and it was significantly higher than the other strain C.V.N in all the tested treatments ($p < 0.05$). However, it was comparable to the growth achieved by C.V.M* in the UV pre-treated and untreated LL which implies that the pre-treatment did not induce a significant change in growth, but it was the strain C.V.M* that showed a significant growth increase compared to its peer C.V.N. The growth C.V.M* in plasma pre-treated LL was 6.8-fold, 3.7-fold, and 18.7-fold higher than that achieved by C.V.N in plasma pre-treated LL, UV pre-treated LL and untreated LL, respectively.

3.5.1.2. Growth measurements by dry weight determination. The dry weight (g/L) was determined for the two tested strains of *Chlorella vulgaris* for all the tested treatments at the end of the experiment. Results showed that the highest dry weight was obtained by the strain C.V.M* grown in plasma pre-treated LL (0.38 g/L) as shown in Fig. 9. A significant increase in the dry weight produced by the strain C.V.M* ($p < 0.05$) in the plasma pre-treated LL was obvious compared to that produced by C.V.N in all the three treatments, where the biomass produced by C.V.M* in plasma pre-treated LL at the end of the experiment was an average of 0.38 g/L which is 4.9, 5.75 and 7.5 times higher than the dry weight produced by the strain C.V.N in plasma pre-treated, UV pre-treated and untreated LL, respectively.

The growth of the two tested strains (C.V.M* and C.V.N) at the end of the experiment (after 30 days) was shown in Fig. 10.

3.5.2. Chemical analysis of landfill leachate

Every five days, samples from each treatment were collected, filtered, and analysed for ammonia-N content, pH, and COD.

3.5.2.1. Ammonia-N analysis. The Ammonia-N uptake by the two tested strains of *Chlorella vulgaris* (C.V.M* and C.V.N) in different LL treatments was monitored continuously every five days throughout the time interval of the experiment (30 days). The results shown in Fig. 11 indicate that the highest decrease in the ammonia-N content was achieved by C.V.M* grown in plasma pre-treated LL at day 25. The strain C.V.M* exhibited significant reduction ($p < 0.05$) in ammonia-N in all the tested LL treatments from Day 0 to Day 30, on the contrary, no significant decrease in ammonia-N concentration was recorded for the strain C.V.N at any of the tested treatments.

The highest reduction in ammonia content was observed at Day 25 of the experiment for the strain C.V.M* in all LL treatments. Therefore, the removal percentage of ammonia-N at Day 25 was shown in Fig. 12 for each treatment coupled with the microalgal strain/control effect. The highest removal percentage was achieved by C.V.M* in plasma pre-treated LL (79 %) which was significantly higher ($p < 0.01$) than its peer C.V.N in plasma pre-treated LL as well as the plasma control. The ammonia removal percentage for the strain C.V.N in plasma pre-treated LL at Day 25 was 39.5 % which is half the amount removed by C.V.M* in

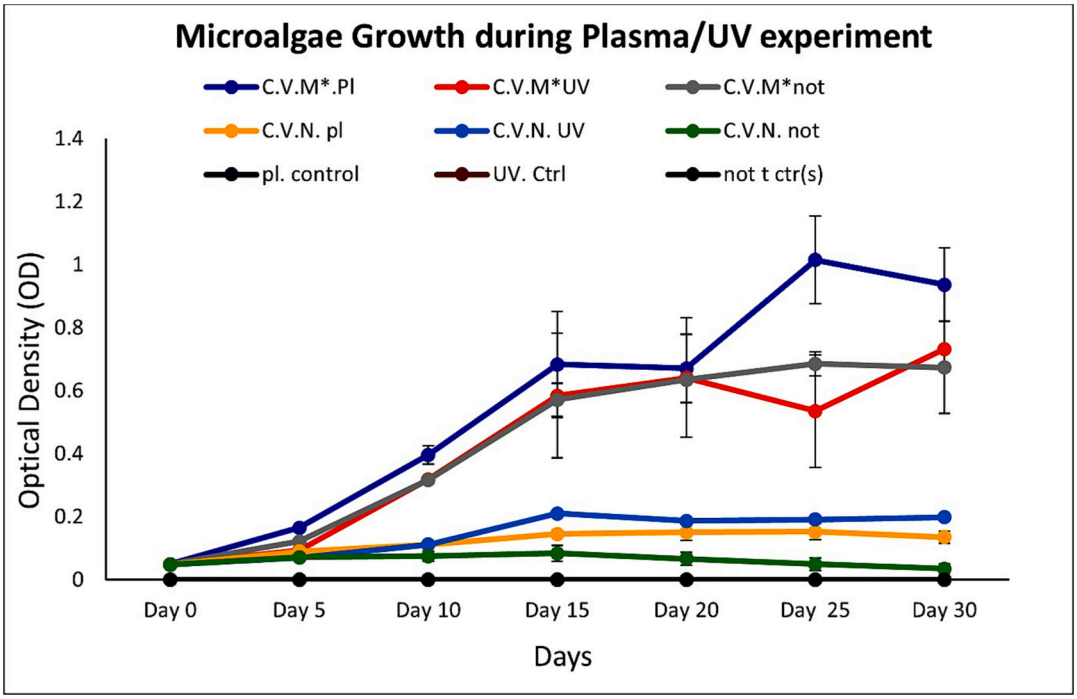

Fig. 8. Growth of the two tested strains of *Chlorella vulgaris* (C.V.M* and C.V.N) in plasma pre-treated, UV pre-treated and untreated LL. C.V.M*.PL (C.V.M*plasma pre-treated), C.V.N.pl (C.V.N plasma pre-treated), pl. control (plasma pre-treated control), C.V.M*UV (C.V.M* UV pre-treated), C.V.N.UV (C.V.N UV pre-treated), UV. Ctrl (UV pre-treated control), C.V.M*. not (C.V.M* untreated), C.V.N. not (C.V.N untreated), not t ctr(s) (untreated control). Mean and standard deviation values of two biological replicates are plotted.

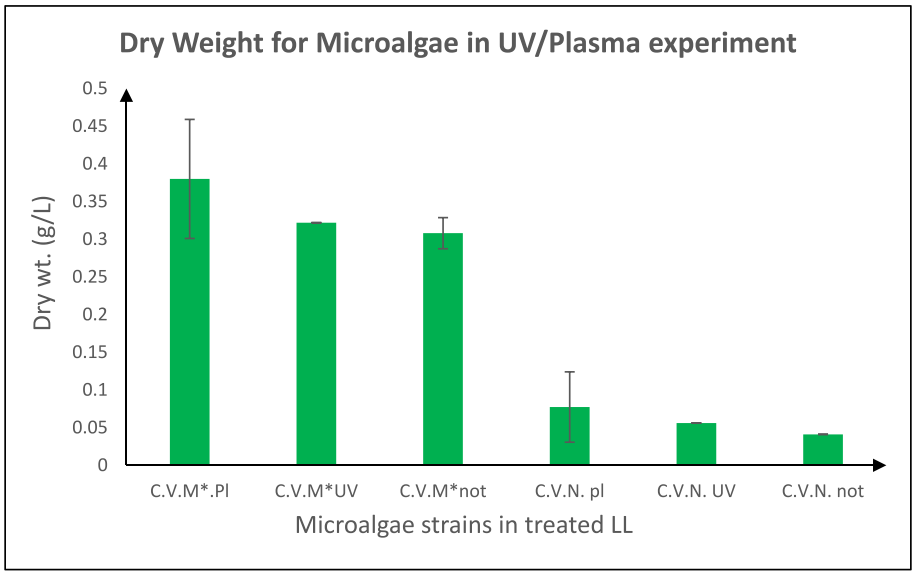

Fig. 9. The dry weight (g/L) at the end of the UV/plasma experiment (Day 30). C.V.M*.PL (C.V.M*plasma pre-treated), C.V.N.pl (C.V.N plasma pre-treated), C.V.M*UV (C.V.M* UV pre-treated), C.V.N.UV (C.V.N UV pre-treated), C.V.M*. not (C.V.M* untreated), C.V.N. not (C.V.N untreated). Mean and standard deviation values of two biological replicates are plotted.

plasma pre-treated LL (79 %).

3.5.2.2. pH measurements. The change in pH values in the experiment was recorded every five days and presented in Fig. 13. A significant decrease ($p < 0.05$) in the pH values from Day 0 to Day 30 was observed for the strain C.V.M* growing in all LL treatments. However, the pH values in case of the strain C.V.N as well as the control of all the treatments increased from Day 0 to Day 30.

3.5.2.3. COD measurements. Change in the chemical oxygen demand (COD) values throughout the time interval of the experiment for the two tested strains of *Chlorella vulgaris* in the different LL treatments was monitored every five days and plotted in Fig. 14. A significant increase ($p < 0.05$) in values of the COD was observed, from Day 0 to Day 30, in case of the strain C.V.M* grown in all different LL treatments. On the other hand, the strain C.V.N did not exhibit any significant change ($p < 0.05$) in COD values from throughout the experiment. Similar to the strain C.V.N, the control also did not show any significant change ($p < 0.05$) in any of the tested LL treatments.

3.6. Genome sequencing for C.V.M & C.V.N*

The two tested *Chlorella vulgaris* replicates/strains (C.V.M* and C.V.N) did not show significant differences when examined microscopically, except that the cells of C.V.N might be slightly bigger than the cells of C.V.M* as shown in Supplementary S6, which made a meticulous differentiation tool on the genomic level essential. Comparing all the SNP (single nucleotide polymorphism) loci of C.V.N and C.V.M*, the genotypes of 347,218 SNP loci were found to be the same in both types. In the same context, when comparing the indels of C.V.N and C.V.M*, 39,509 indel loci were similar between the two. However, the analysis revealed that there are 15,169 SNPs and 2046 indels with varying genotypes between C.V.N and C.V.M* (excluding the deletion loci (./.).

4. Discussion

Combining traditional morphological identification with molecular markers/genetic based identification is considered a reliable method for a powerful identification of the isolated species on a genus and/or species level whilst reflecting their phylogenetic lineages [30–32]. Although identification of algal strains was usually conducted based on the partial amplification of the large subunit rDNA (28S) and/or the small subunit rDNA (18S), followed by subsequent sequencing and NCBI BLAST analysis [33,34], however amplification of the 18S, ITS1, 5.8S and ITS2 regions (to get a better idea of the species identity) was also reported [35]. In the current study, coupling partial PCR amplification of 18S region with entire PCR amplification of ITS region resulted in more confident and accurate results which was further supported by phylogenetic analysis yielding four green microalgal stains which included two strains of *Chlorella vulgaris*, one strain of *Chlorococcum* species and one strain of *Scotiellopsis reticulata*.

Based on the results of a preliminary experiment [36], the concentration 20 % LL was chosen as a challenging concentration for screening the isolated strains for the highest growth and ammonia-N removal capability throughout a 30-day experiment. Two replicates (C.V.M* and C.V.N) of *Chlorella vulgaris* ATFG1 MT137379 were selected for further experiments after achieving the highest growth and/or ammonia-N removal/uptake compared to the other tested microalgae.

Plasma technology is deemed a green technology with no generated postproduction wastes [37]. Plasma was reported to be effective in ammonia-N removal as well as decolourisation of landfill leachate [19]. Comparing the chemical characters of the 20 % LL after plasma/UV pretreatment as well as without treatment prior to the microalgae inoculation revealed that the ammonia-N content showed significant difference following the different pre-treatments, with the lowest ammonia-N content recorded for the plasma pre-treated LL (151.38 mg/L). Zhao et al. [19] also reported that plasma treatment for industrial LL using atmospheric pressure dielectric barrier discharge (DBD) system affected the ammonia-N removal significantly. Different activated species (including ozone) produced by plasma pre-treatment might be responsible for the ammonia-N removal by oxidising the ammonia to form ammonium nitrates [19]. The bench-scale plasma reactor used in this study induced a colour change in the treated LL, as the colour of LL changed from dark brown to yellow after three hours of plasma pretreatment. On the contrary, Zhao et al. [19] reported a change in LL colour from deep grey-black to deep yellow after one hour of plasma treatment and a further change to light yellow after 2 h, whilst almost a complete decolourisation happened after 6 h. Moreover, Singh et al. [20] reported a colour change in the LL after 30 min of plasma treatment. This may be explained by the ability of the activated species

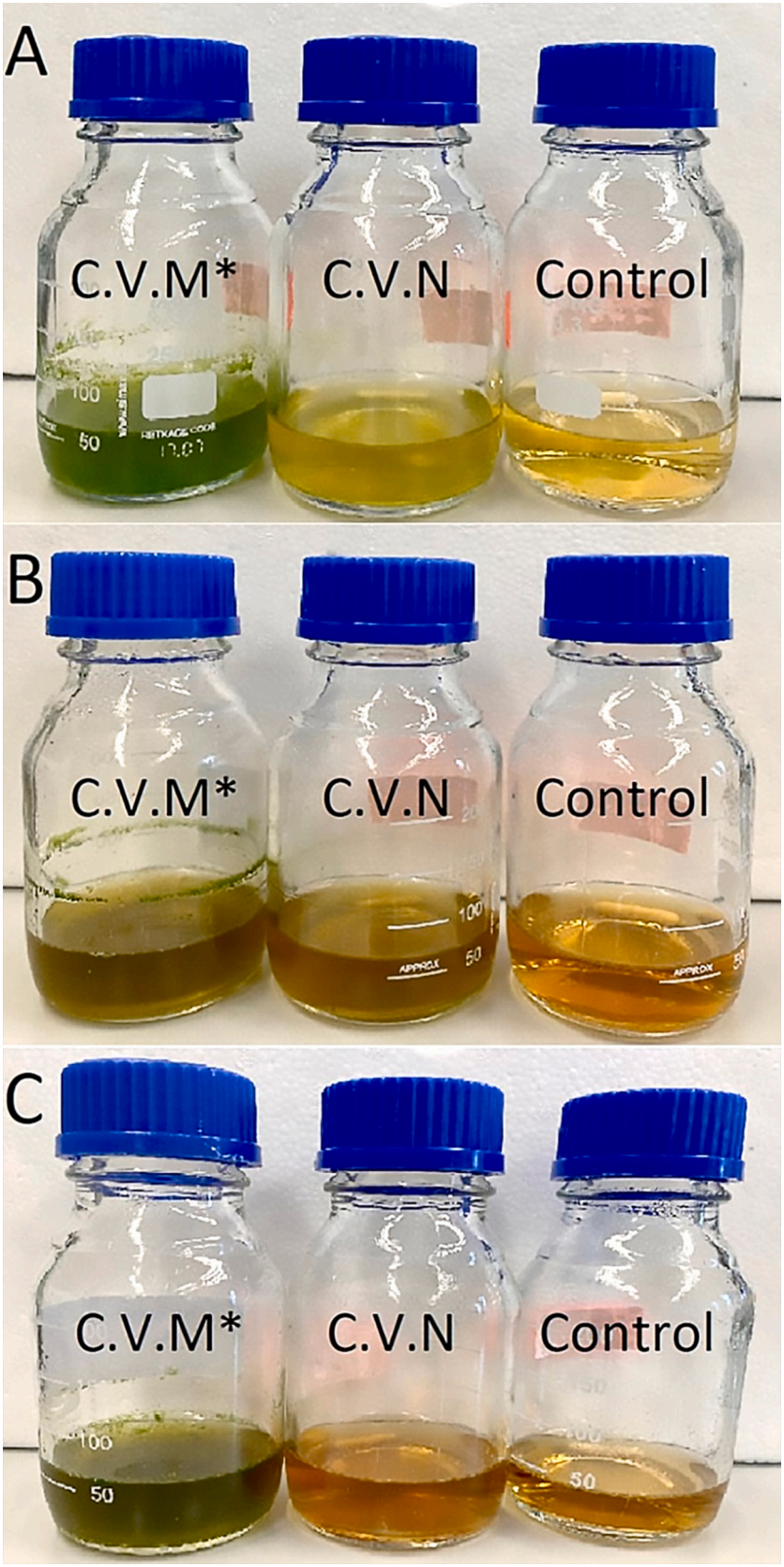

Fig. 10. A picture showing the growth of C.V.M* and C.V.N in all the different treatments; A) plasma pre-treatment LL; B) UV pre-treatment LL C) untreated LL.

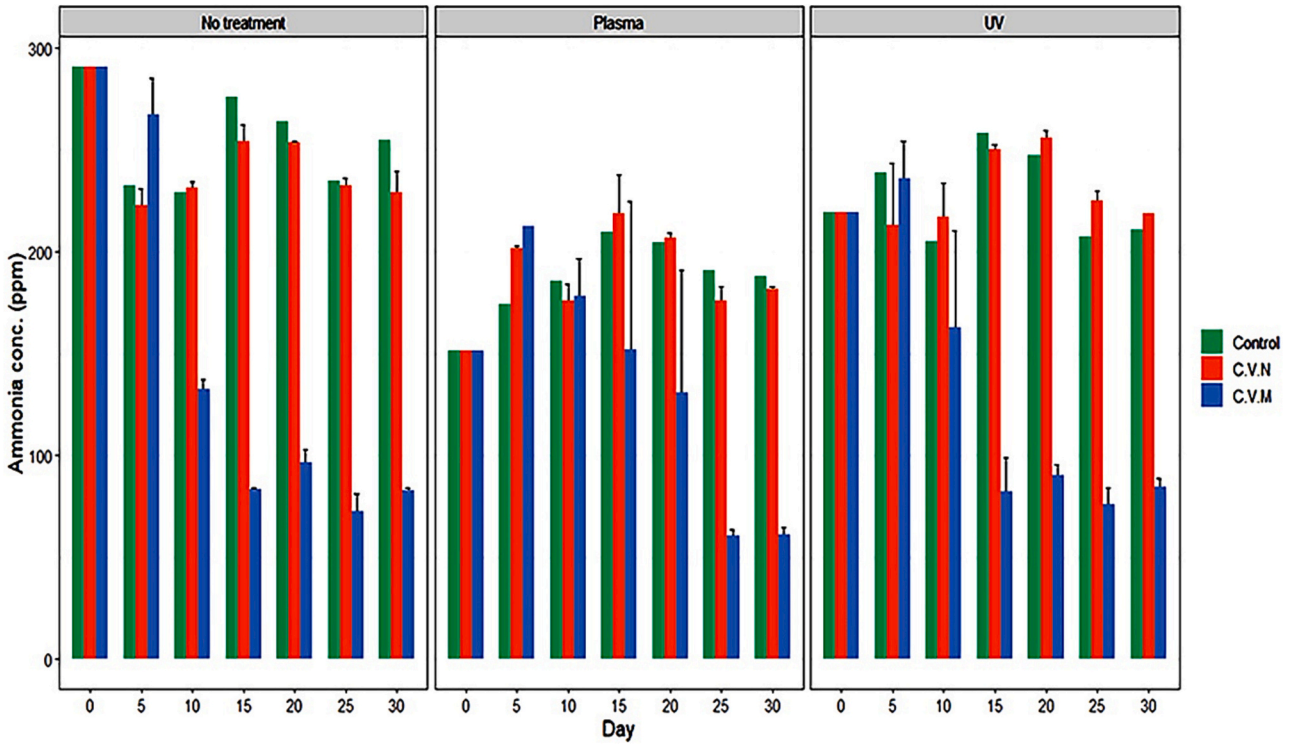

Fig. 11. Difference in ammonia-N concentrations throughout the period of the experiment in different LL treatments with the two tested strains of *Chlorella vulgaris* (C.V.M* and C.V.N). Mean and standard deviation values of two biological replicates are plotted.

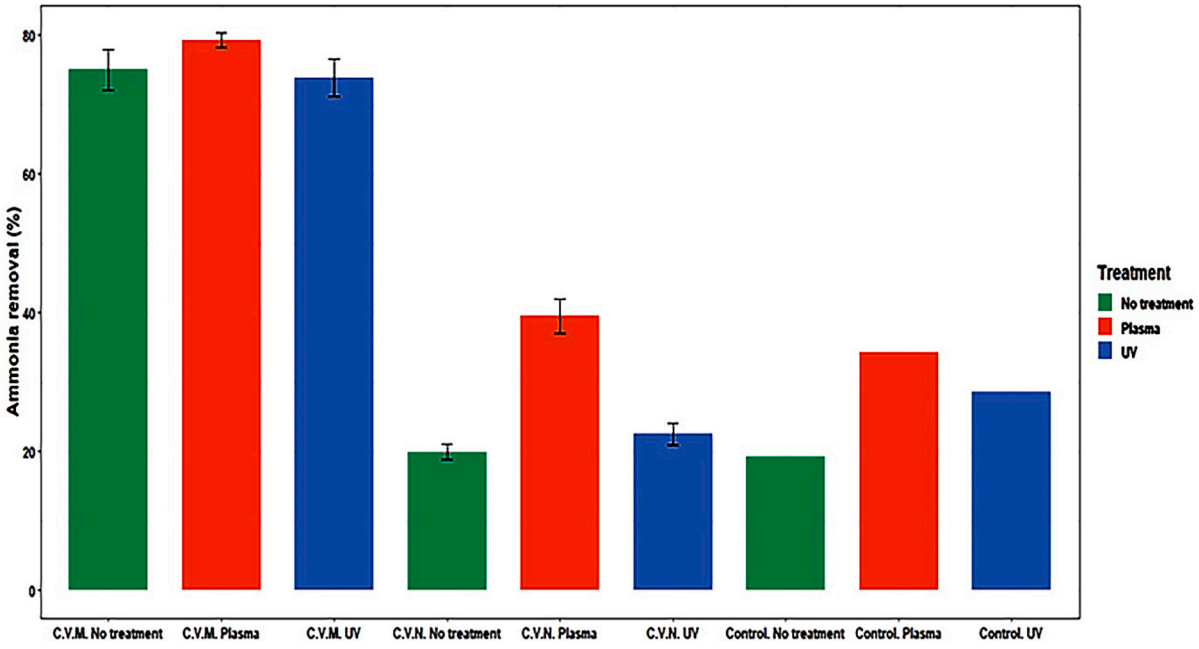

Fig. 12. Comparing the percentage of ammonia-N removal in LL treatments solely (control) and coupled with the microalgal strains tested (C.V.M* and C.V.N) at Day 25 of the experiment. Mean and standard deviation values of two biological replicates are plotted.

produced as a result of plasma treatment to oxidise and cleave the chromophores, thus resulting in a colour change/removal [19,38]. Different time intervals required for the colour change to occur after plasma treatment might be a result of difference in the variable conditions of the plasma bioreactor and/or the produced plasma used in each study.

Combining two or more methods of LL treatments especially physical-chemical and biological methods is widely reported to provide better treatment efficiencies in terms of some contaminants removal such as ammonia-N, organic compounds (as indicated by COD levels) and/or colour, in addition to the possibility of counting for the cost-effectiveness of the whole leachate treatment process [3,4,7,8,39]. Accordingly, an integrated approach of combining biological treatment of LL using the two abovementioned replicates of *Chlorella vulgaris* (C.V.

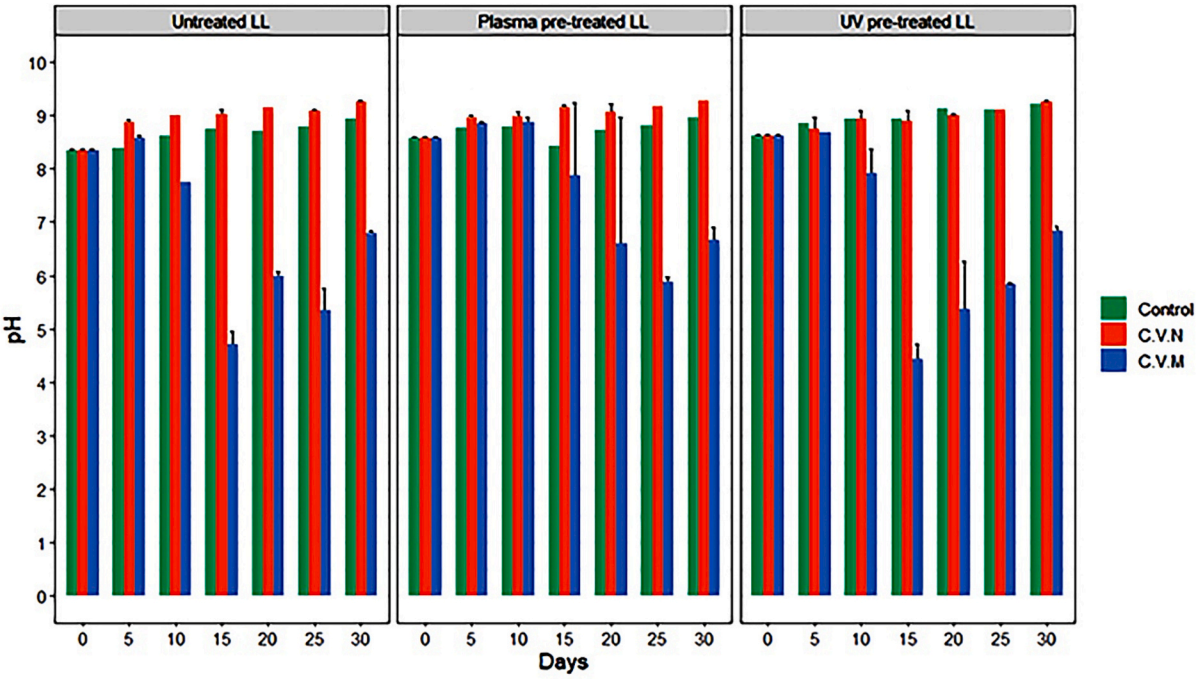

Fig. 13. pH dynamics change throughout the experimental time interval (30 days) as measured every five days for the tested algal strains (C.V.M* and C.V.N) in the different LL pre-treatments. Mean and standard deviation values of two biological replicates are plotted.

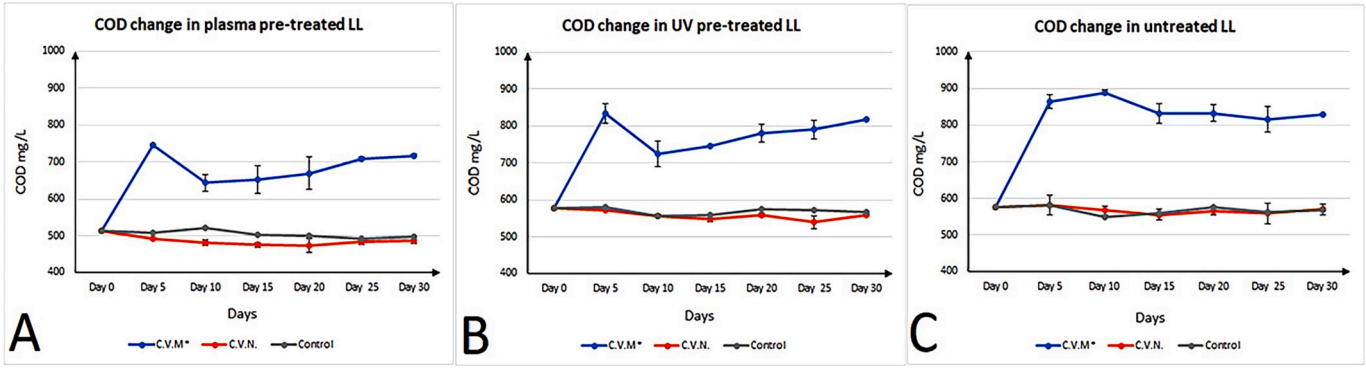

Fig. 14. Change in COD throughout the time period of the experiment (30 days) as recorded every five days for the two tested microalgae (C.V.M* & C.V.N) in the different treatments: A) plasma pre-treatment LL; B) UV pre-treatment LL; C) Untreated LL. Mean and standard deviation values of two biological replicates are plotted.

M* and C.V.N) with a physical pre-treatment of the tested LL using plasma/UV was investigated for its effectiveness in enhancing better algal growth as well as achieving an overall better pollutants removal. Our results indicated that the highest growth was achieved by the strain C.V.M* grown in plasma pre-treated LL in case of both OD and dry weight measurements. Quan et al. [40] also reported an increase in growth rate in case of the green microalga *Scenedesmus* sp. grown in LL pre-treated with ozone compared to that in untreated LL. Quan et al. [40] mentioned a higher biofilm density (18.9 g/m^2) in case of the oxidised (ozone treated) LL compared to that in untreated LL (12.7 g/m^2) and it was concluded that this might be a result of the improvement achieved by ozonisation which helped reducing the macromolecular organics content in LL as well as reducing its chromaticity, as organic macromolecules content and the dark colour of leachate might be possible reasons for hindering microalgal growth by being toxic and preventing light transfer, respectively. However, the interesting increase in growth exhibited by the strain C.V.M* over the strain C.V.N could also be directly related to the robustness of this strain and its tolerance to the tested LL. Whereas, our results showed that the strain C.V.M* outperformed its peer C.V.N by 18.7 times and 7.5 times as estimated by OD measurements and dry weight measurements in untreated LL, respectively, which implies that strain C.V.M* is capable of a better tolerance for the harsh environment of the raw diluted untreated LL (20 %) with all its contents of toxic organic and inorganic compounds as well as its dark colour with lower light transmittance. Nonetheless, plasma pre-treatment although did not induce a significant growth increase in case of C.V.M* compared to other treatments but it did support the highest growth which might be attributed to the abovementioned reasons. This refers to an interesting result in this study where C.V.M* is a robust strain that can effectively grow in diluted LL, however plasma pre-treatment can further improve its growth in leachate.

Ammonia-N (especially in high concentrations) is one of the most important toxicants in LL and, in turn, one of the key parameters during LL treatment. *Chlorella vulgaris* strain C.V.M* exhibited a substantial ammonia-N removal of 75 % when grown in untreated LL with an initial ammonia-N concentration of 290.73 mg/L indicating its tolerance for high ammonia-N concentrations compared to those reported in the literature to exert an inhibitory/toxic effect to microalgal growth i.e.,

concentrations ≥200 mg/L [12], >135 mg/L [41] and >110 mg/L [13]. In their study, Zheng et al. [13] reported the toxic effect of high ammonia concentration (>110 mg/L) on the cell viability, biomass concentration and biomass productivity of the green alga *Chlorella vulgaris* and they stated that both cell viability and biomass concentration of *Chlorella vulgaris* decreased significantly ($p < 0.05$) with increasing ammonium concentration above 110 mg/L. It was further concluded that the threshold for the ammonia toxicity in their study is 110 mg/L and concentrations exceeding this value might induce microalgal growth inhibition by ammonia toxicity. In view of such results, it could be concluded that the strain C.V.M*, which showed positive correlation between growth and significant ammonia removal ($p < 0.05$) in LL with ammonia-N concertation; 151.38 mg/L, 219.43 mg/L and 290.73 mg/L in case of plasma pre-treated LL, UV pre-treated LL and untreated LL, respectively, could be deemed an ammoniacal-N tolerant strain with high capability of thriving in 20 % raw LL with relatively high ammonia-N concentrations.

However, the highest ammonia-N removal was obtained in case of C.V.M* growing in plasma pre-treated LL that together achieved 79 % removal of the total ammonia-N concentration at day 25 of the experiment. The plasma pre-treatment step reduced the ammonia-N concentration from 290.73 mg/L to 151.38 mg/L, after which it was further reduced as C.V.M* growth increased (Day 25) to 60 mg/L. This result might be explained by three possible reasons; the first is the activated species produced by the plasma pre-treatment might have caused ammonia removal by oxidising the ammonia to form nitrates [19] which was evident by the reduction of ammonia concentration by 1.9-fold after plasma pre-treatment, the second is the effect of ammonia removal by the growing strain C.V.M* in LL which was discussed above in detail and the third is a small removal percentage by the effect of volatilisation due to continuous stirring throughout the experiment. However, stirring only accounts for an insignificant removal percentage ($p < 0.05$) as indicated by the control treatments results. Thus, it could be concluded that strain C.V.M* is a highly ammonia-tolerant strain, however applying plasma pre-treatment improved its growth and ammonia-N removal uptake.

It should be noted that there are competing approaches to the destruction of ammonia content in landfill leachate that can be partially achieved by plasma microbubble pretreatment conducted here. Desai et al. [42] removed the ammonia by hot microbubble stripping and reviewed other approaches such as conventional stripping columns and novel stripping approaches, chemical transformation and destruction, as well as absorption. A plethora of microalgae can then grow on the ammonia-depleted LL [43,44]. However, an advantage of pretreatment by plasma-activated microbubbles is the disinfection of the LL [45], leading to the possibility that it can serve as an uncontaminated feedstock for microalgae that produce higher value bioproducts with the microbial consortium of choice, taking advantage of symbiotic engineering [44]. Of course, the advantage of the high ammonia tolerant microalgal strain isolated and identified here is that little pretreatment is needed, and the ammonia titre can serve as a N-source once the level is decreased below the inhibited level by any method.

Simultaneously, with the significant increase in the growth and ammonia-N removal percentage in case of the strain C.V.M*, an observed significant decrease/drop in the pH was recorded. pH is one of the most important factors to be considered when dealing with LL, it could highly influence growth of microalgae in LL [46] as well as different nutrients removal from wastewaters in general [13]. Since there is a close relationship between ammonia uptake and the change in pH dynamics [47]. A significant reduction ($p < 0.05$) in the pH values in case of the strain C.V.M* in all treatments of LL was recorded, these results agree with the findings from the ammonia-N removal experiment mentioned earlier. These results might be explained by Shi et al. [47], who pointed out the possibility of severe drop in the pH accompanying the utilisation of ammonia as the main nitrogen source by actively growing algae where the rapid utilisation of the ammonium ions eventually causes a dramatic pH drop.

COD values can reflect the concentration of both organic and inorganic components of the samples, which are subjected to oxidation, however, usually the organic components are dominant and most interesting [48]. In contradiction to the pH results, the COD values exhibited a significant increase ($p < 0.05$) in case of C.V.M* in all the tested pre-treatments. These results are also in agreement with all the above results where the strain C.V.M* showed a significant growth, ammonia-N removal and pH decrease in all the tested LL treatments. Desai [49] found that the COD concentrations in some treatments had exceeded the control at the end of the experiment where a *Chlorella* sp. was tested for biotreatment in different concentrations of LL (10 %, 25 %, 50 % and 85 %). The significant increase in the COD or the dissolved organic carbon might be an indication of the microalgal growth. When microalgae perform autotrophic metabolism (photosynthesis) they convert inorganic carbon to biomass which will in turn account for the increase in organic matter and thus increase in COD levels rather than accounting for organic carbon removal [11]. Another possibility for the COD increase accompanying high algal growth is the production of extracellular organic matter as the algae grow [11,49]. Hulatt and Thomas [50] estimated the mean maximum amount of dissolved organic carbon (DOC) released as a culture of *Chlorella vulgaris* grows in a photobioreactor to be 6.4 % of the total organic carbon in the culture whilst Zhao et al. [11] in their study reported the extracellular organic matter to account for 1.6 %–9.5 % of the total captured carbon in the culture of *Chlorella pyrenoidosa* used in the study.

The genus *Chlorella* is one of the most common genera of green algae (Chlorophyta) which has wide applications in several fields e.g., biofuel production [51,52]; wastewater treatment [13,53] and as a model in different molecular biology studies [54] as well as several other biotechnological applications. *Chlorella vulgaris* is one of the most promising green microalgae in wastewater treatment in general [13] and landfill leachate treatment in particular [28]. Dramatic increase in the OD measurements, dry weight production and ammonia-N uptake of the strain C.V.M* over the strain C.V.N when grown in 20 % LL, despite being originally replicates for each other and having no sharp differences under microscopic examination nor clear sign of a contaminating organism in the culture, suggested the necessity of performing whole genome sequencing for both, to be able to differentiate between them on the molecular basis. Results indicated a high degree of similarity between the two strains as observed by the 347,218 SNPs and 39,509 indels shared between them, however, the genotypes of 15,169 SNP loci and 2046 indel loci were different. The differences in genotypes might have affected expression of cell cycle genes and/or stress response genes. Unfortunately, due to the absence of GFF/GFF3/GTF files for *Chlorella vulgaris* strains in public databases (at the time of the study), genome annotation could not be performed which prevented us from the possibility of making biological sense of the sequences of both strains when mapped to reference sequences. We could only hypothesize the possibility of occurrence of random mutation allowing the *Chlorella vulgaris* strain (C.V.M*) to show better tolerance for the harsher environment of landfill leachate. Another possible explanation could be the possibility of occurrence of sexual reproduction which might have been triggered by stress conditions and resulted in the production of a more resistant zygote. Although, no sexual life cycle was previously described in the genus *Chlorella* and it has long been assumed to be asexual [54] but recent evidence regarding the presence of meiosis genes in *Chlorella variabilis* NC64A and *Chlorella vulgaris* UTEX 395 was reported by Blanc et al. [54] and Guarnieri et al. [52], respectively. Future work is required to perform genome annotation on the available sequences of strain C.V.M* in order to have a better understanding for the genes responsible for tolerating the harsh conditions of LL and those responsible for the high growth and increased ammonia-N uptake as well as optimising the conditions for their growth in addition to optimising the plasma pre-treatment step thus providing better approach for LL treatment in an efficient, sustainable, and cost-effective way.

5. Conclusions

A strain of *Chlorella vulgaris* CCAP 211/141 (C.V.M*) was proven to be tolerant to the harsh environmental conditions of 20 % diluted LL (*v/v*), with its high NH_3-N concentration (290.73 mg/L) and high pH value (8.6), as it exhibited a dramatic increase in growth by almost 19-fold compared to its peer (C.V.N) when grown in 20 % untreated LL. A significant ammonia-N removal percentage (75 %) was also observed in strain C.V.M* over strain C.V.N. However, this percentage was further improved when LL was subjected to plasma pre-treatment first which helped reduce the initial amount of ammonia-N by 1.9-fold from 290.73 mg/Lto 151.38 mg/L, after which it was further reduced by growth of C. V.M* to 60 mg/L i.e. (79 % removal) with significant decrease in the pH value from 8.6 to 6.67 at the end of the experiment. Complete genome sequencing for both strains revealed different SNPs and Indels which might suggest the possibility of mutation or sexual reproduction that might have possibly conferred the advantages of better adapting to the harsh environment of LL with its high ammonia-N content. The high-ammonia tolerant strain *Chlorella vulgaris* CCAP 211/141 might provide a robust candidate for LL treatment with the possibility of utilising plasma as a pre-treatment step to allow better algal growth and ammonia-N uptake, due to the ammonia reducing effect of the plasma pre-treatment as well as its decolourising effect which might allow better light transmittance thus better light availability for microalgal growth. Future work is required for optimising the integrated plasma-microalgal approach for LL treatment with better understanding, on the molecular level, for the genes responsible for LL tolerance in the *Chlorella vulgaris* strain CCAP 211/141.

CRediT authorship contribution statement

Dr. Farag: design, conceptualization, investigation, writing.

Dr. Holmes: conceptualisation, implementation of plasma treatment regime.

Dr. Gilmour: supervision, conceptualization, methodology.

Prof Zimmerman: conceptualization, supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2023.103345.

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