Article:
Okurowska, K., Karunakaran, E., Al-Farttoosy, A. et al. (Cover date: Jan 2021) Adapting the algal microbiome for growth on domestic landfill leachate. Bioresource Technology, 319. 124246. ISSN: 0960-8524

https://doi.org/10.1016/j.biortech.2020.124246

Bioresource Technology 319 (2021) 124246

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Adapting the algal microbiome for growth on domestic landfill leachate

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HIGHLIGHTS

- An algal-bacterial consortium was enriched from domestic landfill leachate.
- Adapted laboratory evolution was undertaken for 24 months on 20% (v/v) leachate.
- *Chlorella vulgaris* growth rate increased 2.9-fold by adapting its microbiome.
- Adapted consortia reduced nitrate production and increased total organic carbon degradation.
- Bacterial genera were selected that degrade toxic organics and promote algal growth.

ARTICLE INFO

Keywords:
Landfill leachate
Algae microbiome
Adaptive laboratory evolution
PICRUSt

ABSTRACT

We aimed to improve algal growth rate on leachate by optimising the algal microbiome. An algal-bacterial consortium was enriched from landfill leachate and subjected to 24 months of adaptive laboratory evolution, increasing the growth rate of the dominant algal strain, *Chlorella vulgaris*, almost three-fold to 0.2 d^{-1}. A dramatic reduction in nitrate production suggested a shift in biological utilisation of ammoniacal-N, supported by molecular 16S rRNA taxonomic analyses, where Nitrosomonas numbers were not detected in the adapted consortium. A PICRUSt approach predicted metagenomic functional content and revealed a high number of sequences belonging to bioremediation pathways, including degradation of aromatic compounds, benzoate and naphthalene, as well as pathways known to be involved in algal-bacterial symbiosis. This study enhances our understanding of beneficial mechanisms in algal-bacterial associations in complex effluents, and ultimately enables the bottom-up design of optimised algal microbiomes for exploitation within industry.

1. Introduction

It is well understood that strains of algae and bacteria have co-evolved to promote each other's growth in the natural environment, and these can be exploited within industrial processes. For instance, symbiosis for the removal of organic and inorganic waste has been well documented in wastewater treatment processes. Algal cooperation with bacteria, or the algal microbiome, enables performance of more complex metabolic tasks, including the degradation of toxic wastes (Kumari et al., 2016; Luo et al., 2014). However, the mechanisms that underlie the interactions between algae and its symbionts in industrially relevant environments, such as leachate effluent treatments, are not well known (Lian et al., 2018), and therefore the design of well-constructed and robust algal-bacterial consortia remains a significant challenge.

Production of municipal solid waste (MSW) is steadily increasing globally due to growing population, rising affluent lifestyles and industrial/commercial growth (Renou et al., 2008). Landfilling remains a popular method for managing MSW, however, this process produces a potentially toxic leachate. Leachate can vary considerably in composition, depending on the age and type of waste within the landfill, containing both dissolved and suspended organic and inorganic material, high levels of ammoniacal-nitrogen (NH_3-N), heavy metals, and chlorinated organic and inorganic salts (Renou et al., 2008). Ultimately, landfill effluents need to be treated to comply with regulatory standards before discharge to foul sewers or into surface water. Current modular treatment processes rely on multiple stages combining chemical, physical and biological processes, reflecting the complexity and variability of the leachate (Talalaj et al., 2019). Biological processes can target reduction of organic constituents, whereas physical methods, such as reverse osmosis, are more suited to removal of chemical oxygen demand

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https://doi.org/10.1016/j.biortech.2020.124246
Received 27 August 2020; Received in revised form 6 October 2020; Accepted 7 October 2020
Available online 12 October 2020

(COD) and metals. Biological methods can reduce the overall energy requirement for leachate treatment, lower sludge production and chemical use, achieving increasingly strict environmental standards (Rooksby, 2007). Within biological treatment methods, bacterial communities successfully biodegrade toxic components in activated sludge, sequential batch reactors, aerated lagoons and upflow anaerobic sludge blankets (Kurniawan et al., 2010). Recently, the use of microalgae to treat leachate has received increasing interest. They require sufficient concentrations of macronutrients, phosphorus (P), and nitrogen (N) to grow and hence are well suited to domestic wastewaters or in less common cases, leachates with high concentrations of macronutrients (Chang et al., 2018; Cheah et al., 2016; Paskuliakova et al., 2016). A significant advantage of using microalgae is that the biomass can be harvested from the leachate for recovery of nutrients or metals, or even converted into a variety of bio-products, including chemical precursors for bio-plastics and fuels (Cheah et al., 2016; Edmundson and Wilkie, 2013). This can improve the overall economics of treatment, although there are challenges for microalgal growth in landfill leachate. Most landfill leachates are low in macronutrients, have a very dark colouration (limiting light penetration and therefore photosynthesis), and are characterised by high levels of ammonia which are toxic to microalgae (Lin et al., 2007; Pereira et al., 2016). To overcome these challenges, leachates are often diluted to 10% (v/v), pre-treated and/or supplemented with nutrients (Chang et al., 2019; Dogaris et al., 2019; Paskuliakova et al., 2018; Pereira et al., 2016; Tighiri and Erkurt, 2019). Most microalgae leachate cultivation studies use single strains of microalgae, focussing on optimising process parameters, such as photobioreactor (PBR) design. For example, Dogaris et al. (2019) cultivated *Pichoclorum oculatum* in a novel horizontal PBR at 150 L and 2000 L scale, demonstrating high cell density culture growth (1.7×10^9 cells ml^{-1}) although the leachate was sourced from a deep well, with low concentrations of toxic components and supplemented with macronutrients, vitamins and trace metals (Dogaris et al., 2019). Another study designed a membrane based tubular PBR for use with a *Chlorella vulgaris* strain obtained from a culture collection (Chang et al., 2019). This strain was characterised with a robust growth profile and high lipid content. Lin et al. (2007) aimed to identify a more suitable microalgal strain for growth on leachate by comparing the growth kinetics of a freshwater river strain, *Chlorella pyrendoisa* (P), with two isolates from a high ammonia leachate pond, *Chlorella pyrenoidosa* (LK) and *Chlamydomonas snowiae* (LK) (Lin et al., 2007). Although all three strains were able to grow in 10% (v/v) leachate, *C. pyrenoidosa* (LK) was shown to exhibit the highest tolerance, growing in 405 mg L^{-1} NH_3-N.

Combining microalgae with bacteria has distinct advantages. Bacteria are able to degrade complex organic components within the leachate, consuming oxygen (O_2) and producing carbon dioxide (CO_2) through respiration. Concurrently, microalgae are able to fix this CO_2 into organic carbon during photosynthesis whilst producing O_2 to complete the symbiotic process. Interactions can also be more complex, involving exchange of biomolecules including vitamins and phytochromes (Lian et al., 2018). Furthermore, in practice, using pure cultures of microalgae may be uneconomical, as there are naturally occurring microbes (e.g. bacteria, microalgae and fungi) in the effluent and it could therefore require sterilisation prior to treatment. A study by Sniffen et al. (2016) sourced a mixed culture of bacteria and microalgae from a local outdoor pond to treat diluted, unsterilized landfill leachate. They demonstrated a maximum nitrogen removal rate of 9.18 mg L^{-1} day^{-1} with a maximum biomass density of 0.48 g L^{-1} (Sniffen et al., 2016). More recently, Tighiri and Erkurt (2019) cultivated a microalgae-bacteria consortia on 10% (v/v) diluted landfill leachate, and demonstrated more than 90% efficiency in removing nitrate, COD and phenol (Tighiri and Erkurt, 2019). The inoculum was sourced from a local wastewater treatment plant. Kumari et al. (2016) used a specific co-culture of *Scenedesmus* sp. ISTGA1 and *Paenibacillus* sp. ISTP10 to treat leachate, showing degradation of toxic organic contaminants and removal of heavy metals (Kumari et al., 2016). These studies demonstrate that the choice of strains for pure microalgae or microalgae-bacteria consortia for growth on landfill leachate treatment are largely from unrelated environments, culture collections, or even put together as synthetic consortia, which would be difficult to translate to pilot scale.

Algae-bacteria associations or algal microbiomes for industrial growth of microalgae has recently received increasing attention (Lian et al., 2018). However, there are a lack of systematic investigations into optimising the composition of algae-bacteria consortia for specific applications, including efforts to enhance growth on landfill leachate. In this study, an algae-bacteria consortium was enriched from a landfill leachate characterised by a dark brown colour, low levels of phosphorus and nitrate, but very high concentrations of organics, heavy metals and NH_3-N concentrations. Algal growth was characterised in different leachate dilutions, with and without nutrient additions to identify the ideal conditions for adaptive laboratory evolution. The adaptive evolution regime involved regular sub-culturing for 24 months in 20% (v/v) diluted leachate under illumination to support and enhance autotrophic algal growth. A molecular characterisation was undertaken on the composition of the microbial consortium together with an analysis of the microalgal growth rates on leachate before and after adaptive evolution.

2. Materials and methods

2.1. Algal-bacterial consortia enrichment for leachate treatment

One litre of landfill leachate was collected from a leachate pond (Erin Landfill site, Chesterfield, UK 53.2533, −1.3295) on 27th January 2016 in a sterile glass container. Immediately upon receipt in the laboratory, 10 mL leachate was diluted to a final volume of 100 mL with Bold's Basal Medium (BBM) (10% v/v leachate) in 250 mL flasks, with cellulose stoppers to allow gas exchange, and incubated for 42 days at 25 °C with shaking at 150 rpm under a light intensity of 40 μ Einstein m^2 s^{-1}, 12:12 h light dark cycle. The light intensity was measured using Underwater Quantum Scalar Laboratory Radiometer (Biospherical Instruments, San Diego, CA, USA) immersed in water and placed in the centre of the flask. The consortium was subsequently sub-cultured into fresh BBM with 10% (v/v) leachate at a ratio of 1:10. The sub-cultured consortium was incubated for 21 days at 25 °C, with shaking at 150 rpm under a light intensity of 40 μ Einsteins m^{-2} s^{-1}, 12: 12 h light-dark cycle. After a second round of sub-culturing, this culture was referred to as the "original consortium". This culture was characterised for growth on leachate as described in Section 2.3 to identify the ideal conditions for long term adaptive evolution experiments (Section 2.5).

2.2. laboratory-based flask experiments

All laboratory-based cultivation experiments were undertaken in triplicate 500 mL flasks filled with 250 mL diluted leachate (10% or 20% v/v) with distilled water, supplemented with a phosphate source, (0.75 g L^{-1} K_2HPO_4 and 1.75 g L^{-1} KH_2PO_4 (equivalent to 0.5 g L^{-1} dissolved inorganic phosphorus, DIP)), and either with or without a nitrate source (0.75 g L^{-1} $NaNO_3$), both equivalent in concentration to BBM media. They were aerated and mixed using air filtered through a 0.22 μm pore sized filter. Air was introduced using a 6 mm diameter silicone tubing at 0.2 L per minute with an aquarium pump (ACRO 9630). The flasks were exposed to higher light intensity (ca. 150 μ Einstein's m^2 s^{-1}) in 12:12 light dark cycles and grown at 20 °C. Three additional flasks were used as a control where no inoculum was added.

2.3. Growth measurements

Growth was measured using spectrophotometry and reported as optical density (OD) at 680 nm. Samples for OD_{680nm} were blanked against medium filtered with a 0.22 μm syringe (Millex, Watford, UK) prior to measurement on a spectrophotometer (Jenway 6300, VWR,

Lutterworth, UK). The pH was measured using a LAQUA B-712 (Horiba, Moulton Park, UK). For cell counts, a 1 mL sample of the culture was fixed by adding 0.1 mL of Lugol solution (Sigma-Aldrich, Dorset, UK), vortexed and stored at room temperature until counting. Cells were counted using a Neubauer counting chamber (VWR, Lutterworth, UK) at 400× magnification using a light microscope (Zeiss Axiostar Plus, Cambridge, UK). The cell size was measured using microscope images with MATLAB release 2017a (The MathWorks., Inc., Natick, Massachusetts, USA).

2.4. Assessment of light penetration in diluted leachate

Light intensity was measured in 10% and 20% (v/v) diluted leachate in 250 mL flasks. Deionised water was used as a control. The light probe was immersed in the central position inside the flask and secured with a foam stopper. The light intensity was adjusted to 40, 70, 200, 300 and 400 μ Einsteins m^{-2} s^{-1} using a custom-built light jacket and underwater Quantum Scalar Laboratory Radiometer (Biospherical Instruments, San Diego, CA, USA) in a dark room. Average values for light intensity were recorded and compared for both concentrations of leachate.

2.5. Adaptive laboratory evolution

10 mL of the original consortium was added to 90 mL of fresh BBM medium containing 20% (v/v) leachate. The suspension was incubated at 25 °C, with shaking at 150 rpm, under a light intensity of 40 μ Einsteins m^{-2} s^{-1}, 12: 12 h light–dark cycle. After 21 days of incubation, the sub-culturing process was repeated. Successive rounds of sub-culturing were carried out during late exponential growth (approximately every 21 days) for 24 months. The resulting consortium is referred to as the "adapted consortium".

2.6. Leachate chemical analysis

Leachate samples for NH_3-N and DIP measurement were passed through 0.2 μm syringe filters and stored at −20 °C until analysis·NH_3-N content was measured in triplicate using the Modified Nessler Method (Jeong et al., 2013). Leachate samples were analysed in triplicate for DIP content using the ascorbic acid method as described previously (Chian and Dewalle, 1976), where 1 mL of the thawed sample was mixed in a plastic cuvette with 50 μL of ethanol and 50 μL of the combined reagent and absorption was measured at 880 nm.

Removal efficiencies (RE, %) and average removal rate (RR, mg L^{-1} day^{-1}) of NH_3-N was calculated using Eqs. (1) and (2)

$$RE = \frac{X_0 - X_t}{X_0} * 100 \quad (1)$$

$$RR = \frac{X_0 X_t}{t} \quad (2)$$

where X_0 and X_t represents the first and last day of the cultivation period.

Nitrate was measured using colorimetric assay where the nitrate is reduced by vanadium (III) combined with detection by the acidic Griess reaction (Miranda et al., 2001). Briefly, 100 μL of sample was mixed with 100 μL of vanadium cocktail solution and absorbance (540 nm) was measured in a 96 well plate in a plate reader (Tecan Spark, Tecan, Mannedorf, Switzerland) after 2 h incubation. Total organic carbon (TOC) was measured by oxidative combustion-infrared analysis with an automated TOC-V_{CPH} instrument (Shimadzu, Tokyo, Japan), after filtration to remove cells (0.2 μm syringe filters).

For measurement of metals, the leachate was filtered using 0.2 μm syringe filters and acidified with 70% ultra-pure nitric acid (Sigma-Aldrich, Dorset, UK) to 1% (v/v) and stored in the fridge until digestion. Prior to digestion the acidified supernatant was transferred to 60 mL glass tubes that were previously soaked in 1% (v/v) nitric acid. The mix of acids in ratio 4:1 (70% nitric acid and 36% hydrochloric acid, analar grade) was added and digestion undertaken on a dry heating block (Techne DB 30, Staffordshire, UK) at 95 °C for 3 h. The digested samples were centrifuged at 4000 rpm for 10 min. The supernatant was analysed in triplicate for heavy metal content using ICP-MS Perkin Elmer Elan DRC II (MA, USA).

2.7. Microbial diversity and functional analyses (16S and 18S rRNA gene sequencing)

Biomass samples were prepared from the original consortium and adapted consortium, in triplicate, at day 23 of growth. DNA extractions were undertaken using a modified version of the cetyltrimethylammonium bromide method (Karunakaran et al., 2016), where glass beads (425–600 μm) were used in place of enzymatic digestion. PCR amplification, purification and sequencing were performed as described previously (Pandhal et al., 2017) and by RTL Genomics (Lubbock, TX, USA) using an Illumina MiSeq (Illumina Inc. CA, USA). Briefly, the USEARCH algorithm was applied for quality filtering and clustering of merged forward and reverse reads, with chimeras removed using USEARCH detection software in *de novo* mode. Mapping was undertaken using the algorithm in USEARCH with searching performed using an NCBI in-house curated database. Taxonomic information was applied to identify OTU table output values. A low count filter was applied with minimum count 4 with a prevalence filter of 20% (at least 20% of the values should contain at least 4 counts). A low variance filter was also applied to 5% based on interquartile range to remove features constant between the original and adapted consortia and therefore unlikely to be linked to improved growth on leachate. Scaling was performed using total sum scaling. The heat tree analysis uses the mean abundance (in triplicate) to statistically highlight differences between the original and adapted consortium using the non-parametric Wilcoxon Rank Sum test (Foster et al., 2017).

To predict the microbial functional capabilities based on 16S rRNA data, the computational approach PICRUSt (phylogenetic investigation of communities by reconstruction of unobserved states) was applied based on high-quality sequences (Langille et al., 2013). For this process OTU's were close-referenced picked against the Greengenes (v13.5) database at 97% identity for each file. The reliability of the PICRUSt predictions was evaluated using the Nearest Sequenced Taxon Index (NSTI) value. NSTI is the average branch length separating OTUs in each sample from the reference genome with low values implying a close relationship to organisms in the known microbial reference genome databases, representing high accuracy of the predicted KEGG functional groups (Langille et al., 2013). Metabolic functions were predicted by referencing the OTU table to the Kyoto Encyclopaedia of Genes and Genome (KEGG) Orthology (KO) Database. The KO system is the basis for genome annotation and KEGG mapping (Kanehisa et al., 2016). The Global test algorithm was further used for association analyses, which highlighted pathways based on gene onthology databases (Goeman et al., 2004).

3. Results and discussion

In this study, microalgae and associated bacteria were enriched from a landfill leachate pond in the UK. Initially, the growth of the consortium was characterised using diluted leachate (10% and 20% v/v), with and without nutrient supplements, to identify the correct laboratory evolution conditions to observe enhanced algal growth rates. Subsequently, the microbial consortium was subjected to 24 months of adaptive laboratory evolution and the growth characteristics of the new adapted consortium were compared to those of the original consortium. 16S rRNA molecular barcoding was compared between the consortia as well as extrapolation of the metagenomic functionality that could be inferred from the microbial taxonomy data. The aim of this study was to optimise and then characterise the algal microbiome for growth on a complex

landfill leachate media.

3.1. Leachate composition analysis

In order to evaluate the ability of landfill leachate to sustain growth of microalgae consortium, the physicochemical parameters and concentration of NH_3-N, phosphates, nitrates and heavy metals in the leachate were measured. From the measurements, it was evident that several constituents were detrimental to the growth of microalgae. As common with many landfill leachates, concentrations of NH_3-N were particularly high (3960 mg L^{-1}), and likely originates from the decomposition of organic nitrogen (e.g. proteins, urea etc.) (Lin et al., 2007). Although microalgae are able to utilise ammonium as a source of nitrogen, the unionised form, ammonia, is considered toxic as it is uncharged and lipid soluble, easily diffusing across membranes. It can have detrimental impacts on metabolism in phototrophic cells, disrupting photosynthesis by reacting with the photosystem II oxygen evolution reaction core (Drath et al., 2008; Gutierrez et al., 2016). Further challenges for microalgal growth include a low concentration of the macronutrients nitrate and phosphate (1.09 mg L^{-1} and 0.3 mg L^{-1},

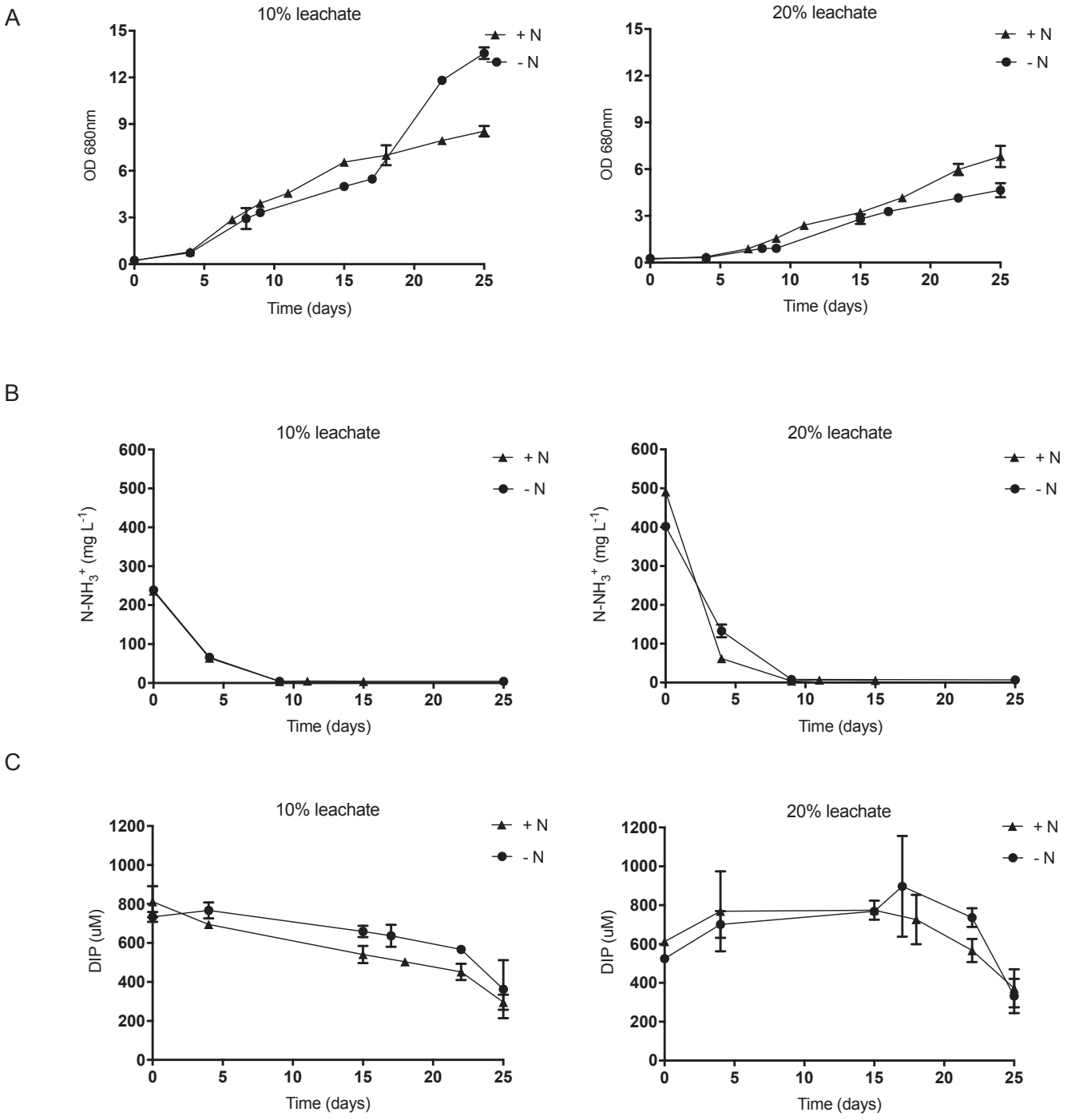

Fig. 1. Comparing growth and nutrients concentrations between 10% and 20% (v/v) diluted leachate, with and without nitrate additions. A: OD_{680nm}, B: NH_3-N concentrations, C: dissolved inorganic phosphate (DIP) concentrations. Mean and standard deviation values of three biological replicates are plotted. Leachate was diluted with distilled water 10% and 20% (v/v) and supplemented with phosphate. Triangles on the graph present data for leachate supplemented with sodium nitrate. Circles on the graph represent data for leachate not supplemented with sodium nitrate.

respectively). Also, the dark brown colouration would hinder light availability for photosynthesis. The concentrations of metals (micronutrient metals and heavy metals) are also relatively high in the leachate, with the latter potentially toxic (Perales-Vela et al., 2006).

3.2. Characterising the growth of original algal-bacteria consortium in 10% and 20% (v/v) diluted leachate

As previously established, landfill leachate can inhibit algal growth and therefore dilutions to 10% (v/v) are often undertaken (Edmundson and Wilkie, 2013; Lin et al., 2007). In addition to toxic concentrations of ammonia and heavy metals, the lack of transparency can inhibit photosynthesis. Light intensity measurements showed a significant reduction in light availability in 250 mL conical flasks filled with 100 mL solution (40–50% reduction in 10% diluted leachate and 60–75% reduction in 20% diluted leachate) and 200 mL solution (47–62% reduction in 10% diluted leachate and 71–80% reduction in 20% diluted leachate). To quantify the impacts of the domestic landfill leachate on microbial growth rates, experiments with 10% (v/v) and 20% (v/v) diluted leachate were undertaken. Due to very low levels of a P-source, i.e. phosphate, supplementation of 0.32 g L^{-1} phosphate was undertaken and compared with and without 0.55 g L^{-1} nitrate additions (Fig. 1). Nitrate additions were tested as it was not clear at this stage whether the enriched algal strain had a preference for nitrate or ammonia as the nitrogen source. The exponential growth rate calculated from OD_{680nm} was 0.16 d^{-1} in 10% (v/v) leachate, whether nitrate was added or not. This decreased to 0.08 d^{-1} in 20% (v/v) leachate without nitrate addition, and to 0.11 d^{-1} with nitrate addition, a significant increase (p-value 0.001) implying nitrate supplementation is more important for faster growth in more concentrated leachate. Growth rate in BBM media (with no added leachate) was considerably faster at 0.21 d^{-1} in the conditions tested. However, the final yields based on OD_{680nm} at Day 25 provides evidence for increased biomass accumulation when nitrate is not added to 10% (v/v) diluted leachate. In 10% (v/v) leachate, the final OD_{680nm} was 13.56 without nitrate additions, 1.6-fold higher than with nitrate additions (OD_{680nm} = 8.54). Conversely, in 20% (v/v) diluted leachate, the final OD_{680nm} was 4.56 without nitrate additions, but 6.82 with nitrate supplementation.

The removal efficiencies (RE) of NH_3-N were also compared across the conditions (Fig. 1B). In 10% (v/v) leachate, a RE of 98.33 ± 0.14% was observed without added nitrate and 98.42 ± 0.16% with nitrate addition (initial NH_3-N concentrations were 236 mg L^{-1}), both within an 8 day period, and not statistically significant. This implied that addition of nitrate did not affect the efficiency of NH_3-N removal in the 10% (v/v) leachate. In 20% (v/v) leachate, also over a 8 day period, RE was 98.02 ± 0.28% without additional nitrate (initial NH_3-N concertation of 402 mg L^{-1}), and 99.24 ± 0.08% with nitrate added (initial NH_3-N of 491 mg L^{-1}). Although statistically significant ($p < .05$), the difference was small, again implying that nitrate additions were not necessary to improve ammonia removal efficiencies.

When calculating removal rates (RR) of NH_3-N, there were notable differences with nitrate supplements, in 20% (v/v) diluted leachate only. In 10% (v/v) leachate, a RR of 26.09 ± 0.04 mg L^{-1} d^{-1} without nitrate addition was comparable to when nitrate was added (RR = 25.75 ± 0.04 mg L^{-1} d^{-1}). However, in 20% (v/v) leachate, a higher rate of NH_3-N removal was observed without nitrate addition, 43.77 ± 0.13 mg L^{-1} d^{-1} and this increased further with nitrate supplements to 53.97 ± 0.48 mg L^{-1} d^{-1}. Therefore, despite higher concentrations of NH_3-N in 20% (v/v) diluted leachate, RR can be enhanced with added nitrate, possibly due to increased microbial growth rates (Fig. 1B), which could explain higher NH_3-N uptake in these conditions.

In 10% (v/v) diluted leachate without nitrate addition, DIP concentrations reduced 48% over 25 days from 734 ± 25 μM to 363 ± 149 μM (Fig. 1D). The addition of nitrate did not change the pattern of DIP concentrations, with a 36% reduction (812 ± 80 μM at day 0 to 296 ± 39 μM at day 25). In 20% (v/v) leachate without nitrate addition, DIP concentrations dropped 63% from 525 ± 18 μM at day 0 to 333 ± 88 μM at day 25, and with nitrate addition, changes in DIP concentrations were comparable, reducing 61% from 613 ± 15 μM to 372 ± 98 μM.

Although the inhibitory effects on algal growth in 20% (v/v) diluted leachate were more prominent compared to 10% (v/v) diluted leachate, growth was still observed, and therefore this dilution was used for the subsequent adaptive laboratory evolution experiments. Moreover, nitrate supplementation was also not undertaken as growth rates were also lower.

3.3. A comparative analysis of the original and adapted algae-bacteria consortia

In an attempt to improve algal growth within the consortium in the higher leachate concentration, an adaptive laboratory evolution strategy was undertaken where the consortium was cultivated with shaking and a light source, with 20% (v/v) diluted leachate providing the only direct heterotrophic carbon source as well as fixed carbon from algal photosynthetic activity. The sub-culturing was undertaken every ~21 days during late-exponential growth phase over 24 months. Subsequently, a batch growth experiment was undertaken to compare the growth characteristics (OD and algal cell counts), algal cell sizes, nitrate, DIP, TOC and NH_3-N concentrations between the original and adapted consortia in 20% (v/v) leachate, supplemented with phosphate only (Fig. 2).

Although the exponential growth rate based on OD_{680nm} showed only a marginal difference between the original and adapted consortium (0.13 d^{-1} versus 0.15 d^{-1}, respectively, Fig. 2A), growth rates based on microalgal cell counts were undertaken as a darker green colouration was evident in the adapted consortium. Cell counts showed that algae in the adapted consortium were able to grow almost three-fold faster (0.20 d^{-1} compared to 0.07 d^{-1}, Fig. 2B). Microalgal cell sizes, did not vary significantly between the consortia, where the original consortium the average length was 3.93 μm ± 0.73 and 3.44 μm ± 0.55 at day 0 and day 25, respectively, and the adapted consortium was 3.64 μm ± 0.66 and 3.61 μm ± 0.67 at day 0 and day 25, respectively.

Surprisingly, DIP concentrations in both consortia did not reduce significantly, even when algal growth was fastest. This could possibly be due to consumed phosphate being replenished by bacterial mineralisation of organic P into phosphates, a process which could be enhanced in the adapted consortium and therefore able to support enhanced algal growth. This is supported by an increase in DIP concentrations in the control flasks, which were uninoculated.

The reduction in NH_3-N concentrations were comparable between both original and adapted consortia (Fig. 2C). In the original consortium, NH_3-N concentrations reduced from 551.5 mg L^{-1} to a mean average of 99.1 mg L^{-1} in 3 days, and then down to 22.7 mg L^{-1} after 11 days. This shows a RE of 95.88 ± 0.85%. Over the same time periods, the adapted consortium demonstrated a slightly higher RE of 97.28 ± 0.30%. RR for NH_3-N were 48.07 ± 0.43 mg L^{-1} d^{-1} and 48.77 ± 0.15 mg L^{-1} d^{-1} for the original and adapted consortia, respectively.

Conversely, nitrate concentrations varied significantly between consortia (Fig. 2D). In the original consortium, nitrate levels were low at the start of cultivation (7.6 mg L^{-1}) but increased to over 900 mg L^{-1} after day 9. Concentrations remained high for 2 days until they fell below the detection limit at day 17. In the adapted consortium, nitrate levels also increased but only approximately three-fold to 21.2 mg L^{-1} after 9 days (similar to control flasks, where no algae were added) and concentrations were lowest at day 35 at 5.6 mg L^{-1}. This surprisingly large increase in nitrate levels in the original consortium cultures could be due to high rates of bacterial nitrification, where NH_3-N is converted to nitrate, but also through increased rates of organic nitrogen decomposition into nitrate. It is possible that bacterial nitrification has been not selected for during the adaption period, and that the microalgae were able to successfully compete with the nitrifying bacteria for NH_3-N, hence reducing overall conversion of ammonia to nitrate, whilst enabling higher algal growth rates (Fig. 2B). There could still be

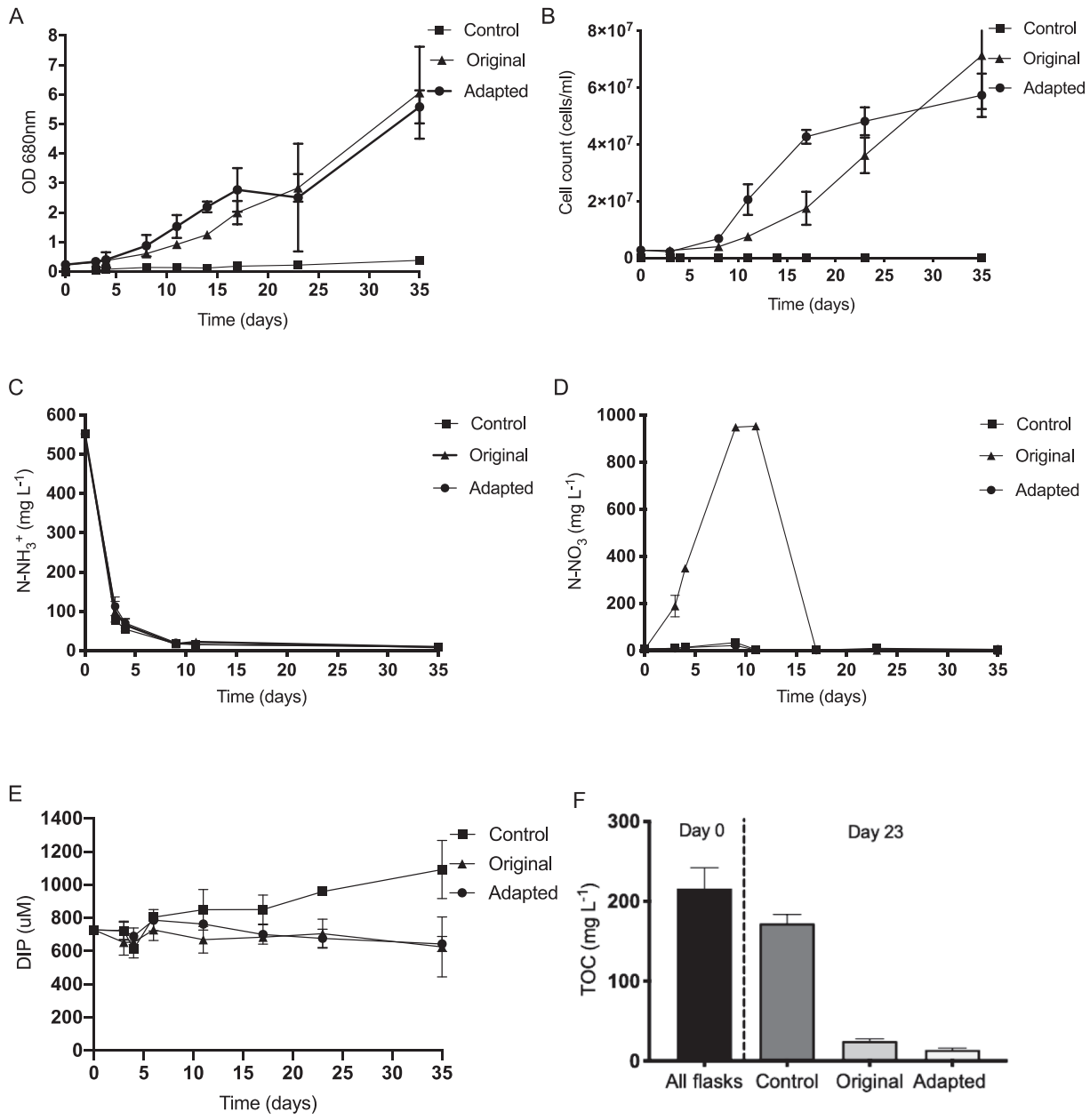

Fig. 2. A comparison of the original enriched consortium compared to the adapted consortium after 24 months. A: OD_{680nm} measurements, B: Microalgal cell counts, C: N-NH_3 concentrations, D: N-NO_3 concentrations, E: DIP concentrations, F: Filtered TOC concentrations.

conversion of NH_3-N into nitrate in the adapted consortium but the algae may have adapted to consume it more readily.

Filtered TOC measurements were measured at the start and end of the cultivation only (Fig. 2F). Initial TOC was low at 216.0 ± 26.2 mg L^{-1} in the in 20% (v/v) diluted leachate, reducing to 24.7 ± 3.1 mg L^{-1} and 13.7 ± 2.3 mg L^{-1} in the original and adapted consortium, respectively. This demonstrated that organic contaminant degradation was occurring during the experiment and improved with the adapted consortium.

3.4. Microbial community diversity analyses

3.4.1. Microbial taxonomic diversity

Microbial taxonomic diversity was compared between original and adapted consortia (in triplicate) from samples taken at day 23 of growth in 20% (v/v) leachate (Section 3.3). The adapted consortia have not only been exposed to the nutrient sources contained within the leachate for an extensive period of time, but also to the presence of eukaryotic algae. Both assessments of 16S rRNA and 18S rRNA reads were undertaken.

For the 18S rRNA analyses to compare eukaryotic communities, there were 100,840 total reads (average counts per sample 16,806,504). One low abundance feature was removed based on prevalence, with one low variance feature removed based on interquartile range. Data normalisation by rarefying was not applied as the variability in sampling depth and data sparsity were not significantly different (Weiss et al., 2017). 18S rRNA sequences were dominated by a *Chlorella vulgaris* strain in both the original and adapted consortia (percentage compositions were 99.6 ± 0.01% and 99.6 ± 0.32%, respectively). *C. vulgaris* has previously shown preferential uptake of ammonium over nitrate, presumably due to no requirement for oxidation–reduction reactions for its assimilation (Pereira et al., 2016). Its presence throughout the adaptive laboratory evolution experiment suggests bacteria have been selected for which can co-exist with the alga as well as utilise the leachate as a growth medium.

For the 16S rRNA analyses, there were 123,024 total reads (average counts per sample 20,504). A total of 13 low abundance features were removed based on prevalence, and 10 low variance features were removed based on interquartile range. Data normalisation by rarefying was not applied as the variability in sampling depth and data sparsity were not significantly different (Weiss et al., 2017).

Abundance profiles using 16S rRNA sequencing data for the original consortium and the adapted consortium are shown in Fig. 3. The genus Pseudomonas dominates in the original consortium (Fig. 3A). Pseudomonas comprises metabolically diverse bacteria with important roles in organic decomposition as well as nitrification. Some species, for example *P. aeruginosa* and *P. putida* are well characterised for their ability to degrade complex hydrocarbons. Adaptive laboratory evolution selected strongly against the Pseudomonas genus (253.53 ± 1.64 fold reduction), for which there could be a variety of reasons. A contributing factor could be that members of this genus have previously been associated with causing rot symptoms in algae (Ashen and Goff, 2000). Although the adapted consortium has more contribution from unclassified and unknown genera, interestingly, Paracoccus strains were enriched (7.10 ± 0.85 fold). Paracoccus is a metabolically versatile genus, well recognised for bioremediation characteristics. Not only this, strains of this genus have been exploited for denitrification properties, helping to remove nitrogen from wastewater, including landfill leachate (Steiner et al., 2019). A statistically relevant depiction of the taxonomic differences (using mean abundance) between the microbial communities at genus level are shown in a heat tree (Fig. 3B). There are other notable differences, including Nitrosomonas sp. which were not detected in the adapted consortium, suggesting that rates of bacterial nitrification would also be reduced. This could explain the large differences in nitrate concentrations in the original versus adapted consortium cultures (Fig. 2D) and suggest that algal based NH_3-N utilisation may have been selected for during the adaptation process. The adapted consortium was enriched for the genus Sphingopyxis, including the species *S. macrogoltabida* (12.83 ± 1.00 fold). The genus is well recognised for species with the capacity to degrade organic contaminants (Shokrollahzadeh et al., 2012) as well as crude oil and kerosene (Kim et al., 2014a, 2014b). More recently, a strain of *S. macrogoltabida* was isolated and characterised with the ability to tolerate toxic concentrations of chromium (Cr VI) (Prabhakaran et al., 2019). The 20% (v/v) diluted leachate contained relatively high concentrations of Cr at approximately 184 μg L^{-1}. Parvibaculum sp. were enriched 11.58 ± 0.99 fold, and although strains belonging to this relatively new genus are known to be relatively difficult to cultivate, strains have been identified with the capability to degrade polycyclic aromatic hydrocarbon, crude oil and surfactants (Schleheck et al., 2011). There is also an increased contribution from the Brevundimonas genus in the adapted consortium (7.27 ± 0.96 fold), for which strains have been isolated previously from leachate and characterised with the ability to degrade synthetic lubricating oils (Morris et al., 2018).

Enrichments could also be explained by direct or indirect associations developed whilst growing with green algae over 24 months. For example, Rhizobia are statistically more prevalent, including the family Bradyrhizobiaceae (5.51 ± 0.99 fold) and genus Rhizobium (10.22 ± 0.98 fold), these have previously been shown to promote the growth of green algae, including *Chlorella vulgaris* (Kim et al., 2014a, 2014b).

3.4.2. Microbial functional analyses

In order to functionally compare how the bacterial composition has

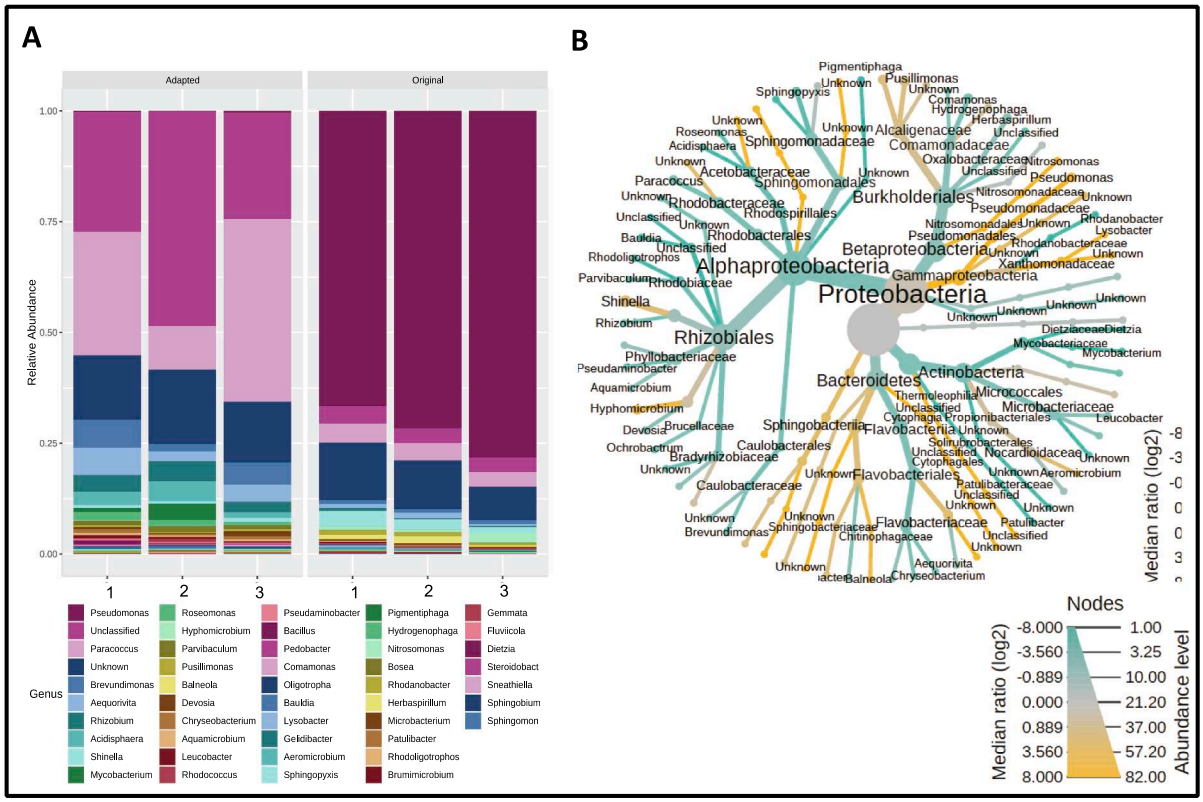

Fig. 3. Microbial diversity comparison between the original isolated consortium and the adapted consortium. (A) The stacked bar chart represents the % abundance of bacteria at genus level in the adapted consortium samples (n = 3) compared to the original isolated consortium at Day 23 (n = 3). Categories labelled as "unclassified" is where the classifier is confident at matching the sequences at a higher taxonomy level above genus level only i.e. below a confidence threshold at genus level. "Unknown" sequences are where sequences can not be matched above the confidence threshold to existing sequences. (B) A heat tree analysis statistically comparing taxonomic differences at genus level. Yellow lines indicate enrichment in the original consortium and green lines represent enrichment in the adapted consortium (n = 3; p < .1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

changed between the original and adapted consortium, a series of analyses were undertaken using 16S rRNA data. Initially, data filtering of samples for functional analysis was performed to remove low quality features and improve downstream statistical analyses. A total of 435 low abundance features were removed based on prevalence and 402 low variance features were removed based on IQR, leaving 3614 features for subsequent PICRUSt analysis.

The PICRUSt approach was applied to predict metagenomic functional content from the marker gene (16S rRNA) data. The PICRUSt algorithm builds on the notion that phylogenetically related organisms are more likely to harbour similar genes. It therefore searches for the most closely related organisms with annotated genomes, assuming that functional information is present within this data, providing insight into microbial functionality.

The NSTI values obtained through PICRUSt were 0.079, 0.081, and 0.082 for replicate original consortia samples, and 0.045, 0.043, and 0.048 for replicate adapted consortia samples, implying a close correspondence with the referenced microbial genome database and therefore high confidence in the predicted metabolic functions of the microbial communities investigated. The stacked area plot in Fig. 4 shows the resulting functional profiles across the triplicate samples from the adapted and original consortia generated using the PICRUSt algorithm. Total hits are shown for each functional category based on Clusters of Orthologous Genes (COG functional category) and where hits belong to multiple groups, they were counted multiple times. The final sum was normalised by the size of each category, and is shown as relative abundance (Fig. 4). This overview shows a collection of comparable COG functional categories between both consortia, however, subtle differences can be visualised. These include an increase in amino acid transport and metabolism in the adapted consortium. There also appears a reduction in cell motility in adapted bacteria as well as defence mechanisms and secondary metabolite biosynthesis, transport and catabolism. These changes are due to adaptation to both the 20% (v/v) diluted leachate media as well as growth with microalgae.

To further investigate associations between bacterial functional categories, the global test algorithm was applied that highlighted predicted K-terms from the KEGG Orthology (KO) database from taxonomic data. The K-term corresponds to the ortholog group for the enzyme, and can be considered functional orthologs. Over a hundred pathways were identified based on K-terms and these were dominated by biodegradation pathways (Table 1 shows the top 25 pathways based on p-values). It is evident from this list that biodegradation pathways are dominant metabolic processes in both consortia.

Classical univariate statistical comparisons between the original and adapted consortium using *t*-test/ANOVA with a p-value cut-off of 0.05 highlighted K-terms enriched during the 24-month adaptation process. The statistically significant bacterial K-terms were mapped onto 262 KEGG reference pathways. These are discussed below from the perspective of leachate substrate utilisation (macronutrient metabolism and biodegradation pathways) and potential interactions with microalgae.

3.4.3. *Nitrogen and phosphorus metabolism*

There were significant differences in the concentrations of nitrate in the flasks where the original and adapted consortia were cultivated (Fig. 2D) and taxonomic assignments showed that no identified strains belonging to Nitrosomonas, a genus associated with bacterial nitrification. Similarly, the PICRUSt analysis highlighted significant differences in nitrate metabolic processes between the consortia and enabled further dissection of the functional pathways involved. Six K-terms with roles in dissimilatory nitrate reduction to ammonia were more prevalent in the original consortium (K00370, K00371, K00374, K00362, K00363) with only one more abundant in the adapted consortium (K02568). These included nitrate reductase subunits with NADH, oxidoreductase and

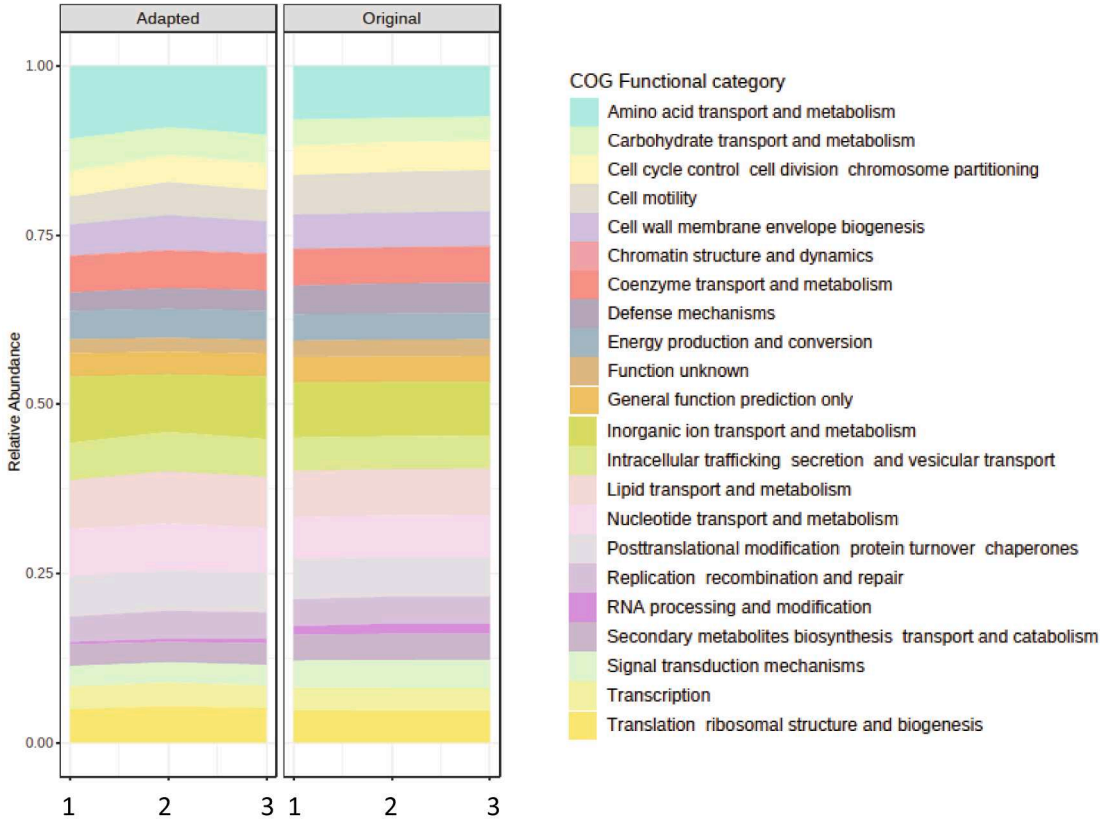

Fig. 4. A stacked bar chart comparing COG functional profiles across triplicate samples from the adapted and original consortium. The plots are based on filtered and normalised data (n = 3).

Table 1
The top 25 KEGG pathways with associated K-terms identified through predicted metagenome hits are listed based on highest p-values.

KEGG Pathway name	Number of K-terms*	P-value
Fluorobenzoate degradation	9	1.8019E−05
Caprolactam degradation	8	2.6376E−05
Biosynthesis of siderophore group nonribosomal peptides	2	2.6608E−05
Limonene and pinene degradation	7	2.6738E−05
Aminobenzoate degradation	9	2.682E−05
Geraniol degradation	5	2.868E−05
Phenylalanine metabolism	19	4.5243E−05
Lysine degradation	12	4.912E−05
C5-Branched dibasic acid metabolism	8	5.0827E−05
Benzoate degradation	39	5.5662E−05
beta-Alanine metabolism	20	7.618E−05
Butanoate metabolism	30	8.3669E−05
Toluene degradation	7	9.7726E−05
Valine, leucine and isoleucine biosynthesis	11	0.00010929
Fatty acid metabolism	26	0.00011104
Xylene degradation	12	0.00015836
2-Oxocarboxylic acid metabolism	26	0.00016736
Propanoate metabolism	31	0.00017052
Tryptophan metabolism	17	0.00017311
Thiamine metabolism	13	0.00022053
Taurine and hypotaurine metabolism	8	0.00022588
Oxidative phosphorylation	9	0.00023119
D-Arginine and D-ornithine metabolism	1	0.00023198
Betalain biosynthesis	2	0.00026086

*K terms link to the KEGG orthology (KO) database, which corresponds to the enzyme orthology group.

cytochrome domains. The nitrate/nitrite transporter was also enriched in this consortium (K02575). Interestingly, assimilatory nitrate reduction was selected for the adapted consortium (K00372, K00366). To support the data from the flask experiments (Fig. 2D), a reduction in complete bacterial nitrification (ammonia to nitrite to nitrate) was predicted within the adapted community (K00370, K00371). This also supports the hypothesis that algal uptake of ammonia increased in the adapted consortium.

The relatively small decrease in DIP concentrations in both consortia was hypothesised to be due to the release of P from organic sources, such as organophosphorus compounds, which were subsequently consumed by the algae. This was corroborated by an enrichment for K- terms involved in phosphonate and phosphinate metabolism in the adapted consortium (K01841, K05915, K06164, K06162, K06163, K06167, K03823). P is a limiting nutrient in most ecosystems, and therefore a plethora of highly efficient P acquisition systems have evolved in nature, targeting a range of all P-containing biomolecules. Although most of these are phosphate esters and anhydrides released into the environment, more recent studies have highlighted the importance of less well studied P-compounds. These include phosphonic and phosphinic acids, that contain highly stable carbon-phosphorus bonds (Yu et al., 2013). It is possible that these are present within landfill leachates, and the action of microbial degradation releases P in inorganic forms bioavailable for algal uptake.

3.4.4. Biodegradation pathways

Association analysis using KEGG K-terms highlighted multiple biodegradation pathways within the bacterial community. A statistical comparison of these showed most were enriched for in the adapted consortium, which was evidenced by a more complete degradation of filtered TOC. However, there were examples where different components of the relevant metabolic pathways were highlighted between both consortia, for example, in benzoate degradation. Benzoates are used as food preservatives and their degradation was recently identified as the most abundant organic contaminant degradation pathway using a metagenomics analysis of a subsurface landfill leachate (Tas et al., 2018). In the original consortium, there was a preference for conversion of benzoate into catechol (K05549, K05550, K05784, K05783). However, once adaptation had taken place, a series of steps involved in the conversion of catechol into precursors for the citric acid cycle were enriched for, through conversion to benzonyl CoA, pimeloyl-CoA and acetyl CoA (K04116, 04117, K07535).

The PICRUSt algorithm also identified that the adapted consortium enriched for naphthalene degradation (K14578, K14584, K14585). Naphthalene is a simple polycyclic aromatic hydrocarbon and considered a contaminant of emerging concern in landfill leachates (Masoner et al., 2014). The K-terms are involved in several pathways including tyrosine metabolism, xylene and benzoate metabolism (Fig. 5). The genes involved in ring removal from the polycyclic aromatic ring to produce salicylate were also statistically enriched. Pathways for the degradation of aromatic hydrocarbon biphenyl were also enhanced in the adapted consortium (K08689, K00462). The bacteria involved in these processes have generated increasing interest since it was observed that toxic man made polychlorinated biphenyls can be biodegraded, and their presence in landfill leachate is well documented (Royal et al., 2003). Further, the enriched degradation pathways in the adapted consortium included those for nicotinate degradation, chlorocyclohexane and chlorobenzene degradation and chloroalkane/chloroalkene degradation (K14974, K01799).

3.4.5. Interactions between microalgae and bacteria

The original consortium is an enrichment of algae and bacteria from landfill leachate, and the cultivation conditions aimed to optimise algal growth within this community. Following 24 months of sub-culturing, it is expected that interactions between algae and bacteria might also be selected for. Although bacteria are capable of minimising the requirement for external CO_2 and essential macronutrients (N and P) for algal growth through regeneration or fixation, they have also been shown to supply algal cells with vitamins (Croft et al., 2006), siderophores (Amin et al., 2009), phytohormones (Segev et al., 2016) and antibiotics (Seyedsayamdost et al., 2014). PICRUSt analysis identified pathways involved in porphyrin and chlorophyll metabolism were highly enriched in the adapted consortium, generating intriguing hypotheses that could be explained by algal-bacterial interactions. Porphyrins are structurally comparable to cobalamin, a known essential biomolecule also referred to as vitamin B12. Many microalgae require exogenous cobalamin as a cofactor for enzymatic reactions, and often rely on bacteria for their biosynthetic capabilities (Croft et al., 2006). We have previously shown that the relationship between a cobalamin-producing bacterium, and its symbiotic green alga, is a complex one, where metabolic trade-offs are required for mutualism (Helliwell et al., 2018). Adverse growth conditions have previously been shown to repress cobalamin-independent methionine synthase in *Chlamydomonas reinhardtii*, leading to cell death (Xie et al., 2013). However, the addition of a cobalamin-producing bacterium *Sinorhizobium meliloti*, reactivated cobalamin-dependent methionine synthase mediated methionine biosynthesis, preventing algal cell death. There is additional supportive evidence in the adapted consortium, through increased predicted abundance of cobalt/nickel transport system permease proteins (ABC transporters) (K02006, K02007, K02008, K10094) in the adapted consortium, a cofactor required in cobalamin biosynthesis.

The metabolism of chlorophyll is further evidence for direct or indirect interactions between bacteria and algae. It is possible that bacteria are degrading and metabolising chlorophyll which would be present within the leachate once algal cells lyse. Interestingly, chlorophyll has been shown to be the major active component in dead algal cells, where it can generate singlet oxygen radicals that catalyse photodegradation of polycyclic aromatic hydrocarbons (Luo et al., 2015). The role of dead algal cells in bioremediation of complex organic molecules is not well understood as the focus has traditionally been on bacterial degradation routes.

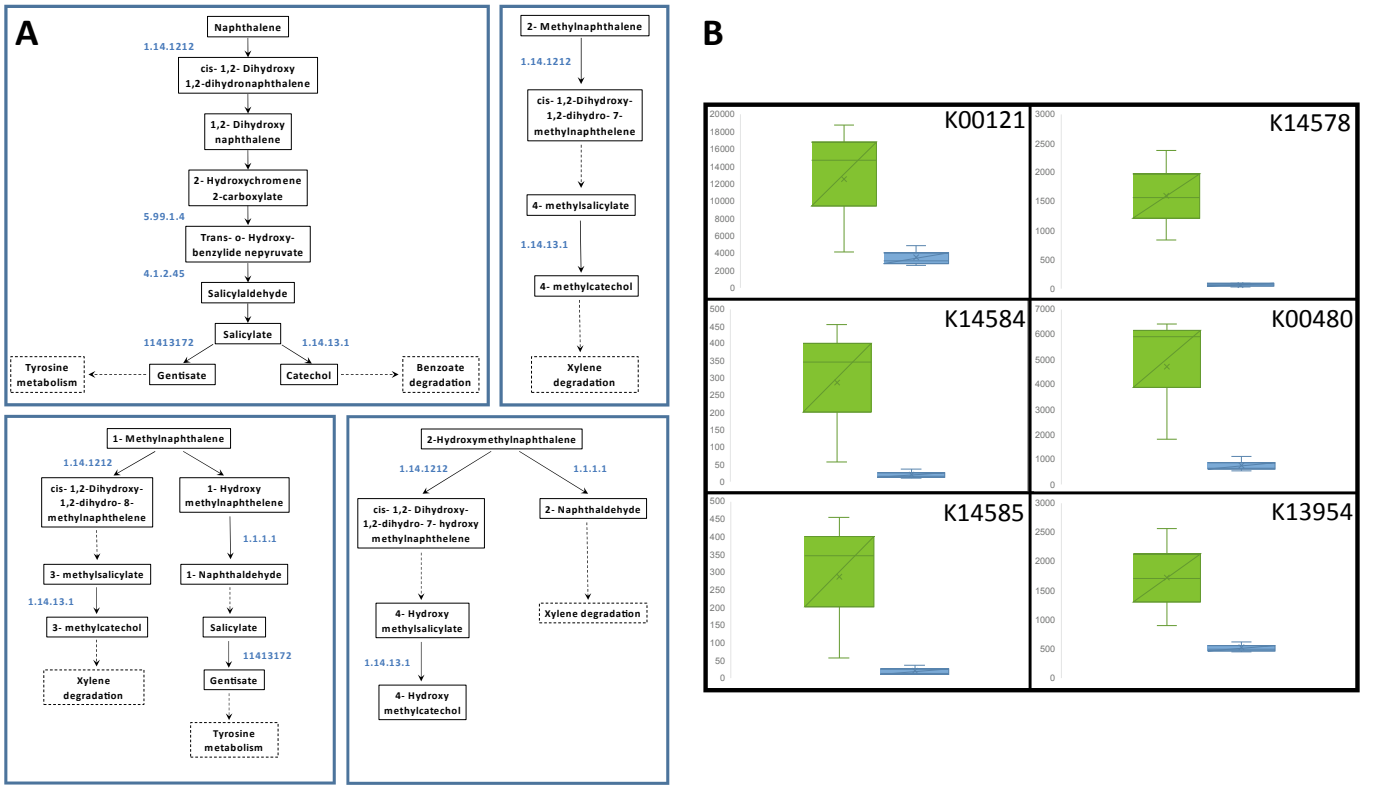

Fig. 5. (A) A metabolic pathway map for naphthalene degradation. The EC (enzyme commission) accession number for statistically significant enrichment of genes encoding enzymes in the adapted consortium are shown in blue. EC numbers are used to link genomes to metabolic pathways for metabolic reconstruction. (B) A selection of box plots of selected K-terms for the original consortium (blue) and adapted consortium (green). The y-axis show counts from the sequencing data. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

This study improved microalgal growth on landfill leachate by optimising the algal microbiome. An enriched microalgal-bacterial consortium was adaptively evolved for 24 months, increasing the growth of *Chlorella vulgaris* over 2-fold. A taxonomic and predicted metagenome analysis revealed enrichment for biodegradation of organic compounds, as well as shifts in macronutrient-resource utilisation, supported by reduced final concentrations of TOC and lower rates of nitrification. This work provides a guided adaptive evolution strategy for enhancing microalgal growth rates in a complex waste stream, and therefore improving treatment rates, as well as the prospect of bottom up engineering of microalgae-bacteria consortia within industry.

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.

Acknowledgments

The authors would like to thank Andrew Fairburn and Neil Bramall for analysing leachate samples on ICP-MS, Dr Thomas Bansagi for writing a script for MatLab calculating cell sizes and counting algal cells from images.

Funding

The authors would like to acknowledge funding from a BBSRC Impact Acceleration Award and NERC Innovation Award (NE/P016820/1).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.124246.

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