

Bioremediation of Landfill Leachate Using Isolated Bacterial Strains

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Abstract Landfilling is one of the most common and widely accepted practices for the disposal of waste throughout the world. Leachate, a major drawback of landfilling, continues to be produced at vast rates and current treatment options are costly and often inadequate. The management of leachate is of economic and environmental importance, due to its potential to cause contamination to ground and surface water. This research focuses on treating leachate in a cost-effective manner through bioremediation. Microorganisms were isolated from landfill leachate (LFL) and screened to determine their ability to remediate a wide range of compounds found in leachate, such as ammonia, phosphate and nitrate. Selected isolates were identified as belonging to the phylum's *Firmicutes*, *Actinobacteria*, and *Proteobacteria*, isolates were inoculated into soil contained in a fixed bed column system. The column system was optimised and used for the treatment of LFL over a 10-hour period. High percentage removal rates were achieved for ammonia (>90%) and removal nitrate and phosphate (>60%). Although EPA discharge limits were not achieved, bioremediation using selected microbial strains represents a cost effective treatment option when compared to conventional methods. Research is now required to further optimise this system to achieve discharge limits for all compounds tested.

Keywords: bioremediation, landfill leachate, wastewater management, municipal solid waste

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1. Introduction

The generation of municipal solid waste (MSW) continues to rise, largely due to global population increase, industrial activities and modern lifestyle [1,2]. Landfilling is the most commonly used method of MSW disposal which results in the production of large volumes of landfill leachate (LFL), the product of water that has percolated through waste picking up the products of degradation. This chemical cocktail can be produced for hundreds of years after the landfill is decommissioned [2,3]. As such, the management of LFL is essential for the protection of the surrounding environment specifically ground and surface water.

The waste industry within Europe has changed dramatically over the past 20 years, due to the implementation of the Landfill Directive 1999/31/ EC [4] and waste management legislation. The Landfill Directive and the Waste Framework Directives [5] directly influence leachate management practices, such as leachate collection, while the Water Framework Directive 2000/60/EC [6] and the Urban Wastewater Treatment Regulation Council Directives 99/31/EC [7] governs and sets discharge limits for wastewater treatment plants (WWTPs) where leachate is often treated. The implementation of these directives has led to a reduction in the amount of waste being sent to landfills. As a result, there has been a dramatic reduction

in the number of landfills in Ireland from two hundred in the mid-nineties to six in current operation [8,9,10]. This significant reduction can be accredited to the closure of smaller landfills directly leading to the production of large volumes of leachate with higher concentrations of pollutants at remaining facilities [9,10,11].

The remediation of LFL is difficult due to the recalcitrant nature of some of its constituents and usually involves the combination of both biological and physicochemical methods. In addition, the varying range of LFL composition also complicates treatment options. For example, biological treatment is hampered by toxic substances and biorefractory compounds, traditional physicochemical treatment, including air stripping and coagulation-flocculation are costly, while treatments such as reverse osmosis only transfer rather than treat the pollution. Biological treatments are more effective at treating leachate from a young landfill (≤ 10 years old), while physicochemical works best for intermediate or mature landfill (≥ 10 years of age). [12,13,14]. Current biological onsite treatment options for LFL include constructed wetland, sequence batch reactor systems, and aerated lagoons [15,16,17,18]. However, in Ireland, the most common treatment practice is to discharge leachate to sewers (51%) or removal by tanker for treatment in WWTPs (48%) with less than 1% being treated on-site. This compares unfavourably to other EU countries, for example, France, where 79% of all leachate is treated onsite [19,20]. The main problem with the

implementation of onsite treatment is the high capital cost, which can range from €260,000 for constructed wetlands to €500,000 for SBR systems, and operational costs [9,10,19]. These options represent significant investment requiring correct planning or retrofitting into existing facilities.

Bioremediation, the process of biologically removing a pollutant from the environment [21,22,23], is a cost-effective and environmentally friendly method of mineralizing most organic compounds in LFL. It is carried out by naturally occurring microorganisms which contribute to degradation and stabilisation of waste in landfill sites by reducing organic compounds to CO₂ and CH₄ under anaerobic conditions [24]. To enhance and improve bioremediation rates it is necessary to isolate and identify microorganisms from landfill locations capable of remediating LFL, particularly its varying toxic pollutants. A study by Latorre et al [26] isolated *Chryseomicrobium imtechense*, *Lysinibacillus fusiformis* and *Acinetobacter* capable of degrading Di-(2-ethylhexyl) phthalate. Work carried out by Xie et al [25] used an aged refuse bioreactor and achieved a reduction in BOD of 95% and total nitrogen of 70%. The results of their pyrosequencing analysis indicated that bacteria from *Pseudomonas*, *Lysobacter*, *Bacillus* and *d-proteobacter*, *Flexibacteraceae* were abundant in their samples, and contributed to these reduction rates. Works by Laing & Liu [27] showed that ANNOMOX bacteria in a bioreactor can reduce ammonia by 60% and nitrogen by 64% and research carried out by Zhang et al [28] describes communities and their biological function in treating LFL.

Isolating microorganisms capable of bioremediating the most common components of LFL, ammonia, phosphate, nitrates, biological oxygen demand (BOD) and chemical oxygen demand (COD) and growing them as pure cultures may result in these strains being harnessed as a cost-effective, natural method of LFL treatment [21,22,23]. The use of microorganisms in bioreactors to treat landfill leachate has been widely reported in the literature particularly in rotating biological contractors, sequence batch reactors and moving-bed biofilm reactors where they have been used for the bioremediation of pollutants including xenobiotic organic compounds (XOC's) and Polycyclic aromatic hydrocarbons (PAHs), as well as removing ammonia [19,28,29,30]. Zhang et al. [28], studied the functional microbial ecology of these reactors in LFL treatment process highlighting the need to have a wide range of microbial species within reactors to achieved optimum removal efficiency.

The aims of the current study were; (1) the isolation and characterisation of microorganism with the potential to bioremediate LFL; (2) the identification of isolates using molecular biology techniques; and (3) to determine if these isolates can be used in the treatment on LFL through bioremediation, in a fixed bed column system.

2. Methods

2.1. Site Description and Leachate Collection

LFL used in this study was sourced from Powerstown Landfill, Co. Carlow, Ireland (52°45'58.46" N, 6°57'20.13" W). The landfill is located 8 km south-east of Carlow

Town in a rural setting and has been operational since 1975. The site consists of three different phases; phase 1 (P1) which operated from 1975-1990, phase 2 (P2) which operated from 1991-2006, and phase 3 (P3) opened in 2006 and is due to close before the end of 2018. P3 consists of four lined cells, surface water settlement pond, leachate tank and green waste composting area. Leachate collection systems are in operation in both P3 and P2. It was decided to use LFL generated in P3 as it is currently in operation and generates a more concentrated leachate than the other phases. Leachate samples were collected mid-November 2015 from the leachate tank (LT) and cell 11 (C11). Further sampling occurred between October 2016 and February 2017. All samples were stored at 4°C prior to analysis.

2.2. Isolation and Characterisation of Bacterial Isolates

Ten-fold serial dilutions of LFL samples LT and C11 were made using sterile distilled water. From these dilutions, 0.1 ml aliquots were spread onto nutrient agar (NA) plates and incubated at 30°C for 2-7 days. Once growth was observed, single colonies were transferred to fresh NA plates, to isolate unique pure cultures for further analysis. These were further characterised using the Gram stain, oxidase test and catalase test [31].

Isolates were assessed for halotolerance using both NA and minimal media (MM) plates supplemented with NaCl (1, 5 and 10%). Isolate resistance to and ability to utilise heavy metals was also determined using, CuCl, ZnCl, CdCl, NiCl, As(NO₃)₃ and FeCl. NA and MM were supplemented with either 5, 10, 25 and 100 mg.l⁻¹ of each metal. Isolates were further characterised by assessing their resistance and ability to utilise NH₄, PO₄ and SO₄. This was carried out by supplementing NA and MM plates with NH₄Cl, KH₂PO₄, and MgSO₄, in stepwise concentrations of 10 mg.l⁻¹ to 1000 mg.l⁻¹. All culture assays were performed in triplicate and incubated at 30°C for 2-7 day. All reagents and chemicals used were supplied by Sigma Aldrich unless otherwise stated.

2.3. DNA Extraction, Bacterial 16S rRNA PCR, Amplified rDNA Restriction Analysis and Phylogenetic Analysis of Isolates

Isolates were grown overnight in LB Broth (LAB M) and 3 ml of culture was centrifuged at 13000 rpm for 10 min. The resultant supernatant was removed and the pellet was retained for DNA extraction using Omega E.N.Z.A® Bacterial extraction kit (VWR Ireland) as per kit protocol. Extracted DNA was visualised by UV excitation after electrophoresis in 1% agarose gels (w/v) 1× TAE (40 mM Tris-base, 1 mM EDTA, 1.14 mM glacial acetic acid; pH 8) gel containing 1 µg ml⁻¹ GelRed™ (Bioscience) with Hyperladder IV (Bioline) as a molecular weight marker.

Bacterial 16S rRNA genes were amplified with the forward primer 27F (5'-GAGTTTGATCCTGGTCAG-3' [32] and reverse primer 1329R (5'-ACGGGCGGTGTGTRC-3 [33]. All PCR reactions (50 µl) were carried out using the GoTaq™ G2 (Promega) kit and contained; 50mM Tris-

HCl (pH 9.0); 50mM NaCl; 5mM MgCl₂; 200µM each of dNTP (dATP, dGTP, dCTP, dTTP), 12.5 pmol of each primer, 200 ng template DNA and 1.25 U Taq DNA polymerase. A 'touchdown' PCR was used to specifically amplify bacterial 16S rRNA genes, with the following conditions: denaturation at 95°C for 10 min followed by 10 cycles of 94°C for 60 s, annealing at 63°C for 60 s and extension at 72°C for 120 s, where the annealing temperature was reduced by 1°C for each cycle; this was followed by 20 cycles of denaturation at 94°C for 60 s, annealing at 52°C for 60 s and extension at 72°C for 120 s, which were in turn followed by a 10-min final extension at 72°C. Negative controls containing no DNA were used, while *E.coli* DNA was used as a positive control. PCR products were visualised as described above.

Amplified rDNA Restriction Analysis (ARDA) was carried out as follows; 5 µl of PCR product was digested with 1U of the restriction endonuclease HaeIII (Thermo Fisher, Ireland) for 3 hrs at 37°C. The resulting DNA fragments were resolved by electrophoresis on 3.5% high-resolution agarose, containing 1 µg ml⁻¹ GelRed™ (Bioscience). Banding patterns were compared by visualisation and grouped into operational taxonomic units (OTUs) as previously described by [34]

PCR products were sequenced by Eurofins Genomics, Wolverhampton. Resultant sequences were analysed using BLASTn searches on NCBI basic local alignment search tool and the Ribosomal Database Project (RDP) (Version 11.5) (classifier function). Similar sequences were downloaded and used for phylogenetic analyses.. Evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model [35], [36]. Phylogenetic trees were constructed using MEGA 7 (Version 7.0.14). Sequences were deposited in Genbank and assigned the accession numbers MG880063-MG880077.

2.4. Preliminary Study into the Bioremediation Potential of Microbes

2.4.1. Influent and Effluent Analysis

Leachate samples were analysed before, during and after all treatments for ammonia, phosphate, nitrate, BOD and COD. All reagent used where of analytical grade and made with deionised water. Ammonia (NH₃-N) was analysed using the phenate method and the concentration read on Shimadzu UV1800 spectrophotometer [37]. BOD was tested over 5 days (BOD₅) and analysed according to

standard methods using a Hanna dissolved oxygen meter [37]. COD was analysed using HACH Lange COD vials. Phosphate (PO₄³⁻) was analysed using molybdovanadate reagent (HACH Lange). Nitrate (NO₃⁻) was carried out using NitraVer® 5 reagent power pillows (HACH Lange). All HACH products were used according to manufactures instruction and measured on HACH DR 6000 UV- spectrophotometer. Percentage removal for each compound was calculated using equation 1 for all three trials.

$$\frac{Co - Ceff}{Co} \times 100 \quad (1)$$

Where,

Co is the initial concentration (mg.l⁻¹),
Ceff is the effluent concentration (mg.l⁻¹).

2.4.2. Trial 1: Effect of the Carrier Matrix

Three PVC columns, Column 1 (C1), Column 2 (C2) and Column 3 (C3) (11 cm ø, 30 cm height, and IC 2850 cm³ each (Figure 1) were utilised in this study. C1 and C2 were packed with c. 1 kg of soil to a height of 20 cm. C3 was packed with sterile soil, autoclaved at 15 psi for 30 min at 121°C, as described for C1-C2.to the same weight and height. Columns C2 and C3 were then spiked with a 500 ml overnight broth culture of 15 previously isolated leachate degrading microorganisms (microbial mastermix) in nutrient broth. The soil mixture was left to incubate for 48 hrs at room temperature (25°C) after which the liquid was allowed to drain off. Leachate (2 L) was then passed through each of the three columns at 10 ml/min over 3.5 hrs with a retention time of 45 min. The composition of the influent leachate is described in Table 1. Effluent samples were collected at 20 min intervals and stored at 4°C.

2.4.3. Trial 2: Effect of Flow Rate

Column operation was further optimised by determining the flow rate which affected the highest percentage pollutant removal efficiency from LFL. Two PVC columns C4 and C5 (dimensions and set up as described above for C1) were utilised in this study. These columns were set up as described above. Leachate (2 L) was passed through C4 and C5 at 10 and 5 ml.min⁻¹, respectively. Column operation and influent and effluent concentrations of ammonia, phosphate and nitrate were determined as described previously.

Table 1. The composition of Powerstown Landfill leachate from 2009-2015, leachates used in this study and the discharge limits set by the EPA

Compounds	Powerstown Landfill 2009-2017*	Leachate used in this study	EPA Limits
Ammonia (mg.l ⁻¹ N)	360-960	790- 1040	≤4
Arsenic (mg.l ⁻¹ As)	25-64	NM	≤0.05
BOD ₅ (mg.l ⁻¹ O ₂)	46-1332	112-170	≤5
Cadmium (mg.l ⁻¹ Cd)	0.2-0.5	NM	≤0.005
COD (mg.l ⁻¹ O ₂)	539-3005	450- 650	≤40
Copper (mg.l ⁻¹ Cu)	5-40	NM	≤0.05
Iron (mg.l ⁻¹ Fe)	2700-11190	NM	≤0.2
Nickel (mg.l ⁻¹ Ni)	60-180	NM	List II substance**
Sodium (mg.l ⁻¹ Na)	510-1280	NM	≤200
Sulphate (mg.l ⁻¹ SO ₄)	61-390	NM	≤200
Zinc (mg.l ⁻¹ Zn)	30-260	NM	≤3
Nitrate (mg.l ⁻¹ N)	NM	89-120	≤50
Phosphate (mg.l ⁻¹ P)	1.2-7.4	3.6-7.25	≤0.4
BOD ₅ : COD	0.07-0.62	0.18- 0.26	

*Sampling didn't occur in 2010 or 2015, **List II substance have a harmful effect on the environment * NM not measured.

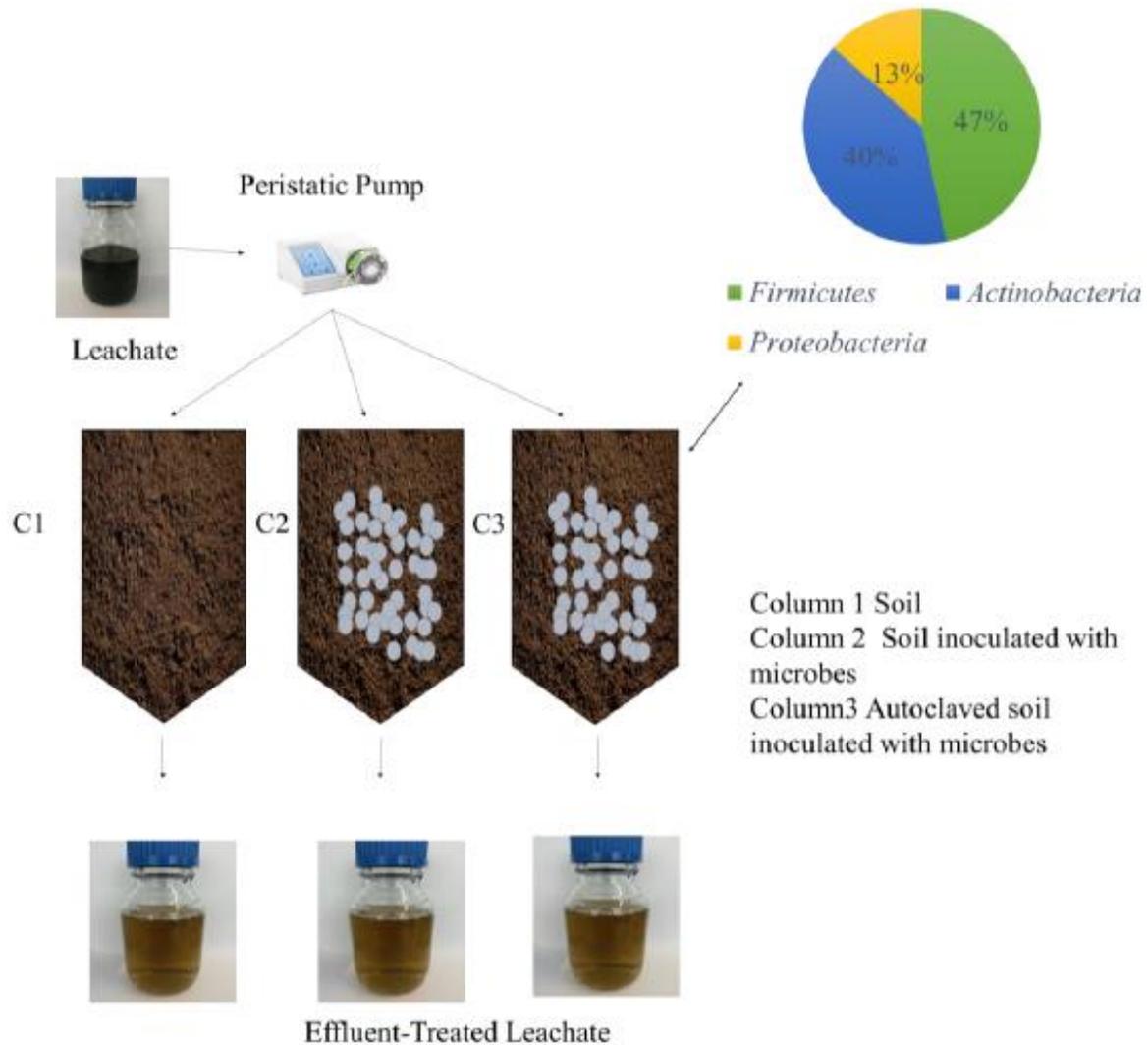


Figure 1. Schematic outline of column system for C1, C2 and C3 utilised in trial 1

2.4.4. Trial 3: Optimised Column Operation

The most effective carrier material and flow rate as determined in trials 1 and 2 were then combined in a further trial (Trial 3) which was operated over a 10 hrs period, with a retention time of 50 min for the treatment of 3 L of leachate. This set-up used soil inoculated with the microbial master mix, while the control contained just soil. The resulting influent and effluent samples collected were analysed as described above. The trial was run in duplicate with samples at each time point being taken in triplicate.

3. Results and Discussion

3.1. Leachate Composition and Characterisation

Leachate from Powerstown was analysed to determine potential treatment options, as well as evaluating the phase of decomposition at the landfill. The results were compared against both previous LFL compositions as determined by Carlow Co. Council during the period 2009-2017 and the EPA discharge limits for each of the

individual components (Table 1). During the sampling (November 2015-February 2017), BOD₅ from Powerstown varied from 112-180 mg.l⁻¹ O₂. The current EPA limit for BOD₅ is set at 5 mg.l⁻¹ O₂ [38]. COD ranged from 450-650 mg.l⁻¹ O₂, with EPA limits set at 40 mg.l⁻¹ O₂. According to both Christensen *et al.*, [39] and Jokela *et al.*, [40] this leachate would classify the landfill in the methanogenic phase, which is determined by a COD range of 500-4500mg.l⁻¹. The BOD₅/COD ratio is used to determine the organic composition of leachate, it is a good representation of waste stabilisation, the transition from early acetogenic phase to the mature methanogenic phase. Ratios between 0.4 and 0.6 are an indicator that the organic matter in the leachate is biodegradable. In mature landfills, this ratio is often in the range of 0.05 to 0.2 reducing as leachate from mature landfills typically contains humic and fulvic acids as well as recalcitrant organic compound, which are not biodegradable [39,41,42,43]. The leachate from this study recorded a BOD₅/COD ratio ranging from 0.18-0.26, indicating a stable leachate which may prove difficult to treat biologically but should respond well to physicochemical treatments [41,42,44].

Ammonia is a common component of LFL which can promote algae growth and accelerate eutrophication in

receiving water bodies. In addition, high concentrations of ammonia can persist for up to 50 years after landfill decommission [10,45] and can decrease the effectiveness of biological treatments such as those employed in wastewater treatment plants (WWTPs) [46,47]. The leachate used in this study recorded an ammonia level range between 790-1010 mg.l⁻¹ (Table 1), these high levels correspond to methanogenic phase [48,49,50]. High levels of ammonia, can impact on biological treatment, resulting in low removal levels for both BOD and COD. To improve the efficiency of treatment, pre-treatment may be necessary to lower the BOD and COD levels.

Nitrates and phosphate were also analysed within this study, as a number of publications discuss these in relation to determining the age/ stage of LFL. Nitrate concentration, in Powerstown leachate, are above the discharge limit set by the EPA (Table 1). Nitrate levels can fluctuate depending on the concentration of ammonia within the leachate. Leachate with high concentrations ammonia concentration, often have high nitrate due to the conversion of ammonia to nitrate during the aerobic process occurring in the landfill. Phosphate level was also determined to be above the EPA discharge limit of 0.4 mg.l⁻¹ P (Table 1). Phosphate levels are considering to be high when compared to the discharge limit as there is a greater than tenfold increase in the level of phosphate in LFL. Nitrate and phosphate can cause contamination to both ground and surface water, and an imbalance in the nutrient cycling process and eutrophication.

3.2. Isolation and Characterisation of Microbes

A total of 96 candidate strains were isolated from initial screening on NA; 52 from LT (LCT) and 46 from C11 (LCC). These were chosen selected on their differing colony morphology, Gram staining, oxidase test and catalase test.

3.2.1. Growth on NaCl

The sodium levels recorded from Powerstown LFL had a mean concentration of 696 mg.l⁻¹ which is equivalent to 1.74 g.l⁻¹ of NaCl, well above the discharge limit set by the EPA of 200 mg.l⁻¹ [38]. To ensure the survival of selected isolates in the bioremediation process, it was deemed essential to use halotolerant microorganisms. The results obtained from this screening indicated that 40 of the 96 isolates were halotolerant i.e capable of growth on NA supplemented with 10% NaCl. These 40 isolates were screened further against other compounds.

3.2.2. Heavy Metal Resistance and Utilisation

In order to achieve successful bioremediation of LFL, it was decided to screen isolates for both their resistance to and ability to utilise heavy metals as a sole carbon source. Results indicated 15 out of 40 isolates were able to grown in the presence of the selected heavy metals tested at concentrations ≤100 mg.l⁻¹. Isolates showed varied results in their ability to grow on MM supplemented with heavy metals (Table 3) with all isolates screened showing growth on one or more of the metals tested at concentrations ≤100 mg.l⁻¹. In particular, LCC32 was capable of growth on all five metals at concentrations of ≤100 mg.l⁻¹, while

three isolates, LCT24, LCT33 and LCC31, displayed growth on concentrations of ≤100 mg.l⁻¹ on three or more metals (Table 2).

3.2.3. Ammonia, Phosphate and Nitrate Utilisation

All 15 strains were resistant to NH₄, NO₃ and PO₄ at concentrations of ≤100 mg.l⁻¹ on NA. In addition, all strains were capable of growth on MM plates containing varying concentrations of NH₄, NO₃ and PO₄ in particular, five isolates, LCT12, LCT33, LCC18, LCC19 and LCT33, were capable of growth at concentration of ≤ 100 mg.l⁻¹ for all three compounds.

3.2.4. 16s rRNA Gene Sequencing and Phylogenetic Analysis

A total of seven bacterial isolates belonged to the phylum *Firmicutes*, containing six *Bacillus* spp. and one *Lysinibacillus* spp. Phylogenetic analysis of these sequences resulted in the formation of two distinct clades (Figure 2) both belonging to the order *Bacillaceae*. The order *Bacillaceae* is a diverse group of gram-positive bacteria within which there are 14 distinct *Bacillaceae* groupings [51]. The two families observed in Figure 2 are the *Bacilli* (first clade) and the *Planococcaceae* (second clade). Martínez and Dussán, [53] and Sharma and Saharan, [52] both observed that *Lysinibacillus* spp. are both phosphate solubilisers and ammonia oxidizers. *Bacillus* species are well known for their bioremediation potential in soil. A study carried out by Safitri et al [54] used a microbial consortium containing *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus coagulans*, *Nitrosomonas* sp., and *Pseudomonas putida* to treat wastewater. This was achieved by inoculating these organisms into the wastewater using 10% of the bacterium to the original volume of wastewater being treated. Safitri et al [54] results showed that this consortium of *B. pumilus*, *B. subtilis*, *B. coagulans*, *Nitrosomonas* sp., and *P. putida* to be effective, resulting in 71% removal of BOD, and 64%, 94.8 % for total suspended solids, and 94.5% for ammonia. All *Firmicutes* isolates used in this study showed great potential for the remediation of heavy metals (Table 2). In particular, isolates LCT 24 and 43 showed resistance to As, Ni, and Fe at high concentrations (Table 2). Leachate from Powerstown Landfill is known for having high levels of nickel (60-180 mg.l⁻¹) and copper (5-40 mg.l⁻¹), therefore it is important to have a range of microbes within the consortium that are resistant to these metals at high concentrations.

Isolates LCT 11, 33 and 48, as well as LCC 19, 29 and 32, belonged to the phylum *Actinobacteria*, a group of microorganisms found in soils with high metabolic versatility and potential for bioremediation [55]. In particular, several studies have indicated their potential for the remediation of heavy metals [55,56,57,58,59]. Specifically, Verma & Singh [60], found that *Brevibacterium casie* was capable of reducing 78% Cr₆₊ and 82% polychlorinated biphenyls in an LB broth medium, indicating that these strain may have the potential to be used in bioremediation. These findings concur with the results of this study (Table 2) which revealed that strains LCT 11, 33 and 48, as well as LCC 19, 29 and 32, show resistance to heavy metals, as well as, ammonia, phosphate and sulphate.

Table 2. Isolates ability to utilise heavy metals as sole carbon source on minimal media

Strain ID	Copper	Nickel	Iron	Cadmium	Arsenic
LCT 10	+	-	+	++	+++
LCT11	+	++	+	++++	++++
LCT12	+	++	-	++++	++++
LCT22	++++	++++	++++	++	++
LCT24	-	++++	++++	++	++++
LCT26	-	++	++++	++++	++++
LCT33	-	++++	++++	++++	++++
LCT42	+	++++	-	-	+++
LCT43	+	++++	++++	++	++++
LCT48	+	-	-	-	+++
LCC18	++++	++++	-	+++	+
LCC19	++++	++++	-	++++	++++
LCC29	-	++++	++++	++	++
LCC31	++++	++++	++++	++	++++
LCC32	++++	++++	++++	++++	++++

Key: Growth on + ≤10 mg. l⁻¹, ++ ≤25 mg. l⁻¹, +++ ≤50 mg. l⁻¹ and ++++ ≤100 mg. l⁻¹.

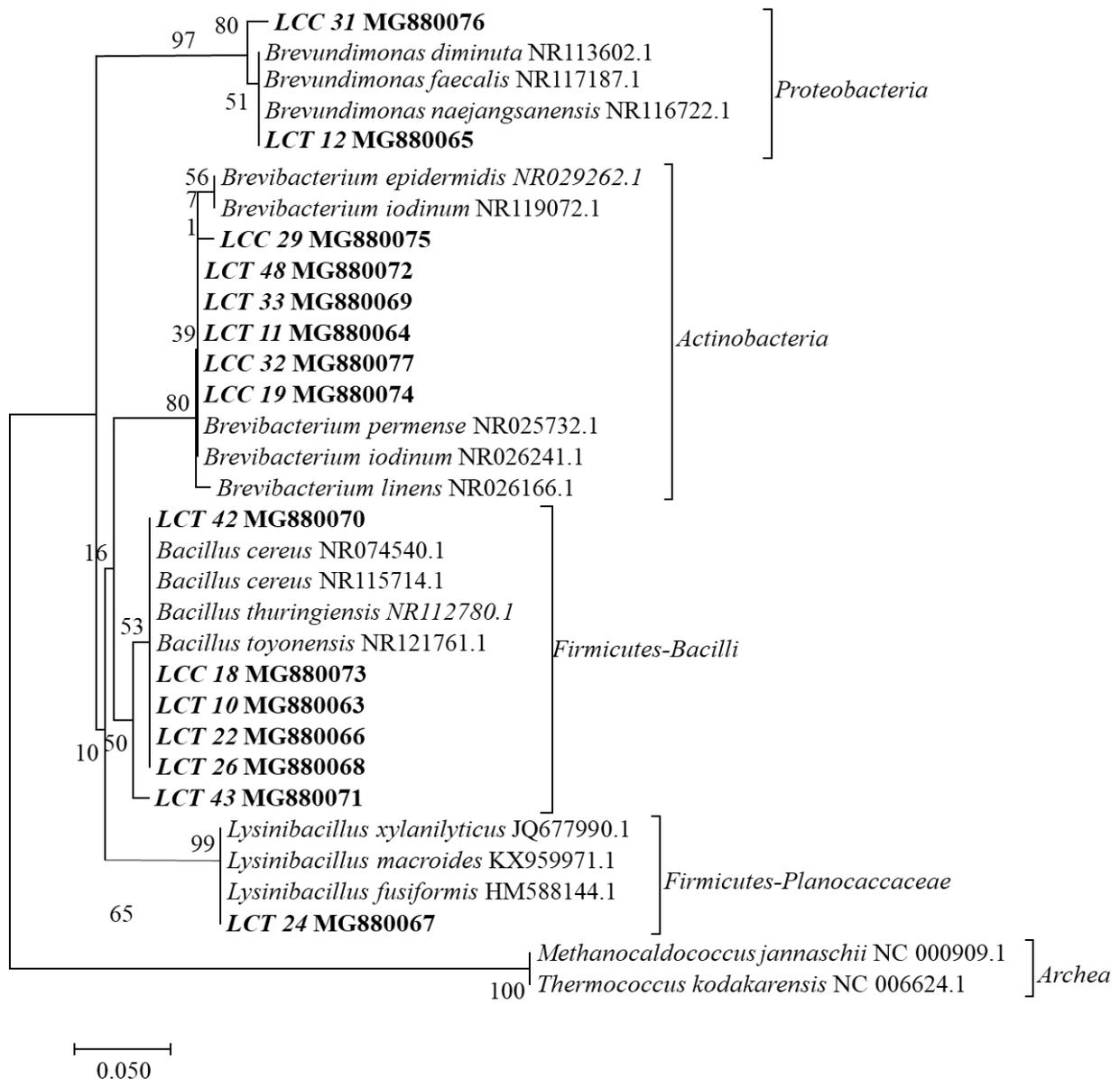


Figure 2. Molecular Phylogenetic analysis by Maximum Likelihood method using MEGA 7 (Version 7.0.14)

For *Proteobacteria* there were two isolates, *Bruvundimonas diminutas* and *Brevundimonas naejangsanensis* (LCT 12 and LCC 31). *B. diminutas*, previously classified as *Pseudomonas* [61], are well known for their bioremediation potential for a wide range of compound including arsenic, nickel, organophosphorus triesters and thioesters and fluorophosphate compounds such as maps and methyl parathion, alongside oil-contaminated wastewater [62,63,64,65]. Other studies have shown *Bruvundimonas* sp. can bioremediate Polycyclic aromatic hydrocarbons and oil [62,66,67]. In addition, isolate LCT 12 was able to utilise Cd, As, NH₃, PO₄ and SO₄ at concentrations of ≤100 mg.l⁻¹, while LCC 31 could utilise Cu, Ni, Pb, As, PO₄ and NO₃ to concentrations of ≤100 mg.l⁻¹.

3.3. Bioremediation Potential of Isolates in a Fixed Bed Column System-Optimisation and Overall Results

3.3.1. Trial 1: Optimisation of the Fixed Bed System-Carrier Matrix

Soil was chosen as a carrier matrix for microorganisms as it is low cost and has been previously used in bioremediation studies treating toxic compounds [21], [23]. Columns containing un-inoculated soil and soil containing the mastermix of microorganisms were compared. Leachate was passed through the column systems and their effluent was analysed. A significant difference of 69% was observed between both soil and soil inoculated with microorganisms in the final percentage removal of ammonia (Table 3) Likewise, for phosphate and nitrate (Table 3) there was a difference in the final percentage removal of phosphate of 26% and nitrate of 15%. In addition, in order to determine the effect the indigenous microorganisms may have on bioremediation it was decided to compare

soil inoculated with microorganisms against autoclaved soil inoculated with microorganisms. The removal rate between soil and microbial master mix and autoclaved soil and microbial mastermix is significant. The final percentage removal achieved for ammonia, phosphate and nitrate are 88, 55 and 35% (Table 3), while the autoclaved soil inoculated with the microbial mastermix recorded slightly reduced removal rates of 81% for ammonia, 46% for phosphate and 31% for nitrates. This deviation was not entirely unexpected as it has been previously reported that autoclaving soil affects its chemical properties, altering pH and affecting the availability of macro-compounds within the soil, while also affecting the physical structure by destroying soil aggregates. It is believed for these reasons the autoclaved soil was not able to hold the inoculum as well as the non-sterile soil [68,69]. Overall from the three possible options soil inoculated with microbial mastermix (C2) was the most effective at removing ammonia, phosphate and nitrates (Table 3).

3.3.2. Trial 2: Optimisation of the Fixed Bed System-flow Rate

Flow rate is an important parameter that influences the removal capacity of fixed bed columns. It is a common hypothesis that the greater the flow rate the lower overall removal in these systems as it determines the pollutant/microorganism contact time [70,71,72]. In Table 4 it can be seen that 5 ml.min⁻¹ shows a greater percentage removal compared to that of 10 ml.min⁻¹. The overall percentage removals at 5 ml.min⁻¹ were 76%, 64% and 36% for ammonia, phosphate and nitrate, respectively (Table 3). The retention time of 5 ml.min⁻¹ was 50 mins compared to 35 minutes for 10 ml.min⁻¹, which effected a longer pollutant/microorganism contact time. As there was a significant difference between 5 ml.min⁻¹ and 10 ml.min⁻¹ for both ammonia and phosphate, it was decided that 5 ml.min⁻¹ would be used in the optimised trail.

Table 3. Influent, effluent and percentage removal achieved by trial 1(C1, C2 and C3) and trial 2 (C4 and C5) for ammonia, phosphate and nitrate. All results are presented as average ± standard deviation

	Trial 1							
	Influent	Column 1		Column 2		Column 3		
		Effluent	% Removal	Effluent	% Removal	Effluent	% Removal	
Ammonia (mg.l ⁻¹ N)	820 ± 0.2	658 ± 0.2	19.7 ± 1.2	92.8 ± 0.3	88.7 ± 1.5	148 ± 0.6	81.9 ± 0.5	
Phosphate (mg.l ⁻¹ P)	4.4 ± 0.2	4.4 ± 0.2	22.3 ± 1.5	2.5 ± 0.3	55.7 ± 1.2	3.0 ± 0.5	46.5 ± 0.5	
Nitrate (mg.l ⁻¹ N)	102 ± 2.3	92.2 ± 0.4	9.7 ± 3.4	65.5 ± 0.6	35.8 ± 2.3	70.2 ± 0.5	31.2 ± 1.2	
	Trial 2							
	Influent	Column 4		Column 5				
		Effluent	% Removal	Effluent	% Removal			
Ammonia (mg.l ⁻¹ N)	869 ± 0.5	208 ± 0.6	76.5 ± 1.2	321.1 ± 0.6	63.6 ± 1.5			
Phosphate (mg.l ⁻¹ P)	4.3 ± 0.2	1.5 ± 0.6	64.1 ± 1.2	1.9 ± 0.5	54.7 ± 1.6			
Nitrate (mg.l ⁻¹ N)	115 ± 1.5	72.5 ± 1.5	36.9 ± 1.2	79.7 ± 1.6	30.6 ± 2.3			

Table 4. Overall results for the removal of ammonia, phosphate and nitrates from soil inoculated with microbes. All results are average ± standard deviation.

	Influent Concentration	Overall Percentage Removal (%)	Effluent Concentration
Ammonia (mg.l ⁻¹ N)	1040 ± 1.3	90.9 ± 1.3	95 ± 0.9
Phosphate (mg.l ⁻¹ P)	7.25 ± 0.5	67.5 ± 0.9	2.36 ± 0.5
Nitrate (mg.l ⁻¹ N)	460 ± 1.2	63.9 ± 1.3	166 ± 1.3

3.3.3. Trial 3: Overall Bioremediation Potential

The use of an optimised fixed bed system to bioremediate ammonia, nitrate and phosphate over 10 hrs was investigated. As previously described ammonia is commonly present at high concentrations in both young and mature LFL [10,19,46,47,73]. Bashir et al [47] notes that ammonia removal is an important concern as its level continues to rise as the landfill ages. In Ireland and the EU, the Water Framework Directive and Urban Wastewater Treatment Directive have stringent regulations for the discharge of wastewater to receiving bodies. These stringent emission limits have caused increasing concern to WWTPs that treat leachate with high ammonia levels and is one of the main reasons why WWTPs are reluctant to treat LFL. This has resulted in over 30 % of WWTPs in Ireland rejecting leachate during the period 2010-2015 [8,10,20]. Leachate used in this trial contained ammonia concentrations of 1040 mg.l⁻¹ N which was reduced to 95 mg.l⁻¹ N, in the final effluent. However, despite the final effluent not reaching the discharge limits (4 mg.l⁻¹ N), there was a significant overall ammonia reduction of 95 % (Table 3). To achieve ammonia levels that are acceptable further studies would need to be carried out including recirculating leachate back through the column system or by using this step as a pre-treatment followed by a physicochemical treatment, such as ammonia air stripping or reverse osmosis. As previously discussed, leachate from a methanogenic stage of the landfill may not respond well to biological treatment and the need for a physicochemical treatment has been shown. It is known that ammonia in the methanogenic phase is quite toxic to microorganisms and can inhibit the biological degradation process. This is why it was essential to have a microbial consortium that can deal with high levels of ammonia.

Phosphate is found at a very low level in leachate from Powerstown Landfill when compared to ammonia and nitrate, 1.2-8 mg.l⁻¹ P (Table 1). However, levels are still above the EPA set discharge limits of 0.4 mg.l⁻¹ P. The main problem associated with phosphate is the contamination of ground and surface waters. When phosphate contaminates ground and surface waters it can cause an imbalance in the nutrient cycling process, eutrophication and blooms of cyanobacteria. Even though phosphate is a concern when it comes to contamination, it is not a major concern for WWTPs in Ireland, in fact, some WWTPs may require the addition of phosphorous as a nutrient for bacterial growth [74]. The microbial consortia used in this study has shown the ability to be resistant to and capable of utilising phosphate at concentrations ≤ 100 mg.l⁻¹ P. In particular, two isolates LCT 24 (*Bacillus vitnamensis*) and LCC32 (*Brevibacterium iodinum*) have the ability to survive in medium with high level of phosphates (<100 mg.l⁻¹ P). A study carried out by Riazonova et al [75] showed that *B. casei*, *B. linens*, and *B. epidermidis* were able to reduce phosphate concentration by 90 %. In total, seven isolates (Figure 2) that cluster within the *Brevibacterium* phylum have the ability to utilise phosphate and give high reduction rates. Phosphate levels of the initial leachate samples were 7.25 mg.l⁻¹ P. Overall phosphate was reduced by 67%, however, discharge limits were not reached (Table 4) indicating further treatment is needed similar to those previously described for ammonia.

Nitrates are the result of the nitrification process by microorganisms, in which biological oxidation occurs to

convert ammonia to nitrite and then to the nitrate. The influent concentration was 460 mg.l⁻¹ N which was reduced by 63% overall. This was the lowest reduction rate achieved, but similarly ammonia and phosphate, discharge limits were not achieved. Final effluent concentration of 166 mg.l⁻¹ N were recorded which are above the discharge limits of 50 mg.l⁻¹ N (Table 1). It should be noted that nitrate readings fluctuated throughout the trial. This may be due to the nitrification process, as when ammonia is treated in aerobic processes it is accompanied by a concomitant increase in nitrate concentrations. Other treatment methods such as physicochemical treatment may be needed for nitrates to counteract this problem.

4. Conclusion

LFL from Powerstown landfill in Carlow, Ireland contains a wide range of bacteria, including *Firmicutes*, *Proteobacteria* and *Actinobacteria*, which displayed great ability to be resistant to a wide range of compounds found within leachate. Bioremediation is a promising treatment option for LFL. The system described and optimised in the current study achieved high percentage removal rates for ammonia (90%), while for both phosphate and nitrate lower percentage removal rates were recorded (67 and 63%, respectively). Overall these reduction rates are promising, but further work is needed to achieve regulatory discharge limits. The findings of this study are as follows, (1) Landfill leachate from Powerstown is in the methanogenic phase of decomposition and contains high levels of COD, BOD and ammonia; (2) Microorganisms isolated from leachate have the potential to utilise a range of heavy metals, ammonia, phosphate and nitrate, common constituents of leachate; (3) The microbial consortium used in this study were capable of reducing ammonia, phosphate and nitrate by 90, 67 and 63%, respectively.

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Abbreviations

LFL	Landfill leachate
MSW	Municipal Solid waste
WWTPs	wastewater treatment plants
NM	not measured
P1	Phase 1
P2	Phase 2
P3	Phase 3
C1	Column 1
C2	Column 2
C3	Column 3
C4	Column 4
C5	Column 5
NA	Nutrient Agar

MM Minimal Media
 XOC's xenobiotic organic compounds
 PAHs Polycyclic aromatic hydrocarbons
 BOD Biological Oxygen Demand
 COD Chemical Oxygen Demand
 EPA Environmental Protection Agency

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